Lung Epithelial Biology in the Pathogenesis of Pulmonary Disease

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Introduction: The Lung Epithelium

Michael Koval and Venkataramana K. Sidhaye

1.1 LUNG ANATOMY

As a specialized organ for handling gas exchange between the bloodstream and the atmosphere, the lung requires several characteristics. Above all, lungs must maintain a barrier between the atmosphere and fluid containing tissues, the so-called air–liquid interface, so that the exchange of oxygen and carbon dioxide can occur [1]. By necessity, this requires that the respiratory tract have a large surface area. To maintain this, the lung is composed of a branching structure (Fig. 1) that has distinct functional zones ranging from the nasopharynx to the conducting airways and then ultimately to alveoli that are sites of gas exchange [2].

Although there are upward of 40 different cell types in the human lung [3], the epithelial cells covering the air-exposed surfaces have central roles in lung function [4,5] and are the topic of this volume. Each of the different zones of the respiratory tract is covered by distinct epithelia that reflect their function in respiration (Fig. 2). The nasopharynx primarily functions as a system to humidify and filter inhaled air as well as provide the sense of smell. The conducting airways begin at the trachea that then bifurcates into bronchi with continued branching into smaller bronchi and then bronchioles. The trachea, bronchi, and some bronchioles are supported by cartilage that provides stability that would not be sustainable without a semirigid scaffold.

The terminal airspaces are known as alveoli and have a larger surface to volume ratio that reflects their function in gas exchange [6]. Central to this function is an alveolar air–liquid interface that must be thin enough to enable free diffusion of oxygen and carbon dioxide. This requires pulmonary surfactant to provide both a critical level of hydration

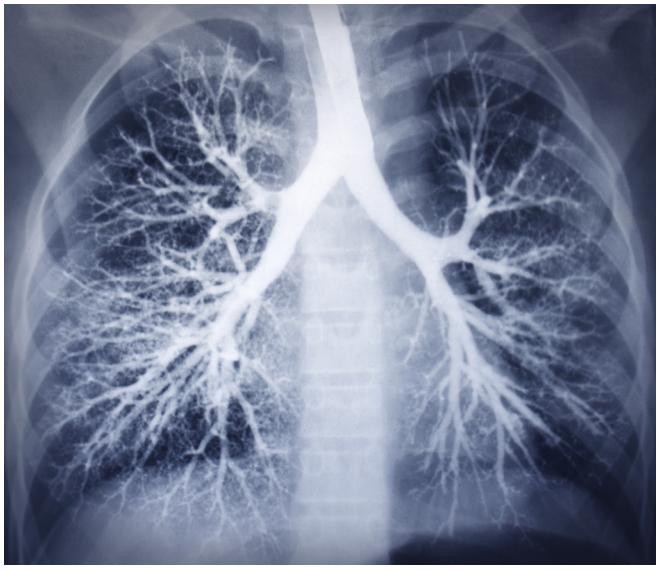

FIGURE 1 **Chest X-ray of healthy adult lung.** A contrast-enhancing agent was inhaled to enable imaging of the bronchoalveolar tree. Note several generations of bifurcations in the transition from trachea to bronchi to bronchioles. Alveolar sacs are not visible in this image.

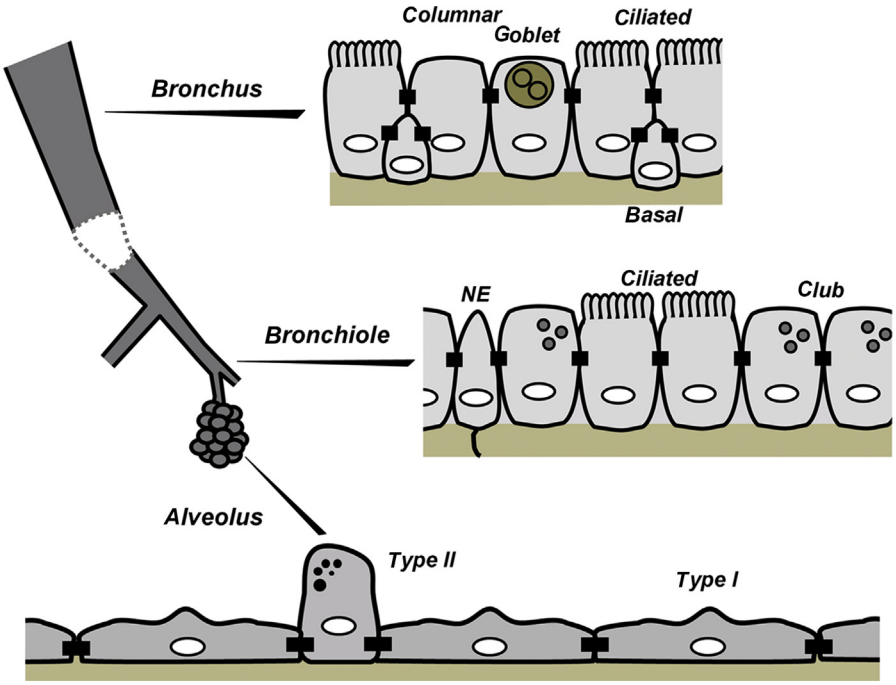

FIGURE 2 Distinct epithelial cell populations in different airspace zones. Representative sections of the respiratory tree corresponding to large airways (bronchus), small airways (bronchiole), and terminal airspaces (alveolus). Ciliated cells are common to large and small airways and facilitate mucus clearance. Goblet cells are mucus secreting cells present in large airways, submucosal glands that also contribute are not shown in this diagram. Basal cells at the basement membrane are multipotent progenitor cells. Club cells are found primarily in transitional bronchioles and both produce surfactant as well as act as multipotent progenitors of the lower airway, neuroendocrine cells (NE) as also shown. The alveolus is dominated by flat type I cells. Type II cells produce pulmonary surfactant and are also progenitors for type I cells. Black rectangles between cells represent intercellular junctions.

and the ability to support low surface tension to facilitate lung expansion and prevent alveolar collapse [7,8]. The smallest bronchioles adjacent to alveoli are also supported by a form of pulmonary surfactant and do not require a cartilaginous superstructure.

1.2 LUNG EPITHELIAL COMPOSITION

The nasopharynx and conducting airways are covered by a pseudostratified epithelium, where each cell has direct contact with the basement membrane [4,5,9]. The majority of airway epithelium consists of ciliated and mucous secreting cells that together constitute the so-called mucociliary elevator that serves to clear particulates and other irritants out of the airspaces. Submucosal glands containing goblet, duct, and serous cells also contribute to mucus production and fluid balance. Airway surface fluid is critically regulated by epithelial ion channels in order to enable mucous clearance to efficiently occur [10]. In addition, airways contain a pool of multipotent progenitor cells, basal cells, located within the epithelial layer, that have a crucial role in airway repair as they are stimulated to proliferate and differentiate in response to injury.

Terminal bronchioles are a transitional airway that bridges the transition from a mucus dominated airspace to the surfactant coated alveoli. In contrast to larger airways and bronchioles, terminal bronchioles lack submucosal glands and goblet cells and instead contain club cells that produce pulmonary surfactant containing surfactant protein A (SP-A), SP-B, and club cell specific protein 10 (CC-10) [11]. Club cells also are progenitors that can self-replicate or differentiate into ciliated or alveolar epithelial cells [12]. In addition to club cells, bronchioles also contain a small pool of multipotent broncholalveolar stem cells (BASCs) that can also participate in repair but have also been implicated in tumor formation [13].

The terminal airspaces are covered by the alveolar epithelium, a heterogeneous monolayer of type I and type II epithelial cells (Fig. 2). Most of the alveolar area is covered by type I cells that are extremely thin to facilitate gas diffusion [14]. Alveolar type II cells are the progenitors for type I cells [15] and also produce pulmonary surfactant, which differs from the surfactant produced by club cells in that it lacks CC-10 and instead contains SP-C resulting in different

biophysical characteristics [16]. Pulmonary surfactant also contains collectins that promote host defense by facilitating clearance of bacteria and other pathogens [17].

Although epithelial cells in the respiratory system show considerable diversity of function, they all have several common features, most notably in that they form a barrier that preserves the air/liquid interface that regulates airspace fluid composition and that protects the host from environmental insults [18]. This is achieved since these cells have apical−basolateral polarity that specifically orients ion channels and transporters so that solutes and proteins can be transported in a polarized manner [19]. They are interconnected by the apical−junctional complex that facilitates intercellular signaling and provides a mechanical barrier that regulates free diffusion between cells [20,21]. These common elements enable the lung epithelium to function in a coordinated manner to facilitate respiration. Furthermore, the epithelium maintains a close interaction with a variety of specialized immune effector cells promoting its participation in local immunity.

1.3 EPITHELIAL DYSFUNCTION IN LUNG DISEASE

The epithelium has emerged as a central focus of several pulmonary diseases. Covered in detail in this volume are pulmonary fibrosis, interstitial lung disease (ILD), chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), asthma, and cystic fibrosis (CF), although each of these conditions varies with respect to certainty of etiology and presents with distinct pathology, it is likely that several overlapping components related to epithelial dysfunction and repair are shared in the progression of different lung diseases.

1.3.1 Genetic Disorders

CF is one of the most widely studied genetic disorders and a multitude of mutations in the CF Transmembrane conductance Regulator (CFTR) gene have been identified [22]. The detailed understanding of how CFTR mutations that affect different functions of this chloride channel has led to significant advances in the ability to treat CF [23]; however, therapeutic strategies are not fully effective and several CF mutations remain intractable to pharmacologic agents.

More recently, mutations in surfactant proteins, such as SP-C, have been linked to increased susceptibility to ILD [16]. In many cases, these mutations lead to cell stress responses, suggesting that other as-yet unidentified mutations may have a similar effect on lung repair and function. However, this may not be universally applicable to protein misfolding mutations. For instance, the F508del-CFTR channel is misfolded and retained in the endoplasmic reticulum (ER) inducing a stress response [24], but defining roles for ER stress in CF has proven challenging.

In addition to overt mutations, genome-wide association studies has led to identification of single nucleotide polymorphisms (SNPs) that have been linked to several lung diseases including susceptibility to COPD [25], ARDS [26], pulmonary fibrosis [27], and CF [28]. Interpreting how genetic polymorphism affects lung disease is not straightforward. For instance, SNPs associated with myosin light chain kinase have been linked to both increased susceptibility to acute lung injury and increased risk of sepsis, yet resistance to asthma [29,30].

Understanding the genetic components to lung disease is critical for the effective application of precision therapy to combat lung disease. However, in addition to genome sequence, epigenetic regulation of gene expression by processes such as DNA methylation, histone acetylation, and regulation by microRNAs add to the complexity of defining the genetic basis for lung disease [31]. Models incorporating these elements of genetic control are needed to fully determine the genetic component of lung disease.

1.3.2 Environmental Insults

One of the clearest risk factors for lung disease is tobacco smoking [32,33]. Although the recent advent of electronic cigarettes has been suggested as a safer alternative that can facilitate smoking cessation [34], this notion has recently been called into question [35]. Marijuana and other smoked drugs can also have deleterious effects on lung function [36] and in developing countries indoor air pollution due to poorly ventilated ovens and furnaces is a significant risk factor for lung disease [37]

Tobacco is most commonly linked to COPD and lung cancer, although increased incidence of asthma has also been connected to tobacco smoke exposure [33,38]. Environmental exposure to toxicants is also a well-recognized risk factor for asthma [39] and is likely to promote COPD and other conditions such as ILD [40]. How environmental insults are mechanistically linked to different lung diseases remains an open question; however, there is likely a strong genetic component that determines how sensitive an individual will be and their pathologic outcome.

In addition to inhaled insults, diet is also likely to have an impact on the development of pulmonary disease [41]. Vitamin D deficiency in particular has been shown to exacerbate CF and asthma [42]. Chronic alcohol ingestion is a recognized risk factor for increased severity and mortality from ARDS [43,44]. How diet interplays with other environmental exposure to cause pulmonary disease is just beginning to be explored, although it represents an area where a relatively easy intervention could have a significant benefit. There is increasing interest in how diet and environmental exposure converge to alter local cellular metabolism. In combination with genetic predispositions, lung epithelial metabolomics is gaining increasing application to understanding how nutrients and toxins affect lung function and susceptibility to disease.

1.3.3 Pathogens

Acute bacterial and viral pneumonia are obviously causative factors in lung disease, predominantly by inducing significant damage to the airway epithelium, leading to ARDS [6]. In severe cases, this is further exacerbated by barotrauma due to ventilator-induced lung injury. Sepsis resulting from bacterial pneumonia remains a serious and difficult to treat complication [45].

In addition to pneumonia, bacterial infections have also been associated with COPD and asthma [46]. As a result of mucociliary dysfunction, CF patients are particularly susceptible to chronic infections that become particularly severe when the bacteria establish biofilms in the lung [47,48]. Deleterious biofilms can also occur in COPD, decreasing the efficacy of antibiotic treatment [46]. In addition, there is increasing evidence suggesting that the lung has an inherent microbiome that promotes lung function [49]. How potentially beneficial microbes are sensitive to specific respiratory diseases remains an open question.

In addition to severe infection, subthreshold infection can also have a deleterious effect on lung function and repair. For instance, respiratory syncytial virus infection can prime the lung to facilitate bacterial colonization [50,51]. Viruses such as gamma-herpes virus induce ER stress and thus cause pulmonary fibrosis, comparable to the effect of misfolded surfactant proteins [52,53]. Chronic human immunodeficiency virus (HIV) impairs lung epithelial barrier function, increasing sensitivity to ARDS even in patients in whom the immunologic effects of HIV are controlled by highly active antiretroviral therapy [54,55]. How viruses impair lung epithelial function remains an active area of investigation.

1.4 EPITHELIA AS A THERAPEUTIC TARGET

Despite the implication of the epithelium in almost every lung disease, there are relatively few epithelial specific therapeutic targets. There are three broad approaches to target impaired lung epithelia. The most direct approach is to use pharmacologic agents that reverse the deleterious function of impaired epithelial cells. The most successful example of this approach is in treatment of CF, where Ivacaftor is used to treat patients harboring the G551D-mutation in CFTR and Orkambi is used to treat F508del-CFTR patients [56]. Although these drugs are true breakthroughs, they do not completely cure CF, leading to the efforts to identify adjunct therapies to further treat CF patients [57]. One can envision other therapeutic approaches to alter other lung epithelial functions, including other ion channels involved in fluid balance, mucus secretion, surfactant secretion or tight junction proteins involved in barrier function. Moreover, strategies to alleviate ER stressor promote autophagy may also prove effective.

Mesenchymal stromal cells (MSCs) provide another approach to target the lung epithelium, since they home to sites of injury [58,59]. MSC integration in host tissues, including lung, does occur albeit at low levels [60]. Instead, a more likely role for MSCs is to transiently migrate through injured tissue to provide a paracrine repair stimulus. The success of this approach in model systems has led to clinical trials testing whether administration of MSCs are effective in treatment of ARDS [61]. Moreover, it was found that that MSCs transfected with a tetracycline-inducible keratinocyte growth factor (KGF) construct had greater efficacy in preventing bleomycin-induced lung injury than nontransfected MSCs [62]. This raises the potential for engineered MSCs as a means to tailor their ability to treat different diseases.

Finally, there have been considerable efforts to identify multipotent stem cells in the lung, which led to the discovery of basal cells, BASCs and alveolar Itga6 + /Itgb4 + cells as well as the finding that club cells have multipotent potential as well [4]. Thus, these cell populations represent an appealing therapeutic target, either through induction of proliferation or by cell transplant in an injured lung. As proof of principal, a fully denuded rat lung matrix was successfully repopulated with human basal epithelial cells, as well as endothelial cells and transplanted into a rat where it functioned for six hours to promote gas exchange [63]. Although the idea of custom-built lungs is a challenging concept, it seems reasonable to identify a regimen where human lung stem cells can be stimulated to repair the lung. This is particularly appealing to treat diseases such as COPD and ILD where the lung is apparently irreversibly impaired and in need of rejuvenation.

1.5 SUMMARY

The lung epithelium performs a fundamental role in maintaining lung health and is critically impaired in several disease processes. This volume seeks to explore roles for the lung epithelium in greater detail and highlights the current state of understanding the effects of epithelial dysfunction in lung disease.

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Chapter 1

Junctional Interplay in Lung Epithelial Barrier Function

Michael Koval

1.1 INTRODUCTION

Epithelial cells line the entire airspace in order to maintain a functional barrier between the outside environment of the atmosphere and inner fluid-filled tissues such as the interstitium and circulatory system. While different zones of the respiratory system have different function and phenotype, lung epithelial cells in general share common features with each other as well as with epithelia present in other tissues. Of particular note are intercellular junctions that enable the individual cells of an epithelial monolayer to coherently function as a unified tissue barrier.

This chapter focuses on the composition, structure, and function of different classes of intercellular junctions found in lung epithelia: adherens junctions, tight junctions, and gap junctions. In addition to general principles, specific roles for these intercellular structures in lung function will be described.

1.2 EPITHELIAL CELL POLARITY COMPLEX

Cells define their basal, or bottom, surface through direct contact with the extracellular matrix mediated by integrin-family transmembrane proteins [1–3]. Integrins provide an organizing point where actin cytoskeletal filaments can attach and be interlinked with other structures in the cell [4]. Among these actin tethered structures is the apical junctional complex (AJC), a multicomponent structure where neighboring cells are in direct contact.

The AJC has several distinct components that serve different functions (Fig. 1.1) [5]. The most apical of these is the polarity complex, which is initiated by transport of a Crumbs-related (Crb) family transmembrane protein to the plasma membrane [6]. There are three mammalian Crb isoforms, of which Crb3 is the most broadly expressed [7]. Crb3 nucleates formation of the AJC by recruiting several cytosolic scaffold proteins, including Protein Associated with Lin Seven (PALS1) and PALS1 associated TJ protein (PATJ) [8]. The Crb3–PALS1–PATJ complex associates with other signaling complexes, including the Par3–Par6-atypical protein kinase C complex, which acts to reinforce polarity by phosphorylating Crb3 and Par3 as well as lateral proteins (e.g., Lgl) and downstream kinases such as MAP kinase [9–11].

An essential role for Crb3 in epithelial development was demonstrated by the observation that Crb3-deficient mice die shortly after birth and have disrupted epithelial morphology [12]. Of particular note, the lung airspaces of neonatal Crb3-deficient mice are filled with debris and the polarity complex is disordered. The effect of Crb3-depletion on epithelia is not limited to the lung as other tissues are also affected.

A specific role for Crb3 in lung development was elucidated using mice with a loxP-flanked Crb3 gene combined with a Cre recombinase driven by a Sonic hedgehog promoter that is active at the earliest stages of lung development [13]. The developing trachea of these tissue-specific Crb3-deficient mice contained largely Keratin5-positive cells suggestive of basal epithelial cell hyperplasia. This was due in large part to the inability of Crb3-deficient cells to retain the Yap transcription factor as part of the AJC [13]. Instead, in the absence of Crb3, Yap translocated to the nucleus where it acted as a transcription factor to influence gene expression. This suggests that regulation of Yap is a key step in lung epithelial differentiation and underscores the role for Yap/Taz/Hippo pathway in regulating lung branching morphogenesis [14,15]. However, given the central role for Crb3 in assembling the AJC, the dysregulation of Yap is not necessarily immediately downstream of Crb3. Instead, YAP may more directly bind to other polarity complex proteins

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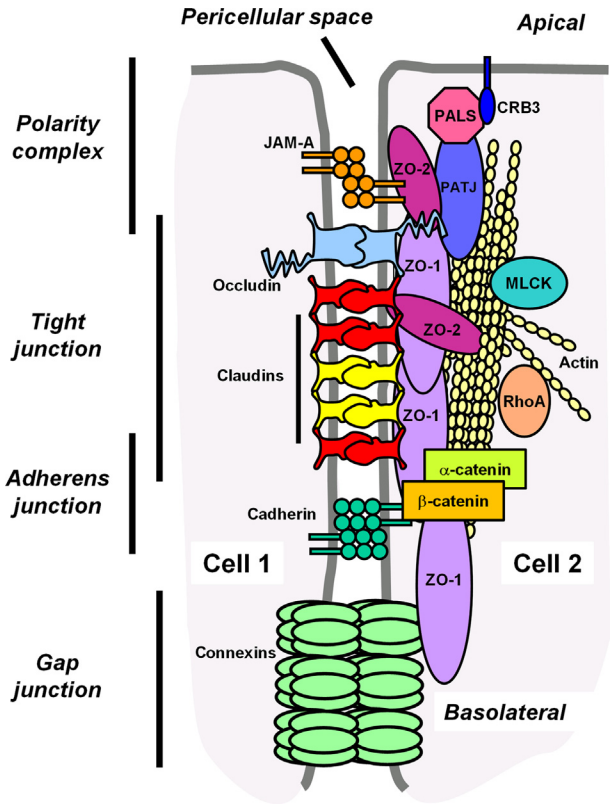

FIGURE 1.1 **The Apical Junctional Complex.** Shown are major functional zones of the Apical Junctional Complex (AJC), including the polarity complex, tight junctions, adherens junctions and gap junctions. A common theme for the structure of the AJC are layers of transmembrane proteins complexed to scaffold proteins, such as ZO-1 and ZO-2, that crosslink them to cortical actin cytoskeletal filaments. Transcellular stability of the junctions arises from interactions of proteins across the pericellular space. Cross regulation of scaffold proteins with different classes of junction proteins also provides structural cues that organize the AJC. Additional scaffold proteins are present in AJCs, but for simplicity are not shown here. Of particular relevance to the regulation of paracellular permeability are head-to-head interactions between claudins on adjacent cells that form paracellular channels. Gap junctions form channels that provide a pathway for transfer of cytosolic molecules from one cell to another. *Modified from Koval M. Structure and function of epithelial and endothelial barriers. In: Muro S, editor. Drug delivery across physiological barriers. Singapore: Pan Stanford Publishing; 2016. p. 3–40 with permission.*

or more broadly to other AJC proteins. Nonetheless, this reflects a common theme with respect to junctions, in that they frequently act as sensors reacting to intercellular contact that regulate genes by sequestering transcription factors in properly assembled AJCs.

1.3 ADHERENS JUNCTIONS

1.3.1 Cadherins

Along with the polarity complex, adherens junctions represent another lynchpin that is necessary for establishment of the AJC [16,17]. Adherens junctions mediate cell–cell contact through transmembrane proteins known as cadherins that bind to each other across the cell junction with high affinity in a calcium-dependent manner [18,19]. Epithelial (E)-cadherin is the canonical classical cadherin most prominently expressed by epithelial cells [20], including lung epithelium [21]. However, there are several other classical cadherin homologues expressed by lung epithelia, including N-cadherin that complements the function of E-cadherin [22].

In addition, the nonclassical cadherins, such as desmocollin and desmoglein, are a structurally distinct subset of cadherin homologues. Nonclassical cadherins form desmosomes, junctional structures distinct from that AJC that mediate a very strong form of intercellular binding and interconnect with intermediate filaments to protect epithelia from mechanical stress [23]. Atypical cadherins are a third group of cadherin homologues that generally are not expressed by lung epithelia [20]. The most broadly expressed atypical cadherin is vascular endothelial (VE) cadherin that is predominantly localized to the circulatory system [24].

1.3.2 Scaffold-Cytoskeletal Interactions

Cadherins recruit cytoplasmic scaffold proteins to adherens junctions, including α-catenin, β-catenin, and p120 catenin [20,25]. It is well established that β-catenin binds directly to the cytoplasmic terminus of E-cadherin [26]. Adherens junction scaffold proteins also facilitate an interaction with the actin cytoskeleton that requires the junction to be under tension [27]. Thus, the polarity complex and adherens junctions act as two cytoskeleton attachment sites to provide loci that orient and stabilize other elements of the AJC.

That α-catenin has the ability to directly interact both with the cadherin/β-catenin complex as well as bind to actin filaments has been interpreted as implicating a role for α-catenin in cross-linking adherens junctions and the cytoskeleton. Experiments showing that cadherin/α-catenin fusion proteins interact with actin support this model [28,29].

However, an indirect role for α-catenin in regulating cytoskeleton/adherens junction interactions has also been proposed and tested. In this model, recruitment of α-catenin to adherens junctions is followed by interactions with formin proteins that dissociate α-catenin from β-catenin to then provide a platform that binds to the arp2/3 complex that subsequently initiates actin filament formation and bundling [25,30]. Although this is not concordant with evidence demonstrating that cadherin/α-catenin fusion proteins can cross-link adherens junctions with the cytoskeleton [28,29], one possibility is that cadherin/scaffold protein chimeras do not reflect a native conformation of α-catenin and instead enable a nonphysiologic interaction with actin to occur [31]. However, different α-catenin isoforms have been shown to differ in the capacity to directly cross-link adherens junctions to filamentous actin [32]. Other scaffold proteins, such as EPLIN, can directly cross-link actin to adherens junctions [33]. Thus, a direct cross-linking role for α-catenin is not necessarily required to tether cadherins to the cytoskeleton. In addition, other functions of α-catenin, such as recruitment of tight junction scaffold proteins [e.g., zonula occludens 1 (ZO-1)], also help organize the AJC [34].

1.3.3 Cross-talk With Wnt Signaling

A common theme in lung injury is a reduction in E-cadherin [35–38]. One direct effect of this is to compromise lung epithelial integrity by disrupting the AJC [21]. Another aspect of decreased cadherin is to release β-catenin and other adherens junction scaffold proteins to perform other functions related to tissue repair. In other words, binding to adherens junctions serves to sequester β-catenin away from other cellular compartments as a regulatory mechanism. For example, β-catenin localized to the nucleus has been demonstrated to act as both a gene repressor and transcription factor [39,40]. There is accumulating evidence that other catenins can also directly regulate gene expression as well [41].

β-Catenin in particular has a well-defined role in regulating Wnt signaling, a secreted protein originally identified as a factor that promotes tissue differentiation in Drosophila since loss of Wnt leads to a wingless phenotype [26,42]. In the absence of Wnt, a kinase cascade organized on the frizzled/LRP5/6 coreceptor complex is activated that induces GSK3β phosphorylation of β-catenin. This, in turn, targets β-catenin to be proteolytically destroyed and unable to function as either a scaffold protein or as a transcription factor. However, when cell secreted Wnt binds to LRP5/6 co-receptors on neighboring cells, this inhibits β-catenin proteolysis. Instead, stabilized β-catenin transits to the nucleus where it regulates gene transcription.

In fact, the β-catenin/Wnt signaling axis is critical for lung development, patterning, and can influence lung epithelial repair [43–45]. While Wnt signaling is critical for normal lung development [46], Wnt signaling in repair from injury been shown to be deleterious, particularly in the context of lung fibrosis where Wnt signaling prevents proper epithelial repair in favor of mesenchyme proliferation, leading to lung scarring [47]. Aberrent Wnt activation may be a common feature of many airway diseases including chronic obstructive pulmonary disease (COPD) and asthma [48,49] suggesting that Wnt inhibitors might be a potential therapeutic approach to treat fibrosing lung disease [50,51]. Nonetheless, since Wnt activation is required to maintain the pool of basal airway stem cells required for airway repair, targeting this pathway needs to be considered with caution, since depleting basal cells would have a deleterious effect on lung repair [52].

1.4 TIGHT JUNCTIONS

1.4.1 Scaffold Proteins and Positioning in the AJC

Tight junctions form the physical barrier that regulates the movement of fluid, ions, and proteins through the paracellular space between cells [53–56]. There are two classes of tight junctions depending on whether they are areas where two cells are in contact (bicellular junctions) or are tricellular contacts where three cells come together (Fig. 1.2). Bicellular and tricellular tight junctions have unique structure and protein composition that confers upon them distinct

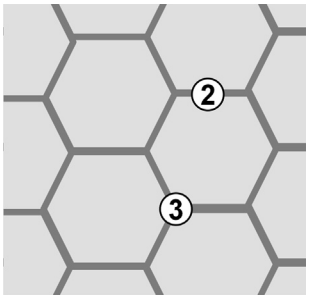

FIGURE 1.2 **Classes of epithelial cell−cell contacts.** There are predominantly two classes of cell−cell contacts in an epithelium, bicellular ② and tricellular contacts ③, where two or three cells, respectively, are in direct contact. The distinction between bicellular and tricellular junctions is most easily resolved in cuboidal and columnar epithelial monolayers. Other classes of epithelia, such as squamous type I alveolar epithelium, do not have clear cut distinctions between these types of junctions due to extensive overlap between extremely flat cells. *Modified from Koval M. Structure and function of epithelial and endothelial barriers. In: Muro S, editor. Drug delivery across physiological barriers. Singapore: Pan Stanford Publishing; 2016. p. 3−40 with permission.*

permeability characteristics [57]. By and large, tricellular junctions are significantly leakier than bicellular junctions, and they also are the most prominent route of leukocyte transmigration across epithelia [58−60]. Tricellular junctions are more prominent in cuboidal and columnar epithelia, the more typical architecture of the airways [46,60]. By contrast, the alveolar epithelium is predominantly squamous and tricellular junctions are less prominent [61]. Instead, alveolar cells show extensive areas of overlap that structurally promote barrier function in the terminal airspaces.

Like most other junctional complexes, tight junctions consist of a combination of transmembrane proteins linked to the cytoskeleton via cytosolic scaffold proteins. The main proteins that determine paracellular permeability are transmembrane claudin-family proteins that form bona-fide ion and water paracellular channels with different permeability characteristics [61−64]. Transport of macromolecules can also occur via an active process where tight junctions remodel to enable transient continuity breaks that allow fluid movement [54].

Tight junction positioning within the AJC is controlled by interactions among scaffold proteins. On the C terminus, almost all claudins have a PDZ binding motif [65,66] which binds to ZO-1, ZO-2, and other compatible scaffold proteins [67,68]. Cross-linking between tight junctions and the polarity complex is mediated by the PATJ scaffold protein [69]. Moreover, direct scaffold protein interactions between ZO-1 and α-catenin provide a direct physical link between adherens and tight junctions (Fig. 1.1).

ZO-1 and ZO-2 have overlapping roles to regulate interactions with the cytoskeleton by direct cross-linking to filamentous actin limiting recruitment of other contractile proteins such as myosin II, myosin light chain kinase (MLCK), and Rho kinase to the AJC [70,71]. MLCK and Rho Kinase activate myosin and actin, respectively to increase tension along the AJC, where the extent of tension is limited by junction associated ZO-1 and ZO-2 [72]. By contrast, aberrant activation of Rho kinase and/or MLCK cause barrier dysfunction by destabilizing the AJC and tight junctions in particular [70,73,74]. Since kinase-driven junction destabilization often occurs in inflammation, targeting Rho kinase and MLCK has been suggested as a therapeutic approach to treat inflammatory diseases, including acute lung injury [75−78].

Transcellular interactions between claudins are either individually weak [79] or require significant activation energy to break into the membrane bilayer in order to fully form [80−82]. However, adherens junctions form fairly readily and are a strong component of the AJC. Given this, adherens junction formation is required as a prerequisite for tight junctions to assemble [83−86]. However, once tight junctions are established, they are highly stable since the individual trans interactions are strengthened as a series of stacked beta sheet dimers cross-linked into a large oligomeric complex [54,82,87].

1.4.2 Claudins and Paracellular Permeability

Over a dozen different claudins have been found to be expressed by airway and alveolar epithelial cells at the mRNA level [88,89]. A broad range of claudin expression reflects diversity of function as well as redundancy in maintaining a critical barrier between the atmosphere and fluid-filled tissues. Claudin expression differs depending on which part of the respiratory tree is examined. For instance, claudin-18 is predominantly alveolar, whereas claudin-8 and claudin-10 are airway-specific claudins [61].

1.4.2.1 Claudin-18

The major claudin expressed in the lung is claudin-18 which is almost exclusively expressed by alveolar epithelial cells. There are two claudin-18 splice variants, where claudin-18.1 is the lung-specific isoform [90–93]. Although this suggests that claudin-18 is critical for lung barrier function, two different claudin-18 deficient mice showed a fairly mild phenotype with respect to lung barrier function [94,95]. There is also a suggestion that claudin-18 deficiency exacerbates the severity of asthma, perhaps through a decrease in airway barrier function [96]. However, since airway epithelial cells express very low levels of claudin-18 compared with alveolar epithelium, the precise mechanistic basis for a role of claudin-18 in lessening the severity of asthma remains to be determined.

Alveolar epithelial tight junctions in claudin-18 deficient mice have disrupted morphology [95] due to reorganization of AJC-associated actin into stress fibers perpendicular to the plane of the cell–cell interface [94] that are associated with increased paracellular flux [89,97–99]. Claudin-18 deficient mice also appear to have decreased P2X7 purinergic receptor expression [100], which may contribute to changes in actin reorganization [101]. This rearrangement of the cytoskeleton is undoubtedly linked to decreased barrier function.

Claudin-18 deficient mice actually have increased fluid clearance due to activation of several ion channels and also showed increases in expression of other lung-associated claudins [94]. The ability of cells to compensate for loss of claudin-18 underscores the redundancy in the mechanisms that regulate lung barrier function. Loss of claudin-18 significantly decreased levels of the early growth response-1 (Egr-1) transcription factor, suggesting one molecular basis for compensation through altered gene expression [102]. Consistent with this possibility, Egr-1 deficient mice are resistant to ventilator-induced lung injury [102] as are claudin-18 deficient mice [94].

In addition to forming a structural component of tight junctions, claudin-18 also regulates post natal lung development, since these mice have considerable type II cell hyperplasia and show evidence of type I cell damage [95]. The molecular basis for this role of claudin-18 is largely unknown at present, however, one potential clue is that claudin-18 expression by lung adenocarcinoma cells limits cell proliferation by modulating the Akt signaling pathway [103]. These observations support models where claudins can have roles beyond simply forming the structural basis for the paracellular barrier. For instance, claudin partitioning into cholesterol-enriched membrane microdomains outside of tight junctions could create functional subcompartments in a manner equivalent to tetraspanins, such as CD81, that form functional membrane domains regulating cell adhesion, migration, and other processes [104].

1.4.2.2 Claudin-4

Claudin-4 also is prominently expressed throughout the lung epithelium [105,106]. The role for claudin-4 in regulating paracellular barrier function has proven difficult to pinpoint, since it is sensitive to the presence of other tight junction proteins and has been found to form either a sodium barrier forming claudin [107,108] or a chloride pore forming claudin [109]. A role for claudin-4 in regulating alveolar epithelial barrier function has been demonstrated in studies correlating increased and decreased claudin-4 with increased and decreased barrier function [110,111].

In isolated perfused lungs from human patients with acute respiratory distress syndrome, claudin-4 levels correlate with increased fluid clearance [112] and are generally upregulated in response to lung injury [111,113]. Nonetheless, as observed for claudin-18 deficient mice, claudin-4 deficient mice also had a mild lung phenotype [113]. Claudin-4 deficient mice have only minor defects in steady state fluid balance, in part because of decreased Na^+/K^+-ATPase activity which decreases fluid transport into airspaces [113].

Nonetheless, claudin-4 deficient mice showed increased sensitivity to mechanical and hyperoxia-induced lung injury. Severely injured claudin-4 deficient mice have an enhanced inflammatory response, as evidenced by increased activation of TNFα and IL-1β [113]. Claudin-4 deficient mice also increased Egr-1 in response to severe injury [113], comparable to what has been observed in claudin-18 deficient mice [94,114]. These observations suggest an as yet unknown cross-talk pathway between claudin-4 and claudin-18 that may also involve other lung tight junction proteins.

1.4.2.3 Claudin-5

Claudin-5 is classically associated with the vascular endothelium [22,105,115] and, in fact, endothelial claudin-5 makes it difficult to detect claudin-5 expression by alveolar epithelial cells in histologic sections [22,92]. However, claudin-5 mRNA and protein have been easily detected in fetal human and rat alveolar epithelial cells [91,110,116–118]. Claudin-5 is also expressed by human airway epithelium [105,106,119].

Somewhat paradoxically, increased claudin-5 expression is associated with a decrease in alveolar epithelial barrier function [98,118,119]. The mechanism of action for this effect of claudin-5 comes from a molecular analysis of alcoholic lung syndrome.

It is well established that chronic alcohol abuse leads to increased susceptibility and severity of acute respiratory distress syndrome (ARDS) in ICU patients [120–123]. After adjusting for smoking and liver dysfunction, ICU patients with a history of alcohol abuse were more than twice as susceptible for ARDS than nonalcoholic patients [122]. In alcoholic lung syndrome, increased susceptibility to ARDS is due to impaired alveolar tight junctions, that is, a leaky lung phenotype [117,124,125].

In a rat model of chronic alcohol abuse, alveolar epithelial associated claudin-5 significantly increased [117]. Using cDNA and shRNA transfection of primary alveolar epithelial cells, it was found that claudin-5 is necessary and sufficient to impair alveolar epithelial barrier function [93]. Consistent with these findings, transfection of claudin-5 into airway cells also diminished transepithelial resistance [119]. Using in situ techniques to measure tight junction protein organization, including super-resolution fluorescence microscopy and the proximity ligation assay, it was found that claudin-5 impairs the ability of claudin-18 to interact with a scaffold protein, ZO-1, thus destabilizing tight junctions [93]. Critically, a claudin-5 extracellular loop peptide mimetic increased the barrier function of alveolar epithelial cells from alcohol fed rats. These data demonstrated that claudin controlled claudin-scaffold protein interactions underlie the decreased barrier function of the alveolus in alcoholic lung syndrome. Whether peptides that can antagonize the deleterious effects of claudin-5 can reverse lung leak in vivo remains to be determined.

1.4.2.4 Claudin-7

Claudin-7 is expressed throughout the respiratory tract during development and in the adult lung [105,106]. Defining roles for claudin-7 in lung epithelial physiology in vivo will require a tissue-specific knockout strategy, since claudin-7 deficient mice die within 10–14 days after birth [126]. Direct roles for claudin-7 in regulating lung epithelial barrier function remain to be fully elucidated, however, alveolar epithelial expression of claudin-7 is increased in response to epidermal growth factor (EGF), which correlates with a significant increase in barrier function [127,128]. It has also been observed that in the gut epithelium of claudin-7 deficient mice there is a significant increase in ENaC and Na^+/K^+-ATPase-$\alpha 1$ that compensates for the loss of claudin-7 by increasing sodium absorption [126]. Since increased sodium absorption leads to a general increase in fluid absorption, especially in lung epithelia [129–131], the ability of claudin-7 to regulate sodium channels provides a potential indirect mode for regulation of lung fluid balance in addition to a direct role in regulating tight junction permeability.

Claudin-7 deficient mice also showed significant inflammation of the gut due to increased expression and activity of matrix metalloprotease-3 (MMP-3) and MMP-7. MMPs are associated with increased severity of lung injury [132] and a role for claudin-7 in regulating lung inflammation is suggested by the observation that MMPs are activated in lung epithelial cells where claudin-7 was knocked down using siRNA [133]. How claudin-7 regulates MMPs is not known, however, claudin-7 localization is not limited to the AJC and instead is distributed throughout the basolateral plasma membrane where it influences formation of focal contacts between integrins and the extracellular matrix independently of its role in junction formation, again reminiscent of a role for claudins in forming functional membrane microdomains [134].

1.4.2.5 Claudin-3

Both airway and type II alveolar epithelial cells express claudin-3 [89,105,106]. By contrast, type I alveolar epithelial cells express fairly low levels of claudin-3. The differential expression of claudin-3 by type II vs type I cells implies that the molecular composition of type I–type II cell tight junctions will differ from type I–type I cell junctions [135]. Moreover, when primary rat type I alveolar epithelial cells are transduced to express claudin-3 levels equivalent to those found in type II cells, their barrier function decreases, suggesting that type II–type I cell junctions have unique permeability as compared with type I–type I cell junctions [110]. Specific roles for claudin-3 in lung barrier function, and whether it plays a unique role in type I–type II cell junctions, remains to be determined.

1.4.2.6 Claudin-8

Claudin-8 is specifically expressed by airway epithelium where it plays roles in fluid resorption [136,137]. In response to glucocorticoids, airway expression of claudin-8 is upregulated that, in turn, increases tight junction permeability to chloride [137]. This provides a paracellular pathway that augments chloride channels and can help promote fluid clearance by increased counterion transport associated with sodium channels and transporters [129].

Interestingly, claudin-8, along with claudin-1, is specifically down regulated in response to tobacco smoke exposure both in vitro and in vivo [136]. Although the acute effects of decreased claudin expression is to alter barrier function, these changes may also underlie changes in airway repair/ remodeling leading to tobacco-related diseases such as COPD. Consistent with this possibility, claudin-8 corecruits occludin, a Marvel domain (MarvelD) transmembrane protein, to tight junctions [137], which may be part of a feedback loop affecting processes such as cell proliferation [138]. Cross-talk between claudins and other tight junction transmembrane proteins is an active area of investigation.

1.4.3 MarvelD Proteins and Tricellular Junctions

MarvelD family proteins are the other major tight junction associated tetraspan transmembrane proteins. There are three MarvelD proteins: occludin, tricellulin, and MarvelD3 [57,139,140]. MarvelD proteins indirectly affect epithelial barrier function by recruiting scaffold proteins and kinases to tight junctions as part of the tight junction protein complex [141,142]. Occludin, tricellulin, and MarvelD3 also directly interact with claudins and may play roles in corecruiting proteins to microdomains as a step in tight junction assembly [143–145]. For instance, occludin binds to caveolin-1 and partitions into cholesterol-enriched membrane microdomains (or lipid rafts) associated with tight junctions [146–148].

By contrast with occludin, tricellulin localizes to tricellular contacts (Fig. 1.2) [149] although overexpression or compensation for loss of occludin can cause it to relocalize to bicellular junctions [57,139,150]. Tricellulin localization to tricellular junctions also depends upon a class of single pass transmembrane Ig-superfamily proteins first identified as lipolysis-stimulated lipoprotein receptors (LSRs), but subsequently renamed angulins [151]. Because of their unique composition, tricellular junctions are the predominant pathway for paracellular solute flux through cuboidal and columnar epithelia, as opposed to bicellular junctions which are the main site for paracellular ion flux [57].

Although there is little known about roles for tricellulin and MarvelD3 in lung epithelial function, loss of occludin occurs in lung injury and diseases attributable to lung barrier dysfunction [152–157]. Moreover, occludin initiates apoptosis when junctions become disrupted consistent with a role in cell regulation as opposed to forming a structural part of the paracellular barrier [141,142]. It seems likely that tricellulin and MarvelD3 will have comparable functions in regulating cell proliferation and apoptosis.

1.4.4 Junctional Adhesion Molecule A

Junctional adhesion molecule A (JAM-A) is another Ig-superfamily transmembrane protein that regulates tight junctions [158]. JAM-A interacts homotypically both by forming cis-dimers in the plane of the membrane as well as trans interactions between cells [159]. JAM-A recruits scaffold proteins to the AJC by binding directly to ZO-2, which nucleates a scaffold to recruit other proteins including Par3, afadin, and ZO-1 [160–162]. This is also a signaling nexus for a complex consisting of PDZ-GTP-exchange factor 1 (PDZ-GEF1) and the small molecular weight GTPase rap2c that act to regulate cytoskeletal turnover [162].

JAM-A deficient mice have defective barrier function in several epithelia including the gut [163] and the lung [164]. Specifically, JAM-A deficient mice are sensitized to mild endotoxemia. Interestingly, this was due to an impaired claudin-18 reassembly during recovery from endotoxin-induced injury. Decreased claudin-18 reassembly was downstream from enhanced disruption of ZO-1 and ZO-2 that occurred in the absence of JAM-A [164]. By contrast with claudin-18, claudin-4 is largely unaffected by loss of JAM-A both at baseline and in response to lung injury. These observations are consistent with a greater interaction between claudin-18 and the tight junction scaffold, as opposed to claudin-4 [110] and underscore the indirect role that JAM-A has in regulating tight junction formation [162].

1.5 GAP JUNCTIONS AND PANNEXIN CHANNELS

1.5.1 Connexins in Lung Epithelia

Gap junctions consist of arrays of channels composed of proteins known as connexins [165]. Gap junction channels serve to interconnect adjacent cells by creating gated high-conductance pores that enable the passive diffusion of small molecules, water, and ions from the cytoplasm of one cell to another [166]. As such, they provide a conduit for intercellular communication that enables individual cells to coordinate their physiologic activity.

Gap junctions serve several functional roles in the lung [167–171]. By electron microscopy, gap junctions are clearly delineated and found to be readily observable between lung epithelial cells [172]. In addition to gap junctions directly interconnecting epithelial cells, there are also heterotypic gap junctions between the epithelium and

endothelium that frequently also include contacts with mesenchymal fibroblasts [172] that can be disrupted in lung diseases such as COPD [173]. More recently, transient gap junctions between alveolar epithelial cells and macrophages [174] or between epithelia cells and mesenchymal stromal cells (MSCs) [175] have been found to modulate inflammation and promote epithelial repair following acute lung injury respectively (refer below).

There are nearly two dozen mammalian connexins [176,177], many of which are expressed by lung epithelia. Connexins selectively form either homomeric channels or heterotypic (mixed) channels with varying permeability [178]. This allows specificity of intercellular networking and also fine tunes channel permselectivity [179].

Several connexins are expressed throughout the lung depending on cell type and stage of differentiation. Immature airway epithelia initially express high levels of Cx26 and Cx43 that decrease upon cell differentiation. Instead, the main connexins expressed by differentiated human airway cells are Cx30, Cx30.3, Cx31, and Cx31.1 [180,181]. By contrast, Cx43 remains prominently expressed throughout the alveolar epithelium. Both type II and type I alveolar epithelial cells express Cx26, Cx43, and Cx46 [182]. However, Cx32 is expressed only by type II alveolar epithelial cells and not by type I cells and, more critically, type I cells are unable to form functional gap junctions with cells expressing only Cx32 [182]. Because type II cells can communicate via Cx32, it is likely to provide a "priority" channel for exclusive communication between type II cells.

Cx43-deficient mouse lungs are arrested in the pseudoglandular or canalicular stage of development, have branching defects and are subject to type II cell hyperplasia, suggesting a crucial role for Cx43 in lung development [183]. How Cx43 regulates lung development is not well elucidated, but is likely to involve control of Wnt signaling, which is regulated by Cx43 [184,185] and has been implicated in lung development and repair [52].

Cx43 deficiency in adjacent tissues can also have a deleterious effect on lung epithelial function. Double knockout mice that are globally deficient in Cx40 and also harboring a VE-specific Cx43 deficiency have spontaneous lung dysfunction, including increased fibrosis and aberrant alveolar remodeling [186]. The Cx40/Cx43 double knockout mice had decreased caveolin-1 and caveolin-2 content and, in fact resembled the phenotype of caveolin-1 deficient mice which had a similar lung phenotype [187,188]. Thus, expression of caveolin-1 may be linked to expression of Cx40 and endothelial Cx43 and vice versa. These data also suggest that functional communication between the vascular and pulmonary epithelial compartments is needed to maintain proper lung epithelial repair.

1.5.2 Regulation of Barrier Function

In addition to forming channels, the connexin C terminus can interact with scaffold proteins such as ZO-1 [189–191], much in the same manner as claudin–ZO-1 interactions described above. This raises the potential for gap junctions to act as a platform for scaffold proteins, signaling molecules and other transmembrane proteins, such as ion channels [192,193]. However in comparison with other elements of the AJC, such as tight junctions, the gap junction proteome is less well defined at present.

Even though connexins do not directly determine paracellular permeability, several lines of data suggest that connexins optimize barrier function. Cx26 has the ability to interact with occludin and thus contributes to tight junction formation in airway epithelial cells [194,195]. Also, the gap junction inhibitors glycyrrhetinic acid and oleamide have been found to decrease barrier function, albeit in rat lung endothelial cells [196].

Cx43 has been found to associate with occludin and claudin-5, as determined by coimmunoprecipitation analysis [196]. Since ZO-1 binds to Cx43 [197] and ZO-1 also binds to claudins and occludin [198], it seems likely that the association of Cx43 with transmembrane tight junction proteins is mediated by ZO-1 as opposed to a direct interaction, although this has not been rigorously tested.

Hepatocytes from Cx32-deficient mice have deficient barrier function that can be rescued by transfecting exogenous Cx32 [199]. Adding back Cx32 enhances ZO-1 and JAM-A localization to the AJC. Interestingly, Cx32 does not bind to ZO-1. On the other hand, Cx32 can interact with the polarity complex protein Discs Large homolog 1 [200] that could help recruit other tight junction proteins.

1.5.3 Pannexins

In addition to connexins, which form channels interconnecting cells, there is a more recently described family of high-conductance channel-forming proteins called pannexins [201,202]. The major substrate transmitted through pannexin channels is ATP released from the cytosol to the extracellular environment which can then stimulate other purinergic receptors to induce intercellular calcium current transmission in airway epithelial cells [203]. In fact, pannexin-1 (Panx1) is the pore forming component of P2X7 purniergic receptors [204]. Thus, ATP released by Panx1 could

amplify the response by stimulating P2X7 to promote additional ATP release. Panx1 and P2X7 receptors are found throughout lung epithelia, including airway epithelial cells [205−207], alveolar epithelial cells [100,208,209], alveolar macrophages [210], and neutrophils [211].

P2X7-deficient mice are resistant to inflammation, asthma, and pulmonary fibrosis, underscoring the importance of these channels in pulmonary disease [212−214]. In the case of inflammatory lung disease, depletion of P2X7 receptors attenuated secretion of proinflammatory hormones including IL-1β and IL-6 otherwise induced by hyperoxia [210].

By contrast, epithelial P2X7 and Panx1 are critical for fibrosis to develop following bleomycin-induced lung injury. Most likely this is due to the effect of ATP release as a "find me" homing signal targeting neutrophils and macrophages to cells undergoing apoptosis [215]. However, other roles for ATP release in exacerbating the epithelial injury response, including generation of proinflammatory cytokines by epithelia or inducing an epithelial-to-mesenchyme transition cannot be ruled out. Regardless, bronchoalveolar lavage fluid from patients with idiopathic pulmonary fibrosis has elevated ATP, suggesting a potential role for pannexins in the etiology of human disease [212].

1.6 PROCESSES REGULATED BY INTERCELLULAR COMMUNICATION

Calcium waves transmitted by the action of gap junctions and pannexins are required to regulate many processes central to lung physiology, including mucociliary clearance, surfactant secretion, and coordination of inflammation.

1.6.1 Mucociliary Clearance

The respiratory tract is continuously exposed to environmental insults. The physical barrier formed by the lung epithelium is one line of defense; however, in the absence of the ability to clear foreign substances, the lung would quickly become overwhelmed. Thus, the airway is lined with ciliated cells which flush particulates and other foreign substances up and out of the airways. Ciliary motion needs to be directional and coordinated in a calcium-dependent manner in order to effectively clear the airways [216,217]. Two transient calcium propagation pathways, which are not mutually exclusive, have been proposed to coordinate ciliary beating. In one model, inositol trisphosphate is intracellularly transmitted through gap junction channels to propagate release of intracellular calcium stores [218]. In a second model, ATP is released to the extracellular space which, in turn, stimulates purinergic receptors via a paracrine pathway to mobilize intracellular calcium in surrounding cells [203,219]. It is also possible that gap junctions and pannexin channels may be coordinately regulated, although how this occurs mechanistically is not well defined [220].

1.6.2 Pulmonary Surfactant Secretion

Type I alveolar epithelial cells are mechanical sensors that transmit calcium transients to type II cells *via* Cx43 gap junction channels [221,222] and by pannexin-mediated ATP release followed by mechanical stimulation or ligand binding to purinergic receptors [223] (Fig. 1.3). Alveolar calcium signaling also has the capacity to adjust surfactant production in response to changes in pulmonary blood pressure [225,226]. Interalveolar gap junctions can also transmit calcium signals [222]. Interalveolar signaling means that trauma to one alveolus, either through partial collapse or a disease state like pulmonary fibrosis can impair surfactant secretion by neighboring alveoli that might have normal morphology.

Increased calcium in the cytosol of stimulated type II cells promotes the fusion of surfactant containing organelles called lamellar bodies with the plasma membrane [227−229]. The major protein constituents of pulmonary surfactant are Surfactant Protein-A (SP-A), SP-B, SP-C, and SP-D. SP-B and SP-C are hydrophobic and directly contribute to the biophysical properties of surfactant [230,231]. By contrast, SP-A and SP-D are members of the collectin protein family and are largely hydrophilic and play key roles in regulating lung inflammation [232]. All of these components are copackaged into lamellar bodies that are then released into the airspace by stimulated type II cells.

The converse to having surfactant secretion regulated by intercellular communication is that impaired signaling will inhibit surfactant production and lamellar body secretion that, in turn, can compromise lung function [222,233]. Given pathologies where intercellular contacts and signaling are disrupted, including ARDS and ventilator-induced injury, it is likely that these conditions will cause surfactant abnormalities that exacerbate the course of the disease.

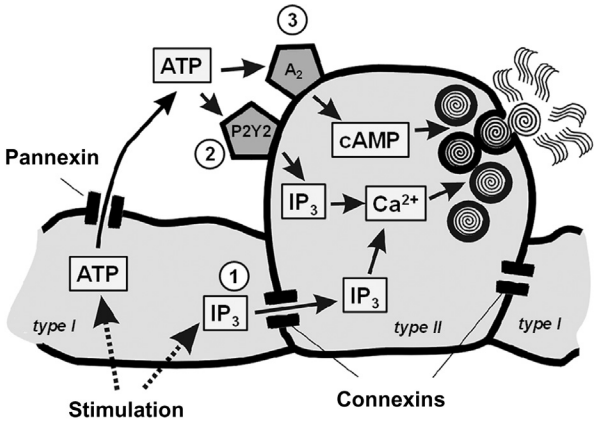

FIGURE 1.3 **Control of surfactant secretion by gap junctions and paracrine ATP secretion.** Depicted here are the gap junction ① and pannexin-mediated ②,③ pathways for intercellular signaling to induce calcium transients. Mechanical stimulation induced by ventilation is shown to generate inositol trisphosphate (IP3) that then diffuses through gap junctions from type I to type II cells to stimulate an increase in type II cell cytosolic calcium that promotes lamellar body fusion with the plasma membrane to cause surfactant secretion. As an alternative pathway, mechanical stimulation of type I cells can stimulate pannexin channels to release ATP that then activates P2Y2 purinergic receptors ② to induce a calcium wave. ATP can also activate protein kinase A through A2 purinergic receptors ③. Other potential receptors (e.g., β-adrenergic receptors), calcium and cAMP transmission through gap junctions, direct mechanical stimulation of type II cells, potential purinergic receptors on type I cells, and interactions between homologous cells are omitted from this diagram for simplicity. *Modified from Koval M. Sharing signals: connecting lung epithelial cells with gap junction channels. Am J Physiol Lung Cell Mol Physiol. 2002;283:L875–L893 with permission.*

1.6.3 Injury and Inflammation

Calcium signaling throughout lung epithelia also has many roles that regulate the severity and extent of the acute inflammatory response. This includes secretion of proinflammatory cytokines and chemotactic factors that recruit immune cells to the airspaces in response to acute lung injury [125]. However, defining roles for gap junctions and purinergic receptor signaling in regulating lung immunity has proven complex, since different systems can demonstrate apparently contradictory outcomes.

ATP release provides a chemotactic signal that attracts phagocytic cells to clear injured cells undergoing apoptosis, suggesting a role for the Panx1/P2X7 axis in inflammation [215]. ATP release by neutrophils may also contribute to this response [211,234]. Interestingly, P2X7-deficient mice are resistant to bleomycin-induced lung injury, show lower levels of proinflammatory cytokine production (e.g., IL-1β) and had significantly less fibrosis [212]. Panx1 inhibitors had a comparable protective effect in the bleomycin injury model as well as attenuating inflammation in response to either bacterial infection or tobacco smoke extract [235,236]. These findings suggest that modulating ATP release may provide a control point to reduce the severity of injury, although whether this will be an effective approach to reverse lung injury seems less likely.

With respect to gap junctions, Cx43 in particular has been implicated in mediating the spatial expansion of inflammation. For example, inhaled *Pseudomonas aeruginosa* activates Toll-like receptor 2 on the airway epithelial surface triggers calcium flux transmitted through gap junctions that, in turn, induces NFκB and MAP kinase signaling and ultimately the release of proinflammatory cytokines [237]. Cx43-mediated calcium waves help amplify signaling by inducing cells that are not in direct contact with a bacterial challenge to have a proinflammatory response [237,238]. This effect only lasts for a short time since gap junctional coupling is attenuated by Cx43 phosphorylation induced by factors such as bacterial lipopolysaccharide (LPS) and secreted TNFα from the inflammation cascade itself [237,239,240].

One implication of a feedback loop involving Cx43 and the immune response is that the inability to down-regulate Cx43 is likely to worsen the extent of inflammation. For instance, airway epithelial cells isolated from cystic fibrosis (CF) patients are unable to down-regulate gap junctional communication which no doubt contributes to pathologic inflammation in the CF lung [239,241,242]. On the other hand, the quorum sensing molecule produced by *Pseudomonas aeruginosa*, N-3-oxo-dodecanoyl-L-homoserine lactone, inhibits gap junctional coupling and also exacerbates bacterial infection [238]. One interpretation of these results taken together is that there needs to be an optimal level of intercellular communication. Too much or too little cell coupling can impair the immune response.

In addition to interconnecting alveolar epithelial cells, Cx43 also enables coupling between alveolar epithelial cells and resident alveolar macrophages [174]. In response to LPS, a calcium signal induced by TLR2 receptor activation in the alveolar macrophages is then transmitted through gap junctions to the epithelium that is not able to be mediated by Cx43-deficient macrophages. Calcium signaling from macrophages stimulates epithelial Akt phosphorylation as a means to modulate inflammation, including dampening secretion of proinflammatory cytokines and strengthening epithelial barrier function [243,244].

By contrast with resident macrophages, epithelial coupling with neutrophils has a proinflammatory effect [245]. Mice expressing a truncated form of Cx43 lacking most of the C-terminus (Cx43M257) show a pronounced increase in neutrophil recruitment in response to LPS instilled in the airway [245]. This is consistent with a role for intercellular communication in neutrophil recruitment, however, in the case of Cx43M257, this response causes excess inflammation since gap junctions containing Cx43M257 are hypercommunicative as compared with wild type Cx43 [246].

Moreover, connexin expression in the alveolus is altered in response to acute lung injury, where both Cx43 and Cx46 increase [182,247], while Cx40 expression decreases [248]. Since Cx46 has relatively limited permeability, one possible role for this increase in Cx46 is to form heteromeric gap junction channels with Cx43 as a means to fine tune gap junctional communication and/or intercellular networking [224,249].

There is considerable research demonstrating that bone marrow-derived MSCs have a protective effect in acute lung injury, leading to a clinical trial [250]. MSCs home to sites of injury where they secrete factors, such as angiopoietin II that promote tissue repair [251,252]. In addition, MSCs have the capacity to interact with injured lung epithelial cells in a Cx43-dependent manner [175]. MSC-epithelial interactions directly promote repair by mediating the transfer of healthy mitochondria from the MSCs to the injured, energy depleted epithelium. Mitochondrial transfer is not through the gap junction channel itself, rather it is mediated by nanotube-dependent pathway facilitated by Cx43-mediated cell−cell interactions [253,254]. MSC-mediated lung repair has considerable potential, not only in the treatment of acute lung injury but also with the possibility of being tailored to promote epithelial repair in other lung diseases.

1.7 SUMMARY AND PERSPECTIVES

As sites of direct contact between cells, intercellular junctions are critical in that they enable individual cells to function as a cohesive unit. Given the essential role of the lung to support respiratory metabolism through the exchange of oxygen for carbon dioxide, the ability of the lung epithelium to maintain a well-regulated air−liquid interface is of paramount importance. Tight junctions provide a key structural element that supports the air−liquid interface by directly regulating paracellular permeability. That tight junctions can withstand several dramatic alterations, including loss of lung-specific claudins, and still function highlights the redundancies and compensatory elements that help insure that lung barrier function is robust and well maintained. This also means that there are multiple proteins and signaling pathways with the potential to be targeted to improve epithelial barrier function in order to enable the lung to withstand or recover from injury.

Tight junctions do not act in isolation and require the infrastructure of the AJC in order to be properly formed and regulated. In fact, the function of each AJC element influences the others in both direct and indirect ways. For example, the ability of the scaffold protein ZO-1 to directly interact with different proteins in tight junctions, gap junctions, adherens junctions, and the polarity complex means that the pool of cellular ZO-1 has multiple simultaneous functions that may be difficult to study in isolation. Other scaffold proteins are likely to provide a similar challenge. Indirect regulation of tight junctions by adherens junctions in mediating high-affinity intercellular binding and by gap junctions by enabling intercellular communication also is difficult to define because interactions between proteins at cell-cell contact sites are complex and sensitive to the phenotype and state of the entire population of cells throughout the epithelial monolayer and not just individual cells.

In addition to serving direct structural functions in epithelia, the AJC also acts as a cell signaling platform that is sensitive to direct cell−cell contact. As mentioned above, the ability of several transcription factors, including Yap and catenins, to shuttle between the AJC and nucleus provides a direct link between intercellular junctions and regulation of gene expression. This function acts, in essence, as a sensor for epithelial integrity. This is particularly important in coordinating epithelial repair in response to tissue damage that, when improperly regulated, can lead to pathologies such as pulmonary fibrosis.

In addition, it is becoming apparent that several classes of transmembrane proteins associated with intercellular junctions can also serve other independent roles that regulate cell function. Of particular note are nonjunctional pools of claudins that appear to influence cell proliferation and differentiation. Defining nonjunctional roles for claudins and other components of the AJC is a major challenge, since they are likely to depend upon their ability to specifically partition and create membrane microdomains as well as simultaneously interact with multiple cytosolic proteins

as well as the cytoskeleton. How the junctional and nonjunctional roles for these proteins are coordinately regulated is also not well elucidated at present. As opposed to being truly independent functions of the same protein, it is more likely that the ability of claudins and other junctional transmembrane proteins to have nonjunctional roles provides a mechanism where remodeling of intercellular contacts acts as a sensor that can influence cell proliferation, phenotype, and other functions.

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Chapter 2

Ion Transport and Lung Fluid Balance

Charles Downs

2.1 INTRODUCTION

The ability to effectively exchange oxygen for carbon dioxide between the blood-gas barrier depends in large part on precise regulation of lung fluid balance. In addition, effective mucociliary function is dependent upon precise regulation of lung fluid balance in the airways. All lung (including nasal) epithelia have the capacity to transport ions. Indeed lung epithelia actively alter secretion or reabsorption of ions to affect lung fluid balance that directly affects mucociliary clearance and/or oxygenation. Therefore, appropriate ion transport and lung fluid balance are necessary for lung function. This chapter will focus on the mechanisms regulating ion transport in lung epithelia and Na^+ transport in particular, altering net fluid transport in the lung. The loss of physiological transport properties in lung epithelia can lead to severe pulmonary disorders such as chronic bronchitis, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and acute respiratory distress syndrome (ARDS).

2.1.1 Overview of the Structure and Function of the Lung Epithelium

The lung can be grossly divided into two major anatomical categories, airways and alveoli. The airways consist of conducting and respiratory airways; they are primarily responsible for the movement of air into and out of the alveoli where gas exchange occurs. Epithelial cells line the surfaces of the airways and the alveoli. In general, the epithelium of the airways is formed in layers, and as such, epithelial cells play a functional role in secretion, selective absorption, transcellular transport, and sensing. In the alveoli, a single layer of epithelial cells cover the alveolar surface and these cells function to facilitate the diffusion of gases, as well as play a critical role in secretory, absorptive, and excretory functions.

Epithelial tissues are designed to form a barrier between two compartments, thereby regulating the composition of the compartments. Lung epithelia are unique because the epithelium separates the internal vascular compartment from an air-filled compartment. As such, the primary physiological purpose of the alveolar epithelium is to facilitate the exchange of oxygen from the airspace into the blood and the removal of CO_2 from the blood into the airspace for exhalation. In the lung, a thin layer of antioxidant rich fluid serves to protect the epithelium from damage. In the airways, maintenance of the height and volume of the liquid is crucial for appropriate mucociliary clearance, and in the alveoli lung fluid balance is tightly regulated to allow effective gas exchange. Indeed, acute flooding of the alveoli with edematous fluid severely impairs gas exchange resulting in a medical emergency requiring urgent intervention.

2.1.2 Overview of Transbarrier Ion and Fluid Transport

The rate of lung fluid absorption is dependent upon ion transport, especially Na^+ transport, across tight epithelia. Fig. 2.1 provides a general schematic for ion and water transport across tight epithelia that serve to maintain lung fluid balance. Salt and water transport across lung epithelia can be divided into four distinct steps. The first step occurs at the apical membrane; lung epithelia are highly permeable to Na^+, with the basolateral membrane conferring high permeability to K^+. Reabsorption of Na^+ at the apical membrane increases intracellular Na^+ concentrations. The electrogenic Na^+/K^+-ATPase pump recycles K^+ and maintain low intracellular Na^+ content needed for the movement of cations across the inner and outer membrane. The epithelial sodium channel (ENaC) is apically located and as such is regarded

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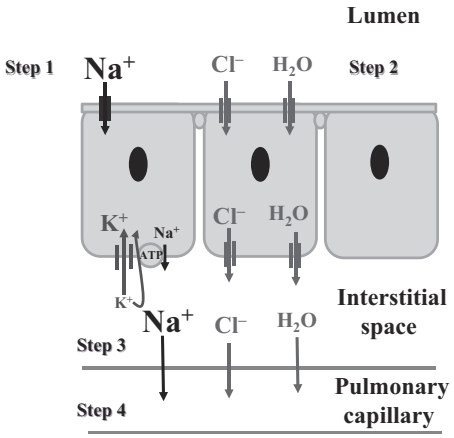

FIGURE 2.1 Four step schematic of net salt and water movement in tight epithelia. Apical lung epithelia are highly permeable to Na^+ and the basolateral membrane is highly permeable to K^+ (Step 1). The basolaterally located Na^+/K^+ ATPase extrudes Na^+ and recycles K^+ to maintain low intracellular Na^+ necessary for cation movement across the plasma membrane. The amiloride-sensitive epithelial sodium channels (ENaC) are located in the apical membrane and activation of these channels is the rate-limiting step in tranepithelial transport. Anions, such as chloride (Cl^-) accompany net Na^+ movement (Step 2). The accumulation of cation and anions in the interstitium creates an osmotic gradient facilitating water movement (Step 3). Starling's forces promote bulk flow of water and salt into the capillary (Step 4).

as the rate-limiting step in transbarrier ion transport. In the second step, anions accompany net Na^+ movement to maintain electroneutrailty. In the third step, anions and cations accumulate in the interstitial space producing an osmotic gradient that drives fluid movement from the luminal space into the interstitium. Finally, in step four, Starling's forces promote water and salt movement into the pulmonary capillary.

2.1.3 Bioelectric Properties of the Lung Epithelium

2.1.3.1 Epithelial Sodium Channel

The ENaC is a member of the Degenerin family of ion channels and is expressed in the apical plasma membrane of several tissues including the colon, kidney, and lung, and represents the rate-limiting step for Na^+ absorption. Classically, ENaC consist of three separate subunits (α-, β-, and γ-ENaC) that are ∼30% homologous to each other. These subunits form a highly selective cation (HSC) channel that favors Na^+ over K^+ (e.g., >40:1) and is sensitive to amiloride in the submicromolar range [1]. HSCs have smaller conductances (∼4−6 pS) and are made up of all three subunits, and are responsible for the unidirectional reabsorption of Na^+ ions from the airway lumen. There are also nonselective cation (NSC) channel isoforms of ENaC. NSC−ENaC channels have larger conductances due to near equal selectivity for Na^+ over K^+ (Na^+/K^+ 1.1:1). Functionally, NSCs serve to maintain airway fluid homeostasis and HSC serve to rapidly resolve edema. However, the molecular identity of HSC and NSC channels remain unclear.

Each ENaC subunit is composed of a large extracellular loop, two transmembrane domains (termed M1 and M2, respectively) and two short intracellular tails. Site-directed mutagenesis shows that pre-M2 region of α-, β-, or γ-ENaC subunits affect amiloride binding and channel conductance [2]. ENaC stoichiometry has been debated, but based on crystal structure of the related acid-sensing channel it appears that ENaC consists of a 1α:1β:1γ complex [3]. In over expression models, α-ENaC knockout mice die within 20 h of birth due to an inability to remove lung fluid [4]. Moreover, normalized expression of all classical ENaC subunits in human respiratory epithelial cells (including trachea, bronchi, and parenchyma) indicates significantly higher levels of α-ENaC subunit expression [5].

In humans, a fourth subunit (δ-ENaC) has been described. δ-ENaC is highly expressed in the testis, ovary, pancreas, brain, and nasal epithelium. The δ-ENaC subunit shares considerable sequence similarity with α-ENaC subunit and forms functional ion channels with β- and γ-ENaC subunits in the pancreas, testes, ovaries, and brain and in smaller amounts in the heart, placenta, lung, liver, kidney, thymus, prostate, colon, and lymphocytes [6−10]. Over expression models show that δ-, β-, and γ-ENaC produce nearly a 10 fold increase in amiloride-sensitive current compared to α-, β-, and γ-ENaC. Therefore, differential expression of α- and δ-ENaC subunits may provide additional pathways to affect ENaC activity [11]. There are two different δ-ENaC isoforms (δ1 and δ2) that have been characterized in human epithelia.

ENaC is the major ion channel responsible for apical Na^+ entry into lung epithelial cells, and as such ENaC is regulated by a wide variety of proteases and second messengers. Proteases include Furin-type proteases and a several serine proteases. Furin-type proteases are Ca^{2+}-dependent convertases that process precursor proteins into biologically active forms. Furin is predominantly located in the Golgi apparatus; however, the furin family of convertases have been found at the cell surface suggesting that ENaC could be cleaved in or near the plasma membrane [12]. Serine proteases possess a serine at their active site, and several types exist within the serine protease family: glycocyphosphatidylinositol-anchored proteases, transmembrane serine proteases, and soluble proteases, such as trypsin and neutrophil elastase. Collectively the serine proteases cleave one or more ENaC subunits to facilitate activation of the channel. The electrophysiological manifestation of ENaC cleavage is observed as an effect on the open probability (P_o), or length of time the channel is open.

A wide range of second messengers and agonists can regulate ENaC activity; however, unlike the proteases, second messengers may also affect the number of ENaC channels in the plasma membrane (n) as well as their P_o. Second messengers include purinergic amines, cyclic adenosine monophosphate (cAMP), reactive oxygen species (ROS), shear stress, CF transmembrane conductance regulator (CFTR) channels, hormones, cytokines, and steroids [13–26]. The multitude of agonists can affect ENaC function under physiological or pathological conditions. Indeed in clinical situations ENaC activity is purposefully manipulated through pharmacological interventions such as inhaled steroids and beta-adrenergic agonists to treat underlying pathology and its associated symptoms.

ENaC in the lung is distinguished from ENaC in other tissues by insensitivity to aldosterone [27]. In the airways, ENaC expression is regulated by glucocorticoids, as such its expression is influenced by changes in inflammation rather than changes in blood volume [28,29]. This has significant implications for our understanding of lung fluid balance in pulmonary diseases, as well as systemic diseases that also affect the lung, in which a variety of cytokines and ROS may be produced.

2.1.3.2 Other Sodium Transporters, Na^+/K^+-ATPase

Although the amiloride-sensitive ENaC is the rate-limiting step in lung fluid balance, and ENaC has been characterized in human airway epithelium [30], other Na^+ channels and pumps are present in lung epithelia. ENaC is the major ion channel involved in the absorption of apical Na^+; however, a nonselective cyclic nucleotide-gated (CNG) cation channel also contributes to apical Na^+ absorption in rat airway epithelium (trachea, bronchi, and bronchioles) [31]. Na^+ is extruded via the basolaterally located Na^+/K^+-ATPase that is ubiquitously expressed in airway epithelia and provides the driving force for apical Na^+ absorption [32,33]. Basolateral Na^+/K^+-ATPase are antiporter enzymes that play an active role in transbarrier transport of Na^+ across lung epithelia. The Na^+/K^+-ATPase pump is composed of α and β subunits that form a heterodimer in the plasma membrane to extrude 3 Na^+ ions in exchange for 2 K^+ ions. The exchange of ions requires α-subunit ATP-coupling, while the β-subunit is required for effective assembly and insertion into the plasma membrane. Na^+/K^+-ATPase play a critical role in resting membrane potential, controlling cell volume, and they serve as important signal transducers.

2.1.3.3 Chloride Channels

The CFTR is the major chloride secreting channel in the human airway, and CFTR is made up of two, six-span membranes-bound regions, each connected to a nuclear binding factor that binds ATP [34]. Cl^- secretion opposes Na^+ absorption to facilitate electroneutrality in which net cation and anion charges are equal. CFTR has been proposed to regulate ENaC, and in CFTR deficient epithelia, as seen in cystic fibrosis, there is accompanying hyperabsorption of Na^+.

Additional Cl^- channels have been characterized in the airway epithelium, including Ca^{2+}-activated chloride channels (CaCCs). CaCCs are present on the apical surface and appear to contribute to Cl^- secretion, although their contribution is modest. Basolaterally located Cl^- channels have been identified in human tracheal epithelium and nasal ciliated epithelial cells. These Cl^- channels appear to be involved in transcellular Cl^- transport processes and in the regulation of apical Cl^- secretion [35,36]. Several chloride cotransporters have been characterized in airway epithelia that function to maintain electroneutrality [$Na^+/K^+/2Cl^-$ (NKCC)] or modulate intracellular pH and Cl^- secretion (HCO_3^-/Cl^- exchanger) [37–39].

2.1.3.4 Potassium Channels

Numerous K^+ channels have been characterized in the human airway and alveolar epithelium. In Cl^- secreting cells, K^+ efflux through basolateral K^+ channels is essential in preventing depolarization and in maintaining the

electrochemical driving force for continued Cl^- secretion. Apically located K^+ channels influence the apical resting membrane potential through contributing to high K^+ concentrations in the airway surface liquid (ASL). K^+ channel taxonomy is based on structure (number of transmembrane domains), method of activation (e.g., voltage-gated), and conductance properties. These K^+ channels are important for regulating epithelial membrane potential and in maintaining the electrochemical gradient for apical Cl^- secretion.

2.1.3.5 *Aquaporins*

The creation of an osmotic gradient through unidirectional transport of Na^+ and Cl^-, as illustrated in Fig. 2.1, drives the movement of water. Aquaporins provide the conduit through which water may travel, and there are 13 known types. Aquaporins are made up of six transmembrane α-helices with amino and carboxy termini located intracellularly. Aquaporins form tetramers in the cell membrane, and an aromatic /arginine selectivity filter selectively allows water molecules to pass while preventing other molecules from entering the pore. The alveolar epithelium is highly water permeable due to its robust aquaporin 5 expression.

2.2 LUNG FLUID BALANCE IN THE AIRWAYS

Appropriate lung fluid balance is required for a healthy lung. A schematic demonstrating the ion channels, pumps and pores involved in maintenance of airway lung fluid balance is provided in Fig. 2.2. Effective innate immune defenses are important to maintain a healthy lung, because the lung is continuously exposed to air-borne particles and various microorganisms. Mucociliary clearance is a part of the innate immune system that functions to cleanse the airways of inhaled pathogens and particles and is vital for a healthy lung. Mucociliary clearance functions are mainly dependent upon ciliary beat and ion transport, and many cell types have been described in the airway epithelium. Ciliated cells contain cilia that are responsible for the transport of inhaled particles and the mucous layer towards the mouth by the direction of their beating cilia. Clara cells and goblet cells secrete ions, mucus, surfactant, and immunoprotective proteins. Basal cells are undifferentiated and serve as stem cells for other airway epithelial cell types such as the ciliated cell. Cell composition along the airway epithelium varies within and between the conducting and respiratory airways,

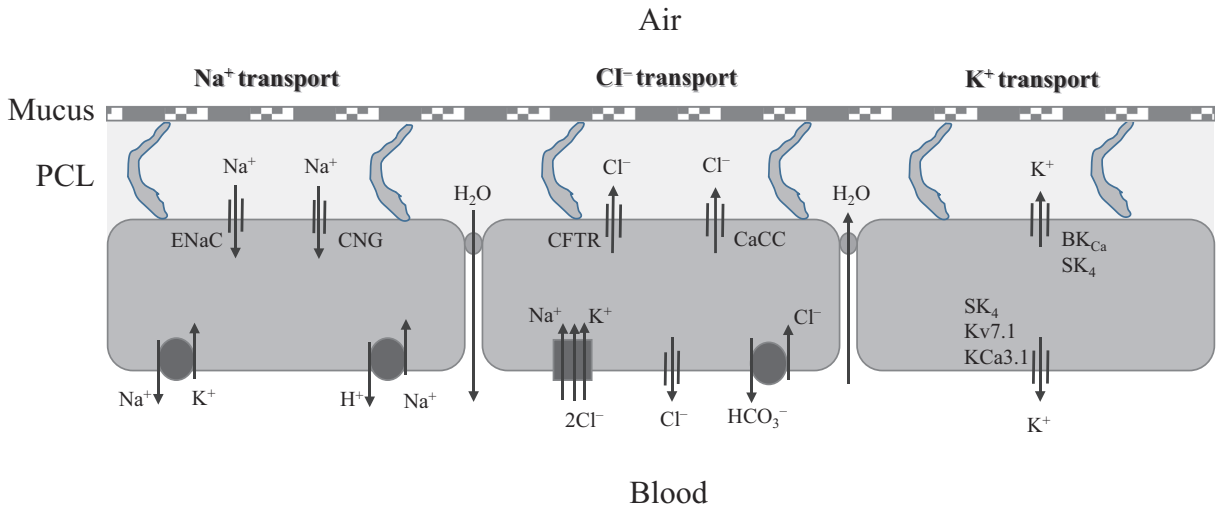

FIGURE 2.2 Schematic of Na^+, Cl^-, and K^+ channels and transporters in ciliated airway epithelium. The apical surface is covered by a periciliary liquid (PCL) that surrounds the cilia and a layer of mucus that traps debris and pathogens. Ciliary beat from cliliated epithelial cells move the debris and pathogens towards the mouth for removal. Volume of the PCL is predominantly regulated by apical Na^+ absorption and Cl^- secretion. Transepithelial Na^+ transport occurs at the apical membrane, primarily through the epithelial sodium channel (ENaC), although apical cyclic nucleotide-gated channels (CNG) might also contribute to Na^+ absorption. Intracellular Na^+ and K^+ concentrations are maintained through the Na^+/K^+ ATPase. Intracellular pH is also regulated through the basolaterally located H^+/HCO_3^- exchanger. Apical Cl^- secretion is mainly mediated through the cAMP-dependent cystic fibrosis transmembrane conductance regulator (CFTR) and to a lesser extent by Ca^{2+}-dependent chloride channels (CaCC). Chloride secretion is further facilitated by $Na^+/K^+/2Cl^-$ cotransporter and the HCO_3^- exchanger; several basolaterally located Cl^- channels have been characterized and are involved in modulation of apical Cl^- secretion. Several K^+ channels, including voltage-dependent (Kv7.1−Kv7.5) and Ca^{2+}-dependent (SK_4, BK_{Ca}, $K_{Ca}3.1$), have been characterized in the apical and basolateral membranes. K^+ channels regulate K^+ concentration in the PCL as well as apical Cl^- secretion.

the frequency of ciliated cells increases progressively towards the periphery, the number of basal cells decreases progressively more distally, and nonciliated cells are also unequally distributed [40].

The ciliated cells occupy an essential role in mucociliary clearance because of their cilia and the ion channels they express. Airway epithelial cells control transepithelial water flow, and play a crucial role in precisely regulating the composition of the periciliary liquid (PCL) surrounding the cilia that is needed for optimal ciliary beat. The PCL together with the mucous layer forms the airway surface liquid (ASL). Maintenance of the height and composition of the ASL is crucial for effective immune function (mucociliary clearance) as the mucous layer with all its trapped particles and pathogens is transported towards the mouth by the ciliary beat. Impaired ciliary beat due to impairment in its regulation results in severe respiratory infections. In addition, the ASL of the conducting airways represents an important component of the innate immunity because it contains immunoreactive proteins, such as the Clara cell secretory protein as well as the surfactant proteins-A (SP-A) and D (SP-D) that are secreted by airway epithelial cells. Clara cell protein is thought to have immunomodulatory functions, and SP-A and SP-D appear to provide a protective role against respiratory pathogens.

The composition and height of the ASL are regulated through coordinated activity of ion channels, pumps and pores. PCL composition is mainly regulated by apical Na^+ absorption through ENaC and concerted activity of the basolaterally located Na^+/K^+-ATPase, as well as Cl^- secretion involving apically located Cl^- channels. CF is the direct effect of a malfunction in the CTFR channel, an apically located cAMP-dependent Cl^- channel that secretes Cl^- into the airway lumen that functions to maintain electroneutrality against Na^+ absorption by the ENaC.

2.2.1 Regulation of Lung Fluid Balance in the Airways

Regulation of the airway epithelial ion channels involved in lung fluid balance can be modulated by a variety of signaling molecules that interact directly with ion channels or bind to epithelial cell receptors that then modulate ion transport via second messengers after activating intracellular signaling cascades. Increasing intracellular Ca^{2+} or cAMP is known to affect ion channel activity. For example, β-adrenergic agonists binding to their β-adrenergic receptor on airway epithelial cells increases cAMP which then activates ENaC to absorb Na^+ [13]; Ca^{2+}-activated Cl^- and K^+ channels, as well as CFTR are regulated by cAMP [41,42].

ROS have been shown to activate a variety of airway epithelial ion channels. ROS are produced under physiological and pathological conditions. For example, superoxide (O_2^-) is generated during cellular respiration, as well as from activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase and NOX) during phagocytosis, cell differentiation, and proliferation. O_2^- has been shown to increase activity of the ENaC in mouse and rat lung [25]. Excessive ROS production has been observed in the airways of persons with CF, smokers, and those with COPD. However, the mechanisms by which ROS influence airway epithelial ion transport are not fully understood.

Purinergic receptor agonists, such as adenosine triphosphate (ATP) and uridine triphosphate (UTP), have been shown to modulate ion channel activity in the airway epithelium. Purinergic receptor agonists act as secretagogues when present in the extracellular space. UTP and ATP activate Cl^- channels. More specifically, in human nasal epithelium, UTP-mediated apical Cl^- secretion has been observed. In mouse tracheal epithelium, ATP (as well as UTP) induces transient Cl^- secretion and sustained inhibition of Na^+ absorption.

2.3 ALTERED LUNG FLUID BALANCE AND DISEASE OF THE AIRWAYS

The importance of ion transport and lung fluid balance in the airway is evident in the pathological features of airway diseases such as CF and COPD. In these disease processes, specific derangements in ion transport affect lung fluid balance and, ultimately, lung function.

2.3.1 Cystic Fibrosis

CF is a heritable, autosomal recessive disorder involving the CFTR gene that encodes for the Cl^- channel necessary for Cl^- secretion to maintain electroneutrality and appropriate lung fluid balance. CFTR also regulates ENaC activity, and when CFTR is absent or ineffective, ENaC becomes over active leading to hyperabsorption of Na^+. The net effect is the dehydration of the PCL and the development of thick, tenacious mucous—that is, an alteration in the lung's innate immunity. In short, altered CFTR function promotes the development of thick, viscous mucous that is difficult to expectorate through impaired regulation of apical Na^+ channels. Impaired innate immunity in the CF lung then allows for bacterial colonization with pathogenic bacteria.

CFTR function is controlled through a variety of regulators. The purinergic amine ATP promotes the recruitment and activation of CFTR to ultimately affect epithelial cell volume. ATP also affects CFTR gating properties by direct interaction with CFTRs cytoplasmic nucleotide binding sites [31,34]. Free radicals affect CFTR function, and it is appreciated that free radicals and oxidative stress, an imbalance between oxidants and antioxidants that results in a loss of redox control, are important moderators in lung disease pathogenesis. In CF, impaired nitric oxide (NO) defense renders airway epithelial cells more susceptible to bacterial and viral pathogens. NO activates CFTR and downregulates amiloride-sensitive ENaC. Moreover, oxidative stress promotes internalization of CFTR and decreases CFTR activity; conversely, oxidative stress appears to promote retention of ENaC in the plasma membrane [14,17,43–45]. CF exacerbations are often caused by respiratory infections. Infection promotes the migration of macrophages and recruits neutrophils into the airways; activated immune cells produce oxidants and exacerbate oxidative stress; thereby further affecting CFTR function.

2.3.2 Chronic Obstructive Pulmonary Disease

COPD is a heterogeneous chronic lung disease characterized by airflow limitation that is not fully reversible. A direct, causal link between altered transbarrier transport and the development of COPD is lacking. However, evidence suggests that ion channels may play a role in disease development. For example, in a murine model in which β-ENaC was overexpressed, mice spontaneously developed chronic bronchitis and emphysema. This observation suggests that hyperabsorption of Na^+ may precede classical lung function changes seen in COPD pathogenesis.

Oxidative stress and inflammation are key features of COPD. A special consideration with COPD is cigarette smoke, the etiologic agent. Cigarette smoke is a highly heterogeneous mixture of over 4000 constituents and contains many free radicals. Cigarette smoke induces airway inflammation and promotes free radical production, as well as the recruitment of immune cells into the airways. These immune cells produce ROS and a variety of cytokines which can affect transbarrier transport in the airway epithelium.

2.4 LUNG FLUID BALANCE IN THE ALVEOLI

In the alveoli, appropriate lung fluid balance is crucial for optimal physiologic function of the lung. As such, alterations in lung fluid balance in the alveoli produces profound alterations in lung function and require immediate medical attention.

The alveolar capillary membrane (ACM) is composed of alveolar epithelial cells that share a basement membrane, and at times fuse with microvascular endothelial cells. The alveolar epithelium is composed of alveolar epithelial type 1 (T1) and type 2 (T2) cells. T1 cells are large, thin squamous epithelial cells that cover 95%–95% of the ACM. T1 cells contain a full complement of ion channels, pumps, and pores. Indeed T1 cells are highly water permeable and play a critical role in the resolution of edema. T2 cells are cuboidal, more numerous than T1 cells and cover $<5\%$ of alveolar surface area. T2 cells also produce surfactant. Microvascular endothelial cells line the capillaries. The ACM is exquisitely thin (0.1–0.2 μm) affording a short gas diffusion distance in order to facilitate gas exchange.

During fetal development, tight junctions form between T1 and T2 cells to provide a barrier between the internal vascular compartment and a fluid-lined airspace. The healthy alveolar epithelium is virtually impermeable to proteins and solutes. Consequently, passage of molecules, fluid, and ions rely on active transport across alveolar epithelial cells by diffusion or active transport to transverse the alveolar epithelium. Therefore, tight junctions play a critical role in the transepithelial paracellular transport.

A thin layer of antioxidant rich fluid lines the alveolar epithelium termed the epithelial lining fluid (ELF) that functions to protect the alveolar epithelium from oxidants. Indeed glutathione is present in micromolar concentrations in the ELF. The amount of fluid on the airway surface represents a balance between the rate at which fluid passively secreted from the vascular space through the paracellular space and tight junctions and the rate at which fluid actively absorbed. Fluid appears in the airways because of the passive movement of fluid driven by hydrostatic pressure out of the pulmonary capillaries across the airway epithelium. The amount secreted is relatively constants because, under normal physiological circumstances, the pulmonary capillary pressure and the permeability of the epithelium are relatively constant. However, under pathological conditions, when either the pulmonary blood pressure is elevated of the permeability of the epithelium is increased by inflammation or disease, passive secretion may increase dramatically. Regardless, even under normal levels of secretion, the alveoli would rapidly fill with fluid were it not for fluid absorption by the alveolar epithelium. Because fluid absorption depends upon the rate of ion transport, regulation of ion transport provides the mechanism by which the amount of airway fluid is regulated.

2.4.1 Regulation of Lung Fluid Balance in the Alveoli

The ability of the alveolar epithelium to mediate alveolar transepithelial transport is very important for the regulation of the ELF, in order to facilitate effective gas exchange. Fig. 2.3 provides an overview of the alveolus and the key ion channels, pumps and pores involved in the regulation of lung fluid balance.

Much like the upper airways, the major driving force for fluid reabsorption in the alveolar epithelium is predominantly provided by the apically located amiloride-sensitive ENaC. Sodium follows the electrochemical gradient that is maintained by the Na^+/K^+-ATPase. This process generates a transepithelial osmotic gradient, which facilitates the osmotic removal of water out of the alveoli into the interstitium. Ion transport across the alveolar epithelium occurs through ion transporting proteins present in alveolar T1 and T2 cells.

Chloride channels (CLC) are present in alveolar epithelial cells, albeit their role in alveolar lung fluid balance is unclear. CFTR and different types of voltage-gated CLC have been described in the alveolar epithelium. Transepithelial Cl^- transporters such as NKCC, K^+-Cl^- cotransporters, and HCO_3^-/Cl^- exchangers have been identified in the alveolar epithelium.

Potassium channels are also present in the alveolar epithelium. K^+ channels function to control the membrane potential, and thus, to maintain the electrochemical gradient that is required for ion and fluid transport. A variety of K^+ channels have been described in the alveolar epithelium, for a review please see Ref. [46].

Alveolar epithelial T1 and T2 cells express a full complement of ion transporting proteins to facilitate transepithelial ion and fluid transport. Key ion transporter proteins in the alveoli include the apically located ENaC, the Na^+/K^+-ATPase, a variety of Cl^- and K^+ channels, as well as aquaporin channels. Regulation of the alveolar epithelial transport proteins can be modulated by a variety of signaling molecules that act directly with the transport proteins or bind to epithelial cell receptors that modulate ion transport via second messengers generated after activating intracellular signaling cascades. These processes are identical to those discussed with airway epithelial cells.

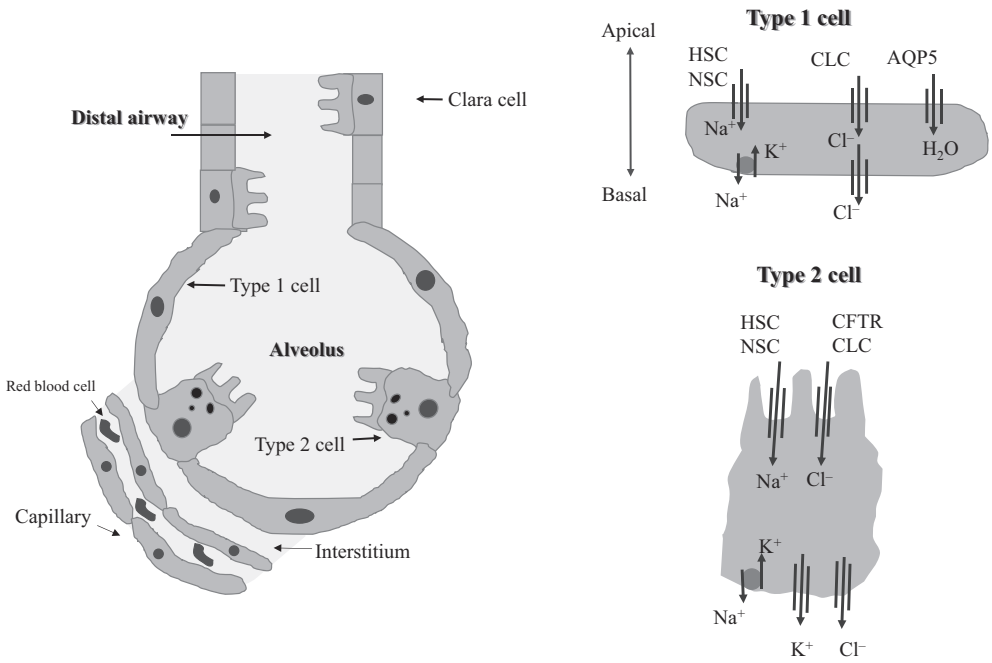

FIGURE 2.3 Diagram of alveolus and ion channels, pumps, and pores in the alveolar epithelium. The alveolus is composed of alveolar epithelial type 1 cells, alveolar epithelial type 2 cells and capillary cells. Type 1 cells are large, squamous epithelial cells that cover ~95% of the alveolar surface area. Type 2 cells produce surfactant and play a role in maintenance of lung fluid balance. Type 1 and type 2 cells contain two isoforms of the epithelial sodium channels (ENaC) (highly selective cation channel (HSC) and nonselective cation channels (NSC)); cyclic nucleotide-gated channels are also present (not depicted). HSC and HSC ENaC contribute to net alveolar Na^+ movement in the alveoli. Chloride channels (CLC) are expressed in type 1 cells, as well as a robust expression of aquaporin 5 (AQP5). Intracellular Na^+ and K^+ concentrations are maintained through the Na^+/K^+ ATPase. The cystic fibrosis transmembrane conductance regulator is apically expressed in type 2 cells, and a variety of Cl^- and K^+ channels are present to regulate Cl^- secretion and K^+ concentration in the epithelial lining fluid.

2.5 ALTERED LUNG FLUID BALANCE AND DISEASE OF THE ALVEOLAR CAPILLARY MEMBRANE

The importance of ion transport and lung fluid balance in the airway is most strikingly evident in the pathological features of diseases affecting the ACM. These are critical illnesses that incur a high mortality rate. The prototype disease is the ARDS.

2.5.1 Acute Respiratory Distress Syndrome

ARDS is a life threatening form of hypoxemic respiratory failure characterized by profound inflammation, noncardiogenic pulmonary edema, and refractory hypoxemia. The pathological features of ARDS are a disruption in the integrity of the ACM and an overwhelming inflammatory response. Early in the pathogenesis of ARDS, there is an influx of protein rich edema fluid into the airspaces. Edema forms in response to an increase in vascular permeability coupled with impaired epithelial ion transport. Collectively these processes disrupt the transbarrier properties of the ACM to impair gas exchange and produce critical illness. Therefore, survival is dependent upon the ability to restore an intact and fully functional ACM.

2.5.1.1 Pulmonary Edema in ARDS

Pulmonary edema in ARDS is a complex phenomenon. In comparison, pulmonary edema that occurs from congestive heart failure occurs in response to increased vascular hydrostatic pressures that drive fluid to accumulate in the airways and alveoli. Therapies that restore lung fluid balance in individuals with congestive heart failure are ineffective in the management of pulmonary edema in ARDS.

Primary causes of ARDS include pneumonia and systemic infections, such as sepsis. There are numerous other causes; despite the range of causal processes, they all share an overwhelming immune response that affects the ACM barrier integrity and alveolar transepithelial transport. A key feature of inflammation is oxidative stress and the importance of oxidative stress on ACM function cannot be over emphasized. Physiologic levels of ROS are necessary for physiologic function and oxidants regulate activity of a variety of ion channels and pumps including ENaC, CFTR, and Na^+/K^+-ATPases [14,16–18,21]. A key feature of oxidative stress is a loss of redox control of cell signaling; this can occur through post-translational modification of ion transport proteins. Antioxidants, such as the abundant glutathione, response to oxidant production in order to prevent injury. As oxidant burden increases, antioxidants are overwhelmed and redox function and control are affected. This is evident by increased levels of oxidized glutathione (GSSG). GSSG modifies protein by attaching to free thiols and can deactivate ion transport proteins. ENaC, as well as other ion transporting proteins, are susceptible to glutathionylation, a post translation modification in which GSSG is added to a protein [18]. Other post translation modifications of ion transporting proteins can occur with oxidative stress or cell injury. Ultimately the consequences are an alteration in the regulation of ion transporting proteins and lung fluid balance. Delineating the specific mechanisms of ion transporter protein disruption is currently an area of intense research.

Inflammation and inflammatory mediators are important in the pathogenesis and resolution of pulmonary edema observed in ARDS. The receptor for advanced glycation end-products (RAGE) is a pattern recognition receptor and member of the immunoglobulin superfamily, and RAGE has many ligands, including lipopolysaccharide. RAGE functions to amplify and perpetuate the inflammatory response. RAGE is constitutively and abundantly expressed in the alveolar epithelium—this is unique as RAGE is down-regulated in all other tissues in the absence of disease. RAGE exists in a variety of isoforms: the full length form, which is involved in the inflammatory response; several slice variants including an endogenously secreted form (esRAGE); and, as an enzymatically cleaved form termed cRAGE. Collectively cRAGE and esRAGE are known as soluble RAGE (sRAGE); sRAGE serves as a decoy signal or antagonist by binding RAGE ligands to prevent feedforward amplification of inflammation. The significance of RAGE in lung injury and ARDS is highlighted by studies demonstrating elevated levels of sRAGE in the BAL fluid of individuals with ARDS [47,48]. Moreover, RAGE signaling activated ENaC [19] suggesting that RAGE, and inflammation, may be involved in transepithelial transport. Furthermore, increasing levels of sRAGE are thought to indicate greater damage to the alveolar epithelium, and may more directly reflect disruption of the ACM.

A variety of inflammatory cytokines and inflammatory mediators have been characterized in the pulmonary edema fluid of individuals with ARDS. Transforming growth factor-β (TGF-β) is elevated in ARDS edema fluid, as well as intercellular adhesion molecule-1 (ICAM-1), tissue necrosis factor (TNF)-α, and Interleukin (IL-) 1, 6, and 8. These cytokines and inflammatory mediators are secreted by inflammatory cells and activated epithelial cells during the

inflammatory response. These cytokines and inflammatory mediators modify ion transporter proteins through a variety of mechanisms. TGF-β promotes degradation of ENaC which contributes to impaired lung fluid clearance observed in ARDS. The cytokines IL-1β and TNF-α increase permeability of the ACM, and these cytokines affect ENaC activity by decreasing mRNA expression of the individual ENaC subunits. The significance of this observation is highlighted by clinical studies in humans showing that elevated levels of IL-1β correlate with a poor prognosis in those afflicted with ARDS.

Although ENaC is considered the rate-limiting step in net Na^+ movement, vectorial Na^+ transport is also affected by changes in activity of the Na^+/K^+-ATPase pump in ARDS. Specifically oxidative stress and ischemia directly affect pump activity and subunit expression, leading to a reduction in vectorial Na^+ transport across the alveolar epithelium. The net effect of inflammation and oxidative stress on Na^+ transport is to reduce vectorial Na^+ transport and to promote pulmonary edema.

ARDS treatment is largely supportive and respiratory support, that is, mechanical ventilation, is required. The initiation of mechanical ventilation appears to offer a modest improvement, at least acutely, in pulmonary edema. The mechanism is unclear; however, higher levels of positive end-expiratory pressure (PEEP) are used to recruit alveoli to improve oxygenation. High levels of PEEP may provide a mechanical force that raises alveolar pressure above vascular hydrostatic pressure to force fluid from the alveoli into the interstitial space. It is also feasible that oxygen-mediated signaling—through respiratory support using mechanical ventilation with supplemental oxygen—may facilitate the activation of Na^+ transporter proteins that are responsible for generating the osmotic gradient necessary for lung fluid clearance.

In addition to mechanical ventilation, inhaled β-adrenergic agonists are often used to treat and/or prevent bronchoconstriction in individualized with ARDS. Mucus accumulation is common in ARDS and β-adrenergic agonists are often used to assist with maintaining an open airway; mucous accumulation produces hypoxia, albeit not usually to the degree seen in ARDS. However, hypoxia can affect ion transporter proteins, leading to a reduction in ENaC and Na^+/K^+-ATPase subunits. β-Adrenergic agonists improve oxygenation and increase cAMP levels in lung epithelia. cAMP activates ENaC to promote lung fluid clearance in a mouse model. However, in large randomized, controlled trials, inhaled β-adrenergic agonists have not proven effective in the treatment of pulmonary edema.

In ARDS, pulmonary edema resolves as ACM integrity is restored. Indeed homeostasis of the lung parenchyma is dependent upon an intact ACM. Restoration of the ACM occurs as damaged T1 cells undergo cell death and are phagocytized by immune cells then T2 cells differentiate into T1 cells to restore the epithelium. T2 cell differentiation is tightly regulated. Therapies aimed at delivering progenitor or mesenchymal stem cells (MSC) to the disrupted ACM hold promise. MSCs offer promise because they can differentiate into lung cells and directly replace damaged cells and tissues. MSCs can also modulate the microenvironment of the ACM by reprograming the inflammatory response to effectively reduce proinjury signals and restore integrity of the ACM.

2.6 SUMMARY

Lung fluid balance is requisite for healthy lung and effective lung function. Transepithelial transport is the direct effect of a series of coordinated events involving ion channels, enzymatic pumps, and pores. Alterations in the function of ion transporting proteins, as well as integrity of the lung epithelium, are involved in disease development and progression. The role of ion transporting proteins in human health and disease remains a source of active investigation and potential targets for therapeutic interventions to treat lung disease.

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Chapter 3

Glucose Transport and Homeostasis in Lung Epithelia

Deborah L. Baines and Emma H. Baker

3.1 INTRODUCTION

Lung epithelial cells, like other cells in the body, have the ability to take up and metabolize glucose to generate energy. The type and distribution of glucose transporters (GLUT) in lung epithelial cells also indicates that GLUT may serve broader physiological functions. One such function, akin to those found in other epithelia, is the control of glucose movement across the lung epithelium and the regulation of glucose concentration in airway surface liquid (ASL). Evidence is emerging that maintenance of low-glucose concentration in ASL is an important component of the innate defense properties of the airway, serving to restrict growth and colonization by inhaled pathogens.

Glucose in human airway secretions is normally maintained at concentrations $\sim$12.5 times lower than plasma by homeostatic mechanisms in lung epithelium that include tight junction barrier function and epithelial glucose transport. These mechanisms are disrupted by chronic lung disease and hyperglycemia, increasing airway glucose concentrations. In cell culture and animal models, disruption of airway glucose homeostasis increases luminal bacterial growth and infection. This can be reversed by drugs that restore airway glucose homeostasis. These findings have implications for the pathogenesis and treatment of respiratory infection in humans with hyperglycemia and chronic lung disease.

3.2 GLUCOSE TRANSPORT AND EPITHELIAL GLUCOSE HOMEOSTASIS

3.2.1 Glucose Transporters

Glucose transport across mammalian cell membranes is mediated either by facilitative transporters (GLUT) which are members of the SLC2A gene family, or by sodium-coupled glucose transporters (SGLT), which are members of the SLC5A gene family. Glucose normally moves through GLUTs by passive diffusion down a concentration gradient generated by the activity of hexokinases (HKs) and glucokinases (GKs), which phosphorylate intracellular glucose and maintain a low-intracellular glucose concentration. In contrast, transport via SGLT is driven by Na^+ and glucose gradients. Thus, in the presence of a Na^+ gradient, glucose can be transported against its concentration gradient [1].

Understanding of the role of glucose transport in the lung and mechanisms that regulate glucose movement across the human lung epithelium, lags far behind that of the gut and kidney. This is partly because research in this area is greatly complicated by regional, species, and developmental differences in pulmonary glucose transport. Research has predominantly focused on the role of SGLT transport as a modifier of lung liquid volume [2,3] and how starvation and diabetes modify glucose transport [1,4]. It is only recently that GLUT transport has received attention as a mechanism for maintaining a nutrient-depleted environment and innate immune function in the airway lumen. Developmental changes in lung epithelial glucose transport also indicate differences in the role of these transport process in fetal and adult lung (refer Ref. [5] for review). Some of these processes relate to cellular proliferation and as such, GLUTs have been a focus for identifying lung cancers and potential targets for tumor growth inhibition.

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3.2.1.1 Facilitative Glucose Transporters (GLUT)

There are 14 known isoforms of the human SLC2A gene family (SLC2A1-14) which encode the facilitative GLUT proteins. Each of these has different substrate (sugar) specificity, transport kinetics, and tissue expression pattern. GLUTs can be divided into three classes according to their sequence homology: Class I includes the classical GLUTs GLUT1-4 [6–9], which also preferentially transport the analog 2-deoxyglucose (DOG) over α-methyl-D-glucopyranoside (AMG), and GLUT14 (a gene duplication of GLUT3) [10]. Class II includes the fructose transporter GLUT5 [11], GLUT7 [12], GLUT9 [13], and GLUT11 [14]; which do not bind cytochalasin B and have no affinity for DOG. Class III comprises transporters GLUT6 [11], GLUT8 [15], GLUT10 [16], GLUT12 [17], and the proton-driven myoinositol transporter HMIT (or GLUT13) [18].

3.2.1.2 Sodium-Coupled Glucose Transporters (SGLT)

There are 11 isoforms of the human SLC5 gene family (SLC5A1-11). SLC5A1 (SGLT1) [19] and SLC5A2 (SGLT2) [20] encode SGLUT proteins. SGLT1 is a high-affinity transporter for D-glucose and galactose [21]. Although similar in sequence, SGLT2 is a low-affinity GLUT and transports galactose less efficiently [22]. Both transporters will also transport the analog AMG. There is a difference in the stoichiometric ratios of sodium coupling between SGLT1 and SGLT2, which are 2:1 and 1:1, respectively [23]. SGLT3 has no glucose transport activity, and as such, has been proposed to function as a glucose sensor [24]. SGLT4 and SGLT5 are primarily mannose transporters and SGLT6 (also known as SMIT2) transports inositol [25].

3.2.1.3 SemiSweet Facilitative Hexose Transporters (SWEETS)

SemiSweet facilitative hexose transporters (SWEETs) and semiSweets are a new class of seven transmembrane hexose transporters originally identified in plants (PFAM PF03083). In *Caenorhabditis elegans,* SWEET1 is a low-affinity GLUT. A human homolog HsSWEET1 has been identified. When expressed in yeast or oocytes, HsSWEET1 exhibited low abundance in the plasma membrane and did not facilitate significant glucose uptake, although weak efflux activity was reported [36]. In human embryonic kidney cells, it localized the Golgi leading to the proposal that it may mediate glucose efflux from this organelle [36].

Excellent Reviews of GLUT Transporters Can Be Found in Refs. [37–40], SGLT [41], and SWEETs [42].

3.3 GLUCOSE TRANSPORTERS IN THE LUNG

The airway epithelium has a complex structure with regional variation. The epithelium lining the nose and the sinuses, the trachea, bronchi and bronchioles, and the alveoli differs in its cellular composition between sites, reflecting the different functional roles it serves. The distribution of GLUTs in the lung similarly differs indicating different roles and functions. We provided a comprehensive review of GLUT and SGLT expression in fetal and adult lung, bronchial, and alveolar epithelial cells from a range of mammalian species and in human cell lines, cultured cells, and tissues [5]. Since then, the accumulation of gene expression data sets in the Gene Expression Omnibus repository for lung epithelia has expanded hugely. This has allowed further analysis of GLUT genes expressed in the lung although, interestingly, it has not yet altered the already identified key players.

3.3.1 Glucose Transporter mRNA

Gene expression studies indicate that there are a number of GLUT transporter genes expressed in the lung epithelium and, where data are available for different species, the pattern of expression is similar in human, pig, rat, and mouse.

In the adult trachea, bronchi and bronchioles GLUTs are the predominant glucose transporter type expressed (Fig. 3.1). The most abundant mRNA transcripts detected are 1, 3, 8, 10, 12, and 13. There is more variation in the pattern of transcripts detected in isolated and cultured primary airway epithelial cells and cell lines. The expression of transporter mRNAs appears to be dependent on the cell type, culture conditions, whether cells are grown at air-liquid interface or submerged culture and length of time in culture. Nevertheless, GLUTs 1, 3, 8, 10, and 12 as well as SGLT1 remain the predominant transcripts. The abundance of GLUT2 transcripts was consistently low. GLUT7 was not identified in the lung. Transcripts for GLUT9 and 13 were relatively abundant in human lung tissue, but less so in mouse. GLUT9 is a voltage-driven urate transporter. Whether it transports glucose or not remains disputed [43]. GLUT13 transports myo-inositol and not glucose.

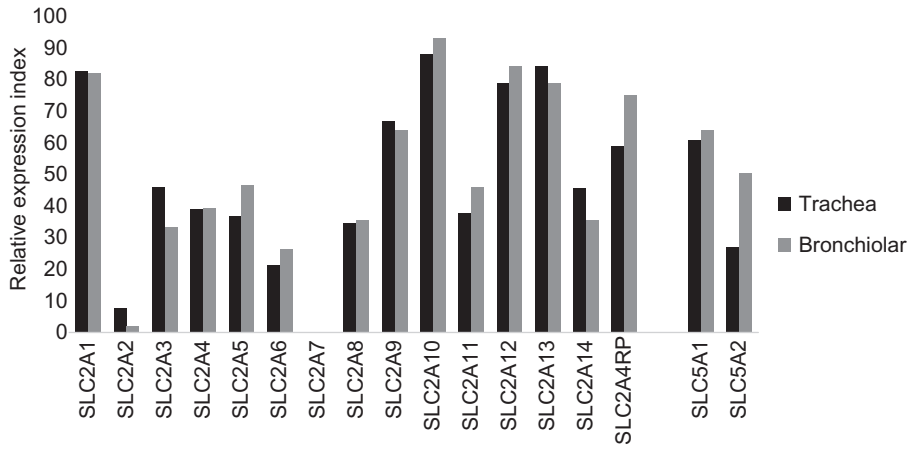

FIGURE 3.1 Glucose transporter expression in human airway. Data are collated from Gene Expression Omnibus (GEO) data sets for human trachea and bronchial tissue available online from the National Center for Biotechnology Information. Relative expression index is a measure of the expression of the specific gene in respect to all other genes in array.

In studies identifying mRNAs by other methodologies, GLUT1, 3, 5, and GLUT8 mRNAs were present in the human and bovine lung, but any role in these tissues has not been defined [44]. GLUT4 mRNA transcripts were present in human, rat, and mouse lung tissue by real-time polymerase chain reaction (RT-PCR) analysis [45]. GLUT10 mRNA was present in human lung by Northern blot analysis and is a highly abundant transcript in human airway tissues from trachea, bronchioles, and alveolar regions and in primary human bronchiolar epithelial cells (HBECs) [16,34,46]. There are three mRNA-encoding isoforms of GLUT11 (GLUT11 A−C) which have different N-terminal sequences. GLUT11A mRNA was present in most tissues, and the presence of GLUT11B mRNA has been confirmed in lung and trachea, although the cellular source of this isoform was not confirmed [37]. GLUT12 transcripts were one of the most abundant in human and mouse lung tissues and cells.

SGLT1 and 2 mRNA transcripts were detected in human, pig, rat, and mouse airway tissues including nasal, trachea, bronchi, and bronchioles in gene arrays. Transcripts were also detected in cell lines such as Calu3. Of the two transcripts, SGLT1 was the most abundant. SGLT1, but not SGLT2, mRNA was identified in distal lung epithelium from adult rats [47,48] and from mouse and human whole lung tissue [49,50] using RT-PCR.

HsSWEET appears to be ubiquitously expressed in microarray analysis of human tissues and cells, although expression in the airway/lungs was relatively low [36].

3.3.2 Glucose Transporter Proteins

The identification of GLUT and SGLT proteins in the lung is dependent on the quality and specificity of antisera used for their detection. Using such tools, several studies have identified GLUTs in the airways and distal lung. High-resolution imaging techniques have also enabled localization of the proteins to be localized to specific cell types and sites within the cells. Interestingly, taking into account the availability of specific antisera, not all mRNAs expressed appear to be translated into proteins. There is also additional discrepancy between poorly expressed mRNA and high-protein abundance.

3.3.2.1 GLUT

GLUT1 protein is widely expressed in fetal lung tissue. It was present in the airway and alveolar epithelium of the fetal rat consistent with its function as a key transporter for glucose uptake into the cell for metabolism [51−53]. Its expression was reported to decline with development of the lung [53]. In fetal rats, GLUT1 mRNA expression increased to maximal levels at gestational day 20 and fell to very low levels by postnatal day 8 [52]. Consistent with mRNA expression studies, GLUT1 protein was observed in the airways at gestational day 19, decreasing in intensity at day 21 [54]. GLUT1 protein was detected in the olfactory epithelium of the adult rat, although there is little evidence for the protein in either the airways or distal lung of the adult rat [55,56]. mRNA abundance for GLUT1 is high in human tracheal and bronchial tissues, but a recent in-vivo study of the upper airway showed that GLUT1 was predominantly localized to the membrane of white blood cells and was not detected in bronchial epithelial cells [57]. The expression of GLUT1 is variable in cultured human airway epithelial cells. In primary human tracheal epithelial cell (HTEC) and HBEC, GLUT1 mRNA is abundant and GLUT1 protein was identified on the basolateral surface of polarized primary HBECs [46]. In contrast, although mRNA was present, protein was not detected in H441 airway cells [58]. GLUT1 expression

was seen in lung cancer cells especially squamous cell carcinomas, and its expression in small cell carcinoma of the lung was associated with reduced cellular differentiation and increased cellular proliferation [53,59]. Thus, evidence indicates that GLUT1 is associated with cellular proliferation/dedifferentiation of lung epithelial cells.

Three independent studies to date have shown, using immunofluorescence and immunohistochemistry, that GLUT2 protein is present in human bronchial biopsies [57,58,60]. Staining for GLUT2 was detected in both apical and basolateral membranes of bronchial epithelial cells. GLUT2 was also detected in rat bronchiolar epithelial cells and in the apical domain of many, but not all, rat ciliated tracheal cells in vivo. It was also detected in solitary chemosensory cells where its distribution was both intracellular and apical [60,61]. GLUT2 was detected in the apical membrane of human H441 cells grown at air-liquid interface, which have a surface epithelial Clara cell like bronchiolar phenotype, although it was not detected in HBEC grown at air-liquid interface [58,62]. There is little evidence for GLUT2 in distal lung tissue. The reasons for the discrepancy in findings between studies are unclear but could arise from the different origin of cells, growth and culture conditions, and the use of different sources of antisera. What is more of a conundrum is that GLUT2 mRNA data do not correlate with the levels of protein detected. The array data indicate that GLUT2 mRNA is either not transcribed or is rapidly degraded in the cell and yet the protein appears to be present and relatively abundant in some airway cells. It is unlikely that the protein detected is not GLUT2 as each of the studies cited used different antisera directed against different epitopes of the protein. There is also emerging evidence that data obtained for mRNA abundance in cells and tissues correlate poorly with protein abundance [63,64]. In cells and tissues, the regulatory processes controlling mRNA and protein are selective and variable. The resultant half-lives of individual mRNA and proteins can therefore differ widely resulting in different retention times in the cell which will impact the amounts detected.

GLUT2 is a key transporter of glucose in the intestine and pancreatic β-cells. It is a low-affinity GLUT (K_d, 17 mM) although it transports glucosamine at much higher affinity. The function of such a low-affinity GLUT in the lung epithelium is difficult to envisage. However, GLUT2 protein abundance was shown to increase in response to proinflammatory stimuli [65] and in the airways of streptozotocin (STZ) and GK deficient diabetic rats [45]. GLUT2 expression was reduced in ciliated cells of obese rats, but remained beneath the cilia in the apical pole of the cells [61]. These findings indicate that GLUT2 is dynamically regulated in the lung. In their review, Leturque et al. proposed that GLUT2 may also act as a glucose receptor with a glucose sensing function [66]. In the airway, GLUT2 was co-localized in ciliated cells and solitary chemosensory cells with the sweet taste receptor protein T1R3 indicating that GLUT2 may indeed play a sensory role in the airway (Fig. 3.2) [60].

The role of GLUT3 in the lung remains elusive. GLUT3 has a relatively high affinity for glucose at 1.4 mM. It is present in olfactory epithelial cells, where it has been postulated to play a role in substrate handling and metabolism

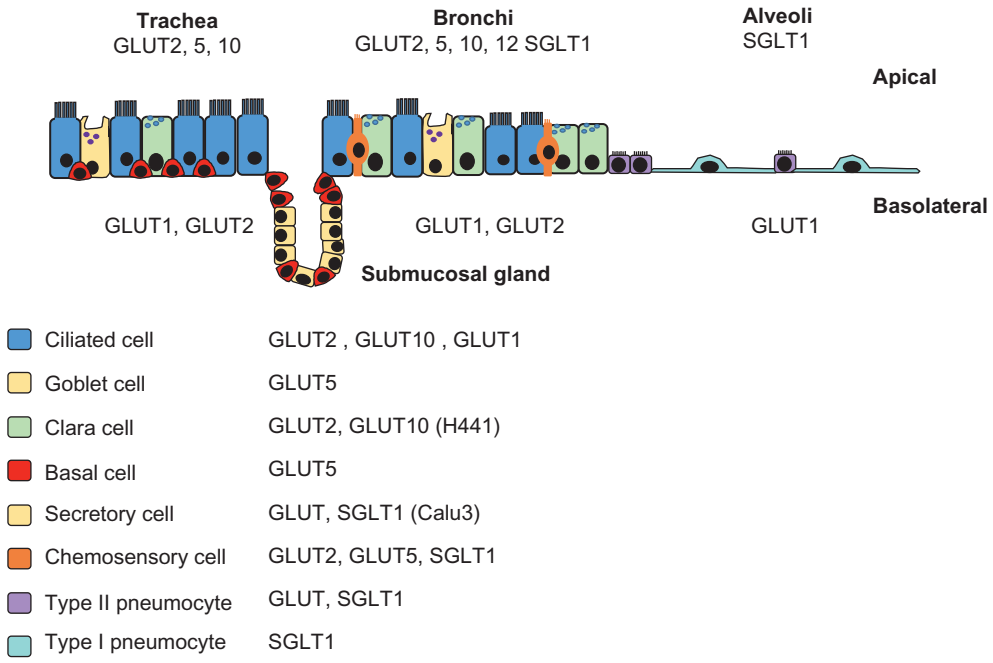

FIGURE 3.2 Distribution of glucose transporters detected in lung epithelium.

[55]. Evidence for GLUT3 in the trachea, bronchi and alveoli is lacking, and GLUT3 protein was not detected in HBEC or H441 airway cells in vitro [46,55,58]. It has, however, been shown to be highly expressed in lung tumor cells which have undergone epithelial to mesenchymal transition and in some lung adenocarcinomas [53,59,67]

GLUT4 mRNA and protein was detected in H441 airway epithelial cells, although not localized to the membrane, even when insulin was present in the medium [58]. GLUT4 protein was not detected in HBEC [46]. Thus, whether GLUT4 is expressed in epithelial tissues in the lung remains uncertain.

GLUT5 is primarily a fructose transporter, although rat GLUT5 transports glucose. It is included here because, consistent with array data, the protein has been detected in the luminal membrane of solitary chemosensing cells and secretory cells of the rat trachea. It was also found in basal cells (Fig. 3.2). Why a fructose transporter would be important in the trachea is currently difficult to answer, but it has been hypothesized that it may be involved in sensing and regulating fructose concentration in the ASL and/or in localized regulation of secretions into the airway lumen [60]. How this might work physiologically is unclear as fructose concentration in the blood is much lower than that of glucose (>0.1 mM) and the K_m of GLUT5 for fructose is >10 mM. Even though blood fructose concentrations have been reported to increase in Type-II diabetes, levels still remain significantly lower than that of glucose (μM compared to mM).

GLUT10 protein was present in the apical domain of HBEC and H441 human airway epithelial cells [46,68]. Further protein localization studies in human lung tissue have yet to be carried out. GLUT10 has a high affinity for glucose (K_m, 0.3 mM). However, in other cell types, there is some controversy as to whether GLUT10 transports dehydroascorbic acid rather than glucose, and what membrane it facilitates transport across (mitochondria, endoplasmic reticulum, or plasma). It has also been proposed to be involved in glucose-dependent regulation of TGFβ [69]. Polymorphisms in GLUT10 are associated with arterial tortuosity syndrome, and therefore much of the work so far has focused on how GLUT10 might influence vascular development [69].

GLUT12 protein is identified in bovine lung and in rat lung bronchioles from day 19, indicating that it is developmentally regulated [54,70]. Indeed, Macheda et al. proposed that GLUT12 was important for glucose transport in proliferating tissues. In human MCF-7 and Chinese hamster ovary cells, GLUT12 was present in the Golgi network and at the plasma membrane and insulin increased its translocation to the membrane [18,71,72]. Whether this is similar in airway cells has not yet been explored.

Proteins for GLUT6, 7, 8, 9, 11, and 13 have not been described in the lung. Of these, GLUT11 remains a potential candidate for a pulmonary GLUT, GLUT11 transports glucose with high affinity when expressed in *Xenopus laevis* oocytes and also transports fructose, but not galactose [32].

3.3.2.2 SGLT1/2

Bodega et al. identified SGLT protein in type-I and type-II alveolar cells of rat and sheep lung [73]. SGLT1 was also present in solitary chemosensing cells of rat trachea, and its abundance did not change in wild type (WT) compared to obese rats. It is much less abundant in the airway than in the distal lung [60,61]. Neither, SGLT1 or SGLT2 protein have been identified in HBEC or H441 cells [46].

3.3.2.3 SemiSweet Facilitative Hexose Transporter

Endogenous HsSWEET protein has not yet been described in human lung tissues.

3.3.3 Glucose Transport

The distribution of glucose transport proteins in the lung indicates that GLUTs predominate in the airway and SGLT in the distal lung. In addition, the cellular distribution indicates that some of these transporters have specific functions in the lung. What is particularly interesting is that several GLUTs are present on the luminal surface of the lung epithelial cell. This implies that glucose (and/or other hexoses) have the potential to move into and/or out of the lung lumen.

The study of glucose uptake across lung tissues and cells has predominantly made use of the pharmacological inhibitors phloretin and phlorizin, cytochalasin B, and analogs of glucose. Unfortunately, these agents lack specificity and there are varying K_i for these inhibitors (refer Table 3.1). Thus, use of pharmacological agents can only really provide a guide to functional transport via GLUT or SGLT. The recent development of specific SGLT2 inhibitors, such as the gliflozins, has made it possible to distinguish between SGLT1/2 function but functional identification of specific GLUT isoforms remains difficult in vivo.

TABLE 3.1 The Key Characteristics of Glucose Transporters GLUT and SGLT

	Sugars Transported	K_m glucose	IC_{50} PT	IC_{50} PZ	IC_{50}CyB	Refs.
Class I						
GLUT1	Glucose	3 mM	49 μM	355 μM	0.44 μM	[26]
GLUT2	Glucose, galactose, mannose, and glucosamine	17 mM	2 μM 1 mM	x	2 μM	[27–29]
GLUT3	glucose, galactose, mannose, maltose, xylose, and DHA	1.4 mM	√	√	0.4 μM	[18]
GLUT4	Glucose, glucosamine, and DHA	5 mM	10 μM	140 μM	0.2 μM	[30]
Class II						
GLUT5	Fructose	(6 mM)	x	x	ND	[31]
GLUT7	Glucose and fructose	0.3 mM	x (200 μM)	x	x (100 μM)	[12]
GLUT9	Glucose, fructose, and uric acid	0.6 mM	ND	ND	x	[32,33]
GLUT11	Glucose and fructose?	0.16 mM	ND	ND	Affinity lower than GLUT4	[32]
Class III						
GLUT6	Glucose	>1 mM	ND	ND	Low	[18]
GLUT8	Glucose, fructose, and galactose	0.3 mM	ND	ND	√ (50 μM)	
GLUT10	Glucose, galactose, and DHA	0.3 mM	√ (100 μM)	ND	ND	[34]
GLUT12	Glucose, fructose, and galactose	ND	ND	ND	√	[18]
GLUT13	Myoinositol	(100 μM)	ND	√	√	[18]
SGLT1	Glucose and galactose	0.4 mM	x	42 μM	x	[35]
SGLT2	Glucose	2 mM (AMG)	x	28 μM	x	[35]

The values of K_m for glucose and IC_{50} inhibitors are given where known. PT, phloretin; PZ, phlorizin; CytB, cytochalasin B; DHA, dehydroascorbic acid; √, activity reported; x, no activity reported; ND, not described. Concentrations in parentheses indicate concentrations used in study.

Distal lung. The most studied form of pulmonary glucose transport to date is SGLT-mediated glucose transport in the distal lung. This is because at this site cotransport of glucose with Na^+ contributes to transepithelial ionic gradients that regulate the osmotic movement of fluid out of the lung lumen. Because of this, activity of SGLT can be ascertained not only by measurement of glucose uptake using radiolabeled glucose analogs, but also by the movement of Na^+ (using electrophysiological methods) or fluid (using dye dilution techniques) across the lung epithelium. In fluid-filled lung models in rats, rabbits, and sheep, glucose instilled into lung fluid is rapidly transported out of the lumen across the epithelium to the interstitium. This process is prevented by phlorizin, which predominantly inhibits SGLT1 and 2 but not GLUTs. Seminal work in this field by Basset and Saumon showed that this glucose transport drives alveolar fluid absorption. The rate of alveolar fluid absorption increases when glucose in the instillate is raised from 0 mM [0.27 pmol/(cm^2 s)] to 10 mM [0.68 pmol/(cm^2 s)]. Alveolar fluid absorption is also slowed by the addition of phlorizin. The contribution of SGLT to total Na^+-driven fluid absorption in either scenario was approximately 50% (with the remaining absorption inhibited by amiloride) [2,3,74,75].

More recent experiments have shown that instillation of phlorizin in glucose-free solution into the lungs of rats or mice results in a slow elevation of glucose concentration in the lung lumen. This indicates that glucose entering the lung lumen, most likely by diffusion from the blood/interstitium, is actively removed by SGLT [76]. Thus, SGLT contributes significantly to glucose clearance and fluid absorption in the distal lung. In vitro, SGLT-mediated transport was demonstrated in freshly isolated alveolar type-II cells from guinea pig [74]. Whilst mRNA, protein and uptake data indicate that SGLT1 is the key transporter in the distal lung, it is only recently, with the development of specific SGLT2

inhibitors for the treatment of diabetes mellitus (DM) (gliflozins), that it has become possible to rule out any contribution of SGLT2 to glucose transport in the lung. The results of these experiments, which are starting to emerge, indicate that this is indeed the case.

There is little evidence of luminal GLUT-mediated transport by the distal lung epithelium. The addition of phloretin which predominantly inhibits GLUTs to the lung instillate did not inhibit glucose or fluid absorption from the lumen of the sheep, rabbit, or rat lung [3,75,77,78]. Neither was there any significant transport of the glucose analog 2-deoxy-D-glucose which is specifically transported by GLUTs in fetal sheep lung [2]. However, both plorizin and phloretin inhibited glucose uptake by isolated type-II alveolar cells from guinea pig lung [74], indicating that GLUT transporters could be present on the humoral but not luminal membrane.

Airway epithelium. Measurement of glucose transport across the airway epithelium is more challenging than in the distal lung. Na^+ transport was used to analyze SGLT function in sheep and human airway epithelium mounted in Ussing chambers by measurement of transepithelial current under short circuit conditions (I_{sc}). In these experiments, normally conducted in the presence of glucose, luminal application of phlorizin reduced I_{sc} by approximately 4% in sheep, but not significantly in human tissue [79,80]. Amiloride inhibited I_{sc} by $\sim$43%. These data indicated that there was some active glucose absorption via SGLT across the luminal membrane of the airway epithelium but that the contribution to Na^+ movement was much lower in the airway than in the distal lung. In vitro, no SGLT-mediated Na^+ currents could be detected in H441 or HBECs. However, there is some enticing evidence that SGLT function was present in polarized cultures of Calu-3 cells, which mimic the submucosal glands. In Calu-3 cells, the addition of phlorizin or removal of glucose from the apical bath solution inhibited $\sim$20% of basal I_{sc} [81–83]. Whether SGLTs are present in human submucosal glands in vivo remains to be seen, but it seems unlikely that SGLTs make a significant contribution to airway glucose transport given the overall lack of evidence of function.

To our knowledge, there have been no studies to date of GLUT-mediated glucose uptake in intact airway epithelium. H441 cells grown at air–liquid interface exhibit polarized ion transport processes. In these cells, phloretin inhibited apical and basolateral glucose uptake [84]. Similarly, in polarized HBEC, 2-deoxyglucose (a substrate for GLUT but not SGLT transport) was taken up across apical and basolateral membranes [46]. Apical uptake correlated with the presence of GLUT2 and/or GLUT10 protein in the apical domain of H441 and HBEC [46,58], although whether these transporters are directly responsible for the luminal uptake of glucose has not yet been confirmed. Basolateral uptake correlated with GLUT2 in H441 cells and GLUT1 in HBEC.

3.3.4 Lung Epithelial Glucose Homeostasis

The observation that airway and alveolar epithelia support diverse glucose transport mechanisms has led to speculation as to why these transporters are segregated to different lung regions. In the airway, the composition of the ASL plays a critical role in the first line of defense against infection. Generation of low-ASL nutrient composition (e.g., glucose) may be crucial for limiting pathogen growth [5,85].

3.3.4.1 ASL Glucose Concentrations

Glucose concentrations are 3–20 times lower in ASL than in plasma. This is in contrast to conditions in the gut and kidney, where luminal glucose concentrations regularly exceed plasma glucose concentrations [86,87]. In humans, glucose concentrations were $<$1 mM in nasal secretions [88] and 0.4 $\pm$ 0.2(SD) mM in the lower respiratory tract, 12.5 times lower than plasma concentrations, which are normally maintained at $\sim$5 mM [89]. In a perfused, fluid-filled, adult rat lung model, ASL glucose was estimated to be 0.5 mM when perfusate glucose was 10 mM [3]. In the chronically-catheterized sheep fetus between 122 and 143 days gestation, lung liquid glucose was $<$0.01 mM with a plasma glucose of 0.19 mM [2]. In vitro H441, Calu3, and HBEC monolayers grown at air-liquid interface had an ASL glucose concentration of 0.3–0.6 mM with corresponding basolateral glucose concentrations of 5–10 mM [46,65,68].

3.3.4.2 Processes that Determine ASL Glucose Concentrations

The current model for airway glucose homeostasis assumes that the concentration of glucose in the ASL is the net result of diffusion of glucose from blood/interstitial fluid across the respiratory epithelium into ASL and removal of glucose from ASL by GLUTs. (Fig. 3.3) [3,58,84,90].

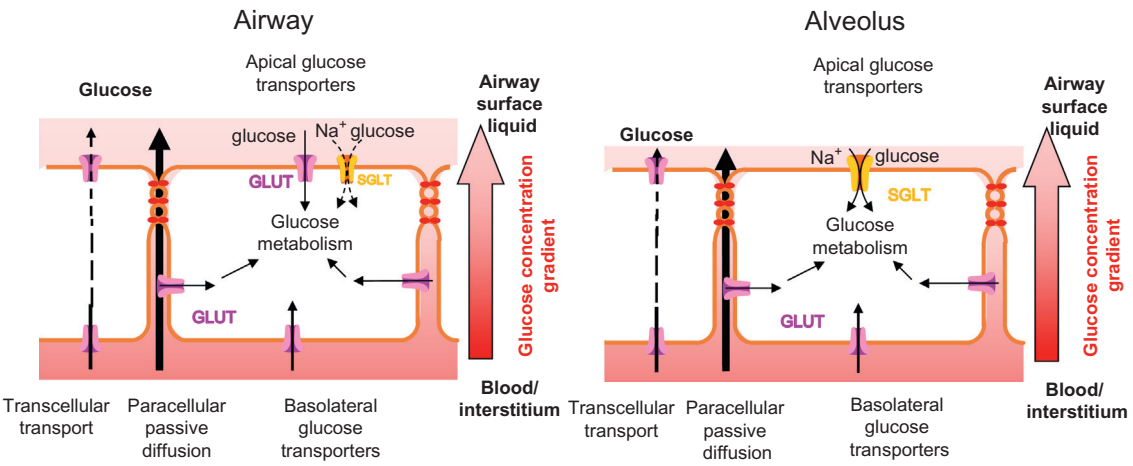

FIGURE 3.3 Processes that determine ASL glucose concentrations in the airway and alveolus.

3.3.4.2.1 Transepithelial Glucose Gradient and Glucose Diffusion

Glucose diffusion across epithelia is determined by epithelial permeability to glucose, the transepithelial glucose gradient and surface area. Given that ASL glucose concentration is lower than that of blood, there is a driving force for glucose to move into ASL. Movement of glucose from blood/interstitial fluid into ASL occurs primarily by paracellular pathways. In vivo, rabbit respiratory epithelium and pig tracheal epithelium were shown to be permeable to L-glucose, an isoform of glucose that is neither transported nor metabolized. More recent work has shown that L-glucose, applied to the basolateral side of H441, Calu3, and HBEC grown at air–liquid interface, moved into the apical compartment in a time- and concentration-dependent manner, consistent with paracellular diffusion [46,62,68,84,91]. These findings imply that glucose diffuses passively across the epithelium via paracellular pathways and that this process is affected by the permeability of epithelial tight junctions to glucose and by the glucose concentration gradient (Fig. 3.3).

3.3.4.2.2 Paracellular Pathways

Tight junctions between epithelial cells determine the permeability of the epithelium [92]. Tight junctions are a complex association of transmembrane proteins, including junctional adhesion molecules, claudins, and occludins, which are linked to cytoskeletal proteins and each other by scaffolding proteins such as zonula occludens (ZO). The abundance of proteins and their localization contribute to the formation of different populations of size- and charge-limiting transcellular diffusion pathways. These pathways have been broadly classified into pore and leak types [93–97]. The pathway(s) by which glucose crosses the lung epithelial tight junction barrier are currently unknown.

Zonula occludens-1 (ZO-1), occludin, and claudins are the main junction proteins determining paracellular permeability [96,97]. Claudins are critical for tight junction formation. Their presence influences leak pathways and charge selectivity of pore pathways. In airway epithelial cells, differential expression of claudin-1, -3, and -5 modified epithelial permeability to large molecules. However, although claudin-2 is important for cation conductivity, it had no effect on the flux of mannitol which is an inert molecule of similar size to glucose [98–100]. Overexpression of occludin increased epithelial permeability to small hydrophilic molecules whilst reducing ion movement [101]. Recent evidence indicated that claudin-1 and occludin were important in determining L-glucose permeability in Calu3 cells [91].

3.3.4.2.3 Transcellular Pathways

The presence of both apical and basolateral GLUTs raises the possibility that glucose can move (via facilitated diffusion) across the lung epithelium via transepithelial routes (as in the gut, albeit with opposing gradients). A comparison of bidirectional flux of L-glucose (which it is not transported and therefore diffuses only by paracellular pathways) and 2-deoxyglucose (2-DOG) (which can be transported and diffuse by paracellular and transcellular pathways) showed that there was 2-DOG flux in both basolateral–apical and apical–basolateral directions. These data indicated that glucose can move by transcellular pathways from blood/interstitial fluid into ASL across airway epithelium [46] (Fig. 3.1). However, maintenance of ASL glucose at concentrations lower than blood glucose indicates that other cellular mechanisms, such as glucose metabolism, restrict the movement of glucose by this route.

3.3.4.2.4 Glucose Transport and Metabolism

The distribution and function of GLUTs in the airway indicates that GLUTs could limit diffusion into the ASL and transport glucose out of the ASL, thus regulating glucose concentration in the ASL. GLUT transporters only transport glucose down a concentration gradient. Thus, a mechanism has been proposed from recent research to explain how GLUT transporters may do this.

Glucose taken up into airway epithelial cells is metabolized rapidly. The first step in the process is the conversion of glucose to glucose-6-phosphate, which is catalyzed by HKs and considered to be the rate-limiting step in glucose metabolism [102,103]. This maintains low-intracellular glucose concentrations providing a driving force for glucose uptake across the apical and basolateral membranes [84]. Uptake across the apical membrane reduces glucose concentration in ASL. Uptake across the basolateral membrane modifies glucose concentration in localized microdomains including intracellular clefts and decreases glucose concentration gradients close to routes of paracellular diffusion [46,84]. Low-intracellular glucose would also limit transcellular movement of glucose and lead to the prediction that ASL glucose would equilibrate with intracellular glucose concentration.

In support of these proposals, phloretin applied to the apical surface of polarized H441 cell monolayers resulted in increased appearance of glucose in ASL. This indicates that glucose moves into ASL by diffusion and that apical GLUT transporters are required for its removal and maintenance of low-ASL glucose [84]. Application of basolateral phloretin also increased appearance of glucose in the apical compartment, providing evidence that basolateral GLUTs limit diffusion [84]. The identity of GLUT transporters in the apical and basolateral domains of the airway epithelium further informs this model. Of the GLUTs thought to be in the apical domain, the low K_m of GLUT 10 (0.3 mM) would be better suited to removing glucose from ASL where glucose concentrations are low. GLUTs identified in the basolateral membrane have higher K_ms (GLUT 1; 3 mM and GLUT2; 17 mM) and would be more suited for transporting glucose at plasma concentrations. The role of HK activity in the regulation of glucose uptake, transepithelial glucose transport, and glucose concentrations in ASL is currently being explored. HKs I, II, and III have been identified in rat lung [104,105], and HKII was present in human lung tumors and A549 alveolar epithelial cells [106,107].

3.3.4.3 Regional Differences in Glucose Homeostasis

The distribution and function of SGLT1 indicates that it does not play a significant part in the removal of glucose from ASL in the upper airway. Nevertheless, the association of SGLT1 with taste receptors (T1R and T2R) in solitary chemosensory cells indicates that it could contribute to a mechanism where glucose concentration in ASL is sensed as part of innate defense (see below).

In contrast, SGLT1 is the key transporter of glucose in the alveolar epithelium. The model can be adapted accordingly (Fig. 3.3). This then raises a question as to why the two regions of the lung may have such different processes for glucose transport. One possible, although as yet unsupported proposal, involves regulation of ASL fluid volume. In the airway, ASL volume is critical for the function of the mucociliary escalator. ASL height is regulated by Na^+ absorption and Cl^- secretion. The transport of glucose via SGLT1 would contribute to Na^+ absorption and drive reduction in ASL height compromising airway function. However, in the distal lung, a very low-ASL volume is required for efficient gas exchange. Thus, the function of SGLT1 in the alveolus may be advantageous by reducing both glucose concentration and fluid volume.

3.3.5 Abnormalities in Lung Epithelial Glucose Homeostasis—Insights from Cell Culture, Mouse Models, and Human Studies

3.3.5.1 Inflammation

The model put forward for glucose homeostasis in the lung infers that changes in paracellular permeability would modify ASL glucose concentration by increasing glucose diffusion (Fig. 3.4). In support of this, apical appearance of L-glucose increased exponentially with a decrease in transepithelial electrical resistance (R_t) across airway epithelial cells in vitro [68,84]. The presence of airway infection and induction of an inflammatory response are known to result in reduced airway R_t [108]. The presence of *Staphylococcus aureus, P. aeruginosa,* and treatment of epithelial cells with proinflammatory mediators [lipopolysaccharide (LPS), TNFα, IL-1β, INFγ] reduced R_t and increased ASL glucose concentration [62,65,91]. In cells isolated from cystic fibrosis (CF) airways, the response was even more potent and consistent with the more proinflammatory response of these cells [62]. In vivo, increasing paracellular permeability

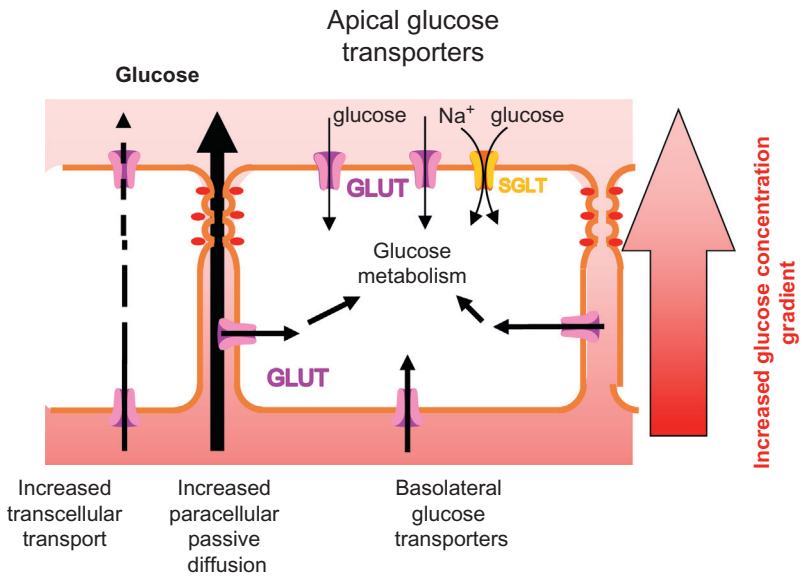

FIGURE 3.4 The effect of inflammation and hyperglycemia on the processes that determine ASL glucose concentrations in the airway/alveolus.

with protamine in rat lungs and LPS in mouse trachea also increased luminal glucose concentrations and glucose permeability, respectively [77,109].

In humans, ASL glucose concentrations are increased (from normal values of $\sim 0.4 \pm 0.2$ mM [89,110,111]) in people with airway inflammation caused by a variety of pathologies including: viral rhinitis [nasal secretions, 1(1−2) mM] [88]; CF [nasal secretions, 1−3 mM [112], distal airway (exhaled breath condensate (EBC)), 2.0 ± 1.1 mM] [89,110,111]; COPD and chronic severe asthma (bronchoalveolar lavage (BAL), values $4 \times$ healthy volunteers) [89,110,111]. Similarly, Lee et al. found glucose concentrations in nasal secretions from patients with chronic rhinosinusitis to be 1.6 ± 0.1 mM [111,113].

There have been many studies showing that inflammation affects the abundance and localization of tight junction proteins in lung epithelia (for review see Ref. [114]). In Calu3 cells, the bacterial-induced reduction in R_t was associated with reduced abundance of tight junction proteins claudin-1 and occludin. Whilst there is still a long way to go in understanding underlying mechanisms, these proteins are candidates for the regulation of glucose permeability [91].

Interestingly, treatment with proinflammatory agents in vitro also increased GLUT-mediated glucose uptake and the abundance of GLUT2 and 10 in airway epithelial monolayers [62,65]. The signaling pathways regulating GLUT abundance are as yet unknown, but could be mediated by cytokine signaling pathways or by elevated apical glucose. Although elevation of glucose transport under these experimental circumstances did not exceed flux and did not prevent a rise glucose concentration, ASL concentrations still remained lower than blood glucose concentrations. This raises the possibility that dynamic regulation of GLUT transporters could mitigate the increased flux of glucose into the ASL during inflammation.

3.3.5.2 Hyperglycemia

Hyperglycemia as a consequence of DM or due to the physiological stress of acute illness increases the diffusion gradient for glucose to move via paracellular pathways into ASL (Fig. 3.4). Elevation of basolateral glucose from 5 to >15 mM in epithelial cell models increased ASL glucose concentrations (up to 3.0 mM) [62,65,115]. It is also possible that elevation of blood/basolateral glucose could increase glucose movement into ASL via transcellular pathways. An increase in the driving force for glucose uptake across the basolateral membrane could increase intracellular glucose concentration, particularly if HK activity reaches maximum capacity. If intracellular glucose concentration rises above that of ASL, then glucose would efflux from the cell via apical transporters. To our knowledge, there is no reported data that describe the effect of hyperglycemia on intracellular glucose concentration or transepithelial glucose transport across lung epithelia at the present time. Nevertheless, as ASL glucose concentrations have consistently been reported to be below that of basolateral glucose, even in the presence of hyperglycemia, this indicates that homeostatic mechanisms remain effective at limiting glucose accumulation in ASL during hyperglycemia.

In vivo, evidence is emerging that ASL glucose is increased in animal models of hyperglycemia/diabetes [77,109,116]. Glucose concentrations are significantly increased in BAL samples from diabetic compared to nondiabetic animals. BAL glucose concentrations were increased from 0.07 mM in WT mice to 0.19, 0.35, and 0.40 mM in GK-deficient heterozygous ($GK^{+/-}$), leptin-receptor deficient (db/db), and STZ-treated diabetic mice, respectively [117,118]. ASL glucose concentrations were 0.02 mM in nondiabetic and 0.16 mM in alloxan-treated diabetic rats [76].

In humans, people with DM have increased nasal (4(2−7) mM (median interquartile range)) [88,89] and distal airway [estimated from EBC] (1.2 ± 0.7 mM) glucose concentrations [88,89]. In patients intubated on intensive care, increasing blood glucose concentrations were associated with increasing glucose concentrations in bronchial aspirates [88,89]. In healthy volunteers, experimental hyperglycemia (10% glucose infusion to increase blood glucose by $\sim$10 mM) increased the glucose concentration of nasal secretions from <1 to 4.8 ± 2.2 mM [119] and of lower airways secretions (EBC) from 0.36 ± 0.27 to 0.75 ± 0.39 mM [88,89].

In cell models and human studies, the combination of inflammatory stimuli with hyperglycemia increases ASL glucose further than either stimulus alone. In Calu3 cells, proinflammatory challenge in combination with elevated basolateral glucose concentrations (mimicking hyperglycemia) raised ASL-glucose concentrations further than either perturbation alone [62]. In humans, people with airway inflammation (CF) and diabetes had greater ASL glucose (4.0 ± 2.1 mM) than people with either CF (2.0 ± 1.1 mM) or DM (1.2 ± 0.7 mM) alone [89]. By contrast in CF transmembrane conductance regulator knockout (CFKO) mice, BAL glucose concentrations were increased by hyperglycemia (from 0.1 mM in nondiabetic to 0.6 mM in streptozocin-treated hyperglycaemic animals), but were not different from BAL concentrations in WT nondiabetic and hyperglycemic animals [118]. Interspecies differences can be explained by the lack of respiratory pathology and airway inflammation in mice compared to humans with CF disease.

3.4 ABNORMAL EPITHELIAL GLUCOSE HOMEOSTASIS AND LUNG INFECTION

In cell culture and animal models, disruption of airway glucose homeostasis increases luminal bacterial infection. Increased ASL glucose is also associated with increased respiratory infection in patients with critical illness who require endotracheal intubation. Mechanisms underlying the relationship between elevated ASL glucose and respiratory infection include direct stimulation of bacterial growth through increased nutrient availability and inhibition of local innate immunity.

3.4.1 Abnormal Airway Glucose Homeostasis and Bacterial Growth

Cell culture. Epithelial cell-bacterial co-culture models have been used to investigate the effect of abnormal airway glucose homeostasis on respiratory pathogen growth/survival. These models use human airway epithelial cell monolayers grown at air−liquid interface. The apical cell surface is inoculated with respiratory bacterial pathogens and glucose concentrations are altered in the medium bathing the basolateral cell surface. After incubation, the apical surface is washed to retrieve the bacteria for analysis.

3.4.1.1 Staphylococcus aureus

In laboratory culture, glucose at concentrations seen in airway secretions stimulated a dose-dependent increase in growth rate of *S. aureus* over and above the effect of nutrient peptone broth [112]. In H441 airway epithelial co-cultures, increasing basolateral glucose from 10 to 20 or 40 mM produced a stepwise increase in apical *S. aureus* 8325-4 colony forming units (CFU) (by 250% and 500%, respectively). Basolateral glucose elevation also stimulated *S. aureus* apical CFU where the bacterial strain (JE2) or epithelial cell type (human primary bronchial epithelial cells) were varied [116]. Infection of epithelial monolayers with *S. aureus* reduced transepithelial resistance and increased paracellular glucose flux.

Apical CFU of *S. aureus* JE2 were increased when either glucose or fructose concentrations were increased in the basolateral compartment [115]. Fructose had a greater effect on *S. aureus* JE2 CFU than glucose. This can be explained by preferential uptake of glucose across apical and basolateral membranes compared to fructose, consistent with the presence of GLUT2/10 limiting glucose accumulation in apical fluid. Where experiments were performed with mutant *S. aureus* JE2 strains with transposon-disrupted sugar transport genes, apical bacterial growth was significantly less when basolateral fructose rather than glucose was elevated. This provides evidence that *S. aureus* directly utilizes sugars present in ASL for growth [115].

3.4.1.2 *Pseudomonas aeruginosa*

In laboratory culture in the absence of glucose, neither minimal growth media nor ASL in vitro supported the growth of *P. aeruginosa*. However, glucose stimulated the growth of *P. aeruginosa* in a dose-dependent manner when added to either of these media [46] or nutrient peptone broth [112]. Low-dose inocula of *P. aeruginosa* up to 2.3 log CFU were effectively killed by HBEC monolayers [46]. However, effective killing of *P. aeruginosa* at inocula of 1.5 and 2.5 CFU was prevented when apical glucose concentrations were increased directly or by pretreatment with the GLUT transporter inhibitor phloretin [46]. In HBEC, increasing basolateral glucose concentrations from 5 to 15 mM increased apical *P. aeruginosa* CFU over 7 h by 81% in non-CF cells and 137% in CF cells [62]. In Calu-3 monolayers, *P. aeruginosa* filtrate reduced transepithelial resistance and increased paracellular glucose flux, increasing apical glucose concentrations from 0.6 ± 0.1 to 1.1 ± 0.1 mM at 5 mM basolateral glucose and to 5.8 ± 0.8 mM at 15 mM basolateral glucose. As in HBEC, an increase in basolateral glucose concentration from 5 to 15 mM increased apical CFU of *P. aeruginosa* on Calu-3 monolayers by $94 \pm 19\%$. However, an increase in bacterial CFU was not seen when basolateral D-glucose was replaced by L-glucose, a nontransportable, nonmetabolizable analog, indicating a direct role for basolateral glucose in stimulating apical bacterial growth [62]. In Calu-3 monolayers, innate immunity was enhanced by increasing the volume or alkalinity of apical fluid by stimulation with forskolin or by removing the overlying mucus layer. Under these conditions, apical growth of *P. aeruginosa* still remained higher with 15 mM compared to 5 mM basolateral glucose. Thus, elevated airway glucose concentrations may tip the balance between bacterial growth and killing in the airway in favor of bacterial growth and infection.

Animal models. In mouse and rat models of respiratory infection, the airways are inoculated via the nose with respiratory bacterial pathogens. After a variable period of incubation, the animals are sacrificed and the lungs are sampled using diverse techniques including BAL or homogenization of lung tissue for quantification of bacterial growth. Bacterial growth is compared in the lungs of WT mice and those with DM. Diabetic mouse models used include leptin receptor deficient (db/db), leptin deficient (ob/ob), and streptozocin-induced hyperglycemia. One study used a rat model with diabetes induced by a single alloxan injection [76].

Diabetic (db/db) mice had significantly more bacteria in BAL 24 h after infection with *S. aureus* than WT mice, and there was a direct correlation between blood glucose and bacterial numbers [116]. *P. aeruginosa* (PAO1 strain) bacterial counts were higher in lung homogenates from db/db and ob/ob diabetic mice and alloxan-treated diabetic rats than in nondiabetic controls 6 h after inoculation [46,76]. Bacterial counts did not differ between db/db and ob/ob mice, indicating that it was the DM rather than the underlying cause of the diabetes that was responsible for increased bacterial proliferation. In support of this, *P. aeruginosa* (PAO1) bacterial counts were also increased in BAL from streptozocin-induced hyperglycemic mice at 24, 48, and 72-h postinoculation and in lung homogenates at 18-h postinoculation compared to streptozocin-naïve controls [118,120].

Elevated glucose concentrations in lung secretions appear to be directly responsible for increased bacterial growth. Olivera et al. showed that phlorizin (SGLT-1 inhibitor) increased BAL glucose concentrations in nondiabetic and diabetic rats [76]. By contrast, isoproterenol increased translocation of SGLT-1 to the alveolar cell membranes and reduced BAL glucose concentrations in diabetic but not nondiabetic animals. In vitro, proliferation of meticillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* was increased in BAL from phlorizin-treated animals, but reduced in BAL from isoproterenol-treated diabetic rats. In the whole group, bacterial proliferation rates were strongly correlated with BAL glucose concentrations. Findings were replicated in vivo, where *P. aeruginosa* CFU in lung homogenates were increased in phlorizin-treated rats and reduced in isoproterenol-treated diabetic animals.

Several investigators have shown that the increased growth of *P. aeruginosa* in the lungs of diabetic mice is dependent on the ability of the bacteria to use glucose as a nutrient source. The WT *P. aeruginosa* strain PAO1 can use glucose as its sole carbon source. Deletion of genes required for glucose uptake and utilization inhibits the growth of PAO1 in media where glucose is the sole carbon source. Growth of WT, but not mutant PAO1, is increased in the lungs of hyperglycemic mice compared to nondiabetic controls [116].

Humans. We identified a direct relationship between elevated airway glucose concentrations and respiratory infection in patients intubated on our medical intensive care unit (ICU) [147]. Patients with elevated glucose concentrations in bronchial aspirates had a 2.4-fold (95% confidence intervals (CI) 1.5 to 3.8) increased risk of any pathogenic bacteria and a 2.1-fold (95% CI 1.2 to 3.8) increased risk of MRSA in respiratory secretions, compared to those without elevated bronchial glucose concentrations. Elevated bronchial glucose concentrations preceded MRSA acquisition in the majority of patients. These findings were recently reproduced by another group in Egyptian patients [148].

3.4.2 Abnormal Airway Glucose Homeostasis and Innate Immunity

The airways are continuously exposed to bacteria through inhalation and aspiration. Bacteria, which are cleared by host immune mechanisms, including mucociliary clearance, antimicrobial factors, and other components of innate and acquired immunity. Glucose can disrupt this balance, predisposing to infection, not only by stimulating bacterial growth, but also by inhibiting local immune functions.

3.4.2.1 Mucociliary Clearance

Four separate clinical studies found that people with DM have impaired nasal mucociliary clearance compared to those without DM [122–124]. Nasal mucociliary clearance was slower in people with insulin-dependent, compared to those with noninsulin-dependent DM and in those with diabetes for more than 10 years. The mechanisms underlying these observations are unclear, although dehydration of respiratory secretions due to generalized osmotic diuresis may play a role. In support of this, occult mucous plugging of the airways has been associated with diabetic ketoacidosis [125]. More recently, Yu et al. found that high glucose at concentrations found in people with diabetes stimulates production of the MUC5AC mucin in a dose-dependent manner, through mechanisms mediated by matrix metalloproteinases (MMP)-9 [126]. MUC5AC is a gel-forming mucin that contributes to the viscoelastic properties of mucus essential for clearance and protection of the epithelium from pathogens and dehydration. However, overproduction of mucins is an important factor in the morbidity and mortality of chronic airways diseases including chronic obstructive pulmonary disease (COPD) and CF [127].

3.4.2.2 Antimicrobial Factors

The ASL contains a complex mix of antimicrobial factors that kill bacteria and act as a first line of defense against infection. Many of these factors are cationic at neutral pH and kill bacteria by disrupting bacterial phospholipid membranes and dissipating the electrochemical gradient [128]. High-ASL glucose concentrations could reduce the activity of antimicrobial peptides by driving acidification of ASL or interfering with regulation of antimicrobial peptide secretion into ASL by bitter and sweet taste receptors (Fig. 3.5).

3.4.2.3 pH

Glucose taken up by airway epithelial cells is rapidly metabolized, maintaining the gradient for glucose movement into the cell. One of the products of glucose metabolism is lactate, even under aerobic conditions, and this is secreted into ASL through monocarboxylate transporters expressed on the apical cell surface [84,129]. In cell culture, an increase in basolateral glucose concentrations increases ASL lactate and phloretin (GLUT transporter inhibitor) reduces lactate appearance. In mouse models, lactate was increased in BAL from diabetic $GK^{+/-}$ mice compared to nondiabetic controls in a manner proportional to blood glucose concentrations [117]. There was no difference in BAL macrophages between groups, indicating that the source of lactate in BAL from diabetic mice was not from an increased number of inflammatory cells.

Monocarboxylate transporters are lactate-H^+ cotransporters; hence, secretion of lactate into airway secretions has potential to acidify ASL. In normal airway epithelium, this is neutralized by secretion of HCO_3^--rich fluid. Bicarbonate secretion is aberrant in CF airway epithelium due to loss of the CF transmembrane regulator. CF airway epithelia are therefore unable to oppose a glucose-induced increase in lactate-H^+ secretion into ASL. Hence, elevated glucose concentrations could drive ASL acidification in CF epithelium. Reduced ASL pH inhibits the antimicrobial activity of ASL and is associated with reduced bacterial killing in the airways [130]. Abou Alaiwa et al. found that reducing pH from 8.0 to 6.8 reduced the ability of key airway antimicrobial peptides human β-defensin-3 and cathelicidin-related peptide (LL-37) to kill *S. aureus* and *P. aeruginosa* individually and reduced synergism between them [131]. Antimicrobial peptides are more cationic in acidic pH, which may alter their peptide structure, changing interactions with bacterial cell membranes and each other to alter antimicrobial activity (Fig. 3.5).

3.4.2.4 Taste Receptor Signaling

Solitary chemosensory cells present in the nasal epithelium express bitter (T2Rs) and sweet (T1R2/3) taste receptors [111,113]. T1R3 has also been found in similar cells in the airway [60]. T2Rs are G protein-coupled receptors activated by harmful compounds including those secreted by bacteria. Activation of T2Rs in nasal solitary chemosensory cells triggers a calcium wave that stimulates secretion of antimicrobial peptides capable of killing a range of respiratory pathogens. Activation of sweet taste receptors by glucose inhibits production of the calcium wave by T2Rs in a

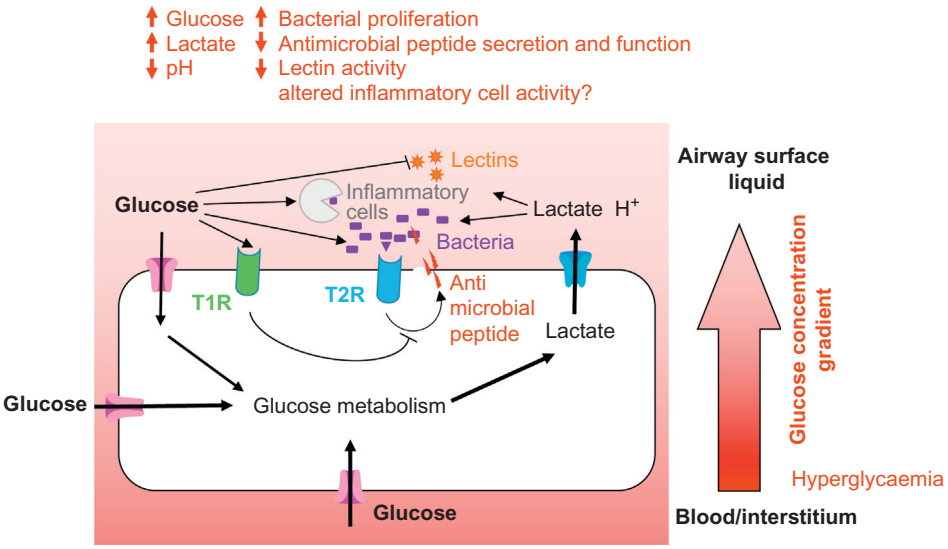

FIGURE 3.5 Effects of abnormal airway glucose homeostasis on infection and local immunity.

dose-dependent manner, and this inhibits antimicrobial secretion. Lee et al. proposed that in times of health, small amounts of glucose in the ASL stimulate T1R2/3, which in turn inhibits T2Rs, regulating secretion of antimicrobial peptides. During infection, bacteria use ASL glucose and secrete products, altering bitter/sweet taste receptor signaling. The fall in ASL glucose reduces the tonic inhibition exerted by T1R2/3 on T2Rs, and the bacterial products directly activate T2Rs, leading to increased secretion of antimicrobial peptides.

This mechanism may be disrupted in people with elevated ASL glucose due to diabetes or epithelial inflammation. Nasal glucose concentrations are elevated in people with acute rhinitis [88] and chronic rhinosinusitis [111] as well as in those with hyperglycemia [119]. Lee et al. hypothesized that elevated ASL glucose disrupts the normal signaling mechanism for the secretion of antimicrobial peptides by preventing sufficient reduction in ASL glucose to remove the tonic inhibition of T1R2/3 on T2Rs. In support of this, they found that it took much longer for *P. aeruginosa* or MRSA to consume the glucose content of a solution containing 1.5 mM glucose than 0.5 mM (Fig. 3.5).

3.4.2.5 *Collagenated Lectins*

Elevated ASL glucose concentrations may disrupt the immune function of C-type lectins, such as mannose-binding lectin and surfactant D [132]. C-type lectins bind oligosaccharides on microbial surfaces, driving complement activation, leading to opsonization, neutralization, and immune activation. In vitro, elevated glucose concentrations inhibit binding of C-type lectin to high-mannose ligands and complement activation via lectin-mediated, but not classical or alternative, pathways [132]. In mice, lung growth of strains of influenza virus sensitive to neutralization by surfactant D was increased in diabetic compared to nondiabetic animals. Viral replication in the lungs was proportional to blood glucose concentrations. Increased replication in diabetic animals was reversed by insulin and abolished by infection with surfactant D insensitive virus [133].

3.4.2.6 *Immune Cell Function*

Glucose in ASL is utilized by inflammatory cells. It is therefore interesting to speculate that the increased flux of glucose into the ASL during inflammation could fuel inflammatory cell function, and that the activity of inflammatory cells in the lung lumen could modify ASL glucose concentration with consequences for taste receptor signaling. Glucose is an important driver of the proinflammatory response in macrophages and influences their phenotype [134,135]. Hyperglycemia was also shown to activate adherent neutrophils. Lactate is known to affect macrophage angiogenic activity, and pH is reported to be important for macrophage recruitment to wound sites. However, whether changes in ASL glucose, lactate, or pH modify local inflammatory cell activity or phenotype (and ultimately their defensive function) is not known.

3.4.3 Implications for Human Health

People with DM are at increased risk of acquiring respiratory infection and have worse outcomes from infection than those without DM. This is a particular problem in people with chronic lung disease where diabetes is a common comorbidity. Although there is little data from clinical studies to determine whether the increased risk of respiratory infection is due to elevated ASL glucose, the common finding of *S. aureus* and *P. aeruginosa* in diabetic airways raises this tantalizing possibility.

3.4.3.1 People With Diabetes Mellitus Without Chronic Lung Disease

People with DM without chronic lung disease are at increased risk of hospitalization, complications, and death from lower respiratory tract infection, including influenza, pneumonia, and tuberculosis.

Influenza. People with DM who develop influenza are more likely to require emergency medical review (odds ratio (OR) 1.68; 95% CI 1.49−1.89) [136], hospital admission (working age adults OR 1.06; 95% CI 1.02−1.10) [137], children (OR 2.34; 95% CI 1.20−4.58) [118], and intensive care support (OR 4.29; 95% CI 1.29−14.3) [138] than people without diabetes. Diabetic patients are more likely to develop complications from influenza, requiring antibiotics or prolonged hospital stay (OR 3.63, 95% CI 1.15−11.51) [139].

Pneumonia. In a primary care study of 39,211 older adults (>65 years), those with DM had a 27%−31% increased risk of hospitalization within 28 days of a diagnosis of community-acquired pneumonia than those without [140]. In an observational prospective study of 19,783 adults, poor glycemic control was associated with increased risk of mortality from influenza, pneumonia, or other acute lower respiratory infections [141].

Tuberculosis (TB). People with DM are around twice as likely as those without diabetes to develop latent TB after contact with an infected person [142]. A metaanalysis of 13 observational studies including 1,786,212 participants with 17,698 TB cases showed that DM was associated with a threefold increased risk of developing active TB infection (relative risk = 3.11, 95% CI 2.27−4.26) [143]. A chronic history of poor glycemic control appears to be an important risk factor for TB. DM is also associated with delays in clearing *Mycobacterium tuberculosis* from sputum during treatment and treatment failure [144]. Diabetic patients have up to fivefold increased risk of death and fourfold increased risk of relapse of TB compared to those without diabetes [145].

S. aureus and P. aeruginosa. Patients intubated on our medical ICU who had elevated glucose concentrations in bronchial aspirates had a 2.1-fold (95% CI 1.2 to 3.8) increased risk of MRSA in respiratory secretions, compared to those without elevated bronchial glucose concentrations. Elevated bronchial glucose concentrations preceded MRSA acquisition in the majority of patients [146,147].

Studies of nasal colonization with *S. aureus* in diabetes have found conflicting results depending on the population studied. One group found that nasal carriage of meticillin-sensitive *S. aureus* (MSSA) was increased in people with DM compared to those without and that risk factors for carriage included insulin treatment, duration of diabetes, and poor glycemic control [148]. Other investigators have found no difference in MSSA colonization in diabetes. In cross-sectional studies, DM is a risk factor for nasal colonization with MRSA [149,150]. A longitudinal study of more than 18,000 patients admitted to acute hospitals identified diabetes as a risk factor for conversion between noncolonized and colonized states [151,152].

Diabetic patients with chronic rhinosinusitis undergoing sinus surgery were more likely than those without diabetes to have *P. aeruginosa* (26% vs 8%; $P = 0.004$) or other gram-negative rods (26% vs 9%; $P = 0.013$) cultured from sinus samples [152].

3.4.3.2 People With Chronic Lung Disease who also have Diabetes

DM is common in people with chronic lung disease. DM affects 3%−37% people with COPD, depending on the population studied, and is 1.4−2.0 times more common in people with COPD than in those without [153]. DM is the commonest complication of CF, affecting around 20% of adolescents and up to 50% adults [154]. During exacerbations of chronic lung disease, people with COPD and CF develop diabetic glucose tolerance and hyperglycemia due to the metabolic stress of acute illness [121,155]. Patients with chronic lung disease who also have DM have more exacerbations with worse outcomes than those without. They also have altered respiratory microbiology.

COPD. COPD patients with comorbid DM are more likely to exacerbate and have more frequent exacerbations than those without diabetes [156]. Fasting blood glucose was positively correlated with exacerbation frequency ($r = 0.55$, $P < 0.001$) [157]. In COPD patients hospitalized for exacerbations, those with DM tended to stay longer (DM, 7.8 days; no DM, 6.5 days) and were more likely to die during (DM, 8%; no DM, 4%) [158] or in the 24 months after

hospitalization hazard ratio 2.25 (95% CI 1.28−3.95) than those without diabetes [159]. Acute hyperglycemia during hospitalization was associated with an increased risk of death or prolonged hospital stay, with the absolute risk of adverse outcomes increasing by 15% (95% CI 4−27) for each 1 mmol/L increase in blood glucose [121]. COPD patients with DM hospitalized for exacerbations are more likely to have gram-negative organisms cultured from sputum [160]. Acute hyperglycemia during hospital admission is associated with increased likelihood of isolating multiple pathogens and *S. aureus* from sputum [121].

Cystic fibrosis. CF-related DM (CFRD) is an independent risk factor for CF pulmonary exacerbations [161,162] and for failure of intravenous [163] or oral [164] antibiotic treatment. Poor glycemic control, measured using glycated hemoglobin (HbA_{1c}), was positively correlated with the number of respiratory infections [165]. Hyperglycemia is associated with an increased risk of lung colonization by *Pseudomonas aeruginosa*. In CF patients with NGT, 83% of those with, but only 44% of those without, intermittent hyperglycemia were colonized with *P. aeruginosa* [166]. Patients with CFRD were more likely than those with normal glucose tolerance to be coinfected with *S. aureus* and *P. aeruginosa*, compared to infection with only one pathogen. Coinfection was associated with an increased frequency of pulmonary exacerbations [167]. CFRD was associated with a 1.6-fold increased risk of multiple antibiotic-resistant *P. aeruginosa* [168]. Colonization with *Burkholderia cepacia* complex was correlated with worsening glucose tolerance category [169].

3.4.4 Drugs that Restore Airway Glucose Homeostasis

Drugs acting on airway epithelial tight junctions, GLUT expression and blood glucose concentrations can restore airway glucose homeostasis and lower ASL glucose.

3.4.4.1 Tight Junctions

Metformin, an AMP-activated protein kinase (AMP-kinase) activator used clinically to lower blood glucose in people with type-2 DM, reduces the permeability of airway epithelial tight junctions to glucose, limiting movement of glucose into ASL.

In H441 cell monolayers, metformin treatment (0.03, 0.3, and 1 mM for 18 h) induced a dose-dependent increase in transepithelial electrical resistance, indicative of an effect on intracellular junctions [116]. This effect was inhibited by compound C, an AMP-kinase inhibitor. Infection of the apical surface of monolayers with *S. aureus* reduced transepithelial electrical resistance and increased paracellular flux of L-glucose (a nontransportable, nonmetabolizable glucose analog). Pretreatment of monolayers with metformin (1 mM for 18 h) before *S. aureus* infection reduced the fall in transepithelial electrical resistance and reversed the effect of *S. aureus* on paracellular glucose flux. Metformin attenuated the rise in ASL glucose concentrations caused by raising basolateral glucose from 10 to 40 mM by ∼50%.

In Calu-3 monolayers, metformin treatment (1 mM for 18 h) increased transepithelial electrical resistance across Calu-3 monolayers and increased expression of the tight junction proteins claudin-1 (by 16 ± 8%) and occludin (by 38 ± 9%) [91]. Addition of *P. aeruginosa* to the apical surface of monolayers significantly reduced transepithelial electrical resistance and induced a 48 ± 6% increase in the rate of paracellular basolateral-to-apical glucose flux. *P. aeruginosa* addition reduced abundance of claudin-1 and occludin and caused the appearance of occludin cleavage fragments. Metformin pretreatment ameliorated the *P. aeruginosa*-induced fall in transepithelial electrical resistance and increase in paracellular glucose flux and reduced the reduction in claudin-1 abundance and appearance of occludin cleavage fragments.

In streptozocin-induced hyperglycemic mice, metformin pretreatment (200 μL 4 mg/mL intraperitoneally daily for 3 days) did not alter blood glucose, but reduced BAL glucose to the same level as in naïve animals [118]. Metformin pretreatment (compared to phosphate-buffered saline) reduced L-glucose flux across ex vivo tracheas from *S. aureus*-infected mice by 40 ± 5% [116].

A number of other drugs have been shown to alter tight junction permeability in airway epithelium. While they have potential to modulate ASL glucose concentrations, to our knowledge this has not been tested.

Glucocorticoids. In Calu 3 cells and transformed human bronchial epithelial cells cultured at air interface, dexamethasone, fluticasone proprionate, and budesonide treatment for 5 days increased transepithelial electrical resistance and reduced epithelial permeability to dextran by potentiating tight junction formation [170]. In primary HTECs and H441 cells cultured at air interface, glucocorticoids decreased paracellular permeability, shifted the ion permselectivity of tight junctions toward Cl^- and upregulated expression of claudin-8, a protein responsible for recruitment of occludin at the tight junctions [171].

1,25-Dihydroxyvitamin D3 (1,25(OH)2D3). In transformed human bronchial epithelial cell monolayers, 1,25(OH) 2D3 partially prevented the decline in transepithelial electrical resistance, reduced occludin expression and redistribution of E-cadherin induced by toluene diisocyanate (TDI). In sensitized mice, intraperitoneal 1,25(OH)2D3 prior to challenge with TDI increased E-cadherin and ZO-1 expression at the cell−cell contact sites [172].

PPAR gamma agonists. In transformed cultures of human nasal epithelial cells, treatment with the PPAR gamma agonist rosiglitazone increased transepithelial electrical resistance and reduced paracellular flux of dextran [173]. Rosiglitazone increased the expression of tight junction proteins occludin, tricellin, claudin-1 and -4 and increased the number of tight junction strands seen on freeze fracture analysis using electron microscopy. Inhibition of these effects by a protein kinase C inhibitor indicates that they may be mediated via a protein kinase C signaling pathway.

Azithromycin. In an immortalized bronchial epithelial cell line (VA10), azithromycin increased transepithelial electrical resistance in a dose-dependent manner and induced processing of the tight junction proteins claudin-1 and claudin-4, occludin, and junctional adhesion molecule-A [174]. Infection of monolayers by *P. aeruginosa* reduced transepithelial electrical resistance and caused tight junction protein rearrangement. These effects were attenuated by pretreatment with azithromycin [175].

3.4.4.2 Glucose Transporters

Facilitative GLUTs. Proinflammatory mediators (TNF-α, IFN-γ, and LPS) enhanced glucose uptake across the apical, but not basolateral, membrane of H441 and HBE monolayers [65]. The increase in glucose uptake was largely inhibited by phloretin, indicating increased GLUT transport that was accompanied by an increase in GLUT2 and GLUT10 abundance. The mechanism underlying inflammation-induced upregulation of apical GLUT transport has not been elucidated, and to date no drugs have been identified that exert this effect. Metformin did not affect glucose uptake by airway epithelial cell monolayers [116]

Sodium glucose cotransporters (SGLT). Oliveira et al. used immunohistochemistry to investigate SGLT1 protein distribution in alveolar cells [76]. In animals treated with isoproterenol, a beta adrenergic agonist that activates the cAMP-PKA pathway, they found increased SGLT1 translocation from the intracellular pool to the luminal membrane. By contrast in animals treated with the inhibitor phlorizin, they were unable to detect SGLT1 in alveolar cell sections. These changes in transporter expression were accompanied by changes in lung glucose homeostasis. Isoproterenol reduced glucose concentrations in BAL from diabetic mice, but not in nondiabetic mice in whom concentrations were already very low. Phlorizin increased BAL glucose concentrations in both nondiabetic and diabetic mice. In clinical practice, beta adrenergic agonists are widely prescribed for patients with airways disease and long acting beta adrenergic agonists reduce the frequency of exacerbations in people with COPD [176]. In contrast, SGLT inhibitors are prescribed to increase renal glucose excretion and ameliorate hyperglycemia. While most of these are highly selective for SGLT2, some are less so and can inhibit SGLT1 [177]. The effect of beta adrenergic agonists and SGLT inhibitors on alveolar glucose transport and ASL glucose concentrations has not been tested in humans to our knowledge.

3.4.4.3 Blood−Glucose Concentrations

In healthy volunteers, experimental hyperglycemia resulted in increased glucose concentrations in nasal and lower airway secretions [89,119]. When the infusion was stopped and blood glucose was allowed to return to normal, glucose concentrations in nasal and lower airway secretions returned to baseline levels. These observations indicate that blood glucose control using insulin or oral hypoglycemic drugs has potential to lower ASL glucose concentrations, although there are no published studies confirming this.

3.4.5 Effect of Drugs that Restore Airway Glucose Homeostasis on Respiratory Infection

Drugs that restore airway glucose homeostasis and limit the appearance of glucose in ASL have potential to reduce or prevent lung infection, particularly in the context of hyperglycemia or inflammation.

In H441 monolayers with basolateral glucose concentrations at 10 mM, metformin pretreatment inhibited apical growth of *S. aureus* in a dose-dependent manner. Metformin completely prevented the increase in *S. aureus* growth induced by increasing basolateral glucose from 10 to 40 mM [116]. Where glucose concentrations were 10 mM on both sides of the monolayer, metformin did not reduce growth of *S. aureus*, indicating that its effect was to prevent glucose movement across the epithelium. Metformin pretreatment also reduced *S. aureus*-induced secretion of cytokines including interferon gamma, IL-1 alpha, IL-6, CXCL9, and TGF-beta. In Calu-3 airway epithelial cell monolayers, metformin

pretreatment inhibited apical growth of *P. aeruginosa* in the presence of 5 and 15 mM glucose and at metformin concentrations as low as 20 μM [62].

In diabetic (db/db) mice, 48 h metformin treatment prior to infection reduced *S. aureus* numbers retrieved from BAL to numbers seen in BAL from normoglycemic mice [116]. This short period of metformin treatment was insufficient to lower blood−glucose concentrations, indicating that metformin was exerting its effect directly on airway glucose homeostasis. In support of this, metformin reduced glucose flux across ex-vivo murine tracheas. In streptozocin-treated hyperglycemic mice, metformin pretreatment strikingly reduced bacterial numbers in BAL after *P. aeruginosa* infection to those seen in nondiabetic animals [118]. In this model, metformin reduced BAL glucose but not blood glucose in diabetic animals, providing further support for an in-vivo effect of metformin on airway glucose homeostasis.

In humans, observational studies and small clinical trials have indicated that people with DM and chronic lung disease who take oral hypoglycemics such as metformin or thiazolidinediones, compared to those taking other hypoglycemic medicines, have less respiratory infections.

Metformin. In an 11-year-retrospective cohort study, using the Taiwan National Health Insurance Research Database, investigators looked at rates of asthma-related hospitalization and exacerbations in people with concurrent asthma and diabetes [178]. They found that patients using metformin had a lower risk of asthma-related hospitalization (OR 0.21; 95% CI 0.07−0.63) and asthma exacerbation (OR 0.39; 95% CI: 0.19−0.79) compared to those taking other medication. Metformin can be given safely to patients with COPD even during exacerbations [179] and, in those with DM, appears to be associated with improvements in lung function [180], symptoms [181], and survival [179]. However, there are no published studies looking at the relationship between metformin treatment and COPD exacerbation rates.

Thiazolidinediones. In a cohort study of U.S. veterans with diabetes and COPD, those taking thiazolidinediones ($n = 7887$) had a significant reduction in the number of COPD exacerbations compared to those taking alternative oral antihypoglycemic medications ($n = 42{,}347$) (incident rate ratio 0.86; 95% CI 0.81−0.92) [182].

Insulin. Several small clinical studies have shown an association between treatment of CF-related prediabetes or diabetes with insulin and reduction in respiratory infection and exacerbations. Lanng et al. performed a retrospective case-control study of 18 diabetic and 18 nondiabetic patients with CF, matched for sex, age, and the presence of chronic *P. aeruginosa* infection [183]. They reviewed parameters of CF clinical status for 6 years before and 2 years after the diabetic patients commenced insulin therapy. Insulin improved lung function in the diabetic patients and reduced the percentages of sputum cultures positive for *Haemophilus influenzae* and *Streptococcus pneumoniae*.

Franzese et al. compared exacerbation rates in the 6 months before and 6 months after commencement of treatment with insulin glargine in four people with CFRD and four CF patients with prediabetes [165]. Six CF patients with prediabetes who did not receive glargine acted as controls. During glargine treatment, exacerbation rate fell in both diabetic (before glargine, 3.8 ± 0.5; during treatment, 1.8 ± 0.9) and prediabetic (before glargine, 2.8 ± 0.5; during treatment, 1.3 ± 0.5) CF patients. Exacerbation rate did not change in control patients who did not receive insulin (first 6 months 3.3 ± 1.2; second 6 months 3.1 ± 0.4).

Mozzilo et al. treated 22 children with CF and abnormal glucose tolerance with insulin glargine in an open label study over 1 year. In the 12 months prior to glargine treatment, participants had 4.1 ± 0.6 exacerbations. This reduced by 42% on glargine treatment to 2.4 ± 0.5 during the 12 months on glargine treatment [184].

3.4.6 Glucose Transporters in Lung Cancer

It is important to note that GLUT transporters are also present in epithelial derived lung cancers particularly nonsmall cell lung cancer such as adenocarcinomas. Glucose metabolism is known to be increased in tumor cells, feeding growth and proliferation, and GLUT1 and GLUT3 have been reported to be upregulated in several studies of lung tumor cells [185]. This has led to investigations into the use of positron emission tomography (PET) to measure glucose accumulation in tumor cells using fluorescent analogs of glucose such as 2-fluoro-2-deoxyglucose (FDG) which is transported but not metabolized. PET scans showed detectable accumulation of FDG in tumors, and it remains an important diagnostic tool in cancer detection and treatment. However, FDG can also be taken up by other metabolically active nontumorigenic cells, such as those at sites of inflammation, including epithelial cells stimulated by pathogens/immune responses (see above). This creates significant background noise and makes the use of FDG-PET to detect early development of tumor cells difficult. There is also evidence that GLUT1/3 expression did not correlate to tumor size or rate of tumor development, and FDG-PET is not a good measure by which to stage lung tumors [186].

The identification of GLUT1/3 in lung tumors has also led to the targeting of these transporters for therapeutic benefit with the use of compounds that inhibit glucose metabolism (For review see [187]). In addition, two naturally derived compounds with antitumorigenic activity have been shown to inhibit glucose uptake via GLUT1. The flavone apigenin

(5,7,4 trihydroxy flavone) and the antimalarial drug dihydro artemisin reduced GLUT1 expression and inhibited lung cell cancer growth [188,189].

What is particularly encouraging about such studies is that the targets for cancer therapy are different to those involved in glucose and fluid homeostasis in the lung, reducing the potential for damaging respiratory side effects.

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Chapter 4

Pulmonary Surfactant Trafficking and Homeostasis

Joseph L. Alcorn

4.1 INTRODUCTION

The metabolic processes responsible for maintenance of life require efficient exchange of oxygen into and carbon dioxide out of the bloodstream, and the alveolus is the site where this exchange takes place in the lung. Alveoli are the numerous and very tiny sacs in the lung that are surrounded by a network of capillaries. Efficient gas exchange in the alveolus is dependent on a very thin cell layer with a very large surface between air and blood. This architecture suggests a fragility that belies the actual resilience of the alveolus in the face of forces endured over a lifetime of breathing requiring repetitive expansion and collapsing of alveoli. A thin liquid lining layer covering the alveolar surface called the hypophase generates high surface tension forces at the air/liquid interface when the alveoli collapse during exhalation and makes the structure of the alveolus unstable. This increased surface tension would make expansion of the alveolus difficult during subsequent inhalation. Without the advent of a biological solution to reduce alveolar surface tension, the lung's primary function to adequately oxygenate blood would not be possible, and the biological solution to this problem is pulmonary surfactant. Surfactant is secreted by specialized alveolar epithelial cells and coats the hypophase to dramatically reduce surface tension at the air/liquid interface. However, the constantly changing alveolar structure and onslaught of damaging environmental factors that accompanies breathing can damage pulmonary surfactant components and structure, adversely affecting surfactant function. Maintenance of this function requires constant regeneration of secreted surfactant. This chapter will discuss mechanisms involved in homeostasis of pulmonary surfactant: the cell solely responsible for biogenesis of surfactant (alveolar type II epithelial cells), the organelles in which surfactant is stored until secretion into the lumen of the alveolus (lamellar bodies (LBs), the components of exocytic pathways responsible for regulated secretion of surfactant and the components of the endocytic pathways involved in recycling of secreted surfactant.

4.2 THE ALVEOLUS

Efficient gas exchange in the alveolus occurs through simple diffusion and therefore is dependent on a very thin cell layer with a very large surface. The main site of gas exchange in the lung occurs in small, sac-like hollow structures called alveoli that are comprised of epithelial cells bound by capillaries and found at the terminal ends of the respiratory tree. With the presence of the hypophase, an alveoli resembles a gas bubble in water: the wet surface of the cells surround a central air space with a diameter that ranges from 150 to 500 μm (250 μm average). The alveolar epithelium is largely composed of two cell types, squamous type I and cuboidal type II alveolar epithelial cells (or pneumocytes) that can be distinguished morphologically and functionally. Type I cells are large and flat cells that have a surface area of about 5000 μm^2 per cell and cover about 95% of the alveolar surface. The remaining surface (5%) of the alveolus is covered by type II cells that have an apical surface area of about 250 μm^2 per cell [1]. Despite the huge difference in size and coverage, type I cells comprise about 8% of the total cells in the lung parenchyma while type II cells comprise about 15%, a 1:2 ratio [2,3]. Adjacent alveoli are separated from each other by the alveolar septum that consists of the basement membranes of alveolar epithelial cells lining the alveolus and endothelium from the capillaries surrounding

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each alveolus. Elastic fibers, type I collagen, interstitial cells and smooth muscle cells can also be found in the septum to give alveoli its structure and elasticity. Alveolar capillaries flowing within the septum come in contact with at least two alveolar structures on each side, maximizing gas exchange. This architecture gives the lung parenchyma a delicate honeycomb-like structure composed of about 480 million alveoli in adult human lung that provides a huge surface area (more than 70 m^2) for gas exchange [4]. The individual properties of the two epithelial cell types that comprise the inner surface of the alveolus are vital in the function, maintenance and defense of this huge area.

4.2.1 Alveolar Type I Epithelial Cells

Type I cells are very thin (0.2 μm thick), quiescent cells with compact nucleus surrounded by a sliver of cytoplasm containing minimal organelles, indicating little metabolic activity [5]. From the nucleus, these cells extend like a thin sheet to cover the capillaries. This leaflet is essentially two plasma membranes forming the apical and basal cell faces with a very small amount of cytoplasm and essentially no organelles in between. The basal membranes of the epithelium and endothelium become fused to form a minimal barrier. This cellular morphology reduces tissue resistance and allows free diffusion of gases at the alveolar surface into the capillary blood. Due to the lack of metabolic activity, it was thought that these cells only passively serve in gas exchange. However, they also form an important barrier between the air in the alveolar lumen and blood surrounding the alveolus and are able to sense microbial products and generate inflammatory responses [6,7]. Despite the lack of organelles in much of the cell volume, there are numerous vesicles implied in the transcellular transport of molecules. Since type I cells express proteins involved in transport of water (aquaporin-5) and ions, they are suspected to be important in the regulation of alveolar fluid and ion balance [8,9]. However, the inability to culture type I cells in vitro and to generate conditional knockout mice has prevented definitive evidence for these functions [10]. With regard to the subject of this chapter, type I cells appear to influence surfactant secretion by type II cells in response to stretch [11,12]. Due to their large size, they also have great potential to be damaged by environmental insults that accompany each breath. However, type I cells do not undergo mitosis and are considered to be terminally differentiated [11]. Thus, damaged type I cells cannot replace themselves and must be replaced by a progenitor cell, the type II cell.

4.2.2 Alveolar Type II Epithelial Cells

Unlike squamous type I cells, alveolar type II epithelial cells are cuboidal with the characteristics of a secretory cell: distinct apical microvilli, a large nucleus, abundant cytoplasm with mitochondria and extensive endoplasmic reticulum (ER) and Golgi apparatus [13]. While type I cells are generally passive in nature, type II cells are highly metabolic, allowing these cells to perform three important activities that keep the alveolus functioning normally: surfactant production, surfactant homeostasis, and repair of the alveolus following injury (type II cells are the progenitor cells for type I cells) [14]. The most distinct morphological feature of type II cells is the presence of LBs in the cytoplasm, the organelle responsible for assembly, storage and secretion of pulmonary surfactant [15]. Although other cells can express some of the components of pulmonary surfactant, the type II cell is the only cell that synthesizes all the surfactant components (lipids and proteins) and assembles them into a structure that allows regulated secretion of functional surfactant [16]. Although the role of type II cells in production and homeostasis of surfactant is the focus of the chapter, a well-tempered type II cell engages in a multitude of functions required for maintenance and optimal functioning of the alveolus. Type II cells are equipped with a number of membrane-bound water channels and ion pumps that are effective in keeping the alveolar space free of excess fluid and ions by transporting sodium and fluid from the apical surface of the alveolus into the interstitium of the lung [13,17]. In this manner, type II cells maintain the hypophase and control its properties, such as pH and the concentration of calcium [18]. Regulation of these parameters is crucial as the hypophase is considered to be the reaction milieu for extracellular biochemical processes including the proper extracellular transformation of secreted surfactant into an efficient, surface tension-reducing film. Type II cells also play a critical role in innate immunity through production of the collectins surfactant protein-A (SP-A) and SP-D, proteins that bind to the surface of pathogens and act as opsonins to facilitate their elimination by alveolar macrophages [19]. In addition, type II cells secrete various cytokines in response to pathogens and alveolar damage to signal recruitment and initiate activation of macrophages to defend the alveolus [20]. Finally, type II cells express high levels of xenobiotic-metabolizing enzymes and are important in detoxifying inspired compounds that may cause alveolar damage [21]. Truly, these four major functions of type II cells: surfactant synthesis and secretion, xenobiotic metabolism; maintenance of the hypophase and regeneration of the alveolar epithelium make the alveolar type II cell the “multifunctional pneumocyte” [22].

4.2.3 Other Cells of the Alveolus

Aside from type I and type II epithelial cells, the alveolar surface also consists of other cell types, the very rare pulmonary brush cell which can be found in some specific regions near the entrance of the acinus, and resident macrophages [23,24]. With respect to alveolar function, resident macrophages play a crucial role in both defense of the alveolus from pathogens (bacteria and viruses) and removal of damaged secreted surfactant. Alveolar macrophages, which are located in the airway lumen, are characterized by high expression of CD11c and the lack CD11b expression, which distinguishes them from macrophages present in other tissue compartments [25]. These macrophages are the predominant front-line innate defense cells in the alveolus that receive pro- and antiinflammatory signals from the alveolar epithelium and are responsible for activating inflammatory responses in order to eliminate invading pathogens [26–28]. Aside from this important function, alveolar macrophages also play an important role in homeostasis of secreted surfactant by endocytosing and catabolizing surfactant lipids and proteins through activation of the granulocyte/macrophage-stimulating factor (GM-CSF) system in the alveolus [29,30]. Although this chapter focuses on the mechanisms of surfactant lipid and protein transport in epithelial cells of the lung, the importance of surfactant catabolism by macrophages cannot be understated and a more complete description of these mechanisms will be considered later in this chapter.

4.3 PULMONARY SURFACTANT

Pulmonary surfactant is the remarkable, highly surface-active lipoprotein complex (phospholipoprotein) that is present in and on the fluid lining the alveolar surface of the lungs. The inaccessibility of surfactant makes its exact in-vivo composition unknown, but based on studies using broncholaveolar lavage and in-vitro lung preparations, the major components of surfactant are lipids (approximately 90% by surfactant mass) and proteins (10% of surfactant mass) (See Fig. 4.1). The lipids that comprise surfactant are mainly amphipathic (both hydrophilic and hydrophobic

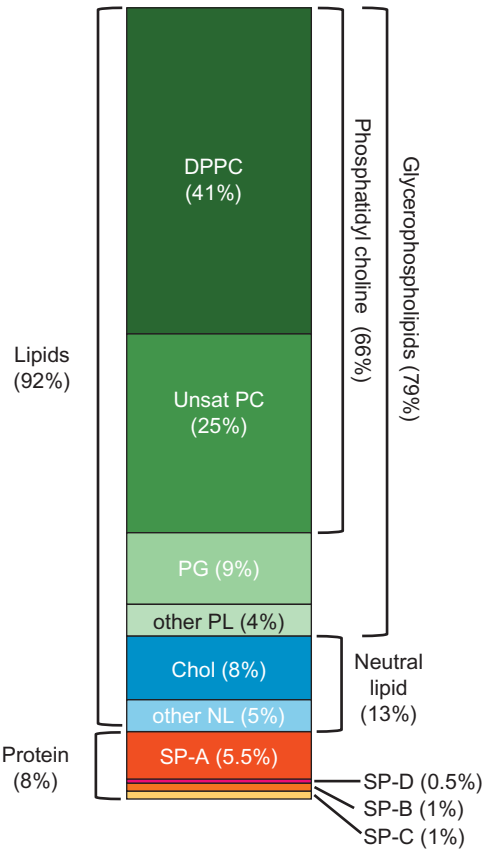

FIGURE 4.1 Composition of pulmonary surfactant. Graphical representation of the contribution of specific lipids and proteins to the mass of surfactant (in percentages). *DPPC*, dipalmitoyl phosphatidylcholine, *Unsat PC*, unsaturated phosphatidylcholine, *PG*, phosphatidylglycerol, *PL*, phospholipids, *Chol*, cholesterol, *NL*, neutral lipid.

properties) with head groups possessing charged qualities [31]. At the air/liquid interface of alveoli, surfactant forms a single layer of lipids with the hydrophilic, polar head groups of the lipids oriented to the interfacial water molecules while the hydrophobic tails face towards the air in the lumen, spontaneously forming a stable film at the interface. This interaction dramatically reduces the net intermolecular cohesive forces of the liquid surface such that surface tension of the hypophase is reduced from approximately 70 mN/m at physiological temperature to close to 0−1 mN/m [32−34]. While it is the unique properties of the main lipid component of surfactant, dipalmitoylphosphatidylcholine, that allows this great reduction in surface tension, other charged lipid species, neutral lipids and proteins participate in the biophysical function of surfactant to reduce surface tension by maintaining the structure of the surfactant film on the hypophase. Physiologically, pulmonary surfactant functions to increase pulmonary compliance, prevent atelectasis (collapse of the lung) at the end of expiration and facilitate recruitment of collapsed airways during inhalation. Pulmonary surfactant also has a nonbiophysical function to protect the lungs from injuries and infections caused by inhaled particles and microorganisms [14,35].

4.3.1 Surfactant Lipids

The biophysical activity of surfactant to reduce alveolar surface tension depends entirely upon its lipid composition. Although the composition of mammalian tissues contain a wide variety of lipid species, the lipid composition of pulmonary surfactant is unusually simple, largely phospholipids (~80% of surfactant by mass), especially the zwitterionic phosphatidylcholines, with some neutral lipids (primarily cholesterol) [36]. However, the contribution of each lipid component to surfactant mass has great impact on both the ability of surfactant to reduce surface tension and to spread over the alveolar surface. Depending on the temperature, a film composed of specific types of lipids can exist in either a fluid liquid-crystalline state or in a solid-gel state, and transition between these two states occurs at the phase transition temperature characteristic to that specific lipid. Since the lipid composition of pulmonary surfactant is relatively simple, temperature can have a profound influence on its structure and function to reduce surface tension. As the body temperatures of mammals range from around 97° to 103°F, the lipid composition of surfactant is highly conserved among mammals, but varies when compared to classes of vertebrates (i.e., reptiles, birds), suggesting that the ratio of specific lipids is altered to provide optimal biophysical activity in the context of the physiological characteristics of specific animals (i.e., body temperature, lung structure) [37,38].

4.3.1.1 Phosphatidylcholines and Dipalmitoyl Phosphatidylcholine

The class of phospholipids that incorporate choline as a head group is called phosphatidylcholine (PC) and is a major constituent of cell membranes that is more commonly found in the outer leaflet of the cell membrane where it plays a role in membrane-mediated cell signaling. In most tissue extracts, PC would be expected to be present at up to 50% of the total phospholipid, but PC is by far the most abundant lipid in surfactant, representing about 60%−70% of total mass, and comprises about three-quarters of phospholipids in alveolar surfactant [38]. PC may be present in both its unsaturated forms (17%) which include palmitoyl−palmitoleoyl phosphatidylcholine (PC16:0/16:1), palmitoyl−oleoyl phosphatidylcholine (POPC) (PC16:0/18:1) and POPC (PC16:0/18:2), or in its saturated forms, that include the disaturated phospholipid, dipalmitoyl phosphatidylcholine (DPPC) (PC16:0/16:0) which constitutes about ~50% of surfactant PC along with significant levels of palmitoyl−myristoyl phosphatidylcholine (PC16:0/14:0) [38,39]. Why the unique prevalence of DPPC in pulmonary surfactant? Since DPPC possesses fully saturated palmitic acid chains, DPPC molecules can be tightly compressed. During exhalation, this compression allows elimination of water molecules from the interface of hypophase of the collapsing alveolus, markedly reducing surface tension [40]. On the other hand, unsaturated phospholipids, with their kinked side chains, cannot reach a similar packing density and unable to reduce surface tension as efficiently.

4.3.1.2 The Acidic Phospholipids: PG and PI

The second most abundant phospholipid in surfactant is phosphatidylglycerol which comprises ~7%−15% of the total phospholipid of surfactant [41]. This anionic species of phospholipids is normally found in only trace amounts in mammalian tissues, so the presence of significant amounts of PG is another interesting feature of pulmonary surfactant and has been suggested to be a "marker" component [42]. Although the role of PG has not been defined, it may play a role in both alveolar stability by promoting even spreading of the surfactant monolayer over the surface of alveoli (the spreading properties of PG are insufficient) and in the innate immune response since PG suppresses viral infection and inflammatory responses in the lung [43−45]. Because of its low levels in surfactants of some air-breathing animals

such as cat and chickens, these roles of PG remain somewhat unclear. However in those cases, PG appears to be replaced by another negatively charged phospholipid, phosphatidylinositol (PI), which has a biosynthetic route in common with PG [36]. In general, when PI levels of surfactant are higher, PG content is lower. Surfactant from fetal lungs usually contains higher levels of PI but this is generally reverted in adult life. Both PC and PI may enhance the adsorption of DPPC to the air−liquid interface because of their anionic charge [46]. With respect to the proteins of surfactant, PG and PI may direct the preferential distribution of SP-B in disordered regions of surfactant membranes and interfacial films through ionic interactions with the positive charges of SP-B [33,47,48].

4.3.1.3 Remaining Phospholipids

The presence (or relative absence) of other phospholipids in pulmonary surfactant relative to levels in other lipid structures again suggests the need for specialized functions and requirements of the proteolipid complex in the alveolus. Although phosphatidylethanolamine (PE) is the second most prevalent membrane phospholipid in animals, it is only present in minor amounts in pulmonary surfactant and although diphosphatidylglycerol (cardiolipin) is abundant in mitochondria, it is absent in pulmonary surfactant. The total mass of these minor phospholipids in surfactant (PE, sphingomyelin (SM), phosphatidylserine (PS), and lysophosphatidylcholine) generally account for less than 5% of the remaining phospholipid content. The specific roles of each of these minor phospholipids are unclear, but they may have a role in the formation of structures such as tubular myelin or be involved in signaling events in surfactant metabolism [41].

4.3.1.4 Neutral Lipids and Cholesterol

Another significant lipid fraction of surfactant are the neutral lipids (8%−10% by mass), which includes mostly cholesterol (80%−90% of the neutral lipid fraction) but also comprises free fatty acids and acylglycerols [38,49,50]. The interactions of cholesterol with surfactant phospholipids and proteins are complex and are crucial for surfactant properties in the alveolus. It seems that the level of cholesterol in surfactant has been evolutionarily optimized for each species [33]. Cholesterol appears to maintain surfactant film fluidity, thereby promoting surface film respreading over the alveolar hypophase [50]. By disrupting the cohesive forces between the phospholipids, cholesterol lowers the phase transition temperature of the surfactant surface film monolayer [51]. However, although cholesterol generally enhances film adsorption, it also increases minimum surface tension, a seemingly paradoxical biophysical property with regards to the ultimate function of surfactant. Minimal surface tension cannot be achieved because cholesterol cannot be easily squeezed out as a surfactant film comprised of both DPPC and cholesterol is compressed [52]. But without enhanced film absorption, the function of surfactant would be compromised, illustrating the delicate balance of the biophysical properties of each component that results in highly efficient surfactant. Interesting, the importance of cholesterol on surfactant dynamics belies its contribution to surfactant mass; the actual level of cholesterol is much lower in surfactant compared to that found in plasma membranes. The remaining neutral lipids, free fatty acids (predominantly palmitate (16:0)) and monocylglycerides, dicylglycerides, and triacylglycerides are suspected to improve surface activity, as well as the rate of surfactant film adsorption. It is believed that these lipids introduce minor packing defects in the structure of the surfactant film that result in altered spreading and packing properties [53].

4.4 SURFACTANT PROTEINS

While the properties of the unique lipids of surfactant are responsible for the high degree of surface tension-reducing activity of the lipid monolayer on the alveolar hypophase, there are significant challenges to the formation of the active monolayer. Surfactant lipids must make their way from the site of synthesis within the cell to the surface of the hypophase, the fragile nature of the monolayer must withstand the constant expansion and contraction of the alveolus, and the monolayer must be protected from the environmental insults that it encounters with each breath. These critical tasks are accomplished through the activity of the surfactant-associated proteins. The protein component of surfactant (approximately 10% by surfactant mass) consists of serum proteins (2%) and four major surfactant-associated proteins; hydrophilic proteins (SP-A and SP-D) that have roles in the nonbiophysical function of surfactant to protect the lung and in homeostasis of surfactant, and hydrophilic proteins (SP-B and SP-C) that are crucial in the biogenesis of LBs, the organelle that stores and transports surfactant, as well the biophysical function of surfactant to spread and to reduce surface tension [54,55]. Although expression of surfactant proteins has been reported in nonpulmonary cells, albeit greatly reduced, the type II alveolar cell is the primary source of surfactant protein expression in the body and is the only cell that expresses surfactant proteins in association with surfactant lipids [56].

4.4.1 Hydrophobic Peptides: SP-B and SP-C

Although surfactant proteins SP-B and SP-C are of relatively low abundance in pulmonary surfactant (comprising only 1% of total surfactant weight each), they are intricately involved in the ability of surfactant to reduce alveolar surface tension and are crucial in the formation and stabilization of pulmonary surfactant films [20]. Due to their extremely hydrophobic characteristics, SP-B and SP-C interact strongly with surfactant lipids and upon secretion of surfactant to the hypophase, these hydrophobic proteins promote the formation and adsorption of the surface film to the air−liquid interface [56]. As the alveolus expands and contracts, the surface film of surfactant lipids is extended and compressed. Upon compression, the lipids move from the surface of the hypophase to the underlying fluid where the monolayer of lipids transitions to multiple bilayer structures. Due to their association with lipids, SP-B and SP-C are integral to the regulation of this movement, and without them, the integrity of the surfactant film on the hypophase is lost, resulting in excessive alveolar surface tension. The importance of SP-B in this role is illustrated by cases of SP-B deficiency in humans and in mice in which the SP-B gene is ablated, resulting in lethal respiratory distress at birth [22,57]. On the other hand, the lack of SP-C is not lethal at birth, its absence is associated with chronic lung diseases [28,29].

Mature SP-B and SP-C arise from proteolytic processing of larger precursors during their transit through the secretory pathway exclusive to type II alveolar epithelial cells. With SP-B, the resulting peptide is 79 amino acids in length of which 52% of the residues are hydrophobic with a substantial number of basic amino acids, resulting in a net charge of +7 [58]. Mature SP-B has been shown to contain a high α-helical content of about 40%−50%, and 6 of 7 cysteine residues in the peptide form intramolecular sulfhydryl bridges such that three individual α-helices are stabilized. The remaining cysteine group forms an intersubunit disulfide bridge to form SP-B homodimers. The resulting structure allows the amphipathic helical motifs of SP-B to lie on surface of the membranes with phospholipid headgroups, and the highly conserved positively charged amino acids interact specifically with the anionic phospholipid phosphatidylglycerol, and parallel to the plane of bilayers and monolayers, as well as in separate, adjacent membranes [59]. Thus, the SP-B dimer stabilizes lipid intermediates during the transition from bilayers to the interface [60]. SP-B binds membranes which leads to destabilization and fusion, and this ability allows the packaging of surfactant phospholipids into LBs and the transition of secreted LBs to the surface film [20]. The mature SP-C peptide is 35 amino acids in length and is extremely hydrophobic due to the fact that 69% of the amino acids are hydrophobic and two cysteine residues near the amino terminus are palmitoylated [20]. The SP-C monomer is composed almost entirely of a rigid α-helical structure that can span a membrane bilayer, and the palmitate moieties that are present on extramembrane N-terminal region can interact with an adjacent membrane or even with the bilayer where SP-C peptide resides [49]. Unlike SP-B, SP-C is an integral membrane protein, and it properties allows for inducing lipid packing perturbations and association of the surface monolayer with bilayers during compression and expansion of the surfactant film [60].

4.4.2 Hydrophilic Multimers: SP-A and SP-D

Surfactant proteins A (SP-A) and D (SP-D) are large, hydrophilic, multimeric proteins that are members of the collectin family of proteins that are collagen-containing C-type (calcium dependent) lectins that recognize and bind patterns of sugar moieties on the surface of pathogens [61]. Due to this ability to recognize and opsonize invasive pathogens, SP-A and SP-D are important components of the innate host defense system of the lung [56]. SP-A and SP-D also have the ability to bind specific lipid in surfactant, which also positions these proteins in surfactant homeostasis.

As its name implies, SP-A was the first surfactant-specific protein to be characterized. SP-A is the major protein in pulmonary surfactant that is associated with surfactant phospholipids and the hydrophobic surfactant proteins SP-B and SP-C, and represents about 5%−6% of the dry weight of surfactant. In its monomeric form, it is an N-linked glycosylated protein with a molecular mass of about 26 kDa [27]. Structurally, an SP-A monomer can be divided into four structural domains: a short N-terminal segment, a proline-rich collagen-like domain, a neck region and a carbohydrate recognition domain. Through the actions of the collagen-like domain, SP-A monomers trimerize. Six trimers then form an octadecamer (18 monomers) 630 kDa in size where disulfide linkage of the trimers through the N-terminal segments results in a base that is followed by a "stalk-like" structure composed of the collagen-like domains with a kink in the middle which provides a bend that allows the formation of a "bouquet of flowers" structure where the carbohydrate recognition domains become the "flowers" ready to bind pathogens [62]. The other hydrophilic protein, SP-D, accounts only for ~0.5% of the total dry weight pulmonary surfactant, but unlike SP-A, it does not appear to be associated with surfactant lipids. The 43 kDa monomers of SP-D have the same basic structure as SPA; an N-terminal segment, a collagen-like domain, a neck region and a carbohydrate recognition domain, but the collagen-like domain does not have a kink. And like SP-A, the N-linked glycosylated monomers trimerize through their collagen-like domains, forming

straight trimers. The difference between mature SP-A and SP-D occurs when for trimers of SP-D are linked through disulfide bridges in the N-terminal section, resulting in a cruciform tetrameric structure with a molecular weight of 520 kDa [19]. Up to eight of these tetrameric structures can undergo further oligomerization, resulting in a large complex formed from 32 trimers. It is believed that the degree of oligomerization of modulates the ability of SP-D to recognize and bind pathogens [21].

The complexity of the structure of mature SP-A and SP-D portends their functional versatility, especially with regards to their crucial roles in neutralization of virus, opsonization and clearance of bacteria and fungi, clearance of apoptotic and necrotic cells, and modulation/control of inflammation, and thus are central in innate immunity and immune functions in the lung, [19,63]. These important roles will not be discussed in this chapter, rather the interactions of SP-A and SP-D with surfactant lipids, surfactant structures and surfactant homeostasis will be discussed. SP-A binds to the most abundant lipid in surfactant, DPPC, with great specifically and avidly while SP-D preferentially binds to a minor surfactant lipid, PI [42,64]. The presence of SP-A inhibits surfactant secretion from type II cells, while enhancing the uptake of DPPC by type II cells [19]. Disruption of the SP-D gene in mice results in progressive accumulation of surfactant lipids and proteins in the alveolar lumen and massive enlargement of intracellular LBs [40]. SP-A appears to have a synergistic, but indirect effect in the enhancement of lipid interfacial adsorption promoted by SP-B and SP-C [65–67]. Mice deficient in SP-A lack tubular myelin, an intermediate of secreted surfactant that enhances surfactant adsorption but is not essential for normal breathing in in vivo models [68]. Thus, although the presence of SP-A and SP-D are not required for normal lung function, but their activities in the lung have a great impact on surfactant integrity and homeostasis.

4.5 LAMELLAR BODIES

When surfactant proteins and lipids are synthesized by type II alveolar epithelial cells, they are transported and stored in lysosome related organelles called LBs, which are the characteristic feature of type II cells. A single human type II cell contains about 200–500 LBs, whereas only about 50–100 LBs are found per mouse type II cell, suggesting that regulation of LB biogenesis, like the lipid composition of surfactant, is dictated by the requirements of a particular species [69]. When visualized by electron microscopy, the surfactant in LBs appears as concentric, extremely compressed stacks of lipid bilayers, or lamellae, surrounded by a limiting membrane [70]. Morphologically these organelles vary considerably in size (100 to 2400 nm) making the mature LB one of the largest secretory granules in any cell type [71]. As with lysosomes and other lysosomal-related organelles, they have an acidic pH ($\sim$5.5), contain soluble lysosomal enzymes and proteins, and have extensive interaction with vesicles that define the endocytic pathway, especially multivesicular bodies (MVBs) [15]. Although LBs resemble a number of other lysosomal related organelles, they are unique in that they are specialized for storage and secretion of their cargo rather than for degradation. Regulation of LB secretion plays an important role in surfactant homeostasis in the alveolus.

4.5.1 Biogenesis of Lamellar Bodies

Surfactant proteins and lipids stored in LBs are generated from two sources, from newly biosynthesized material and from surfactant endocytosed from the lumen of the alveolus, transported to the LBs and reutilized. Biosynthesis of surfactant lipids and proteins is achieved through the same pathways used to synthesize membrane lipids and secretory proteins in all cells. For these newly synthesized surfactant components, the route to the LBs is typical of all secreted components with a few type II cell specific caveats.

As with all secreted proteins, the surfactant proteins are synthesized in the cytoplasm and co-translationally extruded into the ER. Transit from the ER to the Golgi is accompanied with proteolytic cleavage and processing that many secretory proteins encounter. The uniqueness of surfactant occurs during the transport of surfactant proteins from the Golgi by sorting vesicles to downstream destinations. The surfactant proteins most intricately associated with surfactant lipids, SP-B and SP-C, are directed to the MVB before being targeted to the LB. MVBs are formed from early endosomes by the inward budding of the limiting membrane into the lumen [72]. The resulting vesicles can serve as temporary storage compartments for proteins aside from targeting proteins for degradation. The MVB is the organelle where newly synthesized surfactant components and endocytosed surfactant from the lumen of the alveolus are combined and processed during the transit to the LB. The processing of immature SP-B to mature SP-B occurs in both MVBs and LBs, and involves the action of at least several defined proteases (napsin A, cathepsin H, and pepsinogen C) [55]. Mature SP-B has been found to be necessary for the formation of LBs [73–76]. In humans with homozygous mutations that preclude the presence of sufficient functional SP-B and in mice in which the gene coding for SP-B has been ablated, electron

microscopic analysis indicates type II cells containing vesiculated LBs with few or no bilayer membranes. It is believed that mature SP-B facilitates organization of surfactant membranes within in the LB through its abilities involved in maintenance of surfactant on the hypophase; by promoting membrane contacts and fusion [60,77].

Like immature SP-B, posttranslational processing of SP-C occurs in MVBs and LBs. While the identity of all proteases involved in processing of SP-C have remained elusive, involvement of cathepsin H suggests shared machinery with SP-B [78]. It is known that the initial processing events take place in MVBs while the final step takes place in LBs [79]. Since mice lacking the SP-C gene have normal LBs, it is presumed that mature SP-C is not involved in packaging of surfactant in the LB (unlike SP-B) [28]. However, disruption of SP-B results in incomplete processing of SP-C [80]. The orientation of SP-C in the membranes of the small intralumenal vesicles (ILVs) of the MVBs requires SP-B-mediated incorporation of these vesicles into the internal membranes of the LB [81]. In the absence of SP-B, this important step is not achieved.

While the pathway and roles of newly synthesized SP-B and SP-C in LB biogenesis are well known, the pathway of the hydrophilic collectins SP-A and SP-D are less defined. These proteins are posttranslationally modified in the ER and Golgi apparatus [82]. SP-A and SP-D expressed in cells that do not possess LBs are secreted via constitutive secretory pathways in more or less their mature, fully processed forms, and immunolocalization and biochemical studies suggest that SP-A bypasses the LB [83]. However, labeling studies indicate that at least a portion of newly synthesized SP-A makes it to the LB, suggesting that intracellular trafficking and secretion of newly-synthesized SP-A may involve both LB-dependent and constitutive pathways of secretion [83,84]. Very little is known of the relationship of newly-synthesized SP-D and LBs, but it has been suggested that SP-D secretes constitutively independent of LBs [85].

While the synthesis of complete pulmonary surfactant is an exclusive function of type II alveolar epithelial cells, synthesis of surfactant lipids occurs by pathways common to all cell types. The site in which synthesis of all the major classes of lipids occurs from their precursors, including phospholipids and cholesterol, is the ER membranes. The ER enzymes participating in synthesis of most lipid precursors have their active sites on the outer leaf of the ER membranes which face the cytoplasm, raising an interesting topological problem in which the synthesized lipids added to the outer membrane can be translocated to the inner membrane [86]. Pulmonary surfactant has a specific composition of lipids compared to the lipids of the plasma membrane; DPPC, unsaturated PC, PC, and PI are present in larger fractions of the total, and thus the rate of biosynthesis of these specific lipids is regulated to allow the required biosynthesis from known precursors such as fatty acids, dihydroxyacetone phosphate, glyceraldehydes-3 phosphate, phosphatidic acid, and choline [49]. For example, the most important lipid in the function of surfactant is phosphatidyl choline (PC). The rate limiting step for biosynthesis of PC is the activity of phosphocholine cytidyl transferase (CT), and thus modulating CT serves as a major regulatory mechanism for the biosynthesis of PC [49]. CT can be present in soluble (inactive) form or in membrane-bound (active) form, and the transitions between these states is promoted by decreased PC levels or increased levels of fatty acids and diacylglycerol (precursors of PC) in the cell [58,59,87]. LBs do not contain the enzymes required for phospholipid biosynthesis, thus, these lipids must be transported from the site of synthesis in the ER to LBs by at three pathways: vesicular transport, nonvesicular transport, and diffusion of lipids at membrane contact sites [88]. As will be discussed later, recycling of secreted surfactant proteins and lipids from the alveolar lumen to LBs requires the vesicular transport components of the endocytic pathway (ref). The MVB, which serves as the intermediate destination for newly synthesized SP-B and SP-C before transiting to LBs is the point where both the biosynthetic and endocytic pathways for delivery of surfactant components converge. Although vesicular transport of surfactant lipids to LBs via the endocytic pathway has been clearly demonstrated, metabolic labeling studies suggest little evidence for vesicular transport of newly synthesized DPPC or PC from the ER to LBs. Labeled lipids were sequentially detected in the ER, the Golgi and LBs, but not detected in MVBs [89]. In addition, it has been shown that the presence of newly synthesized lipid into LBs is not prevented by inhibition of Golgi vesicular transport, suggesting that a nonvesicular transport pathway moves newly synthesized surfactant lipids from the ER to LBs [90].

DPPC and PC are synthesized in the ER, but there is very little DPPC or PC in the ER, suggesting rapid export of newly synthesized phospholipid. One mechanism of nonvesicular-mediated transfer of phospholipids between donor and acceptor organelles would be through diffusion or facilitated phospholipid exchange at membrane contact sites between the organelles (i.e., ER and LB) [91]. Such a mechanism would require not only transfer proteins to facilitate lipid transfer but also accessory proteins that facilitate the formation of transient contact sites between the organelles. Studies of this mechanism with regard to type II cells and surfactant synthesis are lacking, but it has been proposed that some phospholipid transfer proteins contain domains that could fulfill the both of these roles [92]. Indeed, the activity and levels of several phospholipid transfer proteins with roles in intermembrane transport of PC and PG is enriched in type II alveolar cells and increase as lung maturation and surfactant synthesis increases.

However, ablation of the gene encoding at least one of these proteins in mice had no effect on LB morphology and alveolar DPPC content [93–96]. It is possible that the activities of each of these proteins with regard to transfer of surfactant lipids from the ER to LBs are redundant, but this question and the role of such proteins is clearly important in surfactant homeostasis.

Regardless of the mode of transport of newly synthesized surfactant lipids from the ER to LBs, the incorporation of these lipids into the concentric layers of surfactant lipids that define LBs requires the ATP-binding cassette (ABC) transporter, ABCA3. ABC transporters have been widely recognized to function as drug efflux pumps that protect cells from toxic substances, but the realization of the importance in lipid transport and homeostasis in various cells and tissues came from the identification of the dysfunction of specific ABC proteins in association with human diseases [97]. Such is the case with ABCA3 which is highly expressed in lung (especially in alveolar type II epithelial cells) and is localized to the limiting membrane of LBs [98–100]. When seemingly normal, at-term newborns presented with respiratory distress syndrome and surfactant deficiency that was fatal, genetic analysis showed this condition to be associated with severe, recessive ABCA3 mutations [101,102]. Disruption of the ABCA3 gene in genetically modified mice resulted in neonatal lethality, the absence of LBs in type II cells and a dramatic decrease in production of lung PG and PC [103,104]. ABC transporters are known to move lipids from the cytosolic side of membranes to the inner membranes, so it is likely that the import of some surfactant lipids into the LBs occurs through ABCA3 activity. When expressed a kidney epithelial cell line (HEK293), electron microcopy indicates that ABCA3 localizes to an intracellular vesicle membrane of a multivesicular, LB-like structure of about the same size of a LB that appears to be accumulating lipids, a structure which does not normally exist in HEK293 [105]. All of these findings indicate that ABCA3 is an essential transporter of PC and PG into LBs, and may be involved in the accumulation of surfactant phospholipids in LBs and in generating multivesicular structures.

While the importance of surfactant cholesterol in efficient spreading of surfactant over the hypophase was discussed previously, its synthetic origin in type II cells is not clear. It is known that the internal membranes of MVBs involved in the biosynthesis of LBs are enriched in cholesterol, so the endocytic pathway may be an important source of surfactant cholesterol [55]. It has been shown experimentally that the majority of cholesterol present in LBs is not secreted. As cholesterol levels are regulated independently from surfactant phospholipids and studies show that 83% of cholesterol in the lung comes from the circulation, pulmonary cholesterol probably comes from plasma lipoproteins rather synthesized de novo [50,106]. The internal membranes of MVBs are enriched in cholesterol, and thus the endocytic recycling pathway is likely an important source of cholesterol in pulmonary surfactant. DPPC, the most abundant pulmonary surfactant lipid, has high affinity for cholesterol, so incorporation of cholesterol into surfactant membranes may also be mediated by ABCA3.

4.6 EXOCYTOSIS OF LAMELLAR BODIES AND SURFACTANT

Alveolar surfactant occurs in two major pools: extracellular and intracellular, and essentially all extracellular surfactant lipids and surfactant proteins SP-B and SP-C are secreted via exocytosis of LBs [107]. Once in the alveolar lumen, surfactant lipids and proteins have half-lives of 10 to 20 h, mainly resulting from endocytosis and secretion (recycling) of surfactant between material in the lumen and type II cells [108,109]. Exocytosis of stored surfactant in LBs to the alveolar lumen has been calculated to be 4 to 11 h long, indicating that the entire process between stimulation exocytosis and secretion is very slow [110]. There is an abundance of evidence that implicates Ca^{2+} as a second messenger stimulating LB fusion with the plasma membrane as Ca^{2+}-induced fusion is a general feature of regulated exocytosis [111]. The potent stimulatory effect of increased cytosolic calcium concentration on surfactant secretion has been long recognized [112]. Both chemical and mechanical stimuli generate complex calcium signals that lead extended periods of exocytosis and secretion of LB contents in multiple sequential steps which are correlated to changes in elevations of cytoplasmic calcium concentration [113]. Several proteins associated with SNARE-mediated calcium-induced exocytosis have been identified in type II cells [114]. Annexin II and VII drives LB fusion with the plasma membrane [115]. Annexin A2 is abundantly expressed in type II cells and its interaction with Rab14 is involved in surfactant secretion [116]. Other components include SNARE proteins, proteins that function to disassemble SNARE complexes and two putative regulators of SNARE-mediated exocytosis that are associated with LBs [52].

Once calcium-stimulated fusion of LBs with the plasma membrane of the type II cell occurs, an exocytic fusion pore is formed to allow release of the LB contents into the alveolar lumen. The densely packed membranous structures representing stored surfactant do not readily diffuse through this pore. The relatively insoluble surfactant can remain entrapped within the fused vesicle for many minutes [109,117]. This phenomenon is overcome by a unique property of type II cells. Exocytosis and fusion of LBs results in localized calcium influx at the site of vesicle fusion.

This phenomenon is called "FACE" (fusion-activated Ca^{2+}-entry) and it is mediated through activation of P2X4 receptors that are expressed on the limiting membranes of LBs [118]. P2X receptors are membrane cation channels that are activated by extracellular ATP that are recognized to be regulators of exocytosis and secretion in a wide variety of tissues [119]. The localized influx of calcium from the extracellular space into the cytoplasm mediated by P2X4 receptors at the site of vesicle fusion promotes fusion pore expansion and facilitates surfactant release. It is also thought that this activity mediates fluid resorption from alveolar hypophase into type II cells, which facilitates insertion of surfactant into the air−liquid interphase, thereby "activating" it.

Once the contents of the LB organelle is secreted into the alveolar lumen, they unravel spontaneously in the alveolar fluid to form multilamellar vesicles and highly organized, cross-hatched arrays of intersecting membranes called tubular myelin. This structure supplies the lipids for the surface film, as well as large extracellular membrane layers that are tightly packed to form spherical particles or unilamellated vesicles called large aggregates (LA) [42,120]. Large aggregates and tubular myelin have high surface activity and adsorb very rapidly to the air-liquid interface of the hypophase, promoting spreading of the secreted surfactant. Visually, the surface film at the alveolar air/liquid interface consists of a phospholipid monolayer with a loose network bilayer structures attached to it. In vitro studies suggests that the formation of tubular myelin is dependent on SP-A, which can be detected as electron-dense structures at the corners of the lattice [121]. The findings were confirmed when tubular myelin was not detected in secreted alveolar surfactant isolated from transgenic mice in which the SP-A gene was ablated [68,122]. However, the role of tubular myelin in the function of surfactant to reduce alveolar surface tension is not clear as the pulmonary mechanics of SP-A deficient mice are no different from the mechanics observed in wildtype mice. Perhaps tubular myelin is not absolutely necessary in the function of surfactant, but it may have a role in modulation of surfactant function under various physiological conditions since an increase of tubular myelin-associated SP-A has been correlated with improved lung preservation in ischemia/reperfusion lung injury [64]. Tubular myelin is suggested to have a role in host defense. The association of SP-A with tubular myelin presumably puts this collectin in the first line of defense, but in a configuration that does not disrupt surface activity [123].

4.6.1 Regulated Secretion of Lamellar Bodies

The role of calcium in facilitating the fusion of LBs and secretion of surfactant was discussed above. LB and surfactant secretion, as with many exocytic processes in other cells, is a highly regulated phenomenon that is mediated by numerous biochemical and mechanical stimuli resulting from changes in lung physiology. These include endogenous or exogenous compounds, such as neurotransmitters, hormones, autocrine mediators, paracrine mediators, toxins, and drugs, as well as physical factors such as temperature, pH, and mechanical forces [124].

The primary physiological factor inducing change in surfactant secretion is breathing, or distention of the lung that induces cellular stretch. In an extreme example, stimulation of surfactant secretion occurs within minutes upon hyperventilation [19]. In addition, it is believed that increases in alveolar phospholipid following a single deep breath is due to strain-induced surfactant secretion [124]. As alveolar type II cells are preferentially located in corners of alveoli, they are exposed to maximum distortion. It stands to reason that increased surfactant secretion due to increased ventilation likely occur through direct mechanical stimulation in these cells [125]. Stretch of isolated type II cells in vitro leads to an increase in surfactant secretion equivalent as well as an increase in intracellular calcium levels, which is known to stimulate surfactant secretion [126,127]. Lung distention also affect type I epithelial cells, which respond by activating an intracellular signaling pathway that leads to Ca^{2+} wave generation in lung alveolar epithelium. When alveolar type I-like cells co-cultured with type II cells are mechanically stimulated, the resulting calcium signal in the type I cells is transmitted to neighboring type II cells through an apyrase-sensitive mechanism. This suggests ATP as an extracellular mediator of alveolar cell communications [128]. P2X7 receptors are specifically expressed in type I cells and stimulation of the P2X7 receptor in these cells releases soluble ATP, which acts in a paracrine fashion on type II cells [129]. Thus, ATP produced by type I cells in response to mechanical stimulation triggers surfactant secretion from type II cells [130]. This is a physiologically relevant phenomenon as mice deficient in the gene encoding the P2X7 receptor are less responsive to hyperventilation-induced surfactant release [131]. Therefore, P2X7 receptors in alveolar type I epithelial cells are an important regulator of surfactant secretion and have a role in type I cell and type II cell communications. By this mechanism, the level of surfactant in the alveolar lumen can be adjusted in response to varying levels of ventilation.

It has long been known through mostly in-vitro studies that many agonists and second messengers regulate surfactant secretion in type II cells through three different pathways [85].

1. Stimulation of adenylate cyclase (AC) pathway through activation of ß-adrenergic and adenosine A_{2B} receptors that are coupled to adenylate cyclase via the heterotrimeric, GTP-binding protein G_s results in generation of cyclic AMP (cAMP) followed by activation of PKA, a cAMP-dependent protein kinase.
2. Direct activation of PKC by phorbol esters and cell permeable diacylglycerols (DAGs) or indirect activation of PKC through binding of ATP and UTP to purinergic receptors on type II cells that are coupled to phospholipase C (PLC-ß3) via the heterotrimeric, GTP-binding protein Gq. PLC-ß3 then hydrolyzes PI bisphosphate into DAG, which activates PKC, and inositol trisphosphate (IP3).
3. Stimulation of Ca^{2+}/calmodulin-dependent protein kinase (CaMK) through an influx of extracellular calcium into the cell by the actions of ionophores and mechanical strain. IP3 also promotes mobilization of Ca^{2+}.

Ultimately, activation of any of these pathways leads to protein phosphorylation and although it has been reported that surfactant secretagogues such as TPA (phorpbol esters) and terbutaline (ß-adrenergic receptor agonist) have been reported to lead to phosphorylation of a number of proteins in type II cells, the identity and role of these proteins in stimulating surfactant secretion is unknown [132,133]. Since much of the knowledge concerning the relationship between activation of these pathways by specific agonists and stimulation of surfactant secretion comes from studies in isolated cells or tissues, the physiological relevance of these agents is not clear. However, some physiological inferences can be made. Labor is known to stimulate surfactant secretion which may be the result of β-adrenergic stimulation during labor and birth [134,135]. Although type II epithelial cells are known to express muscarinic cholinergic receptors, cholinergic agonists fail to stimulate surfactant secretion from these cells [136,137]. However, activation of the type II cell β-adrenergic receptors in response to cholinergic agonists such as catecholamines released from the adrenal medulla may indirectly affect surfactant secretion [85]. Perhaps the strongest evidence for physiological relevance of these pathways comes from the example where mechanostimulation of type I cells leads to ATP release, which binds to purinergic receptors in type II cells and stimulates surfactant exocytosis [61,130].

SP-A itself has long been recognized to inhibit surfactant secretion from alveolar type II cells [138,139]. The inhibitory effect of SP-A on surfactant phospholipid secretion is immediate and occurs in the presence of secretagogues discussed above [85]. A more recent study indicates that SP-A does not affect intracellular trafficking of surfactant nor secretagogue-induced LB fusion with the plasma membrane, but SP-A appears to inhibit expansion of the fusion pore [140]. One hypothesis to explain this process concerns the binding of SP-A with its receptor on type II cells, which inhibits calcium signaling [62]. Since pore expansion relies on changes in intracellular calcium, perhaps SP-A modulates local levels of calcium at the fusion/pore sites such that extrusion of the LB contents into the alveolar lumen is prevented.

4.7 ENDOCYTOSIS OF SURFACTANT

Newly secreted surfactant exists as high surface-active large aggregates. Compression−expansion cycling associated with breathing leads to progressive transformation of the large aggregates into much less surface-active forms of secreted surfactant, or small aggregates [131,141]. Formation of these small forms of inactivated surfactant probably result from unfortunate changes to the crucial lipid-protein organization of functioning surfactant or from detachment of small particulate entities from the surface of the hypophase. In addition, surfactant components may be inactivated by oxidation after repetitive exposure to air or by inappropriate incorporation of harmful inhaled materials or harmful materials leaked from capillaries [142,143]. Maintenance of highly functional surfactant on the alveolar hypophase requires efficient removal of spent and inactivated surfactant and incorporation of newly secreted surfactant. In addition to their critical role in exocytosis of surfactant to the alveolar lumen, type II cells have a major role in clearance of lipids and surfactant from the alveolar space [144]. Endocytosis of extracellular lipids and surfactant proteins by type II cells is known to occur predominantly via clathrin-dependent pathways involving the commensurate endocytic pathway that consists of distinct membrane vesicles (early and late endosomes) which are present in all mammalian cells with slight type II-specific modifications (LBs) [145]. Clathrin-independent endocytotic pathways have also been identified that might also be involved in the endocytosis of surfactant [146,147]. Intracellularly, surfactant lipids and proteins are transported differently after endocytosis from the alveolar space. Endocytosed SP-A is targeted to early and recycling endosomal organelles, and a major fraction of this endocytosed SP-A is subsequently exocytosed in a route totally independent of LBs [148,149]. In direct contrast to SP-A, mostly all of the

endocytosed lipids as well as associated hydrophobic SP-B and SP-C proteins are transported toward LBs via a nondegradative route utilizes late endosome related organelles [146,148]. Thus, surfactant endocytosis by alveolar type II cells is not only involved in surfactant clearance from the alveolar lumen, but is also part of surfactant recycling.

An unexpected role of SP-D in pulmonary surfactant homeostasis was not apparent until the gene coding for SP-D was inactivated in genetically altered mice, which resulted in marked accumulation of tissue and alveolar surfactant phospholipids, especially saturated PC [40,108,109]. In the SP-D-deficient mice, sat PC synthesis and secretion by alveolar type II cells was normal as compared wildtype mice, as was catabolism of by the alveolar macrophages [110]. These findings suggested that the lipid abnormalities seen in SP-D-deficient mice are the result of changes in surfactant uptake, catabolism and/or recycling by type II cells. In SP-D-deficient mice, electron microscopy of the large aggregate forms of surfactant demonstrated abnormally dense lipid while atypical multilamellated small vesicles forms were observed by small aggregates of surfactant. When normal type II cells were incubated with both large and small aggregate forms of secreted surfactant isolated from SP-D-deficient mice, endocytosis of was decreased compared to alveolar surfactant isolated from normal mice [109,111]. Finally, preincubation of broncholaveolar lavage material from SP-D-deficient mice with purified SP-D in vitro transformed abnormal surfactant ultrastructure to a structure similar to that seen in wildtype mice. These results suggests that SP-D does not directly interact with type II cells and influence endocytosis of secreted surfactant, rather SP-D regulates surfactant endocytosis by influencing physical structure of secreted surfactant. The mechanisms by which SP-D affects surfactant structure are unclear but it may be dependent upon the known interactions of SP-D with PI and fatty acids [64]. In other studies, it was determined that addition of SP-D to phospholipid vesicles containing PI and SP-B reconstituted highly ordered tubular arrays in vitro. [150]. These findings suggest that SP-D determines the normal ultrastructure of surfactant aggregates in the alveoli, which influences endocytosis of surfactant by type II cells. Ultimately, this activity of SP-D demonstrates its role in maintenance of surfactant pool sizes.

A direct role of SP-D in regulation of lung surfactant homeostasis involving the GPR116 orphan receptor has recently been reported [151]. The GPR116 receptor is a member of the adhesion class of G protein coupled receptors and is highly expressed in the lung and in type II cells [152,153]. Genetic ablation of the gene encoding the receptor in mice results in a pulmonary alveolar proteinosis and emphysema-like pathology reminiscent of pathology seen in mice lacking SP-D. SP-D was biochemically identified as a ligand for GPR116. The data suggest that the GPR116 receptor on type II cells can sense the amount of extracellular surfactant by monitoring one of its protein components, SP-D and regulate the synthesis of surfactant proteins and surfactant lipids, secretion of surfactants into the alveolar lumen and recycling of secrete surfactant.

4.8 ENDOCYTIC VESICLE TRAFFICKING

The role of endosomal trafficking and MVBs in transport of endocytosed surfactant and reincorporation in to LBs for recycling cannot be understated. MVBs are formed from early endosomes by the inward budding of the limiting membrane into the lumen. The resulting intralumenal vesicles can house proteins in temporary storage compartments or serve as a device to deliver the entire protein to the lysosome for degradation. [72]. In the type II cells, the route of endocytosed surfactant to LBs is well described. After Clathrin-mediated endocytosis, surfactant lipids, and proteins are encapsulated into early endosomes that transit the cell and fuse with MVBs. The MVB serves as the point where newly synthesized SP-B and SP-C are combined with recycled material. The MVB transits to and fuses with the composite body which then matures into the LB. The primary responsibility of endosomes is to separate proteins that will recycle to other cellular locales from those that will be degraded in lysosomes, so damaged surfactant destined for catabolism transits to lysosomes rather than MVBs.

4.9 SURFACTANT CATABOLISM

Homeostasis of any biologically-produced entity depends on the balance of biosynthesis and catabolism, and this is no different in the case of surfactant. Secreted surfactant at the interface of the hypophase is subjected to a continuous onslaught of damaging agents during breathing (i.e., oxygen radicals, enzymes from infectious agents, etc.), and damaged, and therefore inactive, surfactant must be removed. In the alveolus, only type II cells and alveolar macrophages endocytose secreted surfactant, and both contribute equally to uptake of saturated PC and SP-A in mice [17]. With regards to surfactant that is endocytosed by type II cells, 50% is recycled and 50% is catabolized, but the mechanisms that determine sorting of surfactant proteins and lipids for recycling to LBs as opposed to degradation have not been identified. On the other hand, 100% of surfactant endocytosed by macrophages is catabolized. The importance of the macrophage in surfactant homeostasis was demonstrated in transgenic mice in which the genes encoding either

GM-CSF, the GM common receptor β-chain or acid sphingomyelinase [29,82,83]. In all cases, saturated PC levels in the lung were significantly increased (4−8 fold). Since synthesis and secretion of saturated PC were normal, the increases in surfactant pool sizes were presumed to be caused by a defect in surfactant catabolism by alveolar macrophages. While phagocytosis of surfactant by alveolar macrophages is not dependent on the physical form of surfactant or presence of surfactant proteins, endocytosis of surfactant by type II cells is more selective [79].

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Chapter 5

Integrin Regulation of the Lung Epithelium

Erin Plosa and Roy Zent

5.1 LUNG EXTRACELLULAR MATRIX IN DEVELOPMENT, HOMEOSTASIS, AND REPAIR

Cell-extracellular matrix (ECM) interactions play a critical role in lung development, homeostasis, and repair. The lung ECM consists of the basement membrane and the pulmonary interstitium. The pulmonary interstitium closely approximates with the lung mesenchyme, provides elastic recoil for the lung, and consists of primarily of fibrillar collagens, elastic fibers, and proteoglycans [1]. The basement membrane lines the basal surface of airway and alveolar epithelial cells, as well as endothelial cells, and intimately connects epithelial cells with their microenvironment. The basement membrane acts as a barrier between cell compartments, guides cell proliferation and differentiation, and provides a reservoir for growth factors. The individual basement membrane components vary throughout development and in response to injury. Laminins, collagen IV, nidogen, and proteoglycans are the primary components of the lung basement membrane.

The role of cell-ECM interactions in lung development is an emerging and understudied field. Numerous murine knockout models and humans with mutations that result in lung disease suggest both interstitial and basement membrane ECM proteins are required for proper lung development. Lung morphogenesis begins early in gestation, by day 25 in human gestation and embryonic day 9−9.5 in the mouse and continues postnatally until day 28 in the mouse and early childhood for humans. Both murine and human lung development proceeds through five well-defined sequential stages (embryonic, pseudoglandular, canalicular, saccular, and alveolar). Accumulation of successive airway generations and formation of a thin gas exchange surface results from two major morphogenic processes, branching morphogenesis, and alveolarization.

5.1.1 ECM Proteins of the Pulmonary Interstitium

In the developing lung, fibroblasts secrete fibrillar collagens and elastic fibers into the developing lung interstitium [1]. Fibrillar collagens play a limited role in development, whereas elastin deposits shape emerging alveolar septa, forming intimate connections with alveolar epithelial cells, and defining the final stage of lung development during alveolarization [2,3].

5.1.1.1 Elastin

Elastogenesis is a critical process during lung late sacculation and alveolarization. In these final stages of lung development, elastin deposition in the lung interstitium provides support for dynamic breathing movements present in postnatal life. Elastin is produced as a precursor, tropoelastin, and is cross-linked by the lysyl oxidase family of enzymes. Association with fibrillin-containing microfibrils and numerous small glycoproteins, such as fibulins, forms mature elastic fibers [4]. The exact identity of the fibroblast population responsible for elastin synthesis during alveolarization is currently unclear, but recent three-dimensional studies of murine alveolarization suggest myofibroblasts that form crests along the developing alveolar septa produce elastin for the mature alveolar septa [3].

Disruptions in elastin synthesis or assembly result in defects in both saccular and alveolar stage lung development [4−8]. Murine elastin deficiency results in saccular stage branching defects and death by postnatal day 3.5 [8].

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Elastin haploinsufficient mice exhibit normal airspace architecture in the newborn period until challenged with mechanical ventilation. Ventilation of these mice at the start of alveolarization produces persistent airspace enlargement [7]. Mice deficient in fibrillin-1 die by postnatal day 14 from aortic aneurysms, respiratory failure, and diaphragmatic collapse. Examination of the mutant lungs reveals large blebs in the distal lung [9]. Similarly, human mutations in fibrillin-1 result in Marfan Syndrome and a portion of affected individuals possess peripheral emphysematous changes in the lung [10]. Mice with impaired elastin-collagen cross-linking due to lysyl oxidase enzyme deficiency demonstrate dilated saccular stage airspaces in late gestation compared to control littermates [11]. Lungs from mice deficient in the related enzyme loxl1 have peripheral airspace enlargement during early alveolarization [12]. In addition, murine deficiency of fibulin-1 and -5, glycoproteins that associate with mature elastin fibers, result in developmental lung defects [13,14].

Elastin synthesis and degradation is tightly regulated in the homeostatic lung. The healthy adult lung exhibits a low level of elastin turnover [15]. However, emphysematous enlargement of distal airspaces occurs with excessive or unopposed elastase activity, as seen in alpha-1 antitrypsin deficiency or other etiologies of chronic obstructive pulmonary disease (reviewed in Ref. [16]).

5.1.1.2 Interstitial Collagens

The developing lung contains fibrillar collagens, primarily collagen I and III, in the fetal lung interstitium. The majority of collagen I and III deposition occurs during the canalicular stage, preceding the bulk of elastogenesis [17,18]. Secreted as propeptide triple helices, fibrillar collagens undergo extensive posttranslational modifications, including hydroxylation, glycosylation, cleavage, and cross-linking [1]. Cross-linking serves to stabilize the triple helix structure and, in later lung development, form a tight lattice with elastin fibers.

The exact role of fibrillar collagens during normal lung development is unknown. The collagen I null mouse is embryonic lethal at embryonic day 12 due to hematopoetic and vascular defects, precluding study of collagen during the bulk of lung development [19]. However, deposition of abundant interstitial collagens was demonstrated as a hallmark pathological finding in preterm infants with classically described bronchopulmonary dysplasia (BPD) and is a variable finding, along with interstitial hypercellularity, in new BPD [20–22]. The mechanisms have not been fully elucidated, but postnatal mechanical stress from positive pressure mechanical ventilation, a well-known risk factor for development of BPD, condenses collagen I and collagen III fibers in the lung parenchyma of preterm infants [18].

The postdevelopmental role of fibrillar collagens in lung disease is an active field of research. Both childhood and adult etiologies of pulmonary fibrosis, a variety of insults in genetically susceptible individuals triggers the expansion of interstitial ECM proteins, particularly collagen I and III (discussed in detail in Chapter 9: The Respiratory Epithelium in COPD). Dysregulated fibrillar collagen turnover decreases wound healing efficiency and disrupts normal alveolar architecture [23–26]. The net deposit of additional interstitial ECM proteins increases tissue stiffness, alters cell behavior, and impairs lung function [27–29].

5.1.2 ECM Proteins of the Lung Basement Membrane

5.1.2.1 Laminins

Laminins, along with collagen IV, provide substantial structural support for the lung and are the primary components of the lung basement membrane. Laminins are heterotrimeric proteins, composed of one α, one β, and one γ chain, and have 16 confirmed or predicted human isoforms formed from five α chains, three β chains, and three γ chains [30]. The fetal lung basement membrane contains laminins with all five possible α chains. In contrast, the adult lung epithelium restricts α chain expression to laminin α3 and α5 [31–33].

Targeted laminin subunit deletions or inhibition demonstrate the requirement for laminins in lung development [34,35]. Inhibition of laminin α1 in fetal lung explants results in branching morphogenesis defects and disrupted epithelial polarity [36]. Global deletion of laminin α5 results in fetal loss in mid-late gestation associated with impaired lobar septation of the lung [37]. Inducible lung epithelial specific deletion of laminin α5 demonstrated additional roles in lung development. These mice died from respiratory failure shortly after birth. However, histological examination of their lungs in late fetal lung development revealed epithelial differentiation defects, dilated distal airspaces, increased apoptosis, and decreased proliferation in whole lung sections [38].

The role of laminin 332 during lung development has been investigated using global and tissue specific murine deletions, fetal lung explants, and in-vitro studies. The expression of laminin 332 localizes exclusively to the basal surface of epithelial cells in the fetal lung basement membrane. In contrast to laminin α1 and α5, the γ2 chain (specific to

TABLE 5.1 Collagen IV Chains in the Lung Basement Membrane

Collagen IV Subtype	Basement Membrane Localization	Human Disease or in vivo Data
α1 α2 α1	Airway, alveolar	Sacculation, alveolarization, epithelial differentiation defects in murine deletion
α5 α5 α6	Airway	
α3 α4 α5	Alveolar	Autoantibodies to α3 chain associated with Goodpasture's disease

laminin 322) is not required for branching morpho genesis or fetal lung epithelial cell differentiation. Histological analysis of lungs from laminin $\gamma 2-/-$ mice demonstrated disorganized hemidesmosomes in the tracheal epithelium, yet these mice retained normal epithelial integrity and lung function [39]. The role of laminin γ2 in the adult lung was not addressed with this genetic approach, as severe skin defects in the laminin $\gamma 2-/-$ mice resulted in perinatal lethality from malnutrition [40]. Laminin 322 is also expressed in the adult lung basement membrane. In this context, lung epithelial specific deletion of laminin α3 demonstrated a role for this laminin isoform in the development of pulmonary fibrosis [41]. These mice lack a developmental phenotype, but exhibit increased mortality, inflammation, and fibrosis following intratracheal bleomycin challenge.

5.1.2.2 *Collagen IV*

Collagen IV is the principal collagen found in the lung BM. Like laminin, it is a heterotrimeric protein. Three separate α chains, selected from six distinct chains, combine to form collagen IV with regional specificity in the lung (Table 5.1). The adult lung under homeostatic conditions expresses the α1(IV) and α2(IV) chains throughout the airway and alveolar epithelial basement membranes. In contrast, the collagen α3(IV), α4(IV), and α5(IV) chains combine to form collagen IV α3α4α5, a specialized form of collagen IV that is restricted to the alveolar basement membrane, as well as basement membranes in the glomerulus, testis, and eye [42]. In addition to the α1(IV) and α2(IV) chains, the airway basement membrane contains collagen α5(IV) and α6(IV) chains [43]. Mice with mutations in the *col4a1* and *col4a2* genes demonstrate the importance of collagen IV during lung development, as these mice exhibit defects in sacculation, epithelial differentiation, and simplified alveolarization, characterized by decreased primary alveolar septa [44].

Goodpasture's disease is a lung and kidney condition involving the α3(IV) chain of collagen IV. Antiglomerular basement membrane autoantibodies bind the α3(IV) chain of collagen IV present in the glomerular and alveolar basement membranes resulting in complement fixation and massive lung destruction [45].

5.1.2.3 *Other Lung Basement Membrane Proteins*

ECM proteoglycans and nidogen, the other two constituents of the lung basement membrane, contribute to basement membrane stability by linking the larger ECM proteins. The roles of proteoglycans and nidogen are less well defined than collagen and laminin. Mutation of the nidogen binding site on laminin γ1 results in late, saccular stage branching morphogenesis defects and perinatal respiratory distress [46]. Consistent with nidogen binding site mutation, deletion of both nidogen isoforms, nidogen-1 and -2, results in perinatal lethality and decreased saccular stage branching. Mice deficient in nidogen-1 and -2 also exhibit differentiation defects in emerging alveolar type 2 cells in the late fetal lung [47].

5.2 INTEGRINS ARE THE PRINCIPAL RECEPTORS FOR ECM

Integrins are a large family of transmembrane heterodimeric proteins that serve as ECM receptors, thereby connecting intracellular and extracellular environments. These receptors are composed of an α and β subunit, and contain a large extracellular domain, a single transmembrane region, and a smaller cytoplasmic tail domain [48,49]. In vertebrates, 18 α and 8 β distinct subunits noncovalently combine as αβ pairs to form 24 different integrin combinations [50]. The extracellular domain binds ECM ligands, whereas the transmembrane and cytosolic domains play a role in modulating the conformation of the extracellular domain, regulate cell signaling, and form physical interactions with the actin cytoskeleton. The intracellular domain mediates its functions by binding cytoplasmic adapter and signaling proteins.

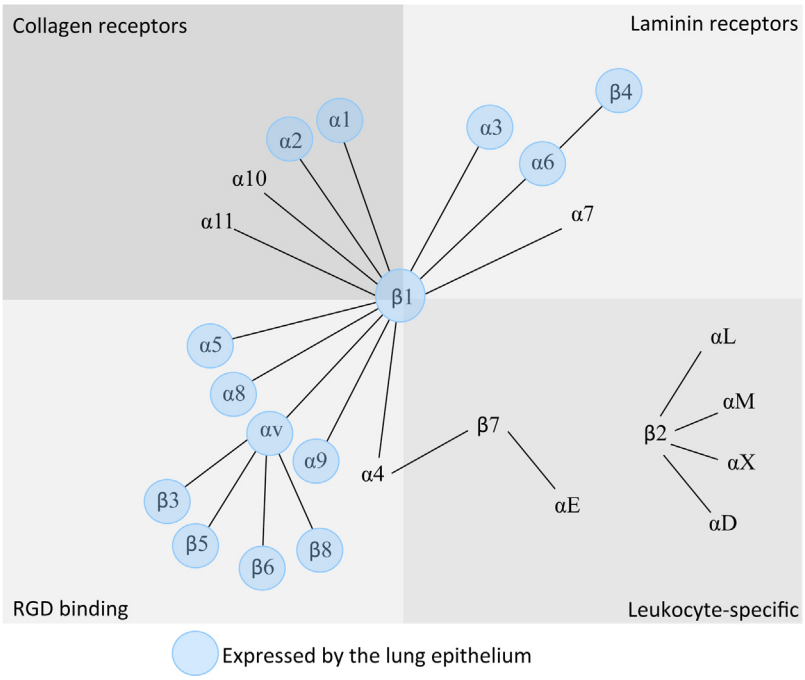

FIGURE 5.1 Integrins expressed in the lung epithelium include collagen receptors, laminin receptors, and RGD binding integrins.

Integrins are required for numerous cell functions such as adhesion, migration, proliferation, differentiation, and apoptosis, as well as cell signaling [48].

Integrins are classified into four major groups by their ability to bind specific ECM ligands. (Fig. 5.1) There is notable redundancy amongst integrins, with multiple integrin pairs binding the same ECM ligand at different affinities. Similarly, a single ECM ligand can bind multiple integrins. One subset of integrins recognizes the arginine-glycine-aspartic acid (RGD) tripeptide sequence on multiple proteins including fibronectin, vitronectin, fibrinogen, osteopontin, or latency associated peptide (LAP) associated with latent TGF-β. The αv integrins, α4β1, α5β1, α8β1, α9β1, and αIIbβ3 comprise the RGD binding group and are expressed by various cell types in the lung, including epithelial and mesenchymal cells [51]. Integrins α1β1, α2β1, α10β1, and α11β1 are collagen receptors, however, only integrins α1β1 and α2β1 are expressed in the lung. The four known laminin receptors are α3β1, α6β1, and α6β4 and α7β1 and α3β1, α6β1, and α6β4 are found in the lung. The remaining integrin subset is leukocyte specific and plays a critical role in adhesion and migration [50].

Integrins are unique receptors because they are found in both an activated an inactive state. This change in conformation is particularly important in cells found in the circulatory system and in processes such as development and injury repair. It is unclear whether all integrins are able to change their conformational state, however integrin activation is an important component in integrin function in multiple cell types. In the resting, inactive state, the extracellular region of the αβ pair is bent with closely associated transmembrane domains, limiting access to the ligand-binding site. Intracellular signaling can induce a conformational change that disrupts the transmembrane domain interaction and extends the extracellular region, thereby exposing the ligand binding site and increasing ligand affinity [52–58]. Binding of many intracellular adapter proteins to β integrin cytoplasmic tails has been shown to regulate this process and the best studied of these are talins and kindlins. The mechanisms of this activation process is described elsewhere [59–63]. The other important way that integrins function is that they mediate the interactions between cells and ECM. Upon integrin-ECM ligand binding, integrins cluster at the cell membrane, recruit intracellular adapter proteins such as talin, vinculin, α-actinin, integrin-linked kinase, and focal adhesion kinase, forming focal adhesion complexes. These large multiprotein complexes, which primarily bind to the intracellular β subunit tails are gatekeepers for intracellular signaling events and regulate cytoskeletal dynamics [64–66].

5.3 INTEGRINS IN LUNG DEVELOPMENT, ALVEOLAR HOMEOSTASIS, AND DISEASE

Similar to morphogenesis of other epithelial branched organs, the fetal lung epithelium expresses primarily laminin and collagen receptors. These receptors bind the major components of the fetal lung basement membrane.

By immunostaining, the collagen receptor integrin α2β1 and the laminin receptors integrins α3β1, α6β1, and α6β4 appear on the epithelial basal and lateral cell membranes in the pseudoglandular and canalicular stages [67–69]. Immunostaining of human fetal lung tissues from the pseudoglandular stage demonstrates scant expression of α5 and αv integrin subunits in the distal lung bud epithelium, whereas these subunits are robustly expressed in the adjacent fetal lung mesenchyme [68]. The expression of integrin αvβ6 is developmentally regulated. Immunostaining of canalicular stage human and primate fetal lung demonstrates moderate distal airway epithelial expression of αvβ6, but no expression in the proximal large airway epithelium. In contrast, there is strong αvβ6 expression in the alveolar epithelium in newborn lung undergoing epithelial differentiation into type I and type 2 alveolar epithelial cells in preparation for alveolarization [70].

The adult lung epithelium expresses an expanded set of integrin receptors compared to the fetal lung epithelium. Epithelial cells in the healthy proximal large airways constitutively express integrins α2β1, α3β1, α9β1, α6β4, αvβ5, αvβ6, and αvβ8 [71,72]. In contrast, healthy distal airway and alveolar epithelium lack integrin α9β1 expression [73–75]. Lung injury, inflammation, or primary cell culture conditions can dynamically change surface expression of epithelial integrins, inducing expression of integrins α2β1, α3β1, α5β1, α6β4, αvβ5, and αvβ6. Induced expression of two of these integrin receptors, α5β1 and αvβ6, is specific to alveolar epithelial cells following injury [70,72,76].

5.4 β1 INTEGRIN—THE CENTRAL INTEGRIN SUBUNIT

The β1 integrin subunit is ubiquitously expressed in numerous cell types and throughout multiple organs. Twelve α subunits (α1, α2, α3, α4, α5, α6, α7, α8, α9, α10, and α11) heterodimerize with β1. Not surprisingly, the global β1 integrin knock out mouse is lethal early in embryogenesis [77]. Therefore, its specific functions in different tissues have been defined by deleting β1 in a tissue specific manner. Using this approach, the in-vivo role of β1 integrin in the lung is just emerging.

Deletion of β1 integrin from the fetal lung epithelium from E9.5 using Shh-Cre results in embryonic lethal mice. The lungs from these mice branch just once at the carina and the two airways end in a mass of multilayered epithelial cells at the end of each main bronchus [78]. Lung epithelial specific deletion one day later in development using SP-C-Cre at E10.5, produces a more modest branching defect. Inducible deletion of epithelial β1 integrin at P0, after branching morphogenesis is complete, demonstrated alveolarization defects distinct from the branching defects [79]. The SP-C-Cre; $\beta1^{f/f}$ lungs exhibited thickened alveolar septa, increased numbers of type 2 alveolar epithelial cells, and increased alveolar macrophages. Interestingly, depletion of β1 integrin- mediated lung inflammation rescued the alveolarization phenotype. These data suggest that β1 integrin is required for alveolarization through the regulation of inflammation. The role of β1 integrin in adult lung homeostasis and repair is currently not well understood.

5.5 LAMININ-BINDING INTEGRINS

The laminin receptors in the fetal lung epithelium include α3β1, α6β1, and α6β4. Mice with a global integrin α3 deletion exhibit branching defects in both the kidney and lung. Large airways fail to taper toward the periphery [80]. Mice with a combined global α3/α6 integrin deletion, in which all the laminin receptors are deleted from the fetal lung epithelium, displayed severe bilateral lung hypoplasia [81]. Similarly, recent reports describe human mutations in α3 integrin that result in a significant lung phenotype. Infants with α3 mutations exhibit skin, lung, and kidney disease but die of respiratory failure by 19 months [82–85]. These mutations cause defects in posttranslational processing and mistrafficking of the integrin α3 subunit. In the well-described A349S mutation, a gain of glycosylation is shown to impair heterodimerization with the β1 subunit, disrupting cell surface expression of α3β1 [82]. The roles of laminin binding integrins in alveolar homeostasis and repair are almost completely unexplored. Lung epithelial specific deletion of α3 integrin lacks a developmental phenotype but these mice do exhibit an increased inflammatory response following bleomycin challenge [86].

Expression of integrin α6β4 is more restricted than that of the other laminin receptors during lung development. It is expressed along the basal membrane of basal epithelial cells that line the trachea during the canalicular stage. This integrin is a critical component of hemidesmosomes and consequently, the β4 null mice exhibit severe skin sloughing from hemidesmosome dysfunction in keratinocytes, as well as perinatal cyanosis. Integrin β4 null mice die from respiratory failure in the neonatal period. However, respiratory impairment occurs due to airway obstruction by sloughed oral and nasal epithelial cells rather than a developmental lung anomaly [87].

5.6 COLLAGEN-BINDING INTEGRINS

The fetal lung epithelium contains primarily one collagen-binding integrin pair, α2β1. The integrin α2 null mouse has a subtle branching defect in the mammary gland, but no appreciable lung phenotype. The mutant mice grow and breed normally without respiratory distress and the lungs examined between 9 and 12 weeks of age appear histologically normal, suggesting that α2β1 has no role in development [88].

Currently, there are no in-vivo studies specifically investigating the role of collagen binding integrins in repair following lung injury. Numerous in-vitro experiments suggest collagen receptors are important for epithelial migration to repair denudation [89–93]. Robust murine models are needed to confirm these findings as cell culture matrix conditions have limited utility in modeling in-vivo integrin behavior.

There is minimal human and murine data to implicate collagen binding integrins in tumorigenicity. In human samples of squamous cell lung cancers, high integrin α1β1 and α2β1 expression is associated with increased metastasis [94]. In a murine model of Kras-induced nonsmall cell lung cancer, deletion of the α1 integrin subunit decreased ERK signaling and tumor cell proliferation, associated with decreased tumor burden and prolonged survival when compared to mice with Kras mutation and wild type integrin α1 [95]. Similarly, in-vitro studies in A549 cells, a nonsmall cell lung cancer cell line, demonstrated that β1 integrin is required for c-met signaling, a proproliferation, and migration pathway overexpressed in numerous cancer cell lines [96–98].

5.7 RGD-BINDING INTEGRINS

RGD-binding integrins are expressed by both lung epithelial and mesenchyme cells. These integrins recognize the RGD motif on a variety of ligands that include large ECM proteins, such as fibronectin or vitronectin. These integrins also serve as receptors for tenascin-C and osteopontin, conditional ECM proteins induced at times of injury. Growth factors and their related peptides also bind select integrins. The αv subset of RGD-binding integrins binds traditional ECM ligands, as well as the RGD motif on LAP associated with latent TGF-β and play a specialized role in the activation of this growth factor.

5.7.1 RGD-Binding Integrins as ECM Receptors

5.7.1.1 α5β1

Integrin α5β1, the primary fibronectin receptor, is expressed in the fetal lung mesenchyme, but not lung epithelium. As such, no role for epithelial α5β1 has been yet identified during lung development. Although murine in-vivo studies of adult lung homeostasis and injury are lacking, numerous in-vitro studies implicate α5β1 in lung epithelial wound healing in which they have been proposed to regulate gap junctions, cell proliferation, and migration [99–101].

As the lung epithelium provides barrier protection against inhaled pathogens, it is not surprising that denudation of the airway epithelium is a risk factor for pathogenic airway infection. Integrin α5β1 is one of several integrins that modulating pathogen adherence. Dedifferentiated primary respiratory epithelial cells have increased susceptibility to *Pseudomonas aeruginosa* infection through α5β1-mediated bacterial adherence [102]. In addition, integrin α5β1 activation by fibronectin enhances *Pneumocystis carinii* attachment to type 1 alveolar epithelial cells [103–105]. Taken together, these in-vitro experiments suggest that integrin α5β1 plays a critical role in wound repair, inflammation and innate immunity in the homeostatic lung, but relevant in-vivo models are lacking.

5.7.1.2 α8β1

The fetal lung epithelium does not express the fibronectin receptor integrin α8β1, but it is present in the fetal lung mesenchyme. As fetal lung mesenchyme partners closely with the epithelium during lung morphogenesis, it is not surprising that deletions in a mesenchymal integrin may result in a disrupted airway morphogenesis. Lungs from integrin α8 null mice have fused medial and caudal lobes and their lungs exhibit subtle branching defects through impaired mesenchymal cell adhesion and migration [106]. Certainly, more studies are needed to define the mechanisms whereby integrins regulate epithelial–mesenchymal interactions during lung development.

5.7.1.3 α9β1

The α9 integrin subunit only binds to β1 and forms the receptor for tenascin-C, osteopontin, VEGF-C, VEGF-D, and the cell surface immunoglobulin vascular cell adhesion molecule-1 (VCAM-1) [107–111]. Integrin α9β1 binds a

cleaved form of osteopontin at a site distinct from the RGD binding site used by other integrins and is only loosely associated with the other RGD binding integrins [112]. In addition, integrin α9β1 does not require the RGD region for binding its other major ECM ligand, tenascin-C. The variety of possible ligands would suggest, α9β1 is expressed in several cell types, including airway epithelial cells, smooth, and skeletal muscle cells, neutrophils, and a subset of endothelial cells. The global α9 null mouse demonstrates a requirement for α9β1 lymph development during lung morphogenesis. Integrin α9 −/− mice die by 12 days of age from fatal congenital bilateral chylothorax [113]. In contrast, no abnormalities were identified in airway structures, presumably due to integrin redundancy in the lung epithelium.

The role for integrin α9β1 in alveolar homeostasis and lung epithelial repair has not been defined. However, in neutrophils, α9β1 integrin enhances cell spreading and cell migration in association with binding of paxillin to the intracellular integrin α9 tail [114,115]. It is possible that a role for α9β1 exists in wound repair, but lung- and epithelial-specific studies are needed to define this role.

5.7.2 RGD-Binding Integrins as TGF-β Activators

In addition to binding the RGD motif in ECM ligands, a subset of the αv integrins also interacts with the RGD motif on LAP of inactive TGF-β, a well-recognized mediator of fibrosis. Under homeostatic conditions, LAP retains TGF-β in an inactive form and requires a change in conformation to release active TGF-β. Integrin αv combines with five β integrin partners, β1, β3, β5, β6, and β8, all of which bind the RGD motif of LAP and have the ability to activate TGF-β. The most extensively studied of these integrins is αvβ6.

5.7.2.1 αvβ6

Generation of the integrin β6 null mouse uncovered a special role for αvβ6 in activation of latent TGF-β. Integrin β6 has a single α integrin binding partner, αv, and is expressed by epithelial cells in the healthy adult lung at low levels. However, injury or inflammatory challenge markedly induces the expression of αvβ6 in a subset of epithelial cells in conducting airways and in alveoli [70]. In addition to binding ECM ligands fibronectin and tenascin-C, Huang et al demonstrated that αvβ6 also binds to the RGD motif on LAP of inactive TGF-β. Adhesion of αvβ6 to LAP facilitates the release of the active form of TGF-β by inducing a conformational change in LAP [116]. Consistent with the known role for TGF-β in the development of fibrosis, β6 null mice are protected against bleomycin-induced pulmonary fibrosis [117,118]. Integrin αvβ6 activation of TGF-β appears to be an important mechanism for fibrosis from a variety of insults. Integrin β6 null mice are also protected from radiation-induced pulmonary fibrosis, but not radiation related mortality [119]. Furthermore, fibrosis related to excessive TNF-α/EGFR signaling is also attenuated in β6 deficient mice [120].

Although alternate mechanisms for TGF-β activation exist, inhibition of integrin αvβ6 has been an important therapeutic target for the treatment of pulmonary fibrosis. Murine studies demonstrate that titratable doses of αvβ6 inhibitory antibody protect against bleomycin-induced pulmonary fibrosis in wild-type mice without inflammatory exacerbation, a side effect of TGF-β inhibition [121]. Follow-up studies of a human αvβ6 inhibitory antibody are underway. A phase-2 safety trial of humanized anti-αvβ6 integrin monoclonal antibody in patients with idiopathic pulmonary fibrosis is currently recruiting (www.clinicaltrials.gov, identifier number: NCT01371305).

Inhibition of αvβ6 holds promise as a potential therapy for pulmonary fibrosis, and recent studies elucidate the molecular mechanisms regulating integrin β6 expression. Tatler et al. identified a putative repressor region on the integrin β6 promoter associated with constitutive binding of the transcription factor Elk1. Loss of Elk1 in $Elk^{-/0}$ mice increased alveolar epithelial β6 integrin expression and exacerbated bleomycin-induced pulmonary fibrosis compared to control mice. The same region of the promoter contained a glucocorticoid receptor. However, treatment with the glucocorticoid receptor agonist dexamethasone only modestly inhibited β6 expression in vitro and failed to significantly decrease β6 expression following bleomycin treatment in vivo [122]. Regulation of αvβ6 appears complex and the mechanisms are currently not well understood.

There is an emerging role for integrin αvβ6 in lung inflammation and allergy. Alveolar epithelial expression of αvβ6 dramatically increases in response to bacterial challenge and at sites of neutrophil influx following lung injury [70,123]. After bacterial exposure, αvβ6 upregulates epithelial secretion of the antiinflammatory cytokine IL-10 [124]. In addition, histological examination of mice homozygous for integrin β6 null mutation reveals increased inflammation, characterized by clusters of lymphocytes surrounding large conducting airways [117].

A study by Sugimoto et al. utilized and in-vivo murine model of allergic asthma to implicate integrin αvβ6 in mast cell dependent airway hyperresponsiveness, a primary component of allergic asthma pathogenesis. In this study, integrin

β6 deficient mice are protected from airway narrowing associated with mast cell-derived proteases from following intranasal OVA exposure due to decreased TGF-β1 activation. Of note, β6-deficient mice exhibit similar mucus metaplasia, BALF inflammatory cells, and airway remodeling as control mice, suggesting that αvβ6-dependent TGF-β1 activation does not play a role in modulating these aspects of the allergic asthma phenotype [125].

Integrin αvβ6 also contributes to wound healing following epithelial injury. In undifferentiated epithelial cells healing denuded epithelium, integrin α6 expression spreads from restricted basal localization to include cell−cell lateral epithelial expression in both early and late repair after wound injury in human bronchial xenograft model [76]. Furthermore, recent in-vitro studies in primary rat AECs demonstrate that IL-1β, a proinflammatory cytokine upregulated in acute lung injury, stimulates αvβ6-induced TGF-β activation, enhancing alveolar epithelial wound closure [126].

5.7.2.2 *αvβ5*

Integrin αvβ5 binds primarily to the RGD sequence of vitronectin and to LAP and is widely expressed in keratinocytes, bronchial and airway epithelial cells, endothelial cells, fibroblasts, osteoclasts, and monocytes. Similar to αvβ6, integrin αvβ5, along with αvβ3, activate TGF-β through a conformation change in LAP, although this mechanism primarily occurs in fibroblasts or mesenchymal cells to promote autocrine TGF-β activation [127−129].

The integrin β5 null mouse informs us about the homeostatic function of αvβ5, as β5 subunit partners only with αv. Integrin β5 null mice develop normally with preserved airway architecture [130]. Due to redundancy in vitronectin integrin receptors, αvβ5 is likely not required for wound healing. Primary keratinocytes isolated from β5 −/− mice have decreased migration and adhesion to vitronectin compared to control primary cells. However, β5 −/− mice retained normal cutaneous wound healing in vivo. Integrin β5 −/− mice lack a lung phenotype in the absence of injury, suggesting compensation by other vitronectin receptors [130,131].

Several studies suggested that epithelial integrin αvβ5 modulates pathogen adherence, in particular infection of adenovirus infection in the respiratory tract [132−134]. However, primary airway epithelial cells from β5 −/− mice have similar infection efficiency compared to control, suggesting adenovirus infection or transfection does not require αvβ5 specifically [130]. A recent study demonstrated that αvβ5 siRNA knockdown in A549 immortilized respiratory epithelial cells decreased adherence of *Pseudomonas fluorescens*, an uncommon human pathogen of low virulence but related to the frequently seen human pathogen *P. aeruginosa* [135]. More studies are needed to define the role of αvβ5 in clinically relevant pathogenic airway infections.

5.7.2.3 *αvβ8*

Similar to αvβ5, integrin αvβ8 binds both vitronectin and LAP. However, distinct from αvβ5, integrin αvβ8 provides a novel mechanism for lung epithelial paracrine TGF-β activation. Rather than a conformational change in LAP due to binding, activation of integrin αvβ8 results in proteolytic cleavage of LAP. Following αvβ8 RGD binding to LAP, epithelial αvβ8 colocalizes with the metalloproteinase MT1-MMP. MT1-MMP then cleaves the LAP-latent TGF-β complex, releasing active TGF-β [136].

In primary airway epithelial cultures from asthmatic children, expression of epithelial αvβ8 is increased compared to control samples [137].

5.7.2.4 *αvβ1*

Recently, Henderson et al. identified as novel role for integrin αvβ1, expressed primarily by the lung mesenchyme, as a TGF-β activator with broad implications for fibrosis in multiple organs. In these studies, targeted murine depletion of the αv subunit in myofibroblasts protects against injury-induced fibrosis in the liver, lung, and kidney [138]. Elegant experiments utilizing deletions of the available β subunits that bind αv failed to protect against fibrosis, thereby excluded αvβ3, αvβ5, αvβ6, and αvβ8 as the critical integrins receptors. Combined with the αv deletion model, these findings implicated the remaining integrin pair, αvβ1, as the critical regulator. Furthermore, inhibition of αv using a small molecule inhibitor protected against bleomycin-induced pulmonary fibrosis and CCl_4-induced liver fibrosis [138].

5.8 SUMMARY

Cell-ECM interactions are required for many basic cellular processes including cell proliferation, differentiation, survival, and apoptosis. Lung epithelial cells connect to the ECM through heterodimeric αβ integrin receptors. The diversity of integrin receptors expressed by lung epithelial cells drives cell behavior and is tightly regulated throughout development and under homeostatic conditions. During lung development, laminin and collagen binding integrins are

required for branching morphogenesis and alveolarization. Epithelial integrins regulate inflammation in the homeostatic lung and likely modulate epithelial repair after injury. Both epithelial and mesenchymal integrins are important modulators of TGF-β activation and the development of pulmonary fibrosis.

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Chapter 6

Epithelial Regeneration and Lung Stem Cells

Shama Ahmad and Aftab Ahmad

In order to provide oxygen to every cell in our body, the lungs work hard by taking millions of breaths a day and inhaling thousands of liters of air. The airway and alveolar epithelial surface is therefore constantly challenged with incoming allergens, toxic chemicals/gases, and infectious agents. The epithelium provides a first line of defense against these toxic agents by multiple mechanisms of protection. These include mechanical activity of cilia, secretion of mucus and surfactant protein, and the innate immunity provided by dendritic cells embedded in the epithelium. Although the normal turnover of airway epithelium is slow, it has an enormous endogenous capacity to repair and replace itself following injury. The stem/progenitor cells that are unique to proximal, distal, and alveolar regions provide this repair capacity.

6.1 LUNG EPITHELIAL STEM/PROGENITOR CELLS

Stem cells are rare cells that have the capacity to regenerate entire lineages and the tissue. The adult stem cells are defined as rare morphologically unrecognizable cells endowed with a high proliferative potential and the lifelong ability to (1) maintain and replenish themselves, (2) generate a large number of functionally differentiated progeny, and (3) replace senescent and damaged cells in the steady state and following perturbation or injury [1]. The ability to regenerate extensively after injury of a largely quiescent lung suggests occurrence of endogenous stem/progenitor cell populations. Their resistance to various chemical injuries in the lung has also identified numerous epithelial cells that fit the definition of a tissue-specific stem cell. However, the search for a multipotent resident stem cell of the lung that can repopulate the whole airway epithelium is still elusive. Mammalian lung is a complex organ ($\sim$40 different cell lineages of which 11 are epithelial) that consists of conducting airway tubes including the trachea, bronchi and bronchioles, and the gas exchange region or alveolar spaces. The trachea and proximal airways are lined by a pseudostratified epithelium comprising basal, secretory (club and goblet), ciliated, and neuroendocrine cells (Table 6.1). Distal conducting airways (bronchioles) are lined by a simple columnar epithelium consisting of club, ciliated, and pulmonary neuroendocrine cells. The alveolar epithelium is populated by cuboidal surfactant secreting alveolar epithelial type II cells and squamous gas-exchanging alveolar epithelial type I cells. Specific stem/progenitor cells maintain the cell lineages of each of these regions.

6.1.1 Stem/Progenitor Cells of the Conducting Airway Epithelium

The proximal tracheobronchial epithelium is populated by two progenitor cell types, the basal and club cells (a specialized secretory cell, also known as the Clara cell). The basal cells (BCs) serve mostly the proximal airways however, the club-like or club cells maintain both bronchial and bronchiolar regions (Table 6.1).

6.1.1.1 Basal Cells

Basal cells, BCs anchor epithelium to the basement membrane. BCs are distinguished from other epithelial cell types by their pyramidal shape and by their distinct keratin expression profile. In the steady state, BCs express primarily keratins 5 and 14. Besides keratins 5 and 14, BCs also express transcription factor p63, nerve growth factor receptor (Ngfr), cell-surface marker podoplanin (Pdpn), a cell-surface lectin GSIb4, and tissue factor (TF) [2,3]. These proteins are

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TABLE 6.1 Summary of Airway Epithelial Lineages and Their Progenitors in Mouse and Human Airways

Airway Region	Cell Lineage	Markers	Self-Renew	Stem/Progenitor
Tracheobronchial	Ciliated	FoxJ1, beta and tubulin 4	?	Club cell, variant club cell, and basal cell
	Secretory club cells	CCSP and CyP450,	Yes	Club cell, variant club cell, and basal cell
	Variant club cell	CCSP	Yes	
	Secretory goblet cells	Muc5A/C, and Muc 5B	?	Club cell, and basal cell
	Neuroendocrine	Cgrp	Yes	Neuroendocrine progenitor, and basal cell
	Basal cells	CK5, CK14, Trp63, Ngfr, podoplanin, and TF	Yes	Club cells, BLPC
	Basal luminal progenitor cell (BLPC)	CK5, CK8, and Trp63	yes	
Bronchiolar	Club cell	Scgb1a1, CCSP, and CyP450,	Yes	Basal cells
	Bronchoalveolar stem cell (BASC)			
	Neuroendocrine cell (PNEC)	Cgrp	Yes	Basal cells
	Basal like cell/Distal alveolar stem cells (LNEPs?)	TRP63 and CK5	Yes	
Bronchoalveolar duct junction, BADJ	Club cells	Scgb1a1	Yes	
Alveolar	Alveolar type I	Aquaporin 5, SPA, SPB, and SPC podoplanin	?	Alveolar type II, BASC, and club cells at BADJ
	Alveolar type II	Lysotracker, aquaporin 1, and SPC	Yes	Alveolar type II, BASC, PNEC, and club cells at BADJ LNEPs
	Bronchoalveolar stem cell (BASC)		Yes	
	Integrin beta 4+ cells		Yes	

unique to BCs and are used as markers for their identification; however, the functional importance of most of these phenotypic markers is unknown. BCs are distributed throughout the human airway. They are abundant in the trachea and up to first six generations of the respiratory track. BCs are also found in the bronchiolar epithelium of the human lung. In rodent lungs, BCs are located primarily in the trachea and bronchi. BCs are also located in the glandular epithelium in which the submucosal glands (SMGs) provide an additional protected niche for them [4,5]. These cells have extensive proliferative potential, self-renewal capacity, and ability to differentiate into club cells and ciliated cells. In an influenza virus injury study, it was suggested that $Trp63^{+}$ BCs might migrate to alveolar region and promote repair in the alveoli [6]. Keratin 5 and 14 positive BCs were also identified in the distal airways and alveolar regions of IPF patients [7]. The molecular mechanisms by which BC self-renew and differentiate are poorly understood. Notch signaling is required for BC differentiation and is highly upregulated during reparative phase in mouse and human tracheobronchial epithelium [8,9]. SMAD signaling was demonstrated to promote differentiation of BCs whereas its inhibition promoted their expansion [10]. Similarly, BMP signaling was important for regeneration of airway epithelium from BCs [11]. Our studies demonstrated that endogenous TF expression and activity in BCs provided survival benefit and a de-novo fibrin matrix on injured/denuded basement membrane for their attachment, proliferation, and subsequent repair function [12,13]. This is important because TF is also a marker for BCs. Importance of TF in maintaining airway

epithelial integrity was further demonstrated in studies in which the low TF-expressing mice had increased hemorrhaging in their airways and were found to be susceptible to endotoxin and viral injuries [14–16]. Similarly, others have demonstrated that Trp63 is important for survival, regeneration, and differentiation of BCs [17]. Hackett et al. identified 1161 genes and approximately 70 transcription factors that were uniquely expressed in the BCs of healthy human bronchial epithelium [18]. How these transcriptional profiles drive BC behavior needs to be further studied. It was demonstrated that in isolated BCs, a Wnt-dependent signaling regulated the BC clone formation [19]. More importantly, this study also demonstrated that an isolated multipotent BC formed clones that were enriched with low cycling cells found normally in their niche suggesting that they are able to form their own microenvironment that is necessary for the maintenance of their stemness [19]. Thus, BCs play a dual role in providing structural support to the epithelium by anchoring it to the basement membrane and maintaining it by repairing and replenishing it during injury. Therefore, they are considered the most important stem cells of the upper airways.

Cole et al. described that in steady-state BCs exhibit two molecular phenotypes, keratin$^{5+/14-}$ and keratin$^{5+/14+}$ in which the later was termed a facultative progenitor cell [20]. This is because the keratin 14 positive BCs in steady state would only self-replicate but upon injury increased keratin 14 expression would render them multipotent. These two subsets were approximately 80% and 20% of the BC population, respectively. Although the steady-state mitotic index of the tracheal epithelial cells is low, about 10% using Ki67 as a mitotic marker, the BC subsets comprised approximately half of all mitotic cells in the mouse trachea [20]. Lineage tracing studies indicated that these steady-state BCs were responsible for maintenance of the BC population. BC phenotype and function also varies in the context of secretory cell injury. Naphthalene-mediated depletion of the tracheal club cell pool initiates an epithelial repair process that is driven by the abundant and broadly distributed BC population. These progenitor cells were keratin 14 positive and were derived from the keratin$^{5+/14-}$ and keratin$^{5+/14+}$ BC pools [20]. Injury-induced increased keratin 14 gene expression was responsible for this altered molecular phenotype. The distribution of keratin 14^{+} reparative cells along the proximal and distal axis and parallel restitution of the secretory cell population indicated that epithelial repair was mediated primarily by BCs rather than through activation of a proximally restricted tissue-specific stem cell. Recent data further indicate heterogeneity of BCs as two functionally distinct BC populations were identified in the proximal airways [21,22]. Accordingly, in the adult lung there are basal stem cells (BSCs) and basal luminal precursor cells (BLPCs). BSCs divide to produce a BSC and a BLPC that differentiates into neuroendocrine or secretory cell [21]. BLPC is further distinguished by its expression of keratin 8 (Fig. 6.1). Currently, this theory is revised as it has been demonstrated that upon injury keratin 5 positive BCs divide

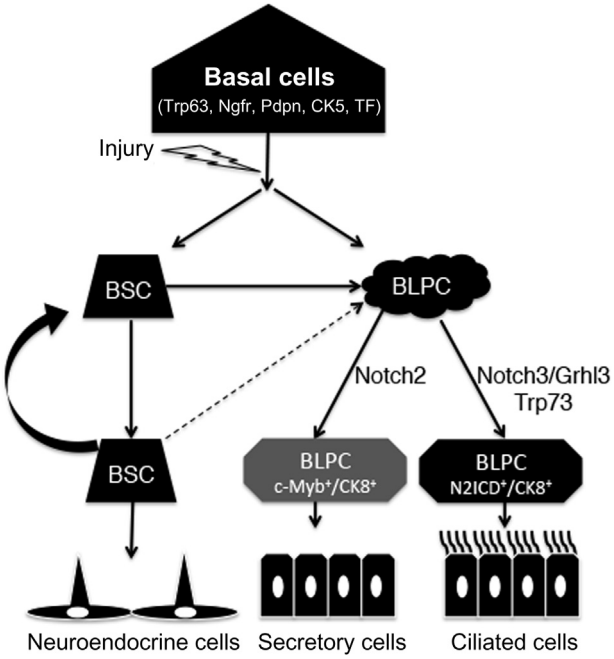

FIGURE 6.1 Injury-induced heterogeneity of basal cells for self-replication and epithelial repair. Basal cells are characterized by expression of Trp63, nerve growth factor receptor (Ngfr), podoplanin (Pdpn), cytokeratin 5 (CK5), and tissue factor (TF). Upon injury, they segregate into a basal stem cell (BSC) and a basal luminal precursor cell (BLPC). BSC self replicates or gives rise to more BLPC or neuroendocrine cells (NEC). BLPC segregate into luminal secretory and ciliated cells to reconstitute the pseudostratified epithelium, which is regulated by N2ICD or c-myb respectively.

into N2ICD$^+$ (active Notch2 intracellular domain) cells and c-Myb$^+$ cells that express cytokeratin (CK) 8. The N2ICD$^+$ CK8 positive cells generate secretory cells whereas c-Myb$^+$CK8$^+$ cells generate ciliated cells [23]. Thus, Notch2 signaling promoted secretory cell differentiation of a subset of BCs. A recent study has demonstrated that Trp73 is a regulator of ciliated cell differentiation from Trp63$^+$ BCs [24]. Similarly, binding of grainyhead like 2 (Grhl2) transcription factor to the promoter region of Notch3 regulated segregation of ciliated cells from BCs [25] (Fig. 6.1). These studies demonstrate that upon injury distinct subsets of basal progenitor cells could arise and contribute to regeneration of the airway epithelium. This heterogeneity of BCs is evolving and needs to be further studied in both homeostatic and injured epithelium. Importance of BCs is also revealed in studies in which their ablation favors differentiation of secretory cells into ciliated cells, suggesting they may regulate behavior of neighboring cells [26]. Potential of BCs to migrate to alveolar region and reconstitute the alveolar epithelium needs to be further confirmed by lineage tracing studies [6]. BCs may also contribute to host defense as they are often target of respiratory pathogens [27]. Thus, BCs play a key role in the epithelial homeostasis and repair of injury to the upper airways.

6.1.1.2 Club Cells

Club cells are the progenitor cells of the trachea and the bronchiolar region, which were identified by their resistance to naphthalene-induced chemical injury [28]. They are dome shaped cubical nonciliated cells that secrete a specific protein belonging to the secretoglobin (SCGB1A1) family called club cell specific protein (CCSP, CC10). Club cell may structurally vary among species and along the proximal to distal axis of the airway epithelium. Thus, studies utilizing oxidant gas exposure and pulse-chase strategies suggested that most if not all rabbit [29] or rat [30,31] club cells have the ability to proliferate in response to injury. The ultrastructural differences between proximal and distal airway secretory cells led to designation of upper airway secretory cells as club-like cells [32]. All mouse airway secretory cells, from the trachea to the terminal bronchioles, express CCSP. Thus, CCSP expression in the mouse is synonymous with the club-like and club cells. However, human proximal airway secretory cells are more readily recognized by expression of mucins such as Muc5Ac. In adult human airways, expression of CCSP is restricted to the terminal bronchioles. These "differences" in expression of CCSP in the adult human and the mouse have led to the conclusion that human airways do not have a constitutive population of nonmucus secreting secretory cells. However, studies in mice suggest that mucus cells are derived from CCSP-expressing cells through a metaplastic transition [33,34]. These mucus cells may be postmitotic, although this is debatable at this point. These studies suggest that the human airway does have secretory cells that are functionally similar, if not molecularly identical, to the CCSP positive mouse club-like and club cells. However, the lineage relationship has not been evaluated in the human.

Upon injury to the epithelium, club cells undergo mitosis for their self-renewal and generation of facultative progenitor club cells. Facultative club cell can differentiate into secretory or ciliated cell to restore the damaged epithelium [28]. Facultative progenitor club cell is also known as the "variant club cell" that expresses SCGB1A1 but not cytochrome p450 (Cyp2f2, which is normally expressed by club cells) [28]. Scgb1a1+Cyp2f2-variant club cells can self renew and differentiate into ciliated cells both during normal homeostasis and during injury [35–37]. Variant club cells are found around the neuroepithelial bodies or at the bronchoalveolar duct junctions (BADJ). Another difference from the nonvariant club cell is the expression of uroplakin3a (Upk3a) in the variant club cell [38]. Fgf10 secreted by the parabronchial smooth muscle cell was also found to be necessary for the regenerative function of variant club cells [39]. Using XB130 knockout mice, it was demonstrated that the club cell proliferation was significantly reduced after naphthalene injury in the knockout as compared to the wild type [40]. Therefore, XB130 may regulate club cell proliferation during epithelial repair. Chronic depletion of club cells by repeated naphthalene exposure can result in development of pulmonary fibrosis [41]. Costaining with proliferation marker Ki67 and CC10 in the bronchial tissue of hypoxia-exposed mice revealed that hypoxia induces a proliferative response in the bronchial club cells. This response was driven by HIF2α-dependent activation of FOXM1 and bronchial epithelial growth factors, the resistin-like molecules, RELMα, and RELMβ [42]. These findings could be a novel mechanism of the adaptation of bronchial epithelium to oxygen fluctuations. Club cells eliminate biological pathogens such as viruses and also confer immunity to the bronchial epithelium against subsequent viral infections [43]. Club cells at steady state play critical role in epithelial barrier maintenance, secretion, and metabolic functions. Thus, club cells have multiple functions that are fundamental to the health of conducting epithelium.

6.1.1.3 Neuroepithelial Bodies

Pulmonary neuroendocrine cells occur as solitary cells or in clusters throughout the respiratory tract starting from the nasal epithelium, in laryngeal mucosa, trachea, and in the terminal airways. These are flask-shaped cells that extend

from the basement membrane to the lumen. They are characterized by calcitonin gene-related peptide (CGRP) expression. Clusters of these cells are referred to as neuroepithelial bodies (NEBs). These NEBs may be formed by certain epithelial neuroendocrine progenitors that "slither" to the airway branch points while undergoing temporary epithelial mesenchymal transition and differentiate into NECs, get clustered and innervated [44,45]. The clustering could be related to the expression of a gene called roundabout receptor (Robo). Inactivation of Robo decreased clustering and increased secretion of neuropeptides leading to increased immune infiltrates that caused matrix remodeling and simplification of the alveoli [46]. Therefore, the NEBs may control lung immune response, and their abnormalities could be involved in the pathogenesis of pulmonary diseases. NEBs are the chemosensors for the lung and detect hyperoxia and hypercapnia. They may also exert paracrine effects on neighboring cells to enhance their proliferation. The NEBs serve as a niche for stem/progenitor cells such as the variant club cells, of the bronchiolar epithelium [47,48]. CGRP positive pulmonary neuroendocrine cells themselves exhibit a potential to self-renew and differentiate into club cells and ciliated cells upon naphthalene injury [49]. However, their role in epithelial regeneration may be limited as their ablation, did not significantly impact club cell regeneration after injury [49,50]. Abnormalities in the NECs have been associated with a number of pediatric lung disorders and diseases indicating that they play important roles in pulmonary growth and function that needs to be further evaluated [51].

6.1.2 Stem/Progenitor Cells of the Alveolar Epithelium

The alveolar sacs are lined by the squamous type alveolar type I cells (or type I pneumocytes) and cuboidal alveolar type II (or type II pneumocytes) cells. The ratio of type I and Type II alveolar cells in the alveoli is 1:2; however, flattened type I cells contribute about 95% of the alveolar surface in which the passive gas exchange takes place. Alveolar type I cells are terminally differentiated; however, emerging studies suggest that they may exit their terminally differentiated nonproliferative state under certain conditions [52,53]. Cuboidal alveolar type II cells have abundant surface microvilli and cytoplasmic lamellar bodies rich in surfactant proteins. The surfactant proteins maintain the structure of the alveoli and protect it from collapsing. Lineage tracing studies have demonstrated that during development both alveolar type cells originate from a common progenitor however after birth alveolar type I cells mostly derive from self-renewing, long-lived, and alveolar type II cell [53]. Whether there is an actual alveolar type II resident progenitor cell or alveolar type II cells are facultative progenitors is still not clear. The stem-like function of alveolar type II cells that express surfactant protein C (sftpc+) is activated by injury such as that caused by hyperoxia [54–57]. Sftpc expressing alveolar type II cells can self-renew or differentiate into alveolar type I cells [58,59]. Alveolar type I and type II cells both contribute toward the immune defense response of the airways against invading microbes [60,61]. Expression of glycoproteins such as stanniocalcin 1 that have antiinflammatory, antioxidant, antiapoptotic, and wound healing properties could contribute toward the type II cell defense actions [62,63]. Thus, the alveolar epithelium is also a source of airway defense as well as repair after injury.

It has been shown that the majority of alveolar type II cell may not be derived from preexisting $sftpc^{+}$ cells [64]. These studies identified a subset of alveolar epithelial cells that expressed laminin receptor integrin $\alpha 6\beta 4$ but not sftpc that acted as the progenitor [64]. Further, these studies also demonstrated that nonlineage marker expressing cells might be involved in the general pulmonary epithelial repair after injury. These are called lineage negative epithelial stem/progenitor cells (LNEPs) that proliferate and migrate widely to injured areas of the epithelium. Repair by LNEPs require Notch signaling to activate deltaNp63 and CK5 cascades followed by subsequent Notch blockade to promote alveolar epithelial cell fate [8]. It was also demonstrated that BCs may migrate to the alveolar region during H1N1 infection to repopulate it but it was not confirmed by lineage tracing [6]. Thus, these studies suggest occurrence of other alveolar epithelial progenitors and open up avenues for future research.

6.2 MECHANISMS/PATHWAYS OF REPAIR BY AIRWAY PROGENITORS

The adult lung is a highly quiescent organ demonstrating a very low turnover. Sonic hedgehog signaling plays a critical role in maintaining airway epithelial homeostasis as its deregulation during injury may cause aberrant repair and regeneration [65]. Similarly, an FGFR1-SPRY2 signaling axis was shown to limit airway epithelial BC proliferation in the steady state [66]. During injury airway epithelial stem/progenitor cell function is largely driven by the external signals produced by microenvironment or niche in which they reside [67]. This has considerable complexity as the lung comprises a large variety of cell types. Moreover, stem/progenitor cell behavior may also be affected by alterations in microenvironment composition such as those found in chronic respiratory diseases such as fibrosis/matrix deposition, angiogenesis, and inflammation. However, a lot of progress has been made in determining the signals that regulate lung

epithelial stem cell proliferation and differentiation [68]. Stem cell behavior such as quiescence, proliferation, or differentiation is regulated by a combined effect of signaling from molecules such as Wnt, Notch, Hippo/Yap, ROS/Nrf2, c-myb, cytokines such as IL-4, IL-13, and IL-6, growth factors (FGF, PDGF, and EGF) produced by the neighboring cells and the cell-to-cell contacts as well as their extracellular matrix [8,9,23,36,69–77]. Hegab et al. recently demonstrated that distal lung epithelial stem cells require coculture with stromal cells to undergo clonal proliferation and differentiation [73]. Of the stromal cells they observed, fibroblasts, were most efficient supporters of their growth. Molecules such as FGF2, FGF9, and FGF 10 and LIF increased their colony formation [73]. Others have also demonstrated that FGF10 signaling may be a critical pathway during lung development as well as in interactions of adult lung epithelial stem cell and their niches [69,78,79]. Histone deacetylase may contribute important proliferative function to the club cells as deletion of HDAC1 and HDAC2 in postnatal club cells in mice inhibits their regeneration after naphthalene-induced depletion [80]. Role of purinergic signaling and coagulation cascade proteins such as TF are other emerging concepts in lung repair and regeneration. Perturbations in these pathways contribute toward pathogenesis of a number of pulmonary diseases [81–84].

6.2.1 Wnt Signaling

Wnt proteins form are a family of highly conserved proteins that regulate cell-to-cell interactions during lung development, adult tissue homeostasis, and stem cell self-renewal and regenerating responses. Signaling by Wnt proteins may modulate functional maturations of airway epithelial cells during prenatal lung development [85,86]. Wnt signaling plays an important role in alveolar type I cell differentiation and lung sacculation [78,87,88]. Molecular analysis of mechanisms regulating SMG development revealed a complex role for the Wnt-β-catenin signaling pathway in bud formation and elongation [89,90]. Wnt proteins also regulate expansion and differentiation of stem/progenitor cells in adult SMG after injury [4]. Wnt and β-catenin is required for self-renewal and proliferation of tracheal epithelial BCs after naphthalene injury [91]. Wnt-β-catenin pathway was recently demonstrated to be involved in adult alveolar progenitor (type II alveolar epithelial cell) cell differentiation [92]. This suggested that Wnt signaling plays an important role in alveolar epithelial regeneration. Deregulation of Wnt-β catenin signaling by inhibition of β-catenin causes fibrosis after bleomycin injury [93]. Others have demonstrated that Wnt signaling may promote bleomycin-induced pulmonary fibrosis and its inhibition may reverse the disease [94]. Increased TGF-β-dependent Wnt 10 expression in the fibroblasts of bleomycin-treated mice was a potential mechanism of fibrosis [95]. This study also demonstrated that increased Wnt expression correlated with adverse outcome in patients with idiopathic pulmonary fibrosis. Thus, Wnt signaling may be important for regenerative responses of epithelial progenitors, but its activation in fibroblasts may lead to fibrosis. Therefore, there is a need to carefully consider these effects while studying potential Wnt-dependent therapies for pulmonary diseases [96].

6.2.2 Notch Signaling

Notch signaling is essential for lung development and plays important roles in airway epithelial regeneration [97]. Disruption of Notch signaling in mice causes abnormal enlargement of alveolar spaces, which is similar to those seen in chronic lung diseases [98]. Notch signaling was also required for maintaining the epithelial and bronchial smooth muscle layer in the distal lung [98]. In the postnatal lung, Notch signaling is required for differentiation of BCs into secretory cells after severe injury [9,23]. This differentiation was shown to be mediated by a Notch ligand JAG1 [99]. More importantly, the transition of BCs into epithelial progenitors that differentiate into secretory cells is mediated by Notch 3 [100]. It was demonstrated that selective endogenous activation of Notch 3 was required to control the pool of Trp63+ BCs that will generate a parabasal cell population having the ability to activate Notch 1 and 2 for ciliated or secretory fate selection [35,100]. Reactive oxygen species (ROS) levels is one niche regulatory mechanism by which the stem cells maintain their quiescence or proliferative states [101]. ROS (ROS)-mediated Notch activation stimulates airway epithelial BC self-renewal and an antioxidant response that scavenges the ROS [76]. Thus, Notch signaling provides important mechanisms for maintenance of airway epithelial progenitor cell functions.

6.2.2.1 Functions of Purinergic Receptors in Airway Repair

Airway epithelium is rich in both P2X and P2Y purinergic receptors [102]. Extracellular ATP and adenosine can activate signaling cascades comprising protein kinases including extracellular signal-regulated kinase (ERKs) and phosphatidylinositol-3-kinase (PI3K) [102]. ATP released from neuroepithelial bodies (NEBs) promotes paracrine

effects on nearby club-like cells by activation of P2Y2 receptors. As NEBs and club-like cells function as pluripotent epithelial stem cells upon injury, purinergic signaling could be critical in the repair after injury [48,103,104]. Further, evidence is emerging that purinergic receptor signaling may promote repair of human airway epithelium [105–107].

6.2.2.2 *Role of Tissue Factor in Airway Epithelial Basal Cell Function*

Coagulation/fibrin deposition in wounds or other sites of injury (e.g., skin) is critical to maintain epithelial barrier integrity. Constitutive TF expression is associated with biological boundaries including skin (where it is also expressed in the basal layer), organ surfaces, vascular adventitia, and epithelial–mesenchymal interfaces, thereby providing a hemostatic envelope that protects organisms against bleeding upon injury [108–110]. It is generally accepted that repair of airway injury is preceded by exudation of plasma that generates a fibrin-rich gel in which restoration of the epithelium proceeds rapidly [111]. Formation of fibrin is initiated by activation of the cell surface TF activation by the serine protease Factor VII ligand. TF-dependent fibrin formation may be required for repair in airway epithelial cell lines [112]. Recently, low TF-expressing mice demonstrated delayed wound healing, comparable to that seen in hemophilic mice [113]. Low levels of TF-enhanced susceptibility to insults and caused alveolar hemorrhage, increased oxidative stress, and potentiation of acute lung injury, hence confirming critical nature of TF expression and its role in limiting airway injury [16]. Low TF-expressing mice are also susceptible to pulmonary and systemic microbial infection further indicating a role for TF in host defense [114,14].

BCs play dual roles in airways, by attaching epithelium to the basement membrane, and by serving as resident progenitor cells [115–118]. TF is a marker for nasal, tracheal, and embryonic BCs [13,118–120]. TF-expressing BCs from nasal airways had several-fold greater colony forming ability than TF-negative columnar epithelial cells [118]. TF expressing tracheobronchial epithelial BCs also demonstrate enhanced colony forming ability [12,13]. Thus, TF expression in human tracheal BCs can improve endogenous BC function and enhance repair after acute airway injury. This strategy of enhancing endogenous stem cell function may supersede stem cell transplantation which often causes lung dysfunction [121]. Our studies support the premise that endogenous TF activity in basal/progenitor airway epithelial cells confers a self-repair capacity to the airway epithelium by initiating formation of a provisional fibrin matrix and improving cell survival (Fig. 6.2) [13].

The conducting airway epithelium serves as the interface between the lung and the environment. This role is reflected in the structural and functional diversity found along its proximal to distal axis. Multiple progenitor cell pools maintain the proximal, distal, and alveolar epithelium regions. However, only subtle differences distinguish them. The magnitude of these differences may increase or decrease in response to injury and the tissue-stem/progenitor cell. Further studies are required to better define these stem cell populations and their regenerative potential to bring the stem cell transplant and regenerative therapies from bench to bedside.

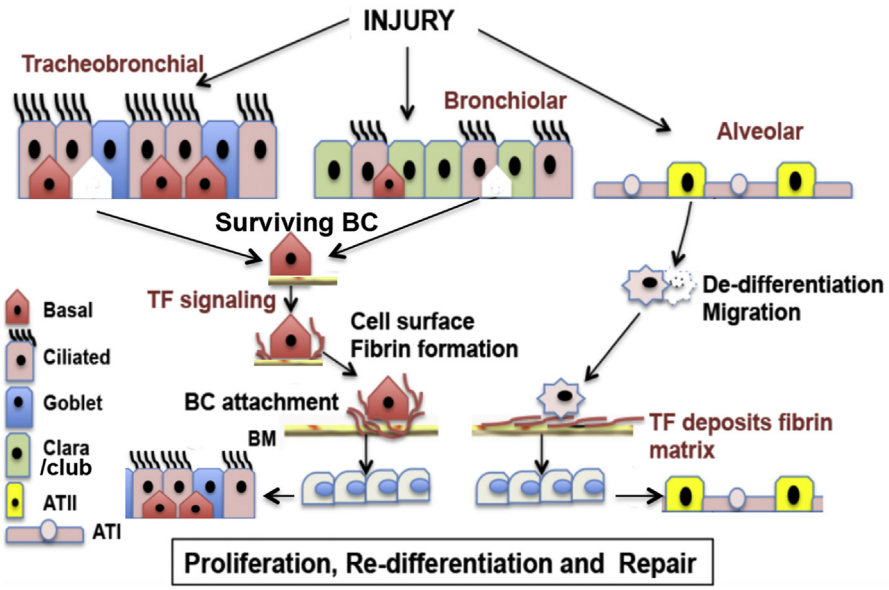

FIGURE 6.2 Conceptual model showing role of tissue factor (TF) in mediating repair of airway epithelial injury by providing an endogenous survival mechanism and exogenous cell surface fibrin matrix for basal cell (BC) propogation and segregation. TF activity may also provide fibrin matrix for the repair function of alveolar progenitors. *BM*, basement membrane; *ATI*, alveolar type I; *ATII*, alveolar type II.

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Chapter 7

The Function of Epithelial Cells in Pulmonary Fibrosis

Shijing Jia and Kevin K. Kim

7.1 INTRODUCTION

Progressive fibrosis is an aberrant wound healing response characterized by dramatic changes in the cellular and extracellular matrix makeup of the lung [1−5]. There is a relative loss of epithelial cells in favor of activated fibroblasts with varying amounts of inflammatory cell infiltrate depending on the specific fibrotic pathology. There is concurrent destruction and distortion of the normal basement membrane architecture which is progressively replaced by a provisional matrix and ultimately by fibrillar collagens, especially type I collagen. Progressive fibrosis can be a devastating consequence of many acute injuries such as fibroproliferation during acute respiratory distress syndrome as well as chronic insults to the lung as with fibrosis in systemic sclerosis. Fibrosis can also progress in idiopathic forms such as in idiopathic pulmonary fibrosis (IPF). Pulmonary fibrosis can predominate in the interstitium as in many primary and secondary interstitial lung diseases or can occur in the airway remodeling that occurs in chronic obstructive lung disease (COPD) and longstanding asthma [6]. Fibrogenesis is of course not limited to the lung but can occur in almost any tissue with similarly profound impact on the normal structure and function of the tissue or organ. This has led some to suggest that collectively fibrosis is the leading cause of death in the United States with estimates that up to 45% of deaths in the developed world are attributable to some type of chronic fibroproliferative disease [7,8]. While this chapter focuses primarily on fibrosis in interstitial lung disease, there are many common overlapping themes to the fibrotic process that represent exciting avenues for further investigation with the goal for developing antifibrotic therapies which could have a very large impact on human health. One of the most prescient themes is the role of epithelial cells in directly and indirectly orchestrating the fibrotic process.

7.1.1 Apoptosis and Senescence

Given the exposure of the lung epithelium to the external environment, various insults, both external and internal, can promote epithelial cell stress, senescence or death. The epithelium is thus poised to react to injurious stimuli by initiating a reparative response in the early stages of its response to injury. Senescence is a process by which cell proliferation is permanently abrogated but the cell remains viable and active [9−12]. In contrast, epithelial cells can also respond to injurious stimuli by undergoing various forms of death. Apoptosis is the process of controlled, programed cell death in response to signaling within or outside of the cell, while necrosis is a more dysfunctional death often in response to direct injury from a pathogen or toxin [13,14].

Alveolar epithelial cell (AEC) injury or death is sufficient to initiate fibrosis. Injury or death to the type II AECs is a critical initiating event in the development of pulmonary fibrosis. A link between AEC defects and the pathogenesis of pulmonary fibrosis was initially suggested in histopathology sections from IPF patients where the type II cells overlying the fibroblast foci consistently demonstrate phenotypic changes including dysplasia, hyperplasia, and apoptosis [15−17]. A causal relationship between these defects and the development of scarring was further suggested by the discovery that patients with familial pulmonary fibrosis harbor mutations in genes that are specifically expressed in type II AECs [18,19], suggesting that epithelial dysfunction is sufficient to induce fibrosis [20,21]. Finally, patients with

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fibrotic lung diseases demonstrate increased levels of lung epithelial cell markers in their serum, suggesting that ongoing epithelial cell injury occurs. This might represent an important biomarker and provide clues to a functionally important role for epithelial cell death during progressive fibrosis [22,23].

Senescence. Senescence can be triggered by DNA damage or counterintuitively, by sustained stimulation of mitogens or oncogenetic activation. DNA damage from various causes such as UV, toxins, or oxidative stress activates an adaptive response to limit genomic instability. The presence of senescence is characterized in part by the downregulation or absence of markers of cell proliferation and increased expression of several senescence associated markers such as β-galactosidase and by the activation of specific signaling pathways to robustly inhibit proliferation. For example, increased expression of pRb, p53, $p16^{INK4a}$, and p21 all function to strongly inhibit cyclin dependent kinases, and increased expression of these cell proliferation signals are often used as markers of senescence [13,24]. Senescence also involves activation of a cellular response mediated by several transcription factors notably, NF-κB and C/EBPβ [9,24]. Many of the respondent genes are secreted factors including proteases, growth factors, and cytokines. The secretory response is often termed "senescence-associated secretory phenotype" (SASP) and may represent initiation of a coordinated repair process as a result of the senescence inducing triggers [25,26]. The SASP enables the senescent cell to rapidly change the extracellular milieu with potential broad impact on the repair versus fibrotic outcome. The exact factors that are released by the SASP cells and how they might influence other cell types are poorly characterized. Several of the SASP factors are ones that are thought to be relevant for epithelial cell paracrine functions during fibrosis discussed below [27]. The role of fibroblast and epithelial cell senescence on fibrosis has not been well established. Markers of senescence, including many of the ones listed above, have been identified in models of lung fibrosis and from lung tissues from patients with IPF. For example, in one study using the bleomycin model of fibrosis, bleomycin-induced DNA damage led to activation of a senescence associated ataxia telangiectasia mutated/ataxia telangiectasis mutated rad3-related (ATM/ATR) pathway within lung epithelial cells, upregulation of $p21^{CIP1}$, activation of NF-κB, and increased production of a number of SASP secretory factors, some of which have been implicated in fibrosis [28,29]. However, a direct role for epithelial cell SASP in promoting fibrosis is still currently lacking. Notably, telomerase activity may act to blunt ATM/ATR induced senescence by limiting telomere shortening. Mutations in telomerase has emerged as a major genetic predisposition for fibrosis, potentially through exaggerated epithelial cell senescence as discussed below [13,14].

Epithelial Cell Autophagy. Autophagy is a cellular response to stress in which intracellular components, including macromolecules and organelles, which have become dysfunctional, are degraded within lysosomes. This response may promote cell survival during stress or may conversely initiate apoptosis [30–33]. During injury and fibrosis many cell types may undergo autophagy, including fibroblasts and epithelial cells. The importance of autophagy during IPF and other progressive fibrotic diseases remains controversial [34]. A number of molecular mechanisms involved in autophagy have also been implicated in fibrosis. In particular, the mammalian target of rapamycin (mTOR) pathway has become a major focus in both studies of fibrosis and autophagy [35]. One study, Patel et al. found evidence for decreased autophagy in IPF lungs, which may have been in response to transforming growth factor beta (TGFβ) signaling [36]. Much of the focus on a potential role for autophagy in fibrosis has been on fibroblast autophagy, but a number of the stressors to the lung epithelium during fibrosis, including ER stress and oxidative stress, are known to trigger autophagy. Epithelial cell autophagy might be involved in initiating epithelial cell apoptosis. Consistent with this notion, several recent reports find that in models of amiodarone induced fibrosis and Hermansky–Pudlak syndrome (HPS), AEC apoptosis is mediated through autophagic processes [37–39].

Apoptosis. Activation of the restorative/preservation pathways such as senescence and autophagy may be insufficient to protect the cell but may instead lead to a process of programed cell death. Apoptosis is typically initiated through an extrinsic or intrinsic pathway which converges on activation of caspase-3 [20]. The extrinsic or ligand-mediated pathway involves a ligand such as FasL or tumor necrosis factor alpha (TNFα) binding to a death receptor such as Fas or TNF receptors which leads to activation of caspase-8 and ultimately caspase-3. The intrinsic or mitochondrial apoptotic pathway is initiated by DNA damage or oxidative stress. This leads to activation of several apoptotic Bcl-2 family members, release of cytochrome C, activation of caspase-9, and ultimately activation of caspase-3. Significantly, these caspases act as proteases and can influence other intracellular signaling pathways through their proteolytic activity. Thus, activation of apoptotic pathways can influence the behavior of cells during or even instead of overt apoptosis [40,41]. There are also a number of caspase independent cell death programs that are less well understood [42–44].

Significantly, TGFβ is a prominent driver of epithelial cell senescence and apoptosis, although the precise molecular pathway is not completely understood [45–48]. The canonical TGFβ signaling pathways converge with the senescence and apoptotic pathways at several points. TGFβ can induce expression of p21, which is prominent in many senescence programs. Furthermore, TGFβ signal activation can lead to formation of reactive oxygen species within the cell, and oxidative stress is a prominent inducer of both senescence or apoptosis [49–51]. Finally, TGFβ can lead to increased

expression of death ligands such as FasL and increase expression of death receptors or other proteins in the apoptotic pathway, all leading to enhanced epithelial cell apoptosis [52].

Inhibition of prominent cell death pathways, such as Fas/FasL and caspases, by genetic deletion or chemical inhibition protects mice from experimental fibrosis [49,53–55]. To directly demonstrate a causal link between type II cell death and lung fibrosis, Sisson et al. generated a transgenic mouse in which expression of the diphtheria toxin receptor (DTR) is regulated by the surfactant protein C promoter (SPC-DTR) [56,57]. The resulting mice thus have high expression of DTR limited specifically on the surface of AECs. Repetitive doses of diphtheria toxin administered to these animals induce targeted caspase-mediated injury and death of the type II AECs [58]. The mice developed significant lung collagen accumulation and histopathologic features of alveolar fibrosis without requiring any other injurious stimulus [57]. This model confirms a direct role for AEC death in the genesis of alveolar scarring; however, the mechanisms that regulate AEC apoptosis during fibrogenesis and the down-stream events that translate the epithelial cell insult into fibrosis are unknown.

Function of senescence/apoptosis in fibrosis. The functional contribution of cell death during repair/fibrosis is unclear and requires further study. Less-controlled cell death as in necrosis may lead to disruption of the epithelial barrier and insufficient expression homeostatic proteins, such as surfactant proteins, FGF7/KGF (keratinocyte growth factor), and prostaglandins, which then leads to insufficient reepithelialization and pathologic wound repair and fibrosis [59]. Indeed, deficiency in certain prostaglandins and mutations in surfactant proteins have been implicated in fibrosis. Apoptosis may similarly lead to loss of normal epithelial function with resulting activation of fibrotic repair. Alternatively, the programed nature of the apoptotic cell may trigger a reparative reaction with secretion of factors to elicit a wound repair response in the surviving epithelial cells and other cell types similar to the SASP [17]. Thus, the impaired AECs may lead to fibrosis through loss of antifibrotic homeostatic AEC functions or through active orchestration by the injured or dying AEC in initiating a fibrotic wound healing response.

7.2 EPITHELIAL–MESENCHYMAL TRANSITION

The possible contribution of epithelial–mesenchymal transition (EMT) to tissue fibrosis was initially proposed over twenty years ago [60] through histological analysis and extrapolation from EMT in other situations. During these last two decades there has been development of sophisticated mouse models and an explosion in the acquisition of fibrotic human tissue and live samples. There has also been considerable progress in understanding the molecular basis and regulation of EMT during embryonic development and in cancer [61–66]. Yet, the role or even occurrence for EMT during fibrosis remains as controversial, if not more controversial, than when it was first proposed. For nearly every organ system there is seemingly convincing evidence both in support of and against EMT as a having a major contribution to fibrosis. Some of the controversies regarding EMT help define and clarify broader uncertainties and controversies in the fibrosis field in general.

Definition of EMT. Epithelial cells are characterized in part by an immobile state with a static, defined shape (e.g., cuboidal, columnar, or squamous). Epithelial cells form a number of well-defined tight cell–cell contacts, which is the foundation of an epithelial lining barrier that is important for the function of many organs. Epithelial cells generally have an apical–basal polarity and produce factors that are secreted from the apical surface into a luminal space. Alternatively, the mesenchymal cell phenotype supports migratory behavior of these cells. The cell–cell contacts are more transient and the cell shape is more fluid and less defined. As the cell migrates, there is formation and turnover of lamellipodia and filopodia. EMT can thus be defined as a process in which epithelial cells lose normal tight epithelial cell–cell contacts, alter cell shape with dramatic cytoskeletal changes, and change polarity from apical–basal to anterior–posterior, all favoring acquisition of a migration/invasive phenotype [67,68]. EMT may simply reflect a process in which there is need for dynamic cellular changes, as might occur during embryonic development, response to injury, and carcinogenesis. These changes must be accompanied by changes in proteins involved in cell contact (e.g., loss of E-cadherin and gain of N-cadherin) and cytoskeletal proteins (e.g., loss of cytokeratin and gain of vimentin and α-smooth muscle actin). There is also loss of proteins normally secreted into the luminal space (e.g., surfactant proteins and hormones) in favor of proteases and extracellular matrix proteins. The presence of EMT has often been defined by detection of a set of biomarkers, with loss of epithelial proteins and gain of mesenchymal markers [69]. Frequently, the acquisition of such dynamic changes in epithelial behavior is reversible. There is broad agreement that EMT occurs and is necessary for normal embryonic development. A role for EMT during cancer metastasis and fibrosis have been proposed, but the significance and occurrence of EMT in adult pathological states remains controversial [70–72]. EMT during these different circumstances has potentially overlapping features but likely also some important differences based on the function of EMT in each condition. These differences have led some to subtype EMT into type I (embryonic

development), type II (fibrosis), and type III (cancer metastasis) [62,69]. Endothelial cells may also transition into a mesenchymal-like phenotype which has been labeled EMT or often categorized within EMT, as these processes have many overlapping features [63,73,74].

EMT Pathways. EMT can be regulated by a number of extracellular ligands and can initiate intracellular signaling cascades upon binding to cell surface receptors, leading to an EMT transcriptional response. EMT is initiated by many ligand/cell surface receptor interactions. TGFβ is a robust and most extensively studied inducer of EMT. Activation of TGFβ signaling can initiate EMT canonical Smad signaling through direct Smad mediated suppression of epithelial genes and transcription of a number of mesenchymal genes [61,68]. TGFβ/Smad signaling can drive the EMT transcription response indirectly through induced expression of a number EMT transcription factors, such as Snail, Slug, Twist, and Zeb, potentially leading to a more robust and sustained EMT response to TGFβ [75–77]. TGFβ overlaps with a number of other EMT pathways. Among the more well-studied EMT ligand/receptor combinations include Wnt/Frizzled, which leads to stabilization of β-catenin [68]. β-catenin signaling has emerged as an important Smad coactivator; so in addition to activating its own target genes through interactions with TCF/LEF, β-catenin can regulate the EMT response through regulation of the Smad complex [78,79]. Other prominent EMT signaling pathways include pathways that are prominent in fibrosis such as Shh/Ptc/Gli and Delta/Notch. Activation of Rho family GTPases, including RhoA, Rac1, and Cdc42 through matrix/integrin signaling has emerged as a prominent regulator of EMT. Activation of these GTPases promotes transcriptional changes and leads to loss of the adherens junction complex, breakdown of the apical–basal polarity, and cytoskeletal rearrangement [80,81]. Activation of small GTPases promotes transcriptional changes through factors such as myocardin-related transcription factor (MRTF). Inhibition of many of these mediators including Rho kinase and MRTF with genetically modified mice or chemical inhibition has been demonstrated to attenuate EMT and fibrosis [82–86]. Hepatocyte growth factor (HGF) was initially described as a "scatter factor" and is perhaps the earliest prototype of a single factor capable of inducing EMT in vitro [87]. More recently, HGF has been shown to both augment or inhibit the cellular response to TGFβ depending on the cell type [88,89].

EMT in Development and Cancer. The role of EMT during development and the controversy of EMT in cancer metastasis is discussed extensively in many other reviews, but a few points regarding EMT in these other circumstances is discussed here to provide context to studies exploring the possibility of EMT in fibrosis [80,81]. EMT during development is well characterized and well accepted [90]. Embryogenesis involves multiple different EMT events that have been well described [67] and occurs throughout embryonic development. For example, EMT occurs early during gastrulation, as epiblasts change phenotypes to migrate and form the primitive streak then, move to the interior of the blastocyst and form the initial mesodermal germ layer [91–94]. Later, a subset of neural tube cells detach and undergo EMT to form the neural crest cells that migrate and eventually give rise to many mesenchymal structures [95,96]. EMT is also well described in later stages of embryonic development. During organogenesis, EMT is involved in cardiac valve formation [97], palate closure [98,99], and formation of the liver, pancreas, and kidney. During organogenesis, EMT is often followed by a reversal, or mesenchymal–epithelial transition (MET), highlighting the plasticity of cells during development [100,101]. For example, in pancreatic development, cells from the developing pancreatic bud undergo EMT and invade into the interstitium, where they undergo MET the form the islets of Langerhans [102]. More durable/permanent EMT also occurs during organogenesis in later embryonic development. Recent studies of lung and gut development suggest that serosal mesothelial cells, which are classic squamous epithelial cells, are a major source of vascular smooth muscle cells, which are classic mesenchymal cells [103,104]. Notably, there is *not* a common epithelial cell progenitor during embryogenesis. Epithelial cells are derived from all three embryonic primordial germ layers. For example, lung airway and AECs are derived from the endoderm, renal tubular epithelial cells are derived from the mesoderm, and epidermal skin cells are derived from the ectoderm. Similarly, mesenchymal cells can be derived from ectoderm (e.g., facial cartilage) and mesoderm (e.g., skeletal muscle) [66,67].

EMT in cancer metastasis is more controversial. Many common solid tumors are of epithelial cell origin, including breast, prostrate, lung, colon, and pancreatic cancers [105]. These malignancies are also among the leading causes of cancer deaths in part due to the ability of these cancer cells to invade and metastasize. Analysis of human tumor samples has identified molecular evidence of EMT with loss of epithelial cell markers, gain of mesenchymal cytoskeletal proteins, and morphological changes consistent with EMT [106]. There is also activation of EMT pathways, such as TGFβ/Smad, Wnt/β-catenin, and expression of EMT transcription factors [107]. Although the early paradigm suggested a progression from a dysregulated cell cycle with transformation followed by invasion/metastasis, recent evidence suggests that EMT and distant metastasis may actually be an early event in cancer progression [108–110]. All aspects of EMT by cancer cells have been demonstrated in vitro in numerous reports, including change in protein and mRNA expression, changes in cell shape and polarity, and increased migration. However, while invasion and metastasis of

epithelial-cell-derived cancer cells in vitro is clear, the contribution of EMT to tumor metastasis in vivo remains more controversial. Some have argued that the migratory or invasive behavior itself is sufficient to define at least partial EMT [61,62,66], while others have argued that amoeboid-like migration could account for metastasis without invoking EMT [111]. Furthermore, the static nature of clinical samples have failed to demonstrate definitive evidence of EMT, and cancer cells from distant metastatic sites often maintain features of the epithelial cell type of origin.

EMT in Fibrosis. Fibrosis is also often characterized by loss of epithelial cells and significant gain of activated fibroblasts, which are protypical mesenchymal cells. The possibility of EMT during fibrosis arose from the overlap between the need for dynamic changes during tissue rebuilding and those found in development. There are also stark similarities in prominent EMT and profibrotic signaling molecules and pathways. For example, TGFβ is the most well-defined profibrotic factor in nearly every tissue, and it is also a prominent inducer of EMT [112] in nearly every epithelial cell type. In the lung, identification of aberrant Wnt/β-catenin signaling in IPF lung samples sparked interest in the possibility of EMT in lung fibrosis [113] given the prominence of this pathway in EMT during development and cancer. Indeed, most factors known to be upregulated during fibrosis have been shown to induce or augment EMT in vitro. Thus, the extracellular milieu in fibrotic tissue is conducive for stimulating EMT. Given the abundance of epithelial cells in tissues like the lung, a subset of epithelial cells transitioning into mesenchymal cells could potentially account for a significant proportion of activated fibroblasts. Furthermore, an important function of activated fibrogenic fibroblasts is to promote further activation of other fibroblasts through release of profibrotic factors. Thus, even transient EMT early after the inciting profibrotic event could initiate a process that leads to more sustained and robust fibroblast responses. As with human cancer samples, identification of EMT in human fibrotic tissue has been challenging given the static nature of the tissue samples which fail to capture dynamic changes in cell phenotype. Many studies have relied on immunostaining to identify cells costaining for epithelial can mesenchymal markers. This technique has inherent limitations [114–116]. The coimmunostaining approach is at best descriptive, potentially identifying expression of two or more markers within a cell but lacking an assessment of the functional contribution of that cell. The coimmunostaining approach likely underrepresents EMT because in most studies of EMT, there is simultaneous downregulation of epithelial markers and upregulation of mesenchymal markers. The window in which a cell expresses sufficient quantities of both epithelial and mesenchymal markers may be brief, and during this transition, the intracellular distribution of these proteins may be atypical. There is also the potential for artifactual staining of overlapping cells giving the appearance of a costained cell. Microscopic approaches such as confocal microscopy have been used to overcome this possibility [114,117] with unclear success. To partially address these issues, several groups have isolated fresh cells by flow cytometry to unequivocally show expression of mesenchymal proteins in epithelial cells and expression of epithelial cell proteins by fibroblasts [118,119]. Single cell RNA sequencing may also help identify the presence of EMT during fibrosis. Newer fate-mapping techniques have enabled more direct tracking and quantification of EMT during animal models of fibrogenesis.

Fate-Mapping. From one of the initial description of the Cre/lox system in mice, use of this system as a powerful method to map or trace the fate of specific cell types in vivo was proposed [120]. Many reviews have described this system extensively. But briefly, for fate-mapping there are at least two transgenes involved. The first is a reporter gene (e.g., green fluorescent protein (GFP) or β-galactosidase (lacZ)) regulated by a strong constitutively active promoter (e.g., CMV). Between the promoter and the reading frame for the reporter gene there is a sequence (e.g., polyA) that blocks expression of the reporter. The blocking sequence is flanked by loxP sites (floxed). The second transgene encodes Cre recombinase, regulated by a cell type specific promoter. Cre is expressed within a specific cell type, which recognizes the loxP sites and removes the "floxed" blocking sequence, enabling permanent expression of the reporter gene specifically within the targeted cell type. Thus, the Cre/lox system has been proposed as a way to engineer cell type specific and permanent labeling of cells in transgenic animals, making it an attractive way to map the fate of cells in response to injury and identify the origin of cells of interest, such as a myofibroblast in fibrosis.

Various Cre-dependent strategies have been used extensively to fate map cells during fibrosis, but with conflicting results. In lung fibrosis, there are at least four different groups that have identified significant numbers of lung epithelial-derived, fate-mapped cells which then coexpress mesenchymal markers after initiation of fibrosis [115,121–124]. Collectively, these groups have used different promoters to drive expression of Cre (Nkx2.1 and SPC), different reporter genes (GFP/LacZ), different techniques to quantify colabeled cells (in-situ tissue staining, single cell suspension followed by flow, cytospin staining, RNA/immunoblot, cell culture), and several models of fibrosis (bleomycin, overexpression of TGFβ, radiation) [78,115,121]. These studies suggest that EMT occurs at least to some extent, although the importance of EMT remains uncertain. However, there is at least one lung fate mapping study that found strong evidence that EMT did not occur at all, using an SPC promoter and a floxed dTomato reporter in the bleomycin model of fibrosis [117]. Using quantitative measures from sorted epithelial cells and costaining, they found no increase

in expression of mesenchymal markers, including type I collagen. They could not identify even a single cell in which there was mesenchymal protein expression within the epithelial cell lineage [117]. This controversy of EMT fate-mapping studies is not limited to the lung. In models of kidney and liver fibrosis, there are similar fate-mapping papers that find significant EMT or absent EMT. The controversy also is not limited to EMT. Both pericytes and fibrocytes have been proposed as sources of activated myofibroblasts and, for both of these cell types, there are fate-mapping studies in favor of and against them as a significant source of myofibroblasts. For pericytes, the difference is probably the greatest. For example in the lung, one report concludes that over two-thirds of myofibroblasts in the lung after bleomycin injury are derived from pericytes [125]. In contrast, the same study that found no evidence for EMT in lung fibrosis also excluded pericytes as the origin of myofibroblasts [117]. Thus, those favoring or opposing the possibility of EMT, fibrocytes, and pericytes as progenitors for myofibroblasts can point to specific fate-mapping publications in strong support of their view. Despite the theoretic promise of this approach, fate-mapping during fibrosis has failed to resolve the controversy regarding the origin of activated myofibroblasts in fibrosis. In part, this likely results from some of the same limitations in analysis of costained tissue sections discussed above. Ultimately, new methods of fate-mapping may resolve this uncertainty and provide greater consensus. For example, new RNA sequencing technology which can be applied to a single cell may determine more definitively if there are any cells which coexpress both classic epithelial and mesenchymal markers [126]. However, even extremely accurate fate-mapping with quantitative measurements may not reveal the functional contribution of subpopulations of epithelial or mesenchymal cells to the process of fibrogenesis. A relatively small number of epithelial-derived cells which express mesenchymal proteins may have an uniquely important function if the mesenchymal proteins are being expressed early after injury or in a unique niche. Conversely, a significant fraction of epithelial cells with high production of mesenchymal genes, such as collagen, may still be redundant or negligible to the overall fibrotic process. Future functional studies will need to clarify the potential importance of EMT as discussed below.

Definition of EMT Revisited. Much of the controversy regarding EMT in cancer, fibrosis, and even specific EMT events during development stems from uncertainties and ambiguities in the definition of EMT. On one extreme, the definition of an epithelial cell might require being derived from a parent epithelial cell and the definition of a mesenchymal cells as being derived from a parent mesenchymal cell. EMT might be defined as a process in which a fully differentiated epithelial cell loses all epithelial cell characteristics and gains all mesenchymal characteristics. This definition excludes the possibility of EMT on a pure semantic basis. On the other extreme, EMT might be defined as any deviation from a classic epithelial cell phenotype with any loss of epithelial protein expression and any acquisition of mesenchymal cell traits or proteins. By this loose definition, type II AEC differentiation to type I AEC or epithelial cell migration to reepithelialize after injury might be regarded as EMT. Indeed, in some circumstances, EMT transcription factors have been shown to be involved in reepithelialization after injury [127]. Indeed, this is reminiscent of developmental organogenesis in which ductal epithelial cells undergo EMT, migrate or invade, then revert back to an epithelial cell phenotype through MET. Whether this is EMT or simply a transient gain of migratory behavior with accompanying changes in a few proteins is a focus of debate regarding the definition. But the dynamic phenotype of epithelial cells is currently more broadly accepted. To circumvent these differences in definition, there is increasing use of the terms "partial" and "full" EMT. But most, if not all, cellular processes are partial, making achievement of "full" EMT difficult, if not impossible. Defining EMT during fibrosis has relied on lists of epithelial and mesenchymal markers [69], but it remains unclear at what threshold loss of epithelial markers or gain of mesenchymal markers would be sufficient to achieve at least partial EMT and what threshold is required for full EMT.

An alternative approach might be to discard the term EMT during fibrosis altogether and focus on the function of cells of different origin. This might be a departure from defining epithelial and mesenchymal cells as different lineages but rather focus on cell phenotype and function during fibrosis. Indeed, similar to the controversy on defining EMT, there is a similar controversy on the definition of myofibroblasts. Some have argued that expression of α-smooth muscle actin itself is not sufficient or even required to define a myofibroblast but rather requires stress fiber formation and a contractile phenotype. Given the heterogeneity among myofibroblasts, the term myofibroblast may better define a phenotype rather than a cell lineage or cell type [128]. Furthermore, while myofibroblasts are an important fibrogenic effector cell, fibrosis is clearly a multicellular process. Depletion of different cell types such as macrophages, epithelial cells, and fibroblasts influence the fibrotic response [129,130]. Similarly, deletion of the TGFβ receptor in fibroblasts, epithelial cells, or bone marrow cells results in abrogated fibrosis [129,131,132]. Injury and fibrotic stimuli clearly elicit a phenotypic response in many cell types which might be viewed as an "activated" state [61,68,133]. While the epithelial cell responses to TGFβ might result in profibrotic effects other than EMT, several groups have shown that epithelial-specific deletion of mesenchymal or EMT transcription factors leads to attenuated fibrosis. For example, hepatocyte specific deletion of Snail attenuates fibrosis in the CCL4 model of liver fibrosis, and deletion of FoxM1 in

AECs attenuates lung fibrosis after bleomycin or radiation [121,134]. Two recent reports found that deletion of Snail and Twist from renal epithelial cells blocked kidney fibrosis [135,136].

Epithelial cell-specific deletion of secreted profibrotic factors and proteins that are thought to be important to the function of activated fibroblasts can also block fibrosis. For example, production of type I collagen may be one of the most important defining functions of fibrogenic effector cells, and deletion of the *Col1a1* gene within lung epithelial cells leads to significant reduction in lung fibrosis after bleomycin [109,137]. Connective tissue growth factor (CTGF) expression has also been used as a marker of activated fibrotic fibroblasts, but several studies have now indicated that epithelial cells may be the major source of CTGF during fibrosis and deletion of CTGF within epithelial cells blocks fibrosis [138–141].

Thus, using a loss of function approach, activated epithelial cells contribute to fibrosis in ways that overlap with other activated fibrogenic effector cells. Whether this is sufficient to be called EMT perhaps depends on the precise definition of EMT. The overlapping versus nonredundant functional contributions of different cell types during fibrosis remains unclear. Attention to the function and activation of different cell types in response to injury or other fibrotic stimuli might reveal novel targets for intervention with better effect and less side effects than targeting single cell types.

7.3 EPITHELIAL GENE MUTATIONS AND FAMILIAL FIBROSIS

Several distinct classes of genetic mutations have been identified in patients with familial pulmonary fibrosis or have been found to be associated with increased risk for fibrotic interstitial lung diseases. While these have been discussed extensively in other reviews [142], they are discussed briefly here to highlight a common theme involving the role of dysfunctional epithelial cell health and pulmonary fibrosis (Table 7.1). While the mechanisms by which these different mutations cause or exacerbate lung fibrosis are still unclear, a common feature is that they all lead to impaired AEC function and survival. The idea that early epithelial cell death can initiate fibrosis is certainly not new and has been confirmed in multiple studies using genetically modified mice. Discovery of genetic disorders in familial pulmonary fibrosis not only confirms direct evidence for the function of these genes in fibrogenesis, but also confirms the notion that normal epithelial cell behavior is necessary for the prevention of lung fibrosis. This conclusion is reinforced by the description of spontaneous pulmonary fibrosis in animal models with isolated defects in epithelial cell function that leads to their death and subsequent lung fibrosis [143].

Surfactant Mutations. Expression of surfactant mutations is limited to AECs. Families with mutations in two different surfactant proteins have been shown to develop pulmonary fibrosis, confirming the important function of epithelial cells in prevention of fibrosis. Surfactant has a critical role in alveolar recruitment and emerging evidence suggest that alveolar decruitment and permanent collapse, or collapse induration, might be crucial for lung fibrosis [144] especially in the context of IPF [145].

A young patient with pulmonary fibrosis and a strong family history of pulmonary fibrosis was first described by Nogee et al. [146]. The histologic characteristics of the patient was similar to desquamative interstitial pneumonia (DIP) with features overlapping with nonspecific interstitial pneumonia (NSIP). Genetic testing on the patient revealed one copy for an intronic mutation of the surfactant protein C (SP-C) gene leading to abnormal splicing. Remarkably, the patient developed lung disease despite carrying only one mutant allele while the other SP-C allele was normal.

TABLE 7.1 Genetic Disorders in Familial Pulmonary Fibrosis

Category of Mutation	Gene Defect	Phenotype
Surfactant mutations	SP-C	DIP, NSIP, UIP/IPF
	SP-A2	UIP/IPF, adenocarcinoma
Telomerase deficiency	TERT	UIP/IPF
	TERC (or TR)	
Hermansky–Pudlak syndrome	HPS1	UIP/IPF
	HPS4	

SP-C, surfactant protein-C; SP-A2, surfactant protein-A2; TERT, telomerase reverse transcriptase; TERC, telomerase, RNA component; TR, telomerase, RNA component; HPS1, Hermansky–Pudlak syndrome 1 protein; HPS4, Hermansky–Pudlak syndrome 4 protein; DIP, desquamative interstitial pneumonia; NSIP, nonspecific interstitial pneumonia; UIP, usual interstitial pneumonia; IPF, idiopathic pulmonary fibrosis.

Further testing indicated that the patient has low levels of pro-SP-C in the lung tissue, suggesting that the abnormal SP-C protein product from the mutant gene might impair the SP-C production from the normal allele. It is speculated that the inappropriately spiced SP-C might disrupt normal trafficking and secretion of the normal SP-C, and that endoplasmic reticulum (ER) stress of the abnormal SP-C might contribute to impaired AEC function in general. Disruption in SP-C as a genetic cause of fibrosis was confirmed in a second larger family with a history of pulmonary fibrosis [18]. Within this family, there was a broad array of pulmonary histologic abnormalities, including some with a usual interstitial pneumonia (UIP) pattern and others presenting with a histologic pattern more consistent with NSIP. Some of the family members presented as early as 4 months old, while others manifested later and were not diagnosed until their six decade, suggesting that other genetic and nongenetic factors strongly contribute to the progression of disease. Genetic analysis of this family revealed a missense mutation in the SP-C gene. Histologic analysis revealed considerable AEC atypia, again suggesting that the underlying mechanism likely involves AEC dysfunction and cell death secondary to an abnormal, highly expressed protein as a initiator of fibrosis.

Similarly, mutations in the surfactant protein A2 (SP-A2) gene were found in several families with IPF [147]. Mutations in SP-A2 have interestingly also been linked to adenocarcinoma. As with SP-C mutations, the mutant SP-A2 may be retained within the ER and cause ER stress [148]. AEC expressing mutant SP-A2 in particular have increased expression of TGFβ potentially as a result of the ER stress induced by this misfolded protein. This upregulation of TGFβ may further contribute to fibrosis [149].

Telomerase Deficiency. Mutations in DNA telomerase have also been identified in cohorts with familial pulmonary fibrosis. Two genes encode the telomerase function, telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC). Each replicative cycle results in a slight shortening of the telomeres, which would limit the replicative capacity of the cell. Telomerase extends the cell's replicative capacity by extending the length of the telomeres at the ends of the chromosomes [150,151]. Thus, patients with deficient telomerase function can present with syndromes associated with early apoptosis such as aplastic anemia and dyskeratosis congenita [152,153]. Given the potential role for apoptosis in pulmonary fibrosis, Armanios et al. [154] analyzed over 70 patients with familial pulmonary fibrosis for mutations in the two telomerase genes and found that 8% of patients in this cohort had mutations in either TERT or TERC. These patients were all heterozygous for telomerase mutations, and the pattern of inheritance and presentation of lung disease was autosomal dominant. Functional testing revealed that these mutations led to impaired telomerase activity, and patients had a diminished telomere length. The authors suggested that the shortened telomere length could lead to AEC apoptosis with subsequent initiation of pulmonary fibrosis. The histologic pattern was noted to be similar to UIP with an age range somewhat older than the families with surfactant mutations. Patients with telomerase mutations were diagnosed with pulmonary fibrosis from 48 to 77 years old.

A second study from Tsakiri et al. [155] similarly found telomerase mutations in patients with familial IPF. This group used an unbiased approach and screened members of two families with pulmonary fibrosis using whole genome single polymorphism nucleotide analysis. This screen implicated a region on chromosome 5p15, which includes the TERT gene. Subsequent analysis identified mutations in TERT in both families. They then compared 44 patients with familial pulmonary fibrosis against a similar number of patients with sporadic IPF for mutations in TERT and TERC and identified several additional telomerase mutations among the cohort with familial fibrosis. Similar to the study by Armanios et al. [154], these patients all had one copy of the mutated telomerase gene and had adult onset IPF. Although the pattern of inheritance was similarly autosomal dominant, there was incomplete penetrance, suggesting other genetic and environmental factors are important for the development of pulmonary fibrosis in patients with telomerase mutations [156]. Indeed, it is noteworthy that patients with familial fibrosis due to telomerase mutations do not always manifest with aplastic anemia or dyskeratosis congenita, suggesting important contributing factors in how the mutations in telomerase manifest clinically. Other insults to the lung epithelium, such as smoking or viral infections, might provoke pulmonary fibrosis in these patients who are genetically at risk. This may also explain the fairly late onset of disease. A recent report found impaired alveolar stem cell function in the setting of telomerase dysfunction [157]. These studies support the notion that familial lung fibrosis can be caused epithelial cell dysfunction or failed epithelial repair after injury.

Hermansky–Pudlak Syndrome. HPS is a genetic disease which can manifest with pulmonary fibrosis as well as albinism, platelet dysfunction, and accumulation of ceroid lipofuscin [158,159]. There are seven different mutations in human HPS genes and the extent of pulmonary fibrosis depends on the specific mutation. Mutations in HPS1, which predominantly affects the Puerto Rican population, and in HPS4 represent the majority of patients with pulmonary fibrosis as a manifestation of their HPS. Histologically, the pulmonary fibrosis seen in HPS is similar to UIP, with extensive honeycombing in the basilar and subpleural regions [160]. Somewhat distinct from classic UIP, there is also significant accumulation of foamy swelling of lipid rich material within atypical appearing AECs. The defects in the HPS genes are thought to lead to abnormal protein trafficking with liposomal accumulation of lipofuscin within AECs,

leading to AEC dysfunction and death as an initiator of pulmonary fibrosis. Mice with genetic deletion of HPS reportedly have exaggerated fibrosis in response to bleomycin due to extensive AEC death [161].

A common theme for these genetic causes of familial pulmonary fibrosis is their impact on normal epithelial cell function. These mutations occur within genes highly expressed within AECs, and abnormal protein expression leads to ER stress or other dysfunctions within AECs, perhaps leading to increased AEC apoptosis. These abnormalities make the patients more susceptible to the development of pulmonary fibrosis. The incomplete penetrance of some of these mutations suggests an important role for environmental factors and contributions from other cell types in the full manifestation of pulmonary fibrosis. Indeed, a recent report found that deficient telomerase activity in bone marrow-derived cells was critical to lung fibrosis in two different models [162]. Overall, these genetic causes of pulmonary fibrosis demonstrate the importance of dysfunctional and apoptotic AECs in initiating fibrosis in familial pulmonary fibrosis.

7.4 PARACRINE FUNCTIONS OF EPITHELIAL CELLS DURING FIBROSIS

7.4.1 Epithelial Cell Cross-talk with Other Cell Types

Epithelial cells line the luminal surface of the airways and alveoli and are thus exposed to the environment. This potentially subjects them to injury from toxins and pathogens in the air. Epithelial cells are thus poised to be first responders to injury and can initiate and orchestrate the response both directly and through recruitment and activation of other cell types. Epithelial cells are well understood to secrete a number of factors which can influence the behavior of other cells in paracrine fashion (Fig. 7.1).

Epithelial–Mesenchymal interactions. Epithelial–mesenchymal cross-talk is essential for normal lung development [163]. This relationship continues to be important because it is a key regulator of repair and fibrosis in response to injury. Indeed, many features of the repair process recapitulates and reinitiates aspects of epithelial–mesenchymal interactions during embryonic development. One of the hallmarks of the epithelial–mesenchymal interactions during development involves Sonic hedgehog (Shh) signaling [164,165]. Shh is a morphogen involved in the development of a broad array of organ systems, including lung branching morphogenesis. The inactive Shh receptors, Patched1 and Patched2, sequester Smoothened (Smo) in the absence of ligand. Shh ligation to Patched leads to release of Smo which then converts the Gli family of zinc finger transcription factors into an active form, resulting in transcription of a number of Shh induced genes. In the developing lung, Shh is expressed exclusively on a subset of respiratory epithelial

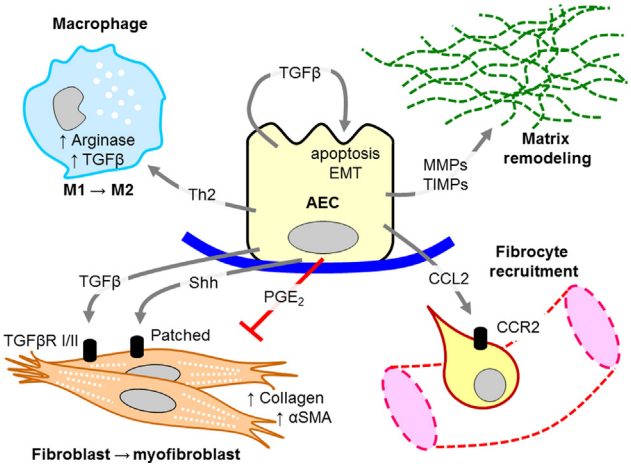

FIGURE 7.1 Epithelial cell paracrine interactions.
Alveolar epithelial cells (AECs) orchestrate the fibrotic response through interaction with other cell types. AECs are responsible for the production and activation of latent transforming growth factor-β (TGFβ), which is a primary profibrotic cytokine. TGFβ signals through dimerization of TGFβ receptor I and receptor II (TGFβRI/II), which leads to phosphorylation of receptor associated Smads. Upon nuclear translocation, the active R-Smad transcriptional complex induces fibroblast-myofibroblast differentiation with increased expression of α-smooth muscle actin (αSMA) and type I collagen. TGFβ can cause AEC autocrine signaling with resulting apoptosis or epithelial–mesenchymal transition (EMT). Other AEC-derived factors such as Sonic hedgehog (Shh) can activation fibroblasts through its receptor, Patched, on the surface of fibroblasts. Alternatively, AECs can produce prostaglandin E2 (PGE2), which inhibits fibroblast activation. AECs can induce profibrotic phenotypes in other cell types, such as inducing an M2 alternatively activated macrophage phenotype through production of Th2 cytokines. AEC production of CCL2 leads to recruitment of profibrotic circulating fibrocytes through its receptor, CCR2. AECs can engage in active matrix remodeling through production of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases.

cells; while the receptor, Patched, is expressed on the lung mesenchyme. This patterned communication is a major regulator of normal lung branching and mesenchymal proliferation. While Shh is not normally active in the adult lung, several studies of human lung tissue from patients with fibrosis and tissue from animal models of fibrosis have demonstrated an increased expression of Shh within epithelial cells, suggesting a reemerging of this pathway [166]. Given its role in development, Shh may be involved in fibroblast proliferation and activation in the adult lung. Several in vitro studies have found increased proliferation and fibrotic matrix protein expression in response to Shh, consistent with a persistent capability to respond to Shh signaling by adult mesenchymal cells [165]. Inhibition of Smo has been shown to be protective in some models of fibrosis. In the lung, the most direct evidence for the role of Shh/Patched signaling during lung fibrosis was recently demonstrated by Hu et al. [166]. The authors showed epithelial cell induction of Shh in response to bleomycin and attenuated fibrosis in mice with mesenchymal cell deletion of Smo, demonstrating that the reemergence of this pathway after injury directly contributes to fibrosis.

Other signaling pathways are clearly involved in epithelial−mesenchymal cross-talk. As discussed below, activation of TGFβ occurs primarily within lung epithelial cells, leading to initiation of one of the best characterized profibrotic pathways within mesenchymal cells and other fibrogenic effector cells [167,168].

Fibroblasts from fibrotic tissue may be more conditioned to have an exaggerated response to signaling from epithelial cells. Recently, Prasad et al. demonstrated in a coculture system that fibroblasts isolated from patients with IPF had aberrant responses to injured lung epithelial cells compared to normal human lung fibroblasts cocultured with injured lung epithelial cells [169]. These differences were due to increased levels of profibrotic factors, platelet-derived growth factor (PDGF)-AA and bFGF (fibroblast growth factor), in the IPF fibroblasts cocultured with epithelial cells. It is unclear if differences in epithelial cells from IPF versus normal epithelial cells might also influence the epithelial−mesenchymal cross-talk.

Epithelial−Fibrocyte Interactions. Fibrocytes are bone-marrow-derived hematopoietic cells that are characterize by coexpression of hematopoietic markers, such as CD45, and mesenchymal markers, such as type I collagen [170]. Adoptive transfer experiments have clearly established an important function for fibrocytes in animal models of fibrosis [171]. However, the precise contribution of fibrocytes to the fibrotic process remains unclear. There is considerable controversy regarding the ability fibrocytes to differentiate into myofibroblasts and directly contribute to type I collagen deposition. Emerging evidence suggests that the primary function of fibrocytes may be more indirect, through paracrine recruitment of other fibrogenic effector cells [170]. Thus, early fibrocyte recruitment may help coordinate the response to injury with the epithelial lung structural cells. Epithelial cells thus have an important role in recruiting fibrocytes. Epithelial cells secrete a number of fibrocyte chemoattractants. For example, mice deficient in the chemokine receptor, C-C chemokine receptor type 2 (CCR2), are protected from several models of fibrosis due to deficient fibrocyte recruitment. Neutralizing antibodies against murine homologs of the CCR2 ligand, CC-chemokine ligand 2 (CCL2), also blocked fibrosis, and AECs were identified as a major source of CCL2 [171,172]. Indeed, fibrocytes express a number of cell surface receptors to ligands secreted by activated or injured epithelial cells, which may represent an early critical step in epithelial-cell-derived recruitment of profibrotic cells to the injured tissue [170,173−175].

Epithelial Cell Recruitment of Alternatively Activated Macrophages. An important role for alternatively activated macrophages has recently been recognized [176]. The function of alternatively activated macrophages may include differentiation into fibrocytes or production of TGFβ and other profibrotic factors. While the full spectrum of macrophage activation is likely more complex, macrophages have traditionally been recognized as becoming activated toward a Th1, classically activated phenotype versus a Th2, alternatively activated phenotype depending on the stimulating cytokines [177,178]. Th2 cytokines such as interleukin (IL)-4, IL-10, and IL-13 induce an alternatively activated phenotype with resulting expression of Ym1, arginase, and TGFβ. Recent reports have shown accumulation of alternatively activated macrophages in human fibrotic tissue and in animal models of fibrosis [179,180]. Gibbons et al. [179] elegantly demonstrated that specific depletion of alternatively activated macrophages strongly attenuated fibrosis, while adoptive transfer of alternatively activated macrophages led to exaggerated fibrosis. Epithelial cells may act to shift the lung to a Th2 balance through direct release of Th2 cytokines such as IL-13 or by inducing expression of Th2 cytokines in other cells types [181,182]. Recently, it was reported that targeting this pathway with tralokinumab, an IL-13 neutralizing antibody, could attenuate lung fibrosis in an animal model, suggesting a potential for therapeutic intervention in IPF [183].

Autocrine Activation of Epithelial Cells. Epithelial cells express receptors for many of the factors that are secreted or activated by the lung epithelial cell itself, suggesting an important role for autocrine signaling. The best example is TGFβ, which is primarily activated by epithelial cells in the lung [184] and is the primary driver of major epithelial cell phenotypic changes, such as EMT and apoptosis, which are likely important for progressive fibrosis [115]. Cues from the environment and signaling from other cell types likely determine the response to the autocrine signal.

7.4.2 Proteins Expressed by the Epithelium Implicated in Fibrogenesis

As discussed above, lung epithelial cell apoptosis can initiate fibrosis, supporting the antifibrotic function of normal epithelial cells. Epithelial cells also secrete profibrotic factors in response to injury. Thus, the dynamic secretory expression profile of AECs is likely an important determinant to the overall fibrotic response. Given the abundance and distribution of epithelial cells in the lung, these cells likely account for a large portion of the production and release of profibrotic cytokines, especially early after the initial insult (Table 7.2).

Transforming Growth Factor β (TGFβ). TGFβ is the most studied and widely accepted profibrotic factor in tissue fibrosis, including pulmonary fibrosis [112]. TGFβ exists in three isoforms, TGFβ1, TGFβ2, and TGFβ3, but the function of TGFβ1 in fibrosis is the best described. TGFβ1 is produced from multiple sources including macrophages, platelets, airway epithelial cells, and AECs. Lung epithelial cells clearly have a unique role in early activation of latent TGFβ which may indeed represent the key regulatory step in activation of the TGFβ signaling pathway [167,185]. TGFβ is secreted in an inactive, latent form in association with a latency-associated peptide (LAP) that prevents receptor binding. Latent TGFβ can interact with the extracellular matrix through latent TGFβ-binding proteins. Even in uninjured tissue there is ample presence of latent TGFβ [186]. Epithelial cell integrins, primarily αvβ6 and αvβ8, bind to LAP release the active portion of TGFβ enabling engagement to the TGFβ receptors which is a heterodimer of two serine-threonine kinases, RI and RII [187–189]. Active TGFβ binds to TGFβRII, promoting receptor dimerization and phosphorylation of TGFβRI (also termed Alk5), which in turn leads to phosphorylation of the receptor-associated Smads (R-Smads), Smad2 and Smad3. These phosphorylated R-Smads form a complex with Smad4 and translocate to the nuclease where it can interact with a number of Smad coregulators to determine the transcriptional response to TGFβ signal activation [68,187]. TGFβ1 ligation to its receptor can also initiate intracellular signaling through a number of canonical non-Smad pathways such as mitogen-activated protein kinase (MAPK) and Rho family GTPases [187]. The cellular response to TGFβ signaling frequently involves activation to a profibrotic phenotype. TGFβ1 leads to fibroblast recruitment, activation, and differentiation into a myofibroblasts phenotye [112]. TGFβ is also a key driver of EMT as noted above. Finally, TGFβ may regulate cell survival by promoting fibroblast resistance to apoptosis while initiating epithelial cell apoptosis [48,190,191]. All of these activities are thought to contribute to progressive fibrosis.

TGFβ induces expression of fibrotic extracellular matrix (ECM) proteins such as fibronectin, type I collagen, and type III collagen. This finding has been reproduced in many reports and spans not only a fibroblast response but also other cell types that have been invoked as potential progenitors to fibrogenic effector cells, including epithelial cells, fibrocytes, and pericytes. A number of other markers of activated fibroblasts are induced by TGFβ, most notably, α-smooth muscle actin (SMA), which is often used as a marker of an activated myofibroblasts. The evidence that TGFβ signaling promotes lung fibrosis is quite extensive and involves data derived from human tissue, animal models, and in-vitro studies [6,112,168].

Increased mRNA and protein expression of TGFβ1 and its receptors have been demonstrated in a wide array of fibrotic lung diseases, including IPF, other interstitial lung diseases (ILDs), and diseases of airway remodeling [112]. Furthermore, specific polymorphisms of the TGFβ1 gene have been associated with IPF [192] and other forms of pulmonary fibrosis [193,194]. Significantly, integrin αvβ6 is strongly upregulated in IPF lung, which may represent a critical regulatory step in activation of TGFβ signaling [195].

Consistent with these observations from human fibrotic disease samples, animal models of fibrosis further solidify a critical mechanistic role for the TGFβ signaling pathway in lung fibrosis. Overexpression of a constitutively active form of TGFβ1 by a transgenic approach or through viral-mediated gene delivery to the lung is sufficient to initiate robust fibrosis in mice and rats without the need for additional injuries to the lung [115,196]. Mice with deletion of major components of the TGFβ signaling pathway are largely protected from experimental fibrosis. For example, mice deficient in β6 integrin are protected in several models of lung fibrosis due to inhibited activation of latent TGFβ. This supports a role for TGFβ in fibrosis, but also highlights the importance of epithelial cells given the specificity of β6 integrin expression on the lung epithelium [167]. Similarly, mice lacking β8 integrin, which is highly expressed on airway epithelial cells, are protected from airway fibrosis in models of COPD [188,197]. Deletion of one of the TGFβ receptors in either fibroblasts or epithelial cells protects mice from bleomycin-induced fibrosis consistent an important role for TGFβ signaling and also demonstrates the need for a multicellular response to TGFβ in order to establish a full fibrotic response [124,131,132]. Studies targeting signals further downstream of the canonical TGFβ pathway demonstrate that mice deficient in Smad3 are also thoroughly protected from bleomycin-induced fibrosis [198]. These finding are not limited to the lung. Indeed, a similar requirement for TGFβ in fibrosis in other organ systems has been demonstrated using a similar, broad approach [199–203].

Given the clear importance of TGFβ signaling in animal models of fibrosis using a genetic approach, a number of studies have sought to target this pathway with molecules or viral-vector-mediated gene delivery to attenuate fibrosis toward the possibility of therapeutic intervention. As noted above, epithelial cell integrin activation of TGFβ may be a

TABLE 7.2 Lung Epithelial-Cell-Derived Secreted Factors that Regulate Cellular Behavior in the Progression of Fibrosis

Family	Protein	Receptor	Signaling Proteins	Function	Potential Therapeutic Targets
TGFβ	TGFβ1, TGFβ2, TGFβ3	TGFβRI, TGFβRII	Smad2/3, MAPK, Rho GTPases	Apoptosis or resistance to apoptosis, expression of ECM proteins (fibronectin, collagen I, collagen III)	Integrin αvβ6 neutralizing antibody, TGFβ neutralizing antibodies, TGFβ receptor inhibitors, Smad inhibitors
EGF	EGF, TGF-α, amphiregulin	EGFR (ErbB1/HER1)	Ras/Raf/MAPK/ERK, JAK/STAT, PI3K/AKT, PLCγ/PKC	Cellular proliferation, differentiation, and apoptosis	EGFR inhibitors (gefitinib, erlotinib)
PDGF	PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, PDGF-AB	PDGFRα, PDGFRβ	PI3K, MAPK, JAK-STAT, Src	Cellular proliferation, differentiation, and survival	Nintedanib, imatinib
Vascular factors	VEGF, ET-1	VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1), VEGFR-3 (flt-4), ETA, ETB	PI3K, AKT, Rho GTPase, FAK	Surfactant production, endothelial cell trophicity, proinflammatory, smooth muscle cell mitogenesis, and fibroblast migration	Nintedanib, ET receptor antagonists (bosentan, ambrisentan)
FGF	FGF2, FGF9	FGFR1, FGFR2	PI3K/AKT, Ras/Raf, MAPK	Epithelial repair, AECII/fibroblast/smooth muscle cell mitogenesis	Nintedanib
CTGF	CTGF (CCN2)	Integrins, LRP6, LRP1	Smad, ERK, FAK	Fibroblast proliferation, chemotaxis, and ECM deposition, tissue wound repair	AntiCTGF antibody (FG-3019)
HGF	HGF	c-MET	Src/FAK, STAT3, PI3K/AKT, Ras/MEK	Tissue repair, epithelial/endothelial cell survival	Recombinant HGF
Eicosanoids	PGE2, LT	EP receptors 1−4, LT receptors	PLC, PKC, cyclic AMP, PI3K	PGE_2: inhibit leukocyte chemotaxis, enhance epithelial cell survival, inhibit fibroblast activation LT: leukocytic accumulation/activation, epithelial cell production of TGFβ, fibroblast activation	LT antagonists (montelukast), COX inhibitors
Inflammatory cytokines	Th2: (IL4, IL13), Th1: (IFNγ, TNFα), CCL2	many	MAPK, NF-κB, STAT3	Activation/recruitment of Th2 (profibrotic) versus Th1 (antifibrotic) cells, Fas-mediated apoptosis	AntiIL-13 antibody (lebrikizumab), antiCCL2 antibody (carlumab), TNF-α inhibitors
Coagulation/Fibrinolysis	u-PA, t-PA, Pg, PAI-1	uPAR, integrins	FXa, plasmin, fibrin	Tissue repair after injury, clot formation and initiation of fibrosis	PAI-1 inhibitor (PAI-039)

TGFβ, transforming growth factor beta; TGFβRII, transforming growth factor, beta receptor II; TGFβRI/Alk5, transforming growth factor, beta receptor I/activin A receptor type II-like kinase; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; TGF-α, transforming growth factor alpha; EGFR, epidermal growth factor receptor; HER1, human epidermal growth factor receptor 1; ERK, extracellular signal-regulated kinases; JAK, Janus kinase; STAT, signal transducers and activators of transcription; PI3K, phosphatidylinositide 3-kinases; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PDGFRα, platelet-derived growth factor receptor, alpha polypeptide; PDGFRβ, platelet-derived growth factor receptor, beta polypeptide; VEGF, vascular endothelial growth factor; ET-1, endothelin 1; VEGFR-1/2/3, vascular endothelial growth factor receptor 1/2/3; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; CTGF, connective tissue growth factor; CCN2, CCN family protein 2; LRP, low density lipoprotein receptor-related protein; HGF, hepatocyte growth factor; c-MET, MET proto-oncogene, receptor tyrosine kinase; PGE2, prostaglandin E2; LT, leukotriene; EP receptors, E-prostanoid receptors; COX-2, cyclooxygenase-2; LTB4, leukotriene B4; TNF-α, tumor necrosis factor alpha; IL, interleukin; Th1/2, T helper 1/2; CCL2, chemokine (C-C motif) ligand 2; CCL12, chemokine (C-C motif) ligand 12; CCR2, C-C chemokine receptor type 2; CXCR4, C-X-C chemokine receptor type 4; IFN, interferon; NK-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; u-PA, urokinase; tPA, tissue plasminogen activator; Pg, plasminogen; uPAR, urokinase receptor; TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; FXa, factor Xa; PAI-1, plasminogen activator inhibitor-1.

critical regulatory step. Neutralizing antibodies to αvβ6 integrin have been developed and have been used to successfully attenuate lung fibrosis in several models, leading to clinical trials currently under way. Other αv integrins such as αvβ8 and αvβ1 are also involved in TGFβ activation, and inhibitors to these integrins also show promise as antifibrosis therapies [195,204]. Other strategies to inhibit TGFβ signaling have targeted preventing TGFβ from binding to its receptor. These studies include neutralizing antibodies to TGFβ itself, overexpression of inhibitors, such as decorin, a natural TGFβ binding protein, and delivery of soluble versions of the TGFβ-binding domain of the TGFβRII [205–207]. These strategies have all been shown to attenuate fibrosis in animal models. One potential limitation of targeting TGFβ as an antifibrosis strategy may be in the prominent antiinflammatory role for TGFβ. Mice with deletion of TGFβ1 and other components of the TGFβ signaling pathway often exert excessive inflammation in the lungs and other organ systems. Whether a balance between these dual roles of TGFβ can be struck to target TGFβ activation and signaling for antifibrosis therapy remains unclear [195].

Epidermal Growth Factor (EGF) Family. The epidermal growth factor (EGF) family of proteins is soluble ligands that are cleaved by proteolytic enzymes from their transmembrane proligand forms and includes EGF, TGF-α, and amphiregulin. The EGF receptor (EGFR), also known as ErbB1 or HER1, is a type I transmembrane receptor tyrosine kinase (RTK) that belongs to the EGFR/ErbB superfamily, which includes three other RTKs: ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. Upon ligand binding, EGFR forms homodimers with a second EGFR or heterodimers with other EGFR/ErbB family members. The receptor dimerization is a critical step for the activation of intrinsic tyrosine kinases and autophosphorylation of the c-terminal specific tyrosine-containing residues that serve as docking sites for a variety of signaling molecules harboring Src homology 2 or phosphotyrosine binding motifs. The recruitment of these proteins then leads to the activation of Ras/Raf/MEK/extracellular signal-regulated kinase (ERK), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), phosphoinositide-3 kinase (PI3K)/protein kinase B (AKT)/mTOR, and phospholipase C (PLC)γ/protein kinase C (PKC) signaling pathways that can affect cell proliferation, differentiation, and apoptosis. This consequently regulates many physiological processes, such as organ development, growth, regeneration, and ion transportation [208]. Lung epithelial cells express both the proligands and the receptor.

TGF-α/EGFR has been reported to be overexpressed in IPF patients [209], and conditional TGF-α overexpression in epithelial cells induced pulmonary fibrosis in mice [210]. This seems to occur via an EGFR pathway dependent paracrine loop between epithelial and fibroblast cells, resulting in excessive collagen production and deposition [211]. Furthermore, TGF-α-null transgenic mice are protected against bleomycin induced lung fibrosis [212], supporting the profibrotic function of TGF-α/EGFR signaling between epithelial cells and fibroblasts.

Amphiregulin is a EGF-like ligand that binds to EGFR and has been shown to be upregulated in epithelial cells in diacetyl induced bronchiolitis obliterans [213]. Recent studies suggest a profibrotic role in TGFβ and bleomycin mouse models [214,215]. Zhou et al. showed that fibroblasts stimulated with amphiregulin proliferate in a dose dependent manner, and silencing expression of amphiregulin with siRNA led to both decreased fibroblast activation in vitro and collagen deposition in vivo in the TGF-β fibrosis mouse model [214]. Therapeutic targets aimed at EGFR (EGFR inhibitors) include gefitinib and erlotinib and were developed for the treatment of lung cancer. Gefitinib is specifically known to cause interstitial lung disease in treated patients, however has also been shown to attenuate pulmonary fibrosis in animal models [216]. This again highlights the likely complex regulatory mechanisms involved in maintaining the balance of epithelial–mesenchymal interactions in fibrogenesis versus repair after injury. Finally, the mTOR inhibitor rapamycin has been shown to limit progression of pulmonary fibrosis in animal models by limiting EGFR signaling pathway activation [211], possibly providing an interesting therapeutic target.

Platelet-Derived Growth Factor (PDGF). The PDGF family is well known to stimulate fibroblast proliferation and activation into myofibroblasts, which likely contribute to the progression of fibrosis [217]. Five different ligand isoforms (PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and PDGF-AB) and two PDGF receptor isotypes (PDGFRα and PDGFRβ) have been described [218]. The PDGFRs belong to the family of transmembrane tyrosine kinase receptors, which are activated by extracellular ligand binding, causing dimerization and subsequent autophosphorylation of the intracellular PDGFR domains. Activation of PDGFR leads to activation of important downstream intracellular signaling pathways that control cell proliferation, differentiation, and survival, including PI3-kinase, MAPK, and the JAK-STAT pathway [219].

PDGF is expressed by lung epithelial cells, and its expression can be induced by thrombin via proteinase-activated receptor (PAR)-1, PAR-3, and PAR-4 [220]. Both PDGF gene and protein expression are upregulated in animal models of pulmonary fibrosis and in patients with IPF [221]. PDGFR-specific tyrosine kinase inhibitors reduce radiation-induced lung fibrosis in mice and vanadium pentoxide-induced pulmonary fibrosis in rats [222]. Imatinib, a PDGFR and c-Abl inhibitor, was shown to reduce bleomycin-induced lung fibrosis and asbestos-induced interstitial pneumonia in mice [222]. However, in a phase-II clinical trial in patients with IPF and mild-to-moderate impairment of lung function treated with imatinib or placebo for 96 weeks, imatinib did not affect survival or lung function [223]. Only a transient improvement in oxygenation was shown at 48 weeks [223].

Nintedanib, a multityrosine kinase inhibitor, has been approved for treatment of IPF in the United States and European Union and has shown benefit in slowing disease progression in patients with IPF [224]. The inhibitor targets the PDGFR, as well as the fibroblast growth factor receptor (FGFR) and vascular endothelial growth factor receptor (VEGFR). The PDGFRs are most implicated in fibrosis based on prior studies, and thus likely is the major receptor family contributing to the drug effect [225].

Vascular Factors. Vascular endothelial growth factor (VEGF) is expressed by epithelial cells and is a crucial factor for the homeostasis of the alveolus via the control of surfactant production and endothelial cell trophicity [226]. VEGF binds specific receptors and coreceptors on multiple cell types including epithelial cells [227]. Three types of VEGF receptors, VEGFR-1 (flt-1), VEGFR-2 (KDR/flk-1), and VEGFR-3 (flt-4), contain tyrosine kinase activity and are activated by the binding of VEGF homo- or hetero-dimers. Coreceptors, including heparin sulfate proteoglycans and neuropilins, lack tyrosine kinase activity but may modify the binding to tyrosine kinase containing VEGFRs or bind VEGF directly to signal a cellular response [227].

In IPF, capillary density is increased in nonfibrotic UIP lesions, and AECs adjacent to these vessels were shown to produce VEGF-B. Furthermore, increased serum VEGF has been shown to be associated with a shorter survival in IPF patients [226]. VEGF gene deletion leads to destruction of the alveolar-capillary barrier in the lung [227]. Nintedanib, a triple angiokinase inhibitor that acts on the VEGF, fibroblast growth factor (FGF), and PDGF signaling pathways, has been approved for the treatment of IPF in the United States and the European Union. Biochemical assays have demonstrated that nintedanib inhibits the kinase activity of all three VEGFR subtypes, by binding to the ATP-binding site in the cleft between the NH_2 and COOH terminal lobes of the kinase domain. However, experimental evidence suggests that tumor cells can circumvent VEGF inhibition by upregulating PDGF and FGF, possibly via hypoxia response signals, to drive compensatory angiogenesis. Thus, a single VEGFR inhibitor may not be a sufficient drug target for IPF [225].

Endothelin-1 (ET-1) is synthesized mainly in the lung tissue and exert their action via stimulation of two receptors, types A and B (ETA and ETB) [228]. ET-1 has proinflammatory, profibrotic, and mitogenic potentialities for smooth muscle cells, myocytes, and fibroblasts. ET-1 and its receptors are expressed by AECs and may have a role in AEC profibrotic activation and EMT [229]. It may be involved in the process of angiogenesis and contribute to endothelial cell apoptosis [228]. Bosentan is a dual ET receptor antagonist previously shown to reduce collagen deposition in the lungs and thus was investigated as a treatment for IPF in a series of clinical trials. The first Bosentan Use in Interstitial Lung Disease (BUILD-1) multicenter, randomized, placebo-controlled trial was published in 2008 showed no difference in 6MWD between the treatment and the placebo groups, though there was a trend toward a lower risk of functional deterioration with bosentan [228,230]. The follow-up BUILD-3 trial published in 2011 showed no difference in the time to death or worsening of IPF with bosentan treatment versus placebo [231]. Ambrisentan, an endothelin A receptor antagonist, was found not effective in treating IPF and possibly associated with an increased risk for disease progression and respiratory hospitalizations [232].

Fibroblast Growth Factor (FGF). There are 18 mammalian FGFs that can be classified into six subfamilies of proteins, which exert paracrine regulation of cellular proliferation, migration, differentiation, and cell growth and survival [233]. The FGFs bind to the extracellular domains of FGF receptors, single-pass transmembrane proteins with three extracellular immunoglobulin-type domains (D1—D3) and an intracellular tyrosine kinase domain, which undergo dimerization followed by autophosphorylation and activation of downstream signaling via FGFR substrate 2, PI3K/Akt, ERK1/2 and Ras/Raf/MAPK pathways. The FGF/FGFR signaling cascade is regulated by TGF-β [222].

Both FGFs and FGFRs are expressed on epithelial cells [234]. In the lung, FGF-2 is a mitogen for AECIIs, fibroblasts, and airway smooth muscle cells; FGF-2 is essential for epithelial repair and maintaining epithelial integrity after bleomycin-induced lung injury in mice [235]. Increased expression of FGF-2, FGF-9, FGFR-1, and FGFR-2 are detected in the lungs of IPF patients [236,237]. On the contrary, FGF-1 and FGF-10 appear to have antifibrotic properties by inducing apoptosis of fibroblasts [238] and AEC repair [239].

The triple tyrosine kinase inhibitor, nintedanib, is approved for treatment of IPF in the United States and European Union. The inhibitor targets the FGFRs, as well as PDGFR and VEGFR, by blocking kinase activity while occupying the intracellular ATP-binding pocket of specific tyrosine kinases [222]. For the mechanisms of action related to PDGFR and VEGFR, refer to above sections.

Connective Tissue Growth Factor (CTGF). CTGF, also known as CCN family protein 2, is a matricellular protein belonging to the CCN family of cysteine-rich signaling proteins [240]. CTGF has been shown to play critical roles in development, particularly the formation of the skeletal system, but also in tissue wound repair, cancer invasion and metastasis, and tissue fibrosis [241]. In the lung, CTGF is produced by epithelial, endothelial, and mesenchymal cells, is mainly mediated by TGF-β and is a potent inducer of fibroblast proliferation, chemotaxis, and ECM deposition [138,242]. Recent evidence suggests that AECs may be the main source of CTGF during fibrosis and epithelial specific deletion of CTGF results in dramatic attenuation of bleomycin-induced fibrosis [141]. Overexpression of CTGF induced

by adenoviral gene transfer results in transient fibrosis in mice; [243] mRNA expression is upregulated in bronchoalveolar lavage fluid of IPF patients [244].

In the bleomycin fibrosis mouse model, coadministration of both TGF-β and CTGF accentuates the extent of bleomycin-induced lung fibrosis compared with administration of either factor alone, suggesting a synergy between the two proteins [242,245]. Bleomycin-induced fibrosis was attenuated by the human antiCTGF antibody FG-3019 [245]. A humanized single-chain variable fragment antibody (scFv) against CTGF inhibited collagen deposition and the severity of alveolitis and fibrosis in the bleomycin mouse model [246]. A phase-I clinical trial of the antiCTGF antibody (FG-3019) has been completed, and a phase-II clinical trial is now underway [247].

Hepatocyte Growth Factor (HGF). HGF, also known as scatter factor, is a paracrine factor produced by mesenchymal cells in an inactive preform which is activated by proteases to form a heterodimer [248]. The HGF receptor was identified to be the ligand for the protooncoprotein RTK c-MET, expressed on epithelial cells and endothelial cells [249,250]. The binding of HGF to its receptor induces activation of the MET tyrosine kinase and subsequent autophosphorylation [251]. HGF/Met signaling activates multiple signal transduction pathways, including the Src/focal adhesion kinase pathway, the p120/STAT 3 pathway, the PI3K/Akt pathway, and the Ras/MEK pathway [252].

Administration of HGF protein or ectopic expression of HGF has been demonstrated in animal models of pulmonary fibrosis to induce normal tissue repair and to prevent fibrotic remodeling. HGF-induced inhibition of fibrotic remodeling may occur via multiple direct and indirect mechanisms, including the induction of cell survival and proliferation of pulmonary epithelial and the reduction of myofibroblast accumulation [248]. Furthermore, bronchoalveolar lavage (BAL) fluid from IPF patients show increased levels of HGF compared with controls, which correlate positively with TGFβ levels [253]. However, lung fibroblasts isolated from patients with IPF demonstrate a reduced production of HGF in vitro [252]. Overexpression of HGF by endotracheal administration of recombinant HGF [254] and electroporation gene transfer [255,256] attenuated bleomycin-induced fibrosis in animal models; therapeutic trials in patients remain to be seen.

Eicosanoids. The eicosanoids are a family of potent, biologically active, lipid mediators synthesized from membrane phospholipid-derived arachiodonic acid and includes prostaglandins, thromboxane, and leukotrienes (LTs), [242]. In general, LTs promote fibrogenic responses, whereas prostaglandin E2 (PGE2), the most abundant prostanoid found in healthy lung, opposes fibrosis [257]. An imbalance of eicosanoids in pulmonary fibrosis favors the production of LTs over PGE_2 [257].

PGE2 is known to inhibit leukocyte chemotaxis, enhance epithelial cell survival, and inhibit fibroblast activation and myofibroblast differentiation. PGE2 production by AECs is predominantly cyclooxygenase-2 (COX-2) dependent [258]. Failure of induction of PGE2 by COX-2 contributes to unopposed fibroblast proliferation and synthesis of collagen, fibroblast transition to a myofibroblast phenotype, and fibroblast resistance to apoptosis, as well as increased AEC apoptosis [242,259]. PGE2 exerts its effects via 4 transmembrane G-protein−coupled receptors, E-prostanoid receptors 1−4 [242]. Targeting these receptors or reconstitution of PGE2 via nebulization [260] may be future therapeutic targets.

In contrast to PGE2, the cysteinyl LTs are well known to promote leukocytic accumulation and activation, stimulate epithelial cells to produce TGFβ, and stimulate fibroblast activation and myofibroblast differentiation. LTs are found at increased concentrations in IPF lung, also due to failure of COX-2 induction and arachidonic acid metabolism by the 5-lipoxygenase pathway into LTs, especially LTB_4 [242,261]. LTs stimulate macrophage production of profibrotic growth factors and cytokines, including FGF, IL-6, IL-8, and TNF-α [261]. Thus, LT antagonists are used in the treatment of asthma. Montelukast, a cysteinyl-leukotriene type 1 receptor antagonist, has been shown to attenuate the development of bleomycin-induced fibrosis in mouse models [262] and may hold therapeutic promise.

Inflammatory Cytokines. Both animal models and human fibrotic lung diseases involve an initial inflammatory response after injury, with many of the cytokines produced and regulated by AECs [4]. IPF is characterized by an imbalance between type 1 helper T-cell (TH1) and type 2 helper T-cell (TH2) cytokines that favors fibroproliferation [242]. TH2 cytokines IL-4 and IL-13 have been described to be profibrotic in numerous reports by induction of TGFβ production and activation of fibroblasts. Inhibition with an anti-IL-13 antibody significantly reduced fibrosis in animal models [183], and the humanized anti-IL-13 monoclonal antibody lebrikizumab is being investigated in the treatment of IPF in a phase II trial (NCT01872689). CCL2, also known as monocyte chemotactic protein 1, is a downstream mediator of IL-13 expressed by lung epithelial cells that binds to its receptor CCR2. Mice deficient in CCR2 and in CCL12, homologous to human CCL2, are protected against fibrosis [171,263]. However, phase II trial of carlumab, a human immunoglobulin G1κ monoclonal antibody that binds and neutralizes human CCL2 did not provide benefit in IPF patients [264].

In contrast, interferon-gamma (IFNγ) produced by TH1 cells directly suppresses collagen synthesis by fibroblasts, antagonizes TH2 profibrotic pathways, and inhibits alternative macrophage activation by inducing nitric-oxide synthase 2 instead of arginase, which attenuates fibrosis [265]. However, the phase III randomized controlled trial to determine the efficacy and safety of recombinant IFN gamma-1b (INSPIRE Trial) showed no improved survival in IPF patients [266].

TNF-α binds its receptors TNFR1 and TNFR2, which induces activation of major intracellular regulatory pathways of cell survival and proliferation in a wide variety of cells via activation of MAPK, NF-kB, and Fas-mediated apoptosis. In the lung, TNF-α has both inflammatory and fibrogenic properties, and it is highly expressed in IPF lung patients. In mouse models, the injection of antiTNF-α antibodies diminishes bleomycin pulmonary injury and fibrosis. Overexpression is associated with increased fibroblast proliferation and activation [267]. TNF-α inhibition with monoclonal antibodies (infliximab, adalimumab, and certolizumab) and circulating receptor fusion proteins (etanercept) are used to treat inflammatory autoimmune diseases. However, a phase II randomized controlled trial of etanercept in IPF showed no difference in primary endpoints as compared to control [268].

Lung epithelial cells also produce IL-1, IL-6, and C-X-C motif chemokine 12 (CXCL12). The IL-6 cell surface receptor induces STAT3 mediated profibrotic signaling in fibroblasts [269], and blockade of IL-6 trans signaling attenuates lung fibrosis in the bleomycin mouse model [270]. IL-1 levels are elevated in animal models of fibrosis as well as in human disease; however, the exact mechanism in its regulation of fibrogenesis is unclear [271]. Finally, the profibrotic CXCL12, also known as stromal-cell-derived factor, attracts fibrocytes to injured lungs and induces CTGF expression in pulmonary fibroblasts, which are both dependent on its cell-surface receptor CXCR4 [272].

Coagulation and Fibrinolysis. The coagulation cascade is one of the first processes activated in tissue injury. Fibrinolysis is initiated by the conversion of plasminogen to plasmin by the plasminogen activators, urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA). The activity of these serine proteinases is regulated by plasminogen activator inhibitor-1 (PAI-1), the expression of which is tightly regulated by a variety of growth factors, including TGFβ, IL-1β, and EGF [273]. Disordered coagulation and fibrinolysis promote extravascular fibrin deposition that characterizes acute lung injury and repair and involves the expression of tissue factor (TF), u-PA, urokinase receptor (uPAR), and PAI-1 by the lung epithelium [274]. In IPF, coagulation is thought to be initiated in the alveolar compartment as a result of increased TF expression by type II AECs and alveolar macrophages [275]. Furthermore, bronchial and pulmonary alveolar epithelium represent important local cellular sources of several coagulation factors and a nidus for Factor X (FX) activation in patients with IPF and in the bleomycin model of lung injury and fibrosis [275]. In mouse models, activation of the major thrombin receptor, proteinase-activated receptor-1 (PAR1), on lung epithelial cells is upregulated after lung injury [276]. Deletion of PAR1 is protective against bleomycin lung injury [277]. PAI-1 overexpression induced greater fibrosis, while mice deficient in PAI-1 are protected from fibrosis via vitronectin binding [278], possibly via abrogated recruitment of macrophages after AECII injury [279] or via regulation of fibroblast apoptosis [280]. Mice with deletion of plasminogen or t-PA resulted in greater fibrosis, and overexpression of u-PA attenuated fibrosis [281–283].

There have been two randomized controlled trials of anticoagulation therapy in IPF patients. The first unblinded, open-label study showed improved survival with the treatment of anticoagulant plus prednisolone versus prednisolone alone in 56 IPF patients [284]. However, the subsequent double-blinded, placebo-controlled trial of anticoagulation with warfarin in IPF showed an increased risk of mortality in an IPF population who lacked other indications for anticoagulation [285]. New classes of oral anticoagulants, the direct thrombin inhibitor dabigatran and the FXa inhibitors apixaban and rivaroxaban, may have antifibrotic properties in addition to their anticoagulant effects [286].

Proteins involved in coagulation and fibrinolysis are activated upon injury and can initiate cross-talk with other injury response pathways which may be important for their role in regulating fibrosis beyond their function in fibrin deposition and clearance. For example, the profibrotic effects of PAI-1 appear to be independent of its function in inhibiting plasminogen activation but may instead be through its interaction with vitronectin, a major provisional matrix protein [278,287]. Consistent with this notion, fibrinogen-null mice are not protected from fibrosis [288].

7.5 REEPITHELIZATION VERSUS PROGRESSIVE FIBROSIS

Injury leads to damage and destruction of the distal lung epithelium. Histologic sections of IPF are characterized, in part, by atypical appearing AECs which may represent hyperplasia and differentiation to restore the damaged epithelium. In the disease state of progressive fibrosis, there may be a failure of this regeneration and restorative process, promoting ongoing fibrogenesis [15].

7.5.1 Stem and Progenitor Cells versus Epithelial Cell Plasticity

There is considerable interest in the potential for mesenchymal and epithelial stem cells in facilitating the response to lung injury, given the potential for lung regeneration and the possibility of using stem cells for therapy [289–295]. Adult stem cells proliferate infrequently, making them "label retaining". Stem cells are at least somewhat undifferentiated and can undergo asymmetric cell division, giving rise to progenitor cells. Progenitor cells are also somewhat undifferentiated and proliferate rapidly in response to injury and can give rise to differentiated cells to replace cell death

in the damaged tissue. An impaired epithelial stem cell response to injury may trigger a fibrotic response and may be the mechanism by which genetic diseases cause lung fibrosis [155,157,296]. In the upper airways, basal cells have been identified as stem/progenitor cells due to their multipotency after injury in fate mapping experiment [297,298]. Basal cells have the capacity for self-renewal as well as the ability to generate multiple epithelial cell types within the pseudostratified mucociliary epithelium [295].

There may be a common stem cell for the distal airway and the alveolar epithelium. Several groups have identified cells at a specific niche within the bronchiolar–alveolar duct junction which have features of stem/progenitor cells, including label-retention, self-renewal, proliferation after injury, and the capacity to differentiate in vitro into cells with features of either club cells, type I AECs or type II AECs [299–301]. These cells were notable of expressing a stem cell marker, Sca1, and coexpressing club cell and AEC markers. The type of injury may direct the differentiation potential of this population; studies from one group demonstrate that a club cell lineage followed in vivo was capable of differentiating into an AEC population after bleomycin but not after naphthalene injury [117,302].

It has been believed for decades that type II AECs can proliferate and differentiate into type I AECs after injury to restore the dead type I AECs and reestablish the extensive surface area required for gas exchange. This was recently definitively demonstrated by several groups using lineage-trace experiments [117,302–304]. Collectively, these studies show that during homeostasis there is relatively little type II AEC proliferation, but after insults with bleomycin, hypoxia, or naphthelene, there is increased proliferation and differentiation into type I AECs. Interestingly, type I AECs are not necessarily terminally differentiated but may retain some plasticity. There is at least one report suggesting that, after injury, type I AECs have the capacity to proliferate and differentiate into type II AECs [305].

The stem cells for AECs may not necessarily be within the AEC population. Recently, a population of cells that were surfactant protein-C negative, but positive for $\alpha 6\beta 4$ integrin, which has previously been used as a stem cell marker, were shown to differentiate into club cells and type I and type II AECs after injury [306]. This report identifies a dynamic population of immature progenitor cells in the adult lung which lack classic AEC markers such as SP-C but can proliferate and expand in response to severe injury and significantly contribute to the repair process. Furthermore, a rare population of lung epithelial cells that are lineage-negative was identified and found to be capable of regenerating the lung epithelium after injury with bleomycin [307]. In this report, a population of cells that was cytokeratin 5-postive but negative for SPC or CC10 was identified. This population of cells was normally quiescent, but proliferates and differentiates into AECs and club cells in response to injury from either influenza or bleomycin. This progenitor repair program required Notch signaling, and failed regeneration led to pulmonary fibrosis.

There remains some controversy on whether lung epithelial stem/progenitor cells are derived from the bone marrow to help regenerate the damaged epithelium. Evidence for this idea is supported in both animal models and human studies of bone marrow transplant recipients. In one study, bone-marrow-derived stem cells from male mice were used to reconstitute irradiated female mice [308]. Evidence for engraftment of hematopoietic-derived cells was found in multiple organs including the lung epithelium using fluorescence in situ hybridization (FISH) for the Y chromosome. Several other groups have used bone marrow from mice overexpressing a reporter gene such as LacZ or GFP to reconstitute wild-type mice and again found evidence of lung epithelial cells derived from hematopoietic stem cells [309–311]. Human studies have analyzed bone marrow transplant recipients and have found evidence for bone-marrow–derived lung epithelial cells [312] using laser-capture microdissection followed by PCR genotyping analysis and FISH for the Y chromosome. More recent evidence, however, questions the reliability of the techniques used to identify bone-marrow-derived lung epithelial cells. For example, marrow-ablated recipient mice reconstituted with marrow from a transgenic mouse in which GFP expression is regulated by the SPC promoter did not demonstrate any GFP expression in the lung, indicating lack of type II AECs derived from the bone marrow, even when the mice were injured with bleomycin [313].

7.5.2 Pathways of Regeneration

Regeneration of the lung epithelial cells involves expression of secreted factors and an epithelial cell response which might include migration, proliferation, and differentiations [314]. As noted, TGFβ is a prominent inducer of epithelial cell apoptosis but can also initiate epithelial cell migration [48]. KGF or FGF7 is a potent lung epithelial cell mitogen and likely has an important role during lung epithelial repair after injury [315,316]. Treating rats with KGF prevented lung fibrosis in one study [317]. Overproduction of KGF has been shown to stimulate epithelial cell proliferation in vitro and leads to AEC hyperplasia in vivo. KGF also increases production of surfactant and promotes epithelial cell differentiation [317–319].

In addition to extracellular matrix remodeling after injury, dynamic changes to receptors to the extracellular matrix may regulate epithelial cell adhesion, migration, and survival during the response to injury, which may be important to the fibrotic versus restoration outcome [184]. AECs are normally attached to the basement membrane, which is rich in

laminin and type IV collagen. AECs express high levels of laminin receptors, including integrins α3β1, α6β4, and α6β1 and collagen integrin α1β1 [320]. AECs also form tight cell−cell contacts, mainly through E-cadherin. Together these matrix and cell interaction are important for the barrier function of the lung epithelium, and changes to these interactions are important for the epithelial cell response to injury [78,122]. Although integrins lack inherent signaling capacity, integrin ligation to specific matrix proteins can initiate intracellular signaling through integrin adaptor proteins, leading to changes in cell behavior including migration and proliferation [321,322]. As noted above, epithelial-cell integrins are likely the critical activator of latent TGFβ, which is the best characterized profibrotic cytokine.

The role of Wnt/β-catenin signaling in lung regeneration and fibrosis is unclear. Aberrant Wnt signaling was demonstrated in IPF tissue several years ago [113]. A number of studies have subsequently explored the potential role for β-catenin signaling during experimental fibrosis. Several studies have found evidence for increased Wnt signaling in the fibrotic lung especially in animal models [323,324]. Inhibition of β-catenin signaling has been shown in some cases to protect against fibrosis [78,325,326]. However, lung epithelial cell specific deletion of β-catenin actually led to increased fibrosis due to increased epithelial cell death [327]. These differences may be due to the pleiotropic effects of β-catenin signaling on epithelial cells, which include promoting cell survival and migration, as well as on promoting EMT [323,328,329].

There is considerable interest in epigenetic regulation during tissue repair and fibrosis, and histone deacetylases (HDACs) in particular have an important role in regulating lung epithelial cell proliferation and differentiation after injury [295,330,331]. HDACs have an important role during lung development, and the epithelial cell response to injury may require reinitiation of developmental programs [332−334]. Mice with genetic deletion of HDAC1/2 have impaired lung epithelial cell regeneration and differentiation after injury [334]. There is also evidence of aberrant HDAC activity in fibrotic lung [335]. Conversely, several reports demonstrate that inhibition of HDAC activity diminishes the fibrotic response and restores epithelial cell homeostasis, suggesting a complex role for HDAC signaling during fibrosis perhaps owing to the number of HDACs and the number of genes they may regulate [336−338].

Notch signaling is critical for normal lung development and has been shown to have an important role during lung regeneration [314]. Notch signaling has been shown to be important for basal cell progenitor effects in the more proximal airway after injury [339] and is likely important for the regeneration of lung epithelial cells in the more distal lung as well [307].

7.5.3 Regeneration of the Damaged Matrix

Injury leads to cell death and destruction of the alveolar basement membrane. The damage is initially replaced by a provisional matrix made up of matrix proteins circulating in the plasma including fibrin(ogen), fibronectin, and vitronectin [287]. The subsequent response by the distal lung epithelial cells dictates the outcome toward regeneration and restoration of homeostasis or fibrosis.

Epithelial cells secrete a number of proteases and protease inhibitors which direct matrix remodeling and can also signal through cell surface receptors. The matrix metalloproteinases (MMPs) are a family of endopeptidases that cleave a number of matrix and nonmatrix proteins that are important for the remodeling process [340−342]. MMP activity is inhibited by a family of tissue inhibitors of metalloproteinases (TIMPs). The importance of MMPs and TIMPs on regulating matrix remodeling after injury have been studied extensively [343,344]. MMP activity may function not only to clear away the damaged matrix but also to foster cell migration facilitating epithelial cell regeneration as well as release growth factors, such as HGF and TGFβ1, which are embedded within the matrix. For example, one report found that mice lacking MMP9 (gelatinase B) had impaired lung epithelial regeneration after bleomycin injury [345]. Several studies have identified an important role of MMP7 (matrilysin) [346]. MMP7 was initially identified in an unbiased microarray screen of patients with IPF. MMP7-null mice were then found to be protected from fibrosis in the bleomycin model. MMP7 has many matrix targets including collagen IV, laminin, and fibronectin, as well as cell surface proteins which may be important for epithelial cell migration such as E-cadherin and syndecan [347,348]. Lung epithelial cells are also known to secrete MMP12, which has also been shown to regulate pulmonary fibrosis in an animal model [49,349,350]. Interestingly, the function of MMP12 in regulating fibrosis may be through regulation of epithelial cell apoptosis, which is important for initiation of fibrosis as noted above [49,351]. Among the TIMPs, TIMP-1 has been the most widely studied in the context of fibrosis. TIMP-1 regulates a number of cellular responses which could be important for fibrosis, although one report overexpression of TIMP-1 within lung epithelial cells did not affect bleomycin-induced fibrosis in mice [352].

As noted above, a number of proteases in the coagulation and fibrinolytic cascades have been implicated in fibrogenesis. Similar to the MMPs and TIMPs, these proteins in the coagulation and fibrinolytic cascade likely function beyond their role in fibrin deposition and turnover but also influence epithelial cell signaling, migration and survival.

PAI-1 is expressed by macrophages and epithelial cells. One of the most durable findings in the fibrosis literature is the protection from fibrosis of mice deficient in PAI-1 [288]. Interestingly, the influence of PAI-1 may not be through its antiprotease activities or through its inhibition of fibrinolysis but rather through its interactions with other proteins such as vitronectin [278,287]. PAI-1 may be an important regulator of epithelial cell migration and survival [287,353]. Finally, uPAR is also expressed on epithelial cells and has also been studied extensively as a mediator of cell migration through its interaction with matrix receptors [354].

7.6 CONCLUSIONS

Tissue fibrosis is a common consequence of many acute and chronic diseases. Collectively fibrosis may be the leading cause of death in the developed world. While much of the fibrosis field has focused on the function of fibroblasts and myofibroblasts, fibrogenesis clearly involves the interaction of multiple cell types. Lung epithelial cells, which are abundant in the healthy lung, have a critical role in orchestrating the response to lung injury (Fig. 7.2). The dynamic changes to epithelial cell behavior, including proliferation and epithelial stem cell differentiation after injury, can

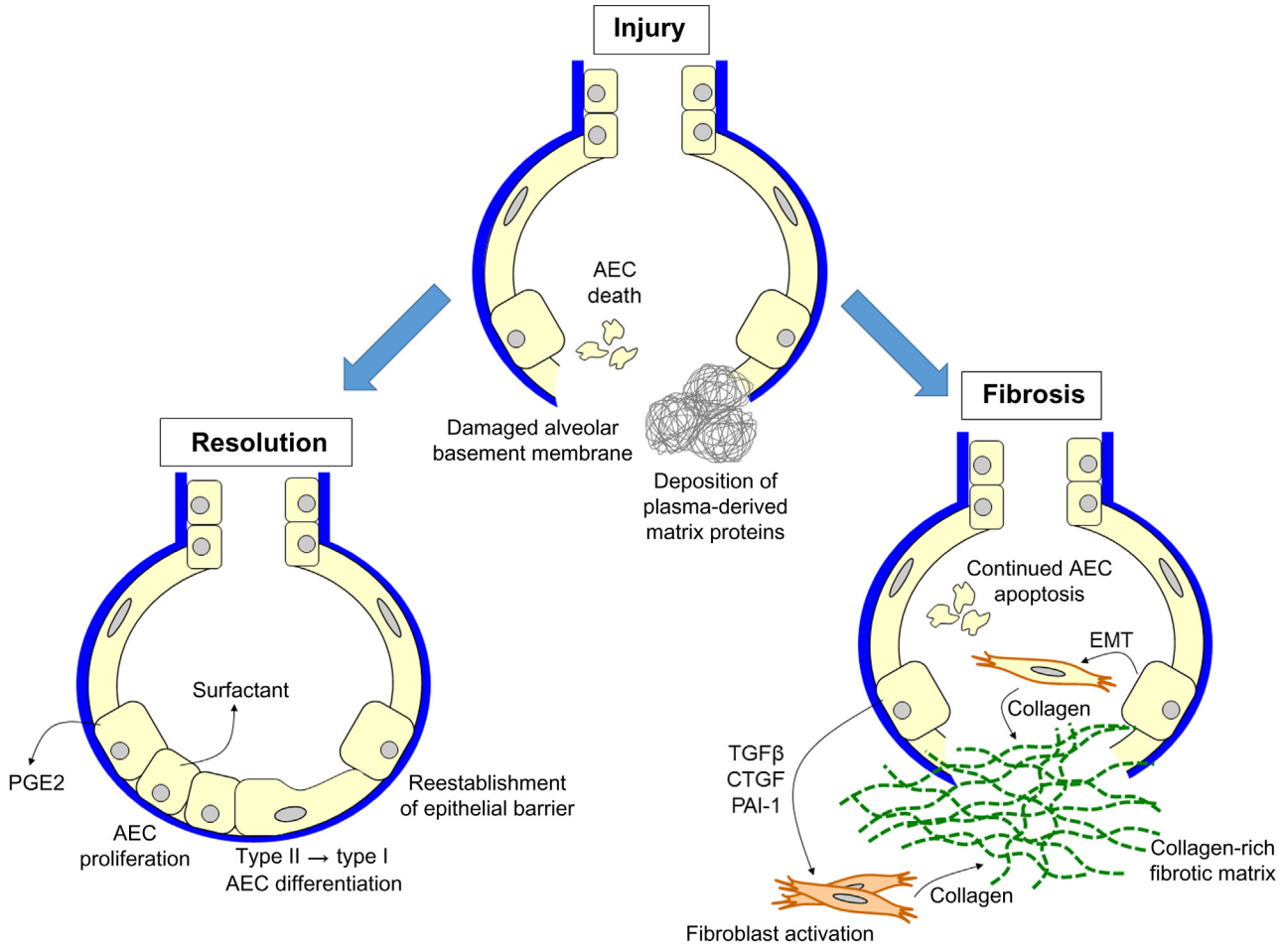

FIGURE 7.2 Epithelial cell functions determine the response to injury.
The lung epithelium can be injured by various insults leading to alveolar epithelial cell (AEC) death and damage to the alveolar basement membrane. This leads to loss of the epithelial integrity, influx of plasma into the alveolar space, and rapid formation of a provisional matrix made up of plasma-derived matrix proteins including fibrin, fibronectin, and vitronectin. Epithelial cells are critical regulators of the response from this initial injury toward resolution or fibrosis. Resolution of injury back to homeostasis involves AEC proliferation and differentiation of epithelial stem/progenitors cells such as type II AEC differentiation into type I AECs. This promotes restoration of the epithelial barrier and resumption of homeostatic functions such as production of surfactant and production of antifibrotic factors such as prostaglandin E2 (PGE2). Epithelial cells can also orchestrate the fibrotic response. Continued AEC apoptosis may result in sustained injury. AECs can also acquire a profibrotic phenotype through epithelial–mesenchymal transition with increased type I collagen production and production of profibrotic factors such as plasminogen activator inhibitor-1 (PAI-1), connective tissue growth factor (CTGF), and transforming growth factor-β (TGFβ). Release of these factors can stimulate paracrine activation of fibroblasts to produce type I collagen, leading to formation of a collagen-rich fibrotic matrix to replace the degrading provisional matrix.

promote resolution and homeostasis. Conversely, through indirect actions, such as apoptosis or secretion of profibrotic factors, and direct actions, such as fibrotic matrix deposition, lung epithelial cells can initiate and propagate a progressive fibrotic response. Understanding the function of lung epithelial cells during fibrosis offers the opportunity to identify novel therapeutic targets.

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Chapter 8

The Role of Epithelial Cell Quality Control in Health and Disease of the Distal Lung

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8.1 INTRODUCTION

The alveolar sacs of the distal lung are lined by a coupled mosaic of two major epithelial subtypes (Chapter 3, Glucose Transport and Homeostasis in Lung Epithelia). The alveolar type 1 (AT1) cell provides the bulk of surface coverage for this region while the alveolar type 2 (AT2) cell represents a multifunctional and metabolically active constituent playing critical roles in the production and maintenance of pulmonary surfactant (Chapter 6, Epithelial Regeneration and Lung Stem Cells), xenobiotic metabolism, and lung repair serving as a progenitor population following lung injury to replace damaged AT1 and AT2 cells [1,2]. In addition, new micropopulations of epithelial cells residing within this niche with stem and/or regenerative capacities are emerging but remain incompletely defined [2,3].

Because of its juxtaposition with the outside world, the alveolar epithelia and in particular the AT2 cell, in the course of performing these key biosynthetic, metabolic, and repair functions central to maintenance of alveolar homeostasis, must also protect itself from the significant stress imparted both by its continuous exposure to exogenous mechanical, metabolic, and environmental factors and by its own high degree of endogenous metabolic activity, macromolecular turnover, and organellargenesis. To manage the considerable threat imposed by these intrinsic and extrinsic challenges to protein synthesis, macromolecular homeostasis, and maintenance of organelle mass and integrity, eukaryotic cells and higher organisms have evolved a complex network of cellular quality control (QC) pathways designed to improve, modify, compartmentalize, and/or remove abnormal molecular and organellar substrates which accumulate during the course of daily activity. As with many other systems (in particular the central nervous system, liver, and pancreas), disruption of this QC network inevitably results in the accumulation of misfolded proteins, complex macromolecules, and/or dysfunctional organelles with elaboration of multiple signaling cascades that can promote disease initiation and/or drive its progression through the triggering of inflammation, cell death, and organ dysfunction. In addition, bystander effects from these events can affect the normal turnover of other resident cellular components further contributing to the resulting lung pathology. While cellular QC 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.

In this chapter, we explore new developments and shifting paradigms for improved understanding of the pathogenesis and molecular mechanisms underlying interstitial lung diseases (ILDs) of the postnatal lung from the view point of dysregulated epithelial cell QC. Many of these insights have been provided by studies of lung disease-related mutations in specific components of the pulmonary surfactant system [including Surfactant Protein C (SP-C), Surfactant Protein A (SP-A), and ATP binding cassette-class A3 (ABCA3), a key lipid transporter] and by genetic derangements in ubiquitously expressed multisubunit complexes, such as adaptor protein 3 (AP-3) and biogenesis of lysosome-related organelles (LROs) complexes (BLOC)-1, -2, and -3, that contribute to membrane and cargo trafficking to LRO, but which have a specific and profound impact on basic AT2 cell biology and secondarily lung remodeling. Furthermore, the AT2 cellular phenotype generated in response to these events and its role as a dysfunctional effector cell in the modulation of disease pathogenesis will be discussed using data on the elicited QC events and signaling pathways generated from a

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variety of in vitro and in vivo models expressing these mutant components. These models will be placed on an expanding larger spectrum of supporting data including translational studies of patients showing similar molecular signatures in both familial and sporadic idiopathic pulmonary fibrosis (IPF) and in preclinical and patient data from other nonsurfactant related AT2 cell dysfunction syndromes associated with either a fibrotic or destructive lung phenotype.

8.2 AT2 CELLS AND THE BIOSYNTHETIC CHALLENGE OF SURFACTANT

The alveolar gas exchange surface is coated with a thin film of *surf*ace *act*ive *agent* (=surfactant), representing a biochemically heterogeneous complex composed of primarily lipids (90% by weight) and protein which function collectively to augment surface tension at the air−liquid interface along the epithelial lining layer [4]. In addition to lipids [principally phosphatidylcholine (PC) with one (lyso-PC) or two (DPPC) palmitic acid side chains], biochemical analysis of surfactant has identified four unique protein components designated surfactant proteins (SP): SP-A, SP-B, SP-C, and SP-D [5]) (Fig. 8.1). 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. [6]). The relatively hydrophilic and more abundant oligomeric proteins, SP-A and SP-D, are members of the larger 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 an essential function with regards to the surface tension activity of surfactant, they play an key role in innate lung host defense (reviewed in Ref. [7]).

AT2 cells synthesize, secrete, and recycle all components of surfactant. When compared to 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, and cytokines) creates a significant burden on cell QC and metabolic demands. Ultrastructurally, AT2 cells lack a dense secretory granule; but instead, transmission electron microscopy (TEM) reveals the presence of cytoplasmic, osmiophilic, lamellated organelles (lamellar bodies = LBs) long recognized as the storage organelle from which surfactant is released into the alveolar lumen [8] (Fig. 8.2). Biochemically, LB resemble other LROs in that they are acidic, express lysosomal markers (CD63; LAMP-1) but also contain surfactant lipids, SP-B, and

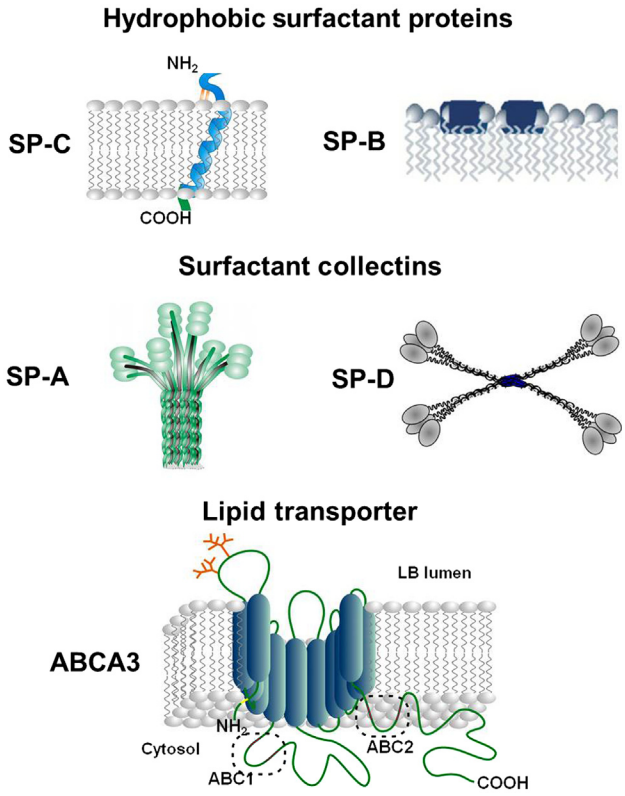

FIGURE 8.1 **Schematic representation of 5 key surfactant system components associated with lung disease in humans**. For additional details, see text.

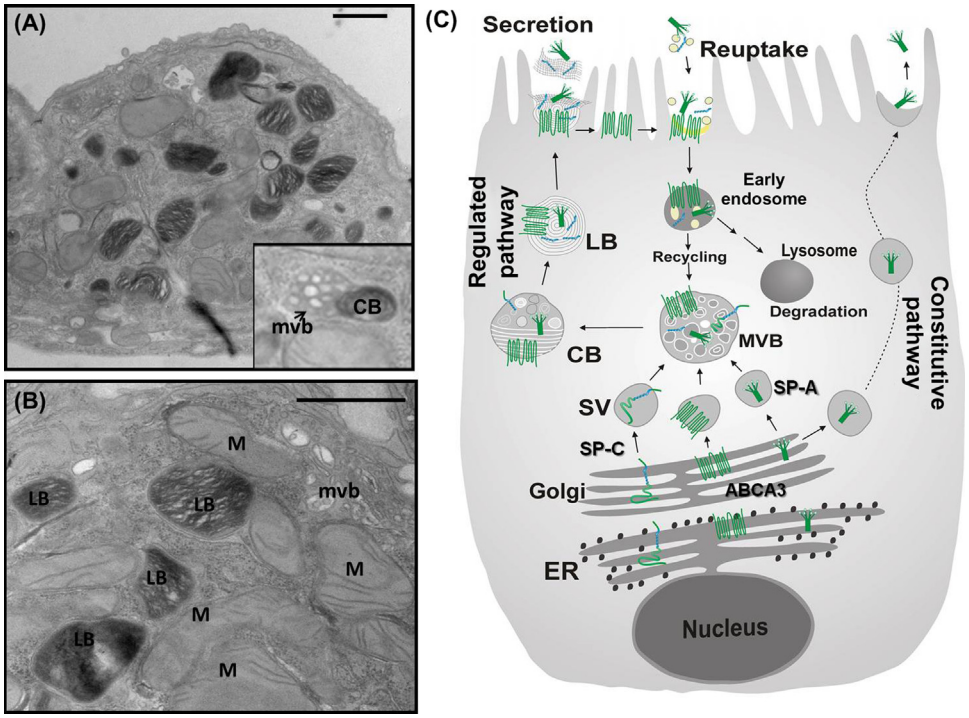

FIGURE 8.2 **The Alveolar Type 2 Cell: A Unique Exocrine Cell. (A) and (B). Alveolar type cell ultrastructure.** Transmission electron micrographs demonstrating key ultrastructural features of alveolar type 2 cells. Low-and high-power TEM of mouse lung section demonstrating multilamellated cytosolic organelles (lamellar bodies = LB), multivesicular bodies (MVB), and abundant mitochondria. Inset shows fusion of a late endsome/mvb with a composite body (**CB**) ("prelamellar body")

(C) Schematic Summary of the 3 Phases Of The Surfactant Lifecycle in the AT2 cell:

Biosynthesis: Along with phospholipids, SP-B, and SP-D, AT2 cells synthesize SP-C, SP-A, ABCA3

SP-C: The human *SFTPC* gene generates a 197 amino acid (21 kDa) proprotein (proSP-C_{21}). ProSP-C_{21} is translocated to the ER, sorted in the Golgi, and enters the regulated secretory pathway where it is processed by four endoproteolytic cleavages of NH_2 and COOH propeptide domains as it transits through small or sorting vesicles (SV), multivesicular bodies (MVB), composite bodies (CB) to lamellar bodies (LB), and the site of the final cleavage.

ABCA3: The product of a 33 exon geneABCA3, glycosylated in the Golgi, is Initially routed to post Golgi sorting vesicles, trafficked via the MVB/CB network to LBs. A pool of *ABCA3* exists on the plasma secondary to exocytotic membrane fusion. *ABCA3* undergoes a posttranslational proteolytic cleavage within the proximal NH_2-terminal region at distal post Golgi compartments.

SP-A: The human *SFTPA* gene gives rise to a 36 kDa monomer which is assembled into an 18-mer petal structure made up of six SP-A trimers. Posttranslational modifications of SP-A include signal peptide cleavage prior to entering the ER, hydroxylation of proline residues, and N-linked glycosylation.

Secretion: Surfactant phospholipid, processed SP-C, SP-B, and SP-A contained in the LB are released by regulated exocytosis; ABCA3 remains in the LB membrane and is recycled. A portion of SP-A and SP-D are secreted constitutively.

Reuptake/Degradation/Recycling: Surfactant proteins (SP-A, SP-B, and SP-C) and lipids are taken up by AT2 cells via endocytosis and are either targeted to the lysosomes for degradation or recycled back to LBs via endosomes and MVBs.

SP-C as well as a portion of the intracellular pool of SP-A [9]. LB release surfactant into the alveolus via regulated exocytosis regulated by a variety of signaling pathways (reviewed in Ref. [10]). In contrast, much of the synthesized SP-A and all SP-D is secreted via a non-LB, constitutive pathway.

In addition to biosynthesis, AT2 cells participate in the uptake, catabolism, and reutilization of surfactant. Protein and lipid components are endocytosed via clathrin-dependent and -independent mechanisms, routed through the central vacuolar system [early endosomes (EE), late endosomes/multivesicular bodies (LE/MVB)] and then sorted to lysosomes for degradation or to LB for re secretion.

Both SP-B as well as the ATP binding cassette-class A3 (ABCA3) transporter, a lipid pump situated within the LB limiting membrane (Fig. 8.1), have been shown to be critical to LB biogenesis as genetically null genotypes from deficiency of either protein are phenotypically marked by a lack of LBs and neonatal respiratory failure in both murine models and human patients. However, in addition to these well-known autosomal recessive, loss-of-function phenotypes, a number of mutations in at least three of these surfactant system components (SP-C, SP-A, and ABCA3) have been associated with the development of interstitial lung disease (ILD) in both children (chILD) and adults [most

frequently usual interstitial pneumonia (UIP) or nonspecific interstitial pneumonia (NSIP)]. As such, these seemingly rare genotypes have provided an important platform and experimental substrates to study the role aberrant QC, cell stress, and cytotoxicity of the distal lung in aberrant injury/repair events critical to the pathogenesis of many parenchymal lung diseases. The principal surfactant system components associated with such a toxic gain-of-function are biochemically and functionally diverse.

SP-C: A Unique Protein Cargo—Of the four SP, only SP-C is an AT2 specific product. 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 5 introns) which produce a 0.9 kb mRNA encoding either a 191 or 197 amino acid 21 kDa proprotein (=proSP-C_{21}) (Fig. 8.3A). The protein form isolated and sequenced from lung lavage fractions (SP-$C_{3.7}$) is an extremely hydrophobic, lipid avid peptide composed of 33−35 highly conserved amino acids containing a high content of Val, Ile, and Leu (~60%−65% of the primary sequence) which self-aggregates in aqueous solution, adopting β-sheet conformation, and forming amyloid fibrils. Thus, SP-C represents a structurally and functionally challenging substrate for the AT2 cell in which the proSP-C_{21} propeptide is trafficked through the regulated secretory pathway as an integral type II bitopic transmembrane (TM) protein ($N_{cytosol}/C_{lumen}$), undergoing four endoproteolytic cleavages of its flanking NH2− and −COOH propeptides to yield the mature, biophysically active 3.7 kDa form found in secreted surfactant (Fig. 8.3B) (reviewed in Ref. [11]). Using epitope specific antisera, both fluorescence immunohistochemistry (Fig. 8.3C) and immunogold EM

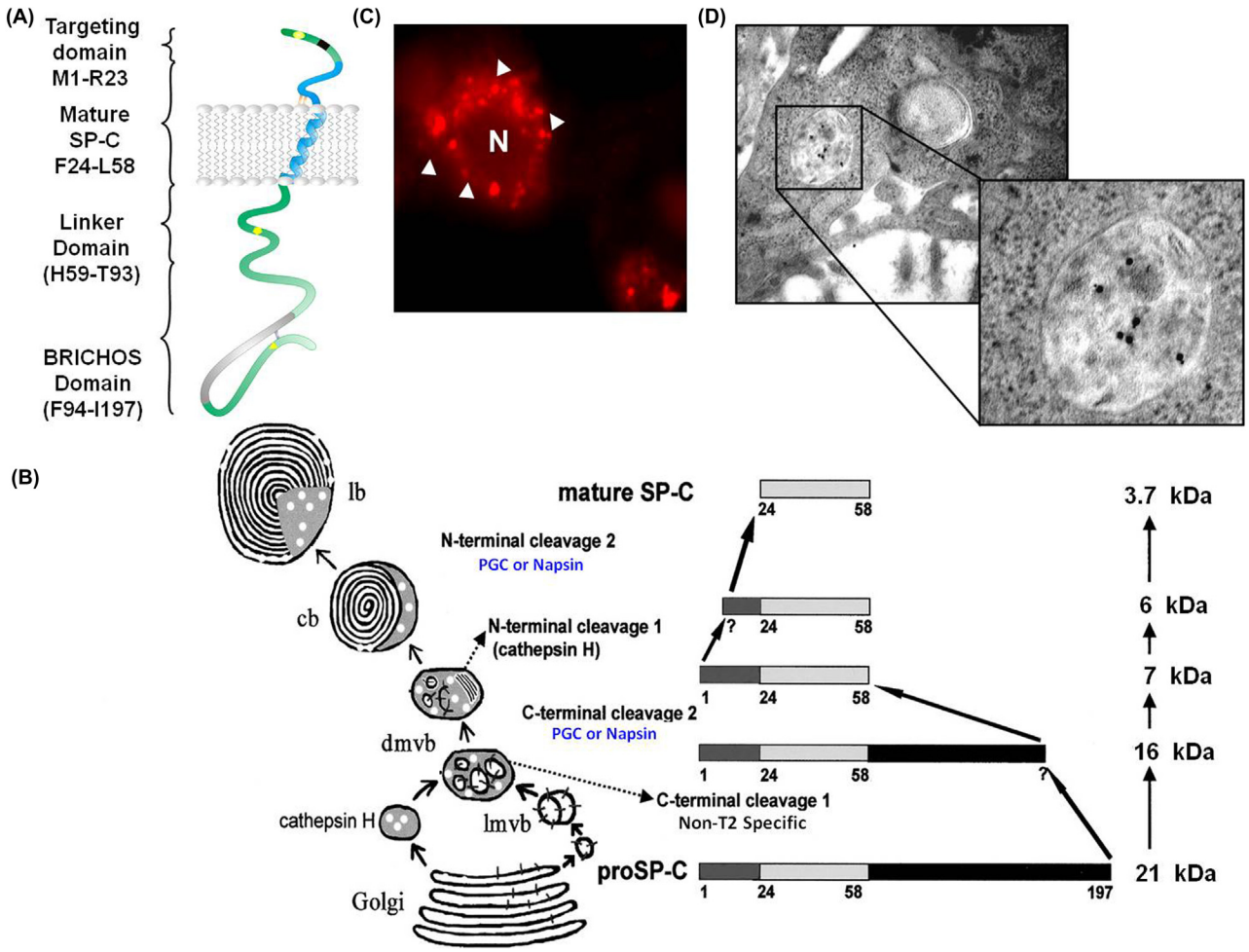

FIGURE 8.3 **Biosynthesis of Surfactant Protein C. (A) Schematic representation of the 197 amino acid SP-C primary translation product containing 4 functional domains**. ProSP-C is an integral transmembrane protein with a Type II (NH2 cytosolic) orientation. **(B) SP-C Proteolytic Processing Steps in Alveolar Type 2 Cells**. At right, the mature SP-C protein secreted by LB is generated from 4 separate cleavages of the propeptide in post-Golgi compartments. The approximate size of each intermediate is illustrated. The compartments and proteases which participate in SP-C processing are shown on the Left. **(C) ProSP-C Expression in AT2 Cells**. Fluorescence Immunohistochemistry of normal mouse lung stained with anti-proSP-C showing localization of proSP-C peptides with lamellar bodies of AT2 cells arrows). N = nucleus. **(D)** Double label Immunogold staining of normal human lung showing co-localization of proSP-C (10 nm gold) and the aspartic protease Napsin A (5 nm) within the lumen of lamellar bodies.

(Fig. 8.3D) as well as subcellular fractionation techniques, can detect proSP-C isoforms in AT2 LBs indicating that the correct routing of proSP-C from the proximal [endoplasmic reticulum (ER) Golgi] to distal (LB) compartments is crucial for mature SP-C biogenesis. Data from our laboratory and others have shown that both normal posttranslational processing events as well as the behavior of disease causing *SFTPC* mutants are influenced (directly and indirectly) by four functional domains and structural motifs contained within proSP-C_{21} (Fig. 8.3A)

1. NH_2 Targeting Domain—Mutagenesis studies have identified and characterized a PPxY (PY) motif within the NH_2 propeptide that is crucial for postER/Golgi proSP-C trafficking [12−14]. The PPDY motif (which serves as a ligand for tryptophan-tryptophan (WW) domains) was shown to specifically interact with two WW domain containing members of an E3 ubiquitin ligase family, Nedd4 and Nedd4-2 (Nedd4-L). Nedd4-2 was subsequently shown to exert the dominant effect on proSP-C transport catalyzing monoubiquitination (a well-known signal for lysosomal targeting of proteins) of proSP-C_{21} at lysine residue 6 of the cytosolic NH_2 propeptide [12].
2. Mature SP-C (TM) Domain—In apolar solvents or phospholipid rich environments the 35 residue mature SP-C domain forms a very stable and rigid α-helix. Functionally, mature SP-C acts as a noncleavable signal anchor peptide for proSP-C21 facilitating its translocation to the ER [15,16]. In addition, a conserved pair of juxtamembrane basic amino acids, lysine (K) and arginine (R) (at positions 34 and 35 of human proSP-C) play an essential role in the determination of the type II TM topology of proSP-C21 [15,17].
3. The BRICHOS domain—the distal proSP-C21 COOH-terminus (residues 94−197), known as the BRICHOS domain, shares a high degree of structural homology with domains identified in over 300 proteins [18]. The term BRICHOS was derived from the original disease-associated family members including BRI2, linked to familial British and Danish Dementia, Chondromodulin-1, related to chondrosarcoma, and SP-C [19]. The structural similarity of these three is punctuated by the fact that each exist as integral type II TM proteins comprised a cytosolic region, a TM segment, a linker domain, and the aforementioned BRICHOS domain containing a pair of positionally conserved cysteine residues. The human *SFTPC* actually encodes four cysteines in its BRICHOS domain. Cysteines 121 and 189 represent the positionally conserved BRICHOS cys residues which are invariant across species and felt to mediate the primary folding function. Mutation of either one of these BRICHOS cysteine residues results initially in proximal (ER) retention and eventual formation of aggregates of unprocessed polyubiquitinated proprotein in vitro [20]. In human proSP-C (but lacking in other species), Johannson et al. have also modeled a second disulfide bridge between cysteine 120 and 148 of human proSP-C but the exact physiological significance is currently incompletely defined (see Refs. [16,21]). Functionally, in addition to promoting proSP-C COOH domain folding, the BRICHOS domain appears to have an intramolecular chaperone-like activity safeguarding the metastable, β-sheet-prone, mature SP-C domain from amyloid fibril formation [22−25]. 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 [26].
4. Linker Domain—As the name signifies, the proximal COOH-terminal propeptide (Residues 59−93) is linearly positioned between the mature SP-C (TM) and BRICHOS domains; and through formation of β-hairpin structure thereby facilitates docking of the BRICHOS domain to the TM segment. Significantly, mutations in the linker domain do not induce cytosolic aggregation of proSP-C but instead result in its mistrafficking and accumulation in plasma membrane, EE, and LE/MVB [27,28] (discussed in detail later).

SP-A: A Multifunctional Collectin—Surfactant Protein A, the most abundant surfactant protein, is a luminal sialoglycoprotein of Mr 28−36,000 that contains a COOH terminal C-type lectin motif, a triple helical collagen domain, and carbohydrate recognition domain (CRD) [7] (Fig. 8.1). In humans, there are two genes (*SFTPA1; 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 collagen-like domain with only one shown to affect protein structure. SP-A is synthesized principally by AT2 cells although message and protein has also been detected in conducting airway epithelia, principally nonciliated (Club/Clara) cells. SP-A has also been localized to extra-pulmonary sites, including salivary glands, lacrimal glands, and the female urogenital tract; however, the exact physiological relevance of its expression in these organs remains to be defined (reviewed in Ref. [29]).

The biosynthesis of SP-A diverges significantly from SP-C (and SP-B). In contrast to posttranslational proteolytic processing of a larger proprotein, generation of the SP-A primary translation product is followed by cleavage of its NH_2 signal peptide, N-linked glycosylation, proline hydroxylation, and ultimately trimeric assembly of SP-A monomers via coiled-coil bundling of α-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 consisting of a higher order 18-mer (Fig. 8.1), is assembled from six trimeric subunits linked by interchain disulfide bonding. A proline residue in the collagenous domain of SP-A monomers

provides a flexible kink that causes the trimers to bend outward in different directions, giving the native SP-A 18-mer a "flower bouquet" appearance.

Metabolic labeling studies have demonstrated that the intracellular trafficking and secretion of nascent SP-A may involve both LB dependent (regulated) [30] and LB independent (constitutive) pathways [31]. Because alveolar SP-A can recycled via endocytosis and reincorporation 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 Lipid Transporter Critical For LB Homeostasis—A key component in the formation of LBs is the ABCA3 glycoprotein, a member of the ABC super family TM transporter proteins that use ATP energy to drive various substrates, ranging from small ions (e.g., CFTR = ABCC7) 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 [32].

Human *ABCA3* has been mapped to chromosome16p13.3 and encodes a 1704-amino-acid protein [33]. Although ABCA3 mRNA is detected in many tissues, the ABCA3 message is highly expressed in AT2 cells and ABCA3 protein is predominantly associated with the LB limiting membrane [34,35]. ABCA3 has been shown in vitro to transport PC, phosphatidylglycerol, sphingomyelin, and cholesterol into lysosomes of model cell line systems [35–39]. Morphologically, transmission EM of lungs from homozygous null mutations of *ABCA3* reported in neonates with respiratory failure as well as the ABCA3 knockout mouse has shown replacement of normal LB structures with abnormal electron dense inclusion bodies demonstrating its functional importance as one of the critical regulators of LB biogenesis and lung surfactant metabolism [36,38,40].

The structure of ABCA3 is schematically shown in Fig. 8.1. The ABCA3 protein structure includes two TM complexes composed of six domains each, coupled with two cytosolic ATP binding cassettes (ABC1, ABC2) also termed nucleobinding domains. In addition, two Walker domains A and B are present in each ABC domain on the cytosolic face while one major and two minor extracellular loop domains (ECDs) per each TM complex face the LB lumen.

To date studies have identified two trafficking domains in the molecule: (i) xLxxKN Targeting Motif—nearly all ABCA subfamily of transporters share significant homology in their N-terminal domain, including a highly conserved motif (xLxxKN) [32] which we have shown to be essential for directing these proteins to post-Golgi compartments [41]. Since each ABCA transporter has a unique subcellular destination and function, subsequent sorting of ABCA3 to LB requires additional yet to be identified signal or signals; (ii) N-linked-Glycosylation-site directed mutagenesis of two asparagine residues within the first NH_2-terminal luminal loop of the transporter impairs protein stability, disrupts its anterograde trafficking resulting in proteasomal degradation and cell stress [42].

Taken together, while the cuboidal shaped AT2 cell comprises only 3%–5% of the alveolar surface area, it constitutes 60% of total alveolar epithelial cells (10%–15% of total lung cells) and represents a highly 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 overlap in multiple subcellular compartments of the secretory, endocytic, and degradative (lysosomal) pathways [43]. As such, the complexity of these cargo and their atypical organellar features also render the AT2 cell highly susceptible to dysfunction from QC challenges and thus potential participants in lung injury and aberrant repair associated with parenchymal lung diseases.

8.3 A NEW "OLD" HYPOTHESIS: ALVEOLAR EPITHELIAL DYSFUNCTION AND PARENCHYMAL LUNG DISEASE

Pulmonary fibrosis (PF) represents a heterogeneous group of postnatal interstitial lung disorders characterized by pathological distortion of distal or peripheral pulmonary architecture that ultimately results in scar formation, organ malfunction, disruption of gas exchange, and respiratory failure. In adults, 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 [44]. In the United States, the annual incidence of IPF is increasing and currently estimated to affect 5–16/100,000 individuals with a prevalence of 13–20/100,000. The disease is more common in men and increases significantly with age. IPF is characterized histologically by a pattern of UIP, in which the peripheral

subpleural parenchyma shows evidence of fibroblastic foci, traction bronchiectasis, and microscopic honeycombing lined by hyperplastic AT2 cells interspersed with areas of normal or nearly normal lung tissue. Familial forms of PF (FPF) are well described and associated with a number of inheritable mutations that affect a variety of surfactant components, intrinsic QC systems, and key structural components of the AT2 cell (detailed below). FPF also present in the pediatric population and are part of the larger spectrum of childhood interstitial lung disease (chILD) [45,46]. 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.

The role for a dysfunctional distal lung epithelium at risk in the pathogenesis of ILD/chILD represents a rebirth of a concept first put forth by Haschek and Witschi in the late 1970s. Their seminal work in support of an "epithelial hypothesis" of the pathogenesis of lung fibrosis challenged the then contemporaneous view of lung fibrosis as an "inflammatory disease" [47,48]. While chronic inflammation as a primary driver in ILD pathogenesis had persisted in some circles (and may remain highly relevant in the pathogenesis of some forms of PF that develop in association with collagen vascular diseases), it is not a prominent feature in biopsies of IPF patients [49]. Coupled with the low efficacy or even harmful effects of immunomodulatory therapies shown in many clinical trials involving these patients [21,50–52], a paradigm shift away from the so-called "alveolitis" theory began to develop resurrecting a pivotal role for alveolar epithelial cells (AEC) and specifically AT2 cell dysfunction as a proximal component in the generation of a fibrotic lung phenotype.

The "epithelial injury/abnormal wound repair" model, rechampioned in the early 2000s [49], is illustrated in Fig. 8.4. IPF development and progression was proposed to be initiated and sustained by microfoci of repeated cycles of injury to a susceptible lung epithelium occurring within a larger dysfunctional repair process reminiscent of aberrant wound healing described in other organs such as skin, kidney, and liver [53,54]. Inflammation and the local immune milieu can still be important modifiers of PF pathogenesis whose regulation and magnitude of contribution to the entire process still remains to be contextually defined.

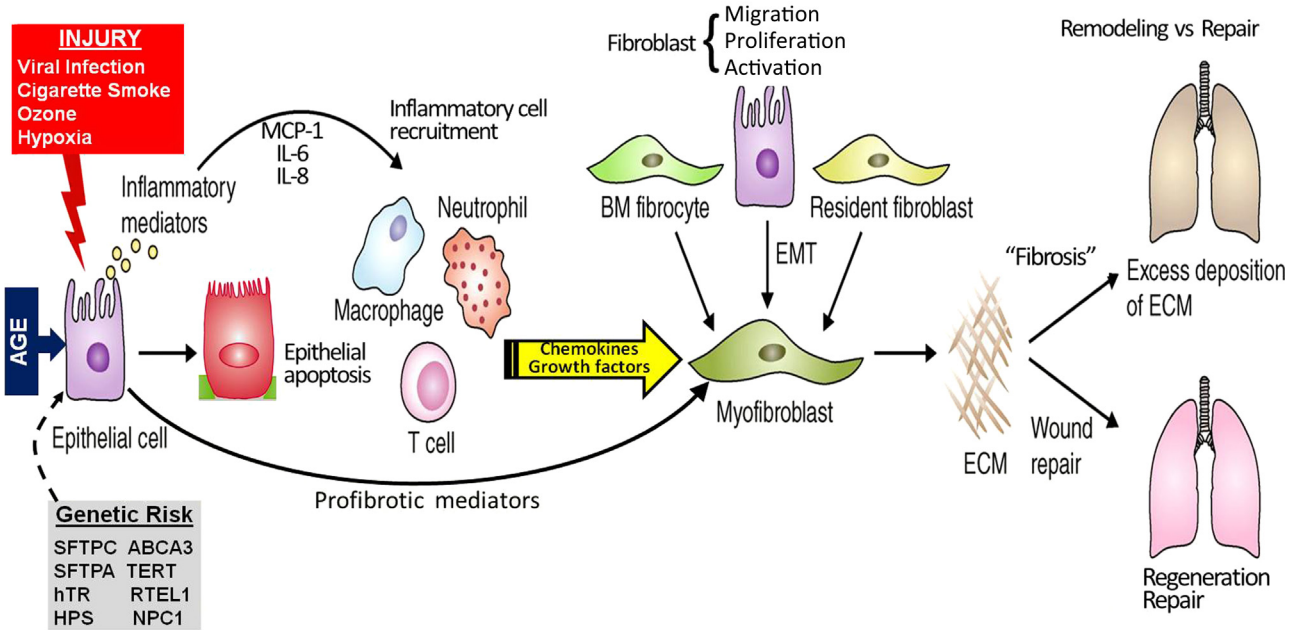

FIGURE 8.4 Model For Pathogenesis of Pulmonary Fibrosis. The pathogenesis of IPF has four distinct stages: (1) Injury to epithelial cells by a "two-hit" process composed of susceptibility imposed by intrinsic factors (genetic mutations, age) and exogenous injury by factors (tobacco smoke, oxidative stress, infection) release inflammatory mediators and profibrotic factors (TGF-β); (2) An inflammatory cell migration phase that both amplifies the epithelial injury as well as alters mesenchymal composition. Key events include entry of leukocytes (e.g., neutrophils, macrophages, and T cells) into both the interstitium and alveolar space. The recruited leukocytes and the epithelium secrete profibrotic cytokines such as IL-1β, TNF, IL-13, and TGF-β; (3) a fibroblast migration/proliferation/activation phase composed of fibrocytes from the bone marrow, resident fibroblasts that proliferate and differentiate into myofibroblasts releasing extracellular matrix components (ECM), and transdifferentiated epithelial cells that may adopt a mesenchymal phenotypic = EMT; (4) a tissue remodeling and resolution phase-activated myofibroblasts can promote wound repair, leading to wound contraction and restoration of blood vessels. However, fibrosis often develops if any stage in the tissue repair program is dysregulated or when lung-damaging stimuli persist.

More recently, the "postmodern" link between intrinsic epithelial cell dysfunction and IPF/ILD postulated by Haschek and Witschi has been established based on multiple lines of preclinical experimental evidence and clinical findings involving rare gene variants:

1. Monogenetic diseases such as Hermansky–Pudlak syndrome (HPS) are characterized by dysfunctional AT2 cells and ILD [55]. Mutations in nine different HPS genes encoding for important trafficking and structural proteins such as BLOC-1,-2, -3 as well as AP3 complex members result in disruption of intracellular cargo and organelle trafficking leading to dysfunctional LROs in various organs. LBs, the LROs of AT2 cells, show marked increase in size due to over accumulation 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 [55]. 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 MCP-1 in which effector cell accumulation precedes the development of fibrotic remodeling [56–58].
2. When aberrantly activated, lung epithelial cells can also produce virtually all the known mediators responsible of fibroblast migration, proliferation, and activation with the subsequent exaggerated EMC accumulation and destruction of the lung parenchyma [49,59].
3. In addition to intrinsic epithelial dysfunction, depletion of AT2 cells (recognized as key progenitor/stem cells in the distal lung) can induce 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 increases in lung collagen deposition. However, these effects appear to be dose dependent as lower levels of DT expression were insufficient to promote such changes in some models [2].
4. AT2 cell dropout via apoptosis is paramount in mouse models of lung fibrosis generated by single or repetitive exposures to bleomycin. Inhibition of apoptosis in these models using caspase inhibitors, Fas/FasL pathway blockade, or genetic deletion of proapoptotic Bcl2 proteins (BID) attenuates fibrosis [60].
5. Some of the most compelling evidence for the role of AT2 cells in fibrotic remodeling is derived from studies linking patients with UIP or chILD histopathology with mutations in alveolar epithelial cell-restricted genes involved in the production of pulmonary surfactant. In 2001, Nogee et al. first reported a mutation in the COOH-terminal region of Surfactant Protein-C gene (*SFTPC*) that resulted in deletion of exon 4 and its 37 amino acids (SP-C exon4) in both an infant and mother with ILD [61]. Shortly after, a second report described a different *SFTPC* mutation resulting in substitution of glutamine for leucine at position 188 of the SP-C propeptide (SP-C^{L188Q}) in large kindred of whom the biopsies of 11 adults showed IPF/UIP patterns and NSIP patterns were present in three chILD. Using a variety of model systems, we and others subsequently demonstrated that expression of SP-C exon 4 or SP-C^{L188Q} in vitro resulted in intracellular SP-C protein aggregation and cell death by apoptosis. To date, more than 60 (*SFTPC*), and 150 *ABCA3* mutations, as well as two mutations in the Surfactant Protein A2 gene (*SFTPA2*) have been identified in affected chILD and adults [40,62–64]. The link was further strengthened by translational evidence in which the signatures of protein aggregation, ER stress, altered macroautophagy, and apoptosis present in model systems expressing not only *SFTPC* mutations but also *SFTPA* and *ABCA3* disease variants are also present in the lungs and AT2 epithelia of sporadic and familial IPF patients [65–68]. The overwhelming majority of affected patients with *SFTPC*-, *SFTPA*-, and *ABCA3*-related ILD reported to date have been heterozygous for the mutant allele suggesting a toxic gain-of-function mechanism.

Thus, substantive evidence currently 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.

8.4 CELLULAR QUALITY CONTROL

The heterozygous expression of a mutant allele of a surfactant component which is sufficient to promote pathological lung remodeling is reminiscent to observations reported for a seemingly diverse set of chronic, degenerative disorders in a variety of organ systems including the central nervous system, pancreas, and liver all of which are associated with expression or altered processing of a mutant protein conformer that unmasks either an intrinsic ability to self-associate (aggregate) and/or be deposited in nonnative subcellular or tissue compartments [69–72]. How cells respond to the production of these conformers and how mutant proteins induce cytotoxicity, contribute to local tissue inflammation, and/or participate in aberrant repair remain critical questions confronting cell biology and medical therapeutics.

In order 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 QC 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 or to remove defective, irretrievably broken, or unwanted substrates. In addition, signaling pathways emanating from many of these component pieces serve to modulate the overall composition of the QC network.

A recent NHLBI workshop ("*Malformed Protein Structure and Proteostasis In Lung Diseases*") highlighted the potential role that disorders of protein folding, trafficking, and degradation play in the pathobiology of lung disorders and their associated complications [69]. By extension, recent published discoveries have also implicated organelle dysfunction and turnover, in particular of mitochondria or the endosomal−lysosomal system, in the pathogenesis of some age-dependent (e.g., IPF), age-accelerated (e.g., Hermansky−Pudlak Syndrome (HPS)-associated fibrosis), and environmentally modified (e.g., tobacco, ozone, and asbestos) parenchymal lung disorders pointing to a critical role for the need to edit and remove damaged organelles to maintain cellular health. A major conclusion put forth by the participants was that studies into the mechanisms and pathways underlying protein (and organelle) QC in the lung and the coupling of alterations in QC to lung disease has been largely underdeveloped.

By contrast, much of the progress made in our understanding the role of QC pathways in disease pathogenesis has been derived from studies of a seemingly diverse set of chronic, degenerative nonlung-based disorders involving the central nervous system, 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) [73,74]. Similarly, liver disease from expression of the mutant Z allele of Alpha-1-Antitrypsin (Z-A1AT) is associated with ER retention and cytosolic aggregation of Z-A1ATprotein in hepatocytes leading to their eventual death [71]. The sheer magnitude of biosynthetic capacity for insulin and other proteins (e.g., islet amyloid peptide) in islet cells of the endocrine pancreas requires an equally robust QC system which appears to fail in models of Type II diabetes [75,76]. Furthermore, disorders such as Parkinsons 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 QC [77]. Finally, lipid storage disorders such as Niemann−Pick C (NPC) disease which are marked by severe neurodegeneration and lung disease have been shown to acquire a defective cell QC phenotype (principally impaired macroautophagy) [78].

A wealth of basic, preclinical, and translational studies using abnormal protein isoforms associated with these various diseases as substrates has permitted the definition of generic and cell specific responses to misfolded proteins, mistrafficked cargo, and dysfunctional organelles. The major components of cellular QC responses defined by these results are schematically shown in Fig. 8.5 and include

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" [79]. Proteostasis refers to the control of concentration, formation, binding interactions, quaternary structure, and location of individual proteins making up the proteome by readapting 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 events in a prototypical QC response to aberrant conformers include

- *The Unfolded Protein Response (UPR)*—As the initial response, highly specific and complimentary signaling pathways have evolved to ensure that ER protein folding capacity is not overwhelmed, and have been collectively termed the unfolded protein response (UPR) (reviewed in detail in Ref. [80]). The UPR pathways are modulated by three ER TM protein sensors: inositol-requiring enzyme 1 (IRE-1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6) (illustrated in Figs. 8.5 and 8.6). Their activation by abnormally folded proteins initiates one or more of three corresponding signaling cascades with two functional objectives: (1) expand ER refolding capacity by generating, translocating, and binding select transcription factors (X-box binding protein 1 [XBP1], ATF-6p50, and ATF4) to nuclear ER stress responsive elements (ERSE) to upregulate a variety of chaperones including Bip (GRP78); GRP94; calnexin and protein disulfide isomerase (PDI); (2) Promote translation attenuation at the level of the ribosome primarily through increasing levels of phosphorylated eif2α.

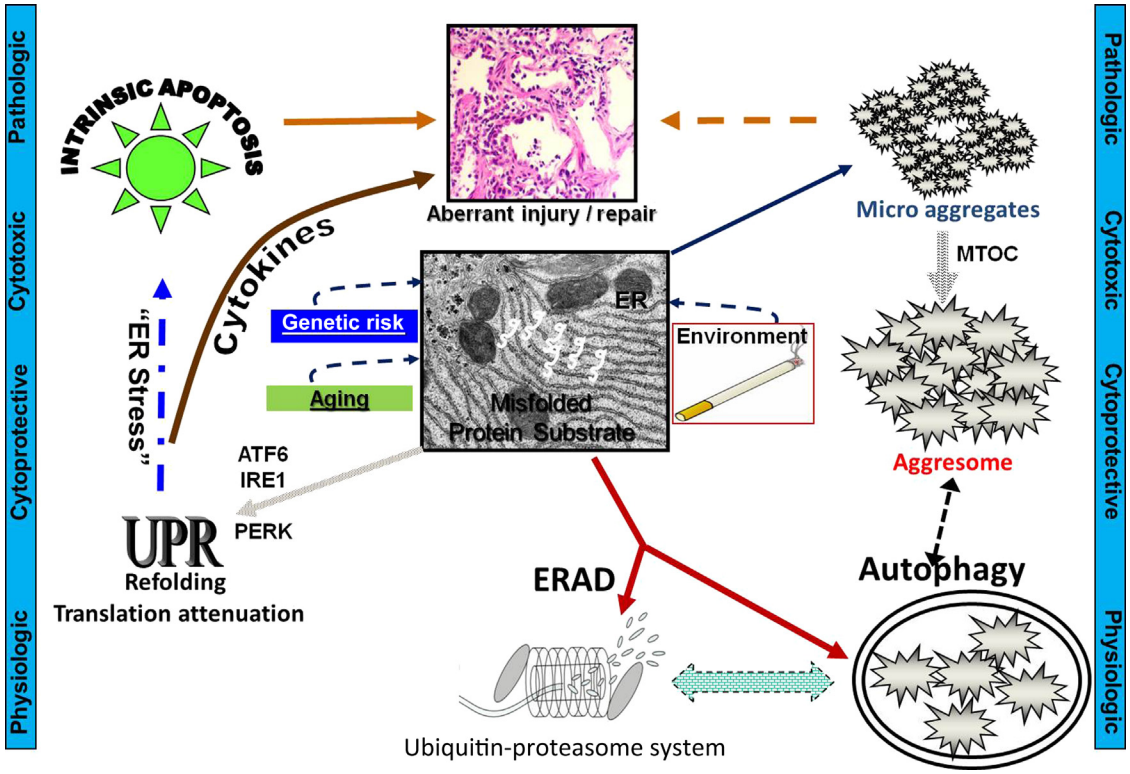

FIGURE 8.5 Schematic representation of proteostasis network utilized by cells in response to the expression of misfolded or unassembled proteins. The proteostasis network responds to the presence of increase protein folding demand either from translation of mutant conformers or simply increased demand for wild-type proteins. The misfolding can be exacerbated by age or environmental factors. The first line network components include:

Unfolded Protein Response (UPR): Three ER based (proximal) sensors for misfolded proteins, IRE1, PERK and ATF6 are activated by misfolded conformers to initiate the downstream signaling of the UPR. The presence of increasing load of misfolded proteins or demand to form nativeconformers within the ER disrupts control of these sensors normally held in check by BIP and initiates up to 3 downstream signaling pathways (see Fig. 8.6) to attempt to expand proteostatic capacity and decrease translational burden.

ER-Associated Protein Degradation (ERAD)— Involves a multistep process consisting of recognition of misfolded cargo by ER resident proteins (e.g., EDEM, PDI, Bip/Grp78) followed by their retrotranslocation out of the ER through translocons and subsequent polyubiquitination mediated by cytosolic E1/E2/E3 ubiquitin ligases. Targeting of ubiquitin decorated conformers to the 26S proteasome results in eventual degradation.

Autophagy: Cytosolic macroaggregates are targeted for degradation by autophagy. Activation of a cascade of upstream events (not depicted) mediated by a complex of 30 atg gene products initiates isolation membrane assembly, formation of a phagophore, and its eventual elongation envelop cytosolic ubiquitinated cargos including protein aggregates and mitochondria, leading to the formation of double membrane autophagosome which fuses with lysosomes resulting in an acidified and functional autophagolysosome to promote degradation of internalized cytosolic content.

Aggresome formation: As a last coping mechanism for a failure of the UPR/UPS/autophagy network to control mutant protein levels aggregate forms of misfolded protein are transported in a microtubule dependent manner to the microtubule organizing center (MTOC) near the nucleus forming aggresomes.

Failure of this network can result in ER stress inflammatory signaling or apoptosis and contribute to tissue injury/remodeling (detailed in text).

Significantly, molecular signatures generated by these events can be used to detect UPR activation in cells and in tissues: (1) IRE1 activation is heralded by appearance of spliced XBP1 mRNA or XBP1 protein; (2) PERK activation can be confirmed by detection of phosphorylated eIF2α, CHOP/GADD153 expression, and appearance of the transcription factor ATF4; (3) a 50-kDa cleavage fragment of ATF6 (ATF6-p50) can be used to assess engagement of this pathway. The relative contributions of each UPR pathway to the proteostatic response are protein substrate and cell specific.

- *Ubiquitin-Proteasome System (UPS)/ER-Associated Protein Degradation (ERAD)*—If the UPR is unsuccessful at restoring protein conformation, misfolded or unassembled protein targets are subsequently extracted from the ER by retrotranslocation and ultimately destroyed by the UPS depicted in Fig. 8.5 [81,82]. In many cell types, ERAD, utilizing the 26S proteasome represents the principal compartment for protein degradation machinery catabolizing about 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

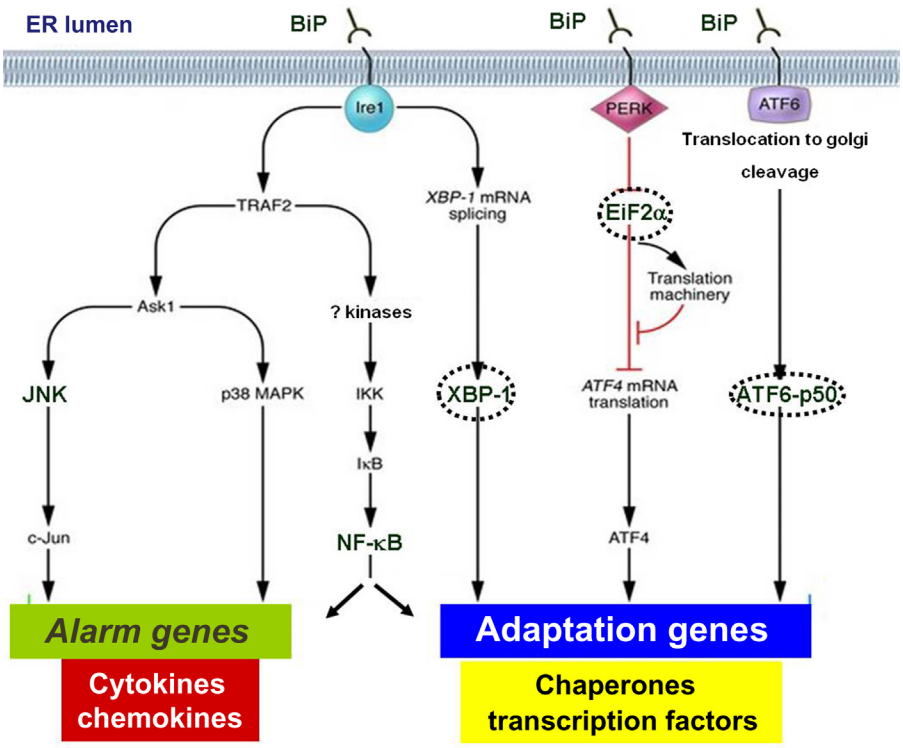

FIGURE 8.6 **Schematic representation of signal transduction events associated with the UPR and ER stress.** The ER chaperone Bip/Grp78 binds the transmembrane sensors Ire1, PERK, and ATF6, preventing their activation. Unfolded proteins in the ER cause Grp78 to release Ire1, PERK, and ATF6. Upon Grp78 release, Ire1 and PERK oligomerize in ER membranes. Oligomerized Ire1 binds TRAF2, signaling downstream kinases that activate NF-κB and c-Jun (AP-1), causing expression of genes associated with host defense such as cytokines. The intrinsic ribonuclease activity of Ire1 also results in production of XBP-1, a transcription factor that induces expression of genes involved in restoring protein folding or degrading unfolded proteins. Oligomerization of PERK activates its intrinsic kinase activity, resulting in phosphorylation of eIF2α and in overall suppression of mRNA translation. but also promoting translation of a selected group of mRNAs, including ATF4 which induces expression of genes involved in restoring ER homeostasis. Titration of Grp78 of ATF6 allows its translocation to the Golgi apparatus for proteolytic processing to release a 50 kDa active form ATF6, which controls expression of UPR genes.

peptides. In addition to a role in protein QC, protein degradation by the proteasome is essential for diverse cellular functions such as apoptosis, transcription, cell signaling, and immune responses (reviewed in Refs. [83,84]. 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/ Mg^{2+} is present [85]. 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. [86]).

The 26S proteasome used for 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 [87]. In addition, environmental factors such as cigarette smoke (CS) can directly impair proteasome activity in lung cells (without affecting proteasome expression) resulting in accumulation of polyubiquitinated proteins [88].

- *Autophagy*—The process of macroautophagy (hereafter referred to as autophagy (Atg)) was originally described as a cellular response to fasting starvation. However, an important emerging concept is that Atg 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) [89,90]. An extensive discussion of the autophagic process is beyond the scope of this chapter but can be found in several excellent reviews [91–93]. In brief, Atg is a dynamic process controlled by at least 30 known Atg genes whose assembly and turnover ultimately results in the enclosure of cytosolic substrates and organelles by generation of isolation membranes (phagophores) which mature to form double membrane-bound vesicles ("autophagosomes") readily identified by transmission 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 Atg for misfolded protein is dependent on the recognition of ubiquitinated aggregates by ubiquitin-binding receptors such as p62/sequestosome-1 (p62/SQSTM1) [94].

Various reports have confirmed the existence of a link between ERAD/UPS and Atg in the clearance of misfolded proteins [95,96] as perturbations in the flux through either pathway can affect the activity of the other system [97]. Evidence also exists for cross-talk between the UPR and Atg mediated by several pathways including an eIF2α/ATF4 axis and JNK1 [98,99].

- *Aggresomes, JUxta Nuclear Quality control compartment (JUNQ), and insoluble protein deposit (IPOD)*—Although still incompletely resolved, current consensus has put forth that the bulk of direct cellular toxicity from mutant protein conformers can be attributed to nonnative, soluble protein oligomers and that the formation of large aggregates is likely cytoprotective. In order to limit toxicity from misfolded proteins accumulating in the cytosol or nucleus that escape degradation by either the UPS and/or Atg, mammalian cells contain an additional cellular QC defense that involves the recognition, segregation, and active compartmentalization of ubiquitylated protein oligomers/microaggregates via transport in a microtubule dependent manner to pericentriolar, membrane-free, vimentin-enwrapped perinuclear structures termed aggresomes [100]. Published data has revealed that in addition to the protein aggregates, both Atg components (Atg proteins) and lysosomes can also be directed to aggresomes in a process regulated by the microtubule deactylase HDAC6. It has been suggested that aggresomes may then function as a substrate delivery and concentration system to improve the efficiency of bulk autophagic degradation [101,102].

  Recently, our understanding of cellular aggregate deposition sites has been further expanded by the discovery of two other distinct types of deposition sites that can concurrently exist within a single cell [103] Like aggresomes, one site is located in close proximity to the nucleus and functions as a quality control center that attracts chaperones and proteasomes in order to either refold or degrade misfolded aggregated proteins. This type of deposition site, which contains ubiquitylated misfolded proteins, was termed "*JU*xta *N*uclear *Q*uality control compartment" (JUNQ) and is a dynamic structure that rapidly exchanges proteins with the cytosol. The other aggregate deposition site, located near cytosolic vacuoles, was termed "insoluble protein deposit" (IPOD), and sequesters immobile substrates that have no ubiquitin signal. The interrelationships of aggresomes, JUNQ, and IPOD to each other and to the UPS and macroautophagy in mammalian cells remain to be established.
- Chaperone mediated autophagy (CMA)—In recent years, while macroautophagy has received the bulk of attention as an important cellular QC pathway, a second highly selective, but lower capacity pathway has emerged which can contribute to degradation of intracellular proteins in lysosomes. CMA substrate proteins are selectively targeted to lysosomes and translocated into the lysosomal lumen through the coordinated action of chaperones located at both sides of the membrane and a dedicated protein translocation complex (reviewed in Ref. [104]). The selectivity of CMA permits timed degradation of specific proteins supporting a regulatory role for CMA in enzymatic metabolic processes and subsets of cellular transcriptional programs. In addition, CMA contributes to cellular QC through the selective removal of damaged or malfunctioning proteins. To date, CMA events or their contribution to distal lung epithelia QC has not been reported.

Quality Control for Dysfunctional Organelles: The Example of Mitophagy—In addition to the utilization of macroautophagy for selective removal of polyubiquitinated protein aggregates (aggrephagy), Atg can target and remove specific subcellular components (selective Atg) 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 photo-irradiation, this and only this mitochondrion will be degraded [105]. In addition, global disruption of mitochondrial function in cells treated using an uncoupler such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) 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. [106,107]). 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 ($\Delta\psi$), 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 Atg machinery commissioned for misfolded protein removal. For example, the ubiquitin-binding adaptor p62/SQSTM1, 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.

ER Stress, Aggregation, and Apoptosis: Consequences of Failed QC—If the integrated proteostatic program (combined efforts of UPR, ERAD, and Atg) fails to control mutant protein levels and/or dysfunctional organelles are not removed by processes such as mitophagy, a series of deleterious consequences for the cell can ensue which can include any or all of 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 UPR driven "alarm signals" that overlap with some of the same signaling transduction cascades associated with innate immunity [108,109]. Termed "ER stress", UPR sensors (particularly IRE1) can then activate downstream both MAP kinases such as c-Jun N-terminal kinase (JNK) as well as NF-kB to promote the elaboration of proinflammatory cytokines, chemokines, and growth factors from disaffected cells (Fig. 8.6) [110,111]. The proof of principle that a balance between adaptive UPR/alarm ER stress signals 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 [112,113].
2. Intrinsic Apoptosis—In mammalian cells, ER stress signaling can trigger an internally mediated programed cell death event that is death receptor (Fas/FasL) independent [108,114]. Termed "intrinsic apoptosis," this has been shown to occur via a variety of signaling cascades. One well-known pathway involves the cleavage and activation of the ER-membrane-localized caspase-4/12 [115]. In a second, the protein complex generated by IRE1 binding with TNF-receptor-associated factor 2 (TRAF2) recruited to the ER membrane signals downstream effectors such as JNK [116]. In some systems, a PERK driven pathway mediated via the transcription factor ATF4 and/or an IRE1-based cascade mediated through XBP-1 can result in the upregulation of C/EBP-homologous protein (CHOP), a transcriptional repressor of expression of prosurvival Bcl2 proteins. In addition, an ER-mitochondria death-signaling cascade mediated through a Cytochrome *c*/apoptotic proteasome activating factor 1(APAF1)/caspase 9 apoptsosme mechanism has been described in a variety of model systems [117]. Further downstream integrative death signaling can involve activated caspase cascades that vary among cell types, by pathway and by inciting 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. [118]). Similarly, amyloid-beta (A-beta) also induces apoptosis via activation of caspases 4/12 and then caspase 3 in neuroblastoma cell lines [119] however neurons expressing misfolded polyQ72 huntingtin, undergo caspase-8-mediated apoptotic events [120]. As will be detailed below, expression of aggregation prone (misfolded) mutant isoforms of *SFTPC* mutants has been shown to activate many of these same pathways in lung epithelial cell lines.
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 histopathologic appearance of inclusion bodies, amyloid fibrils, and protein macroaggregates in the cytosol or within the nucleus [74,121–123]. As will be discussed in detail subsequently, given the propensity of SP-$C_{3.7}$ to form β-amyloid [22], it is not surprising that expression of some *SFTPC* mutations results in generation of SDS insoluble proSP-C macroaggregates in vitro [124,125] as well as intracellular amyloid formation in vitro and in vivo [25].
4. Mitochondrial Dysfunction—In addition to extrinsic injuries, endogenous ER stress from disrupted proteostasis can induce mitochondrial dysfunction through both physical and functional communications between mitochondria and the ER network. The accumulation of uncoupled/damaged mitochondria ± changes in cellular ATP levels can further exacerbate apoptotic events (reviewed in Ref. [126]).

Aging, Cellular Quality Control, and Parenchymal Lung Disease—The effects of aging on cellular QC capacity have been well documented, particularly in the CNS where perturbations to proteostasis and mitochondrial function are hallmarks of age-related neurodegenerative disorders [127]. 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. [128]). Aging has been shown to markedly down regulate autophagic activity [129]. Given their key role in processing cellular constituents, either to the lysosome for degradation or to other cellular compartments for immune activation, Atg, and the UPS represent important cytoprotective strategies against aging, especially for postmitotic cells. As proof of concept, expansion of either proteasome activity or Atg in the CNS extends lifespan and protects organisms from symptoms of proteotoxic stress [130].

Aging is a known risk factor for IPF [44]. 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. [131]). Telomerase mutations have

been described in FPF cohorts but also have appeared in some populations of patients with chronic obstructive pulmonary disease (COPD) [132,133]. 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 [134].

In addition to these pathways, several lines of evidence link age-dependent alterations in cell QC, AT2 cell function, and susceptibility to fibrosis. Aged mice are susceptible to fibrosis from herpes virus infection with increased expression of ER stress markers [135]. In a preliminary report, lung tissue from aged mice displays decreased proteasomal activity and alterations in 26S proteasome assembly [136]. Recently, Bueno et al. 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 [67]. This was associated with both an up regulation of ER stress markers and a down regulation 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 [67]. Interestingly, in a transgenic mouse model of atg5 deficiency, a 90% decrease in Atg 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 [137]. Collectively, these data, suggest that aging has marked effects on both the proteostasis network and Atg-mediated processes 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 QC.

8.5 EPITHELIAL DYSFUNCTION IS INDUCED BY SURFACTANT PROTEIN C MUTATIONS

The three ILD associated components of the surfactant system (SP-C, SP-A, and ABCA3) opportunistically provide a series of unique and 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 pulmonary fibrosis.

SFTPC mutants, being the first group reported, represent a starting point with which to explore the role of AT2 dysfunction in both FPF and sporadic IPF. Since the initial description of two clinical *SFTPC* mutants, SP-C$^{\Delta Exon4}$ and SP-C^{L188Q} in the early part of this century [61,138], over 60 additional mutations have been found in association with parenchymal lung disease. These have been localized to all four domains of the SP-C propeptide (Fig. 8.7). We have previously provided a classification scheme grouped on the basis of their functional behavior and domain location within the proSP-C sequence (summarized in Table 8.1) [139]. The QC responses along with other adaptive and maladaptive changes revealed by their expression produce two major cell endophenotypes:

1. *SFTPC BRICHOS Mutations: Aggresomes, ER Stress, Cytokines, and Apoptosis*—The Group A_1 mutations appear to be prone to misfolding and tend to cluster around the conserved cysteine residues (cys^{121}/cys^{189}) in the SP-C BRICHOS domain. As illustrated in Fig. 8.8, the following features emerge in SFTPC BRICHOS expressing cells:

   Aggresome Formation—When either of the prototypical SP-C$^{\Delta Exon4}$ and SP-C^{L188Q} 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, are not transported anterograde to LB, and demonstrate a high propensity to form perinuclear intracellular aggregates. Laboratory-based alanine mutagenesis of either or both cysteines produces aggregation prone isoforms that colocalize with components of the MTOC consistent with their sequestration within "aggresomes" whose formation can be blocked with microtubule inhibitors, exacerbated by proteasome inhibitors, and rescued by the chemical chaperone 4-phenylbutyric acid (4-PBA) [20,140]. Aggresome formation has also observed within histological samples from some clinical BRICHOS mutant patients [141].

   Additional mutations in the more proximal BRICHOS domain termed Group A_2 (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 [142,143].

   The sole Group C mutant reported to date (P30L) is also ER retained and polyubiquitinated and thus presumably also a substrate for ERAD) [11,143]. To date, whether this isoform is also aggregate prone or can activate the UPR has not been reported.

   ER Stress—Using a variety of in vitro expression models, we and others have shown that misfolded BRICHOS conformers (both SP-C$^{\Delta Exon4}$ and SP-C^{L188Q}) are capable of activating one of more of the three UPR sensors, IRE1/XBP1, ATF6, and PERK/eIF2α in both cell lines and primary rodent AT2 cells [124,125,144,145].

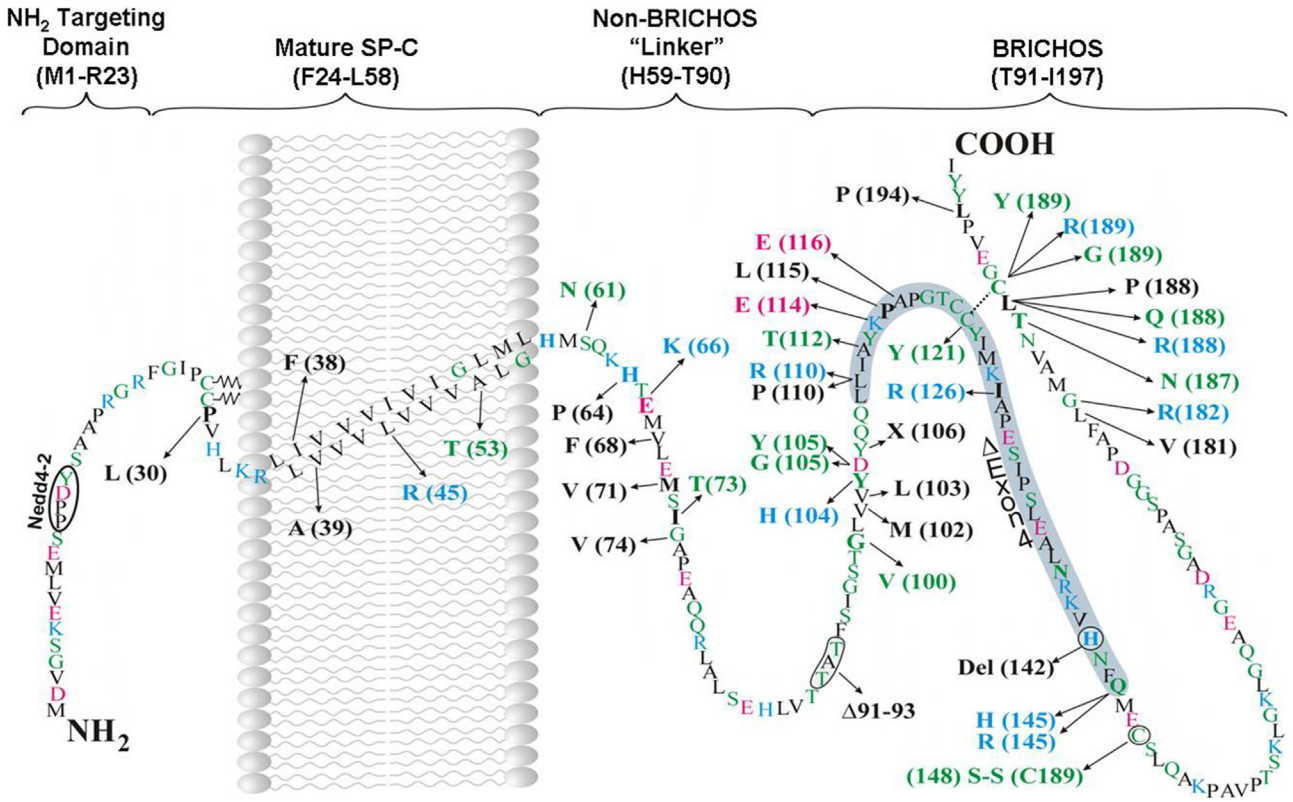

FIGURE 8.7 **Spatial distribution of published mutations within the human proSP-C primary translation product associated with interstitial lung disease.** The orientation of proSP-C as a bitopic integral transmembrane protein within the ER membrane is represented. The majority of disease associated SP-C mutations (Group A) map to the BRICHOS domain of the COOH flanking propeptide (Residues 91−197). Additional mutations in the COOH propeptide (Group B) are found in the "Linker Domain H^{59}-T^{93}. To date only one mutation (hSP-C^{P30L}) is found in the NH2 targeting domain located on the cytosolic side of proSP-C. Rare missense substitutions are also noted in the mature SP-C domain encompassing residues F^{23}-L^{58}.

The ultimate failure of the UPR to correct mutant protein load and alleviate ER stress appears to induce a series of downstream events which then contribute to AT2 cell dysfunction and promote aberrant injury/repair responses.

Generation of 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 (NFκB) [145]. Stably transfected cell lines expressing SP-$C^{\Delta Exon4}$ appear to adapt to chronic ER stress via an NF-kB -dependent pathway and suggests this may be a prosurvival response, but one capable of both elaborating additional inflammatory mediators and of being exploited by second hits such as viral infections [159].

Apoptosis—Given the role of AT2 cells as progenitors [1], their depletion poses a threat to the overall capacity of the lung for repair/regeneration. ER stress induced by *SFTPC* folding mutants can induce intrinsic apoptotic death via induction of numerous pathways including caspase 4 activation, mitochondrial cytochrome *c* release, c-Jun N-terminal kinase (JNK) signaling, and caspase 3 activation [124,125,144,145]. Interestingly, apoptosis induced by pharmacologic proteasome inhibition or overexpression of an SP-C BRICHOS mutant can be significantly inhibited by angiotensin receptor blockade (saralasin) or the Angiotensin II counteregulatory peptide Ang1-7 showing that ER stress-induced "intrinsic" apoptosis in human AECs may involve autocrine pathways such as the ANGII/ANG1-7 system [160].

Alterations in Cell Phenotype—Although somewhat controversial as to its 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

TABLE 8.1 Classification of Clinical Surfactant Protein C Mutations

Mutation (Domain)	Subcellular Localization/Trafficking	Cellular Responses (In Vitro)	Lung Phenotype (In Vivo)	Clinical Diagnosis	References
SFTPC					
Group A1					
ΔExon4 (BRICHOS) **L188Q** (BRICHOS) **G100S** (BRICHOS)	ER retention → Aggresomes Intracellular aggregates ERAD requires Erdj 4/5 MG132 Blocks Degradation 4-PBA improves aggregates	(+) ER stress (+) Apoptosis (+) Incomplete or absent proSP-C processing (+) IL-8 expression (+) Polyubiquitinated isoforms	Absence of mature SP-C (humans) Arrested Lung Development (mice) ER stress (humans; mice) ↑Sensitivity to bleomycin (mice) Epithelial cytotoxicity	NSIP (Children) IPF/UIP (Adult)	[61,62,124,125, 138,140–149]
Group A2					
L110R (BRICHOS) **P115L** (BRICHOS) **A116D** (BRICHOS)	EEA-1 (+); Syntaxin2 (–) Intracellular aggregation	↓ PC secretion (+) Aberrant processing ↓ cell viability ↑ HSP response (+) Congo Red aggregates	Phenotype not reported	Unspecified ILD Unspecified ILD Unspecified chILD	[142,143]
Group B1					
E66K (Linker) **I73T** (Linker)	Plasma membrane → EE → LE/MVB	(+) Aberrantly processed protein (+) Late autophagy block ↓ mitophagy ↑ dysfunctional mitochondria	↑ Phospholipid; ↑SP-A, PAS positive staining Biopsy: PM and EE localization Misprocessed SP-C (BAL) Misprocessed SP-B (BAL)	NSIP/PAP (Child) IPF/UIP (Adult)	[62,150–157]
Group B2					
Δ91–93 (Non-BRICHOS)	Plasma membrane[$] EEA1 (+) compartments[$]	Not reported	↓ BAL SP-B ↑ BAL SP-A, ↓ surfactant surface tension (+) intracellular aggregates (+) Congo red staining	NSIP/PAP	[26,158]
Group C					
P30L (N-terminal)	(+) ER Retention	↑ Bip expression (+) Polyubiquitinated isoforms	Phenotype not reported	Unspecified ILD	[11,62,143]

Abbreviations: NSIP, nonspecific interstitial pneumonia; *PAP*, pulmonary alveolar proteinosis; *UIP*, usual interstitial pneumonia; *IPF*, idiopathic pulmonary fibrosis; chILD, interstitial lung disease of childhood; *RDS*, respiratory distress syndrome of newborn; *CPI*, chronic pneumonitis of infancy; *4-PBA*, 4-phenylbutyric acid; *PC*, phosphatidyl choline; *LRO*, lysosome-related organelle; *EEA1*, early endosomal antigen-1; *HSP*, heat shock response.

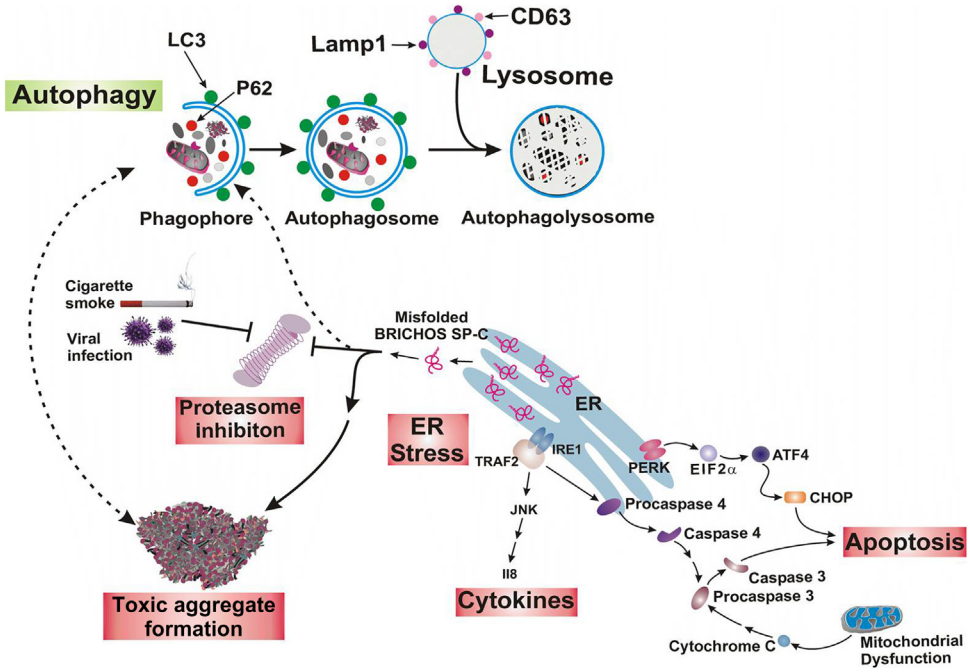

FIGURE 8.8 Pathways to generating an AT2 cell at risk by expression of SFTPC BRICHOS mutants. Misfolded BRICHOS proteins in which ER QC attempts fail at refolding are initially targeted for retrograde translocation, ubiquitinated, and directed to the 26S proteasome for degradation. However proteostasis perturbations occur when several or all events occur: (i) Defective UPS from direct toxicity of mutant conformers; this can be exacerbated by second hits such as cigarette smoke and viruses or use of proteasome inhibitors; (ii) Synthetic capacity exceeds ERAD. This results in secondary QC events in which microaggregates of protein could be directed to macroautophagy and/or aggresomal compartmentalization (not shown). Currently, the exact contribution of macroautophagy to proteostasis in AT2 cells is undefined.
The formation of toxic macroaggregates and/or the induction of ER stress marks the state where the UPR and other QC pathways can no longer maintain proteostasis leading to a cascade of cellular disruption and injury that includes: (1) ER stress-dependent inflammatory signaling by mediated by recruitment of TRAF2 by IRE1 to activate JNK that promotes upregulation and release of cytokines; (2) Induction of intrinsic apoptotic pathways by ER stress-induced activation of either the PERK/EIF2α/ATF4 network to trigger C/EBP homologous protein (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. For additional details, see text.

by ER stress induced either pharmacologically or using *SFTPC* mutants has been shown. In primary rodent AT2 cells, Zhong et al. 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-C$^{\Delta Exon4}$ in this model largely recapitulated these effects. Tanjore et al. reported similar findings following induction of ER stress by overexpression of the SP-C^{L188Q} mutant and identified SMAD2/3 signaling as a mediator of these events [161]. Significantly, both studies demonstrated a role for SRC family kinases and suggest that therapies based on mitigating ER stress may have pluripotent benefits.

2. *SFTPC Trafficking Mutations: A Phenotype of 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 [150,151,158]. 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 nonspecific interstitial pneumonia (NSIP) [151]. Interestingly, the identical SP-C^{I73T} mutation (both inherited and sporadic) has reappeared in several other published reports of parenchymal lung disease in chILD and adults and is now felt to be the most common *SFTPC* mutation [62,152]. Collectively, the Group B mutations (E66K; I73T; Δ91−93) [150,158] all reside within the linker domain of *SFTPC* (Fig. 8.7). Although presentation and course can vary [153,154], overlapping features that emerge include interstitial lung remodeling with intraalveolar accumulation of surfactant (lipids, SP-A, and SP-B), the presence of aberrantly processed proSP-C, and abnormal AT2 cell cytoplasmic inclusions on EM suggesting abnormalities in surfactant homeostasis [27,150,151,162].

The cellular phenotype induced by Group B *SFTPC* mutants is also distinct from that of Group A_1 (Table 8.1). Functionally, Group B *SFTPC* mutants have been defined by in-vitro expression studies (predominantly utilizing

SP-C^{I73T} as the model substrate) demonstrating a unique and markedly aberrant intracellular trafficking pattern for proSP-C first directly to the plasma membrane with subsequent internalization and accumulation within EE and LE/MVB [28,140] (Fig. 8.9A). 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 EE of AT2 cells [151]. Recently, two separate preliminary studies using knock-in 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 [155,163].

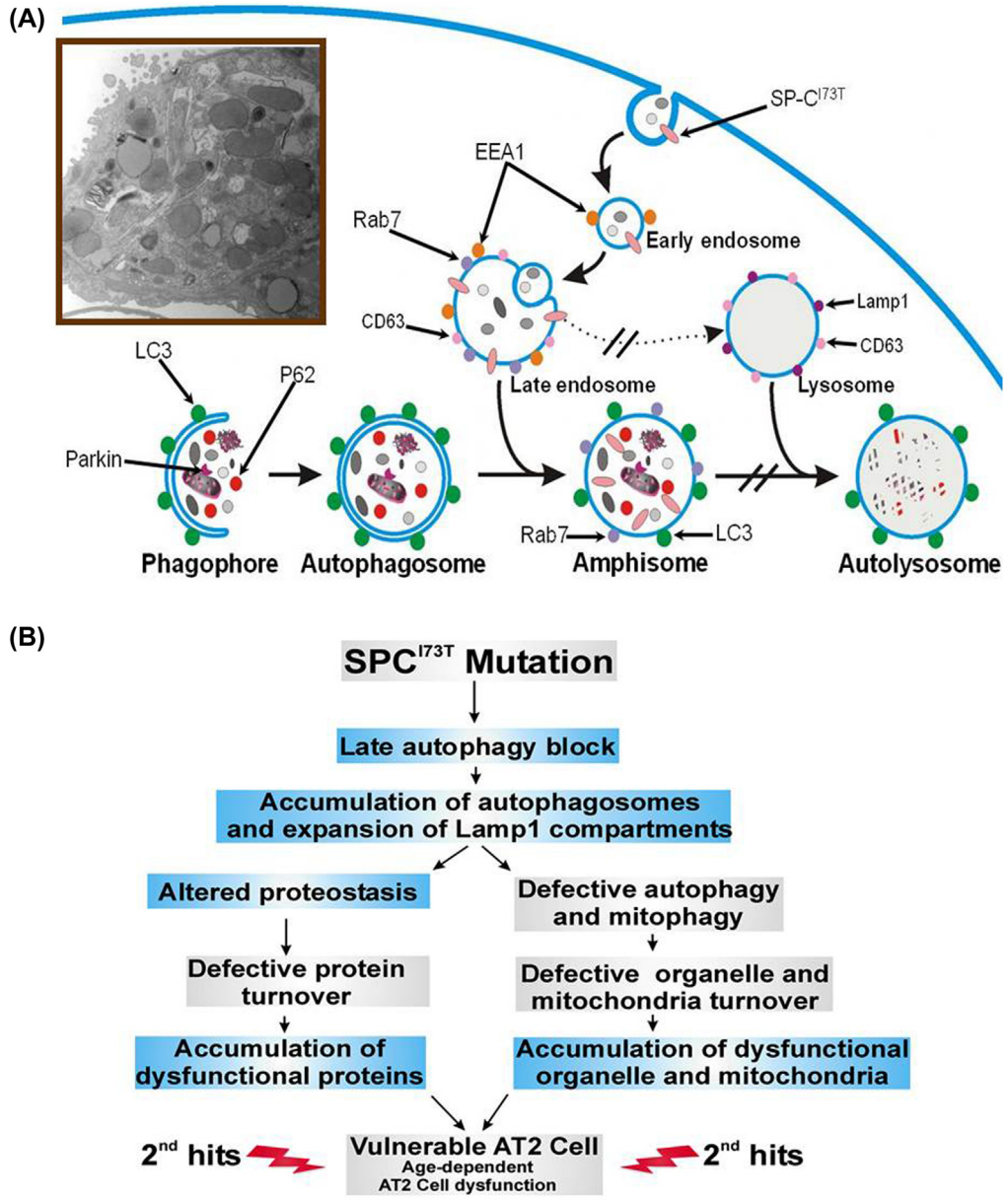

FIGURE 8.9 Pathways to AT2 cell injury/dysfunction induced by non-BrRICHOS mutant SFTPC isoforms. (A) Linker domain (non-BRICHOS) proSP-C mutations (Group B) residing in the COOH terminus outside BRICHOS domain of proSP-C reported to date are initially mistargeted to the plasma membrane via a default constitutive pathway. Because of a failure to be processed, proSP-C in the plasma membrane (PM) is subjected to re-internalization and trafficked through early endosomes to 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 as manifested by vesicle populations enriched in non-degraded autophagy proteins including LC3 and P62, as well as Parkin, a protein critical for autophagy-dependent removal of dysfunctional mitochondria (i.e., mitophagy). INSET: TEM showing the consequences of such a block marked by accumulation of abnormally large autophagic vacuoles containing non-degraded organellar and proteinaceous debris. (B) Proposed scheme for cellular dysfunction and creation of an epithelium at risk from SP-C^{I73T} expression in AT2 cells.

The link between this mistrafficking behavior and cellular dysfunction is novel and distinct from SP-C BRICHOS mutants as it does which does not involve ER stress [125]. Using cell lines stably expressing hSP-C^{I73T}, we have shown that hSP-C^{I73T} induces a novel QC phenotype punctuated by a striking disruption of macroautophagy characterized by the accumulation of dysmorphic, autophagic vacuoles containing organellar and proteinaceous debris [27] (schematically shown in Fig. 8.9). 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 [27]. Biochemically, hSP-C^{I73T} cells exhibited increased expression of Atg8/LC3, SQSTM1/p62, and RAB7 consistent with a late block in autophagic vacuole maturation which was confirmed by Atg flux studies. The observed disrupted Atg 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 Atg-dependent proteostasis and mitophagy induced by expression of SP-C^{I73T} is reminiscent of cellular QC phenotypes observed in organellar storage disorders such as Niemann–Pick disease [78,164] which has been associated with a premature development of fibrotic lung disease.

8.5.1 Aberrant Cellular Responses to Other Surfactant Component Substrates

The cellular phenotypes described above are not idiosyncratic to *SFTPC* mutants as 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 et al. using genome wide linkage scanning, reported on two kindreds with pulmonary fibrosis and lung adenocarcinomas to 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.

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 [37,165]. Designated as "Type I" (see Ref. [139]), this mutant subgroup can enhance expression of Bip, increase XBP1 splicing, and induce apoptosis in vitro [166]. Recent work with both the Type I clinical *ABCA3* mutants as well as laboratory generated glycosylation deficient constructs suggest that these misfolded isoforms are substrates for ERAD [42,166] and Atg (S. Mulugeta, unpublished observation).

Taken in total, data generated using mutant surfactant component substrates are highly supportive of a common model of cellular dysfunction in association with altered QC.

8.5.2 AT2 Quality Control Responses to Surfactant Component Mutants

An important concept, especially in the context of QC issues discussed above, is in that the cellular pathology and molecular signatures of ER stress, apoptosis, cytokine generation induced by various *SFTPC* genotypes must invariably result from of an imbalance in or failure of the cellular QC network (ERAD, macroautophagy, translation attenuation, mitophagy, etc.) to accommodate the offending protein conformers and dysfunctional organelles. Very little is currently known about the actual QC repertoire of AT2 cells. Using microarray analyses, Dong et al. identified genes specifically induced in response to expression of SP-$C^{\Delta Exon4}$ and SP-C^{L188Q} and showed that two BiP cochaperones, endoplasmic reticulum localized DnaJ homologues ERdj4 and ERdj5, selectively bind to mutant but not wild-type proSP-C or to non-BRICHOS SP-C^{I73T} and were required for successful retrotranslocation and proteasomal degradation of BRICHOS mutant SP-C substrates [146]. It is presently unclear why Group B (Linker domain) mutant *SFTPC* proteins evade recognition by AT2 QC and traffic abnormally to distal compartments.

The failure to control levels of mutant surfactant component substrates is not due to a lack UPS activity or autophagic repertoires. In a preliminary report, we have shown that while primary human AT2 cells in culture exhibit a high level of proteasomal activity and demonstrate the capacity to increase macroautophagy above basal levels in response to cardinal stimuli (starvation, mTOR inhibition, proteasome inhibition), these cells appear to primarily rely upon ERAD for initial control of either misfolded *SFTPC* conformers or in a more general manner, of other substrates such as polyQ huntingin [167]. Similarly, Maitra et al. have shown that primary mouse AT2 cells in vitro rely almost exclusively on MG132 sensitive proteasomal activity but not 3-methyladenine inhibitable Atg to clear ER retained SP-A2 mutants [168]. Therefore, while not appearing to have a primary role in basal proteostasis, the function of Atg either as a back-up to ERAD and/or in the QC of dysfunctional AT2 organelles such as mitochondria, LB remains an important area for further investigation.

8.6 LINKING EPITHELIAL DYSFUNCTION IN VITRO TO FIBROTIC REMODELING IN VIVO

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 expressing various degrees of a variety of SFTPC mutant isoforms. Bridges et al. reported that transgenic mice constitutively expressing SP-C ^{exon4} exhibit a gene dose-dependent arrest of lung morphogenesis, show induction of Bip expression, and display markers of aberrant proSP-C processing [147]. More recently, low level, conditional expression of a different BRICHOS mutant, SP-C^{L188Q}, in AT2 cells of adult mice at levels approximately 10−20% of that of *sftpc* endogenous gene expression produced ER stress [169]. 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 [169].

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 et al. demonstrated evidence of UPR activation markers (including BiP, EDEM, and XBP1) in FPF patients expressing SFTPC^{L188Q} mutations as well as in samples from both one non-*SFTPC* FPF patients, and in sporadic cases of IPF [68]. Korfei 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 pathologic features of UIP [65]. Thus, chronic ER stress in the alveolar epithelium likely represents a broad mechanism for the pathogenesis of ILD [170].

A role for failed autophagy in IPF—In translational studies using lung tissue samples from IPF patients, two separate groups have provided evidence of impaired Atg as defined by increases in LC3, p62, and polyubiquitinated protein expression and decreased numbers of autophagosomes [66,171]. These findings, when combined with the aforementioned work on AT2 QC and aging [67], emphasize the potential importance of better understanding in detail the full repertoire of AT2 QC pathways as well as the implications of impaired Atg, mitophagy, and mitochondrial dysregulation in contributing to a dysfunction lung epithelial phenotype to parenchymal lung diseases such as IPF.

Beyond ER Stress and Autophagy: Dominant Negative or Loss-of-Function Effects—Patients heterozygous for the SFTPC$^{\Delta Exon4}$ mutation do not express mature SP-C [61]. 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 dominant negatives to direct wild-type proSP-C away from normal routing and into aggregates [172]. 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 [173] but exhibit enhanced sensitivity to bleomycin fibrosis [174] and RSV infection [175]. However, when backcrossed onto a different strain (129/Sv), the absence of SP-C is associated with spontaneous inflammation and lung remodeling [176] suggesting that SP-C deficiency is a potential disease modifying factor. Significantly, 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 [151,177].

In a related fashion, when expressed in primary rodent AT2 cells, the disease causing human *SPA2* mutants are not secreted [168]. 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) [63]. While 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, homozygous expression of functionally impaired *ABCA3* mutations (designated Type II mutations see Ref. [139]) have been associated with in severe loss-of-function phenotypes [37,165,178]. Affected patients as well as four separate lines of *ABCA3* null mice with constitutive absence of protein expression demonstrate deformed LBs, and die from respiratory distress shortly after birth [38,179−183] indicating that a critical role of this protein in production of pulmonary surfactant dominates the early lung phenotype during transition to air breathing.

Interestingly, hypomorphic expression or delayed downregulation of ABCA3 expression results in more chronic forms of lung disease. A mouse model of conditional *ABCA3* deletion in AT2 cells, resulting in partial survival and

emphysema in adulthood, demonstrated its role in surfactant homoeostasis modulation beyond the neonatal period [184]. Similarly, 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 [156]. 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 [185], not surprising given their overlapping biology within the AT2 cell.

Environmental Factors: Second Hits Affect For Lung Quality Control and Remodeling—Substantial clinical variability in the age of onset and disease severity in both surfactant mutation associated (SP-C, ABCA3, and 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 QC 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 CSs [186,187] and cigarette smoking is one of the strongest associated risk factor for the development of DPLD [188]. CS exposure induces ER stress and proteasomal dysfunction in alveolar epithelial cell lines in vitro [88,189] and signatures of UPR activation have been detected in the smoke exposed human lung in vivo [190].

Similarly, emerging evidence also implicates human herpes viruses (including herpesvirus-8; Epstein-Barr virus; and 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 [68,191–194]. 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 [135]. In human IPF, CMV antigens and the UPR transcription factor XBP-1 were colocalized in alveolar epithelial cells from an individual with UIP [68]. More recently, sampling of asymptomatic relatives of patients with familial IPF showed evidence of herpes virus infection and ER stress [195].

In a recent clinical study, IPF exacerbations were significantly associated with antecedent 6-week increases in air pollution exposures of ozone and nitrogen dioxide suggesting that air pollution may contribute to the development of this clinically meaningful event [68,196]. Although mechanism was not assessed, given the oxidative–nitrative stress applied by these agents, it seems likely that additional proteotoxic and mitochondrial stress to the QC network in the distal lung from the enhanced generation of "challenged substrates" by these exposures may be contributing factors.

8.7 QUALITY CONTROL ISSUES IN OTHER PARENCHYMAL LUNG DISEASES

In addition to IPF, FPF, and some forms of chILD, it is becoming clear that disruption of epithelial cellular QC likely plays a broader role in the development of other fibrotic, environmental, and obstructive (destructive) parenchymal lung diseases.

Asbestosis—Asbestos exposure results in pulmonary fibrosis (asbestosis) whose histology resembles that of UIP by mechanisms that are not fully understood. AEC apoptosis and mitochondrial abnormalities have been implicated in the pathogenesis of pulmonary fibrosis after exposure to an array of toxins, including bleomycin and asbestos. In addition, it is well-known that asbestos exposure can induce oxidative stress and mitochondrial DNA damage [197]. While a rodent model of chrysotile-induced asbestosis used transmission electron microscopy to document abnormalities in both AT2 ER morphology and cytosolic inclusion bodies [198], the interplay between AT2 QC, its disruption, and apoptosis in the development of asbestosis is incompletely defined. Recently, Kamp et al. demonstrated that amosite asbestos fiber exposure of human A549 and rat primary AT2 cells increased ER stress component expression including IRE1 and XBP-1 as well as promoted ER Ca^{2+} release [199]. These events which were attenuated by both Eukarion-134, a superoxide dismutase/catalase mimetic or overexpression of Bcl-xL and suggest that asbestos triggers an AT2 ER stress response and subsequent intrinsic apoptosis that is mediated in part by ER Ca^{2+} release. Additional translational studies assessing these pathways in samples from patients with asbestosis will still be required.

Hermansky–Pudlak-Syndrome Related Fibrosis—Known as HPS-associated interstitial pneumonia (HPSIP), this complication invariably develops in the fourth–fifth decade of life in patients harboring mutations in HPS1, HPS2, or HPS4 genes [200–202] and is a leading cause of death in these patients. Histologically, the lungs of patients with HPSIP are typically characterized by an increase in size and number of LBs in AT2 cells [55]. Giant LBs are also observed within the AT2 cells of *HPS1/2* double-mutant mice [203], which develop age related spontaneous lung fibrosis associated with elevated markers of AT2 ER stress (CHOP) and apoptosis [204]. In addition, a role for intracellular trafficking defects in HPS AT2 cells as a driver of AT2 apoptosis and fibrosis has been supported by data from Young et al. used bone-marrow chimeras to show an increased sensitivity to bleomycin-induced pulmonary

fibrosis in HPS1 or HPS2 null mice that was mediated by caspase dependent apoptosis in AT2 cells [57]. Transgenic epithelial-specific correction of the *HPS* defect significantly attenuated these findings thus linking AT2 trafficking defects to epithelial dysfunction and fibrosis. The role of defective AT2 cell QC in the HPS lung phenotype has been further supported by recent findings that key Atg proteins, including LC3-II and p62, are increased in HPS1/2 mice lungs, that in vitro knockdown of *HPS1* results in increased LC3II and p62 accumulation in association with an increase in proapoptotic caspases, and that these events could be attenuated by overexpression of LC3-II [205]. Taken together, the data to date suggest that defective AT2 QC plays a critical role in parenchymal lung remodeling in *HPS*.

Cigarette smoke exposure and COPD—COPD is a disease of increasing prevalence in aging populations, characterized by progressive destruction of lung parenchyma and chronic inflammation of the airways and whose pathogenesis remains incompletely defined. CS, which exposes the lung to high concentrations of reactive oxidant species (ROS), is the major environmental risk factor for COPD and oxidative stress-induced apoptosis occurs in alveolar cells in models of emphysematous lung destruction. CS appears to have a major impact of lung epithelial QC. In vitro, CS exposure is known to induce proteostasis imbalance through impairment of both epithelial proteasomal activity [88,206] and possibly macroautophagy [207,208]. Molecular signatures of this proteostatic in balance have been detected both in vitro and in vivo. Aggresome formation, increases in the level of polyubiquitinated protein isoforms and p62, and enhanced ER stress pathways have all been reported [190,209,210]. Interestingly, treatment of chronically CS exposed mice with the Atg activator carbamezpine can abrogate aggresome formation and attenuate airspace destruction.

8.8 UNANSWERED QUESTIONS, NEW APPROACHES, AND OPPORTUNITIES

Over the past two decades much progress has been made in understanding the pathogenesis of IPF. There is now substantial evidence to show that the AT2 epithelium is a pivotal regulator of lung injury, inflammation and repair. Although *SFTPC, SFTPA*, and *ABCA3* mutations are rare (2%−20% in most ILD cohorts) [211,212], understanding the spectrum of cellular phenotypes, clinical endophenotypes, and the molecular mechanisms underlying the genesis of the AT2 cell dysfunction caused by these mutations adds value to understanding the broader mechanisms that underlie the pathogenesis of sporadic IPF, chILD, and potentially other parenchymal lung diseases.

The body of literature generated utilizing surfactant protein mutations as an investigative platform has revealed the role for both ER stress and alterations in cellular QC pathways in epithelial cells in IPF pathogenesis (Fig. 8.10). 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 further 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 current data 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. In addition, the well-known associations of telomerase mutations with familial IPF [132], which can also be thought of as a "QC 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 [213,214].

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 [53,215,216]. On the basis of the preclinical discovery and clinical trials to date, it seems likely that future therapeutic options for IPF should involve directed therapies aimed at engaging multiple targets to mitigate pathways in all four phases of pathogenesis: epithelial cell dysfunction, inflammatory amplification, fibroblast activation, and matrix deposition. This approach is unlikely to be 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 in order to identify personalized therapies based on pathogenesis not histology. Finally, given the protracted temporal realities of drug development, it may be worth considering that the overlap of many of the QC pathways discussed above with those seen in other chronic degenerative diseases (Alzheimer's disease; Huntington's disease, cystic fibrosis, and α1-antitrypsin deficiency) could be leveraged for lung 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 devastating parenchymal lung diseases.

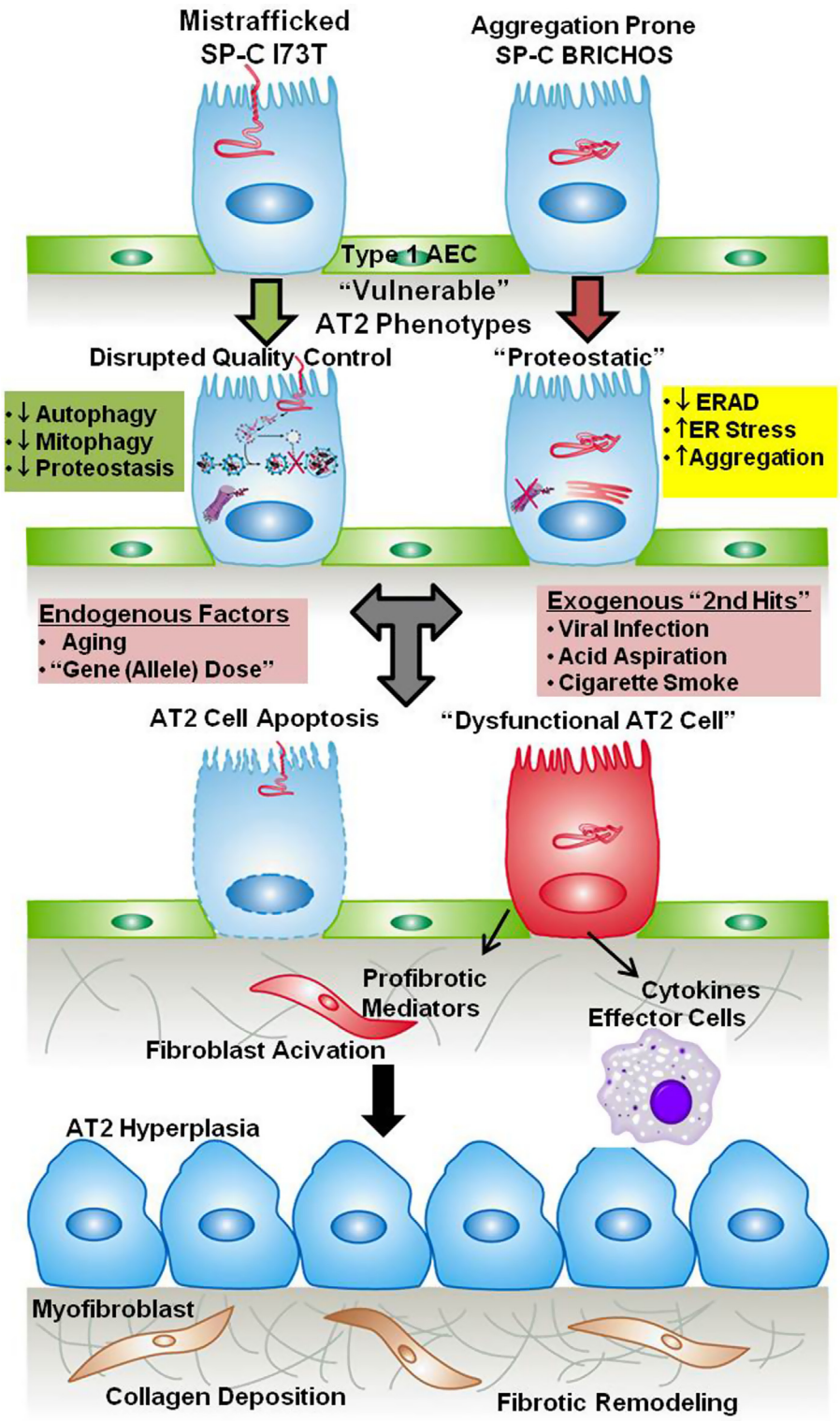

FIGURE 8.10 **Pathogenesis *SFTPC* mutation driven IPF**. Cells expressing these prototypical substrates in combination aging act through pathways that converge to produce a "vulnerable" alveolar epithelial cell (AT2) population. Exposure of vulnerable AT2s to secondary recurrent or persistent injurious stimuli leads to enhanced AT2 apoptosis, as well as aberrant activation of developmental programs and stress response pathways with recruitment of inflammatory cells (secondary amplification) and fibroblast modulation. Ongoing AT2 dysfunction impairs normal injury-response mechanisms, leading to scar formation and progressive loss of lung architectural complexity. Over time, this culminates in progressive fibrotic remodeling and clinically evident IPF.

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DISCLOSURES

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Chapter 9

The Respiratory Epithelium in COPD

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9.1 INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is defined as a preventable and treatable state characterized by progressive airflow limitation that is not fully reversible. This airflow limitation develops in part as a process of abnormal response of the lungs to noxious particles or gases [1]. Although the main culprit worldwide is cigarette smoke, other sources of air pollution, biomass fuel, and occupational exposures are also likely to contribute to a substantial burden of COPD [2]. In the early 2000s, COPD was the fifth leading cause of death globally; however, the prevalence is increasing secondary to smoking uptake and pollution in developing countries, and World Health Organization estimates predict that by 2030 COPD could be the third leading cause of death [3]. As the first line of exposure to the noxious particles and gases implicated in the pathophysiology of COPD, the respiratory epithelium plays a central role in the development of the disease. The characteristics of epithelial cell response, be it ordered or disordered, to these insults, leads to the remodeling of the airways and air spaces that are characteristic of COPD. Key to understanding the abnormal response of epithelial cells is to understand the role that the airways epithelium has in providing a protective barrier from the environment. The barrier functions fulfilled by the respiratory epithelium includes physical, chemical, and immunological roles. As we seek to demonstrate in this chapter, it is the breakdown in the regulation and control of these functions that is the key to the pathophysiology of COPD.

Although inextricably linked, in COPD there are distinct pathologic processes occurring in the airway and the alveoli. In the airways, a remodeling process, characterized as "excessive tissue repair" by Hogg and Timens [4], leads to an airway with an altered epithelial lining, airway wall tissue fibrosis, smooth muscle hypertrophy, and inflammatory cell infiltration [4]. In contrast, the process in the alveoli has been thought of as "insufficient tissue repair [4]" and is characterized by the development of emphysema; defined as "abnormal permanent enlargement of air spaces distal to terminal bronchioles, [and] accompanied by destruction of their walls without obvious fibrosis." [5]

In this chapter, we will describe the morphological changes that occur to the respiratory epithelium in COPD. We will then outline the response of the epithelial cell to inhaled noxious particles highlighting the important role of the epithelial cell as a physical, chemical, and immunologic barrier. Along the way, we will explore the molecular mechanisms implicated in the pathological changes involving and orchestrated by the respiratory epithelium.

9.2 AIRWAY REMODELING

In health, the bronchial mucosa is composed of a pseudostratified mucociliated epithelium. Depending on the location of the airway, the composition of cells making up this epithelial lining changes. In the larger airways, ciliated cells make up the majority of this epithelium and are accompanied by mucous secreting goblet cells and basal cells (thought to be the progenitor cell of the large airways). As the airways branch into more distal smaller generations, the pseudostratified epithelium changes into a simple cuboidal type. Goblet and basal cell numbers decline and are replaced by serous secreting cells and clara cells (thought to be the progenitor cells of the small airways) [6,7]. In COPD, both the morphology and function of this airways epithelium are altered. This involves both goblet cell hyperplasia

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(as defined by increase in number of this cell type) and metaplasia in which cells of a different phenotype (i.e., ciliated cell) transition into a secretory goblet cell. The epithelium is also metaplastic in other ways as seen by differentiation into mesenchymal and squamous cell-types [8]. These changes are accompanied by loss of effective ciliary functioning, fibrosis of the small airways, and an inflammatory infiltrate consisting of neutrophils, macrophages, and lymphocytes that is seen in the airway wall and accumulated in the airway lumen [4]. In the "chronic bronchitis" phenotype seen in COPD, the mucous cells are hyperplastic and hypersecretory leading to chronic cough and sputum production. Notably, this phenotype can be seen in the absence of significant airflow obstruction [4]. This may reflect the extent to which the increased mucous production is seen primarily in isolation in the larger airways, or accompanied by other processes involving small airways remodeling.

9.3 EMPHYSEMA

The changes seen in the alveoli in COPD are characterized by tissue destruction, with progressive loss of alveolar septae, the extracellular elastic matrix that gives the airspaces their shape and function, and the capillary endothelial cells that abut the airspaces creating a surface for gas exchange. In contrast to the pathology seen in the airways, emphysema occurs without obvious fibrosis [4,9]. In similarity to the airways, the changes in emphysema are also accompanied by an inflammatory infiltrate [9]. In terms of the distribution of emphysematous changes, there is a predilection for different parts of the lung architecture depending on the underlying process. In smoking-related diseases, centrilobular emphysema is the most common pathological correlate and has an apical predominance. This is in contrast to alpha-1-antitrypsin deficiency in which a panacinar pattern with basilar predominance is seen. Paraseptal emphysema may be seen in isolation in young healthy nonsmokers and may only become apparent after presentation with spontaneous pneumothorax [10]. *Much like the phenotypic/pathologic changes seen in "chronic bronchitis," emphysema alone does not necessarily indicate the presence of underlying airflow obstruction* [4]. Rather, the final expression of airflow obstruction in COPD is dependent on the interaction of a number of pathophysiologic processes including: mucous plugging, narrowing of the small airways (2 mm or less) secondary to remodeling, and the contribution of decreased ability of those airways to remain open without the elastic tethering forces from intact alveolar structure and elastic matrix [4,9]. Now that we have defined the morphologic changes that occur with COPD; we will focus on how these changes signify and in some cases lead to dysfunction of respiratory epithelium.

9.4 EPITHELIAL DYSFUNCTION IN COPD

To understand the development of COPD, one must consider the role of the epithelium in protecting the body from inhaled noxious particles and examine how breakdown in that function may lead to pathologic outcomes. There are number of ways in which epithelial cells fulfill their barrier function (see Table 9.1). The first and perhaps most intuitive is a physical barrier that involves the junctional proteins and ion channels that regulate epithelial permeability as well as the ciliated and mucus producing cells that create an effective mechanism for the clearance of noxious particles. A healthy epithelium requires both a properly functioning physical barrier, as well as an ability to restore proper barrier functions in response to injury. A chemical barrier is also present and in addition to the proteins that make up mucus, the epithelium secretes a number of other chemical products with antimicrobial and antioxidant properties. Finally, the epithelium serves as a central coordinator of the immune response with important functions in innate immunity and in stimulating and guiding an adaptive immune response.

9.4.1 Physical Barrier Dysfunction

At the epithelial level, barrier integrity is maintained by tight junctions (TJs), adherens junctions, and desmosomes [11]. In particular, it is the TJ and adherens junction that localize to the apico-lateral side of the polarized epithelial cell and closely regulate permeability. Together, they form a crucial protective barrier known as the apical junctional complex (AJC). The TJ closely regulates paracellular ion transport and the adherens junction promotes formation of the TJ and regulates cell-to-cell adhesion [12]. The TJ is composed of multiple proteins that interact with the intracellular actin network and include claudins, occludins, junctional adhesion molecules as well the zona occludens (ZO). The adherens junction is composed of transmembrane E-cadherin, which then interacts with intracellular β-catenin and α-catenin to connect with the cell microtubule and actin network [12,13]. The cadherin/β-catenin complex is also involved in cellular signaling pathways that are important for cell proliferation and differentiation and has a critical role in epithelial repair in response to injury. This cell signaling is controlled in part by intact cell polarity. Under conditions of normal

TABLE 9.1 Epithelial Dysfunction in COPD

	Description	Important Mediators
Physical Barrier Disruption		
"Leaky" intercellular junctions	Breakdown of tight junctions from altered epithelial specifications	CS, ROS, IL-1β, and PTEN
Mucociliary dysfunction	Goblet metaplasia; ciliary dysfunction, mucous hypersecretion, and altered mucous composition	TGF-β, IL-13, EGFR, CFTR, NF-κβ, IL-1β, IL-17A and TNF-α CS, ROS
Altered epithelial specification	Squamous cell metaplasia, epithelial-to-mesenchymal transition	TGF-β, EGFR, Wnt/β-catenin, and MMP-9
Chemical Barrier Dysfunction		
Altered secretion of innate antimicrobial particles	Decreased/altered lyoszyme, lactoferrin, defensins and LL-37	TLRs, CS, and ROS
Immune Dysfunction		
Mucosal IgA deficiency	Failure to transport IgA across epithelial barrier	pIgR
Immune activation via pattern recognition receptors	Chronic stimulation of PRR with PAMPs and DAMPS	CS, ROS, LPS, Acrolein, and NF-κβ
Cytokine/chemokine	Proinflammatory, COPD-specific profile	TNF-α, IL-1, IL-8, IL-6, GCSF, LTB4, and MCP-1

CS, cigarette smoke; *ROS*, reactive oxidative species; *PTEN*, Phosphatase and tensin homolog; *pIgR*, polymeric immunoglobulin receptor; *TGF*, transforming growth factor; *EGFR*, epidermal growth factor receptor; *CFTR*, cystic fibrosis transmembrane receptor; *TLR*, toll-like receptor; *ROS*, reactive oxidative species; *PAMP*, pathogen-associated molecular pattern; *DAMP*, damage-associated molecular patterns; *LPS*, lipopolysaccharide.

polarity and function, E-cadherin interacts with and retains epidermal growth factor (EGF) receptor (EGFR), localizing it to the basolateral side of the epithelium, and limiting its association with its epithelial ligand (EGF) [11,13]. The EGFR pathway is critical in COPD. In response to injury it is a stimulator of a number of important processes promoting mitosis, cell migration, and epithelial cell differentiation [17]. In COPD, where there may be conditions that lead to chronic injury, the central role of EGFR is supported by the observation that increased EGFR activity is a key mediator in many of the pathologic consequences seen in small airway disease and remodeling (see Fig. 9.1). This includes breakdown of barrier function, goblet cell hyperplasia/metaplasia, mucous hypersecretion, and epithelial-to-mesenchymal transition (EMT) [8,14–16]. The AJC is also involved in the Wnt/β-catenin pathway. When intracellular β-catenin is freed from association with transmembrane E-cadherin, it translocates to the nucleus and activates the canonical Wnt/β-catenin pathway to play a critical role in cell proliferation [16].

In response to cigarette smoke and specifically in the disease state COPD, there is evidence of an ongoing injury to the epithelium causing a breakdown of barrier function leading to a "leaky" epithelial barrier. This is an effect that has been shown both in vivo [17,18] and in vitro [18,19]. In addition to a direct cytotoxic effect of cigarette smoke [20], oxidative stress from smoking-related injury and chronic injury has been shown to disrupt epithelial TJs through protein modification, such as phosphorylation of TJ proteins [21]. There is also evidence of a more "targeted" effect of cigarette smoke-induced changes on epithelial intercellular junctions via an EGFR-dependent pathway. Cultured bronchial epithelial cells exposed to cigarette smoke showed disassembly of TJs and showed increased expression of downstream actors in the EGFR pathway as demonstrated by phosphorylation of the mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase (ERK) molecules [14]. In addition, in cells cultured both from subjects with COPD and healthy controls, a "leaky" barrier in response to cigarette smoke exposure was confirmed by showing a decrease in resistance across the epithelium. This effect could be rapidly reversed by EGFR inhibition, providing further evidence of a primary role for EGFR in regulating intercellular junctions. Interestingly, this effect, as well as delayed restoration of barrier function in wounded cells exposed to cigarette smoke, was not found to be different between those with COPD and healthy controls [22]. However, there is evidence of specific dysfunction of the epithelial barrier in those with COPD versus smokers without COPD. Gene mapping of epithelial cells obtained from healthy smokers and smokers with COPD, as compared to nonsmokers, showed marked downregulation of gene expression of critical components of the AJC. This downregulation was even more pronounced and consistent in the cells from COPD smokers

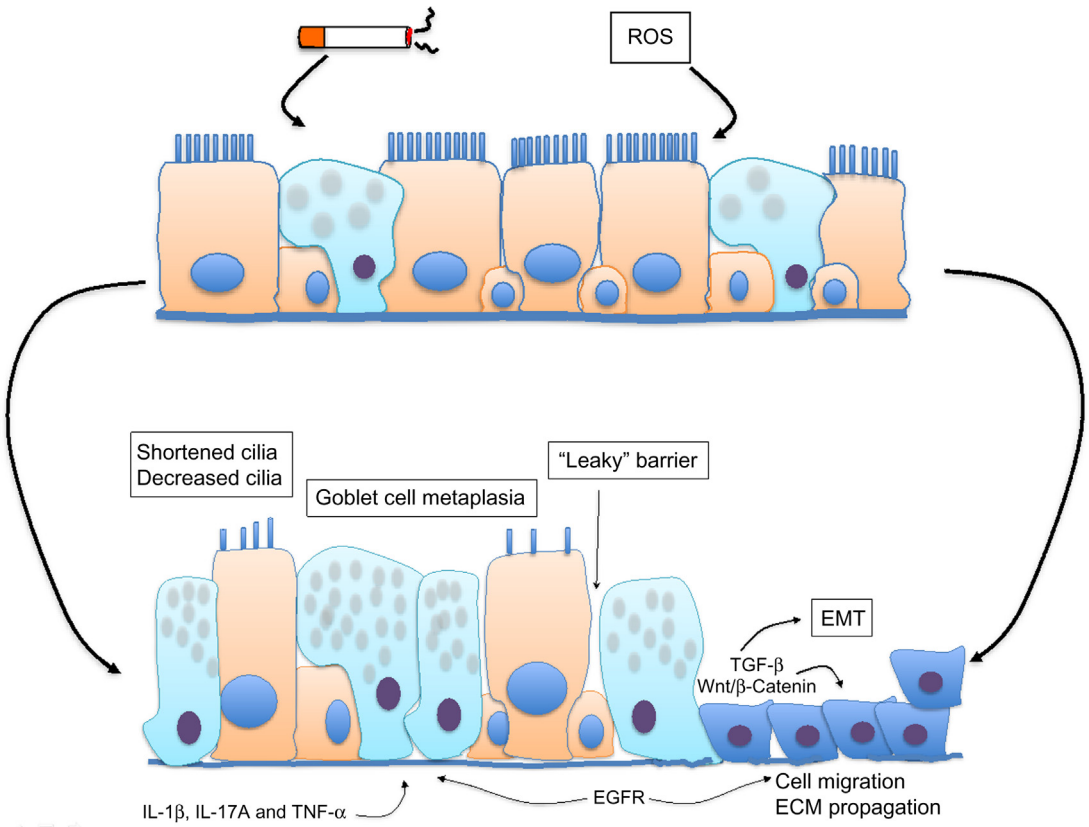

FIGURE 9.1 A damaged epithelial barrier. In COPD, the epithelial barrier is not as effective in the setting of inflammatory and smoking-induced damage to intercellular junctions leading to a leaky barrier. In part, this leads to overactivity of repair pathways resulting in epithelial-to-mesenchymal transition (EMT), goblet metaplasia, and squamous metaplasia. Mucus hypersecretion is seen in the setting of goblet cell metaplasia/hyperplasia and is stimulated by chronic inflammation. Failure of mucociliary clearance is further contributed to by shortened and decreased number of cilia on remaining ciliated cells.

compared with the cells of healthy smokers, providing evidence of an altered phenotype [22]. This alteration in the AJC genetic profile was accompanied specifically by a strong decrease in the expression of phosphatase and tensin homolog (PTEN) and other transcription factors such as FOXO3A in the PTEN pathway [22]. This suggests that PTEN may be an important regulator of epithelial cell polarity [23], and this decrease in expression provides evidence that smoking may induce a fundamental dysfunction in the epithelial programing to maintain polarity. In addition, PTEN may be regulated via a number of mechanisms including increased levels of IL-1β, which induces AJC disassembly of endothelial cells via suppression of PTEN activity [24]. Whether it be from prolonged exposure to cigarette smoke or a genetic predisposition of the epithelium from those with COPD to accumulate injury, there does appear to be a disease-specific effect that persists even after smoking cessation [25]. Finally, there is evidence showing that although protein levels of E-cadherin and ZO-1 were expressed at a lower level in the epithelial cells of smokers and COPD subjects, the mRNA levels remained normal [30]. This suggests that in some patients/scenarios, there may be either protein degradation by the metalloproteinases that are produced in excess in COPD, or decreased translation of junctional proteins in the setting of cellular dysfunction [30].

What are the consequences of this leaky barrier? There are now multiple lines of evidence showing that bacterial pathogens including *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, and *Haemophilus infuenzae* are more adherent to receptors found in the basement membrane [26–28]. Therefore, exposure of basolateral receptors to bacterial/viral particles may lead to increased susceptibility to infection and microbial colonization, as is seen in COPD. This is likely to play a role in the exacerbation state of COPD and in initiating and sustaining the inflammatory mechanisms that drives the disease process forward [7,34]. The disruption of the intercellular junction also has important consequences related to inappropriate initiation and propagation of cellular signaling. This includes signals for proliferation and inhibition of dedifferentiation via the EGFR and Wnt/β-catenin pathways. This has important implications for goblet cell hyperplasia and metaplasia as will be discussed below. In other aspects of small airways disease, the EGFR

pathway, which is both inappropriately stimulated by lack of barrier function and simultaneously may drive further TJ disruption, serves to autoamplify the effect of an initial barrier disruption. In times of injury, this may be beneficial, as the cells attempt an EGFR-dependent repair process, but in COPD this effect may be prolonged and lead to the "excessive tissue repair" as posited by Hogg and Timens [4].

9.4.2 Mucociliary Dysfunction

In health, the respiratory track has an efficient mucociliary clearance mechanism to expel noxious and infectious particles from the lower respiratory tract. Function of this mechanism depends on a number of factors. One is the proper composition and quantity of the airway surface liquid (ASL). ASL is composed of two strata, one an upper viscoelastic layer composed of secreted mucins that sit on a second periciliary layer composed of membrane-bound glycoproteins that serve to provide lubrication for beating cilia. The composition of the viscoelastic layer is regulated by the expression of mucin genes (MUC), and the viscosity is determined in part by the water content as regulated by transmembrane ion channels such as the cystic fibrosis transmembrane conductance regulator (CFTR), and the apical membrane Na+ channel [29]. In addition to the proper quantity and composition of this fluid, intact ciliary function is also required to clear mucous out of the lower airways.

In COPD, mucociliary dysfunction occurs via a number of mechanisms. There is mucous hypersecretion both from goblet cell hyperplasia and metaplasia as well as ongoing stimulus for mucous release. There is altered composition of the mucin produced with changes in both types of mucin expression and the pH of this substance. In addition there is decreased ciliary function from both shortening and loss of cilia. Taken together, this leads to marked abnormalities of mucociliary clearance, causing increased mucous in airways which contributes to mucous plugging and airflow obstruction. This feature of mucous hypersecretion is not universal in COPD but is seen in the "chronic bronchitis" phenotype, and when present, may portend a more rapid decline in FEV1 (forced expiratory volume) over time [4,30].

In COPD, the number of goblet cells seen in the bronchi and bronchiolar epithelium are markedly increased. This is thought to be both a hyperplastic phenomenon as well as a metaplastic process, as both ciliated and clara cells have been shown to undergo metaplastic change into secretory-cell phenotypes [15,31]. It is likely that this initial proliferation and differentiation into mucous producing cells is an early attempt of the airway to respond to a damaging insult by creating the mucous needed to clear out pathogen and cell debris. However, in COPD the inflammatory stimulus may persist, especially in the setting of ongoing smoking, and lead to the constitutively increased quantity of mucous secreting cells seen in chronic bronchitis [15]. Although the mechanisms of goblet cell metaplasia have not been entirely elucidated, much focus has been placed on signaling via EGFR and IL-13 [15,32,33]. EGFR activation has been shown to be a required step in mucous metaplasia in response to a number of insults including cigarette smoke, a process in part attributed to the effects of oxidative stress [34]. EGFR expression is also shown to be higher in the epithelium of airway tissue sampled from COPD patients, even in the absence of current smoking [35]. In one study, evidence for a two-step model of ciliated-to-goblet cell metaplasia was found. In this schema, EGFR-induced antiapoptosis via a PI3K/Akt pathway, is followed by IL-13 induced differentiation of ciliated cells into goblet cells [33]. There are several important transcription factors involved in this process including, thyroid transcription factor 1 (TTF-1), Sam pointed domain-containing ETS transcription factor (SPDEF), and forkhead transcription factor (FOXA2) [15,36]. SPDEF has been shown to be involved in ciliary-to-goblet cell metaplasia via an IL-13 pathway [37]. TTF-1 (also known as airway epithelial-specific transcription factor NK2 homeobox 1 (NKX2-1) decreases mucous metaplasia by inhibiting SPDEF and maintaining levels of FOXA2 [38]. Another pathway that has been shown to be involved in goblet cell metaplasia is the Notch pathway, which is important in the final differentiation of ciliated epithelial cells [39]. Notch pathway expression has been shown to be altered in the epithelium of those with COPD [40,41], and in vitro studies have shown sustained Notch-1 and Notch-3 signaling to be implicated specifically in the differentiation of cells to a secretory phenotype [42]. Although mucous metaplasia is also a feature of asthma [8], the extent to which this process is different in asthma versus COPD has yet to be clearly delineated.

In addition, to goblet cell metaplasia and hyperplasia in COPD, there is also an increased quantity and altered composition of mucous. Mucin glycoproteins are the main component of epithelial mucous and are large glycosylated viscoelastic macromolecules. Mucin biosynthesis requires several processes including transcription of MUC genes to mRNA, translation into mucin-protein backbones that are then modified by glycosyltransferases prior to being transferred across the golgi apparatus for packaging into a secretory granule that resides at the apical portion of the goblet cell [43]. Although the human genome contains >20 MUC, expression of 12 of these mucin subtypes has been detected in the human respiratory tract. In health, the predominant mucins are MUC5AC expressed in goblet cells and MUC5B from mucosal glandular tissue [43]. In COPD, MUC5B mRNA is expressed in both goblet and glandular cells, and the ratio

of MUC5B/MUC5AC is increased [43]. The implications of this are not entirely clear, but in chronic bronchitis the mucus produced is less acidic compared to normal subjects, and this may lessen the inherent antimicrobial properties of mucin [44]. In addition, also contributing to decreased mucociliary clearance is the increased viscosity of the mucus found in COPD and a number of other obstructive lung disease such as cystic fibrosis (CF). Much like CF, in COPD this increased viscosity of mucus is thought in part to be related to CFTR dysfunction and studies have shown smoking-induced decreases in expression of CFTR in epithelial cells [45], and CFTR expression is altered in areas of squamous metaplasia and EMT [7,46]. Finally, accompanying the mucus metaplasia and mucous hypersecretion, there is an increase in mucus-to-serous ratio of submucosal glands. This is seen in COPD but not in other mucus producing disease states such as asthma [31]. Given that the serous glands are a rich source of antimicrobial products, this may have an impact on the innate immunity of the mucus barrier and on the subsequent bacterial colonization of the airways seen in COPD [31,47].

Broadly speaking, a chronic inflammatory stimulus is thought to be responsible for the overexpression of MUC genes. Specifically, many lines of evidence have pointed to stimulation of the NF-κB pathway by cytokines including IL-1β, IL-17A, and TNF-α [48]. The EGFR pathway in addition to being integrally involved in mucous metaplasia is also thought to be involved in increased MUC expression in response to tobacco smoke and oxidative stress [34,49]. This role of EGFR may be amplified by the expression of matrix metalloproteinases (MMPs) and specifically MMP-9 (produced by both bronchial epithelial cells and macrophages). When MMP-9 is overexpressed in response to acrolein in cigarette smoke, it acts to cleave EGF ligand on the cell surface, thus allowing it to stimulate EGFR and MMP-14 (a membrane-bound activator of other secreted MMPs) [50–52]. Mucin production is also upregulated by a number of bacterial pathogens [53] and by stimulation of toll-like receptors (TLRs) by microbes and endogenously and exogenously produced noxious particles [8,54]. In particular, cigarette smoke appears to act synergistically with other environmental irritants such as lipopolysaccharide (LPS) to increase mucin production even further [55]. Finally, many of the same stimulators for mucous production also drive mucous secretion from the goblet cell, although this process is less well understood [53]. Tobacco smoke, bacterial pathogens, oxidative stress, TNF-α, and neutrophil elastase have all been shown to stimulate secretion from the goblet cells [53,55–57].

Breakdown of the ciliated cells themselves also further contributes to mucociliary dysfunction. Epithelial cells exposed to cigarette smoke have an over 70% decrease in the number of ciliated cells and also show a shortening of the cilia that remain [58–60]. The exact mechanisms of this ciliary shortening have not been fully elucidated [61,62]. One mechanism under investigation involves autophagy that is dependent on histone deacetylase 6 (HDAC6) [60]. In general, HDAC6 is upregulated in the airways of COPD patients where it may act to target damaged and misfolded proteins for proteasomal degradation [60]. In the case of ciliary shortening, HDAC6 was found to colocalize with α-tubulin (a ciliary protein) at the apical surface of the epithelial cell prior to cytoplasmic translocation in which α-tubulin then associated with LC3B, a protein active in autophagy. In addition, the HDAC6 enzyme is not just upregulated in the epithelium of those with COPD, but at an epigenetic level there is evidence of hypomethylation of the HDAC6 gene, suggesting a fundamental alteration in gene expression [60]. Finally, there is also evidence of ciliary motor dysfunction with a study showing a decreased frequency of ciliary beating of nasal mucosal ciliated cells in subjects with COPD compared to healthy and at-risk controls [63].

Taken together, the hypersecretion of mucous in the setting of goblet cell metaplasia stimulated by ongoing respiratory insults leads to a chronic bronchitis phenotype in COPD. This is characterized by chronic cough with excessive mucus production. Mucin plugging the airways in conjunction with airway fibrosis and narrowing is shown to be closely associated with airflow obstruction. It is also likely that the overproduction of mucus is tightly connected with the bacterial colonization seen in COPD with potential changes in the local microbiome [64]. This then provides a positive feedback mechanism by which propagation of ongoing inflammatory stimulus leads to further mucociliary dysfunction.

9.4.3 Airway Repair Dysfunction

As mentioned above, a key process in COPD airflow obstruction is the remodeling of the airways, and in particular small airways (<2 mm) [65]. Although this involves both the epithelium as well as deeper structures of the airway wall (i.e., smooth muscle hypertrophy and airways fibrosis), it is the response of the epithelial cell to injury that may contribute to a number of the pathologic defects. When injured, the airway epithelium initiates a repair process in an attempt to restore normal function and structure. In brief, epithelial repair as currently understood involves four processes: (1) cellular dedifferentiation, (2) migration of the dedifferentiated cells to create a barrier over the area of

injury, (3) proliferation of basal cells at edge of the wound which then serve to repopulate the damaged epithelium, and (4) redifferentiation into the proper balance of epithelial subtype (i.e., ciliated/goblet cell etc.) [7,36,66].

In the dedifferentiation stage, EMT may be the predominant activity. There are three types of EMT: type I seen in development, type II seen in tissue repair, and type III seen in metastatic transformation of cancer cells [67]. In this section, we will be discussing Type II EMT, which is signified at the cellular level by loss of epithelial markers including E-cadherin and gain of mesenchymal markers including vimentin, desmin, fibroblastic-specific protein 1/S100A4, and alpha smooth muscle actin [68]. At a functional level, EMT involves loss of cell polarity, altered intercellular adhesions, and transformed cell-to-matrix interactions, which lead to a subsequent gain of migratory function [36]. This allows for spreading of cells over areas of damaged epithelium and for the secretion of the extracellular-matrix (ECM) products needed to restore a functioning epithelial barrier [66]. The pathways leading to EMT are complex, but at a fundamental level are thought to be driven by growth factors such as TGF-β, EGF, and fibroblast growth factor [66,68]. EMT is also facilitated by the activity of MMPs and specifically MMP-9 that allows for breaking of epithelial cell connections to the basement membrane and ECM, thus allowing for cell migration [68]. Although thought to be an important aspect of "normal healing," EMT when constitutively active in response to various inflammatory signals may favor formation of fibrosis via overactive propagation of ECM materials [68].

In COPD, the question of whether EMT plays a major role has been debated [69]. However, there are now a number of studies that support the presence of persistent EMT in COPD. Cells obtained from the large and small airways of COPD subjects show increased expression of mesenchymal cell markers and decreased expression of epithelial cell markers [70–73]. Notably, this expression of EMT was highest in COPD tissues even when compared to tissue from control patients with active smoking [70]. There are also data that implicate this process directly with airway fibrosis and show that EMT features in the bronchial epithelium of subjects with COPD correlates with severity of airway obstruction [73]. This persistence of EMT may reflect a number of processes that have been implicated in the pathogenesis of COPD including a chronic inflammatory insult from smoking, upregulation of various cytokines, and an overproduction of MMPs [36]. This may favor overexpression of both the TGF-β and EGFR pathways, which have been shown to contribute to EMT. Binding of TGF-β to its receptor leads to dimerization of the TGF-β receptors type I and II which then act via a Smad-dependent pathway by which Smad-2 and -3 complex with Smad-4 and translocate to the nucleus to serve as a transcriptional regulator of target genes [67]. The inhibitory limb of this pathway acts via Smad-7 and Smad-6 that inhibits Smad-2/-3 activation and is induced in response to TNF-α, IFN-γ as well as by cigarette smoke itself. Given that this pathway is both activated and inhibited by some of the same components (cigarette smoke and TNF-α), there is likely a precise balance of TGF-β stimulation and receptor density that determine the final predominant effect [4]. Further evidence of this being an active process in COPD is from data showing that TGF-β levels are higher in the small airways of patients with COPD as compared to healthy controls [74], and that TGF-β has been associated with COPD development in genetic studies [75,76]. In addition, Smad-6 and Smad-7 levels (inhibitors of Smad-2 and 3) are decreased in smokers with COPD [77,78]. In terms of the stimulant for increased TGF-β, this is thought in part to be from cigarette smoke-induced oxidative stress [70]. It has also been shown that the induction of EMT in the setting of TGF-β activity is potentiated by TNF-α [79] and IL-1β, which are both overexpressed in COPD [80]. The Wnt/β-catenin pathway is also active in the process of EMT via both TGF-β-dependent and independent pathways. Recently, Heijink et al. provided evidence that Wnt-5B is stimulated by cigarette smoking and serves as an activator of EMT via a TGF-β/Smad3-dependent pathway [81]. In contrast, nicotine directly stimulates the Wnt/β-catenin pathway to promote EMT in a TGF-β-independent manner [82]. Taken together, there is now strong evidence that EMT stimulated by TGF-β-dependent pathways and cigarette smoke likely plays a major role in the propagation of the "excessive tissue repair" and tissue fibrosis seen in COPD.

Squamous metaplasia is another kind of metaplastic event seen in COPD and is found in the bronchial epithelium of COPD subjects [83]. These metaplastic cells may act in a fundamentally different manner with an altered elaboration cytokines in response to stimuli. In particular, squamous cells may be a source of increased IL-1β that further drives the EMT-associated fibrosis and remodeling of small airways via the TGF-β pathway discussed above [84]. In addition, both squamous metaplasia and EMT are accompanied by fundamentally altered cell polarity and adhesion allowing for EGFR activation that further promotes fibroblast proliferation, secretory-cell hyperplasia, and smooth muscle hypertrophy.

Although stated in passing above, the role of MMPs is crucial in COPD where it has major consequences for the development of emphysema. Relatively less is known about the role of MMPs in airway remodeling. As previously discussed above, MMP-9 has a role in both the stimulation of EGFR via cleavage of it's EGF-ligand from the cell surface, activation of MMP-14, and in degrading ECM to allow for epithelial migration. MMP-7, known as matrilysin, has different effects depending on whether it is secreted apically at which time it activates defensins (secreted molecules

with inherent antimicrobial products) or on the basal side of the cell in which it acts to degrade matrix proteins and has been shown to be crucial in reepithelialization after injury where it is posited to have a role in shedding the E-cadherin intercellular connections [85,86]. MMP-12 is a molecule produced in both airway epithelium and airway smooth muscle cells. Although the role of MMP-12 in human COPD has yet to be worked out, it has generated great interest given that in animal models involving MMP-12 inhibition and/or genetic knockout, there is resistance to developing both emphysema and small airway remodeling [87].

Taken together, the breakdown of barrier function via epithelial permeability and impaired mucociliary clearance both contribute to setting up the chronic injury state that is likely needed to propagate the excessive tissue repair and remodeling that occurs in the airways of COPD. During this process, the airway lumens narrow in response to the mucin and inflammatory infiltrate in the airway lumen and from the remodeling in the airway wall culminating in the airflow obstruction characterizing COPD [65].

9.4.4 Alteration of the Immune Barrier

Epithelial cells play a key role in orchestrating the immune response in COPD. This includes innate functions such as secretion of antimicrobial enzymes and peptides, as well as elaboration of cytokines in response to the sensing of microbial products (see Fig. 9.2). In this section, we will focus on the role of the epithelial cell in facilitating this immune barrier. We start by focusing on the role of the epithelium in innate immunity in COPD. As mentioned above, the epithelium secretes a number of products with antimicrobial activity. In the airway secretions, there is epithelial elaboration of the large enzymes lactoferrin and lysozyme, small cationic peptides such as β-defensins, the cathelicidin LL-37 and CCL-20, and the immunoglobulin IgA that all have innate antimicrobial activity [88,89]. This contrasts with the products secreted by alveolar epithelial cells, which must elaborate proteins that do not alter the function of surfactant that helps keep the alveoli from collapsing. Alveolar fluid proteins involved in innate immunity and secreted from alveolar epithelium include surfactant proteins (SP) A, B, and D. In the airspaces, SPs act as pattern recognition receptors (PRRs), leading to further cell signaling in response to contact with particles from viruses and bacteria [90,91]. The innate response of the lung in COPD is altered. In some circumstances, the response may be deficient and yet in other ways there appears to be increased activity in response to cigarette-smoke induced damage and oxidative stress. The response is powerful and even in the setting of adaptive immune deficiency, as seen in B- and T-cell deficient mice, the innate response is sufficient to lead to the pathological changes of COPD [92].

There are a number of perturbations in the spectrum of secreted antimicrobial epithelial products in COPD. Lysozyme and lactoferrin are the most abundant antimicrobial product in the airways. Lysozyme acts to breakdown bacterial walls via cleaving of glycosylated bonds, whereas lactoferrin acts as a chelator depriving organisms of iron [8]. Although in some studies both lysozyme and lactoferrin have been shown to be increased in airway secretions of patients with COPD [93], lower levels of lysozyme has been associated with increased risk of exacerbation [94]. In contrast, the role of defensins is less well understood. Defensins are a group of molecules with antimicrobial properties against a broad array of gram positive, gram negative, viral, and fungal pathogens. In addition, they are thought to have chemotactic effects on immune cells, and an ability to activate dendritic cells (DCs). Human β-defensins (HBD) are expressed from bronchial epithelial cells. HBD-1 is constitutively expressed by the epithelium and is upregulated in both COPD and asthma, whereas HBD-2, -3, and -4 are regulated by TLR expression via the NF-kβ pathway [95,96]. Studies of β-defensin levels in the lungs of those with COPD have yielded seemingly conflicting results. There are studies showing that smoking decreases the activity of β-defensin in the central airways thereby impairing innate immunity [97], whereas others have shown upregulation of β-defensin in the peripheral lung tissue [98]. In a more recent study, central airways were shown to have a decrease in HBD-2 activity despite increased TLR-4 activity, whereas in the distal airways from the same subjects both TLR-4 and HBD-2 activity were increased [99]. It may be that the expression of HBD-2 at different tissue levels reflects a breakdown of the initial innate immune barrier allowing for colonization of the smaller airways, and a subsequent deregulated inflammatory response. There are also likely to be alterations in the function of the cathelicidin LL-37, a small cationic peptide with antimicrobial properties. This protein has been shown to undergo citrullination in response to tobacco smoke, which leads to a decrease in ability to neutralize LPS, but an increased ability to attract neutrophils [100]. It is also likely that for LL-37, as well as a number of the other molecules involved in innate immunity, there is a transition in function during the course of COPD progression. For example, one study showed that in earlier stages of COPD, LL-37 levels are increased in bronchiolar lavage and epithelial lining fluid and whereas in later stages LL-37 levels were decreased [101]. This may reflect a transition of the underlying tissue phenotype in the setting of chronic injury.

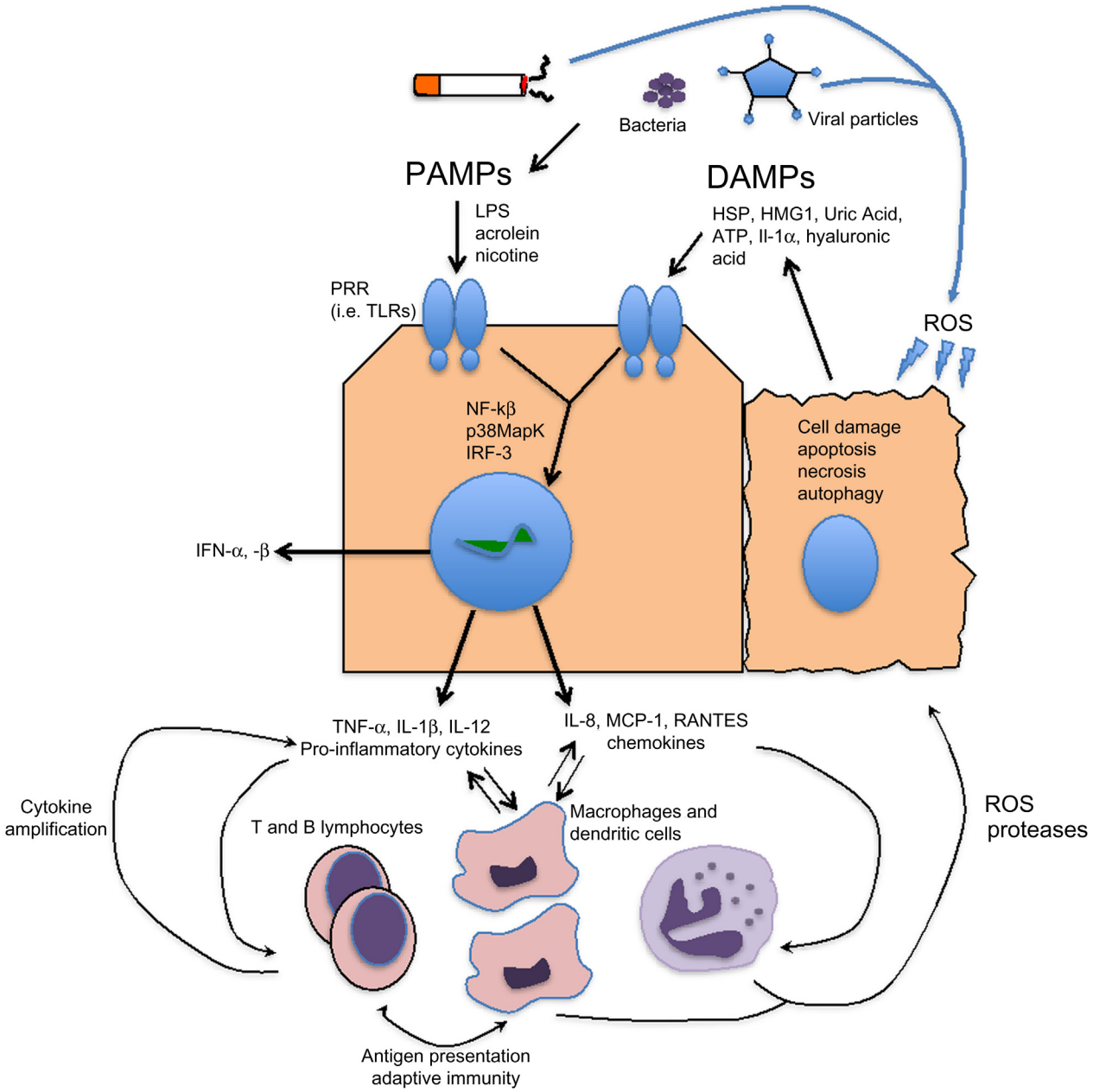

FIGURE 9.2 The epithelial cell as the initiator of innate and adaptive immune processes. Pattern-associated molecular patterns (PAMPs) join with damage-associated molecular patterns (DAMPs) to stimulate pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) on the apical cell surface. PAMPs include elements in cigarette smoke, lipopolysaccharide, and other viral and bacterial products while DAMPs include materials from cell damage from reactive oxidative species (ROS), autophagy, and other processes involving cell damage. PRRs then initiate signaling via NF-κβ, MAPK, and interferon-regulatory pathway 3 (IRF3), leading to production of proinflammatory cytokines and chemokines that initiate further innate and adaptive immune processes. *HSP*, heat shock protein; *HMGB1*, high-mobility group box 1.

IgA is a secreted immunoglobulin that participates in humoral immunity by scavenging various pathogens on mucosal surfaces and within epithelial cells without causing cell damage [102]. The respiratory epithelium has a key role in IgA immunity by facilitating active transport across the cell via a polymeric immunoglobulin receptor (pIgR) [103]. In COPD, this process is disturbed by the alteration of a normal pseudostratified mucociliary epithelium. The metaplastic dedifferentiated epithelium expresses pIgR at reduced levels or not at all, leading to reduced IgA transport across the epithelium [104]. This finding is correlated with increased viral particles of Ebstein Barr Virus (EBV) and cytomegalovirus (CMV) in the small airways, and an increase in lymphocytic infiltration. Paradoxically, although IgA secretion is

markedly impaired in an abnormally differentiated epithelium, IgA production may actually be increased. The epithelium in COPD propagates signaling that stimulates class switching of B cells into IgA-producing plasma cells [105]. This is thought to be accomplished by increased elaboration of IL-6, BAFF, and APRIL proteins, which interact with the IL-6 receptor and the BAFF−APRIL receptor, Transmembrane Activator and Calcium-modulator and cyclophilin ligand Interactor, on B cells [105]. Taken together this leads to increased levels of subepithelial IgA, but less active IgA is seen on mucosal surfaces.

Another key role of the epithelial cell in immune barrier is the early recognition of foreign particles via PRR. PRRs recognize pathogen-associated molecular patterns (PAMPs) from exogenous noxious particles and damage-associated molecular patterns (DAMPs) from endogenously produced products and respond to coordinate a protective response. PRRs include TLR, NOD-like receptors, and RIG-I-like receptors and are expressed by alveolar macrophages, DCs, and epithelial cells [89]. PAMPs include elements of microbes including LPS and flagella that stimulate TLR on epithelial cells but also may be stimulated by noninfectious noxious particles. DAMPs are molecules released by cells in response to cell damage and include uric acid, ATP, high-mobility group box 1 (HMGB1), heat shock protein, IL-1α, and hyaluronic acid. In response to stimulation of PRRs by PAMPS and/or DAMPs, a number of proinflammatory cytokines are elaborated including (TNF-α, IL-1β, and IL-12), chemokines (IL-8, MCP-1, and RANTES), endothelial adhesion molecules (E-selectin), costimulatory molecules (CD80 and CD86), and antiviral cytokines (IFN-α, -β). It is this response that begins a cascade of activity leading to the attraction of inflammatory cells to the area of injury and a priming of their response [4,89]. Elaboration of the cytokines and chemokines after activation of PRRs involves the activation of the NF-κβ pathway via MAPK or NOD signaling, and the elaboration of interferons via an interferon regulator factor-3 pathway [91]. DAMPs such as HMGB1, uric acid, and extracellular ATP have been shown to be higher in bronchiolar lavage fluid of COPD patients versus smokers without COPD [106−108]. The increased levels of DAMPs likely reflect cell damage from cytotoxic cigarette-smoke-induced damage and oxidative stress. In addition, PRR signaling can also be induced directly by components of cigarette smoke including LPS, acrolein, and nicotine [89]. DAMPs also appear to be released through the process of autophagy, and there is increasing evidence that this plays an important role in COPD and specifically emphysema [89,109]. Although the precise mechanism of each component has yet to be worked out, taken as a whole PRR signaling via stimulation from various sources including pathogens in colonized airways in the setting of decreased innate immunity, exposure of receptors to noxious gaseous particles, as well as the build up of cell breakdown products creates a selfsustaining inflammatory milieu that may be at the heart of tissue damage in COPD.

The proinflammatory signals described above lead to an influx of adaptive inflammatory cells. As T and B cells gather in areas of airway inflammation, they eventually form lymphoid follicles that are seen in the airways of smoker and provide microscopic evidence of an adaptive immune response [4]. Not normally present in healthy lungs, these lymphoid follicles are found in the airways of 5% of smokers and those with mild-to-moderate COPD with the prevalence of lymphoid follicles increased to 25%−30% of airways among those with severe or very severe COPD [65]. The crucial link between the innate and adaptive arm of the immune system in this setting is the DC. DCs are antigen-presenting cells and in response to the appropriate stimuli influence CD4 + T-helper cell differentiation and CD8 + cytotoxicity, both features seen in COPD [89]. In COPD, several subsets of DCs are found in the epithelium, where they express costimulatory signals for CD8 + differentiation of T cells. This increase in DC accumulation also exhibits a positive correlation with worsening airflow obstruction [110]. Although the exact nature of the epithelial influence on DC and macrophage function is unknown, epithelial cells do secrete chemokines such as CCL-20 which serve to attract these cells [111] and given the close proximity are likely to have other influences as well. In addition to the chemotactant properties, the epithelium also likely potentiates the function of the cells it attracts. For example, epithelial cells were shown to act in a paracrine fashion to increase the release of inflammatory mediators from resident macrophages [112]. In addition, has already mentioned above, the epithelium is able to influence specific class switching of B cells [105].

Although already eluded to in a number of examples, it is worth explicitly stating the important role of epithelial cell elaborated cytokines and chemokines in COPD. It is the cytokine and chemokine profile that determines the behavior of the underlying tissue, and this is regulated tightly by the epithelium. As already discussed, TNF-α, IL-1, IL-8, IL-6, and GCSF released from epithelial cells promote recruitment of inflammatory cells [113,114] and are shown to be upregulated in COPD [115−117]. In terms of chemokine activity, IL-8, GCSF, and LTB4 are potent attractors of neutrophils, whereas molecules, such as MCP-1 and LTB4, serve as chemotactants for macrophages [117]. Furthermore, rather than a nonspecific inflammatory response, the cytokine/chemokine profile in COPD is unique when compared to other inflammatory airways diseases such asthma and favor a disease-specific effect [113].

Finally, although mentioned a number of times above, the airway epithelium in COPD is exposed to persistent oxidative stress from reactive oxidative species found in and induced by cigarette smoke. Oxidative damage can directly or

indirectly stimulate NF-κβ pathways leading to further elaboration of proinflammatory molecules. Epithelial cells are protected against this stress by a number of antioxidants including glutathione (GSH) that is normally secreted in response to oxidative stress [114]. In COPD, this reserve may be exhausted and there is evidence that persistent cigarette smoke exposure depletes and modifies GSH in a fashion to make it less effective. A modified less effective reserve of GSH has been shown to occur to a greater degree in cells cultured from those with COPD versus cells from never smokers [118].

Taken together, the elements mentioned above all work together to create the immune dysfunction that is seen in COPD. Although the initial responses of the epithelium to injury may be beneficial, is it is likely that in COPD this process becomes irreparably altered leading to a chronic inflammatory process that drives cell dedifferentiation and "excessive tissue repair" leading to airways remodeling, as well as a stimulus for the chronic injury seen in emphysema.

9.5 THE ROLE OF THE EPITHELIUM IN THE DEVELOPMENT OF EMPHYSEMA

Emphysema is another phenotypic characteristic of COPD, although one that does not develop in all smokers. In some cases, this process occurs at the same time as small airways remodeling and in other cases emphysematous changes without mucous hypersecretion or marked remodeling may predominate. There are a number of pathological processes involving the epithelial cell that are hypothesized to lead to emphysema. The prevailing theory for many years was that of an inflammatory insult arising from chronic exposure to cigarette smoke leading to a protease−antiprotease imbalance causing extracellular-matrix destruction [119]. However, recent investigations have also shown the importance of a number of other factors including direct cytotoxicity from CD8 + T cells, cellular stress in response to persistent damage, autophagy, and decreased cell maintenance leading to apoptosis in the setting of lung aging and cell senescence (see Table 9.2). In the next section, we will focus on a number of these mechanisms as they relate to the alveolar epithelium.

The initiation of the protease−antiprotease theory of emphysema stemmed from two major observations. One was that the instillation of papain, a proteolytic meat tenderizer, into the lungs of rats induced emphysema [120], and two was the association of early emphysema in those with a genetic deficiency in the neutrophil elastase inhibitor α1-antitrypsin (AAT) [121]. Although there is now considerable interest in a more complex theory of emphysema pathogenesis, the role of proteases remains important. In particular, experimental models have shown the importance of neutrophil elastase and MMP-12 in the characteristic degradation of the elastin scaffold supporting the alveoli. Once the breakdown of matrix products begins, this process is then amplified by the chemotactant properties of elastin fragments, leading to a positive feedback loop [9,122]. Although much attention has been given to MMP-12 and neutrophil elastase being secreted by macrophages and neutrophils, both proteases and antiproteases may also be secreted directly by the epithelium. For example, MMPs are one such group of proteases produced in part by the pulmonary epithelium, though the contributions of epithelial cell versus inflammatory cell-elaborated proteases are not well understood. MMPs have a broad array of functions, some involved in breakdown of ECM, including the collagenases (MMP-1, -8, and -14), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, and -11), and the elastases (MMP-7 and -12) [6]. MMP-12 is probably the best studied MMP in COPD/emphysema. Although it has been shown to be upregulated in response to cigarette smoke in animal models [87,123,124], data in humans have been conflicting with some studies providing

TABLE 9.2 Pathophysiology of Emphysema

Mechanism	Important Mediators
Protease-antiprotease imbalance	MMPs, TIMPs, SPLFI, ELAFIN, and AAT
CD8 + cytotoxicity	Perforin and granzyme B
Cellular stress/oxidative damage	Nrf2, RTP801, and NF-κβ
Altered cell repair/maintenance	VEGF and Wnt/β-catenin
Autophagy	HDAC
Cellular senescence/cellular aging	Telomeres, IL-6, IL-8 and TNF-α

MMP, matrix metalloproteinases; *TIMP*, tissue inhibitor of matrix metalloproteinases; *SPFLI*, secretory leukocyte protease inhibitor; *ELAFIN*, elastase specific inhibitor; *AAT*, alpha-1 antitrypsin; *VEGF*, vascular endothelial growth factor, *HDAC*, histone deacetylases.

evidence for increased MMP-12 expression [111,125], and other finding no increase or association with COPD [126]. However, in animal models evidence of MMP-12 involvement is strong, with MMP-12 knockout mice showing resistance to the development of emphysema and fibrosis as mentioned above [87]. The involvement of other MMPs in the development of emphysema is less clear, though there is posited role of MMP-9 in both small airways disease and emphysema [124]. It is also known that MMP expression is influenced by cytokines/chemokines secreted in part by epithelial cells [87]. IL-1β induced from lung epithelium in mice led to an increase in MMP-12, MMP-9, and neutrophil elastase as well as pathologic changes such as disruption of alveolar septae, dilation of airways, and increased thickness of the airways [127]. TNF-α, from macrophages, also has a key role by way of a mechanism in which MMP-12 induces TNF-α release from macrophages and an upregulation of chemokines MIP-2 and MCP-1 (possibly from epithelial cells). This leads to subsequent neutrophil chemotaxis and elaboration of neutrophil proteases [128]. The role of TNF-α in emphysema is also further supported by a study showing that up to 70% of alveolar airspace enlargement may be driven by TNF-α [129]. The pulmonary epithelium is also the major source of two antiproteases, secretory leukocyte protease inhibitor (SLPI), and elastase specific inhibitor. These two molecules are both potent inhibitors of neutrophil elastase and a number of other proteases [6]. Pulmonary epithelium also secretes another class of proteases called cathepsins (K, L, and H) that have been shown to degrade elastin and inactivate SLPI and AAT [6], indicating a direct involvement of the epithelium in the milieu of protease/antiprotease activity. Finally, another important group of antiproteases are the tissue inhibitors of matrix metalloproteinases (TIMPs). In human subjects with COPD, TIMP-1 has been shown to be increased and has been immunolocalized to the respiratory epithelium. In addition, the ratio of MMP-9/TIMP-1 has been shown to be increased in COPD subjects versus those with idiopathic pulmonary fibrosis, supporting an imbalance playing a role in excessive tissue destruction leading to emphysema [130,131].

As a smoking-related lung disease, any theory or model of COPD must take into account the initial response of the tissue to exposure to noxious particles. In pulmonary emphysema, the initial response to cigarette smoke triggers the innate immune system through PAMPs and DAMPs. This initial response is brought on not only by toxins in noxious inhalants but also by endogenously derived oxidants such as peroxynitrite ($ONOO^-$) that is linked to alveolar damage [132]. In emphysema, the role of initial inflammatory pathway upregulation is not as straightforward as it would seem as upregulation of NF-κβ pathways induced by PRR stimulation may be short-lived [133]. In addition, there is the intriguing finding that mice with knockout of TLR-4, a major PRR leading to proinflammatory NF-κβ, were protected from emphysema [134]. One possible explanation of these findings are that in part it is the epithelial cell response to chronic noxious particles that primes important cell repair mechanisms. It would seem that a balance of activation without overexpression or constitutive activation is necessary for normal cell repair [9]. This is supported by the finding that activity of the NF-κβ pathway reduced cell apoptosis in a model of autophagy-induced cell death [62]. Furthermore, it is the initial epithelial response to oxidative and inflammatory stress that may be the key. In recent years, two pathways have garnered interest in their role in this initial response. RTP801 is a stress-induced molecule that when upregulated may lead to increased cell apoptosis and as an inhibitor of the mTOR pathway, decreases cell growth and protein synthesis [133,135]. In response to cigarette smoke, RTP801 expression in alveolar epithelial cells was increased and found to be both necessary and sufficient for the activation of NF-κβ. Mice without RTP801 were protected from early smoke-induced inflammation and importantly were protected from emphysematous changes at a 6-month follow-up [135]. The response of tissue to inflammatory and oxidative stress also is likely highly dependent on the activity of the master antioxidant transcription factor Nrf2, with decreased expression of this factor being linked to the process of aging [136]. In a COPD mouse model, Nrf2 knockout mice were found to have higher levels of RTP801 and had a predisposition to develop alveolar cell (epithelial and endothelial) apoptosis and emphysema [137].

The early stresses brought on by cigarette smoke may eventually lead to progression of disease via altered or inefficient cell maintenance programs. This effects all the structures of the alveolar air space including alveolar epithelial cells, endothelial cells, and fibroblasts. Whether this cell destruction seen in emphysema is initiated in the epithelium and then subsequently involve the ECM and endothelial cells, or whether as some have put forth it is primarily a vascular disease with subsequent involvement of the alveolar epithelium and ECM is unknown [133]. One of the most important pathways in cellular repair as it relates to emphysema is the vascular endothelial growth factor (VEGF) pathway. In animal models, genetic ablation of VEGF or treatment with a VEGF inhibitor led to a ceramide-dependent apoptosis and pathologic changes consistent with emphysema [138]. Furthermore in human emphysema, VEGF mRNA expression is significantly reduced [139], and multiple studies have pointed to the association of genetic polymorphisms in VEGF with COPD [140,141]. VEGF expression is induced by hypoxia-inducible factor 1α (HIF-1α), which in the endothelial cell leads to expression of nitric oxide and prostacyclins. Investigators have shown decreased expression of HIF-1α from human emphysema tissue and provided evidence of a pathway in which oxidative stress inhibits histone deacetylase 2 (HDAC2) leading to increased p53 levels that inhibit HIF-1α [142].

In contrast to the "excessive tissue repair" seen in small airway remodeling where the Wnt/β-catenin was hypothesized to be overactive, in emphysema there is evidence of decreased Wnt/β-catenin activity in both animal and human tissue [143]. A recent study evaluating the function of the FAM13A gene, which has been consistently shown to be associated with COPD [144,145], showed that FAM13A protein interacts with PP2Ab to influence β-catenin degradation [146]. Furthermore, the under activation of this pathway may also be driven by smoking-associated upregulation of the Wnt suppressor, secreted frizzled-related protein 2 [147]. Why there is a difference in Wnt/β-catenin signaling in the two lung compartments (airways versus airspaces) is unknown.

In addition to lack of growth factors, epithelial and endothelial cells may be more susceptible to proapoptotic signaling. The sphingolipid, ceramide, a protein involved as a second-messenger in apoptotic pathways, was shown to be induced by lack of VEGF and was more effective at inducing apoptosis in epithelial and endothelial cells as compared to macrophages. At the same time, ceramide also seemed to inhibit alveolar macrophage phagocytic efficiency resulting in a defect of clearance [148]. In the setting of defective lung maintenance, differential survival of inflammatory versus structural cells and lack of clearance of apoptotic debris may be one mechanism for ongoing inflammatory damage even after cessation of the original stimuli.

Another process that has garnered recent attention in the pathogenesis of COPD is the role of autophagic cell death. Chen et al. showed that autophagy was an active process in human pulmonary epithelial cells. They also provide evidence of a possible epigenetic modification of HDACs leading to downregulation of these molecules and the upregulation of Erg-1 that increased expression of the autophagic protein LC3b. They were also able to show that inhibition of autophagy decreased epithelial cell apoptosis [61]. The induction of autophagy in COPD appears to be an early event and is followed by apoptosis in later stages [61]. This fits with the evolving notion that prolonged autophagy or an altered autophagy process leads to prolonged stress on the cell which then influences the eventual cell death via capsases or necrosis [61,109,149].

Finally, in COPD and in particular emphysema, there has been an increased interest in the role of aging and cellular senescence. Cellular senescence provides organisms with critical protection against neoplastic changes as cells that accumulate injury and cellular stress become senescent leading to growth arrest. Although this initially may be beneficial, as senescence may allow for cell clearance and regeneration, in the setting of tissue aging this may not be possible, leading the accumulation of senescent cells [150]. In COPD subjects, endothelial and epithelial cells showed higher expression of important markers of aging such as p16, p21, and senescence-associated β-galactosidase [151,152]. In addition, in a recent study of peripheral blood from COPD subjects, shortened telomere length and increased p21, both markers of aging, were associated with COPD, and the shortening of telomeres was correlated with worsening lung function [153]. So, what are the consequences that aging tissue has in COPD? For one, senescent cells, and in particular senescent progenitor cells, have been shown to have less ability to undergo regeneration; a process which may involve aberrant Wnt signaling [154]. This could account in part for which in emphysema the alveolar cells are predominantly Type I pneumocytes, as the progenitor Type II alveolar cells have been shown to be early targets of cell senescence [155]. There is also evidence that senescent cells have a proinflammatory phenotype that may serve to propagate further tissue damage in COPD. Alveolar cells that underwent induced senescence were found to be more likely to elaborate IL-6, IL-8, and TNF-α, whether stimulated by LPS or not, via a NF-κβ pathway. This indicated a fundamental proinflammatory dysfunction [156], with a "senescence-associated phenotype" that acts in a paracrine manner. This also creates a positive feedback loop as the elaboration of cytokines such IL-6 and IL-8 may further influence the development of cellular senescence [157–159]. Taken as a whole, the final outcome of cellular apoptosis and the expression of emphysema is likely to be a complicated balance of cell breakdown in response to stress and aging in the setting of depleted growth factors and paracrine activation of damaging proinflammatory pathways.

One of the seeming contradictions in the pathophysiology of COPD is that the "excessive tissue repair" seen with small airway remodeling and fibrosis coexists with an "insufficient tissue repair" resulting in destruction of the alveolar air spaces in the same patients. Broadly speaking, it is convenient to speak about and study the two mechanisms as separate entities, whereas the true interaction between the two is likely to be far more intricate and complex. Contemporary evaluation of lung tissue does indicate that the two processes may be more interdependent than previously thought. A recent examination of lung tissue in COPD patients showed a significant destruction of terminal bronchioles, with the remaining airways showing fibrosis, prior to the detection of emphysema [160]. This is a finding that has also been corroborated by using new CT techniques that detect physiologically significant nonemphysematous air trapping attributed to small airways disease prior to the radiologic detection of emphysema and show that a tissue-destructive process may be occurring in a similar manner in airways at the same time as fibrosis [161]. Whether a spatiotemporal effect of tissue response [162], two separate disease processes occurring at different tempos [163], or a synergistic effect, more research needs to be done to fully elucidate the connection between small airways disease and emphysema.

9.6 THE EPITHELIUM AS THERAPEUTIC TARGET

As a central actor in the pathogenesis of COPD, the epithelium is an important target for novel COPD treatment. The current therapeutic landscape for COPD includes inhaled bronchodilators, inhaled corticosteroids (ICS), and oral PDE4 inhibitors. These therapies have been shown to reduce exacerbations, improve exercise tolerance, and increase quality of life. However, as of this writing, none of the aforementioned therapies have been shown to decrease decline in FEV1 or mortality [164–166]. Targeting the dysfunctional epithelium and protecting the epithelium from further damage is an important component of developing disease modifying COPD therapeutics.

Global initiative for chronic obstructive lung disease treatment guidelines recommend long-acting bronchodilators as first-line maintenance therapy in COPD. This class of medications includes β-receptor agonists and muscarinic receptor antagonists. Although the primary mechanism of action of these drugs is to stimulate airway smooth muscle relaxation, they have also been shown to have secondary effects on the epithelium itself. In vitro, the long-acting beta-agonist salmeterol altered human bronchial epithelial response to stimulation with IL-4 and TNF-α, by reducing epithelial production of cytokines and adhesion molecules [167]. Currently used bronchodilators have also been shown to have some activity in modulating TGF-β pathways. The TGF-β pathway has garnered much interest as a therapeutic target in COPD. As described above, activation of TGF-β is integrally involved in the development of small airways disease and mucus hypersecretion. However, the use of TGF-β inhibitors in COPD have been approached with caution as the detrimental effects of TGF-β-associated remodeling in the airways may be balanced by the beneficial effects of TGF-β in protecting against emphysema [168]. However, both β-agonists and muscarinic antagonist were shown to reduce TGF-β-dependent neutrophilic inflammation [169]. In addition, the β-agonist salmeterol was shown to activate the CFTR that may be dysfunctional in COPD. This effect was blocked by increased TGF-β activity again suggesting a further potential benefit TGF-β-inhibition in conjunction with current therapies [170]. In terms of reducing TGF-β-associated EMT, muscarinic antagonists have been shown to have activity in this area [171]. More recently, macrolides antibiotics, such as azithromycin, have been employed in patients with frequent exacerbation [172], and in addition to antiinflammatory properties, may also exert therapeutic effect in part via reduction of EMT and mucous production [173,174]. Another mainstay in therapy for COPD are ICS. Glucocorticoids act to reduce inflammatory pathways, by suppression of proinflammatory genes. Although in other inflammatory airways diseases such as asthma, this may be a very effective approach, in COPD the inflammatory process is largely steroid-resistant [175]. This may account for the lack of disease-modification with steroid treatment [164]. In part this "steroid resistance" is thought to be secondary to oxidative stressed-induced reduction of HDAC2 via a phosphoinositide 3-kinase-d (PI3Kd) pathway [176]. HDAC2 is a crucial deacetylase that in response to glucocorticoid receptor stimulation acts in the nucleus to decrease expression of proinflammatory target genes. HDAC2 levels decrease with progression of COPD and in response to viral-associated exacerbation [177,178]. This reduced efficacy of HDAC2 may be reversed in part by treatment with existing therapies. Theophylline, a phosphodiesterase (PDE) inhibitor has been used in the treatment for over 80 years and is still popular worldwide as a bronchodilator [179]. In addition to PDE-inhibitory activity leading to bronchodilation at higher doses, lower dose theophylline has been shown to inhibit PI3Kd in a PDE-independent manner and hence enhances HDAC2 activity [180]. In a small trial, low-dose theophylline when co-administered with low-dose ICS was shown to reduce indices of inflammation and increase FEV1 in a cohort of COPD patients [181]. Larger trials are now underway to further evaluate whether steroid/low-dose theophylline combinations will be an effective strategy to reverse glucocorticoid resistance [182]. Drug development with PI3Kd inhibitors is an active area of development. With this therapy, the hope is that by reversing the proinflammatory inactivity of HDAC2 the profound inflammatory process in COPD may be lessened and the ability of glucocorticoids to tamper this process may be restored [168]. There have also been efforts to directly target inflammatory pathways and the inflammatory mediators secreted and initiated by the respiratory epithelium. Areas currently under investigation for targeting in COPD include NF-κβ inhibitors, p38 MAPK inhibitors, and JAK inhibitors [183,184]. Attempts at blocking various cytokines and chemokines have included anti-TNF and anti-I-1 therapy for which efforts so far have been either ineffective and/or hampered by serious side effects and the neutrophil chemokine receptor CXCR2 inhibition [168].

In addition to trying to reduce TGF-β-associated airways remodeling, there have also been a number of other areas of investigation into pathways that may decrease small airways remodeling and mucous hypersecretion. The EGFR pathway has been investigated extensively in terms of the role of EGFR in mucous hypersecretion. There have been trials of EGFR inhibition, but unfortunately these were limited by side effects at the doses that were effective in reducing mucous stores [185]. PPAR-γ agonists, such as rosiglitazone, are currently approved for use in diabetes and have shown promise for use in COPD [186]. These agents have been shown to reduce mucous secretion, have reduced fibrosis in a bleomycin-induced lung injury model, and also have promising antiinflammatory effects [187,188]. Although these drugs have yet to be tested in COPD, they present an exciting opportunity in this disease.

9.7 CONCLUSION

As we have sought to show throughout this chapter, the epithelium is centrally involved in the pathogenesis of COPD. As the first line of defense to inhaled pathogens, the epithelium initiates a number of attempts at repair and protection. However, after prolonged insult, these processes become dysregulated. This sets up the inflammatory stimulus and structural dysfunction that leads to constitutive activity in pathways such as EGFR and TGF-β in airways remodeling. In emphysema, an imbalance of protease/antiprotease activity, inflammatory-associated breakdown of tissue maintenance and perhaps accelerated tissue aging lead to tissue destruction. Central to the question of why some people develop COPD, whereas others with similar exposures do not, is the response of the epithelium to chronic insults. This response is the outcome of a complex set of interactions between genetic susceptibility, epigenetic modification, and ongoing environmental insults. Furthering our current understanding of these processes promises to shine much needed light on the underlying pathophysiology of COPD and heralds possibilities for more targeted and disease modifying therapies.

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Chapter 10

Acute Respiratory Distress Syndrome

Rachel L. Zemans

10.1 INTRODUCTION

In 1967, Ashbaugh et al. described a cohort of patients with the acute onset of severe hypoxemia and bilateral infiltrates [1], a syndrome that became known as the acute respiratory distress syndrome (ARDS). ARDS is now defined as bilateral infiltrates and hypoxemia attributable to noncardiogenic pulmonary edema of acute onset. The severity of ARDS is classified by the degree of hypoxemia, as quantified by the ratio of the partial pressure of oxygen in the arterial blood to the concentration of oxygen in the inspired air (P:F ratio). A P:F ratio of 200−300 is defined as "mild" ARDS, a ratio of 100−200 is "moderate" ARDS, and a ratio of <100 is "severe" ARDS [2]. A large study conducted in 1999−2000 estimated the incidence of ARDS in the United States at 190,000 [3,4], although the incidence has likely decreased since then [5]. ARDS is a heterogeneous syndrome, resulting from diverse etiologies in the context of diverse host factors. Etiologies include pneumonia, which is the most common cause [6], sepsis, aspiration, trauma, pancreatitis, transfusion related acute lung injury (TRALI), and ventilator-induced lung injury (VILI). Patient risk factors for the development of ARDS include alcohol abuse [7] and tobacco use [8,9]. Genetic susceptibility certainly plays a role as well [10]. Mortality from ARDS ranges from 22% to 40%, but is likely decreasing [3,6,11].

One critical pathophysiologic hallmark of ARDS is injury to the alveolar epithelium [12]. The normal alveolar epithelium forms a tight barrier that restricts the passage of fluid and solutes, thus maintaining dry airspaces. During ARDS, epithelial injury impairs barrier function, allowing flooding of the airspaces with protein rich edema fluid [13] that results in the severe hypoxemia and bilateral infiltrates that characterize ARDS clinically. Although endothelial permeability is enhanced in ARDS, epithelial permeability is necessary for alveolar flooding [14]. In addition to the enhanced permeability, epithelial injury also results in impairment of other epithelial functions. For example, ARDS is characterized by surfactant deficiency [15−19], leading to atelectasis of alveoli, which increases shunt and therefore compounds hypoxemia. In addition, epithelial injury results in an impaired ability of ion and fluid transport, resulting in a decreased ability to clear edema fluid [20]. Direct injury to the alveolar epithelium occurs in some cases of ARDS, but much of the injury to the epithelium in ARDS is inflammatory injury. Significantly, the epithelium itself plays a role in the inflammatory response via production and secretion of mediators that serve host defense functions and recruit and activate inflammatory cells. The extent of epithelial injury correlates with severity of disease [21,22], and repair of the epithelium is predictive of clinical recovery, including survival [20,23]. Unfortunately, there are no specific pharmacologic treatments that limit injury or promote repair of the epithelium in ARDS [24,25]. ARDS therapy is limited to supportive management [26,27]. Two critical breakthroughs in the treatment of ARDS have been low tidal volume ventilation, which attenuates epithelial injury [28], and fluid restriction, which minimizes edema formation and thus improves oxygenation [27]. These approaches have dramatically improved survival from ARDS, which has recently been reported to be as low as 22% [11].

This chapter will focus on the role of the alveolar epithelium in the pathogenesis of ARDS. It will be divided into four sections: (1) Mechanisms of injury of the alveolar epithelium, (2) role of alveolar epithelium in inflammation and host defense, (3) mechanisms of repair of the alveolar epithelium, and (4) mechanisms of fluid clearance by the alveolar epithelium.

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10.2 EPITHELIAL INJURY

At baseline, the alveolar epithelium consists of alveolar type (AT) I and ATII cells. ATI cells are thin cells that cover 95%–98% of the alveolar surface area and play a critical role in barrier function, gas exchange, and fluid reabsorption [29]. ATII cells produce surfactant and play a role in host defense as well as serve as an important progenitor during homeostasis and during repair after injury. The alveolar epithelium forms a tight barrier that limits the passage of fluid and proteins from the bloodstream into the airspaces and also prevents the entry of toxic substances and pathogens from the environment into the body. This barrier is created by a continuous epithelium comprised ATI and ATII cells. In addition, tight junctions between adjacent epithelial cells seal the barrier but regulate paracellular permeability via pores. Epithelial tight junctions comprise primarily transmembrane proteins such as claudins and occludins. Claudins play a particularly important role in maintaining barrier function of the alveolar epithelium [30]. The size of the pores in the epithelial cell tight junctions is much smaller than those in the endothelium, such that most of the resistance to macromolecular (i.e., albumin) occurs at the level of the epithelium [31]. The tight barrier formed by the alveolar epithelium is responsible for maintaining dry airspaces during health. During ARDS, the epithelial barrier is damaged. Endothelial injury also occurs in ARDS, but injury to the endothelial alone leads to interstitial edema, which can then be cleared by lymphatics. Injury to the alveolar epithelium, which is more resistant to injury than the endothelium [14], is required for the flooding of the airspaces observed in ARDS. Disruption of the alveolar barrier not only allows for the influx of edema fluid into the lungs but also facilitates bacterial dissemination into the bloodstream, which can result in sepsis and multiorgan failure.

10.2.1 Opening of Tight Junctions

Epithelial barrier dysfunction is partially due to opening of tight junctions [32]. For example, expression levels of claudins and occludins are decreased in alveolar epithelial cells isolated from animals after lung injury [33,34]. Interestingly, experimental models of lung injury are characterized by increased expression of one the tight junction protein, claudin 4 [35]. Claudin 4 levels correlate with barrier function [32], and claudin 4 directly increases epithelial barrier function [36], likely a compensatory mechanism to restore barrier function after lung injury. Interestingly, alcohol loosens tight junctions in part by downregulating expression of certain claudins [37]. Alcohol has also been shown to increase expression of claudin 5 [38], which has long been known to paradoxically decrease epithelial barrier function [39]. Recently, this seemingly paradoxical effect has been elucidated—claudin 5 impairs the interaction between claudin 18 and ZO-1, which is critical to barrier integrity. These effects likely contribute to the baseline decreased alveolo-capillary barrier function observed in humans and animals who ingest alcohol [37,40,41] as well as their predisposition to the development of ARDS [7]. Another mechanism that triggers disassembly of epithelial tight junctions during lung injury is PTEN. Deletion of PTEN from ATII cells results in decreased claudin 4 and E-cadherin expression, resulting in enhanced alveolar flooding [42].

10.2.2 Epithelial Cell Loss

In addition to impaired tight junction integrity, loss of barrier function in ARDS is a result of epithelial cell death. The loss of epithelial cells as a dominant feature of ARDS has been recognized since the classic ultrastructural studies performed by Bachofen and Weibel in the 1970s [12,43]. ATI cells are particularly susceptible to injury [12,43], but in severe cases of injury or in response to etiologies that specifically target ATII cells, there is ATII cell death also [44]. More recent studies have revealed biochemical evidence of epithelial injury, particularly ATI cell injury, during ARDS [45–53].

Epithelial cell death can occur by apoptosis or necrosis or perhaps other forms of cell death, depending on the insult [54,55]. The lungs of ARDS patients [56] and animal models of lung injury [57] reveal evidence of alveolar cell apoptosis. Apoptosis is a programed cell death that is induced by engagement of death receptors on the cell surface (extrinsic pathway) or by mitochondrial injury (intrinsic pathway) [58]. Engagement of the death receptor Fas by its ligand FasL is a classic extrinsic apoptotic pathway. Alveolar epithelial cells from ARDS patients express Fas. FasL levels are elevated in the lungs of in ARDS patients, particularly those who died [56,59], and animal models of acute lung injury [60] and induce epithelial cell death [59,61,62]. In animal models of ARDS, prevention of apoptosis with pharmacologic inhibitors or genetic methods attenuates lung injury [63–66]. Epithelial necrosis occurs in response to acid aspiration or stretch due to mechanical ventilation.

Mechanisms by which the epithelium is damaged vary depending on the cause of ARDS and can be direct, such as in the case of acid aspiration or lytic viral infection of epithelial cells, or indirect, such as in the setting of trauma or sepsis, in which case a systemic inflammatory response results in inflammatory damage to the epithelium.

10.2.3 Direct Epithelial Injury

There is a component of direct injury to the alveolar epithelium in some causes of lung injury. Animal models of lung injury that appear to be involved in direct epithelial injury include hyperoxia [67]. Hyperoxia induces alveolar epithelial cell apoptosis and necrosis [68] in part via the generation of reactive oxygen species [69]. Acid aspiration certainly causes direct injury to the epithelium, although there is likely a secondary inflammatory insult. It has long been known that lung overdistention by mechanical ventilation can cause lung injury [70], a phenomenon that has become known as VILI [71]. In animal models, high tidal volume ventilation induces epithelial injury, as demonstrated by ultrastructural evidence of epithelial damage [72,73], and low tidal volume ventilation reduces epithelial injury [28]. In fact, ARDS patients ventilated with low tidal volumes have decreased markers of epithelial injury [21]. In VILI, mechanical stress can lead to a necrotic epithelial cell death [74–76]. In addition, mechanical stress can activate specific inflammatory signaling pathways in epithelial cells, which in turns induces the influx of immune cells that can cause a secondary injury [77–79]. In fact, arguably the single most important therapeutic advance in the treatment of ARDS patients has been the use of low tidal volume ventilation, which has resulted in a significant reduction in mortality [26]. In addition to overdistention, VILI is partially attributable to repeated opening and closing of alveoli [80], a phenomenon that has been termed "atelectrauma." Atelectrauma directly compromises epithelial barrier integrity via disruption of tight junctions [81] and adherens junctions [82]. Improvements in outcome observed in trials of high positive end-expiratory pressure [83] and prone positioning [84] are likely related to minimization of atelectrauma. In addition, as discussed above, hyperoxia causes epithelial injury. Therefore, some component of VILI may be attributable to high oxygen tension of inspired air [85], although overdistention is the predominant mechanism underlying VILI.

Viral infection, particularly with influenza, causes both direct and inflammatory injury. As alveolar epithelial cells, especially ATII cells, express the sialic acid residues that influenza virus uses as receptors, these cells are targets of viral infection. Hemagglutinin (HA) expressed on the surface of the virus binds to sialic acid residues on the surface of the epithelial cells, leading to endocytosis of the virus. After viral replication inside the epithelial cell, viral particles are released and infect neighboring cells. Viral infection results in ATII cell death [44,86,87]. Some strains of influenza virus preferentially infect upper airway epithelial cells due to the specificity of their HA for the specific sialic acid residues on the tracheal epithelial cell surface; other strains, such as avian influenza viruses preferentially infect ATII cells due to the affinity of their HA for the ATII sialic acid residues [88–91]. This preferential infection of upper vs lower respiratory tract epithelial cells explains why some viruses cause tracheobronchitis and others are more likely to cause pneumonia and ARDS. Conversely, the specific tissue tropism of a particular virus determines the likelihood of human-to-human transmission and therefore the likelihood of initiating a human pandemic, with those strains that preferentially infect the upper airway epithelium being more transmissible due to the ease of aerosolization from sneezing and coughing, as compared to those strains that preferentially infect the alveolar epithelium [90].

Bacterial infection induces a robust inflammatory response that in turn causes epithelial injury. However, some bacteria can also cause direct epithelial injury via lysis of epithelial cells by their exotoxins. Pseudomonas is notorious for causing epithelial cell cytotoxicity via exoenzymes [92,93]. The mechanism by which pseudomonal exoenzymes cause epithelial cell death are multiple and include phospholipase signaling that results in interference with integrin survival signaling, leading to anoikis [94]. Exoenzymes also activate intrinsic apoptosis pathways [95]. In fact, strains of Pseudomonas that express certain exoenzymes cause more severe lung injury and enhanced mortality in patients compared to strains that do not [96]. The *Escherichia coli* virulence factor hemolysin forms transmembrane pores in host cells, including epithelial cells [97], resulting in cell lysis. Gram positive infections can also directly induce epithelial cell death via toxins, such as pneumolysin, a virulence factor of *S. pneumoniae* that forms pores in host cells, resulting in cell lysis [98,99]. Bacteria have other mechanisms by which they directly cause epithelial cell death besides exoenzymes. For example, several bacteria have recently been shown to cause epithelial cell death via acetylation of mortality factor 4 like 1 protein (Morf4l1), rendering it resistant to ubiquitination and degradation [100]. Bacterial derived hydrogen peroxide can cause DNA damage, leading to apoptosis of alveolar epithelial cells [101].

10.2.4 Inflammatory Epithelial Injury

10.2.4.1 Neutrophils

Regardless of whether there is a component of direct epithelial injury by certain insults, inflammatory injury to the epithelium is almost universally implicated in the pathogenesis of ARDS. The inflammatory cell type most strongly implicated in epithelial injury is the neutrophil [102]. With rare exception [103,104], neutrophils are highly prevalent in the lungs of ARDS patients [105] (Fig. 10.1). Furthermore, neutrophil numbers correlate with mortality from ARDS [105]. Circulating neutrophils are recruited to migrate across the endothelium, through the interstitium, and across the epithelium into the airspaces in response to a chemotactic gradient [106]. During migration, in response to a variety of inflammatory mediators, neutrophils become primed and then activated. Activated neutrophils serve a critical role in host defense via phagocytosis and release of toxic mediators that kill invading pathogens. It is possible for neutrophils to migrate into the lung without causing tissue injury [14,107,108]. However, there is substantial evidence, mainly from animal studies, that neutrophils are not only microbicidal, but can cause significant bystander injury to the lung, including the alveolar epithelium, particularly when their activation is excessive and dysregulated as is the case in ARDS [109]. In animal models of ARDS induced by LPS [110], live bacteria [111], virus [112], acid [113], surfactant depletion/VILI [114], TRALI [115], VILI [77], or pancreatitis [116], depletion of neutrophils or blockade of neutrophil influx prevents epithelial injury, as measured by leakage of protein and fluid from the bloodstream into the bronchoalveolar lavage (BAL) fluid. In the oleic acid and hyperoxia models of ARDS, tissue injury is independent of neutrophils [117,118], as these are models of direct injury. Moreover, in in vitro coculture and transmigration systems, neutrophils cause injury to alveolar epithelial cells [109,119] (Fig. 10.2). Mediators of neutrophil-induced lung injury include proteases, oxidants, antimicrobial peptides, and NETs. The role of each of these in causing epithelial injury will be discussed.

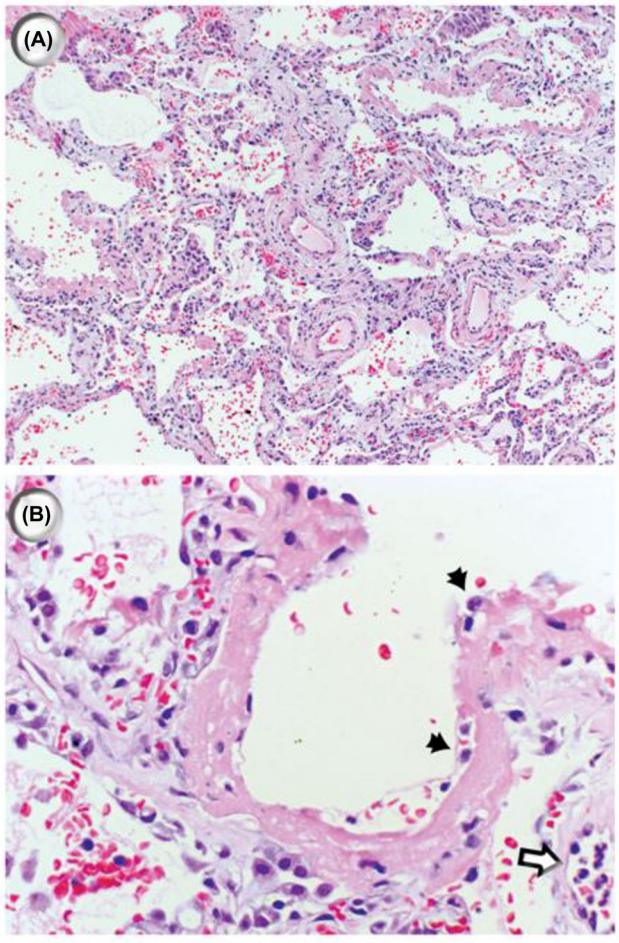

FIGURE 10.1 (A) Histologic image of a lung of a human ARDS patient with gram-negative Sepsis. Shown are features of diffuse alveolar damage, including hyaline membranes, inflammation, intra-alveolar red cells and neutrophils, and thickening of the alveolar–capillary membrane. (B) High power image reveals dense hyaline membrane and diffuse alveolar inflammation. Polymorphonuclear leukocytes are imbedded in the proteinaceous hyaline membrane structure (*black arrows*). The *white arrow* points to the edge of an adjacent alveolus containing polymorphonuclear leukocytes. Images provided by K. Jones, M.D., UCSF. *Adapted from Ref. [393].*

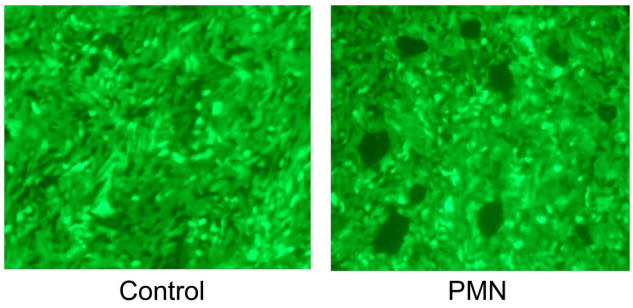

FIGURE 10.2 An in vitro model of inflammatory epithelial injury. Lung epithelial cells were cultured on the underside of inverted transwell inserts. A chemoattractant was placed in the bottom chamber and neutrophils were placed in the top chamber so that neutrophils were induced to migrate in the physiologic basolateral to apical direction. In this model, neutrophils migrate in clusters and this migration results in epithelial cell death, leading to the round holes in the epithelial monolayer. *Adapted from Ref. [393].*

10.2.4.1.1 Proteases

Neutrophils release from their granules a variety of proteases, particularly serine proteases, which can injure the alveolar epithelium. The prototypical serine protease released by activated neutrophils is neutrophil elastase. Because of its potent antimicrobial properties, elastase plays an important role in host defense [120,121]. Well known to cause injury to the alveolar basement membrane during pathogenesis of COPD [122], elastase is also implicated in tissue injury, including epithelial injury, during ARDS. Elastase levels are elevated in the BAL fluid of ARDS patients and correlate with disease severity [123–125]. In animal models of ARDS, elastase inhibitors limit lung injury and improve survival [79,126,127]. For example, elastase inhibition attenuates neutrophil recruitment and epithelial permeability in an animal model of ischemia-reperfusion-induced lung injury [128], LPS [129,130], and Pseudomonas pneumonia [126]. As elastase is required for the migration of neutrophils, with all their toxic mediators, into the lungs [131], it is unclear from these animal studies to what extent elastase directly injures the epithelium. However, in vitro studies suggest that elastase directly causes epithelial injury both via degradation of intracellular junctions [126,132,133] and by inducing epithelial cell death [134]. These injurious effects of neutrophil elastase on the epithelium likely underlie epithelial permeability and impaired gas exchange observed in animal models [126,135]. Elastase similarly injures the endothelium during ARDS [136,137]. Interestingly, elastase also facilitates the ability of certain pathogens to infect epithelial cells [126,138], so increased lytic infection could be another mechanism through which elastase engenders epithelial cell injury. Unfortunately, despite the pivotal role of neutrophil elastase in tissue injury, elastase inhibitors have failed to consistently improve outcomes in ARDS [139–141]. Reasons for this are multifactorial but likely include the heterogeneity of both patient and etiologic factors in ARDS as well as the complexities of regulation of neutrophil migration, priming, and activation in response to many inflammatory mediators including but not limited to elastase, which interact with each other and with neutrophils in a tightly regulated fashion with precision in terms of spatiotemporal regulation, as well as the paradoxical effects of elastase on tissue injury and host defense. Paradoxically, neutrophil elastase has also been implicated in epithelial repair [133].

Other neutrophil serine proteases, such as cathepsin G and proteinase 3, have been shown to cause tissue damage [142], including endothelial cell death [143] under some circumstances. Although the role of these proteases in epithelial injury in the setting of ARDS is not well understood, isolated animal [144,145] and invitro studies [135,146,147] suggest that these proteases contribute to epithelial permeability, including cell death [148,149].

10.2.4.1.2 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are another class of serine proteases that are secreted by neutrophils and macrophages during ARDS and contribute to lung injury. MMP levels are elevated in the lungs of ARDS patients [150–152] and some MMPs portend a poor prognosis [153]. In general, inhibition or genetic deletion of MMPs attenuates lung injury [154–157], although the mechanisms are not fully elucidated and are likely multifactorial and vary depending on the specific MMP. For example, MMP7 has been shown to be critical for neutrophil migration into the airspaces by cleaving syndecan-1, a heparin sulfate proteoglycan necessary for the establishment of a neutrophil chemokine gradient [158]. Although mice deficient in either MMP3 or MMP9 are protected from acute lung injury, MMP3 but not MMP9 is critical for neutrophil influx [159]. MMPs clearly degrade the extracellular matrix [160], an effect that might facilitate neutrophil influx and/or lead to secondary loss of epithelial cells. MMP7 can cleave E-cadherin from the adherens junctions of epithelial cells [161], and MMP9 disrupts epithelial tight junctions [162]. Some MMPs are actually protective against lung injury [163]. In summary, although the role of MMPs in inflammation is fairly well understood [164], whether MMPs, like elastase, directly induce epithelial injury via cleavage of intercellular junctions or inducing cell

death in the setting of acute lung injury is not fully known [165]. MMPs can also paradoxically enhance epithelial survival [166], and some have been implicated in epithelial repair [161,167,168].

10.2.4.1.3 Peptides

In addition to proteases, the neutrophil contains peptides that have antimicrobial properties but can cause lung injury. One class of antimicrobial peptides that has been implicated in inflammatory lung disease is the defensins [169]. Defensins are elevated in the lungs of ARDS patients [170] and have been shown to induce epithelial permeability [135,171] and cell death [147,172].

10.2.4.1.4 Oxidants

Reactive oxygen and nitrogen species derived from neutrophils and macrophages (as well as epithelial cells and endothelial cells) clearly contribute to lung injury during ARDS [173]. The lungs of ARDS patients have high levels of oxidants [125,174–177] and low levels of antioxidants [178], and the degree of oxidative stress correlates with mortality [179]. It has long been known that oxidants cause lung injury in animal models [64,180–187]. However, as with the other toxic mediators, because oxidants have multiple effects on many cell types, the precise effect of various oxidant species on the alveolar epithelium during ARDS is difficult to discern. Several studies have demonstrated that oxidants induce epithelial cell death [64,66,85,188–195] as well as disassembly of tight junctions [196–198]. Of course, endogenous mechanisms are in place to limit oxidant-mediated damage. For example, hypoxia inducible factor (HIF) signaling protects against oxidant-induced epithelial barrier dysfunction [196]. Interestingly, pharmacologic activation of autophagy attenuates oxidant-induced epithelial cell apoptosis [199]. Another mechanism through which oxidants induce lung injury is by exacerbating the destructive effects of elastase by oxidizing and inhibiting endogenous elastase inhibitors [200]. Initial studies of antioxidants in ARDS patients were promising [201], with improvements in barrier function oxygenation, ventilator dependence, and length of stay in the intensive care unit (ICU); however, subsequent studies revealed no benefit and some even suggested harmful effects [202–206].

10.2.4.1.5 Neutrophil Extracellular Traps

In addition to proteases and oxidants, neutrophils effect tissue damage through the formation of NETs, which involves the release of DNA and histones [207]. Potently microbicidal, NETs are also toxic to host cells. The effects of NETs on tissue damage, including epithelial cell death has been observed in multiple animal models of ARDS [208–213] and sepsis [214], as well as in vitro studies [215]. Histones are elevated in ARDS patients [216].

10.2.4.2 Macrophages

Although neutrophils are the classic inflammatory cell associated with tissue injury in ARDS, recruited monocyte-derived macrophages are proinflammatory and can affect significant tissue injury. In influenza infection, blocking recruitment of monocyte-derived macrophages into the lung is protective against tissue injury without impairing viral clearance [217]. One mechanism underlying this phenomenon is the production of TRAIL by monocyte-derived macrophages, which interact with death receptors on the surface of infected epithelial cells, inducing epithelial cell apoptosis [218,219]. Cellular inhibitor of apoptosis protein cIAP2 is an endogenous protective mechanism that limits TRAIL-induced epithelial cell death [220]. Recruitment of a specific monocyte-derived dendritic cell population also contributes to tissue injury during viral infection in part by presenting antigen to cytotoxic CD8+ T cells [221]. Virus-specific CD8+ T cells are critical for clearing virus but do so via contact-dependent death of epithelial cells, either via perforin/granzyme-mediated cell lysis or Fas-dependent apoptosis [222]. Given the role of monocytes in lung injury, a human trial was performed in which monocytes were depleted from human subjects via leukapheresis, followed by treatment with inhaled LPS. Monocyte depletion had no benefit as measured by BAL protein or inflammation [394]. The failure of monocyte depletion to improve outcomes may be partially related to a paradoxical beneficial effect of macrophages after LPS-induced injury. In mice treated with LPS, recruited macrophages are protective against epithelial injury via IL1 receptor antagonist production, which attenuates epithelial cell apoptosis [223]. Resident alveolar macrophages are also protective against epithelial injury in an influenza model of lung injury [210]. The conflicting effects of macrophages on epithelial injury suggest that further investigation is needed to discern the effects of various macrophage populations, each with plasticity in their phenotypes, on tissue injury in different settings [224].

10.2.4.3 Platelets

In addition to leukocytes, platelets have recently been recognized to play an important role in lung injury [209,225]. Although platelets may not directly injure the alveolar epithelium, they likely potentiate neutrophil-mediated tissue damage [226].

10.2.4.4 Additional Mediators

Clearly, there are additional mediators that induce epithelial cell injury, such as TGFβ [227] and angiopoietin 2 [228]. Severe hypoxia may also be a mechanism driving alveolar epithelial cell apoptosis in the setting of ARDS [229]. Hypoxia may impair junctional integrity [230] and induce apoptosis [229], leading to epithelial permeability [231], although HIF can also protect against apoptosis [232]. There are also endogenous mechanisms in place that limit epithelial injury. For example, proresolving lipids, critical for the resolution of inflammation [233] have recently been shown to limit alveolar epithelial cell apoptosis [234], as has survivin [235]. Growth factors such as keratinocyte growth factor (KGF) [236] and granulocyte macrophage colony stimulating factor (GMCSF) [237,238] protect against epithelial cell death. Hyaluronan expressed on the epithelial cell surface limits epithelial cell apoptosis, contributing to recovery of the epithelial barrier after lung injury [239].

10.3 ROLE OF EPITHELIUM IN HOST DEFENSE & INFLAMMATION

Resident alveolar macrophages initiate the host defense response to pathogens. They phagocytose opsonized pathogens and, in the case of viral infections or intracellular bacterial infections, they clear infected epithelial cells via efferocytosis [86]. In turn, resident alveolar macrophages, as well as ATII cells [240], produce a variety of inflammatory mediators, including neutrophil chemokines that induce the influx of neutrophils. Neutrophils are also capable of clearing virus-infected epithelial cells, thus limiting propagation of viral infection [86]. Depletion of resident alveolar macrophages or neutrophils generally results in impaired ability to contain infection, in turn increasing mortality [241,242]. Cytotoxic CD8+ T cells are a critical component of host defense against viral or other intracellular pathogens, destroying any infected cell. The role of the professional innate and adaptive immune cells in host defense in ARDS induced by pneumonia/infection has been reviewed in detail elsewhere [222,243].

Although the innate immune system is clearly critical for host defense in ARDS caused by infection, the role of epithelial cells in host defense has become increasingly recognized. Although more is known about the role of the airway epithelium than the alveolar epithelium in host defense [244–246], and the antimicrobial functions of the airway epithelium contributes to maintenance of relatively pathogen-free alveoli [245], alveolar epithelial cells are likely to possess similar host defense capabilities as the airway epithelium.

First of all, the physical barrier created by the alveolar epithelium is the first line of defense against invading pathogens, preventing their entry into the systemic circulation. Second, the epithelium secretes various factors with important immune functions. Besides neutrophils, the epithelium is an important source of antimicrobial peptides [245,247,248]. In addition, surfactant proteins (SP) produced by ATII cells line the alveoli and contribute to the constitutive antimicrobial milieu of the lung that precedes activation of the innate immune system. SP-A and SP-D are collectins, which have several antimicrobial properties. First, they opsonize bacteria, viruses, and other pathogens, thus enhancing clearance by phagocytes [249,250]. In addition, they can activate phagocytes to uptake pathogens independently of serving as opsonins [250]. They can also bind to Toll-like receptors (TLRs), triggering proinflammatory cascades [250]. Finally, SP-A and SP-D can directly kill pathogens [251], in part by acting cooperatively with NETs [252]. A comprehensive review of the role of SP in the immune response has been performed elsewhere [250]. Surfactant phospholipids can have immune functions as well [253].

In addition to constitutive secretion of immunomodulatory proteins, the epithelium also plays a critical role in the initiation of the innate immune response to invading pathogens [254]. Pathogen-associated molecular patterns (PAMPs) expressed on invading microorganisms as well as damage-associated molecular patterns (DAMPs) derived from injured host cells are recognized by pattern recognition receptors (PRRs) on resident lung cells. Although alveolar macrophages are a crucial resident lung cell in the response to invading pathogens, epithelial cells also express PRRs and respond to PAMPs and DAMPs [244]. Epithelial cells respond by producing chemokines, which in turn recruit inflammatory cells, initially neutrophils and then inflammatory macrophages [255,256]. Also, proinflammatory cytokines such as TNFα, IL6, and IL1β produced by the alveolar macrophages in response to PAMPs and DAMPs also act via paracrine signaling to induce epithelial cells to produce chemokines [257].

Both bronchial [258,259] and alveolar [256,260,261] epithelial cells express the important class of PRRs, the TLRs, and respond to TLR stimulation by synthesizing neutrophil chemokines. In fact, in some circumstances, the responses of epithelial cells to TLR ligands play a more important role in host defense than the response of leukocytes [262]. Alveolar epithelial cells can produce many neutrophil chemokines, including IL-8 [263,264] and its ortholog in rodents KC [263], as well as SDF-1 [265], all of which play an important role in neutrophil influx into the lungs during acute lung injury. Alveolar epithelial cells are the primary source of CXCL5 or Lix [266–268], which also contributes to neutrophil recruitment in animal models of acute lung injury [269,270]. Parenthetically, CXCL15 or lungkine is produced by bronchial epithelial cells [271,272]. Alveolar epithelial cells can also produce monocyte and lymphocyte chemokines, including CCL2 [256,257,263,273,274], CCL7 [275], CCL5 [274,276], and CXCL10 [274]. In addition, ATII cells are likely the primary source of GMCSF during lung injury [237,256,277,278]. GMCSF levels are elevated in the BAL fluid of ARDS patients [279] and stimulates immune function of macrophages and neutrophils [280], as well as dendritic cell function [281]. Epithelial cells also directly communicate with professional immune cells during an inflammatory response. One important study demonstrated that a subset of alveolar macrophages modulates the immune functions of alveolar epithelial cells by Ca^{2+} waves conducted through gap junctions between the two cell types [282]. As mentioned, alveolar epithelial cell products have been demonstrated to regulate dendritic cell function in an influenza model [281].

Most of the literature on alveolar epithelial cell immune and inflammatory function ascribes these functions to ATII cells. However, as ATI cells are difficult to study both in vitro and in vivo and many of the papers discerning immune/inflammatory function of alveolar epithelial cells were performed in vitro, when ATII cells rapidly transdifferentiate into ATI cells, it would not be surprising if we come to discover that ATI cells also have important immune/inflammatory functions. In that context, one recent report revealed that ATI cells activate inflammatory pathways during pneumonia [268].

10.4 REPAIR

10.4.1 Epithelial Repair

As mentioned, ATI cells are particularly susceptible to injury, although ATII cells can be damaged in the case of severe injury, particularly when the offending insult preferentially affects ATII cells. After mild–moderate lung injury resulting in loss of ATI and/or ATII cells, surviving ATII cells are activated to proliferate to replace lost ATI and ATII cells and ultimately transdifferentiate to replace lost ATI cells [283–288].

Several growth factors have been identified to induce ATII cell proliferation during repair after lung injury, including KGF, HGF, EGF, and GMCSF. KGF has been considered the prototypical ATII cell mitogen [289], having been shown both in vitro [290] and in vivo [291,292] to promote ATII cell proliferation. KGF also promotes epithelial cell spreading [293] and protects against epithelial cell damage [236]. Interestingly, KGF can also resolve paracellular permeability, in part by enhancing actin cytoskeletal function near the apical junctions [294]. Because of these effects, KGF has been shown to have beneficial effects in human [295] and animal [296–298] models of lung injury. HGF also potently induces ATII cell proliferation in vitro [290,299,300], although its role in repair after acute lung injury in vivo has not been firmly established. GMCSF is produced mainly by the alveolar epithelium and functions via autocrine signaling to promote ATII cell proliferation and barrier restitution [237,277,278]. GMCSF has also been shown to be an endogenous mechanism limiting epithelial cell death [237,238]. Of course, GMCSF stimulates myeloid cell differentiation and immune function [301]. Given the cytoprotective/reparative and immune functions of GMCSF, several clinical trials have been performed [302,303]. Although not definitive, results have been promising enough that GMCSF continues to be the subject of current and planned trials for ARDS [24]. EGF has also been shown to promote ATII cell proliferation during regeneration in various settings [285,304–306]. The proliferative response that ensues to replace lost epithelial cells in response to these growth factors can sometimes result in excessive numbers of ATII cells. Excessive ATII cells then undergo apoptosis to restore normal cell numbers [307,308].

The role of these growth factors in ATII cell proliferation begs the question of their cell source and the role of signals from other alveolar cell types in epithelial repair. KGF and HGF are likely produced in large part by the alveolar fibroblasts [290]. Alveolar lipofibroblasts have recently been identified as constituting the niche for ATII stem cell function [286], suggesting that they may promote ATII cell proliferation after injury, perhaps via KGF and HGF secretion. EGF may derive from alveolar macrophages [304]. Endothelial-derived factors also contribute to epithelial repair, either directly or indirectly. Elegant studies by Ding, Rafii, and colleagues demonstrated that during lung regeneration after pneumonectomy, endothelial cell-derived MMP14 cleaves heparin-bound EGF, allowing it to signal through

EGFR to induce ATII cell proliferation [306]. Interestingly, endothelial cell MMP14 production is triggered by CXCL12 released from platelets, demonstrating that platelets also contribute to epithelial repair [309]. Whether these phenomena occur during repair after acute lung injury or whether there are additional endothelial-derived factors that promote epithelial repair after lung injury remains to be determined. Foxp3+ regulatory T cells, known to promote the resolution of inflammation [310], have recently been shown to induce ATII cell proliferation [311]. This effect may be partially mediated by expression of KGF by the Tregs [312]. Macrophages contribute to ATII cell proliferation, for example via secretion of TNF, which stimulates epithelial production of GMCSF [278]. Even neutrophils, while causing epithelial injury, promote ATII cell proliferation [133]. Although speculative, it would not be surprising if every cell type present in the alveolus after lung injury, whether resident or recruited, contributes in some way, either directly or indirectly, to epithelial repair.

In addition to secreted growth factors, a number of transcriptional pathways have been identified that promote ATII cell proliferation after lung injury. Wnt/β-catenin signaling, known to regulate lung development [313], is activated in ATII cells after lung injury [133,314,315] and promotes ATII cell proliferation [133,316]. β-catenin signaling is also protective against epithelial cell death during lung injury [314,315]. A landmark paper revealed the importance of FoxM1, a member of the FOX family of transcription factors, in ATII cell proliferation after lung injury [317]. In ARDS, despite the multitude of endogenous mechanisms that promote ATII cell proliferation, proliferation may be impeded by various mediators such as elevated CO_2 [318].

Although ATII cells are primarily responsible for reepithelializing the denuded alveolar surface after mild−moderate epithelial injury, in more severe cases of injury, particularly when ATII cells themselves are highly injured, other cell types can be mobilized to regenerate the alveolar epithelium [44] (Fig. 10.3). Several different cell types have been identified as progenitors of the alveolar epithelium after injury. Club cells, a differentiated bronchiolar epithelial cell responsible for the production and secretion of a secretoglobulin protein, have been shown to differentiate into ATII cells after injury induced by bleomycin [286,287,319], a chemotherapeutic agent that directly injured ATII cells and can render them unable to proliferate or even become senescent [320]. A subpopulation of club cells that also express the ATII cell marker SPC (SPC) has been identified. These cells, designated as bronchoalveolar stem cells (BASCs), are imputed to efficiently give rise to ATII cells after injury [321]. Interestingly, the endothelium supports the differentiation of BASCs into ATII cells via secretion of paracrine factors [322]. Influenza causes a severe injury to the alveolar epithelium, with ablation of huge numbers of ATII cells as the influenza virus HA specifically recognizes sialic acid residues on the ATII cell surface [88−91]. In response to this immense loss of the ATII cell progenitor, a population of basal-like [keratin (K)5+ Trp63+] cells preexisting in the alveoli repopulates the alveolar septa [44]. This population may derive from preexisting K5+ basal cells [87] or from a rare "lineage negative" progenitor that expresses integrin β4 and appears to exist dormant in the alveoli under conditions of homeostasis and mild injury [44,323]. A K5+ population also arises after bleomycin-induced injury [324]. Although this population is capable of differentiating into ATII cells under certain conditions (i.e., pharmacologic inhibition of Notch signaling), this

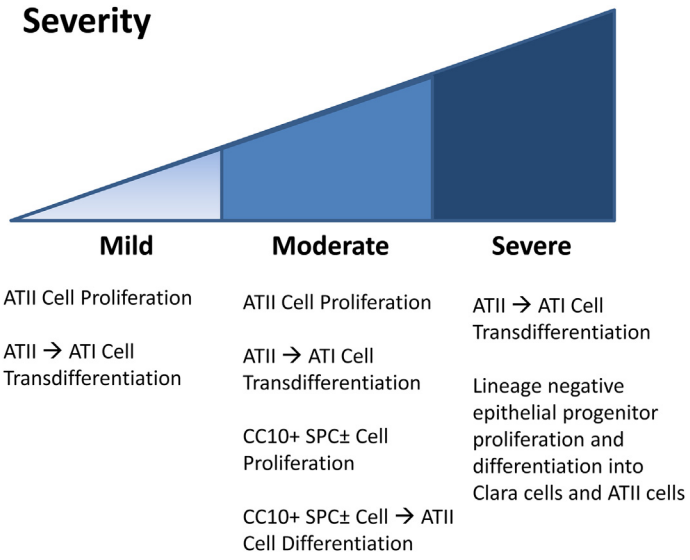

FIGURE 10.3 The specific epithelial progenitors responsible for reepithelializing the alveolar surface after injury depend on the severity and nature of the injury. In mild-moderate injury, ATII cells are the principal progenitor. In moderate–severe injury, CC10 + SPC + / − cells also serve as progenitors. In severe injury, basal-like lineage negative epithelial progenitors (LNEP) also contribute to reepithelialization. *Adapted from Ref. [44].*

reparative response to influenza infection does not by default restore normal alveolar architecture [44]. Still the response of these cells is so dramatic that pharmacologic agents to drive their differentiation into ATII cells and ultimate ATI cells could be a plausible therapeutic approach to lung regeneration after severe injury. Another unlikely progenitor of the alveolar epithelium is the ATI cell. Long assumed to be terminally differentiated, a recent study revealed that ATI cells are capable of generating new ATI and ATII cells to regenerate lung after pneumonectomy [325]. Transdifferentiation of an ATI cell to an ATII cell is a remarkable feat considering the difference in morphology between the two cell types. It is unknown whether ATI cells directly transdifferentiate into ATII cells or whether they first dedifferentiate to a bipotent progenitor capable of an ATI or ATII cell fate, as has been observed during lung development [285,326]. Moreover, whether ATI cells are important progenitors for lost ATI and ATII cells after acute lung injury remains to be determined. In sum, many epithelial cell types in the lung, perhaps with the exception of ciliated cells, can serve as progenitors for alveolar epithelial cells destroyed during injury. These studies have provided increasing awareness of the remarkable plasticity of lung epithelial cells that was not previously fully recognized. Much of this information derives from novel methodology allowing the tagging of a given cell type with a marker that persists in all progeny [327]. This methodology allows for lineage tracing, i.e., determining cell fate of various progenitors. The extent to which such progenitors exist in the human lung and are mobilized after injury in human ARDS remains to be determined, although one report suggests that some of these progenitors do repopulate the alveoli in human pulmonary fibrosis [328].

Overall, the evidence to date suggests that if ATII cell largely survive, they can effectively replace lost ATI and ATII cells after lung injury. However, in the setting of severe ATII cell loss, as occurs in influenza infection [44,87] or due to drugs or toxins [320,329] or genetic defects [330] which selectively kill ATII cells or impair their regenerative capacity including by inducing senescence, or the experimental ablation of ATII cells [331] results in an inability of the ATII cells to restore lost cells. In these cases, either a variety of alternative progenitors are mobilized to differentiate into ATII and ultimately ATI cells (Fig. 10.3) and/or abnormal repair ensues, resulting in pulmonary fibrosis or emphysema [332–336]. In fact, a subset of ARDS patients exist in whom normal repair fails, leading to fibroproliferation, characterized by excessive fibroblasts and matrix deposition [337,338]. Fibroproliferative ARDS carries a high mortality rate [339].

The identification of these alternate epithelial progenitors has set the foundation for additional investigation into the mechanisms of epithelial repair after lung injury. As the heterogeneity within individual cell types is increasingly being recognized [285,286,326], an outstanding question in the field of lung repair and regeneration is whether there are subpopulation of progenitor cells, whether ATII cells or these alternate progenitors mobilized under certain circumstances, that have enhanced capacity for proliferation and differentiation after lung injury [340–342]. One example of this is a recently identified subpopulation of ATII cells that express Sca1 was recently identified to proliferate with enhanced efficiency after lung injury [343]. In addition, studies to further identify the molecular mechanisms that underlie transition from one cell type to another during repair after lung injury, and whether these transitions are direct or proceed through an undifferentiated progenitor, are needed. In addition to in vivo lineage tracing studies, in vitro differentiation of induced pluripotent stem cells will provide additional insight into regulatory pathways [341,344].

As discussed, ATI cells are particularly susceptible to injury, although in certain circumstances ATII cells are also damaged. After epithelial injury, lost cells are replaced via proliferation of ATII cells or, in the case of severe injury, alternate progenitors. Lost ATI cells are then replaced through transdifferentiation of ATII cells into new ATI cells. The critical role of the ATII cell as a progenitor for ATI cells has been known since the 1970s, when experiments using radiolabeled thymidine that tags proliferating ATII cells, with the subsequent observation of new labeled ATI cells [283]. Further support for this phenomenon derived from the transdifferentiation of primary ATII cells into ATI cells during cell culture [345–347]. Recently, the role of the ATII cell as the primary stem cell to regenerate lost ATI and ATII cells after injury has been confirmed using modern lineage tracing techniques [285–287]. The molecular mechanisms underlying the transdifferentiation of ATII cells into ATI cells are unknown, although there is some evidence that β-catenin signaling may be involved [315,343,348,349]. Again, whether ATII cells directly transdifferentiate to ATI cells or proceed via a bipotent progenitor [285] is also unknown. Other progenitors are capable of differentiating into ATI cells [44,87,350], although it is unknown whether this differentiation is direct or proceeds through an ATII-like cell and whether ATII cells derived from these other progenitors are as efficient at transdifferentiating into ATI cells. Transdifferentiation of ATII cells into ATI cells requires a remarkable degree of cell spreading and flattening. Mechanisms that have been identified to promote ATII cell spreading include β-catenin [351], KGF [293], TGFα [352], TGFβ [314,353,354], PTEN inhibition [355], and IL-1β [356]. Unfortunately, during lung injury, these potent endogenous mechanisms exist to drive ATII cell spreading can be impaired. For example, in addition to causing epithelial injury, mechanical strain such as that induced by overdistention during mechanical ventilation has been shown to impair epithelial cell spreading during wound repair in vitro [76].

Although mesenchymal stem cells (MSCs) are unlikely to serve as epithelial progenitors during repair after lung injury [341], exogenously administered MSCs appear to provide a multitude of beneficial effects in ARDS models [357]. In preclinical models of acute lung injury, MSCs mitigate lung permeability and improve survival [358]. The mechanisms through which MSCs confer protection in ARDS are likely multifactorial and almost certainly are related to paracrine effects of the MSC secretome on the alveolar epithelium. One report suggested that MSC-derived angiopoitin-1 enhanced barrier function in a cultured ATII cell monolayer [359]. Additional factors that may act in a paracrine fashion on the injured lung include IL1ra, TSG-6, IGF1, prostaglandin E2, and KGF [357]. A remarkable study demonstrated that MSCs can transfer mitochondria to epithelial cells via gap junctions, enhancing the bioenergetics of the epithelial cells, which in turn promotes barrier function [360]. Endogenous MSCs are unlikely to be recruited to the injured lung to effect repair [341]. However, because of the multitude of beneficial effects of MSCs observed in preclinical models, MSCs are currently being tested in clinical trials of ARDS [361,362]. Although not yet established to have benefit, trials of MSCs for ARDS and other diseases suggest that they are safe.

10.4.2 Resolution of Inflammation

Resolution of lung injury requires not only repair of the injured tissue but also clearance of inflammatory cells to prevent ongoing tissue injury. Despite inflammatory mediators that enhance neutrophil lifespan [363], neutrophils eventually become apoptotic. Neutrophil apoptosis is triggered in part in response to regulatory T cells that are recruited to the lungs late in the injury time course [310]. Apoptotic leukocytes are recognized and ingested by tissue macrophages via specific surface receptors on the cell membrane [364]. Dying cells are cleared, usually by professional phagocytes such as alveolar macrophages by a process called “efferocytosis” [365]. The dying cell expresses “find me” and “eat me” signals that attract the macrophages and bind the phagocytic receptors, respectively. ATII cells contribute to efferocytosis, as SP bound to the surface of apoptotic cells enhance their uptake by macrophages [366]. Because this process is extremely rapid, the number of visible apoptotic leukocytes at sites of tissue inflammation is usually low, and probably underestimates the extent to which apoptosis is occurring [367]. Inflammatory macrophages must also die and be cleared during the resolution of acute lung injury [368]. Finally, debris from epithelial cells that have died and sloughed off the alveolar surface must be cleared.

As apoptotic cells contain antigens that can be recognized by autoantibodies, failure of the clearance of apoptotic cells, which leads to secondary necrosis, can result in autoimmunity [369,370]. In addition, efferocytosis of apoptotic cells induces an antiinflammatory phenotype in macrophages, which further promotes the resolution of inflammation [371]. A group of lipid mediators celled resolvins are critical regulators of the clearance of inflammatory cells and debris after lung injury. The mechanisms by which resolvins contribute the resolution of inflammation has been reviewed elsewhere [233].

Epithelial cells themselves can function as nonprofessional phagocytes under certain circumstances, clearing dead or dying epithelial cells. This occurs in a Rac1-dependent fashion in the airway epithelium, with failure of this process resulting in exacerbated and prolonged inflammation in an asthma model [372]. Whether alveolar epithelial cells also function as nonprofessional phagocytes, clearing dead or dying alveolar epithelial cells with resultant antiinflammatory effects during the resolution phase of ARDS, remains to be determined.

10.4.3 Reabsorption of Edema Fluid

During the resolution of ARDS, once the pathogens have been killed, the barrier is sealed, and inflammatory cells and debris have been cleared, edema fluid must be reabsorbed. In fact, alveolar fluid clearance correlates with positive clinical outcomes including survival [20,23,373]. It was established long ago that fluid clearance is driven by active ion transport [374]. $Na+/K+$ ATPases located on the basolateral surface of alveolar epithelial cells pump $Na+$ out of the cell into the interstitial space in exchange for $K+$. The resultant low intracellular $Na+$ levels generate a $Na+$ gradient that drives passive transport of $Na+$ from the alveolar fluid into the cell via an apical epithelial $Na+$ channel (ENaC). This resultant transepithelial sodium gradient then drives water reabsorption [31]. Although historical evidence pointed to ATII cells as primarily responsible for alveolar ion and fluid transport [375], it is now known that ATI cells express both the $Na+/K+$ ATPase and ENaC [376], and it has recently been demonstrated using state-of-the-art in vivo genetic models that ATI cells account for at least half of alveolar fluid clearance [377,378].

Unfortunately, not only is the amount of alveolar fluid dramatically increased after lung injury, but the rate of alveolar fluid clearance is also decreased in both animal models of lung injury [379] and ARDS patients [20]. One likely mechanism underlying the impaired fluid clearance observed in lung injury is the effect of hypoxia, which triggers

endocytosis and degradation of the Na+/K+ pump [380,381] as well as impaired expression and activity of ENaC [382]. This may occur via HIF signaling as an adaptive mechanism to conserve ATP in the setting of limited oxygen supply [380–383], perhaps as a mechanism to conserve ATP under hypoxic conditions. In addition, the elevated CO_2 present in the lungs of ARDS patients have also been shown to downregulate Na/K pump function [384,385]. A recent elegant study by Peteranderl et al. revealed that macrophage-epithelial cell crosstalk during lung injury further contributes to Na+/K+ downregulation. They demonstrated that epithelial derived type I interferon increased macrophage expression of TRAIL, which then drives Na+/K+ downregulation in an influenza mouse model [386]. Proinflammatory cytokines such as IL-1β also induce downregulation of ENaC [387].

Overexpression of the Na+/K+ ATPase has been shown to augment fluid clearance after lung injury in animal models [388]. β-agonists can also enhance alveolar fluid clearance, by increasing expression and function of both the Na+/K+ ATPase and ENaC [31]. Therefore, clinical trials were performed to determine whether inhaled β-agonists might improve clinical outcomes by enhancing alveolar fluid clearance in ARDS patients. Unfortunately, these trials have revealed no benefit of β-agonists in ARDS patients [389,390]. The failure of improvement may be a result of desensitization of the alveolar epithelial cells to β-agonists [31] or ongoing leak due to persistent barrier dysfunction.

10.5 CONCLUSION

In summary, the alveolar epithelium plays a critical role in the pathogenesis of ARDS. During homeostasis, the epithelium forms a tight barrier that maintains dry airspaces. Barrier function is attributable to both the continuity of ATII and ATI cells as well as the tight junctions that form between them. During ARDS, this barrier is disrupted due to impairment of tight junctions and destruction of epithelial cells. Such epithelial injury can be directly induced by certain insults, such as viral infection and acid aspiration but is also largely attributable to the toxic mediators of inflammatory cells, particularly when inflammation is excessive and dysregulated. In addition to serving barrier function, the alveolar epithelium also plays an important role in host defense and inflammation during lung injury via production of factors that are directly microbicidal or recruit and enhance the microbicidal function of professional immune cells. In patients who survive ARDS, the alveolar epithelial repair ensues. ATII cells are the primary progenitor cell responsible for repopulating the damaged epithelium after mild–moderate lung injury. However, in cases of severe injury, particularly when the ATII cells are destroyed or their proliferative capacity is impaired, alternate progenitors are mobilized. Once the epithelium is repaired, epithelial cells must clear edema fluid to restore dry airspaces, although some of the mechanisms that promote fluid clearance are impaired during ARDS. Most ARDS survivors remarkably recover lung function [391]. However, failed epithelial repair results in a fibrotic response and/or patient mortality. Although lung protective ventilation and fluid restriction have improved outcomes in ARDS [11], clinical trials aimed at attenuating inflammatory injury, promoting epithelial repair, or promoting fluid reabsorption have not yet yielded effective therapeutic approaches. Optimism remains that enhanced understanding of the cellular and molecular mechanisms underlying the pathogenesis of ARDS will ultimately yield effective therapies to prevent [392] or promote the resolution of lung injury.

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Chapter 11

Epithelial Barrier Dysfunction in Asthma

Janelle Veazey, Tim Smyth and Steve N. Georas

11.1 EVIDENCE FOR BARRIER DYSFUNCTION IN ASTHMA

There are multiple components of the airway epithelial barrier that protect against the development of asthma including airway surface liquids, secreted peptides, mucus, the mucociliary escalator, and junctional complexes that form between neighboring epithelial cells. Junctional complexes include apical tight junctions (TJ) and adherens junctions (AJ), the compositions of which were summarized in previous chapters. Defects in airway surface liquids and mucociliary clearance have been implicated in the pathogenesis of asthma and will not be discussed further in this chapter [1,2]. Instead, we will focus specifically on evidence that defective structure and function of epithelial TJ and AJ is a feature of epithelial cells in asthma. Future research investigating the relationship between mucus composition, mucociliary clearance, and junctional complex structure and function will be needed to understand how these different processes relate to each other.

Airway biopsies are not routinely obtained from asthmatic subjects. Furthermore, unlike patients with chronic obstructive pulmonary disease, asthma patients rarely die or undergo lung transplantation due to respiratory failure. Consequently, airway tissue from asthmatic subjects is not routinely available but only obtained during bronchoscopy for research purposes. A few research groups have obtained primary airway biopsies or epithelial brushings from asthmatic compared with nonasthmatic donors and studied the structure of epithelial TJ and AJ. Table 11.1 summarizes the results of seven studies to date, noting the different approaches that have been used by different groups [3–11]. The structure of junctional complexes was investigated in tissue biopsies (or cadaveric lung samples) using immunohistochemistry with antibodies directed against specific TJ or AJ components [3,6,9,10]. This approach has the advantage of visualizing junctional complexes in their native context and avoids potential artifacts introduced by propagating cells in vitro in cell culture. When comparing biopsies from subjects with asthma compared with nonatopic or atopic controls, de Boer et al. found statistically significant reductions in α-catenin, E-cadherin, and ZO-1 that was localized to the superficial epithelium [3]. Reduced ZO-1 expression was also observed in bronchial biopsies by Xiao et al. [6]. In cadaveric lung samples obtained from subjects with asthma, Hackett et al. reported that E-cadherin and β-catenin expression was reduced when compared with lung tissues from nonasthmatic controls [9,10]. Sweerus et al. found that claudin-18 mRNA expression was reduced in epithelial brushings from asthmatics compared to control subjects and implicated defective expression of this TJ protein in enhanced epithelial permeability [11].

In other studies, epithelial brushings were obtained from asthmatic and nonasthmatic subjects, and cells were propagated in vitro at air–liquid interface (ALI). This approach has the advantage of allowing functional studies of the epithelial barrier, for example, by measuring transepithelial electrical resistance (TEER) and paracellular permeability. Three studies reported that TEER was reduced in epithelial monolayers obtained in this manner from asthmatic donors as compared to healthy control subjects [20,21,24]. Similar results were observed using nasal epithelial cells obtained from subjects with chronic rhinosinusitis and nasal polyposis, which demonstrated reduced TEER and higher permeability than tissues or cells from control subjects [25]. Xiao et al. also found that permeability was higher in airway epithelial monolayers grown from bronchial brushing of asthmatic subjects compared with controls, especially those with moderate and severe disease [24]. However, other studies reported that TEER was unchanged in epithelial cells obtained asthmatic, compared to control subjects [19,22,23]. Reasons for these apparent discrepancies between studies are not known but may relate to differences in subject characteristics or cell culture conditions used. The use of epithelial cells propagated in vitro has the potential to introduce artifacts due to defects in cell growth or differentiation.

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TABLE 11.1 Evidence for Defects in Airway TJ and AJ Structure in Asthma

Year	Tissue Source and Subject Characteristics	Key Findings	Reference
2008	Bronchial biopsies from adults with asthma or atopy	• Statistically significant reduction in expression of α-catenin, E-cadherin, and ZO-1 in superficial epithelial cells from asthmatics • Slight reduction in α-catenin in biopsies from atopic nonasthmatics	[3]
2009	Bronchial brushings from children with asthma	• No significant difference in TEER in asthmatic epithelium	[4,5]
2011	Biopsies and brushings from mild, moderate, and severe asthmatics	• Reduced junctional protein expression in asthma, especially ZO-1 • Reduced barrier function, especially in moderate and severe asthmatics	[6]
2013	Bronchial brushings from adults	• Reduction in baseline TEER asthmatic epithelium	[7]
2013	Bronchial brushings from adults	• Reduced TEER at baseline in epithelial cells from severe asthmatics • No evidence of Timothy grass extract-induced barrier dysfunction	[8]
2013	Cadaveric lungs and bronchial brushings from adults	• Reduced E-cadherin and β-catenin in cadaveric lung sections • No baseline difference in TEER in asthmatic epithelial cells	[9,10]
2016	Bronchial brushings from adults	• Statistically significant reduction in Claudin-18 mRNA in asthmatic epithelial cells	[11]

A: asthma, NA: nonasthma.
ALI: epithelial cells from brushings or lung digests propagated in vitro in defined culture medium for several weeks.
ECIS: electrical cell impendence sensing.

For example, defects in barrier function observed in epithelial monolayers grown in vitro for several days or weeks could be due to defective expansion or differentiation of epithelial precursors, rather than intrinsic defects in the structure and function of AJ or TJ. The fact that treatment with epidermal growth factor restored barrier function toward normal in cells from asthmatic donors supports the idea that some of the barrier defects reported in cell monolayers are potentially reversible [24]. Regardless of mechanism, taken together, the studies summarized in Table 11.1 strongly suggest that defects in airway epithelial barrier structure and function are frequently observed in asthma.

The need to use bronchoscopy to obtain airway epithelial cells or airway biopsies from asthmatic subjects is not a practical approach for widespread clinical studies. Consequently, there is a current need to develop noninvasive approaches to measure airway barrier dysfunction in asthma. One strategy involves measuring the concentration of club cell secretory protein (CCSP) in circulation. CCSP, which is also known as uteroglobin or CCSP 16 (CC-16), is normally secreted apically into the airway lumen by epithelial cells. Increased serum or urine CCSP concentrations have been used to infer the presence of defective epithelial barrier integrity (allowing translocation of luminal CCSP into intraepithelial lymph channels and ultimately to the circulation) [9] and have been observed after exposure to ozone [10], cold-dry air challenge [11], and following respiratory syncytial virus (RSV) infection in children [12]. Surprisingly, serum levels of CCSP were found to be lower in two different cohorts of asthmatics compared to healthy controls [12,13]. This could reflect improved barrier function in long-standing asthma (e.g., due to airway remodeling) but is difficult to reconcile with the studies identifying defects in airway epithelial junction and structure described in Table 11.1. Alternatively, CCSP levels may be lower in asthma because the number of CCSP + epithelial cells is decreased in the asthmatic airway [14]. Until other non-invasive approaches are developed, bronchoscopy with airway biopsy or epithelial brushing remains the most direct way to measure airway barrier function in asthma.

11.2 CAUSES OF EPITHELIAL BARRIER DYSFUNCTION IN ASTHMA

Epithelial barrier defects in asthma could be caused by genetic or acquired defects in the expression of junctional complex proteins. Two studies to-date have identified genetic variants in junction-related proteins associated with asthma. First, Koppelman et al. performed linkage analysis in 200 Dutch asthmatic patients with the goal of identifying genes associated with bronchial hyper-responsiveness (BHR) [15]. These investigators identified sequence variants in protocadherin-1 (PCDH1) that were associated with BHR and replicated in seven (out of eight) additional cohorts. Protocadherins are believed to play a role in cell adhesion and organ development, but more research is needed about their exact role in asthma pathophysiology. Kozu et al. demonstrated that PCDH1 colocalized with E-cadherin in

16HBE airway epithelial cells, and that PCDH1 knockdown reduced the expression of both TJ and AJ, leading to reduced TEER and greater paracellular permeability [16]. Therefore, PCDH1 appears to play a key role in maintaining epithelial junctional structure and function. The second genetic association study implicating a junction-related protein in asthma was a genome-wide association study of genes associated with risk of asthma exacerbation in Danish children. This analysis identified a coding variant in cadherin-related family member 3 (CDHR3) that was associated with an almost twofold increased risk of hospitalization for asthma [17]. The exact function of CDRH3 is not entirely clear. Interestingly, Bochkov et al. reported that type C rhinoviruses (RV), a major cause of asthma exacerbation in children, preferentially bind this CDHR3 genetic variant in transfected epithelial cell lines [18]. These interesting studies suggest that by promoting viral adhesion and infectivity, genetic variants in a junctional complex protein could increase susceptibility to asthma exacerbations.

Aside from the association between PCDH1 and CDHR3 polymorphisms and asthma, no other GWAS or genetic association study to-date has identified mutations in other junctional proteins in asthmatic subjects, and it seems likely that most defects in barrier function in asthma are acquired rather than genetic. Supporting this contention, exposure to diverse environmental allergens, pollutants, and pathogens has been shown to compromise airway epithelial barrier integrity in numerous studies (for reviews, see Refs. [19,20]). Multiple different mechanisms for inducible barrier dysfunction have been described, which can be broadly divided into those that involve decreased mRNA expression or protein translation of a junctional gene product, and those that involve expression-independent redistribution away from the cell membrane (e.g., via endocytosis). One of the first demonstrations of the effects of an allergen on the epithelial TJ was reported in 1995 by Herbert et al. [21]. These investigators found that allergens isolated from the house dust mite *Dermatophagoides pteronyssinus* increased permeability across sheets of bovine airway epithelium [21]. Subsequent studies suggested that house dust mite proteases directly cleaved cell surface occludin, resulting in defective barrier function [22,23]. These influential studies laid the groundwork for the "protease hypothesis" of allergies and asthma. This hypothesis is built on the observation that many allergens contain serine or cysteine proteases and suggests that allergen-induced barrier dysfunction is a defining feature of these diseases, resulting in greater penetration of inhaled allergens into the subepithelial space, and subsequent allergic sensitization. Support for the protease hypothesis comes from in vitro studies of epithelial monolayers demonstrating protease-dependent disruption of epithelial barrier function by house dust mite allergens [22,23], fungal proteases [24], and different pollens [25,26]. However, other studies have reported that allergens can induce barrier disruption in a protease-independent manner [7,27,28] (e.g., dependent on reactive oxygen species [29]). Furthermore, a careful analysis of different house dust mite extracts concluded that protease activity correlated poorly with their ability to cause either barrier dysfunction in vitro or mucosal sensitization in a mouse model of asthma [30]. Therefore, more research is needed to understand exactly how proteases (and other enzymatic activities) associated with inhaled allergens affect the respiratory epithelium in human subjects. We need to be careful about extrapolating experiments conducted in vitro with epithelial monolayers, often exposed to supraphysiologic concentrations of allergen extracts, to events that occurs in the airway in real-world conditions. Since inhaled allergens deposit in the respiratory tract at very low concentrations, surrounded by epithelial lining fluids enriched in antioxidants and protease inhibitors, they are likely "neutralized" or cleared in most subjects. Perhaps subjects with defects in antiproteases and other epithelial defense mechanisms are more susceptible to the barrier disruptive effects of inhaled allergens.

In addition to allergens, other studies have shown that different components of air pollution including ozone [31,32], diesel exhaust particles [33,34], and ambient particulate matter [34,35] can induce barrier dysfunction in epithelial monolayers grown in vitro. One of the first demonstrations of this phenomenon was reported by Yu et al., who exposed canine bronchial epithelial cells to ozone at relatively low levels (0.5−0.8 ppm). Interestingly, ozone exposure decreased TEER and increased paracellular permeability as determined using mannitol as tracer [31]. Bayram et al. confirmed this effect using primary bronchial epithelial cells obtained from human donors and in fact reported that cells from asthmatic subjects were more susceptible to the adverse effects of ozone on barrier integrity [32]. Mechanisms by which ozone and other pollutants compromise airway barrier integrity are not clear. Many of the adverse effects of ozone and other inhaled pollutants are mediated by reactive oxygen intermediates, but definitive evidence linking these signaling intermediates with barrier dysfunction in epithelial cells in asthma is currently lacking.

Infections with respiratory viruses can lead to barrier dysfunction by causing epithelial cell death, either due to direct cytopathic effect or indirectly via immune cell cytotoxicity. Recently, several reports have shown that respiratory viruses can lead to barrier disruption without causing epithelial cell death. As many viral particles are shed basolaterally, junctional dysfunction should facilitate virus escape and infectivity of neighboring cells and might represent an evolutionary strategy used by viruses to facilitate their replication [36,37]. There appear to be multiple molecular mechanisms by which respiratory viruses decrease junctional integrity that are virus- and possibly cell-type specific. For instance, in Caco-2 cells, Coxsackie virus causes occludin internalization via macropinocytosis, dependent on the Rab GTPases [37]. Influenza virus can disrupt barrier integrity by a variety of mechanisms. For example, in MDCK cells, the NS1 protein of

influenza virus indirectly disrupts epithelial tight junctional complexes by binding the adaptor proteins Scribble and DIg-1 [38]. Short et al. studied the effects of influenza on alveolar epithelial cells grown on the top of Transwell inserts with endothelial monolayers grown on the bottom [39]. Influenza infection resulted in a marked decrease in epithelial barrier function independently of endothelial cells, without affecting cell viability. Using immunofluorescence microscopy, these investigators observed no differences in the expression of zona occludin-1, β-catenin and E-cadherin between influenza and mock-infected cells. While there was a trend toward reduced expression of both occludin and junctional adhesion molecule, the most striking effect of influenza infection was loss of claudin-4 staining. Since claudin-4 is important for barrier integrity, these results suggest a molecular mechanism by which influenza infection may lead to lung injury. Hershenson et al. showed that infection with RV leads to epithelial barrier dysfunction activity dependent on the NADPH oxidases (NOX), since RV-induced barrier dysfunction was inhibited by the NOX antagonists [40,41]. Interestingly, the apical/basal translocation of bacteria was significantly enhanced following RV infection. This suggests that virus-induced junctional complex dysfunction might contribute to the clinical observation that RV and other respiratory viral infections are frequently followed by bacterial superinfection [40]. RSV infection causes bronchiolitis in children and has been associated with asthma susceptibility later in life [42]. Kilani et al. provided one of the first demonstrations that RSV infection results in enhanced epithelial permeability. These investigators found that the barrier disruptive effects of RSV were attenuated with a neutralizing antibody directed against vascular endothelial growth factor and concluded that RSV promoted epithelial permeability indirectly [43]. Studies from Singh and Imani demonstrate that p38/MAPK (mitogen-activated protein kinase)-driven cytoskeletal remodeling contributed to RSV-induced barrier dysfunction [44]. Research from our lab showed that RSV results in sustained decreases in TEER and increased paracellular permeability in 16HBElo airway epithelial cells (as well as primary epithelial cells grown at ALI). We found that the barrier disruptive effects of RSV were not associated with cell cytotoxicity but were dependent on sustained activation of protein kinase D (PKD) [45]. PKD was originally known as PKCμ, but this molecule was renamed since it has different structure and substrate specificity than other PKC family members [46]. PKD regulates cell shape and motility in part by controlling actin dynamics, and in support of a role for PKD in cytoskeletal remodeling, we found that PKD inhibition prevented RSV-induced phosphorylation of the actin-binding protein cortactin [45]. In a separate study, the double-stranded RNA polyI:C also induced barrier dysfunction and AJC disassembly in a PKD-dependent manner [47]. This was not dependent on production of soluble factors but rather involved a cell-intrinsic signaling mechanism dependent in part on toll-like receptor 3 [47]. Taken together, these studies suggest that PKD could be involved in barrier dysfunction caused by respiratory viruses and potentially other stimuli. Indeed, PKD has been shown to be necessary in potentiating MyD88-dependent MAPK and NFκB activation downstream of multiple toll-like receptors [48]. PKD activity has also been linked to changes in junctional protein expression. For example, the Tang lab demonstrated that expression levels of PKD, and especially PKD3, in bronchial epithelial cells correlated with barrier permeability and decreased claudin-1 expression [49]. PKD has been shown to colocalize with E-cadherin showing that this enzyme is intimately associated with intact junctions [50]. Taken together, these results suggest that PKD may be a master regulator of epithelial junctional integrity, and antagonizing PKD activity in the context of viral infection or allergic airway inflammation could enhance defective barrier activity.

11.3 IMPLICATIONS OF EPITHELIAL BARRIER DYSFUNCTION FOR ASTHMA IMMUNOLOGY AND THERAPY

Airway epithelial cells are at the front and center of the asthma playing field. For example, epithelial-derived soluble mediators including interleukin (IL-25), IL-33, and thymic stromal lymphopoeitin (TSLP) have been implicated in promoting the differentiation of CD4 + Th2 cells. Th2 cells produce the cardinal cytokines implicated in many cases of allergic "Th2 high" asthma including IL-4, IL-5, and IL-13. Exactly how the observed defects in epithelial junctional protein structure and function contribute to immune pathogenesis is not clear, but several possibilities can be envisioned. First, defective junctional integrity could result in activation of signaling cascades in epithelial cells, leading to the production of proinflammatory mediators. This is best established for the case of epidermal growth factors, where loss of junctional integrity allows apical (luminal) growth factor to translocate paracellularly and bind basolateral receptors. Sustained dysfunction of epithelial junctions could also lead to epithelial dedifferentiation programs, such as epithelial−mesenchymal transition. One possibility is that sustained defects in epithelial integrity lead to airway remodeling, a pathologic process characterized by deposition of collagen and other extracellular matrix proteins that occurs in long-standing asthma. Another possibility is that defects in barrier integrity translate into greater uptake of inhaled allergens by subepithelial immune cells, such as antigen-presenting dendritic cells (DC). After acquiring inhaled antigens, DC traffic to local lymph nodes where they present their cargo to naïve lymphocytes and provide signals that initiate CD4 + differentiation. Support for a link between epithelial barrier dysfunction and Th2 immune responses

comes from studies of Heijink et al. who found that knockdown of E-cadherin enhanced the production of TSLP and TARC, cytokines, and chemokines that initiate and promote Th2 immunity [51]. Since the Th2 cytokines IL-4 and IL-13 also decrease barrier integrity [52], these observations led us to propose the existence of a "vicious cycle of leak" (Fig. 11.1). According to this model, epithelial barrier dysfunction induced by different environmental exposures facilitates the uptake of inhaled allergens by mucosal DC. Epithelial-derived soluble mediators, such as TSLP produce

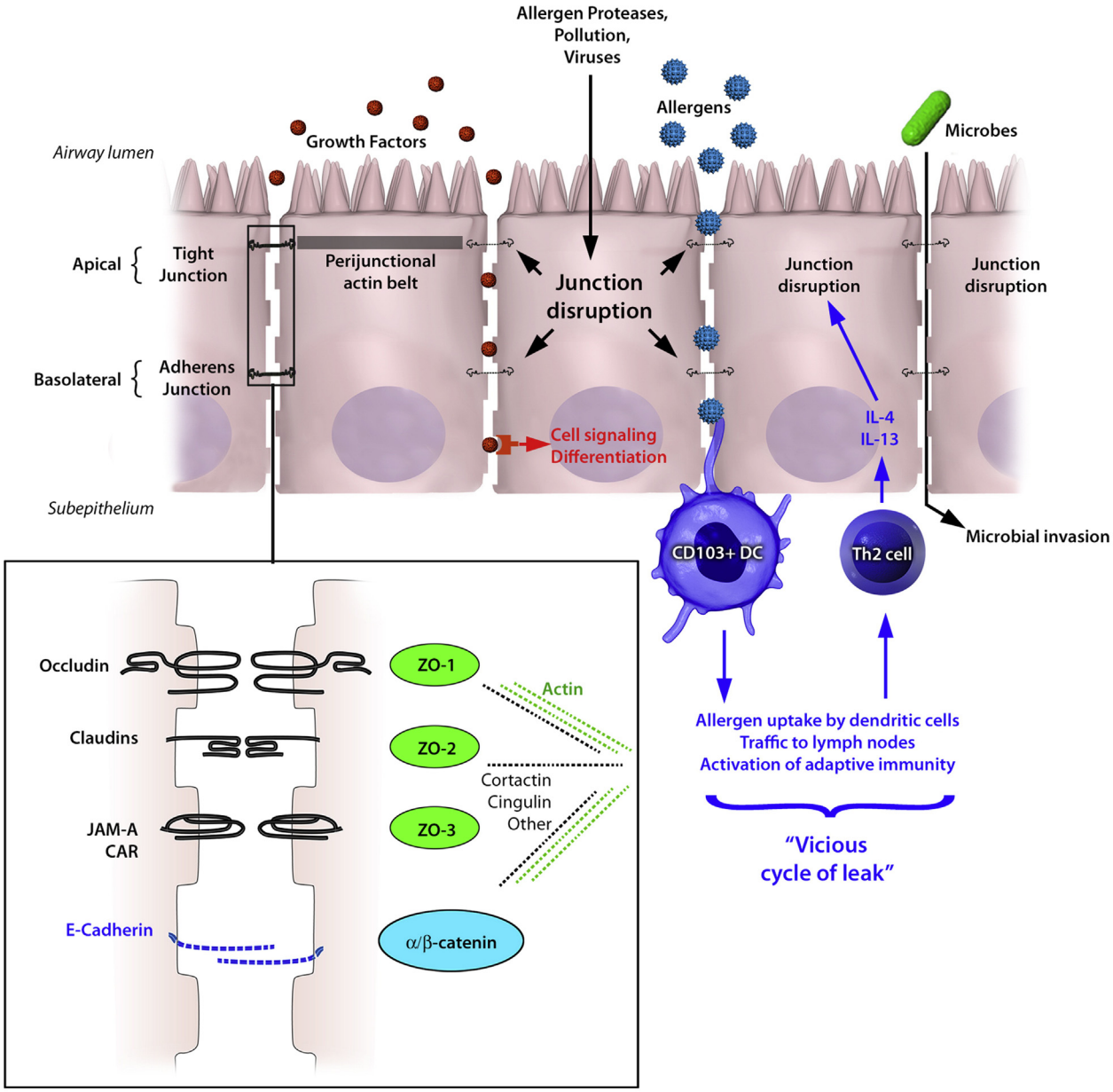

FIGURE 11.1 Airway epithelial cells are joined by tight junctions and adherens junctions, which are intimately linked with perijunctional actin filaments. The inset shows an enlarged schematic of protein−protein interactions in tight junctions (black text) and adherens junctions (blue text), including ability of ZO proteins to interact with intracytoplasmic domains. The inset also indicates that junctional proteins are linked to the actin cytoskeleton (green dashed line) via several potential adaptor proteins (black dashed line). Inhaled allergens, air pollutants, and respiratory viruses can cause to dysfunction of epithelial junction resulting in greater outside/in permeability. Barrier dysfunction can lead to epithelial cell signaling or differentiated, since it will allow apical growth factors constitutively present in epithelial lining fluids (red dots) to interact with their basolateral receptors. In the presence of intact epithelial junctions, these ligand/receptor interactions are prevented. Barrier dysfunction will also allow greater sampling of luminal allergens (blue stars) by intraepithelial dendritic cells (DC). Allergen-induced Th2 responses can induce a vicious cycle of leak, since Th2 cytokines perpetuate junctional dysfunction. Another consequence of leaky epithelial barriers is increased microbial invasion (green oval), which might predispose susceptible asthmatics to exacerbations or lung infections. *Reproduced from Georas S.N., Rezaee F. Epithelial barrier function: At the front line of asthma immunology and allergic airway inflammation. J Allergy Clin Immunol. 2014;134(3):509−520, with permission.*

by epithelial cells with dysfunctional junctions, imprint on local DC the capacity to instruct Th2 differentiation. After trafficking to local lymph nodes, DC promote the development of Th2 cells that travel back to airways and perpetuate the existence of barrier dysfunction by virtue of T-cell-derived IL-4 and IL-13 [20]. In future studies, it will be important to determine whether these immune pathways operate in subsets of asthmatics, especially those with severe disease or prone to frequent exacerbations.

We currently have only a rudimentary understanding of the relationship between epithelial barrier dysfunction and asthma pharmacotherapy. One counter-intuitive possibility to keep in mind is that defective barrier function could actually enhance the efficacy of inhaled medications such as beta-agonist bronchodilators, by increasing their penetration into airway smooth-muscle cells. Fluticasone propionate and budesonide, two inhaled glucocorticoids commonly used in moderate−severe asthmatics, prevent epithelial barrier disruption caused by the viral mimetic polyI:C [53]. Interestingly, Kozu et al. reported that expression of PCDH1 (the protocadherin family member identified in a genetic study of BHR, discussed above) is also induced by the glucocorticoid dexamethasone [16]. Therefore, induction of barrier integrity may be a common property of multiple glucocorticoids.

In conclusion, multiple lines of evidence have established that the expression and function of both TJ and AJ are defective in asthma. Genetic variation in two genes (PCDH1 and CDHR3) is to blame in some subjects, but many environmental exposures can induce acquired defects in different junctional components. Different exposures act by different mechanisms, with increasing evidence pointing to expression-independent downregulation of cell surface expression of TJ and AJ involving PKD. The development of noninvasive ways to measure epithelial barrier integrity in human subjects will be an important advance and will help decipher the clinical implications of this phenomenon. This will also help identify subjects who will benefit from therapeutic agents that restore barrier function in the future.

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Chapter 12

Cystic Fibrosis: An Overview of the Past, Present, and the Future

Samuel A. Molina and William R. Hunt

12.1 INTRODUCTION TO CYSTIC FIBROSIS

Cystic fibrosis (CF) is an inheritable lethal disorder characterized by progressive obstructive pulmonary disease and pancreatic insufficiency [1]. The pulmonary disease, which ultimately leads to increased mortality, is punctuated by thick mucus production, excessive inflammation, reduced pathogen defense, small-airways obstruction, and recurrent exacerbations [2]. The gene defect underlying the disease traditionally has been described as coding for an aberrant protein that is defective in chloride ion transport in epithelial cells [3,4]. However, the epigenetic and microbiological interactions associated with this gene defect are profound and create a complex multisystemic illness [2].

While only a relatively small number of people exhibit the classical symptoms of CF, the carrier rate for this genetic mutation is prominent in the population, particularly amongst those of Northern European descent [5]. CF confers a very high burden in terms of morbidity, healthcare utilization, and mortality. Recent research also suggests that even asymptomatic genetic carriers for CF may be at risk for subclinical physiological derangements, which can be exacerbated by external stresses and other environmental triggers [6,7]. This chapter explores the epithelial cell dysfunction in relation to the genetic and phenotypical manifestations of CF.

This chapter will present our current understanding of CF in regards to its epidemiological and clinical impact with a particular focus on the genetic and protein defect within airway epithelial cells. The CF transmembrane conductance regulator (CFTR) protein functional defect will be reviewed both in terms of its classical activity as a chloride anion channel and also its complex behavior as a transporter and its interactions with epithelial sodium channels (ENaC). As CF is punctuated by excessive, but ineffective, inflammation, the current understanding of the complex interplay between CF airway epithelial cells and innate immunity will be explored. While lung disease is the largest driver of mortality in CF, it is a systemic illness. The most common comorbidity associated with CF is a unique diabetes (CF-related diabetes, CFRD), which when present significantly exacerbates the lung dysfunction seen in this disease. This chapter will review the impact of CF-related diabetes and current understanding of its effects on airway epithelium. Animal models of CF will be compared and contrasted in regards to their phenotype and relative pros and cons as CF remains a lethal disease and animal model research will likely still be required to advance understanding of this fatal disease. Finally, common therapeutic interventions for CF airway's disease will be reviewed as well as future therapeutic pathways.

12.1.1 Historical Context of Cystic Fibrosis

CF is the most common lethal inheritable disorder amongst individuals of Northern European descent, though it is by no means racially unique [8]. While it is a multisystemic illness, morbidity and mortality are primarily attributed to recurrent respiratory infections leading to early obstructive lung disease and eventual respiratory failure and death [2,8]. Although advances in basic science and clinical therapeutics have improved mortality, the average life expectancy for someone living with CF remains substantially reduced at only 39.2 years [8].

While current medical terminology describing CF was only developed in the 20th century, there are various reports of Northern European folklore that have been presumed to describe manifestations of CF as far back as the 17th century. Modern medical understanding of CF as a distinct clinical entity began in 1938 with Dr. Dorothy Anderson's description of

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pancreatic glandular duct mucus plugging seen during autopsy in several malnourished infants, which she described as "CF of the pancreas" [9]. Subsequent study of CF recognized glandular mucus obstruction was generalized throughout the body, and in 1946, Dr. Anderson et al. further recognized that CF was a genetically inheritable disease with an autosomal recessive pattern [10,11]. Four years later, a young pediatrician, Dr. Paul di Sant'Agnese, observed many children presenting with severe heat exhaustion were also afflicted with CF. This led to the discovery of excessively salty sweat as a pathologic feature in CF, the characteristic that had previously been described in ancient folklore [12,13]. This discovery led to the development of the sweat chloride concentration test as a diagnostic tool to identify individuals with CF in 1959, which remains a cornerstone diagnostic criterion for CF to this day [14]. The primary defect in CF was finally identified in 1983 as a chloride transport deficiency in epithelial cells [3]. Further studies also observed excessive epithelial sodium resorption in CF airways [15,16]. With better mechanistic understanding of CF, therapeutic development was finally able to advance beyond supportive care measures. Despite the genetic pattern for CF being described in 1946, it took 43 years to identify the gene responsible for the genetic defect [17–19]. Since discovery of the epithelial ion transport defect in CF and subsequently the causative gene, there has been an exponential increase in pharmaceutical and therapeutic development.

12.1.2 Epidemiological Impact of Cystic Fibrosis

Though it is likely underreported in developing countries, CF affects at least 70,000 persons worldwide with approximately 38,000 affected in the United States and 36,000 individuals in the European Union [8,20]. As CF adheres to an autosomal recessive pattern, similar rates of prevalence exist between males and females. Carrier rates for the CF genetic mutation are high amongst Caucasians. Approximately, 1 in 25 US Caucasians are heterozygous carriers for the CF genetic mutation, which accounts for a CF birth rate of 1 in 3000 in this population [21]. However, the disease is not racially unique, though prevalence of CF genetic mutations vary among the races. The prevalence for classical CF in Hispanic Americans is 1 in 9200, in African-Americans is 1 in 15,000, and for Asians 1 in 35,000 [21]. This gives an overall CF prevalence of 1 in 3900 in the United States with nearly 1000 new births each year. While the prevalence of CF is lower than other obstructive lung diseases, such as chronic obstructive pulmonary disease and asthma, CF carries a substantial burden in terms of healthcare dollars, morbidity, and mortality [22].

When CF was first described by Dr. Anderson in 1938, the median life expectancy was less than a year [2]. Indeed, for the majority of the last century, CF has been identified as a pediatric disease, with many patients not living past their 18th birthday. An improvement in therapeutics and healthcare utilization has significantly improved survival with CF. Today, more than half of the CF population is now over the age of 18, and by some estimates more than 70% of the population will be adults by 2025 [23]. In addition to medical advances, initiation of newborn screening has improved early disease recognition and management.

Newborn screening for CF is now performed in all 50 states in the United States and many European countries [24]. As one of the earliest manifestations of classical CF is pancreatic dysfunction, most newborn screens test for immunoreactive trypsinogen (IRT) [25]. IRT is the inactive precursor protein of the protease trypsin. As CF pancreatic dysfunction is punctuated by reduction in pancreatic enzyme release, IRT is often not converted to its active form in individuals with CF, and therefore, its concentration in the serum is much higher. Elevated IRT levels prompt secondary screening test such as DNA mutational investigation as well as sweat chloride testing for confirmatory analysis. Newborn screening currently captures about 64% of all new CF diagnoses in the United States [8].

Even if not captured by the newborn screening program, new diagnoses of CF are made in patients under the age of 2 years old 75% of the time [8]. There is a minority of patients who are diagnosed in adulthood. Many of these patients have differences in prevalence of certain CF mutations, lower rates of pancreatic dysfunction and better lung function when compared to patients who are diagnosed before the age of 2 years, which helps explain why their disease may go undetected until their adult years [26].

12.1.3 Clinical Manifestations of Cystic Fibrosis

Symptoms of recurrent respiratory abnormalities and/or pancreatic exocrine dysfunction are usually prevalent early in classical CF [8]. Clinical manifestations of CF have traditionally been ascribed to loss of proper cellular location of a chloride channel [27], which will be discussed in greater detail later in this chapter. As the majority of mortality in CF is from respiratory failure, the disease is often thought of as a pulmonary disease. While it is true that most deaths in CF are attributable to respiratory complications, it is important to remember that CF is a systemic illness with manifestations of multiple comorbidities (Fig. 12.1). Many of these comorbidities also have complex interactions with lung function.

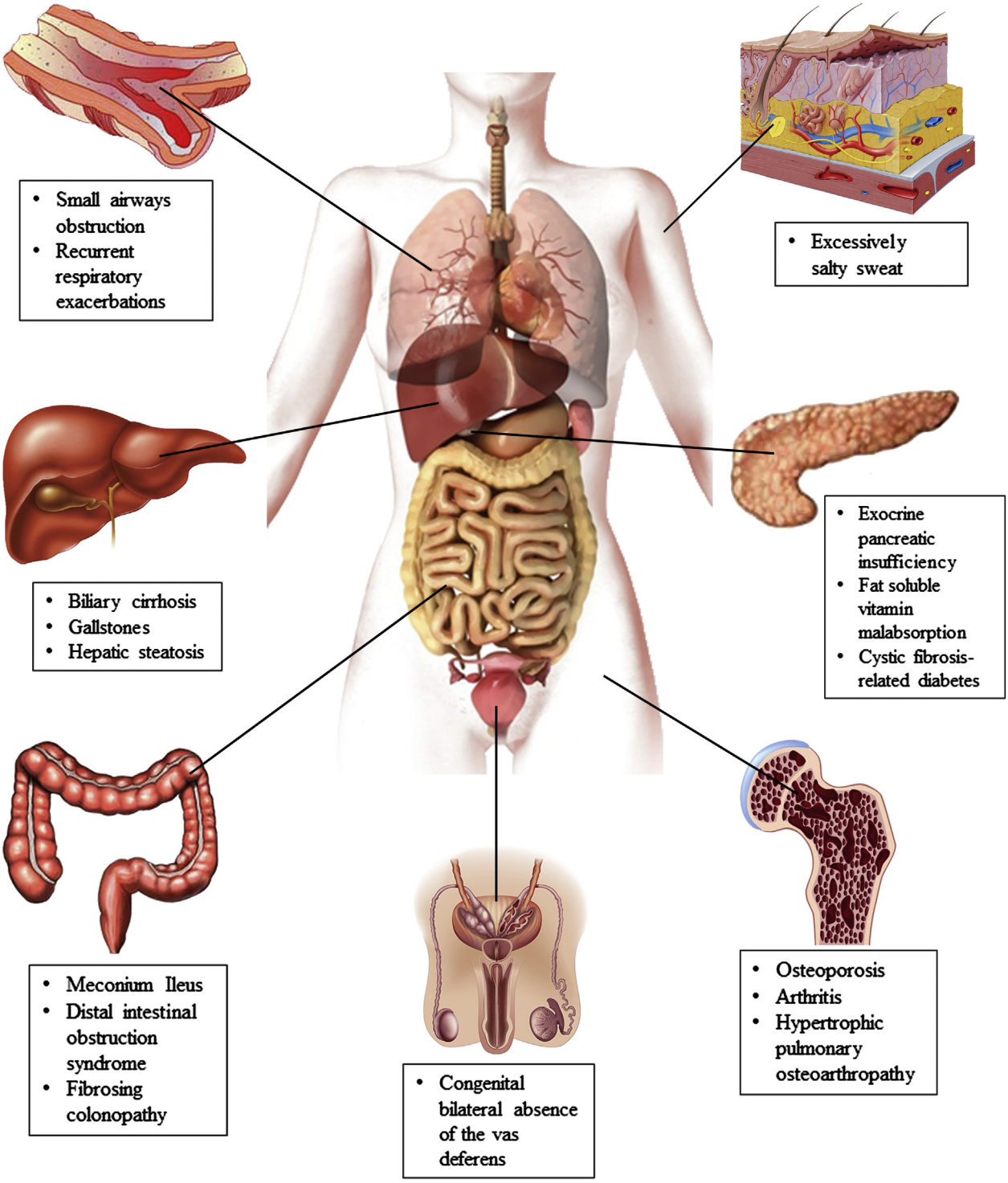

FIGURE 12.1 Common clinical manifestations of cystic fibrosis. While cystic fibrosis is primarily characterized by a progress obstructive pulmonary disease and exocrine pancreatic dysfunction, many organ systems can be affected. Variations in prevalence of extrapulmonary symptoms are not completely understood, but evidence suggests some disease manifestations are related to the severity of the underlying CFTR mutation as well as environmental and genetic modifiers [5].

Pulmonary manifestations of CF include thickened mucus secretions, small-airways plugging, exaggerated neutrophilic inflammation, and recurrent respiratory infections [1]. The cycle of infection, inflammation, and airways damage ultimately leads to bronchiectasis with distortion of small-airways architecture. Chronic bacterial respiratory infection is common, with the majority of patients showing colonization with *Staphylococcus aureus* even before the age of 2 years old [28]. *Pseudomonas aeruginosa*, an important CF pathogen that confers worse mortality, becomes the most prevalent respiratory microorganism in the second and third decade of life [8]. Respiratory symptoms are punctuated by intermittent acute exacerbations. While there is no stringent universally agreed upon criteria to define an acute CF respiratory exacerbation, they are often characterized by an increase in quality and quantity of cough and a decline in pulmonary function. These recurrent exacerbations are associated with the exaggerated decline in pulmonary function seen in CF [29]. Ultimately, end-stage chronic respiratory failure can develop with resting hypoxemia, dyspnea, and hypercapnia.

As previously described by Dr. Anderson, there is often early glandular mucus obstruction and fibrotic destruction of the pancreas [12]. This leads to exocrine pancreatic dysfunction with a significant reduction in pancreatic enzyme release [30]. This causes poor absorption of macro and micronutrients. Patients often experience steatorrhea, the malabsorption of dietary fat, without appropriate pancreatic enzyme supplementation. In addition, the fat-soluble vitamins (A, D, E, and K) are often under absorbed and must be supplemented. Ultimately, pancreatic endocrine function can also suffer with the development of CFRD [31]. This is a unique form of diabetes associated with CF. More than 34% of CF patients over the age of 18 will develop CFRD [8]. Its onset is profound in terms of morbidity and mortality. CFRD is the most common comorbidity associated with CF [8]. There is also evidence to suggest the onset of CFRD is associated with critical changes within the lung environment. As such, this comorbidity will be highlighted later in this chapter.

In addition to malabsorption, the gastrointestinal luminal tract also produces thickened mucus secretions and is at higher risk for obstruction [32]. Approximately, 10% of infants with CF will present with meconium ileus, a condition where the first fecal material of a newborn fails to pass and causes distal intestinal obstruction [8]. Similar pathology can occur in adulthood, termed distal intestinal obstruction syndrome, in which there is a partial or complete small-bowel obstruction. The hepatobiliary system is also affected with 80% of CF patients demonstrating some form of focal cirrhosis and almost 3% developing diffuse cirrhosis [8].

There are a number of other clinical manifestations that occur either as a direct complication of the loss of CFTR protein function or as an indirect consequence of the disease pathophysiology. For example, nearly all men with CF have congenital bilateral absence of the vas deferens [33]. Rates of osteopenia and osteoporosis (early bone loss) are higher in individuals with CF [34]. Sinus disease is common and often complicated by the same microorganisms that are found in the respiratory tract [35].

12.1.4 CFTR Gene and Protein Defects

Previously recognized as an autosomal recessive genetic disorder, the causative gene mutation for CF was discovered in 1989 [17–19]. The gene was found to reside on the long arm of chromosome 7 and codes for the CFTR [36]. CFTR is a transporter in the ATP-binding cassette family that functions primarily as a chloride channel on the apical surface of epithelial cells, though there is also evidence that it may transport bicarbonate and glutathione [37]. In addition, it appears to have important regulatory interactions with ENaC [15,16]. One of its key regulatory mechanisms is maintenance of the airway surface liquid (ASL), which will be detailed later in the chapter.

There are over 1800 known mutations of the CFTR gene, though the effects on CFTR protein function with many of these mutations have not been extensively explored [38]. Mutations range from complete absence of CFTR protein production, to production of functional CFTR, but in insufficient quantities. Mutational prevalence varies depending on race and worldwide region. For example, $3120 + 1G \rightarrow A$, a splice-site mutation, is the second most common mutation found in Native Africans [39]. The most common CFTR mutation found in the US population is ΔF508, which is a loss of phenylalanine at codon 508 [40]. This results in a CFTR protein that is misfolded and subsequently never reaches the cell surface. Nearly 90% of individuals with CF in the United States have at least one copy of ΔF508, with nearly 50% of patients being homozygous for this mutation [8].

To create a conceptual framework for these mutations, they historically have been categorized into classes based on functional impact (Fig. 12.2). This categorization provides some context to the mutational manifestations and it is still used today. However, there has been some recent concern regarding the functionality of categorizing mutations in this manner. In some instances, it is an oversimplification of a mutational impact as there are some mutations that can lead to multiple CFTR protein functional defects [41]. Many CFTR mutations that have been discovered do not yet have categorical classifications described [41]. Also, genotype does not always correlate with phenotype in

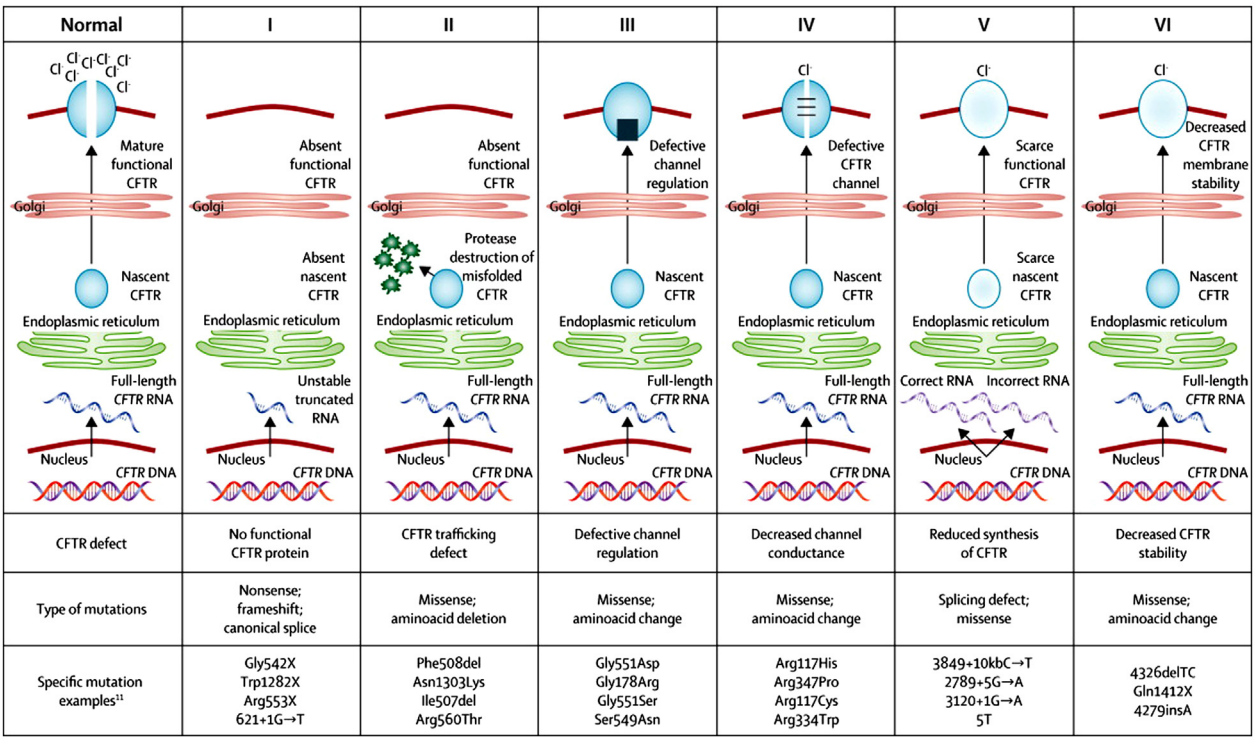

FIGURE 12.2 Classes of CFTR Mutants. *Reproduced, with permission, from Boyle MP, De Boeck K. A new era in the treatment of cystic fibrosis: correction of the underlying CFTR defect. Lancet Respir Med 2013;1:158–63.*

CF [42]. While class I–III mutations are generally associated with higher sweat chloride concentrations and worse pancreatic dysfunction compared to class IV–VI mutations, the correlations with lung function and mutational class are much less complementary [41]. For example, patients homozygous for ΔF508, a class II mutation, have significant variability in their lung disease from individual to individual. As CFTR protein modulators become a more important aspect of the therapeutic regimen for CF (see Sections 5 and 6), this historical classification of CFTR function may need to be updated.

12.2 MODELS OF CYSTIC FIBROSIS DISEASE

The importance of functional and physiologically relevant disease models is evident by their contribution to discovery of the pathophysiological mechanisms that drive any disease. CF is no exception as various mouse models have proved to be critical in understanding both lung and gut manifestations of the disease [43]. Mouse models have been the "workhorse" for decades; however, a simple deletion of the CFTR gene in the mouse does not fully reproduce how CF manifests in humans. Rising out of this discrepancy was a major effort to create new in vivo disease models in rats, ferrets, and swine where the CF pig is believed to best replicate the human manifestations [44]. As in vivo models are important for understanding whole animal pathophysiology, in vitro models comprised of epithelial and immune cells are just as important for identifying and understanding molecular mechanisms that contribute to CF and become potential therapeutic targets. This section will define important in vivo and in vitro models that have made significant contributions to understanding the disease mechanisms contributing to CF.

12.2.1 In Vivo Models of Cystic Fibrosis Disease

The CF mouse was introduced in the early 1990s and various approaches were used to generate at least 15 strains that attempted to faithfully replicate human CF pathophysiology [45], including one knockout mouse that targeted ENaC instead of CFTR [46]. Mice differ from humans in a number of ways with the most obvious being size and lifespan. Another important difference between mice and humans is that mouse airways do not contain submucosal glands. As opposed to mice, accumulated evidence in pigs suggested that submucosal glands contribute to CF airway disease

where defective CFTR-dependent mechanisms caused mucus tethering and resulted in defective mucus release from submucosal glands [47,48]. Mice also lack the spontaneous and progressive airway colonization of infectious bacteria that is a hallmark of the human manifestation of CF disease. Although CF mice do not obtain spontaneous airway infection, they do mount an exaggerated immune response similar to human CF disease.

The CF ferret model was developed to address deficiencies in the mouse model such as lifespan and inability to acquire spontaneous lung infections. The presence of submucosal glands and goblet cells throughout the ferret airway aided interrogation of the pathobiological aspects of the human lung [49]. The CF ferret model demonstrated gastrointestinal obstruction and pancreatic architectural distortions similar to what is observed in human CF disease [50,51]. Due to the development of spontaneous pancreatic dysfunction, CFTR knockout ferrets have also served as an important model for CFRD. Kits exhibited decreased first-phase insulin secretion, prolonged glucose clearance times, and poorly regulated blood sugar levels [51,52], similar to human CFRD. An advantage of the ferret model in CFRD research is the development of spontaneous pancreatic disease as compared to the induced diabetic disease state in the rodent model [53]. It is conceivable that studying the gut−lung or pancreatic−lung axes in the ferret model of CFRD could uncover important pathophysiological mechanisms that would not be evident in the rodent.

The ferret airway is comprised similar cell types as the human airway complete with submucosal glands. Mucociliary clearance of CFTR knockout ferrets resembled that of both CF humans and CF pigs [44,51,54,55]. Mucociliary clearance is the movement of mucus from the distal airways to the proximal airways with the eventual expulsion of mucus into the pharynx and esophagus. This is achieved by tracts of ciliated airway epithelial cells that direct the beat of their cilia in a unidirectional and coordinated manner; this process is termed the mucociliary escalator. In CF, the mucociliary escalator and clearance effect is impaired by both periciliary fluid layer (PCL) and ASL dehydration due to the primary chloride transport defect of mutant CFTR.

Closest yet to the human lung and gastrointestinal tract are swine models [44,56,57]. Pigs are an important species for studying CF, and possibly other lung diseases, for a number of reasons as explained by Stoltz et al. [58]. Meyerholz explains why pigs are important in detail [58a], however, a few examples specific to CF are worth highlighting: (1) pigs live a much longer lifespan compared to rodent, ferret, and the zebrafish models; (2) pig lungs resemble human lungs with respect to size and structure; and (3) CF pigs succumb to gastrointestinal and pancreatic disease similar to human patients with CF [51,56].

A hallmark difference between rodent and pig models is the ability of the pig to acidify the airway similarly to how human airways acidify in CF disease. Mice lack this ability because mice do not express a key hydrogen−potassium exchanger, the nongastric H^+/K^+ adenosine triphosphatase (ATP12A) protein. Pigs lacking CFTR exhibit airway acidification to a similar degree that CF human airways acidify [59], which contributes to bacterial colonization of pig airways [60]. Increased colonization is aided by changes in biochemical and electrostatic interactions of secreted glycoproteins, such as mucins, with bacteria when ASL pH is acidified [61]. These important findings could not have taken place without a more faithful human model such as the pig and have led to the understanding of how CF airways are susceptible to recurrent and chronic bacterial infections.

12.2.2 In Vitro Models of Cystic Fibrosis Disease

Epithelial monolayers have been the mainstay of molecular research in the field of CF. The gold standard is the primary human bronchial epithelial cell directly isolated from patients, cadavers, or rejected lung transplant tissue [62,63]. Cells isolated from the trachea are also used as these can be isolated at the same time as bronchial cells and provide increased number of cells for an investigator to use. An attractive alternative to the tracheobronchial epithelial cell is the primary human nasal epithelial cell. Nasal epithelia are easily obtained from patients during a routine visit to the clinic and are minimally invasive compared to obtaining cells from an intact lower airway. Current methods of propagation allow patient-derived epithelial cells to grow exponentially while retaining their ability to properly differentiate.

Much of the early work on the function and regulation of CFTR was investigated in various epithelial cell lines that overexpressed CFTR to supraphysiologic levels. Many of these cells were isolated from cancerous tissue, isolated from donor lungs that were subsequently artificially immortalized, or common laboratory airway cell lines virally transduced to overexpress mutant CFTR. Questions have been raised as to the usefulness of cell lines that overexpress proteins regarding their true ability to faithfully replicate cell physiology of the disease [64]. Cell lines can be problematic when studying key physiological processes due to their origin or their deviance from normal physiology. For example, cancer-derived cell lines are a poor choice to study the disease-related changes in metabolism due to the Warburg effect and disruptions to cell cycle dynamics [65−67]. Another example comes from studying the effects of endoplasmic

reticulum stress in cell lines that overexpress CFTR beyond what is physiologic, which has the possibility of triggering off-target effects like ER stress. Heterologous expression systems such as human cell lines expressing ferret CFTR exhibit species-specific differences in the processing and steady-state levels of matured CFTR [68], which may not reflect the physiological control of CFTR expression and function.

Critical to studying the physiological context of CFTR function are new cell culture techniques that allow the efficient amplification of human primary cells, such as conditional reprograming [69–72]. Conditionally reprogramed cell (CRC) culture allows for multiple population doublings of cells (25 + doublings) which ultimately, under the right culture conditions, will differentiate into mature mucociliary cultures that demonstrate electrophysiological characteristics of low passage primary cultures [72,73]. By using this conditional reprograming method, large scale expansion of human primary-like cells can be differentiated back to near-original primary cell states for testing in a variety of applications. Unlike induced pluripotent stem cells, CRCs do not have to be transfected with exogenous genes or need to be treated with various growth factors to propagate. Thus, the CRC method holds vast promise for large scale, high-throughput screening methods that are faster and cheaper than using low passage, traditionally grown human primary cells or any form of pluripotent cells.

Personalized medicine in the form of individual patient-directed therapy based on genotype of both the patient and the infectious organism is becoming the preferred method of treatment, especially for rare CF genotypes. By utilizing cell models that resemble unique patients, unique treatment regimens for patients with rare mutations can be developed [74,75]. Systems that utilize primary human airway cells from patients are actively being developed [76]. From lung-on-a-chip, to recellularized tracheal scaffolds and induced pluripotent stem cell-derived organoids, drug-screening efforts of rare mutants are being rapidly developed. Screening methods have improved to rely on imaging analysis of swelling organoids after drug treatment and stimulation, which is compatible with high-throughput methods.

The power of personalized medicine can be realized with the utilization of organoids from patient-derived samples. Rectal biopsies from patients with the rare and uncharacterized genotypes G1249R/F508del and F508del/R347P were used to create intestinal organoids and test the effects of recently developed CFTR modulators [75]. Dekkers et al. [75] were able to show the power of patient-derived intestinal organoids in personalized medicine by directly, and accurately, predicting patient response to the available pharmaceuticals through organoid swelling assays. The organoids showed a positive correlation with clinical outcomes, specifically, percent predicted forced expiratory volume at 1 s (FEV_1), airway resistance at a flow of 0.5 L/s (Raw0.5), sweat chloride concentration, and nasal potential difference. These results demonstrate that the idea of personalized medicine can be realized through the power of screening patient-derived organoids to predict individualized therapies.

Although much progress has been made in the realm of CF-specific disease models, future therapeutic approaches will be challenged by the plethora of rare mutants where there are only a handful of patients each. The hope is to overcome this challenge by utilizing patient-derived organoids to predict effective treatment strategies to improve patient outcome.

12.3 EPITHELIAL CONTRIBUTIONS TO INNATE IMMUNITY IN CYSTIC FIBROSIS

Adaptive immunity plays an important role in airway diseases garnering much attention in the CF research community and the innate immunity contributions of the epithelium play an important role in the first line of defense in airway infection and disease. A layer of mucus lines the airway epithelium and is the first line of defense against inhaled particulate and microorganisms. Below the mucus layer lies the epithelium. The epithelium is where direct interactions with infections agents trigger events such as the secretion of antiinfective proteins (e.g., defensins and antimicrobial enzymes) and chemotactic agents that both resist infection and recruit key immune cells to the area of infection (e.g., antimicrobial peptides and cytokines) [77]. Neutrophils must migrate through the epithelium to access the airway, where infectious insults are neutralized [78]. The epithelium plays an important role in modulating fluid clearance in the airway, which directly affects the ability of infectious agents to take hold (Fig. 12.3).

12.3.1 Compositional Changes in Airway Surface Liquid

The movement of both ions and water defines the epithelial surface liquid composition where an imbalance of either sodium or chloride results in defective ASL and PCL composition and movement [79]. CF directly alters the composition of ASL though a feedback system of dysfunctional mutant CFTR channels and imbalanced ion transport [80,81]. Regulation of ASL height and composition by extracellular adenine nucleotides, including ATP, ADP, AMP, adenosine, and inosine, and is influenced by activation of luminal purinergic receptors [82,83]. Activation of both the purinergic

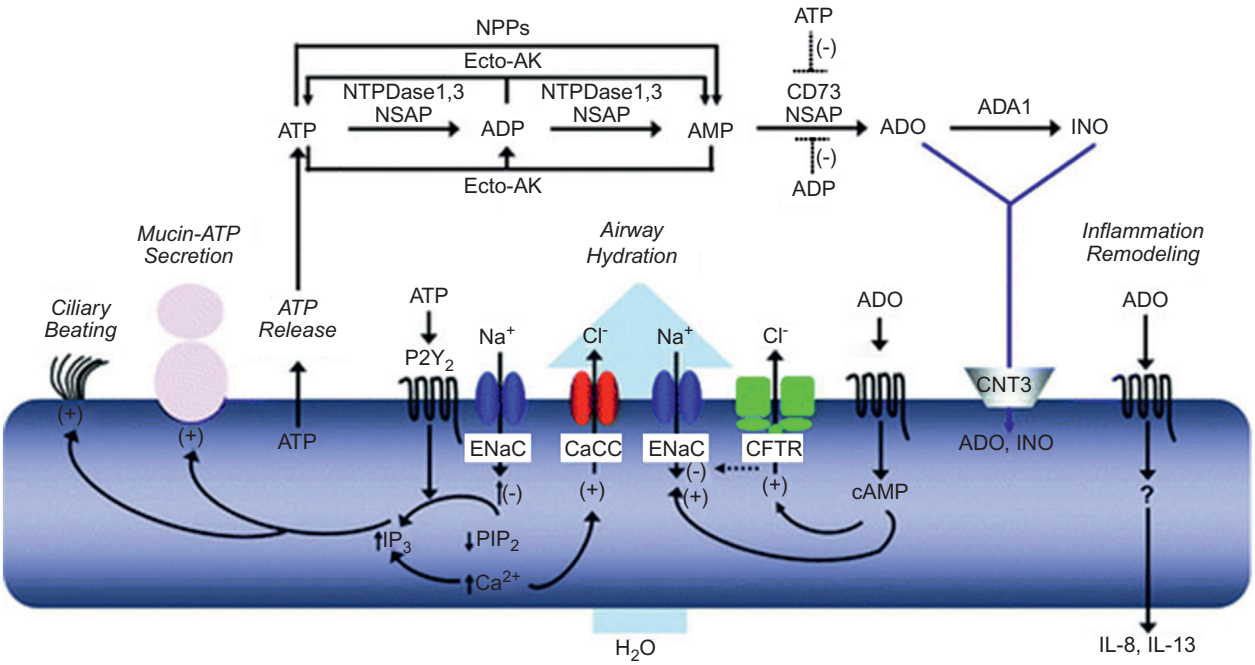

FIGURE 12.3 The "Purinome" of human airway epithelia. The apical surface concentrates proteins specialized in the regulation of ATP and adenosine levels named ectonucleotidases. Although the enzymatic network is positioned above the surface for clarity, all enzymes are membrane proteins. This network regulates the availability of ATP for P2Y2 receptors and adenosine for A2B receptors, which mediate various epithelial functions involved in airway defenses against infection: ciliary beating activity, airway hydration and inflammation [84]. *Purinergic Regulation of Respiratory Diseases, Mechanisms Regulating Airway Nucleotides, 55, 2011, pp 17–49, Maryse Picher, Springer Science + Business Media B.V., With permission of Springer.*

P2 receptor directly by ATP and the P1 receptor indirectly through the ATP hydrolysis product adenosine results in chloride secretion, movement of water, and coordinated ciliary beat frequency [84,85]. Extracellular enzymes either anchored to the luminal face of the epithelial cells or free-floating in the ASL modulate the available purinergic signaling molecules [84,86] Fig. 12.3.

Multisubunit proteinaceous ATP-releasing Pannexin-1 (Panx1) channels [87] that reside in the apical membrane of air-matured human airway cells seem to be responsible for supplying the bulk of ASL purines [88]. Short-term exposure to oxidative stress in the form of hydrogen peroxide inhibits Panx-1 channels from functioning during hypotonic stress and IFN-γ treatment, suggesting that ATP release is impaired during an inflammatory state [89]. For CF, this implication suggests that when faced with chronic inflammation the airway epithelium may not regulate purine release properly, which negatively influences ASL regulation.

Loss of CFTR function results in dehydration of the ASL and the PCL causing a decrease in the liquid height, which effectively immobilizes the mucus layer. The dehydration of the PCL contributes to the inability of cilia to beat thus causing mucus to accumulate which dramatically reduces mucus removal [80,81]. The stagnant mucus contributes to mucus plugging of the small airways and serves as a rich microenvironment for fungi and microbes [47,90,91]. Since CF lungs are in a state of chronic inflammation, simply correcting the CFTR defect may not adequately allow the PCL/ASL layers to reregulate to normal function. The level of CFTR correction needed to ensure ASL regulatory feedback normalization to allow constant and efficient clearance of airway mucus remains unknown and is an active area of research.

12.3.2 Changes in Airway Surface Liquid pH

The pH of a porcine non-CF airway in vivo is 7.2, where in air-matured airway epithelial cultures the pH is near 7.4. In contrast, in vivo CF pig airway ASL pH was near 6.9 where in cultures the pH was near 7.1 [60]. In CF neonates, nasal ASL pH was near 5.2 and was lower than child or adult CF nasal ASL pH, which measured near 6.4. There were no detectable differences between non-CF and CF child and adult nasal ASL pH although the variation was high [92],

which could be due to confounding factors like diet, environmental, and chemical exposures, medication (over-the-counter or prescribed), or habits like smoking or bathing. Airway pH has proven to be difficult to measure; however, understanding the role that CFTR plays in regulating airway pH may uncover therapeutic options.

CFTR is known to transport anions such as bicarbonate (HCO_3^-) and thiocyanate (SCN^-), where both contribute to the control of airway bacteria [47,93,94]. Both the pH and the ion composition of the ASL have been shown to influence the innate ability of the ASL to kill bacteria due to their contribution to secreted antimicrobial peptides such as lactoferrin and defensins [95–99]. Studies in the porcine airway have given strong evidence to support that the loss of CFTR directly contributes to a decrease in the pH, but not the ionic strength of the ASL, which inhibits bacterial killing [60]. Airway enzymes like lactoperoxidase, which creates the antimicrobial hypothiocyanate ($OSCN^-$), are not as active in ASL that is depleted of the CFTR-transported SCN^- [100,101].

In addition, studies in the β-ENaC mouse model of CF have implicated that restoration of thiocyanate in the airway could serve as a therapeutic option for CF patients [102]. Therapies that improve the biochemistry of the ASL are likely to improve bacterial clearance and help resolve chronic inflammation; however, the innate ability of the airway epithelium to control bacterial colonization is complemented by adaptive immunity. Tackling both the innate and adaptive immunity components together is likely to improve the treatment outcomes of CF patients and slow the destruction of lung function that occurs over a patient's lifetime.

12.3.3 Neutrophil Interactions

The main component of the adaptive immune system that is activated in the CF airway is the neutrophil. Neutrophils work to clear infections by releasing damaging substances such as reactive oxygen species, proteases, and cytokines into the airways [103–105]. Neutrophil elastase is a destructive protease and in the airway it decreases ciliary beat frequency and increases epithelial permeability [106,107], induces mucin production [108,109], and induces cytokine production from the epithelium [110,111]. CF neutrophils that enter the airway undergo a metabolic adaption which upregulates many stress-associated pathways and prolongs their lifespan to unusual lengths. In CF neutrophils, these upregulated pathways are related to metabolism of energy sources (amino acids and sugars) and prosurvival pathways (mTOR and CREB) which correlate with the active release of human neutrophil elastase (HNE) [112]. Elastase and other proteases present in the airway can produce metabolites, which conceivably can feed neutrophils and bacteria and possibly complicate the resolution of neutrophil activity or unintentionally aid bacterial growth or biofilm formation. Thus, HNE represents an active target for a therapeutic strategy [103,104].

Whether or not neutrophil activity is the sole source of epithelial dysfunction remains an unlikely scenario due to the variety of substances, both infectious and noninfectious, found in ASL from chronically infected patients. The cytokines that are released from activated neutrophils affect the airway epithelium in numerous ways. The proinflammatory cytokines IL-1, IL-6, IL-8, IL-17, CCL10, and CXCL10 are secreted from the CF neutrophils in response to infection and produce inflammatory effects in airway epithelial cells as well as serve to recruit and activate other immune cells [113–116]. Many proinflammatory cytokines induce defects in the epithelial barrier function that separates the interstitial serous lung from the airway lumen. Studies show that the airway epithelial tight junction barrier is weak in CF-affected cells and became weaker when epithelial cells were exposed to bacteria or the cytokines that are secreted in response to bacterial infection [117–119]. The epithelial barrier is important to regulate ion and fluid balance as well as solutes like growth factors, cytokines, and metabolites in the airway.

12.3.4 Nutrient Availability in the Airways

Glucose is found in the airways of chronically ill patients [120] and in the lavage fluids of CF patients [121,122]. The epithelia are known to possess apical nutrient transporters, which are suspected to function as part of the innate immune system acting as a mechanism to clear nutrients from the airway fluid [123]. Epithelial cells [118,124–126] and neutrophils [127] possess and/or change their nutrient transporter activity according to the nutrients available in airway fluid.

Two mouse studies have shown that streptozotocin-induced diabetic animals have increased glucose in their lavage fluid. Hunt et al. demonstrated this in the CF-knockout mouse [53], and Gill et al. demonstrated this in non-CF mice [128], where both models suggest that insulin signaling is important in controlling airway glucose availability. The direct loss of CFTR function has been implicated in causing oxidative stress and signaling dysfunction in the pancreas, which leads to impaired insulin release and eventually beta cell death [129,130]. The loss of insulin or insulin signaling may be the critical point as to how the airway epithelium maintains low nutrient availability in the airway

fluids; however, the effects of insulin and other growth factors as well as glucoregulatory hormones in the airway are currently poorly understood.

12.4 CYSTIC FIBROSIS-RELATED DIABETES AND THE AIRWAY EPITHELIUM

The most common comorbidity associated with CF is CF-related diabetes [8,131]. CFRD is a distinct clinical entity from Types I and II diabetes mellitus [132]. It is a relative insulin deficiency leading initially to unsuccessful postprandial glucose control, which can eventually devolve into sustained fasting hyperglycemia [133]. Its prevalence increases with age and nearly 50% of patients with CF over the age of 30 will develop this comorbidity [131]. The onset of CFRD is a significant event as its development is associated with a quicker decline in lung function and an increased mortality [134]. As CFRD usually presents insidiously without fasting hyperglycemia, it is important to actively screen for it. Yearly oral glucose tolerance testing is recommended for all patients with CF after the age of 10 [135]. While CFRD is associated with microvascular complications as seen in other types of diabetes, the associated detriment to lung function is what drives the increased mortality [136,137].

12.4.1 Cystic Fibrosis-Related Diabetes Lung Pathology

The onset of CFRD is associated with accelerated decline in lung function, more frequent acute pulmonary exacerbations and increased mortality [138,139]. Even patients with glucose intolerance ("prediabetes") who do not meet strict criteria for CFRD demonstrate significant declines in their pulmonary function several years before frank CFRD is achieved [140,141]. A murine model demonstrated increased levels of IL-2 and IL-10 in CFRD mice compared to normoglycemic CF controls [142]. In a similar murine model of CFRD, diabetic cystic fibrosis mice demonstrated an exaggerated, but ineffective neutrophilic response to an acute pseudomonal pneumonia when compared to normoglycemic CF and diabetic wild-type controls [53]. One receptor important in the inflammatory response is the receptor of advanced glycation end-products (RAGE). RAGE has multiple ligands and its activation causes production of inflammatory mediators [143]. RAGE expression is upregulated in lung tissue from CF mice and antagonism of the receptor was associated with decreased inflammatory markers [144]. Advanced glycation end-products, the primary ligand for RAGE, has been found to be significantly elevated in human subjects with CFRD and were associated with worse lung function [145]. There is some evidence to suggest there is a dysfunction in the airway glucose barrier with CFRD. Baker et al. found that humans with CFRD had elevated concentrations of glucose in exhaled breath condensate as well as elevated breath-to-blood glucose ratios in subjects with CFRD compared to normoglycemic controls [146]. Similar findings have also been seen in a murine model of CFRD, with CFRD mice showing elevated levels of glucose in bronchoalveolar fluid at baseline [53]. Early insulin therapy in some cases has been associated with improved lung function, but the underlying mechanism of lung dysfunction associated with the onset of CFRD remain incompletely realized [147,148].

12.4.2 CFTR, Insulin, and Glucose in Cystic Fibrosis-Related Diabetes

The effects of CFRD on the airway are unknown at present. Current research is focused on insulin secretion and whole-body glucose homeostasis utilizing the ferret [52,149,150], and pig models [58,151] as well as clinical research studies involving CF patients [152–156]. Mouse physiology can lend insights to some, but not all, pathological consequences that are found in humans. Evidence of differences in CFRD physiology between mice and humans was again highlighted when Fontés et al. [157] found that ΔF508 homozygous mice did not display insulin secretion problems or abnormal pancreatic organization in contrast to pig and human studies. However, ΔF508 mice did exhibit abnormal insulin sensitivity and a decrease in β-cell mass when aged, similar to the vertebrate models and to humans. This discrepancy highlights the advances made in the pig and ferret models of CF toward understanding the natural history of CFRD.

In the ferret, age plays a major role in the development of CFRD. Kits become fully weaned by the age of 6 weeks, are fully developed by 4–6 months, and have a finite lifespan of 5–10 years. During the first 16 weeks of life, ferrets develop basic skills related to feeding, mobility, and visual acuity. Yi et al. [149] defined the age-dependent development of CFRD into four major phases of blood glucose derangement: I, normal; II, very elevated; III, near normal; and IV, elevated. Phase I occurred from birth to about 4 weeks of age. Phase II occurred in the second month of life, lasting another 4 weeks. After the eighth week of life, Phase III began and lasted for roughly eight additional weeks, where then Phase IV manifested through the remainder of the study, roughly 52 weeks in duration. During Phase II, fasting glucose was consistently higher in CF kits than the non-CF kits and blood glucose averaged

180 mg/dL (10 mM) in the CF kits where it was 125 mg/dL (7 mM) in the non-CF kits. In contrast to this impaired resting blood glucose were the other phases that exhibited normal glucose concentrations that were not significantly different. Hyperglycemic clamp studies in Phase III kits demonstrated problematic early and late insulin secretion and hyperinsulinemic euglycemic clamp studies suggested that insulin sensitivity was maintained [149]. Therefore, studies in the young ferret point to β-cell loss as the culprit for decreased insulin production but do not fully explain the decreased insulin secretion in response to hyperglycemia [52,149,150].

Pancreatic destruction is apparent in CF fetal and newborn piglets as marked by higher rates of apoptosis, increased Ki-67 proliferative markers, and increased α-smooth muscle actin fibers that are indicative of incomplete or reorganizing pancreatic development [158]. As in the airway, CFTR-mediated regulation of pH in the pig pancreatic and bile fluid secretion is apparent. Similar to humans, exocrine pancreatic enzyme secretion is impaired in the CF pig as well [159]. Similar still to humans, pigs developed spontaneous lung infection, airway mucus plugging, and unresolved chronic inflammation that occur early in human life and continue with age. All of which seemed to correlate with the inability to resist bacterial infection from an early age [160]. Interestingly, as exocrine pancreatic disease progressed in the pig, insulin-producing endocrine islet cells did not reduce in number or organization. As insulin remains to be produced in the pig pancreas, the secretion of insulin was found to be impaired resulting in abnormal control of blood glucose levels with CF pigs having higher blood glucose concentrations and lower rates of insulin secretion [151]. Much of the pathophysiology identified in CFTR knockout pigs and ferrets match those observed in human CF and CFRD, however, differences and controversies remain in observed age-related decreases in human insulin sensitivity and reduced glucose depletion rates [161–163].

While CFRD is an active topic of research since it has the most deleterious effect on patient outcome today, the direct consequences of CFRD to airway physiology remain unknown. Much of what has been discovered in the ferret and pig is also true in humans, such as decreased first and second phase insulin release, spontaneous hyperglycemia with age [152], impaired glycemic control and tolerance [153,154,156], and the correlation with worse pulmonary function [155]. The exact effects of years of undiagnosed impaired glucose tolerance, hypoinsulinemia, and hyperglycemia on peripheral tissues like the lung remain mysterious. Further research is needed to define the natural history of CFRD and the effects that impaired insulin secretion have on peripheral tissues like the lung in order to properly address the rapid decline in lung function that occurs with CFRD diagnosis.

12.5 THERAPEUTIC INTERVENTIONS FOR CYSTIC FIBROSIS DISEASE

For many years, the primary therapies used to treat CF have targeted the downstream consequences of CFTR dysfunction. These therapies have aimed to restore normal ASL, improve mucus rheology, suppress chronic respiratory infections, or decrease airway inflammation. Many of these therapies have been very beneficial, demonstrating significant improvements in pulmonary function, and decreases in respiratory exacerbations in clinical trials [164]. Their employment, along with early diagnosis and improved nutritional supplementation, has played a significant part in improving the overall life expectancy in CF. When the causative CFTR gene was finally discovered in 1989, there was much enthusiasm that directed gene therapy may provide an ultimate cure for CF. Unfortunately, successful gene therapy has remained elusive, though there are some recent promising advances (see Section 12.6). Finally, just in the last several years, a watershed event occurred in CF therapeutics with the development and registration of the first CFTR protein potentiator, ivacaftor. This was followed quickly by approval for a combination CFTR potentiator/corrector, ivacaftor/lumacaftor. With the development of these drugs there are now clinically effective therapeutics that target the underlying protein defect. Nevertheless, only about 50% of the CF population have a CFTR mutation that qualify them to potentially benefit from these new medications [8]. As such, downstream therapies remain an important component of the therapeutic arsenal in CF care (Table 12.1).

12.5.1 Airway Surface Liquid Restoration

As previously detailed in this chapter, the ASL is a critical component of innate airway defense. It is comprised of two layers, the periciliary layer and an overlying layer of mucus [165]. The periciliary layer is composed mostly of water and is maintained through ion regulation and liquid secretion/reabsorption by superficial airway epithelial cells. Its height and viscosity are important components in ensuring appropriate lubrication of epithelial cilia. The overlying mucus layer is composed primarily of secreted mucins. It functions to trap any exogenous airway pathogens or particulate matter. The underlying periciliary layer then ensures appropriate cilia beat function in order to propel mucus and its ensnared exogenous material up and out of the airways. As discussed previously, in CF the periciliary layer

TABLE 12.1 Therapeutics Available for Chronic Cystic Fibrosis Lung Health

Treatment Category	Mechanism of Action	Clinical Benefit
Rehydration of ASL[a]		
7% Hypertonic saline	Increases osmotic gradient with exogenous NaCl	Improves lung function, quality of life and reduces exacerbations
Mannitol[b]	Nonabsorbable sugar alcohol	Improves lung function
Mucolytic		
rhDNase	Catalyzes the hydrolytic cleavage of extracellular DNA in airway mucus	Improves lung function and reduces exacerbations
N-Acetyl-L-cysteine	Breaks down disulfide bonds; provides cysteine and a precursor to glutathione	Uncertain benefit
Antiinflammatory		
Oral corticosteroids	Suppresses multiple inflammatory genes	Can improve lung function, but not recommended for routine use due to risk for long-term side-effects
Inhaled corticosteroids	Localized exposure to lung with suppression of inflammatory genes	Not recommended for routine use unless there is concurrent asthma or ABPA[c]
Azithromycin	Multiple pathways; suppression of ERK 1/2?	Reduces exacerbations and improves lung function (strongest benefit if chronic infection with *P. aeruginosa*)
Inhaled antimicrobial		
Inhaled tobramycin	Macrolide antibiotic	Improves lung function, quality of life, and reduces exacerbations
Inhaled aztreonam	Monobactam antibiotic	Improves lung function and quality of life
CFTR Modulator		
Ivacaftor	CFTR potentiator	For specific gating mutations[d]; improves lung function, weight, and reduces exacerbations
Lumacaftor	CFTR corrector	Used in combination with ivacaftor for patients homozygous for ΔF508, can improve lung function

[a] *ASL, airway surface liquid.*
[b] *Mannitol is currently approved for use in Europe, but not the United States.*
[c] *ABPA, allergic bronchopulmonary aspergillosis.*
[d] *G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, S549R, R117H.*

is substantially reduced. This is due to lack of adequate CFTR chloride secretion in superficial epithelial cells and hyperabsorption of sodium from the periciliary layer through unregulated ENaC channels [165]. The consequence is reduced height of the periciliary layer and significant attenuation of mucus transport [166]. As such, therapies aimed at restoring the ASL have been developed with several available for clinical use.

12.5.1.1 Hypertonic Saline

Of the available medications targeting ASL rehydration, hypertonic saline has been the most studied. Development of inhaled hypertonic saline as a therapy for CF came about somewhat serendipitously. A group of CF clinicians in Australia recognized CF patients who spent more time near the ocean reported improved respiratory symptoms and less frequent acute pulmonary exacerbations [167,168]. Researchers hypothesized the increased salinity of the ocean air aided in restoration of the ASL and improved mucus clearance. Knowledge of increased sodium resorption in the periciliary layer in CF disease preceded this observation. Indeed, previous in vitro studies had demonstrated an increase in mucus clearance with the addition of hypertonic saline [169,170]. Small, short-term pilot studies had demonstrated a potential benefit with inhaled hypertonic saline in vivo [171–174]. However, they also demonstrated a short duration

of action, causing concern for the long-term efficacy of hypertonic saline administration. The anecdotal Australian ocean-goer experience created optimism that long-term efficacy could be achieved leading to the largest, longitudinal study of hypertonic saline administration.

Elkins et al. were able to recruit and randomize 164 patients with CF across 16 centers in Australia to receive 4 mL of either 7% hypertonic saline or 0.9% saline twice daily for 48 weeks [167]. The primary outcome measure was the rate of change of lung function over the study period, which did not differ significantly between groups. However, important secondary outcomes were significantly different. Specifically, the group receiving hypertonic saline had fewer acute respiratory exacerbations and higher absolute values of pulmonary function in terms of both forced vital capacity (FVC) as well as FEV_1. Significantly, exposure to inhaled hypertonic saline did not alter respiratory bacterial isolates between the groups or worsen markers of inflammation as measured by sputum levels of IL-6, IL-8, IL-10, and tumor necrosis factor-α [167]. Concurrently, Donaldson et al. from the University of North Carolina demonstrated in a smaller, short-term trial that administration of inhaled hypertonic saline four times daily was associated with sustained increases in mucus clearance as well as improvements in lung function (FEV_1 and FVC) and respiratory symptoms [175]. Inhalation of 7% hypertonic saline solution has since become a mainstay of CF care with over 60% of adult patients partaking in this therapy [8].

As these prior clinical trials excluded patients under the age of 6 years old, a large randomized, placebo-controlled trial was developed to study hypertonic saline administration in infants and children with CF [176]. Prior studies had demonstrated no exaggerated decline in pulmonary function in children receiving hypertonic saline and relatively good adherence to the medical regimen [177,178]. Unfortunately, no significant differences in pulmonary exacerbations, respiratory symptoms or antibiotic treatment days were found in children under the age of 6 years receiving hypertonic saline when compared to placebo [176]. These negative results may have been related to the relatively limited clinical disease seen in this patient population, choice of placebo, the study duration (48 months) or possible effects of viral infections seen commonly in this population age. Nevertheless, hypertonic saline administration in infants seems relatively well tolerated and improved mucus clearance may have long-term effects in lung function maintenance beyond the length of this study. As such, a clinical trial is underway exploring effects of hypertonic saline administration in this patient population on lung clearance index and small-airways architectural changes with an estimated study completion date in April 2018 (ClinicalTrails.gov, NCT02378467).

12.5.1.2 Mannitol

Mannitol is a nonabsorbable sugar alcohol, which is available as a dry powder. As an osmotic agent it exerts effects on ASL rehydration by drawing water into the airway lumen, increasing the ASL and improving mucociliary clearance [179,180]. Unlike hypertonic saline, which requires specialized equipment for nebulization and is time consuming, inhaled dry powder mannitol is easily transportable, requires no electricity for administration and typically can be administered in a shorter time frame compared to hypertonic saline. A caveat to inhaled mannitol administration is it can induce significant bronchospasm. Mannitol is one of the agonist agents used for a bronchial challenge test; a provocative test designed to assess for airway hyperresponsiveness seen in asthma and other conditions of airway hyperreactivity [181]. However, for patients with CF that do not demonstrate airway hyperreactivity with exposure to inhaled mannitol, this therapy may provide a useful therapeutic alternative to hypertonic saline.

There have been two large multicenter, randomized controlled phase III trials to study inhaled mannitol in CF [182,183]. Patients were only included in the study if they demonstrated no significant bronchial hyperreactivity to an initial mannitol bronchial provocation test. Patients over the age of 6 years old meeting this criteria with mild to moderate lung disease (30% or $40\% \leq FEV_1 > 90$) were randomized to receive either 400 mg of dry powder mannitol or a subtherapeutic dose of 50 mg dry powder mannitol (control) inhaled twice daily for 26 weeks. Both studies also had a subsequent 26 week open-label trial following the first 26 week randomization study. Both studies used measurements of FEV_1 (absolute value at 26 weeks or change in FEV_1 over 26 weeks) as their primary outcome. In both studies, patients were prohibited from concurrently using hypertonic saline. However, they were allowed to use other chronic CF therapies available at the time. Both studies demonstrated a sustained improvement in pulmonary function with the use of therapeutically dosed inhaled mannitol. There was also a reduction in acute exacerbations in the treatment group, though this met statistical significance in only one of the two studies. Neither study demonstrated a significant difference in adverse events between the two groups. However, there was a higher percentage of adverse events that led to withdrawal from the study in the experimental group in one of the trials (6.5% vs 1.7%). As these studies included adults and children, a separate post-hoc analysis of pooled data from both studies only including subjects aged 18 years and older was performed [184]. Analysis revealed

similar findings to the original larger studies. Adults receiving therapeutic doses of inhaled mannitol had significant and sustained improvements in lung function at 26 weeks. Interestingly, therapeutic response to the study drug at 6 weeks was predicted of continued response at study-end (correlation slope = 0.726, $P < 0.0001$). There was also a meaningful trend toward reduction in acute pulmonary exacerbations in the experimental group, though this did not meet statistical significance. Again, researchers noted an increase in treatment-related adverse events leading to drug discontinuation, but overall there were no differences in the number of adverse events between the two groups [184]. A subsequent phase II trial examining inhaled mannitol effects on lung function and sputum properties in children aged 6−17 years old has been completed and currently under analysis (ClinicalTrails.gov, NCT01883531). Based on these data, inhaled mannitol has been approved for use in CF in Europe. Inhaled mannitol has never been compared head to head with inhaled hypertonic saline or dornase alpha. It has not been regulated in the United States and a further safety and efficacy trial examining its effects in adults with CF is underway in North America with expected completion date in December 2016 (ClinicalTrails.gov, NCT02134353).

12.5.2 Breaking Up the Mucous with Mucolytics

In addition to reduced height and increased viscosity of the periciliary layer, the overlying mucus layer in CF is also significantly thickened. This is in part due to the effects of dehydration caused by dysfunctional CFTR, but also exacerbated by aberrant neutrophil function [185]. CF lung disease is characterized by significant infiltration of the airways by neutrophils. These leukocytes are present in large numbers, yet are unable to clear chronic bacterial infections from the airways [104]. These short-lived airway neutrophils release substantial amounts of extracellular DNA, which serves to thicken the mucus making it more difficult to be expectorated [185]. As such, therapeutic mucolytic agents have been developed for CF.

12.5.2.1 DNase Alpha

Dornase alpha (rhDNase) is a recombinant human deoxyribonuclease. Aerosolized dornase was one of the earlier lung directed therapies proposed for CF [186]. Shak et al. were able to clone, sequence and express rhDNase I. They then exposed samples of CF sputum to rhDNase, which significantly reduced sputum viscosity compared to controls [187]. This quickly led to small phase I and II trails of aerosolized rhDNase in patients with CF that demonstrated the medication was relatively safe and was associated with short-term improvements in lung function [188−191]. Subsequently, a large randomized, double-blind, placebo-controlled trial examined the effects of inhaled rhDNase administration in patients with CF [192]. A total of 968 patients with CF over the age of 5 years old were randomized to receive either 2.5 mg of rhDNase once daily, 2.5 mg of rhDNase twice daily or placebo for 24 weeks. Acute pulmonary exacerbations were reduced in both groups receiving rhDNase compared to placebo. There was also significant and sustained increases in pulmonary function with FEV_1 increasing nearly 6% in both groups receiving rhDNase. The experimental groups did have higher rates of laryngitis and voice alterations, but the majority of these cases resolved by 21 days in the course of the study [192]. A subsequent safety and efficacy study of 320 patients with CF receiving either once daily rhDNase or placebo over 12 weeks corroborated these earlier findings [193]. A two-year long clinical trial of rhDNase administration in children aged 6−10 years old with mild CF lung disease also demonstrated improvements and maintenance of lung function as well as a 34% reduction in acute pulmonary exacerbations in the experiment group [194]. In small studies, rhDNase has been shown to improve lung ventilation inhomogeneity in children aged 6−18 years [195]. Administration of inhaled rhDNase has also been associated with improved weight gain in children with CF under the age of 2 years. Administration of rhDNase has also been associated with reductions in markers of airway inflammation. The Bronchoalveolar Lavage (BAL) for the Evaluation of Antiinflammatory Treatment (BEAT) was a 3-year study in 105 patients with CF and early lung disease [196,197]. Those patients with high levels of airway neutrophils at baseline were randomized to receive either rhDNase or no treatment. Compared to the groups receiving inhaled rhDNase, the no treatment group developed higher levels of airway neutrophilic infiltrates, DNA, elastase, interleukin-8, myeloperoxidase-8, and myeloperoxidase-9 compared to those receiving rhDNAase [196,197]. A smaller, short-term study of slightly older patients (mean 16.8 years) failed to show any significant changes in BAL neutrophil numbers in patients taking rhDNase, but the study was significantly shorter in duration compared to the BEAT trial (1 month vs 3 years), and nevertheless, the patients in the experimental group still had significant improvements in lung function [198]. Given the preponderance of evidence for the efficacy of inhaled rhDNase, the CF Foundation recommends anyone with CF and mild lung disease 6 years of age and older be considered for inhaled rhDNase therapy [197].

12.5.2.2 N-Acetyl-Cysteine

N-Acetyl-L-cysteine (NAC) is a mucolytic and important precursor to glutathione, the most prominent small biothiol in lung extracellular redox regulation [199,200]. NAC administration provides sulphydryl groups, which act on and break down disulfide bonds found in mucus glycoproteins creating smaller glycoprotein subunits and thereby reducing the mucus viscosity [201]. In addition, epithelial cell cultures exposed to NAC have demonstrated reduced sodium absorption, which may also aid in improving mucus rheology [202].

Recent safety and efficacy trials have yielded conflicting results with the use of NAC in CF. In a recent phase II double-blind, placebo-controlled trial, 70 subjects with CF received either NAC or placebo orally thrice daily for 24 weeks [203]. Compared to those on placebo, subjects taking oral NAC had no more significant side-effects and demonstrated more stability in lung function. However, there were no statistical differences in acute pulmonary exacerbations or markers of pulmonary inflammation [203]. Similarly, Skov et al. demonstrated subjects with CF receiving high-dose oral NAC (2400 mg/day) over 4 weeks demonstrated a trend toward improved lung function compared to controls along with statistically significant increases in plasma levels of ascorbic acid [204]. Conversely, a small phase II trial in Germany randomized subjects with CF to receive low dose (700 mg/day) or high dose (2400 mg/day) oral NAC for 12 weeks and demonstrated no significant improvements in clinical function or markers of inflammation [205]. Given the lack of robust clinical data regarding the use of NAC in CF, the CF Foundation Guidelines do not advocate for or against the chronic use of this therapy [206].

12.5.3 Antiinflammatory Agents in Cystic Fibrosis

12.5.3.1 Steroids

In addition to the known pulmonary inflammatory state seen very early in CF, it was observed that children with CF who had lower serum IgG levels had improved lung function when compared to aged-matched CF controls with normal or elevated levels of IgG [207]. Corticosteroids were the first antiinflammatory therapy employed for CF. Corticosteroids have multiple immunomodulatory effects including reducing NF-κB activity, leukocyte adhesion and activation, and inhibiting or down regulating a host of inflammatory intermediaries [208]. Oral steroids are efficacy and early clinical trials demonstrated improvements in lung function, body mass index, and reduced exacerbations [209,210]. However, long-term potential risks associated with chronic oral steroid administration including osteopenia, hyperglycemia, adrenal suppression, and growth retardation limit their clinical use [208]. Unless, a patient has concurrent asthma or allergic bronchopulmonary aspergillosis, the CF Foundation Guidelines actively recommend against use of chronic oral steroids.

With the hope of achieving the therapeutic benefits of oral steroids while limiting the systemic side-effects, inhaled corticosteroids have been proposed for use in CF care. Unfortunately, there have been no large prospective clinical trials that have demonstrated a significant benefit with inhaled corticosteroid therapy in CF patients without concurrent asthma [211,212]. Furthermore, use of inhaled corticosteroids has been associated with reduced height and increased risk for lung function decline [213]. The CF Foundation no longer recommends the routine use of inhaled corticosteroids in patients without concurrent asthma or allergic bronchopulmonary aspergillosis [206]. In support of that, a large, multicenter trial of 171 patients with CF who underwent randomized withdrawal of inhaled corticosteroid demonstrated no significant adverse effects on lung function or antibiotic use in the group weaned to placebo over a 6-month period [212].

12.5.3.2 Ibuprofen

Prior studies had demonstrated beneficial effects of steroids in CF, but at the risk of significant side-effects. As such, nonsteroidal immunomodulatory agents have been explored. Ibuprofen is a nonsteroidal antiinflammatory drug that acts as a cyclooxygenase inhibitor thereby inhibiting prostaglandin synthesis. Early murine studies demonstrated ibuprofen administration attenuated significant inflammation associated with a rat model of chronic pseudomonal lung infection [214]. This led to further human clinical studies. The most recent 2016 Cochrane Database analysis for ibuprofen use in CF included three randomized, placebo-controlled clinic trails [215]. Metaanalysis demonstrated high-dose ibuprofen use was associated with a slower rate of annual decline in lung function as well as a reduction in intravenous antibiotic usage, particularly in younger children [215]. At present, the CF Foundation Guidelines recommend chronic use of oral ibuprofen to achieve plasma concentrations of 50−100 μg/mL to slow the loss of lung function for individuals with CF under the age of 18 years [206]. Nevertheless, there is only limited use of ibuprofen in actual clinical practice

(about 3%−5% of the CF population) given the logistical difficulties of obtaining accurate pharmacokinetic studies as well as concern for possible long-term renal and gastrointestinal side-effects [8,216,217].

While these moderately sized clinical trials showed encouraging results for ibuprofen use in CF, the underlying immunomodulatory effects remains incompletely understood. Recent data has demonstrated a defect in microtubule acetylation and polymerization in CF epithelial cells, which may alter inflammatory signaling [218,219]. Further, cell culture experiments exposing CF epithelial cell lines as well as primary nasal epithelial cells to high-dose ibuprofen demonstrated restoration of microtubule reformation rates similar to wild-type cells as well as induced extension of microtubules to the cell surface [220]. The effects of ibuprofen were mimicked by inhibition of adenosine monophosphate-activated protein kinase, suggesting the antiinflammatory properties imparted by ibuprofen on CF pathology were potentially through regulation of microtubules. Ibuprofen may also act as a CFTR corrector, though data is limited. Carlile et al. found that CF epithelial cell lines had increased short-circuit current when exposed to high-dose ibuprofen. In addition, they found that a murine model of ΔF508 CF mutation demonstrated partial restoration of CFTR trafficking [221].

12.5.3.3 Azithromycin

Azithromycin is a macrolide antibiotic that has both antimicrobial and antiinflammatory properties [222]. It has shown benefit and been employed in diseases with neutrophilic-inflammation including obliterative bronchiolitis, chronic obstructive pulmonary disease, and CF [223]. Its effects within the lung environment in CF have not been completely elucidated, but multiple potential therapeutic pathways have been proposed. An exhaustive review of the theoretical immunomodulatory effects of macrolides is beyond the scope of this chapter, however, excellent review papers exist [223]. Azithromycin likely exerts some of its antiinflammatory effects through modulation of the ERK 1/2 pathway, inhibiting NF-κB activity, an important transcription factor in the inflammatory response [223]. In an in vitro model of CF epithelial cells, azithromycin exposure has been shown to reduce tumor necrosis factor-α (TNF-α) mRNA and protein levels. In addition, NF-κB and specificity protein 1 DNA-binding sites were reduced [224]. Azithromycin has also been shown to decrease lung bacterial burden and pulmonary inflammation (neutrophil infiltrate) in CF mice infected with pseudomonas [225]. In a small human study, children with CF chronically infected with *P. aeruginosa* were treated with azithromycin for 3 months. Following completion of therapy, measurements of pseudomonas adherence to buccal epithelial cells in the patients were significantly reduced to levels similar to controls [226]. Similarly, in a coculture of CF bronchial mucins with *P. aeruginosa*, addition of azithromycin to the culture significantly reduced pseudomonas adherence to the mucins in most strains that were tested [227].

The theoretical immunomodulatory benefit and these early in vitro experiments prompted larger clinical trials for the use of oral azithromycin in CF. These trials included both patients that were chronically colonized with pseudomonas as well as those who were not [228−232]. Several large trials demonstrated significant improvements in pulmonary function (FEV_1 and FVC) in patients taking azithromycin [230,231]. Most of these randomized clinical trials also demonstrated significant reductions in acute pulmonary exacerbations rates in groups receiving azithromycin regardless of their pseudomonas status [228,230−232]. There has been some recent concern that administration of azithromycin may interfere with the therapeutic effects of inhaled tobramycin (a commonly utilized inhaled antibiotic therapy in CF care) [233]. However, these data have not been substantiated in large prospective trials, though a multicenter, randomized, placebo-controlled trial to study the effects of adding azithromycin to inhaled tobramycin therapy is currently in development (ClinicalTrails.gov, NCT02677701). Current guidelines now recommend at least consideration of long-term use of azithromycin (500 mg orally thrice weekly or 250 mg orally daily) for all patients with CF regardless of their pseudomonas status [206].

12.5.4 Inhaled Antibiotics

Long-term antibiotic suppressive therapy has shown clinical benefit in individuals with CF once chronic pulmonary infection with *P. aeruginosa* occurs. Inhaled antibiotics have been used most frequently with the goal of obtaining localized therapeutic benefit at the site of the lungs, while limiting the potential long-term side-effects of systemic antibiotic administration [234]. There are several formulations of inhaled antibiotics with antipseudomonal properties used in CF including tobramycin, an aminoglycoside, and aztreonam, a monobactam, both of which are regulator approved drugs in the United States and much of Europe. An exhaustive review of the clinical data associated with the efficacy and approval of these inhaled antibiotics in CF care is beyond the scope of this chapter, but exists in detail in several recent review articles [235,236]. Tobramycin and aztreonam have demonstrated improvements in lung function

and reductions in pulmonary exacerbations in patients with CF chronically infected with *P. aeruginosa* and moderate to severe lung disease [206]. They are both recommended by the CF Foundation for individuals with mild to severe lung disease, though the optimal inhaled antibiotic regimen is unknown [206].

Sputum cultures are often substituted as a surrogate for lower respiratory cultures and are grown on agar plates selective for pathogens previously identified as important in CF clinical outcomes. However, as molecular diagnostic techniques have become more sophisticated over the years, the understanding of the microbiome within the context of CF has become far more complex [237–239]. The current understanding of the microbial diversity within the CF lung is very limited. Further, how presently used inhaled antibiotics manipulate the larger microbiome and effect microbial diversity and interaction with epithelial cell function remains an important unexplored topic.

12.5.5 Modulators of CFTR Protein Trafficking and Channel Function

Prior to the development of CFTR modulators, available therapies only targeted downstream consequences of CFTR protein dysfunction. The therapeutic goal of a CFTR corrector is to rescue CFTR protein up to the epithelial cell surface. A CFTR potentiator increases the effectiveness of CFTR protein channel activity if it is already in the correct location [240]. The development of clinically effective modulators has occurred only very recently with ivacaftor receiving regulatory approval by the FDA in 2012 [241]. With the advent of high-throughput screening to identify potential CFTR modulators and early clinical success with ivacaftor and ivacftor/lumacaftor, there is great enthusiasm that further exploration and development of CFTR potentiators will significantly alter the natural progression of CF lung disease.

12.5.5.1 Ivacaftor

Utilizing a cell-based fluorescence membrane potential assay, high-throughput screening was performed on 228,000 unique compounds to identify CFTR potentiators [242]. Ivacaftor (known as VX-770 in its preclinical trials) was chosen for further clinical study based on its CFTR selectivity and pharmacokinetic profile [243]. In vitro analysis of cell cultures demonstrated that ivacaftor exposure increased CFTR channel open probability in cells expressing ΔF508, a processing mutation in which the CFTR protein does not traffic up to the cell surface, and in cells expressing G551D, a gating mutation in which there is defective channel regulation. In addition, exposure to ivacaftor in epithelial cells expressing G551D CFTR mutation demonstrated significant increases in chloride conductance, reduction in excessive sodium and fluid resorption at the apical surface, and increased cilia beating [243]. While its mechanism of action is not completely understood, evidence suggests ivacaftor binds directly to the CFTR protein and exerts its effects in an ATP-independent manner, which is also independent of dimerization of the CFTR nucleotide binding domains [244–246].

Two-phase III trials, ENVISION and STRIVE, established the clinical effectiveness and secured regulatory approval of ivacaftor for patients with CF and at least one G551D mutation [247,248]. STRIVE, the larger of the two trials, enrolled 161 patients older than 12 years of age with G551D mutation [248]. Participants were randomized to either ivacaftor or placebo for 48 weeks. Subjects taking ivacaftor demonstrated statistically significantly increases in lung function with percent predicted FEV_1 increasing by 10.8% compared to the control group. The ivacaftor group also experienced a decrease in pulmonary exacerbations by 55% and a significant and sustained increase in weight. The subjects receiving ivacaftor had significant decreases in their measured sweat chloride concentrations supporting the mechanism of action did improve CFTR function [248]. Similar findings were seen in the ENVISION trial, which randomized subjects ages 6–12 years to either ivacaftor or placebo [247]. Both of these trials rolled into a long-term (96-week) open-label extension, which demonstrated two important findings. First, patients receiving ivacaftor during the initial blinded study, maintained improvements in lung function (FEV_1) and weight throughout duration of the open-label trial. Second, subjects who originally received placebo during the blinded portion of the trial and later switched to ivacaftor demonstrated increases in lung function at 48 and 96 weeks in the open-label trial [249]. While the clinical improvements observed in patients taking ivacaftor were very exciting, patients with a G551D gating mutation who may benefit from this therapy only account for about 4% of the total CF population. There was hope that ivacaftor would also be efficacious for individuals homozygous for ΔF508 so that nearly 50% of the total CF population would benefit from the drug [8]. Unfortunately, a randomized, double-blind, placebo-controlled trail with open-label extension failed to demonstrate any clinically relevant changes between subjects homozygous for ΔF508 taking ivacaftor compared to controls [250]. Efficacy of ivacaftor has also been explored in other non-G551D CFTR gating mutations and subjects demonstrated similar improvements in lung function, weight, and sweat chloride concentrations as had been seen in

initial clinical trials in subjects with G551D mutations [251]. Ivacaftor is orally bioavailable and now FDA approved for individuals with CF with selected gating mutations (G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, S549R, and R117H), which account for approximately 7% of the population [241].

12.5.5.2 Ivacaftor/Lumacaftor

It was hypothesized that ivacaftor's limited effects in patients homozygous for ΔF508 was related to the processing defect of CFTR. That is, the ΔF508 mutation causes a trafficking defect whereby the CFTR protein never reaches the cell surface where it can be effectively acted on by ivacaftor [250]. Another CFTR modulator discovered by high-throughput screening, lumacaftor (also known preclinically as VX-809), demonstrated improved CFTR processing through the endoplasmic reticulum and increased chloride secretion in human bronchial epithelial cells homozygous for ΔF508, though levels were still reduced compared to that of wild-type CFTR [252]. It was hypothesized that lumacaftor would act as a CFTR corrector and rescue CFTR up to the apical cell surface where ivacaftor could work as a potentiator on the dysfunctional protein and increase its chloride conductance. Following a phase II safety and tolerability trial for lumacaftor monotherapy in subjects with CF, a phase II safety trial with ivacaftor/lumacaftor combination therapy demonstrated small, but statistically significant reductions in sweat chloride concentrations in subjects taking dual therapy [253]. Significantly, there were no significant adverse-events compared to placebo. This led to two-phase III randomized, double-blind, placebo-controlled trials with the use of combination ivacaftor/lumacaftor for individuals homozygous for ΔF508 [254]. A total of 1108 patients were enrolled and randomized to receive study drug or placebo for 24 weeks. Patients who received combination therapy had modest, but statistically significant improvements in lung function compared to placebo (2.6%−4% increase in percent predicted FEV_1). In addition, combination therapy was associated with a 30%−39% reduction in pulmonary exacerbations compared to the control group, though the rate of adverse-event associated drug discontinuation was slightly higher in the combination therapy group (4.2% vs 1.6%) [254]. Combination ivacaftor/lumacaftor (marketed as Orkambi) was FDA approved in the summer of 2015 for individuals 12 years and older with CF and ΔF508 homozygous CFTR mutation.

With the approval of Orkambi, approximately 50% of the cystic population potentially qualifies for CFTR modulator therapy. However, Orkambi has not proven to be as efficacious for individuals homozygous for ΔF508 mutation as monotherapy ivacaftor is for individuals with a gating mutation, such as G551D. In addition, therapy does not completely reverse the underlying CFTR protein dysfunction, and it remains to be seen what the long-term consequences of these medications will ultimately be on CF morbidity and mortality. As such, the CF Foundation continues to explore new modulators and other downstream therapies through their drug development pipeline.

12.6 FUTURE DIRECTIONS OF THERAPIES FOR CYSTIC FIBROSIS

While the discovery and development of clinically relevant CFTR modulators has been a significant breakthrough in CF care, the disease remains progressive with a significantly reduced lifespan. Further studies to discover more efficient and mutation-specific CFTR modulators remain an important component to the CF therapeutic development pipeline. In addition, new therapies are continuing to be explored aimed at previous therapeutic targets including restoration of ASL, improving mucus rheology and immunomodulation. The most current CF Foundation sponsored drug development pipeline can be found at https://tools.cff.org/research/drugdevelopmentpipeline (Table 12.2).

12.6.1 Future Therapies for Airway Surface Liquid Restoration

12.6.1.1 Denufosol

Denufosol is a P2Y [2] receptor agonist. As discussed earlier in this chapter (see Section 12.3) P2Y [2] mRNA is located primarily in epithelial cells and stimulation leads to CFTR-independent increases in chloride and water secretion as well as cilia beat frequency [255]. An inhaled formulation of denufosol tetrasodium (previously identified as INS37217) was developed and demonstrated fair tolerability in phase I and II clinical trials [256,257]. Early phase III clinical trials demonstrated modest, but statistically significant increases in lung function associated with administration of denufosol [258]. Unfortunately, the TIGER-2 phase III trial compared denufosol to placebo in 466 patients with CF and found no significant changes in pulmonary function or reduction in pulmonary exacerbations over a 48-week period [259]. Following this, the open-label extension was terminated and there are no current trials exploring denufosol or other P2Y [2] inhibitors in CF currently (ClinicalTrials.gov, NCT00846781).

TABLE 12.2 The Cystic Fibrosis Therapeutic Development Network Pipeline

Treatment Category	Mechanism of Action	Clinical Phase
Rehydration of ASL[a]		
Denufosol (INS37217)	P2Y [2] receptor agonist	Phase III open-label extension was terminated; no active trials underway
VX-371 (P-1037)	Inhaled epithelial sodium channel antagonist	Phase II trial goal completion in summer 2017
Mucolytic		
OligoG	Chelation of divalent cations and disruption of biofilms	Phase IIb trials for patients with and without Burkholderia infection
Antiinflammatory		
CTX-4430	Leukotriene A4 hydrolase inhibitor	Phase II trials with goal completion in winter of 2017
JBT-101 (resunab)	Synthetic endocannabinoid CB2 receptor agonist	Phase II trial actively recruiting with goal completion in April 2017
GS-5745	Anti-MMP9[b] antibody	Phase II trial is planned, though not yet recruiting. Goal completion is April 2019
Inhaled Antimicrobial		
Inhaled levofloxacin	Fluoroquinolone antibiotic	Phase III trial has been completed
Inhaled lipsomal amikacin	Aminoglycoside antibiotic	A phase III trial for therapy of nontuberculous mycobacterium was completed in 2015
AeroVanc	Inhaled Vancomycin powder	Phase II trial for MRSA therapy completed
Gallium	Disrupts iron-dependent biological processes	Phase II trial underway
Nitric oxide	Inhaled nitric oxide	Phase II trial planned
CFTR Modulator		
VX-661	CFTR corrector	Phase III trial with VX-661 in combination with ivacaftor for subjects homozygous for ΔF508 is currently underway
Ataluren	Read-through agent; over-rides premature stop codons	Active phase III trial for patients with nonsense mutations who are NOT taking aminoglycosides
FDL169	CFTR corrector	Phase Ib trial completed in July 2016; Phase II trial for subjects homozygous for ΔF508 is being planned
QBW251	CFTR potentiator	Phase II trial terminated in November 2015
N9115	Increases *S*-nitrosoglutathione	Phase II trial for patients concurrently on Orkambi is underway
Riociguat	Stimulates soluble guanylate cyclase	Phase II trial for subjects homozygous for ΔF508 is underway
QR-010	Repairs CFTR mRNA	A phase Ib trail is underway

[a]*ASL, airway surface liquid.*
[b]*MMP9, Matrix metallopeptidase 9.*
Modified from The Cystic Fibrosis Foundations Drug Development Pipeline. URL: https://www.cff.org/Trials/pipeline Accessed July 29, 2016.

12.6.1.2 VX-371

VX-371, formally known as P-1037, is an inhaled ENaC antagonist developed by Vertex Pharmaceuticals and Parion Sciences in collaboration with the CF Foundation Therapeutics Development Network, currently, in phase II clinical trials (https://tools.cff.org/research/drugdevelopmentpipeline, ClinicalTrials.gov, NCT02709109). Prior studies utilizing amiloride did not demonstrate appreciable clinical benefit, potentially a consequence of the short half-life of amiloride

[260]. VX-371 has demonstrated a potentially more favorable pharmacokinetics profile and the phase II clinical trial is currently enrolling with goal data completion in June 2017 (ClinicalTrials.gov, NCT02709109).

12.6.2 Future Therapies for Modulating Mucus

12.6.2.1 OligoG

OligoG is a derivative of seaweed alginate oligosaccharide that demonstrates mucolytic properties through chelation of divalent cations and disruption of biofilms [240,261]. It also has been shown to interact directly with *P. aeruginosa* cell walls, altering cell surface charge and bacterial motility [262]. In addition, in a murine model of CF, OligoG administration has been demonstrated to improve intestinal transit times and survival [263]. Inhaled OligoG is now being actively studied in a phase IIb clinical trial in CF patients with and without burkholderia infection (ClinicalTrials.gov, NCT02157922, NCT02453789).

12.6.3 Immunomodulation of the Airway Epithelium

12.6.3.1 CTX-4430

CTX-4430 (Celtaxsys) is a Leukotriene B4 (LTB4) antagonist (https://tools.cff.org/research/drugdevelopmentpipeline). LTB4 is released by macrophages and neutrophils and is important in the inflammatory response within the lung environment [240]. A previous LTB4 receptor antagonist, BIIL 284, was associated with reductions in pulmonary neutrophils, but increases in *P. aeruginosa* numbers in a murine model of chronic pseudomonal lung infection [264]. A phase II trial utilizing BIIL 284 had to be terminated early due to increased serious adverse events and pulmonary exacerbations associated with BIIL 284 administration [265]. CTX-4430 acts as an inhibitor of Leukotriene A4 Hydrolase (LTAH4), which is the key enzyme in the production of LTB4 [266]. A phase II clinical trial studying CTX-4430 use in CF is currently recruiting with the goal of data completion in December 2017 (ClinicalTrials.gov, NCT02443688).

12.6.3.2 JBT-101

JBT-101 (Resunab) is a synthetic oral endocannabinoid-mimetic compound that selective binds to the CB2 receptor, present mostly on cells involved in inflammation [267]. Because it is preferential to CB2, it is not associated with the psychoactive effects seen in CB1 receptor activation. A phase II safety, tolerability, pharmacokinetics, and efficacy trial for JBT-101 use in CF is actively recruiting subjects with the goal completion date of April 2017 (ClinicalTrials.gov, NCT02465450).

12.6.4 CFTR Modulators in Development

12.6.4.1 VX-661

VX-661 is a corrector similar to lumacaftor. In combination with ivacaftor, it has been shown to increase CFTR channel activity in vitro [268]. A 12-week, double-blind, placebo-controlled trial in patients homozygous for ΔF508 mutation receiving ivacaftor and VX-661 combination therapy demonstrated fair tolerability and increases in lung function compared to controls [269]. There are currently seven active or planned clinical trials to further study VX-661 in combination with ivacaftor for the treatment of CF (ClinicalTrials.gov, NCT02565914, NCT02392234, NCT02508207, NCT02347657, NCT02953314, NCT02730208, and NCT02412111).

12.6.4.2 Ataluren

Ataluren is a read-through agent that over-rides premature stop codons found in class I CFTR gene mutations [270]. Small, short-term safety, and efficacy studies have been performed and demonstrated improvements in nasal epithelial chloride transport with administration of ataluren [271,272]. A phase III trial was then undertaken enrolling patients over the age of 6 years with nonsense CFTR mutations to receive either oral ataluren or placebo [273]. A total of 238 patients were enrolled and randomized. In whole group analysis, there were no significant differences between groups in terms of lung function or pulmonary exacerbations. However, a post-hoc subgroup analysis found patients who were not on inhaled tobramycin while they were receiving ataluren had a significant increase in pulmonary function with

a 5.7% mean increase in FEV_1 compared to placebo [273]. Ataluren is structurally similar, but chemically distinct to gentamicin, an aminoglycoside [274]. As tobramycin, a common inhaled antibiotic utilized in CF, is also in the aminoglycoside family, it was hypothesized that concurrent administration may interfere with the function of ataluren [273]. A subsequent phase III trial of ataluren use in CF patients not on chronic inhaled tobramycin therapy is currently underway with estimated completion date in June 2019 (ClinicalTrails.gov, NCT02107859).

12.6.4.3 Other Modulators

There are several other modulators that are in very early stages of clinical development. QBW251 is another potentiator similar to ivacaftor. A phase II trial was performed and terminated in November 2015. Data from the trial was presented at the 2016 American Thoracic Society International Meeting and demonstrated oral QBW251 was safe, but demonstrated no improvements in lung function or sweat chloride concentrations in patients homozygous for ΔF508 [275]. No further trials with QBW251 are active at this time. N9115 is a first in its class modulator that increases levels of *S*-nitrosoglutathione, which are significantly reduced in individuals with CF. A phase II study of N9115 as adjunct therapy in patients also taking Orkambi is currently underway (ClinicalTrials.gov, NCT02589236). Other potential modulators include riociguat, which stimulates soluble guanylate cyclase, and QR-010, which is designed to repair CFTR mRNA (TDN URL). Both are in early phase I or II trials (ClinicalTrials.gov, NCT02564354, NCT02532764, and NCT02170025).

12.6.5 CFTR Gene Therapy

Since the CFTR gene was initially discovered in 1989, there has been great interest both in the lab and clinic in exploring gene therapy with the aim of curing this life-limiting disease. There have been over 20 clinical trials studying gene therapy for CF [276]. Unfortunately, effective gene therapy has remained elusive. There are a number of barriers that will have to overcome in order for gene therapy to become a viable therapy for CF care. Gene vectors have to bypass innate lung defenses and the substantially thickened mucus within the CF lung in order to be transported to epithelial cells within the conducting airways for gene transcription to occur [276]. Nonviral vectors may be degraded by lysosymes limiting their gene transmission [276]. Viral vectors also have their own complications. For example, adenovirus vectors rely on expression of coxsackie-adenoviral receptors, which are not always expressed on the apical surface of airway epithelial cells [276]. However, there have been some recent encouraging clinical data with an inhaled liposomal-gene formulation [277]. In a recent phase IIb trial, subjects were randomized to receive either inhaled pGM169/G67A, a CFTR gene-liposome, or saline every 5 days for 12 months. There was a modest, but statistically significant, increase in FEV_1 at 12 months in the treatment group compared to placebo. Effects were seen as early as 1 month after therapy began and were not limited by CFTR mutation type [277]. Further trials are under development to ascertain the most efficient dosing administration of gene-vector as well as other gene-vectors such as a pseudo-typed lentivirus [240].

12.6.6 Personalized Medicines

There has been a recent watershed in CF therapeutics with the development of high-throughput screening and identification of effective CFTR modulators. Development of mutation-specific modulators has made CF an example of what is possible with personalized medicine. However, nearly 50% of the CF population do not carry a genetic mutation amenable to the modulators currently available. While ΔF508 mutation heterozygosity is prevalent in the United States, there are over 1800 CFTR mutations now identified. More so, CFTR mutational incidence varies depending on geography and heritage. Many drug regulatory agencies like the FDA require robust clinical safety and efficacy data before consider approval of pharmaceuticals. Obviously, robust clinical trials are difficult in rare mutations that may be found in less than 1% of the population. As mutation-specific personalized medicine becomes more realized for CF, and other rare disease, the scientific community will have to develop new paradigms to adequately study drug safety and efficacy for very rare mutations ($n = 1$). New disease model systems may need to be developed to better predict clinical response. For example, intestinal organoids have recently been developed and may be an effective preclinical predictor of personalized response to experimental CF therapeutics [278]. In addition, novel trial end-points may need to be explored to find ones that are potentially more sensitive at detecting biochemical therapeutic response. There have been great advances in CF therapeutics; however, the disease remains progressive and life-limiting. Further exploration

of the epithelial cell dysfunction and cellular interactions will be critical to better mechanistic understanding of these disease and eventual development of a cure.

12.6.7 Concluding Remarks

CF is a progressive disease that affects 70,000 people worldwide and presents itself as a genetic disorder with more than 1,800 mutations in CFTR, the gene responsible for the primary defects observed in patients. CF is a multiorgan disease affecting the major systems like the gastrointestinal tract, the liver, skin, bone, the reproductive tract, the pancreas, and the small airways of the lung. More than half of CF patients will develop CFRD by the age of 30 years which severely affects patient lung function. Although patients can expect to live to the age of 40, on average, those who are diagnosed with CFRD are likely to experience a rapid decline in lung function. Understanding this decline and the driving mechanisms behind the rapid loss of lung function will allow advanced therapeutic options to help patients preserve their lung function and prevent or prolong the need for a lung transplant.

Using the mouse, ferret, and pig animal models, pathophysiologic mechanisms will be uncovered sooner since the ferret and pig have demonstrated human-like CF disease in most organs examined. Significantly, both the pig and ferret acquire spontaneous lung infection, mucus plugging, and pancreatic disease, however, the effect of CFRD on lung function of these animal models remains to be reported. Much of the work that has been reported has focused on the pancreatic defect as a means to understand how the exocrine and endocrine functions are lost during age-related CF disease progression. The effect of the loss of insulin secretion early in life for humans seems to correlate with a loss of insulin sensitivity and the gain of fluctuating hyperglycemia. Treatment of frank CFRD patients with recombinant insulin aids whole-body glucose homeostasis; however, there is currently no way to identify the damages or changes to organs that have already occurred by the time frank CFRD is diagnosed and treated. Similarly, no published data suggests that CFRD-related damages could be reversed with treatment. How the currently available CFTR channel modulators affect CF disease progression remains to be reported as well. In addition, the CFTR modulator drugs are only available to a handful of CFTR mutation classes and patients who are adults over the age of 18 where not every patient responds positively.

Personalized medicines and screening methods offer the promise of finding the right drug combination for each individual patient based on responses from their own cells. Methods to harvest patient samples have improved and now organoids can be made from various epithelial sources. Every effort should be taken to ensure the new therapeutic pipeline of CF medicines take advantage of the recent developments in disease models. These advances in cell culture, coupled with the advances in various drug development pipelines, will usher in a new era of personalized medicines to those who suffer from airway diseases each year.

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