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Claudins: gatekeepers of lung epithelial function

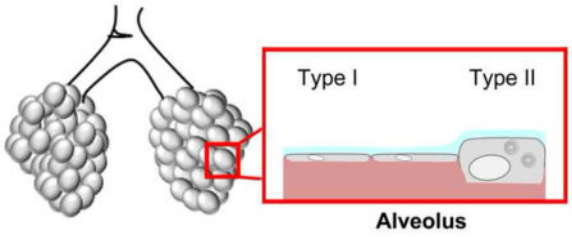
Barbara Schlingmann[*], **Samuel A. Molina**[*], and **Michael Koval**[†]

Department of Medicine, Division of Pulmonary, Allergy, Critical Care and Sleep Medicine and Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322

Abstract

The lung must maintain a proper barrier between airspaces and fluid filled tissues in order to maintain lung fluid balance. Central to maintaining lung fluid balance are epithelial cells which create a barrier to water and solutes. The barrier function of these cells is mainly provided by tight junction proteins known as claudins. Epithelial barrier function varies depending on the different needs within the segments of the respiratory tree. In the lower airways, fluid is required to maintain mucociliary clearance, whereas in the terminal alveolar airspaces a thin layer of surfactant enriched fluid lowers surface tension to prevent airspace collapse and is critical for gas exchange. As the epithelial cells within the segments of the respiratory tree differ, the composition of claudins found in these epithelial cells is also different. Among these differences is claudin-18 which is uniquely expressed by the alveolar epithelial cells. Other claudins, notably claudin-4 and claudin-7, are more ubiquitously expressed throughout the respiratory epithelium. Claudin-5 is expressed by both pulmonary epithelial and endothelial cells. Based on *in vitro* and *in vivo* model systems and histologic analysis of lungs from human patients, roles for specific claudins in maintaining barrier function and protecting the lung from the effects of acute injury and disease are being identified. One surprising finding is that claudin-18 and claudin-4 control lung cell phenotype and inflammation beyond simply maintaining a selective paracellular permeability barrier. This suggests claudins have more nuanced roles for the control of airway and alveolar physiology in the healthy and diseased lung.

Graphical Abstract



[†]Corresponding author: Michael Koval, Emory University School of Medicine, Department of Medicine, Division of Pulmonary, Allergy, Critical Care, and Sleep Medicine, 205 Whitehead Biomedical Research Building, 615 Michael Street, Atlanta, GA, 30322, Phone: 404-712-2976, mhkoval@emory.edu.

[*]Denotes shared first authorship

Keywords

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1. Introduction

Epithelia form selective barriers that regulate inner luminal organ spaces, including those found in the respiratory system. Key among the structures which promote epithelial barrier function are tight junctions, structures at cell-cell interfaces composed of transmembrane, cytosolic and cytoskeletal proteins that interact in a coordinated manner to regulate tissue barriers. The core tight junction transmembrane proteins are the claudins. Claudins on adjacent cells associate with each other to form paracellular channels of varying permeability that create a barrier by regulating the paracellular compartment between cells. Importantly, although the cell composition varies throughout the respiratory tree, epithelial tight junctions provide a unified cell-mediated barrier between airspaces and fluid containing tissues.

Several reviews on tight junctions and claudins in the lung have been published over the last few years [1–3]. This review provides an update to current knowledge about the importance of claudin diversity for lung barrier function. This is underscored by recently characterized claudin knockout mouse models demonstrating that multiple claudins play a crucial role in preserving lung barrier function.

2. Multiplicity of epithelial cells lining the respiratory tract

As a conduit for gas exchange, the respiratory tract consists of functionally distinct segments ranging from the conducting airways to the terminal airspaces where gas exchange occurs (Figure 1). The conducting airways are further divided into functionally distinct anatomical segments: the upper, lower, and distal airways. The upper airway consists of the nasal and oral cavities, pharynx, and larynx and is predominantly involved in sensory function, air humidification and gross filtration of airborne particulates. The lower airway begins with the trachea which then branches into two primary bronchi. Primary bronchi extend and branch through secondary and tertiary bronchi followed by 5–20 generations of bronchioles. The bronchial tree provides a semi-rigid cartilaginous framework to support the terminal airspaces that have the flexibility and compliance required for air influx and efflux.

The main airspace surface of the trachea, as well as conducting airways distal to the trachea, are lined by a pseudostratified epithelium which includes ciliated cells, mucus secreting goblet cells, columnar cells, serous cells and basal cells which altogether form a permeability barrier. Conducting airways are also peppered with submucosal glands containing goblet, duct, and serous cells which contribute to mucus secretion. A major function of the lower airway is clearance of particulates from the lower airspaces. This is facilitated by the concerted efforts of goblet cells and ciliated cells that serve as a “mucociliary escalator” moving foreign material upward and out of the lung [4]. Regulation of the airway surface liquid (ASL) underneath the mucus layer is critical to proper

mucociliary clearance [5]. Airway epithelial cells maintain ASL balance by the concerted action of plasma membrane channels (which regulate transcellular fluid and ion transport) and tight junctions (which regulate the paracellular route). Roles for claudins in regulating paracellular ion and water diffusion are described below.

Conducting lower airways are kept open to the atmosphere by supportive cartilaginous rings which gradually thin as the airway diameter decreases. At the most distal portion of the conducting airways are terminal bronchioles, the smallest branches of the conducting airway with a diameter of 5 mm or less. Terminal bronchioles interconnect the conducting airways with the terminal airspaces, known as alveoli, where gas exchange occurs. In contrast to the conducting airways, the terminal airspaces of the alveolar epithelium are coated with pulmonary surfactant which serves to reduce the surface tension of the air filled alveoli under the pressure of the fluid filled tissue. Thus, bronchioles serve as transition zones from the mucus dominated airspace as reflected by their distinct cellular composition [6, 7]. In contrast to larger bronchioles which are dominated by ciliated and goblet cells, terminal bronchioles lack submucosal glands and are enriched for club cells (formerly known as Clara cells, [8]). Instead of mucus, club cells are secretory cells that produce a form of pulmonary surfactant containing surfactant protein A (SP-A), SP-B and Club Cell specific protein 10 (CC-10) [9]. The surfactant produced by club cells differs from surfactant produced by alveolar epithelial cells, which lacks CC-10 but instead contains SP-C and therefore has different surface active properties [10].

The terminal airspaces are covered by the alveolar epithelium, which consists of type I and type II alveolar epithelial cells (Figure 1). Although type II alveolar epithelial cells constitute 60% of the alveolar cells, they only cover <5% of the alveolar surface because of their size and cuboidal shape [11]. Conversely, type I alveolar epithelial cells are large, squamous and extremely thin to allow the diffusion of gases between the terminal airspaces and capillaries. Given their large surface area, type I cell junctions are mainly responsible for alveolar epithelial barrier function [3]. However, pulmonary microvascular endothelial cell tight junctions also contribute to alveolar barrier function by maintaining a tight seal to prevent leakage from the bloodstream. Failure of the microvascular barrier while the alveolar epithelial barrier is maintained leads to tissue edema [12, 13], whereas failure of the alveolar epithelial barrier causes airspace flooding and increases susceptibility to acute respiratory distress syndrome (ARDS) [14]. Besides the prevention of fluid leak, alveolar epithelial cells maintain lung fluid balance by regulating ion transport to promote fluid resorption, comparable to conducting airway epithelium [15–17].

A key point in considering intercellular junctions throughout lung epithelia is that their nature as intermixed cell populations (such as pseudostratified monolayers) means that they are enriched for heterocellular interfaces between cells of different phenotype. Thus, in order to form functional tight junctions, cells of different phenotype that are in direct contact need to express compatible claudins. Consistent with this, Flynn et al. [18] showed by confocal immunofluorescence microscopy that claudin-1, -3, and -7 localize to tight junctions formed between goblet and ciliated airway epithelial cells. Moreover, type I and type II alveolar epithelial cells were shown to have multiple claudins localized to tight junctions despite having differential claudin expression [19, 20].

3 Claudin structure and composition in tight junctions

Tight junctions are protein complexes that connect neighboring cells with each other to create a barrier that regulates paracellular diffusion of ions and solutes. Morphologically, tight junctions encircle the cell and connect them with their surrounding cells [21]. Freeze fracture electron microscopy showed that tight junctions appear as a meshwork of strands at the apical end of the lateral cell membrane that differ in complexity and continuity, provoking the conclusion that the amount of strands correlate with the degree of sealing. However, studies have shown that the number of horizontally arranged strands does not always correlate with increased barrier function [22, 23] suggesting that the protein composition [24] and dynamics [25] within tight junctions are crucial for regulating barrier function.

3.1 Claudin family proteins

Claudins are a family of tetraspan transmembrane proteins that form the structural basis for tight junction permeability [26–28]. Topographical prediction for all claudin proteins demonstrate the same secondary structure: four transmembrane (TM) domains, N- and C-terminal domains (NT and CT, respectively) oriented towards the cytosol, two extracellular (EC) domains and a one short intracellular loop domain. In humans, there are 27 claudins which range in size from 207 to 305 amino acids (with apparent molecular weights between 21 and 34 kDa). Based on sequence homology, mammalian claudins are sub-divided into so-called classic and non-classic claudins [29]. The classic claudins show a high degree of structural similarity, particularly in the second EC domain, and generally have a short C-terminal cytosolic domain. By contrast, the second EC domain of non-classic claudins has more heterogeneity [29]. Non-classic claudins also tend to have longer C-terminal domains, suggesting the potential for increased interaction with cytoplasmic scaffold proteins. In the lung, the most prevalent classic claudins confirmed to be expressed are claudin-1, -3, -4, -5, and -7 [1, 30]. Claudin-1 expression in the developing and adult human lung is strikingly enriched in the lower airway bronchial epithelium throughout development but not in the small transitional airways, unlike claudin-4 and -7 which are expressed throughout the airway [31, 32]. Other classic claudins likely to be present include claudin-8 and claudin-10 [33–36]. By far, the most prevalent non-classic claudin in lung is claudin-18 which is highly and specifically expressed by the alveolar epithelium yet undetectable in normal airways [1, 37].

In order to form a functional barrier, claudins interact with each other within the same cellular membrane (polymeric *cis*-interactions) and between two cells via head to head interactions (polytypic *trans*-interactions). Claudin interactions can be classified as homomeric (polymeric *cis*-interaction) and homotypic (*trans*-interaction) when the polymer consists of the same claudin. Heteromeric or heterotypic claudin interactions occur between two different claudins either in the same cell or between two adjacent cells, respectively.

Claudins have also been categorized by whether they have the capacity to form paracellular channels (pore forming) or whether they restrict paracellular permeability (sealing claudins) [29, 38]. Lung epithelial cells tend to favor expression of sealing claudins (e.g. claudin-1, -3,-4, -5, -7 and -18), and do not express high levels of pore forming claudins (claudin-2,

-10, -15) [1, 3]. Since the surface liquid that lines the lung epithelium is critical to maintain function, sealing claudins are likely to serve an important role in limiting uncontrolled leak of water, ions, and metabolites between the apical and basolateral compartments. However, there is a report that the pore forming claudin-10 is specifically expressed by club cells, although the functional significance of this is not known at present [34]. Also, whether claudin-7 is an anion channel forming or sealing claudin is somewhat controversial [38] and likely depends upon the context of expression since cells co-expressing compatible claudins have the capacity to form paracellular channels with unique permeability characteristics. For example, claudin-4 and claudin-8 interact to form anion channels [39]. Also, claudin-16 and claudin-19 have an obligate heteromeric interaction required to form cation channels [40]. Roles for heteromeric and/or heterotypic interactions between claudins in maintaining a healthy barrier in the human lung remain to be determined.

3.2 High resolution structure of claudins

Based on orientation and functional evidence, claudin extracellular domains have been demonstrated to regulate paracellular permeability and *trans* claudin-claudin interactions between adjacent cells [41, 42]. Understanding the basis for extracellular claudin-claudin interactions was illuminated when the structure of mouse claudin-15 was determined with a crystal diffraction resolution of 2.4 Å [43] (Figure 2). In this structure, it was shown that claudins are formed by four TM domains that form a left-handed four helix bundle. Except for the TM3 domain, the length of the other TM domains matched the diameter of the lipid bilayer underscoring that claudins are firmly embedded into the plasma membrane. Interestingly, the EC domains of claudin-15 were not loops but in fact formed a β-sheet structure that consists of five β-strands. Four of these β-strands are formed by the EC1 domain and the fifth β-strand is provided by the EC2 domain (Figure 2). Cysteine residues within EC1 stabilize the β-sheet structure, as predicted by biochemical analysis [44]. The EC1 domain was suggested to be responsible for the charge-selective permeability of claudins [44, 45]. This hypothesis is supported by the structure of claudin-15 [42]. Homology modeling revealed a similar EC conformation for other ion selective channels such as claudin-10b [43].

3.3 Structural determinants of claudin-claudin interactions

Earlier studies suggested homo- and heterotypic claudin interactions are determined by the EC domains [46–48]. Suzuki et al. [43] found variable regions within the EC domains between the β-strands, variable region 1 (V1, between β-strand 3 and 4) and variable region 2 (V2, between TM3 and β-strand 5), suggesting that V1 and V2 loop regions were involved in hetero- and homotypic interactions of claudin-15 [42] (Figure 2). *Cis* interactions were suggested to be mediated by interactions between EC1 and TM3. Residue M68 located in the EC1 helix fits into a pocket formed by residues F146, F147 and L158 located in the extracellular part of TM3 and the beginning of the fifth β-strand allowing to form a polymer [42]. In addition, the structure revealed that the claudin-15 monomer contains complementary electrostatic potentials on opposite sides of the molecule which allow claudin-15 to form a linear polymer (*cis* interactions) within the same membrane. These findings are in apparent contrast to studies of claudin-2 which inferred a role for TM2 in *cis*-interactions [49], although this could reflect claudin-specific differences in regulating *cis*

interactions. Moreover, posttranslational modifications such as palmitoylation that promote partitioning into cholesterol-enriched membrane microdomains also have the potential to influence claudin *cis* interactions [50].

3.4 Regulation of claudin assembly by other tight junction proteins

High resolution structural models of claudins do not yet incorporate other components of tight junctions which are critical for tight junction assembly [51]. This includes other classes of transmembrane proteins known to regulate tight junction formation, such as MARVEL proteins (e.g. occludin [52–54]) and Ig superfamily proteins (e.g. Junctional Adhesion Molecule-A (JAM-A) [55]; Coxsackie and Adenovirus Receptor (CAR) [56]).

Occludin, an important regulator of tight junction stability and function, is under the transcriptional control of TTF1/NKX2.1 [57], which is a critical transcription factor required for lung development that also regulates transcription of claudin-1 [57] and claudin-6 [58]. Although this suggests the potential for coordinate regulation of occludin and these claudins, roles for claudin-1 and claudin-6 in lung development are not known at present.

Occludin also biochemically interacts with claudins in tight junction strands [52, 59]. Consistent with a role in regulating paracellular permeability, decreased lung occludin has been associated with several conditions known to promote pulmonary edema, including exposure to environmental toxicants, ventilator induced lung injury (VILI) and chronic exposure to alcohol [60–64]. Also, rats pretreated with a PKCα inhibitor (bisindolylmaleimide) were protected from VILI, which correlated with an increase in total lung occludin protein [65]. Whether diminished occludin expression associated with VILI has an effect on claudins was not determined nor was it determined whether occludin was necessary for protection from VILI.

Dozens of different cytosolic proteins have been found to associate with the tight junction complex [66, 67]. Cytosolic tight junction proteins can be subdivided into those containing PDZ binding domains and those that do not. PDZ binding proteins primarily serve as a structural backbone to crosslink junction-associated proteins, although several also act as transcription factors [68]. Non-PDZ containing proteins are mainly kinases, GTPases, phosphatases, and transcription factors that control tight junction assembly as well as cell proliferation and differentiation [69, 70].

Cytosolic PDZ domain containing scaffold proteins include Zonula Occludens (ZO) -1, -2, and -3 and the PALS/PATJ polarity complex which interacts with crumbs family transmembrane proteins [68, 71]. Interestingly, crumbs3-deficient mice have a significant lung phenotype, where the lungs are filled with proteinaceous material and there is significant epithelial blebbing, however, tight junctions in crumbs3-deficient mice appear morphologically normal [72]. Whether lung epithelial tight junctions are impaired in crumbs3-deficient mice or whether their claudin composition is altered has not yet been determined.

ZO-1 and ZO-2 are the most widely studied scaffold proteins. They are known to directly bind to PDZ motifs on the extreme C-terminus found on nearly all claudins and function to

link them to the actin cytoskeleton [73]. Roles for ZO-1 and ZO-2 in promoting lung epithelial barrier function are inferred by decreased ZO-1 and ZO-2 associated with conditions where the alveolar barrier is impaired [61, 62, 64, 74–76]. Nonetheless, ZO-1 and ZO-2 provide more than an inert scaffold for tight junction assembly, since MDCK cells deficient in both ZO-1 and ZO-2 were still able to form tight junctions with a transepithelial resistance comparable to wild type cells [77]. However, it was shown that the permeability to small molecules was significantly increased in ZO-1+ZO-2 deficient cells due to a higher apical contractility of the perijunctional actin-myosin ring [77]. Previously, it was hypothesized that paracellular flux of molecules larger than the actual claudin pore is accomplished by partial strand breaks that are regulated through contraction of the perijunctional actin-myosin ring [78], but elimination of the more active perijunctional actin-myosin ring in the ZO-1+ZO-2 deficient MDCK cells could not diminish the increased paracellular permeability [77]. These findings suggest a likely role for ZO-1 and ZO-2 in regulating claudin dynamics and turnover by controlling interactions with the cytoskeleton as opposed to merely serving as a direct crosslink between claudins and filamentous actin [3, 69].

Scaffold proteins underlie the mechanism behind the observation that JAM-A deficient mice have permeability defects in both the gut [79] and the lung [80]. The effects of JAM-A deficiency on the alveolar epithelium are claudin-specific as revealed by the effects of mild systemic endotoxemia, which specifically disrupted the assembly of claudin-18 into tight junctions, but claudin-4 was unaffected [80]. This was downstream of the effects of endotoxemia on ZO-1 and ZO-2, which were significantly more disrupted in JAM-A deficient mice than wild type controls and consistent with a model where claudin-18 is more tightly associated with these scaffold proteins than claudin-4 [19]. Moreover, these data suggest that, in contrast with occludin, JAM-A does not directly interact with claudins but instead, JAM-A serves to recruit cytoplasmic scaffold proteins where they promote assembly of claudins and other transmembrane proteins into tight junctions [81].

ZO-1 also provides a link between airway barrier function and the Cystic Fibrosis Transmembrane Regulator (CFTR) chloride channel [82, 83], which is important for maintenance of airway surface liquid composition [5, 84, 85], mucociliary clearance [86, 87], and innate immune responses. [4, 88, 89]. There are considerable data demonstrating that airway epithelial cells derived from CF patients have deficits in barrier function as compared to cells expressing wild type CFTR [90–92]. However, measures of tight junction permeability such as transepithelial resistance are sensitive to whether primary cells are being examined or a cell line model is being used. The method of immortalization can also influence barrier function, so results obtained *in vitro* do need to be interpreted with caution [93]. Biochemical data support a role for CFTR directly interacting with ZO-1 via a PDZ motif [82] as well as indirectly through another scaffold protein, Na^+/H^+ exchanger regulatory factor 1 (NHERF1) [83]. This parallels the scaffold protein recruitment function of JAM-A and provides a putative mechanism for impaired epithelial paracellular barrier function in CF. The pathologic consequences of a deficit in airway epithelial barrier function remain to be determined, but changes in tight junction permeability are likely to contribute to the overall fluid imbalance that is a hallmark of CF [5].

4. Roles for specific claudins in lung epithelial function

4.1 Claudin-18

There are two main splice variants of claudin-18, one of which is mainly expressed in the lung (Claudin-18.1) and the other is predominantly expressed in the stomach (Claudin-18.2) [37]. The stomach and lung isoforms of claudin-18 differ slightly in their NT, TM1 and EC1 domains. The EC1 domains of the lung and stomach isoforms are 87% identical, however differences in key specific EC1 amino acids might result in subtle changes in specificity of paracellular permeability. Based on MDCK cells overexpressing the stomach isoform, claudin-18.2 has a selective sealing function against sodium and protons but not chloride [94]. Whether claudin-18.1 has a comparable selectivity remains to be determined.

Claudin-18.1 in the lung is overwhelmingly expressed by the alveolar epithelium, suggesting a key role in regulating pulmonary barrier function [35, 95]. Thus to investigate roles for claudin-18.1 in lung barrier function, two different groups produced claudin-18 deficient mice, one of which was a deletion of exons 2 and 3 [96] and the other was a 17.3 kb deletion spanning exons 1–5 [97]. Surprisingly, in both cases, claudin-18 deficient mice were not only viable but had a mild apparent phenotype with respect to lung barrier function. This is due to a significant increase in alveolar fluid clearance rate in claudin-18 deficient mice associated with increased epithelial sodium channel (ENaC) and Na^+/K^+-ATPase activity as well as the activation of the CFTR chloride channel [96]. This increase in fluid clearance also enabled the mice to be resistant to VILI [96]. Although increased channel function correlated with loss of claudin-18.1, it is not known how these pathways are mechanistically linked.

Despite the ability of claudin-18 deficient mice to maintain lung fluid balance, a general permeability defect was demonstrated by assays *in vivo* using labeled macromolecules such as albumin as tracers that revealed a significantly higher rate of paracellular flux between the airspace and circulation [96, 97]. Interestingly, changes in paracellular flux for tracers such as dextran was not observed in the stomach of claudin-18.2 knockout mice [98], indicating a context or isoform dependent aspect of claudin-18 function in lung as opposed to stomach.

Transmission electron microscopy revealed that junctional architecture between alveolar type I cells is disrupted in the absence of claudin-18, where type I-type I junctions appeared ruffled and splayed as compared to the normal overlapping junctions as observed in wild type mice [97]. Consistent with a role for claudin-18 in regulating alveolar tight junction morphology, Li et al. [96] found by immunofluorescence that junctional ZO-1 had areas exhibiting enlarged zones of separation (or gaps) in the absence of claudin-18 as opposed to normal cells which had few areas with disrupted tight junction morphology. These changes in tight junction morphology correlated with changes in the actin cytoskeleton, where claudin-18 deficient alveolar epithelial cells had an increase in formation of perinuclear aggregates with radial fibers that were connected with the plasma membrane [96]. Actin stress fibers have previously been associated with tight junction destabilization and increased paracellular flux [20, 99–101]. These data also indicate that claudin-18 is likely to regulate tight junction turnover which may be a mechanism to directly control paracellular

flux, which is underscored by the finding that in claudin-18 deficient mice, claudin-3 and claudin-4 were markedly increased while occludin was significantly decreased [96]. A potential clue to the mechanism for this effect comes from the observation that plasma levels for the pro-inflammatory cytokine IL-1β are significantly increased in claudin-18 deficient mice [98]. IL-1β has been demonstrated to cause pulmonary inflammation and is associated with emphysema and aberrant airway remodeling [102, 103], impaired airway barrier function [104], and decreased claudin-18 incorporation into alveolar epithelial tight junctions [105].

How claudin-18 deficiency leads to changes in lung ion channel activity and function is not known however, there are significant changes in cell architecture and gene expression as a result of loss of claudin-18 [96, 97]. In particular, loss of claudin-18 significantly decreased in mRNA levels of the transcription factor early growth response-1 (Egr-1) [106]. Egr-1 deficient mice also show lower sensitivity to VILI, suggesting that alveolar ion channels may be under the control of Egr-1. LaFemina et al. [97] also showed that claudin-18 is important for postnatal lung development. By gross histology, claudin-18 deficient mice showed impaired alveolarization after 4 weeks post natal as compared to 3 day old claudin-18 knockout mice which were largely normal in appearance. Bronchiolar lavage fluid of claudin-18 deficient mice was enriched for the alveolar type I cell type specific protein podoplanin, indicating significant type I cell damage [97]. Claudin-18 knockout mice had alveolar type II cell hyperplasia as well, suggesting a potential deficiency in alveolar type I cell differentiation. Clearly claudin-18 plays diverse and complex roles in regulating the alveolar epithelium, and perhaps lung epithelial differentiation in general, which is likely to be an active area of investigation over the next decade.

4.2 Claudin-4

Claudin-4 is expressed by epithelial cells throughout the respiratory tree, as detected by immunohistochemistry of fetal and adult lungs [31, 32]. The effects of claudin-4 on paracellular permeability are context dependent. Depending on overall tight junction composition, claudin-4 has been shown to serve either as selective sodium barrier without affecting chloride selectivity [107, 108], as a chloride pore forming claudin [39] or as a barrier forming claudin [19].

Studies using an adenoviral expression system to increase claudin-4 expression by primary rat alveolar epithelial cells within the physiologically relevant range showed a ~30% increase in transepithelial resistance without any changes in either the P_{Na}/P_{Cl} permeability ratio or paracellular flux [19]. Conversely, siRNA knockdown of claudin-4 in human distal lung epithelial cells decreased transepithelial resistance [103]. However, alveolar epithelial cells from claudin-4 knockout mice lacked a change in transepithelial resistance or ion permeability whereas paracellular flux to large fluorescent dextran (155 kDa) tracers was increased [109]. The difference between acute, partial claudin-4 depletion in human cells and long term complete claudin-4 deficiency may reflect compensatory remodeling of tight junctions or may be due to cell or species specific differences in tight junction permeability, comparable to what was observed in claudin-18 deficient mice. Also, whether claudin-4 has

the same influence on paracellular permeability in airway cells *versus* alveolar cells has not been directly examined.

Physiologically, claudin-4 has been associated with a protective effect against lung injury [19, 103, 110]. In patients, increased claudin-4 levels were associated with better lung fluid clearance and decreased physiological respiratory impairment [110]. Consistent with this, claudin-4 is upregulated 12–16-fold in response to mechanical trauma (VILI) or hyperoxia in mice [103, 109]. One hypothesis for the beneficial effect of claudin-4 in promoting fluid clearance is that it provides a pathway for paracellular chloride efflux to balance sodium transport through channels such as ENaC [15].

Lungs of claudin-4 deficient mice are morphologically normal and have minor defects in steady state fluid balance, which was largely attributed to decreased Na^+/K^+-ATPase activity [109]. Nonetheless, claudin-4 deficient mice showed increased sensitivity to VILI and lung injury due to hyperoxia, where lungs became significantly edematous. However, these results were confounded by the mice being on a mixed background of BALB/c, 129S6/SvEvTac and C57BL/6 [109] which is known to have an impact on severity of acute lung injury and oxidative stress [111]. Analysis of mice on a uniform background will enable strain dependent differences in roles for claudin-4 to be elucidated.

A protective role for claudin-4 was also inferred using a blocking peptide derived from *Clostridium perfringens* enterotoxin (CPE), which exacerbated the injurious effects of high tidal volume mechanical ventilation on alveolar permeability and impaired fluid clearance [103]. These data led to the expectation that claudin-4 deficient mice would have a serious impairment in lung epithelial barrier function. However, as was the case for claudin-18 deficient mice, loss of claudin-4 resulted in a mild lung phenotype even in response to injury [109].

Gene expression analysis of injured claudin-4 deficient mice revealed increased activation of genes downstream of TNF and IL-1β, however, in order to see this effect, the mice needed to be stratified into “high” vs “low” injury groups based on having post injury bronchioalveolar lavage (BAL) fluid protein greater than 1.2 mg/ml [109]. Severely injured claudin-4 deficient mice also showed increased Egr1 [109]. These differences intriguingly parallel the injurious effect of increased IL-1β and protective effect of low Egr1 in claudin-18 deficient mice [96, 98], which further supports roles for lung claudins in regulating cell physiology beyond control of paracellular permeability.

4.3 Claudin-7

Claudin-7 is expressed throughout the respiratory tract during development and in the adult lung [31, 32]. The permeability characteristics of claudin-7 have proven to be difficult to elucidate, indicating that claudin-7 function is largely context sensitive [38]. A role for claudin-7 in restricting paracellular chloride permeability was initially inferred by examining LLC-PK1 cells overexpressing claudin-7 which showed a simultaneous decrease in chloride and increase in sodium permeability [112]. By contrast, claudin-7 depletion of mIMCD-3 cells resulted in a 40% increase in chloride permeability and a 45% increase in sodium permeability, suggesting a more general sealing function for claudin-7 [39]. As a

further complication, claudin-7 permeability appears to be sensitive to phosphorylation by WNK4 kinase. WNK4 kinase phosphorylation of claudin-7 correlated with increased chloride permeability [113]. However, it was not established whether this was due to a conformational change in claudin-7 or whether phosphorylation altered the ability of claudin-7 to incorporate into tight junctions.

Claudin-7 deficient mice die within two weeks after birth [114] and so their lung phenotype has not been interrogated. Claudin-7 deficiency leads to an overall defect in water homeostasis and the mice suffer from chronic dehydration [114]. Furthermore, both the small and large intestine of the claudin-7 knockout mouse showed signs of inflammation downstream of increased expression and activity of extracellular matrix remodeling enzymes matrix metalloprotease-3 (MMP-3) and MMP-7, which was recapitulated by silencing claudin-7 by siRNA in a lung epithelial cell line [115]. MMPs in general are associated with increased severity of lung injury [116] suggesting a potential mechanism where loss of claudin-7 could amplify ARDS. Conversely, EGF has a protective effect with is associated with increased claudin-7 and claudin-4 expression and function [117].

In claudin-7 deficient mice, expression of other claudins that are important for kidney function were not affected, although claudin-1 localization in the gut is impaired, primarily due to a defect in formation of a complex with claudin-7 and integrin α2 [115]. However, there were several changes in ion and water channels, including induction of ENaC, aquaporin-2 and Na^+/K^+-ATPase-α1 which together led to increased sodium absorption. Increased sodium absorption, in concert with decreased chloride secretion, underlies a defect in airway surface liquid (ASL) that is a hallmark of CF [118, 119]. The concordance between the claudin-7 and CFTR-deficient epithelium raises the possibility that claudin-7 may play a role in regulating apical chloride, perhaps by reducing paracellular chloride flux. Consistent with this, in CF there is a 4-fold decrease in passive intestinal chloride absorption that acts as a potential compensatory mechanism [120]. Whether this occurs in the airway and whether the paracellular route plays a role in regulating chloride balance is not known at present. However, this also raises the potential for a therapeutic approach to CF by targeting claudin expression as a means to either reduce chloride efflux from the apical surface and/or augmenting paracellular chloride flux from the basal to apical surface as a means to compensate for loss of chloride secretion by CFTR.

4.4 Claudin-5

Claudin-5 is most prominently expressed by the vascular endothelium and the pulmonary microcirculation is no exception [30, 31]. The phenotype of claudin-5 deficient mice reflects a function for this claudin in sealing the vasculature, since these mice have a selective blood brain barrier permeability defect which allows small molecules (<0.8 kDa) to penetrate into the cerebral spinal fluid resulting in immediate perinatal lethality [121]. Claudin-5 is also clearly expressed by the human airway epithelium [31, 32, 122]. The high levels of claudin-5 expressed by the endothelium tend to obscure the ability to detect claudin-5 expression by alveolar epithelial cells in histologic sections [30, 35], however, claudin-5 mRNA and protein are readily measurable in isolated fetal human alveolar epithelial cells [95]. Rodent alveolar epithelial cells also express claudin-5 [19, 123–125].

The alveolar air-liquid barrier reflects the combined function of tight junctions in both the pulmonary microvasculature and the alveolar epithelium [17, 126, 127]. Because of the dual nature of this barrier, the close apposition of alveolar endothelial and epithelial tight junctions and the fact that both tissues express claudin-5 has led to some apparently contradictory data related to roles for claudin-5 in regulating alveolar barrier function. In brief, claudin-5 is likely to have a protective effect by increasing the barrier function of the endothelial part of the barrier [128–133]. However, increased claudin-5 expression *decreases* alveolar epithelial barrier function [100, 122, 125].

Related to the endothelial barrier, there was no effect on barrier function at baseline in studies using pulmonary artery endothelial cells *in vitro* where claudin-5 was silenced using siRNA [128]. However, cells treated with claudin-5 siRNA had increased permeability to fluorescein (0.4 kDa) after thrombin treatment as compared to controls expressing normal levels of claudin-5. Simvastatin, which normally bolsters endothelial barrier function, also had diminished efficacy as demonstrated by increased paracellular flux of fluorescein into the alveolar space, although transepithelial resistance and large molecule permeability were not affected [128], consistent with the claudin-5 deficient mouse model [121]. Critically, intratracheal claudin-5 siRNA administered *in vivo* did not change small molecule permeability at baseline and had only a small protective effect on endotoxin induced lung injury [128]. Nonetheless, total lung claudin-5 tends to inversely correlate with severity of lung injury. For example, studies assessing different mouse strains to acrolein-induced acute lung injury (ALI) showed that claudin-5 expression was increased in more injury-resistant strains as compared to injury-sensitive strains [132]. In HIV-1 infected patients with interstitial pneumonitis, claudin-5 was down-regulated in lung tissue [133]. Further experiments in a mouse model of HIV-infection confirmed this finding which was associated with increased lung infiltration of CD4+ and CD8+ monocytes in HIV-1 infected mice [133].

A potential clue to the dual nature of the alveolar barrier comes from studies using the cancer chemotherapeutic agent imatinib that promotes blood brain barrier function and thus might be expected to have a protective effect in ARDS [134]. However, imatinib treated mice have a complex response to lung injury in that they are protected from endotoxin induced lung injury but have an increased sensitivity to VILI [135]. Although the mechanism underlying this divergence in response is not known at present, we speculate that this is due to differential regulation of the endothelial and epithelial parts of the alveolar barrier.

In support of a deleterious role for claudin-5 on the epithelial barrier, several studies have associated increased claudin-5 expression by lung epithelial cells with decreased barrier function [100, 122, 125]. This is particularly striking in alcoholic lung syndrome, which is associated with increased susceptibility to ARDS [136–139]. In a rat model of chronic alcohol abuse, claudin-5 protein expressed by type II alveolar epithelial cells was significantly increased [124]. This increase in claudin-5 persisted in model type I cells derived from “alcoholic” type II cells which also exhibited impaired barrier function as compared with cells derived from control fed rats. A more direct demonstration of claudin-5 was revealed when airway cells were transfected to overexpress claudin-5, which decreased

transepithelial resistance [122]. These data are suggestive; however, further work is needed to identify a molecular basis for the ability of claudin-5 to impair alveolar epithelial barrier function and to assess whether specifically targeting epithelial claudin-5 is a therapeutically viable option for promoting lung barrier function.

4.5 Claudin-3

Claudin-3 is expressed in the developing human lung in both the airway and alveoli. Expression in the developing and adult human lung is ubiquitous upon transition to the canalicular period of branching bronchi biogenesis (weeks 16–28) and beyond, staining most epithelia present in the respiratory tree including type II alveolar epithelial cells [31, 32]. By contrast, claudin-3 expression by type I cells is relatively low compared with other lung epithelia in general and especially in comparison with type II cells [20, 31, 32]

Adenoviral expression of claudin-3 in primary rat model type I alveolar epithelial cell at the level found in type II cells led to decreased transepithelial resistance, increased short circuit currents, and higher paracellular flux of calcein (0.6 kDa) and dextran (10 kDa) [19]. This contrasts with other studies, where increased claudin-3 expression by other types of epithelial cells and NIH/3T3 fibroblasts has an overall sealing function [140, 141]. These apparently conflicting data are likely to reflect a context sensitivity for claudin-3 function that differs for alveolar epithelial cells vs. other cell types. Moreover, claudin-3 is primarily found in type I-type II cell junctions in the alveolus. Since type II cells cover less than 5% of the alveolar surface [11], type II-type I tight junctions provide a relatively small part of the alveolar barrier as compared to type I-type I tight junctions which have vanishingly low levels of claudin-3 [20]. This suggests the hypothesis that claudin-3 enables type I-type II cell junctions to have unique characteristics distinct from type I-type I cell junctions [3]. Interestingly, claudin-3 is unique in that it mediates several heterotypic *trans* interactions between claudins, including claudin-1, claudin-2 and claudin-5 [142, 143]. By contrast, claudin-3 and claudin-4 do not have a *trans* interaction, however, they do interact as *cis*-heteromers [142, 144]. Specific roles for claudin-3, and particularly whether its unique heterotypic/heteromeric compatibility is needed to form fully functional type I–type II cell junctions, remain to be determined.

5. Summary and perspectives

There is a growing literature addressing how individual claudins regulate lung barrier function, ranging from *in vitro* studies to claudin-deficient mice. Analysis of transgenic mice deficient in two different key lung epithelial claudins, claudin-18 and claudin-4, had a surprisingly mild phenotype with respect to barrier function. Of course altering claudin expression to elucidate function is subject to compensatory changes in other tight junction and channel proteins and thus make it difficult to use these approaches to fully interpret specific roles for specific claudins in control of lung barrier function. Moreover, the fact that the lung can withstand large changes in claudin expression likely reflects the critical nature of the air-liquid barrier for gas exchange and blood oxygenation in that there are several overlapping mechanisms to prevent airspace flooding and clear fluid from the airspace.

Claudin-4 and claudin-18 deficient mice also show phenotypes beyond a simple deficit in barrier function. Alveolar epithelial gene expression is significantly changed in both cases and there is an increase in pro-inflammatory responses, which make the lung more susceptible to injury. It remains to be determined whether increased inflammation is directly downstream to changes in barrier function. Alternatively, inflammation could be independent of changes to tight junction permeability and instead due to changes in the control of lung cell phenotype due to loss of claudin-4 or claudin-18.

There are hints that other claudins also may have non-junctional roles in regulating lung inflammation as well. For instance, claudin-1 was detected in human airway smooth muscle cells, which lack tight junctions, where gene transcription correlated with improved cellular survival and proliferation upon stimulation with pro-inflammatory cytokines IL-1β and TNF-α [145]. What this means for the airway epithelial barrier is unknown at present, but claudin-1 may have a role in the pathogenesis of asthma, based on the location of expression by airway smooth muscle cells.

The morphologic abnormality in claudin-18 deficient mice is profound and suggests that alveolarization may be under the direct control of claudin-18. A role for claudin-18 in alveolar formation also raises the possibility that interfering with claudin-18 expression and/or function may underlie diseases such as pulmonary fibrosis or chronic obstructive pulmonary disorder (COPD), where alveolar morphology is pathologically disrupted.

Roles for claudins in airway barrier function remain relatively less explored than for the alveolus, particularly when considering claudins beyond the most prominently expressed alveolar claudins. For instance, claudin-1 and claudin-8 were specifically down regulated in so-called healthy smokers, which was recapitulated in cigarette smoke extract treated airway cells *in vitro*, suggesting that changes in claudin expression may presage more severe lung disease or cancer [33]. Expression of the pore forming claudin-10 by club cells [34] may reflect a unique niche for increased paracellular flux in the most distal part of the airway. For instance, increased paracellular flux between club cells could play a role in transmitting chemotactic signals to promote homing of immune cells for transmigration into the terminal airspaces. Whether this is the case remains to be determined.

As mentioned above, there are several indications that tight junctions are diminished in CF, which is related to at least two roles for CFTR, namely, chloride channel activity and structural recruitment of scaffold proteins to the tight junction complex. With respect to decreased chloride secretion, the paracellular route provides the potential to regulate ion homeostasis by either promoting chloride diffusion or limiting apical chloride efflux. Whether this is therapeutically targetable, particularly in the absence of CFTR structurally integrated into tight junctions remains to be determined. Also, the deleterious effects of misfolded mutants of CFTR need to be considered in addition to the loss of CFTR activity, since ER stress and disruption of protein quality control can potentially have a negative impact on claudin assembly into tight junctions. Understanding the mechanistic basis for claudin trafficking and assembly will help elucidate whether this is the case.

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Abbreviations

ARDS	acute respiratory distress syndrome
ASL	airway surface liquid
CAR	Coxsackie and Adenovirus Receptor
CC10	club cell specific protein 10
CFTR	cystic fibrosis transmembrane conductance regulator
COPD	chronic obstructive pulmonary disorder
EC	extracellular
ENaC	epithelial sodium channel
JAM-A	Junctional Adhesion Molecule-A
MMP	matrix metalloprotease
PALS	proteins associated with Lin-7
PATJ	Pals1-associated tight junction protein
PDZ	PSD95, Dlg1, ZO-1
SP	surfactant protein
TM	transmembrane
VILI	ventilator induced lung injury
ZO	Zonula Occludens

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Highlights

- Airway and alveolar epithelial barrier function require tight junctions with distinct properties
- Claudin-deficient animal models reveal redundancy in mechanisms which maintain air-liquid barriers
- Claudins have roles beyond forming tight junctions that regulate lung morphogenesis and inflammation

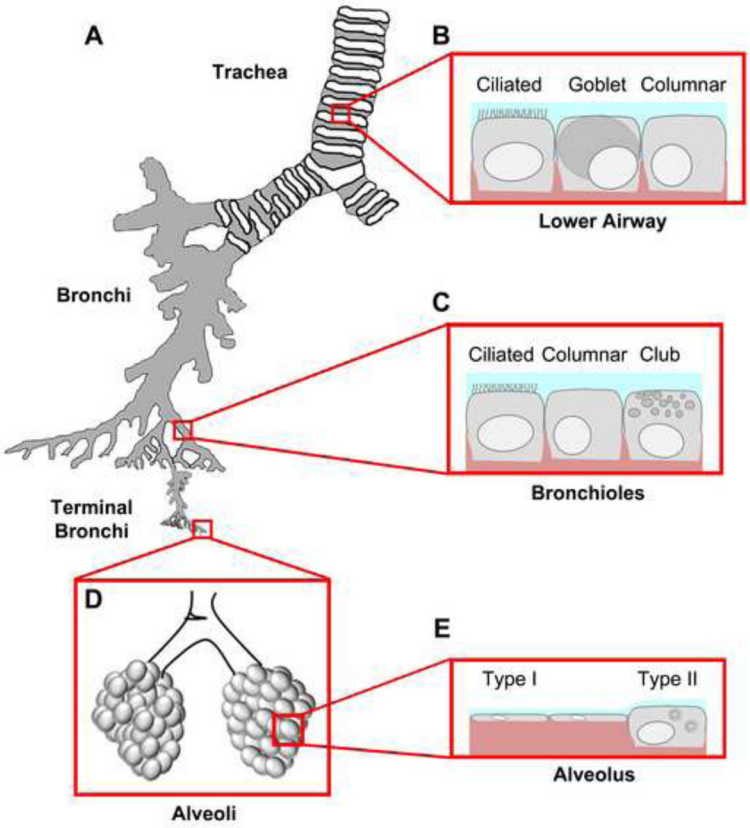

Figure 1. Epithelial diversity along the respiratory tree

A. Airways are divided into four main segments: the trachea, the branching bronchi, the terminal bronchi, and the alveolar space. Each segment contains a unique mix of cell types that have specialized functions. B. The lower airway, proximal to the bifurcation of the left and right bronchus, consists of mostly ciliated cells whose main function is to sweep mucus secreted by goblet cells (blue) out of the airways. Columnar and other cells (e.g. serous cells) also contribute to the airway barrier. Basal cells (not shown) are localized to the basement membrane but do not contribute to the tight junction barrier. C. Distal to the tracheal bifurcation are bronchiolar cells that consist mainly of ciliated, columnar and club cells. Club cells secrete a specialized form of pulmonary surfactant as opposed to mucus and provide a transition zone between the airway and alveolar space. D. The alveolar space is the location of gas exchange and consists mainly of squamous type I and cuboidal type II cells. Tight junctions between these cells form at apical cell-cell interaction sites. The alveolar sac maintains surface tension through surfactant secreted by type II cells preventing alveolar collapse. E. Gas exchange occurs efficiently through type I cells, which make up the vast majority of alveolar surface area.

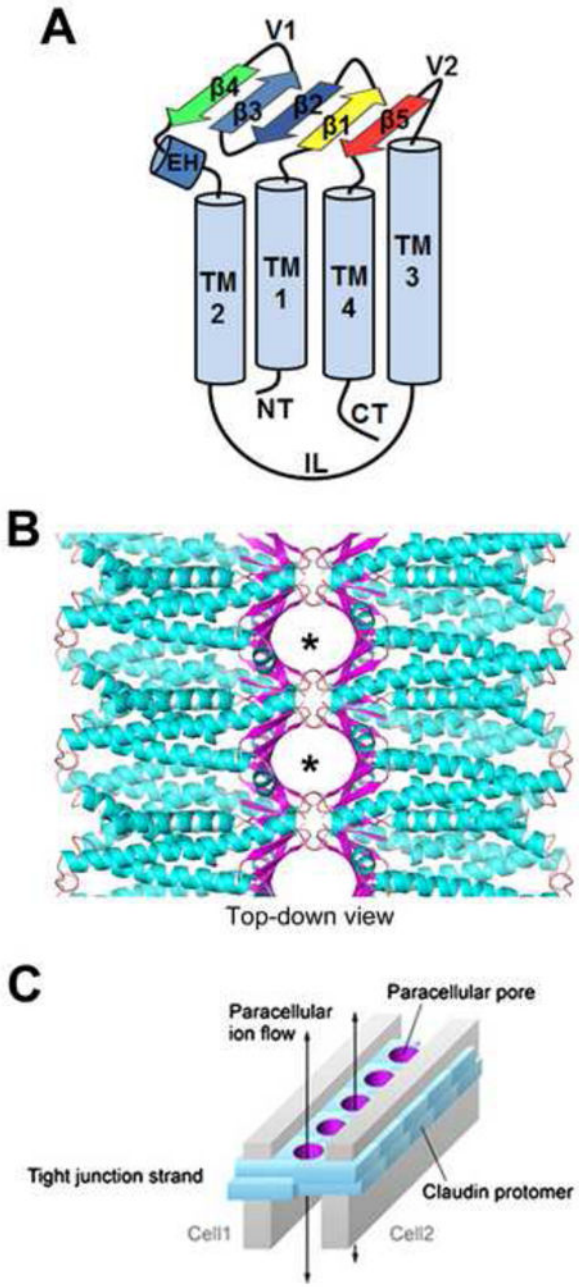

Figure 2. Structure of claudin ion selective pores

A. Claudin proteins are multi-pass transmembrane proteins that contain intracellular amino terminal (NT) and carboxy terminal (CT) ends, four transmembrane domains (TM1-4), an intracellular loop (IL) and an extracellular (EC) β-sheet domain where interactions between claudins occur. The EC domains consist of a small extracellular α-helix (EH) and five anti-parallel β-strands (β1–5) which form the interacting β-sheet. Based on this structural model, two variable region loops (V1 and V2) are positioned to regulate heterotypic interactions. B. The EC β-sheet (purple) interacts to form paracellular ion or metabolite selective pores (asterisks), where the specific amino acids of the β-sheets comprise the pore lining residues that confer ion/molecule selectivity. C. A simplified schematic of the paracellular pore

structures (purple) formed by homo- or heterotypic interactions between claudins. Figure modified from [42] with permission.