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Roles for claudins in alveolar epithelial barrier function

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Abstract

Terminal airspaces of the lung, alveoli, are sites of gas exchange which are sensitive to disrupted fluid balance. The alveolar epithelium is a heterogeneous monolayer of cells interconnected by tight junctions at sites of cell-cell contact. Paracellular permeability depends on claudin-family tight junction proteins. Of over a dozen alveolar claudins, cldn-3, cldn-4 and cldn-18 are the most highly expressed; other prominent alveolar claudins include cldn-5 and cldn-7. Cldn-3 is primarily expressed by type II alveolar epithelial cells whereas cldn-4 and cldn-18 are expressed throughout the alveolar epithelium. Lung diseases associated with pulmonary edema, such as alcoholic lung syndrome and acute lung injury affect alveolar claudin expression which is frequently associated with impaired fluid clearance due to increased alveolar leak. However, recent studies have identified a role for increased cldn-4 in protecting alveolar barrier function following injury. Thus, alveolar claudins are dynamically regulated, tailoring lung barrier function to control the air-liquid interface.

Keywords

tight junction; acute lung injury; acute respiratory distress syndrome; alcoholic lung disease; sepsis

Introduction

Gas exchange between the lung airspace and the circulatory system is necessary to support respiration in mammals. In order for gas exchange to occur, the lung must maintain a highly specialized barrier between the atmosphere and fluid filled tissues. The airspace is not completely dry, rather, it is covered by a highly regulated thin layer of fluid known as the air-liquid interface. Lung epithelia maintain the air-liquid interface by providing both a physical barrier to prevent leakage into airspaces and active transport of excess fluid^{1, 2}. Clinically, during acute respiratory distress syndrome (ARDS), failure of the lung epithelial barrier leads to airspace flooding significantly decreasing the efficiency of gas exchange that exacerbates the severity of acute lung injury^{3, 4}. The terminal airspaces of the lung, known as alveoli, provide the physical barrier to paracellular fluid permeability.

The alveolar epithelium is heterogeneous and consists of two different cell types, type I and type II alveolar epithelial cells (Fig. 1). Type I cells are a squamous epithelium which cover over 90% of the alveolar surface area.⁵ These large thin cells are the primary site of gas exchange between the airspaces and pulmonary capillary vasculature. Type II cells are interspersed throughout the alveoli and serve several functions. Most critically, type II cells produce pulmonary surfactant, lowering surface tension of the air-liquid interface to

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maintain open airspaces. Type II cells can also differentiate into type I cells in response to injury. As shown in Figure 1, the vast majority of alveolar intercellular junctions are between adjacent type I cells, however, there are also heterocellular interfaces between type I and type II cells.

There are several types of intercellular junctions between alveolar epithelial cells. These include tight junctions, adherens junctions, gap junctions and desmosomes, each serving a distinct function.⁶⁻¹⁰ Tight junctions are the most critical determinant of epithelial barrier function although evidence suggests that other junctions, particularly adherens junctions, contribute to barrier function by regulating tight junction assembly.^{9, 11, 12}

Tight junctions are a network of interwoven strands forming a ring around the lateral plasma membrane. Claudins, a family of tetraspan transmembrane proteins, form the structural and functional basis for control of tight junction permeability.¹³⁻¹⁷ The extracellular domains of claudins interact with extracellular domains of claudins on adjoining cells to form a barrier that restricts ion, solute and liquid trafficking between cells via the paracellular pathway.¹⁸⁻²¹ There are over two dozen mammalian claudins that have tissue specific patterns of expression that determine tight junction permeability.

Claudins require additional protein components in order to be assembled into tight junctions. They are directly tethered to the actin cytoskeleton via cytosolic scaffold proteins that interact primarily with the C-terminal domain.^{22, 23} ZO-1 and ZO-2, are the best characterized scaffold proteins and have been shown to promote and regulate claudin incorporation into tight junctions.^{24, 25} Tight junctions are also controlled by other transmembrane proteins. For example, the tetraspan transmembrane protein occludin interacts with claudins to regulate tight junction assembly and barrier function^{19, 26}. However, occludin is not an absolute requirement for a high resistance barrier and acts as a pro-apoptotic signal when junctions are disrupted, suggesting an important role for occludin in cell signaling.^{27, 28} Immunoglobulin-fold transmembrane proteins, including Junction Adhesion Molecule A (JAM-A), also regulate claudin expression and tight junction permeability.²⁹ Although claudins are one part of the multiprotein complex required to form tight junctions, they nonetheless function as the primary structural component that controls paracellular permeability and the tight junction barrier.

Claudin expression by the Alveolus

At least 14 different claudins are expressed at the mRNA and protein level by alveolar epithelial cells.^{17, 30} The predominant claudins expressed by the alveolar epithelium are cldn-3, cldn-4, and cldn-18.³¹ However, other claudins expressed by alveolar epithelium can also influence alveolar barrier function, e.g. cldn-5 and cldn-7 which are associated with decreased and increased alveolar barrier function, respectively (see below)^{32, 33}. Claudin expression is not uniform throughout the alveolus, instead, type II and type I alveolar epithelial cells have distinct patterns of claudin expression³¹ (Table 1). The most prominent difference is that type II cells express over 17 fold more cldn-3 than type I cells. By contrast, cldn-4 expression by type II and type I cells is comparable at baseline, although cldn-4 is upregulated during acute lung injury (see below). Type II and type I cells also express comparable levels of cldn-18, which is specifically expressed by alveolar epithelium and is absent from the upper airways. There are two cldn-18 splice variants, cldn-18.1 found primarily in the lung and cldn-18.2 expressed in the stomach^{34, 35}.

Resistance to Triton X-100 extraction is a commonly used biochemical assay that correlates with the incorporation of transmembrane proteins into junctional complexes.³⁶⁻³⁹ Using this approach, cldn-18 is significantly more insoluble (~75 % insoluble) as compared with cldn-3 (~40% insoluble) or cldn-4 (~30% insoluble).⁴⁰ Although the basis for enhanced cldn-18

resistance to detergent extraction is not known at present, the C-terminal domain of cldn-18 is roughly twice as large as that of cldn-3 or cldn-4, which could provide a more effective template for scaffold proteins to bind and crosslink cldn-18 to the cytoskeleton. Whether this is the case remains to be determined, however, this would suggest that association of cldn-18 with cortical actin is an important contributor to alveolar barrier function.^{31, 41} Consistent with this, pro-inflammatory hormones decrease cldn-18 expression and assembly into alveolar epithelial tight junctions *in vitro* which correlates with decreased barrier function⁴² (Table 2). Moreover, cldn-18 expression is decreased in murine models of sepsis using cecal ligation and puncture and bleomycin induced lung injury, which is expected to compromise alveolar barrier function.^{43, 44}

Alcoholic lung syndrome impairs alveolar barrier function

Chronic alcohol abuse is a clinically significant risk factor for the development of ARDS.^{45–47} A key root cause of this effect is that prolonged ethanol ingestion induces a significant oxidant load driving the alveolar epithelium to produce transforming growth factor beta (TGF- β) which has a deleterious effect on alveolar barrier function⁴⁸ and primes the lung for an amplified response to acute lung injury.^{49, 50} Production of TGF- β in response to alcohol further exacerbates oxidant stress by inhibiting glutathione transport into the airspaces, impairing the anti-oxidant capacity of the lung.^{51, 52}

In an otherwise healthy alcoholic, ion channels (e.g. amiloride sensitive sodium channels) can compensate for compromised barrier function and maintain a proper air-liquid interface.^{1, 53} However, because the alcoholic lung is already under a significant oxidant burden,¹⁷ it is highly susceptible to the effects of a so-called “second hit”, such as direct trauma or inflammation due to sepsis. As a result of a second hit, alveolar barrier function in the alcoholic lung is further compromised overwhelming mechanisms of fluid clearance that are already near capacity.

In addition to affecting oxidant load, TGF- β has a direct influence on alveolar epithelial function by promoting epithelial-to-mesenchyme transition (EMT).^{54–56} EMT induced by TGF- β directly affects tight junctions through increased expression of transcription factors such as snail which repress cell polarity and claudin expression.^{57, 58} In fact, chronic alcohol ingestion decreases expression of several claudins, including cldn-1, cldn-7, and cldn-18⁵⁹. Alcohol also decreases expression of other alveolar tight junction proteins, including occludin and ZO-1, which are likely to contribute to a leaky lung phenotype⁶⁰. In addition to the changes to tight junction protein expression, tight junction formation is also impaired in response to chronic alcohol exposure (Fig. 2). The overall decrease in ZO-1 expression in the alcoholic lung is likely to contribute to decreased tight junction formation, since a decrease in the scaffold impairs the ability of claudins to stably incorporate into tight junctions²⁵.

In parallel to alcohol-induced decreases alveolar epithelial cell claudin expression, alcohol surprisingly increases cldn-5 expression⁵⁹. The effect of alcohol on cldn-5 requires an as yet unknown post-translational mechanism of regulation, since alveolar cldn-5 mRNA remains unchanged by alcohol exposure⁵⁹. Several studies have correlated increased cldn-5 with increased lung epithelial paracellular permeability^{32, 61, 62}, suggesting that increased cldn-5 expression is a critical aspect of impaired barrier function in the alcoholic lung. Consistent with this potential mechanism, we have found that transducing normal alveolar epithelial cells with YFP-cldn-5 decreases barrier function *in vitro* (C.E. Overgaard and M. Koval, unpublished results). How cldn-5 could decrease alveolar epithelial barrier function is not known at present. Nonetheless, the ability of cldn-5 to reduce alveolar barrier function is likely to be tissue specific, since cldn-5 is necessary for maintaining the blood brain

barrier and overexpression of cldn-5 by low resistance epithelia can increase barrier function^{63, 64}. Defining roles for cldn-5 in the pathology of alcoholic lung disease will require understanding how cldn-5 interacts with other alveolar epithelial claudins and tight junction proteins.

Cldn-4 expression correlates with increased alveolar fluid clearance

A role for cldn-4 in response to acute lung injury was first implicated in studies where cldn-4 was found to be acutely upregulated by mice in response to ventilator-induced lung injury (VILI)⁶⁵. The increase in cldn-4 correlated with decreased severity of injury and was specific, as other claudins, including cldn-3 and cldn-18, were unchanged in response to VILI⁶⁵. Interestingly, alveolar epithelial cldn-4 expression is downregulated in sepsis that is likely to increase the severity of lung injury⁴³. Moreover, cultured alveolar epithelial cells show considerable variation in endogenous cldn-4 expression, even within the same monolayer. This suggests that cldn-4 expression is more sensitive to cell phenotype or microenvironment as opposed to other claudins that are more uniformly expressed and regulated, such as cldn-18.^{32, 66}

A functional role for cldn-4 in lung fluid clearance *in vivo* was confirmed using a peptide fragment derived from *Clostridium perfringens* enterotoxin (CPE), which binds to cldn-3 and cldn-4 with high affinity.^{67, 68} Intratracheal instillation of a CPE peptide decreased lung cldn-4 content and rendered the lungs more sensitive to VILI.⁶⁵ Recently, cldn-4 was found to be associated with increased alveolar fluid clearance rates in *ex vivo* perfused human donor lungs.⁶⁹ The extent of lung injury was inversely correlated with cldn-4 expression, supporting a clinically relevant role for cldn-4 in protecting the lung from damage along with improved fluid clearance.

Differential effects of cldn-3 and cldn-4 on alveolar barrier function

Cldn-3 and Cldn-4 are the most closely related by amino acid homology.⁶² However, since cldn-3 and cldn-4 differ dramatically in their pattern of expression in the alveolus, this suggests that these two claudins serve different roles in alveolar barrier function. To directly test this, alveolar epithelial cells cultured on permeable supports were transduced to specifically increase expression of YFP-tagged versions of either cldn-3 or cldn-4.⁴⁰ Expression of YFP-cldn-3 or YFP-cldn-4 has no effect on the expression or localization of other claudins (Fig. 3). However, YFP-cldn-3 and YFP-cldn-4 have differential effects on alveolar epithelial barrier function (Fig. 4). Alveolar epithelial cells transduced with YFP-cldn-4 increased trans epithelial resistance (TER) by nearly 50%, providing direct evidence that increased cldn-4 improves alveolar barrier function⁴⁰. By contrast, cells transduced with YFP-cldn-3 show a decrease in TER from $\sim 550 \text{ Ohm} \times \text{cm}^2$ to $\sim 400 \text{ Ohm} \times \text{cm}^2$ (Fig. 4). Thus, cldn-3 and cldn-4 have differential effects on alveolar epithelial barrier function.

The context of claudin expression influences their function, since claudin-claudin interactions alter paracellular permeability⁷⁰. For instance, increased cldn-3 augments barrier function of a low resistance clone of Madin Darby Canine Kidney (MDCK) epithelial cells from $\sim 50 \text{ Ohm} \times \text{cm}^2$ to $\sim 100 \text{ Ohm} \times \text{cm}^2$, most likely by interacting with cldn-2 which acts as a pore forming claudin⁷¹. This contrasts with the observation that cldn-3 decreases alveolar epithelial barrier function⁴⁰. In this light, it is interesting that cldn-3 is capable of a broad range of heterotypic interactions with other claudins, as opposed to cldn-4, which appears to be restricted to homotypic interactions with cldn-4^{61, 62}. Whether cldn-3 and cldn-4 have fundamentally different roles in directing tight junction assembly and whether this affects paracellular permeability remains to be determined. However, given that cldn-3 is mainly localized to type II-type I cell interfaces in the alveolus (Fig. 1), it seems likely that type II-type I tight junctions differ from type I-type I

cell junctions in paracellular permeability. Determining whether this is the case will require novel methods to measure alveolar tight junction permeability *in situ*.

Conclusions and perspectives

The strong correlation between *cldn-4* and improved lung fluid clearance makes this an appealing therapeutic target for the prevention of ARDS by improving fluid clearance from airspaces. This is particularly appealing for prevention of lung injury during sepsis, where *cldn-4* is downregulated⁴³. In fact, *cldn-4* transcription is under the control of the grainyhead-like 2 (*Grhl2*) transcription factor which is activated during development in several epithelia, including lung^{72, 73}. Whether *Grhl2* is expressed by the adult lung and whether it is activated endogenously in response to injury or through a pharmacologically activated pathway remain unknown at present. It is also unclear whether *Grhl2* can overcome the effects of transcription factors that are activated in EMT that suppress claudin expression, including *snail*, *slug* and *twist*^{74–76}. This could be a critical issue when attempting to target *cldn-4* in alcoholic lung disease, where TGFβ promotes EMT in the alveolus.

Even if *cldn-4* is upregulated, is this sufficient to improve fluid clearance when expression of other claudins is impaired? For example, in the context of decreased *cldn-18*, which is the major alveolar epithelial claudin, increasing *cldn-4* may not augment the alveolar barrier enough to maintain a proper air-liquid balance. Moreover, in the case of alcoholic lung disease, increased *cldn-5* may antagonize *cldn-4*. This could be due to a direct effect on *cldn-4*, if *cldn-5* heteromerically influences *cldn-4* assembly or function. Alternatively, *cldn-5* may compete with *cldn-4*, and other claudins, for interaction with scaffold proteins. *Cldn-5* may also recruit specific subclasses of scaffold proteins to tight junctions that could influence assembly as well. In these models, differential affinity for scaffold proteins by claudins dictate the priority, or stability, of claudin integration into tight junction strands through competition for binding to scaffold proteins. In essence, claudin composition could control recruitment of scaffold proteins to tight junctions, the converse of models where scaffold proteins control claudin incorporation junctional strands²⁵. If this is the case, then steady state tight junction composition is dictated by coordinated bi-directional interplay between claudins and scaffold proteins.

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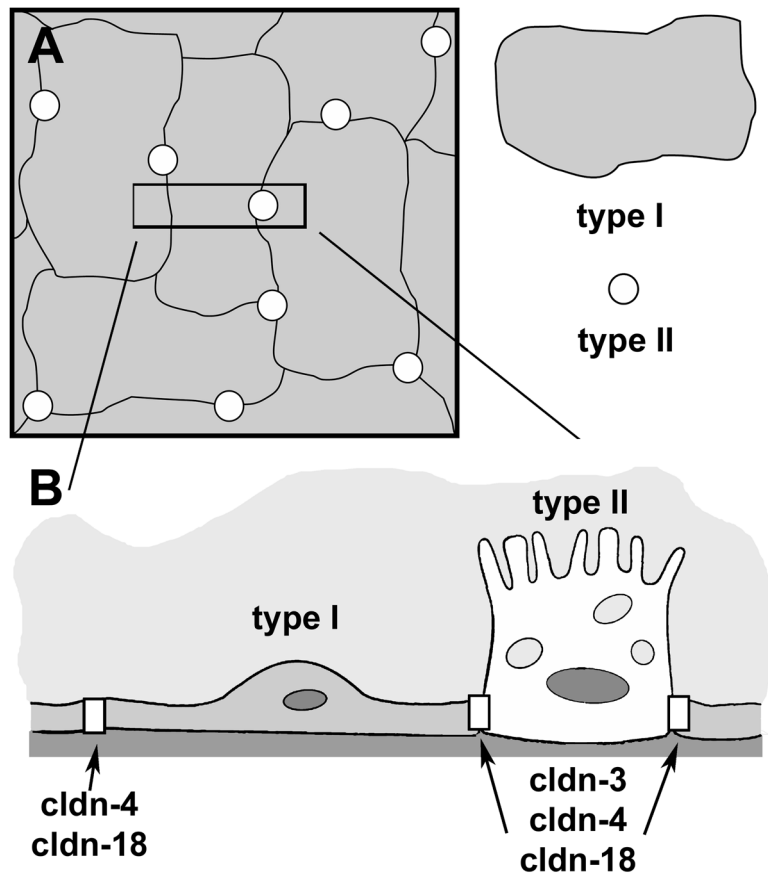
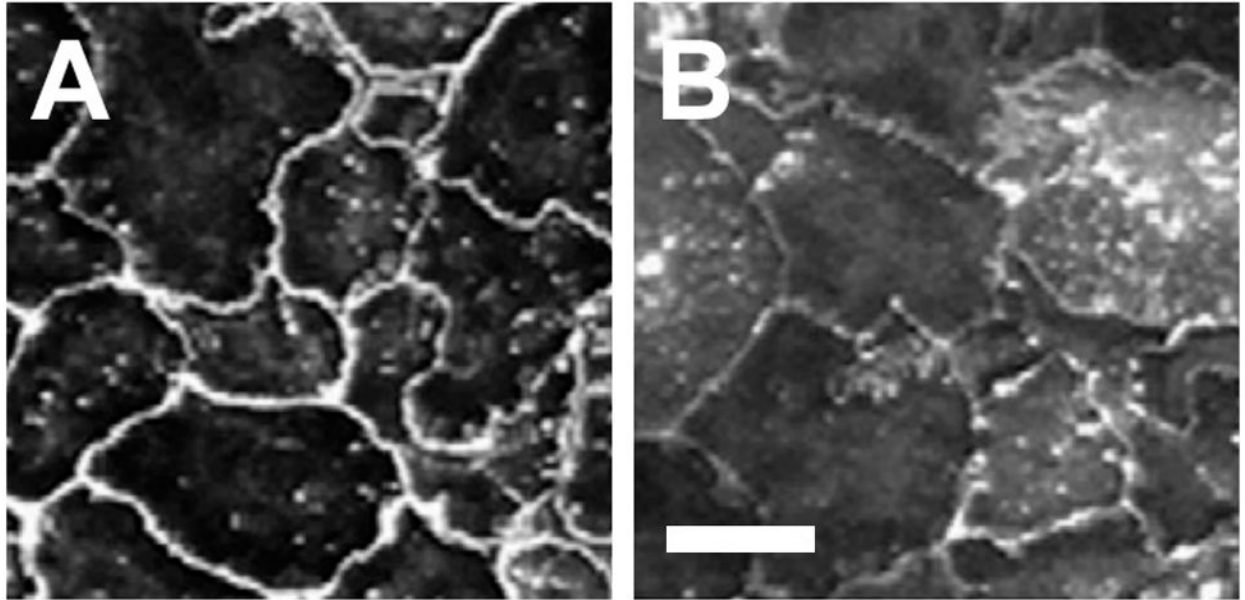


Figure 1. Alveolar epithelial cells

A. En face view of alveolar epithelium, showing the relative size and number of type I and type II cells. B. Cross section of the area delineated by the box in A. Shown are type I-type I and type I-type II cell junctions which have distinct claudin composition. The major difference is the presence of high levels of cldn-3 at type I-type II cell tight junctions.

control**alcohol****Figure 2. Alcohol impairs assembly of claudins into tight junctions**

Model type I alveolar epithelial monolayers were derived from primary cells isolated from either control (A) or alcohol fed (B) rats which were cultured for 6 days and then immunolabeled for cldn-7. In contrast with control alveolar epithelial cells, where cldn-7 prominently localized to sites of cell-cell contact (A), cells isolated from alcohol fed rats had impaired claudin assembly (B) which correlated with impaired barrier function. Bar = 10 microns. Adapted from Fernandez, et. al. ⁵⁹.

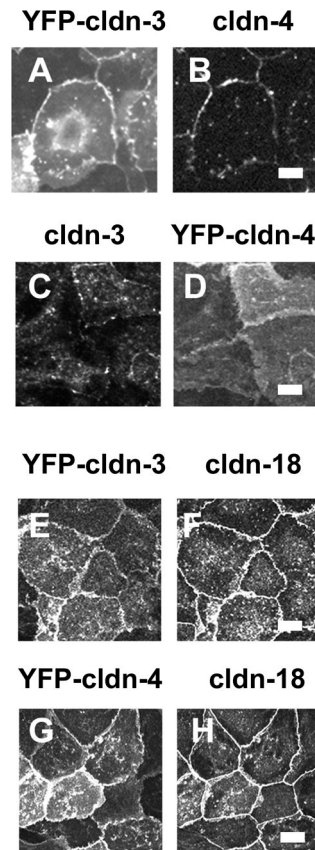


Figure 3. Increasing cldn-3 or cldn-4 expression by alveolar epithelium does not affect tight junction morphology

Model type I alveolar epithelial cells transduced with YFP-cldn-3 (A, B, E, F) or YFP-cldn-4 (C, D, G, H) were fixed and immunostained for cldn-4 (B), cldn-3 (C) or cldn-18 (F, H). YFP-cldn-3 and YFP-cldn-4 localized to the plasma membrane at sites of cell-cell contact. E-H. Increasing expression of either cldn-3 or cldn-4 had little effect on cldn-18 localization (F, H). Bar, 10 micron. Adapted from Mitchell, et. al. ⁴⁰.

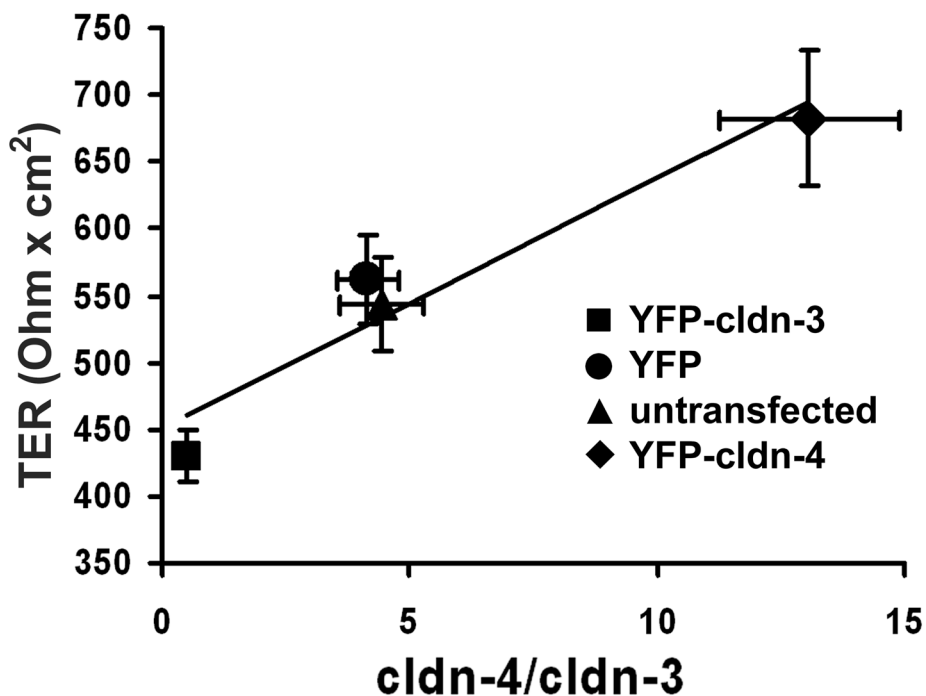


Figure 4. Differential effect of increasing cldn-3 or cldn-4 on alveolar epithelial cell barrier function

Model type I alveolar epithelial cells transduced with YFP-cldn-3 (■), YFP-cldn-4 (◆), YFP-control virus (●) or untransfected controls (▲) were assessed for the effect of altering claudin expression on barrier function, as determined using transepithelial resistance (TER; Ohm \times cm²) (y axis). The expression ratio cldn-4/cldn-3 was determined by immunoblot (x axis) demonstrating that there was a linear relationship between cldn-4/cldn-3 ratio and TER ($r^2 = 0.93$). Cells expressing increased cldn-3 had significantly lower TER than either control cells or cells expressing increased cldn-4 ($P < 0.05$). Increasing cldn-4 also significantly increased barrier function ($P < 0.05$). Adapted from Mitchell, et. al. ⁴⁰.

Table 1

Alveolar epithelial claudin expression

	Human Fetal ^{77, 78}	Human Adult ^{42, 69, 79}	Rat Type I ^{31, 32, 65}	Rat Type II ^{31-33, 65}	Mouse Type II ^{44, 80}
Claudin-3	RP*	P	RP#	RP#	P
Claudin-4	RP	P	RP	RP	P
Claudin-18	RP	P	RP	RP	P
Claudin-5	RP	P	RP	RP	P
Claudin-7	RP		RP	RP	P
Claudin-10b	R		R	R	P
Claudin-12			R	RP	
Claudin-15			R	RP	
Claudin-19			R	R	

* R = mRNA expression detected; P = protein expression detected

Rat type II cells express ~17-fold more cldn-3 than type I cells, cldn-4 and cldn-18 expression is comparable.^{31, 32, 65}

Other claudin mRNAs expressed by rat alveolar epithelial cells: cldn-9, cldn-11, cldn-20, cldn-22, and cldn-23 (Ref 31).

Table 2

Changes to alveolar epithelial claudin expression in disease

	Alcoholic lung syndrome ⁵⁹	Ventilator induced lung injury ⁶⁵	Inflammation ⁴²	Sepsis-induced ARDS ⁴³	Pulmonary Fibrosis ⁴⁴
Claudin-3	decreased	unchanged	unchanged	unchanged	decreased
Claudin-4	unchanged	increased	unchanged	decreased	decreased
Claudin-18	decreased	unchanged	decreased	decreased	decreased
Claudin-5	increased		unchanged	unchanged	decreased
Claudin-7	decreased			unchanged	decreased