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Claudin Expression in Alveolar Epithelia Influences Barrier Function:

The Effect of Extracellular Matrix on Claudin Expression

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MK Findley
B.A., Agnes Scott College, 2006

Advisor: Michael Koval, Ph.D.

An abstract of
A thesis submitted to the Faculty of the Graduate School of Emory University
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Abstract

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By MK Findley

The lung is the essential respiration organ in all air breathing animals. The alveoli are the physical site of the exchange of carbon dioxide for oxygen. Lung injury can drastically damage alveolar epithelium, leading to respiratory failure. This respiratory failure is caused by a loss of barrier function of the alveolar epithelia and a subsequent flood of the alveolar space with proteinaceous fluid.

The model I present here hypothesizes that injury to the alveolus stimulates a change in the extracellular matrix of the alveolar epithelium. In turn, localization and expression of tight junction proteins found in the alveolar epithelium are significantly altered. These changes in the tight junction are sufficient to alter the barrier function of the alveolus, thus leading to the flooding of the alveolar space.

Following lung injury there is a dramatic alteration of the extracellular matrix. This remodeling directly impacts the expression and function of tight junction proteins, leading to either healthy or impaired lung regeneration. In this thesis we hypothesize that although a fibronectin enriched provisional matrix accumulates during acute lung injury and this matrix promotes rapid reformation of alveolar barriers, these barriers are suboptimal as compared with barriers more slowly produced by cells on native matrix.

Our results show that cells grown on a fibronectin matrix, representative of an injury matrix, show a significant change in tight junction protein expression that is correlated with changes in barrier function. We test the hypothesis that altering the expression level of a single claudin is sufficient to induce changes in barrier function.

The data in this thesis supports the conclusion that despite the ability to form a rapid barrier after injury, cells on fibronectin ultimately have suboptimal barrier function as compared with cells on native matrix. These results will have an impact on understanding a number of health issues. For example, events occurring before lung injury can also significantly impact the severity of injury without drastically damaging the alveolus. Chronic alcohol abuse is known to increase the risk of acute lung injury by 2-4 fold. Chronic alcohol ingestion promotes fibronectin expression in the lung and is associated with changes of tight junction protein expression. These events lead to a decrease in the barrier function of the alveolus leaving the lung more susceptible to injury and exaggerating the lung's injury response, often leading to increased mortality.

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Chapter 1. Introduction to the Pulmonary System and Lung Injury

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In this thesis I present a model that hypothesizes that injury to the lung stimulates a change in the extracellular matrix composition of the lungs. In turn localization and expression of tight junction proteins found in the lungs are significantly altered. These changes in the tight junction are sufficient to alter the barrier function of the lungs, leading to flooding of the alveoli and impaired pulmonary function.

Chapter One and Chapter Two will review the pulmonary system, lung injury and extracellular matrix as it relates to would healing. Chapter Three will provide a detailed current review of the regulation and roles for the claudin-family of tight junction proteins. Chapter Four will discuss the methods used in the following chapters. Chapter Five will discuss how the extracellular matrix influences alveolar epithelial claudin expression and barrier function and Chapter Six will discuss the manipulation of claudin expression in alveolar epithelia and its affect on barrier function. I will conclude in Chapter Seven with a discussion of the work and how it relates to the field.

I. The Pulmonary System

The lung or pulmonary system is the essential respiration organ in all air-breathing animals. Its principal function is to transport oxygen from the atmosphere into the bloodstream, and to release carbon dioxide from the bloodstream into the atmosphere. A mosaic of specialized cells facilitates this exchange of gases. The physical sites of exchange are millions of tiny, exceptionally thin-walled air sacs called alveoli (Figure 1A). Alveoli are lined with epithelium and contain some collagen and elastic fibers. The elastic fibers allow the alveoli to stretch as they fill with air during inhalation and spring

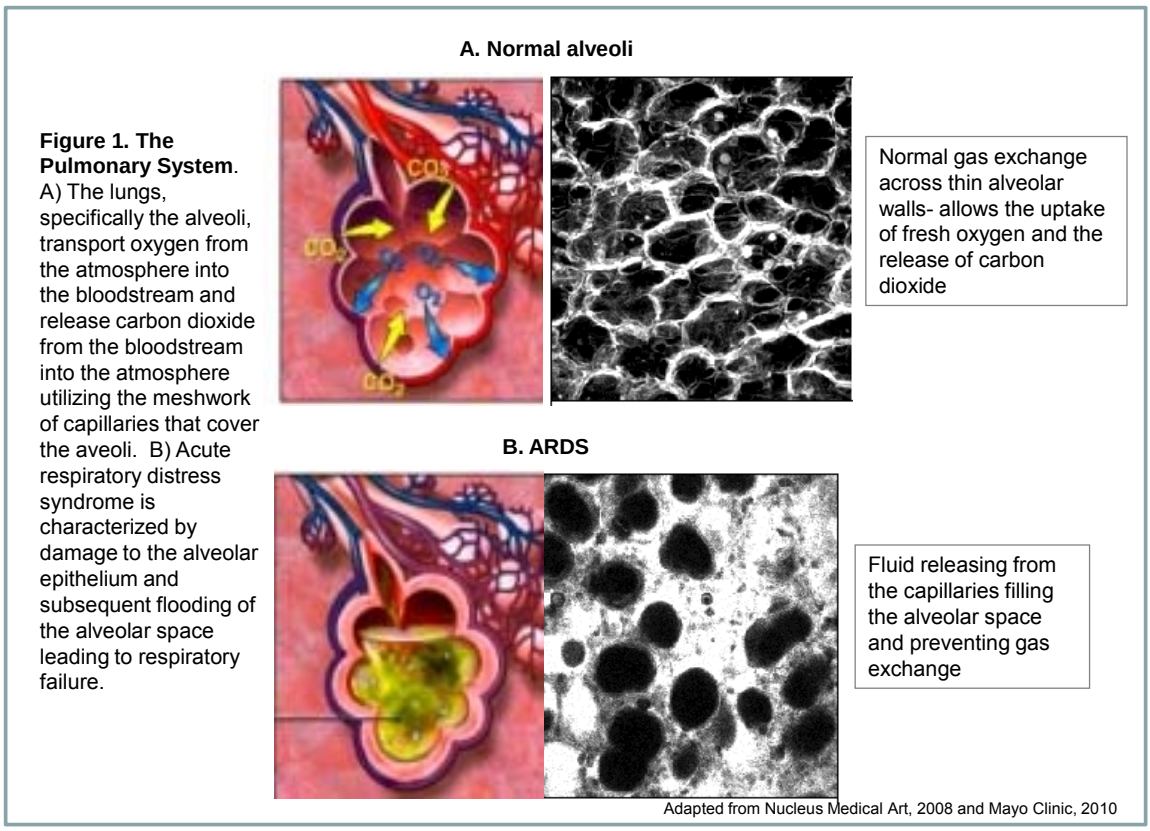

Figure 1. The Pulmonary System. A) The lungs, specifically the alveoli, transport oxygen from the atmosphere into the bloodstream and release carbon dioxide from the bloodstream into the atmosphere utilizing the meshwork of capillaries that cover the aveoli. B) Acute respiratory distress syndrome is characterized by damage to the alveolar epithelium and subsequent flooding of the alveolar space leading to respiratory failure.

back during exhalation to expel the carbon dioxide-rich air. The alveolar membrane is the gas-exchange surface. The interalveolar septum is the close-meshed capillary network that is between adjacent pulmonary alveoli. In normal lung tissue, the interalveolar septum is covered by alveolar epithelium, contains endothelial cells of the capillaries and is supported by a basement membrane of extracellular matrix and connective tissue [227]. This is illustrated in Figure 2A. Each human lung contains about 300 million alveoli. Each alveolus is wrapped in a fine mesh of capillaries covering about 70% of its surface area to facilitate oxygen uptake. An adult alveolus has an average diameter of 200 to 300 microns that increases in diameter during inhalation [1, 2].

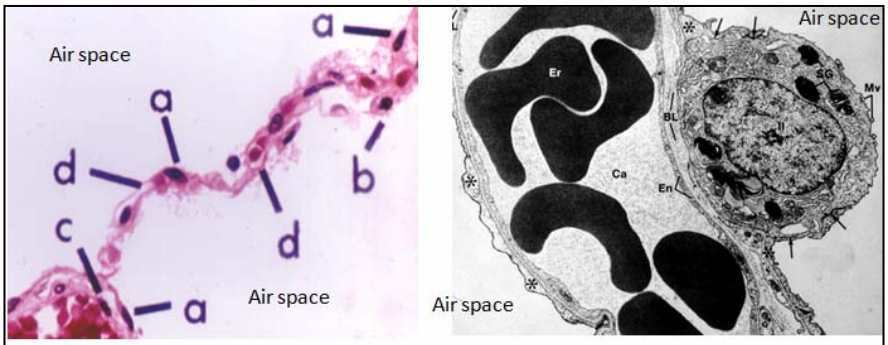

Figure 2A Light (left) and electron microscopic (EM, right) illustrating the an inter-alveolar septum and the respiratory barrier between alveolar air space and the blood in alveolar capillaries. The letters in the right panel indicate both epithelial and endothelial cells of the inter-alveolar septum. The EM image illustrates that air space and blood are separated by type I epithelial cells (*), endothelial cells of the capillary and an intervening connective tissue basement membrane (BL). The right half of the EM features a secretory type II epithelial cell responsible for secretion of alveolar surfactants. (Images contributed by W. Sale, Cell Biology, Emory University)[1]

The focus of this thesis is specifically on the alveolar epithelium. The alveolar epithelium is composed of two distinct cell types (Figure 2B). Type I cells are squamous alveolar cells and form the structure of the alveolar wall. These structural cells are relatively flat and have a much greater surface area than their counter part Type II cells. Type II or great alveolar cells are cuboidal pneumocytes. They synthesize and secrete pulmonary surfactant. This surfactant serves to lower the surface tension of water and allows the membrane to separate increasing the capability of gas exchange. Type I and Type II cells are present in approximately equal numbers; however, Type I cells comprise ~95%

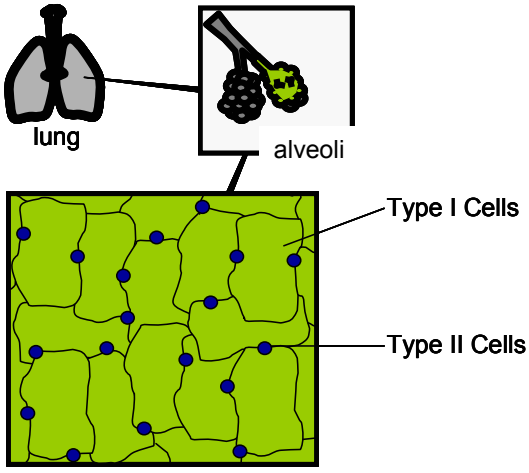

Figure 2. Alveolar Epithelium. Type I cells are relatively flat and form the structure of the alveolar wall. Type II cells are more cuboidal and synthesize and secrete pulmonary surfactant.

of the alveolar epithelial barrier *in vivo* [3]. Defects or injury to any of the alveolar barrier components could lead to the pathology discussed in the following section.

II.Lung Injury

The theme of my thesis is that certain injuries to the lung can lead to the disruption of the extracellular matrix basement membrane and result in dysfunction of the epithelial tight junctions. In the following sections I will briefly discuss the nature of lung injury, extracellular matrix, and epithelium and tight junctions.

There are multiple causes of lung injury and disease such as asthma, emphysema, cystic fibrosis, and pneumonia. I will focus on acute respiratory distress syndrome (ARDS) and a less severe form of ARDS called acute lung injury (ALI). ARDS is a severe form of lung injury that occurs in response to major insults such as trauma, sepsis, gastric aspiration, and multiple blood transfusions [4-6]. A cardinal feature of ARDS is diffuse damage to the alveolar epithelium with subsequent flooding of the alveolar space with proteinaceous fluid. These events lead to respiratory failure and frequently result in multiple organ failure. Even with the current best medical practices, the mortality of ARDS is in the range of 40%-50% [4, 6] (Figure 1B).

Although the precise incidence of ARDS in the United States is unknown, the estimate is that as many as 150,000 cases occur each year [6]. Patients with impaired alveolar fluid clearance are three times more likely to die from ALI/ARDS than patients with an optimal ability to clear lung fluid [7, 8]. Significantly, greater than 85% of patients with ALI/ARDS have at least a partial defect in lung fluid clearance. Thus

treatment regimens that improve alveoli barrier function, and thus lung fluid clearance, have the potential to be a valuable therapeutic tool to improve patient outcome.

Lung fluid balance is regulated by the concerted action of the pulmonary epithelial and endothelial barriers [9]. See figure 2B for an electron micrograph of the endothelial-epithelial alveolar barrier. From the epithelial side, there are two major factors that affect the ability of the alveolus to clear fluid from airspaces. This includes the active clearance of water and ions by specific transporters expressed by alveolar epithelial cells [6, 10-15] and the extent of permeability between alveolar epithelial cells [16-18]. Of note, there are also intracellular transport mechanisms in the apical and basal lateral membranes that will not be discussed in this thesis. The extent of permeability between alveolar epithelial cells is the paracellular permeability and is controlled by tight junctions. These junctions create a barrier to free diffusion between cells. In this thesis we hypothesize that following acute lung injury there is an alteration of the extracellular matrix that initiates changes in the expression of tight junction proteins and ultimately induces a shift in alveolar barrier function.

Chapter 2. Extracellular Matrix

Chapter 2. Extracellular Matrix

In response to acute lung injury, lung extracellular matrix composition is dramatically altered [186]. The extracellular matrix (ECM) is the extracellular part of animal tissue that provides structural support to the animal cells as well as performing various other important functions. The ECM can provide support and anchorage for cells, segregate tissues from one another and regulate intercellular communication. Formation of the extracellular matrix is essential for processes like growth and wound healing. The ECM is composed of an interlocking mesh of fibrous proteins and glycosaminoclycans (GAGs) [19]. This thesis will focus on the fibers laminin, collagen and fibronectin and the role they each play in wound healing in the alveolar basement membrane. (Figure 3). The alveolar basement membrane, also known as the basal lamina, separates the endothelium from the epithelium in the alveolus and helps to organize the cells of the alveolar respiratory barrier. Wound healing is intricate process that involves multiple proteins. We choose to focus on laminin, collagen and fibronectin ECM component because laminin is representative of a native alveolar basement membrane, and destruction of the alveolar basement membranes induced during injury is followed by significant increased expression and deposition of fibronectin and type I collagen which are the basis of what we label the “provisional” matrix [187-189].

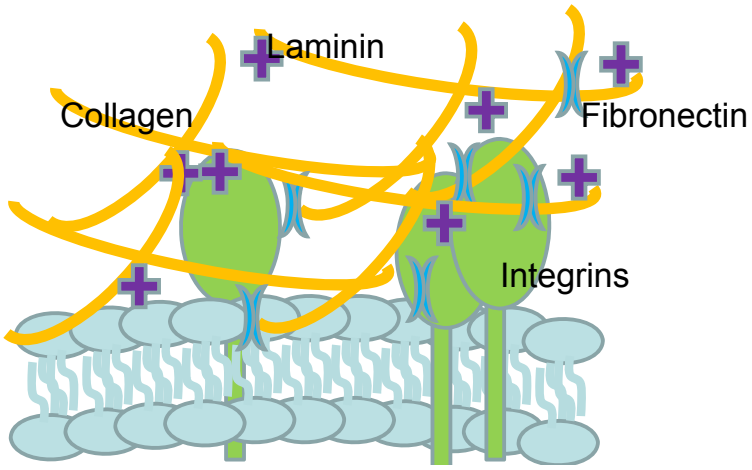

Figure 3. Extracellular Matrix. The extracellular matrix is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface that produced them.

I. Laminin

Laminins are major proteins of the basal lamina or basement membrane, a protein network creating a foundation for most cells and organs in healthy cells. The laminins are a family of glycoproteins and are an integral part of the structural scaffolding in almost all tissues. Laminins influence cell differentiation, migration and adhesion as well as phenotype and survival [20-23]. Laminin is a 500 kDa glycoprotein composed of three chains: A (400 kDa), B1 (210 kDa), and B2 (200 kDa). (Figure 4A) Laminin is in contact with a variety of epithelial and mesenchymal cells that include muscles, adipocytes and neurons [24, 25]. Purified laminin exhibits disparate cellular activities, including promotion of proliferation, attachment, chemotaxis, inhibition or enhancement of neuronal precursor cells and induction of both collagenase type IV and tyrosine hydroxylase enzymes [26].

In the lung laminin acts as a structural and adhesive protein; it spans the basement membrane and binds to various substances on the surface of cells. Binding to laminin elicits cell-specific responses such as migration and differentiation [229]. When

the extracellular matrix is reorganized, as in lung injury, laminin's important structural and regulatory roles are lost. In the studies of this thesis alveolar epithelial cells will be grown on a laminin matrix that is representative of a native matrix.

II. Collagen

Collagen expression is increased after injury and is considered a component of the provisional injury matrix [187-189]. Collagens are the most abundant protein in the ECM and actually the most abundant protein in mammals, making up 25% to 35% of the whole body protein content. The collagen molecule is a subunit of larger collagen structures known as fibrils. Each fibril is made up of three polypeptide strands of left-handed helices twisted together into a right-handed coiled coil or "super helix." (Figure 4B) Collagens are present in the extracellular matrix as fibrillar proteins and give structural support to resident cells. Collagen is secreted in a precursor form that is then cleaved by procollagen proteases to allow extracellular assembly [27, 28]. The fibrous backbone of ECM is made of collagens [29, 30]; depending on the source of the ECM, the collagens may be represented by fibrillar (types I, II, III), nonfibrillar (e.g. type IV) or the so-called FACIT (fibril-associated collagens with interrupted triple helices) collagens [31], in various combinations. Type I collagen forms a relatively rigid, linear fibrillar network, whereas type IV collagen is assembled into a complex network, found mainly in basement membranes. The FACIT collagens, by becoming integrated into collagen fibrils via their collagenous domains and interacting with other components of ECM through their noncollagenous modules, have been speculated to enhance organizational stability of the ECM [26, 31].

In the lung collagen IV is found in the basement membrane. Its turnover is a dynamic process that is necessary for the maintenance of normal lung architecture [228]. With injury, fibroblasts secrete excess collagen I and other factors such as TGF-β which inhibits collagenase thus disrupting the maintenance of the normal lung architecture [228]. In the studies of this thesis alveolar epithelial cells will be grown on a collagen matrix that is representative of an injury matrix.

III. Fibronectin

Fibronectin is the main component of the provisional injury matrix [187-189]. Fibronectin is a high molecular weight extracellular matrix glycoprotein that binds to integrins. Fibronectins bind both collagen and cell surface integrins, causing a reorganization of the cell's cytoskeleton and facilitating movement [32-24]. Cells secrete fibronectins in an unfolded, inactive form. Binding to integrins unfolds fibronectin molecules, allowing them to form dimers so that they can function properly [26]. Fibronectins play a major role in cell adhesion, growth, chemotaxis and differentiation. They are also important in the process of wound healing and embryonic development [32-34]. This 540-kDa glycoprotein is a dimer composed of two similar polypeptide chains. Each polypeptide subunit of fibronectin is made up of repeating modular domains; these include two cell binding domains in addition to the unique modules interacting with collagen, heparin, and fibrin (Figure 4C). Subtle variations are introduced by alternative splicing [26].

Fibronectin expression is greatly increased upon injury to the lung and previous in vitro studies have demonstrated that fibronectin promotes wound healing [190] and

stimulates the ability of alveolar epithelial cells to form high resistance tight junction barriers after four days in culture [191]. In the experiments that follow fibronectin matrices are representative of a provisional injury matrix

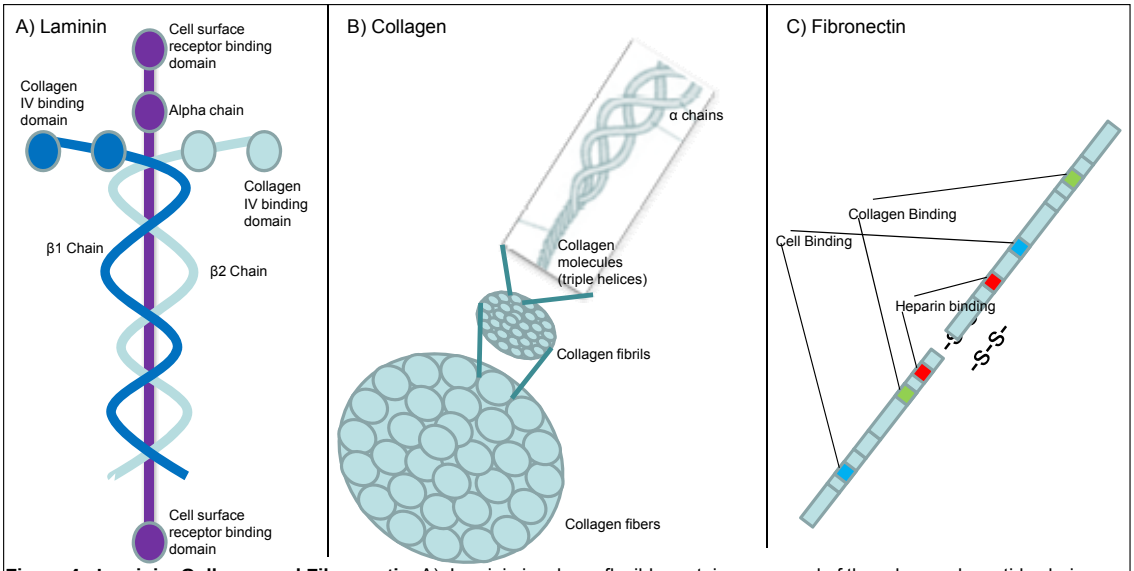

Figure 4: Laminin, Collagen and Fibronectin. A) Laminin is a large flexible protein composed of three long polypeptide chain (alpha, beta and gamma) arranged in the shape of an asymmetric cross and held together by disulfide bonds. Several isoforms of each type of chain can associate in different combination to form a large family of laminins. B) The primary feature of a typical collagen molecule is its lung, stiff, triple-stranded helical structure in which three collagen polypeptide chains, called alpha chains, are wound around one another in a rope like superhelix. C) Fibronectin is a dimer. The two polypeptide chains are similar but generally not identical (being made from the same gene but from different spliced mRNAs). They are joined by two disulfide bonds near the C-termini. Each chain is almost 2500 amino acids long and is folded into five or six domains connected by flexible polypeptide segments. Individual domains are specialized for binding to a particular molecule or to a cell.

IV. Wound Healing

Part of the foundation of my thesis are the diverse studies indicating that in response to acute lung injury, lung extracellular matrix composition is dramatically altered [186] and fibronectin-enriched "provisional" matrix is introduced. Several studies have demonstrated that in the lung, this matrix promotes wound healing [187-189]. Experiments discussed in later chapters will examine the effects of the fibronectin matrix on aspects of wound healing specifically associated with the alveoli, such as barrier function.

Wound healing is an intricate process in which an organ repairs itself after injury. Once the injury has occurred, the classic model of wound healing is divided into three sequential, overlapping phases: 1) inflammatory, 2) proliferative and 3) remodeling. Within minutes post-injury, platelets aggregate at the injury site to form a fibrin clot. During the inflammatory phase, bacteria and debris are phagocytosed and removed. Factors are released that cause the recruitment and division of cells involved in the proliferative phase. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction [35-38].

In the first two or three days after injury, fibroblasts from normal tissue migrate into the wound area from its margins. The fibroblasts use the fibrin scab formed during the inflammatory phase to migrate across the wound, adhering to the fibronectin. Fibroblasts then deposit ground substance into the wound bed, followed by collagen, to which they can adhere for migration. By the second or third day after the injury fibroblasts produce collagen. Collagen production continues rapidly for two to four weeks, after which its destruction matches its production so its levels plateau. Collagen deposition is important because it increases the strength of the wound; before it is laid down, the only thing holding the wound closed is the fibrin-fibronectin clot, which does not provide much resistance to traumatic injury [39, 40].

Granulation tissue is a fibrous connective tissue that replaces the fibrin clot. It functions as rudimentary tissue and begins to appear two to five days post wounding and continues growing until the wound bed is covered. Granulation tissue consists of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts and

components of a new provisional ECM. The provisional ECM is different in composition from the ECM in normal tissue and its components originate from fibroblasts. Such components include fibronectin, collagen, glycosaminoglycans, elastin, glycoproteins and proteoglycans. Its main components are fibronectin and hyaluronan, which create a hydrated matrix and facilitate cell migration. Later this provisional matrix is replaced with an ECM that more closely resembles that found in non-injured tissue [36, 40-42].

Previous in vitro studies have demonstrated that fibronectin promotes wound healing [190] and stimulates the ability of alveolar epithelial cells to form high resistance tight junction barriers after four days in culture [191]. However, the effect of the extracellular matrix on composition of the tight junction, the structure that controls alveolar barrier function, has not been determined. The next chapter discusses the tight junction, specifically the claudin family of proteins that form the physical barrier for paracellular permeability. This information provides support for the model that, injury to the alveolar epithelium induces changes to the extracellular matrix and these changes are correlated to changes in tight junction localization and expression. We hypothesize these changes are sufficient to induce the changes in barrier function that are related with injury.

Chapter 3. Tight Junctions and Claudins

Adapted from: Regulation and roles for claudin-family tight junction proteins. Findley MK, Koval M. IUBMB Life. 2009 Apr; 61(4):431-7. Review.

Chapter 3. Tight Junctions and Claudins

In the model proposed in this thesis, injury to the alveolar epithelium induces changes to the extracellular matrix and these changes are correlated to changes in tight junction localization and expression. This model is founded in published work that shows that fibronectin promotes wound healing [190] and stimulates the ability of alveolar epithelial cells to form high resistance tight junction barriers [191]. However, additional studies show if left unopposed, fibronectin-mediated events lead to excessive inflammation and fibroblast proliferation, resulting in permanent disruption and of normal alveolar structure [213]. This work tests the hypothesis that alterations to the extracellular matrix induce changes in tight junction protein localization and expression and that these changes in the tight junction are sufficient to alter barrier function. The structure, function and control of the claudin family of tight junction proteins are discussed below.

I.Tight Junctions

Epithelial and endothelial cell monolayers form barriers to separate organs into functional subcompartments. This distinct compartmentalization and isolation from the external environment is crucial for the function of organ systems in multicellular organisms. Cells control these selective barriers by regulating the movement of water, ions and proteins across the monolayer, thus generating polarity of cellular structure and function [43, 44]. The movement of ions and molecules between cells is known as paracellular permeability and is regulated by sites of cell-cell contact known as tight junctions. The tight junction is a complex of transmembrane and peripheral proteins that

is tethered to the cytoskeleton [43, 45-47] (Figure 5). Here we will focus on the claudin family of tight junction proteins.

While tight junctions require the coordinated activity of several different proteins, the specificity of tight junction permeability is regulated by transmembrane proteins known as claudins, a name derived from the Latin *claudere*, which means "to close" [48-52].

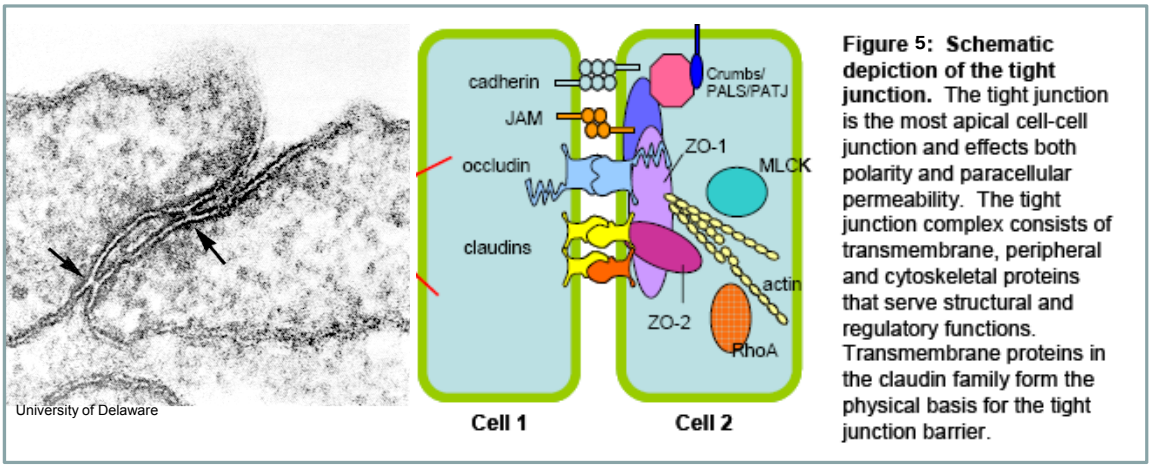

Figure 5: Schematic depiction of the tight junction. The tight junction is the most apical cell-cell junction and effects both polarity and paracellular permeability. The tight junction complex consists of transmembrane, peripheral and cytoskeletal proteins that serve structural and regulatory functions. Transmembrane proteins in the claudin family form the physical basis for the tight junction barrier.

II. Claudin Superfamily

Members. To date, 23 distinct human claudins have been identified. Claudins are 20-27kDa transmembrane proteins that span the bilayer four times, where the N- and C- termini are oriented towards the cytoplasm and there are two extracellular loop domains [53] (Figure 6). For at least two claudins, claudin-10 and claudin-18, alternative splicing can also generate claudin isoforms with different properties [54, 55]. Although not directly addressed in this thesis, major questions about the claudins include their function in paracellular selectivity, their role in the tight junction, the functional regulation of claudins and the role claudins play in disease.

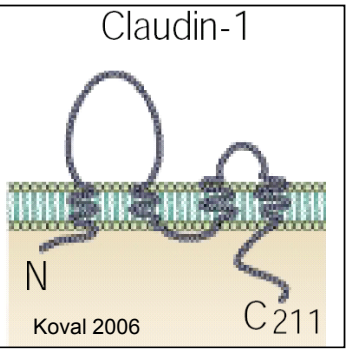

Figure 6 : Claudin Structure. Claudins are 20-27 kDA transmembrane proteins that function in the tight junction. They span the bilayer four times, where the N- and C- terminus are oriented towards the cytoplasm and there are two extracellular loop domains.

Claudin tetraspanin structure. Little is known about the relatively short, 7-amino acid, N-terminus. The cytoplasmic C-terminus sequence varies considerably in length (from 21-63 residues) and sequence between isoforms. All claudins have a C-terminal PDZ binding motif that enables direct interaction with tight junction cytoplasmic proteins such as ZO-1,-2, and –3, multi-PDZ domain protein (MUPP)-1 and PALS-1 associated TJ protein (PATJ) [56-59]. The interaction with tight junction cytoplasmic scaffolding proteins, like ZO-1, indirectly links claudins to the actin cytoskeleton which stabilizes the tight junction, ultimately affecting tight junction turnover and permeability [60].

The first extracellular loop of a claudin is approximately 52 residues and influences paracellular charge selectivity [51, 61]. This loop domain has a signature motif (Gly-Leu-Trp-x-x-Cys-(8-10aa)-Cys) which is highly conserved [62] and is also found in closely related proteins, such as epithelial membrane proteins (EMPs, PMP22, MP20) [49] as well as the more divergent y5 subunit of voltage-gated calcium channels [62]. The cysteines in this signature motif may form an intramolecular disulfide bond to stabilize protein conformation, however this remains to be determined [48]. The second extracellular loop of a claudin is shorter (16-33 residues) and less well characterized.

Molecular modeling suggests the second extracellular loop is folded in a helix-turn-helix motif which participates in claudin-claudin interactions [63].

Claudin function in paracellular selectivity. The primary role of claudins appears to be the regulation of paracellular selectivity to small ions. Claudins form selective pores to one population of ions while at the same time hindering the passage of ions of the opposite charge [64]. For example studies have shown that overexpression of claudin-7 decreases paracellular permeability to Cl^- and increases paracellular permeability to Na^+ [226]. The evidence for this also includes mutagenesis approaches in which claudin-7 mutants were made by replacing negatively charged amino acids with positively charged amino acids in the first extracellular domain which resulted in increased Cl- permeability [226]. The majority of claudin isoforms that have been tested lead to an increase in TER. Dilution potentials show that in most cases this increase in TER is due to selective decreases in cation permeability but this is not always the case [65-70]. It has been assumed that the reason there are more than 24 claudin isoforms is that each has distinct permeability properties, but this has not turned out to be the case [65-70]. Many claudins have shown fairly crude differences in paracellular selectivity, suggesting that there must be other important differences between them and raising the possibility that claudins may have additional roles. Additional roles for claudins could include cell-matrix interaction, scaffolding functions, cell-cell adhesion, and crosstalk with signaling; however, these models will require additional testing

.

III.Claudins Role in Tight Junctions

Tight junctions were functionally identified by electron microscopy as structures that blocked the movement of horseradish peroxidase across the blood-brain barrier [71]. Subsequent freeze fracture analysis showed that tight junctions are composed of a network of strands of which claudins are the major transmembrane constituent. These strands are roughly the diameter of a gap junction channel [72-75]. This has led to the suggestion that claudins are organized into a basic hexameric unit, similar to connexins in gap junctions. Claudins have been shown to form stable oligomers in native gel electrophoresis increasing in molecular mass up to the equivalent of a hexamer [74] . It still remains to be determined whether claudins oligomerize into subunits prior to incorporation into tight junction strands.

Claudin-claudin interactions. Epithelia and endothelia typically express multiple claudin isoforms in a tissue specific manner, suggesting the potential of claudin isoforms to intermix in tight junction strands. This claudin-claudin intermixing has the potential to control barrier permeability; however, testing this hypothesis has proven to be difficult because of the complexity of claudin intermixing. Claudins can interact in two different ways: laterally in the plane of the membrane (heteromeric) or head to head binding between adjacent cells (heterotypic) (Figure 7). Through using fibroblasts as claudin-null backgrounds

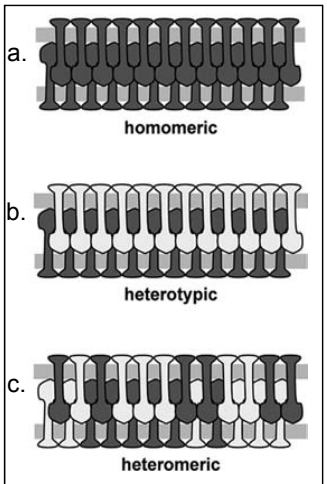

Figure 7. Arrangement of multiple claudins in single tight junction strand. Strands can theoretically be composed of either a) a single claudin-homomeric b) different claudins interacting through heterotypic head-to-head binding or c) different claudins interacting via heteromeric binding in the plane of the membrane.
Koval 2006

[74], some progress has been made in defining heterotypic claudin binding. It has been found that the heterotypic compatibility is extremely complex, suggesting multiple surfaces of the extracellular loop domains regulate claudin heterotypic compatibility. Little is known about motifs that control heterotypic compatibility. For example, although claudin-3 and claudin-4 are heteromerically compatible when expressed in the same cell, they do not heterotypically interact despite having extracellular loop domains that are highly conserved at the amino acid level. Claudin-1 and –5 are heterotypically compatible with claudin-3 but do not heterotypically bind to claudin-4 [76]. However, single point mutations in the first extracellular domain of claudin-3 to convert Asn(44) to the corresponding amino acid in claudin-4 (Thr) produced a claudin capable of heterotypic binding to claudin-4 while still retaining the ability to bind to claudin-1 and –5. Thus, heterotypic claudin-claudin interactions are very sensitive to small changes in the extracellular domains[76].

Very little is known about claudin heteromeric binding. Claudin homomultimers composed of up to six monomers have been observed biochemically [73, 74, 77] and lateral interactions of claudin-5 within cells has been detected using fluorescence resonance energy transfer (FRET) [78]. All interactions described thus far have involved claudin-3, suggesting that these heteromeric interactions may be an exception and that homophilic claudin interactions are more common.

Claudin-occludin interactions. Occludin is another tetraspan transmembrane protein of the tight junction. For many years occludin was assumed to be the protein which formed the physical basis of the tight junction barrier; however, transgenic occludin deficient mice were found to be viable and have normal barrier

function [79, 80] leading to the current idea that claudins play a significant role in forming the physical barrier of tight junctions. It is unknown whether occludin interacts with claudins, but is still thought that occludin helps to regulate barrier function. Studies show that peptides that mimic the occludin extracellular loop domains can disrupt epithelial barrier function by enhancing junctional disassembly [81-83]. Claudin extracellular loop peptides also bind to occludin [84]. Transfection of cells with an occludin construct with a C-terminal truncation does not affect TER but does increase the paracellular flux of uncharged molecules [45] . These studies suggest the interaction of occludin with the claudins of the tight junction strands.

Claudin-scaffold protein interactions. Claudins and other transmembrane tight junction proteins interact with several cytosolic scaffold proteins, serving as a platform to recruit signaling proteins and to link junctions to the cytoskeleton [85]. Most claudins interact with zona occludens (ZO) –1, -2 and –3 through a conserved C-terminal YV domain [56]. Some claudins have also been shown to interact with MUPP1 and PATJ [57, 59, 86, 87]. These interactions are claudin tail dependent and induce changes in strand morphology [88, 89]. Claudin-ZO protein interactions are required for tight junction assembly and can alter epithelial barrier function. In cultured epithelial cells, in which the expression of ZO-1 and ZO-2 was suppressed, cells had a complete lack of tight junctions. When exogenously re-expressed in these ZO-1/ZO-2 null cells, ZO-1 and ZO-2 promoted claudin recruitment to tight junctions where claudins formed functional tight junctions thus restoring barrier function. The ZO proteins dimerize, interact with claudins and initiate the polymerization of claudins into tight junction strands and also direct the correct localization of these tight junction strands [60].

IV.Functional Regulation of Claudins

The properties of claudins are influenced by environmental cues, developmental changes and physical disruption of cell-cell contacts. Functional regulation can occur at the level of posttranslational modification and at the level of gene expression. Conventionally it was thought that paracellular permeability was constitutive and unregulated but increasing evidence suggests that claudins can be modified, suggesting that paracellular permeability responds to physiological stimuli.

Internalization of claudins. It is known that individual tight junction strands are stable based on the lack of recovery when tight junctions containing EGFP-tagged claudins are photobleached [90]; however, the tight junction complex itself is dynamic. The network of tight junctions is constantly remodeled [90]. In measuring tight junction protein dynamics, mathematical modeling shows that the majority of claudins are stably localized at the tight junction but in contrast the majority of occludin diffuses rapidly within the tight junction [91]. ZO-1 molecules are also highly dynamic but rather than diffusing within the plane of the membrane, most exchange between membrane and intracellular pools. This constant remodeling suggests that the dynamic behavior of tight junction molecules may contribute to tight junction assembly and regulation [91].

This remodeling is often facilitated through endocytic internalization. Compared to other tight junction proteins, claudins are internalized by a unique mechanism. The tightly opposed membranes of the tight junction are endocytosed together into one of the adjoining cells. During this internalization the claudins separate away from occludin, JAM and ZO-1 and generate claudin-enriched vesicles [92-94]. This claudin internalization is then further assisted by clathrin-mediated endocytosis [95, 96]. Claudin

internalization is one physiological mechanism for regulating epithelial barrier function. Studies show that interferon-γ induces claudin endocytosis and increases tight junction permeability [97, 98]. Toxins such as *E.coli* cytotoxic necrotizing factor-1, *H. Pylori* associated factors, and *Clostridium perfringens* enterotoxins (CPE) also induce claudin internalization [99-101]. CPE induces claudin endocytosis and increases paracellular permeability by binding to the second extracellular loop domain of claudins [102].

Palmitoylation of claudins is required for tight junction formation. Palmitoylation is the covalent attachment of fatty acids to a protein. Palmitoylation of claudins potentially plays a role in barrier function, tight junction assembly and claudin oligomerization. Signature di-cysteine palmitoylation motifs are conserved throughout the claudin protein family [103]. Palmitoylation of claudins is required for tight junction formation. Palmitoylated claudins also more efficiently portion into detergent resistant membranes as compared to non-palmitoylated mutants, suggesting lipid rafts play a role in tight junction assembly [104, 105]. Other tetraspanins similar to claudins are also palmitoylated [106, 107] enhancing their partitioning and oligomerization [106, 107]. This tetraspanin palmitoylation occurs in the Golgi Apparatus suggesting that it is also a potential location for claudin oligomerization [108]. It is unknown where claudins oligomerize; however, claudins may oligomerize in the Golgi Apparatus in a manner analogous to the connexin family of gap junction proteins [49].

Phosphorylation of claudins affects paracellular permeability. Claudin post-translational modifications by phosphorylation can induce alterations to the paracellular permeability of tight junctions to both ions and non-charged molecules. Claudins are known to be direct substrates for kinases such as cAMP activated PKA, PKC and myosin

light chain kinase [109-111]. Claudin endocytosis and turnover can be enhanced by phosphorylation which causes a decrease in barrier function [98, 112]. Threonine phosphorylated claudins decrease detergent solubility suggesting a change in tight junction strand structure. The claudins move to the cytosol and increase paracellular permeability most likely due to the lack of claudin targeting to the membrane [110]. Claudin dephosphorylation, for example through PP2A, has the opposite effect and enhances epithelial barrier function [113].

Other studies have conflicting observations and have associated claudin phosphorylation with enhanced barrier function. For example, Claudin-1 phosphorylation in epithelial cells is required for proper barrier function and dephosphorylation by PP2A negatively regulates the tight junction by enhancing claudin solubility in detergent and increasing paracellular permeability [113]. These conflicting roles for phosphorylation are a topic for further study in the regulation of claudins.

Transcriptional regulation in epithelial-mesenchymal transformation. Epithelial-mesenchymal transition (EMT) is involved in embryonic development and repair of epithelial injury. EMT is associated with changes in claudin expression and regulation. The Eph-ephrin axis functions in controlling EMT [114]. The Eph-ephrin axis may directly control claudin function by binding and phosphorylating claudins. EphA2 and ephrin-B1 both bind the first extracellular loop of claudins. EphA2 phosphorylates a conserved C-terminal tyrosine residue after binding. This binding and phosphorylation is accompanied by an increase in paracellular permeability [115]. This suggests a direct link between Eph receptors, ephrins and down-regulation of tight junction proteins that occurs during EMT [116, 117]. In epithelial cells cultured to study

EMT, overexpression of the transcription factor SNAIL both induces EMT and downregulates junctional proteins such as occludin and claudins. SNAIL directly represses the transcription of claudin-3, -4, and –7 as well as occludin [116, 117]. Claudin and occludin genes contain E-box motifs in their promoter to which SNAIL can directly bind and repress transcription. SNAIL can also downregulate claudin-1 without affecting transcription, suggesting that it might also regulate claudin translation [118, 119].

V.Implication of Claudins in Disease

Defects in claudins have been implicated in several diseases, many which have been mimicked by mouse knockout models. Claudin-1 is expressed in the skin where it is an essential epidermal water barrier [120] and in cholangiocytes of the bile duct. Claudin-1 null mice have skin permeability defects [120] and in humans, mutations in claudin-1 cause neonatal icthyosis and sclerosing cholangitis [121]. Claudin-14 null mice are deaf [68] and, in humans, claudin-14 mutations also cause nonsyndromic deafness [122, 123]. In vitro, claudin-14 mutations render the protein unable to form junctions [123]. Claudin-14 is thought to play a role in the cation-restrictive barrier that maintains the normal endolymph ionic concentration that bathes the outer hair cells of the cochlea in the ear [68]. Mutations in claudin-16 lead to recessive renal hypomagnesaemia [124, 125]. Mutations in claudin-19 show a similar deficiency in regulating magnesium reabsorption [126]. Although it was initially hypothesized that claudin-16 directly regulated paracellular magnesium permeability, in fact, claudin-16 interacts with claudin-19 to increase paracellular selectivity to sodium [69, 127, 128]. This leads to a model

where a gradient of claudin-16/claudin-19 expression in the thick ascending limb of the kidney establishes a NaCl concentration gradient which creates a lumen positive transepithelial diffusion potential that drives paracellular magnesium reabsorption, as opposed to a direct change in magnesium permeability. Claudin disruptions also play an important role in diseases that are exacerbated by decreased barrier function such as inflammatory bowel syndrome [95, 129-132]. Hepatitis C entry into cells is also facilitated by claudins. CD81 is a tetraspanin that uses claudins as a co-receptor for Hepatitis C virus binding and entry into cells [133, 134]. This suggests that CD81 may have a role in tight junctions and claudin assembly but this is not yet known.

Claudins role in cancer suggests functions beyond the regulation of permeability. Changes in claudin expression are often associated with epithelial cancers and may potentially play a role in their pathogenesis. Tight junction structure and function are often found altered in human carcinomas. Some studies have found that claudins are downregulated in some cancer types [135-139]. The decreased expression of claudins in tumorigenesis is in agreement with the hypothesis that tumorigenesis is accompanied by tight junction disruption and loss of cell-cell adhesion. This process could then play a role in the loss of differentiation, uncontrolled proliferation, loss of cohesion and invasiveness [140]. However, other studies have shown that certain claudins are upregulated in cancer [140-150]. In these studies claudin overexpression seems to be an early event in carcinogenesis, and upregulated claudin expression can often be found in precursor lesions [140, 151, 152]. Although not absolute, claudin-7 seems down regulated in metastatic cells and claudin-4 tends to be upregulated in metastatic cells [135-139, 141, 142, 144, 147, 148, 151-153] . Whether changes in

claudin expression are a cause or a consequence of carcinogenesis is not clear. The observed discrepancy in claudin expression in tumorigenesis could be related to tissue-specific differences in claudin function or tissue microenvironmental features [140]. The diversity of claudins' roles in cancer suggests that they may play a role beyond that of the regulation of paracellular permeability.

Clostridium toxin has been shown to be an anti-tumor agent because it specifically interacts with the extracellular loop of claudin-3 and claudin-4, is internalized and kills cells and recognizes claudins upregulated in tumors [146, 149, 154]. The structure of the loop binding domain of clostridium toxin is now known, which suggests the possibility of designing other claudin specific interacting proteins [155].

Claudins are affected by growth factors and cytokines. Growth factors and cytokines give different responses depending on the combination of stimulus and claudin isoforms that are analyzed. Epidermal growth factor has been shown to alter tight junction barrier function and increase the expression of claudin-3 and –4 but decrease the expression of claudin-2. These changes are mediated by MAPK activation and the MAPK signaling pathway negatively controls claudin-2 expression [156].

Inflammation is associated with transcriptional and post-transcriptional regulation of the tight junction barrier through cytokines [157]. Inflammatory bowel syndrome causes a strong increase in the expression of claudin-2 while normal colons have low levels of claudin-2 [131, 158]. Other inflammatory cytokines such as tumor-necrosis factor (TNF)-α, interferon-γ and interleukin (IL)-13 downregulate claudins and induce a marked increase in paracellular permeability in epithelial cells in culture [131, 159].

It has been assumed that the reason there are more than 24 claudin isoforms is that each one has distinct permeability properties but this has not turned out to be the case. This raises the possibility that claudins may have other roles. Increasing evidence suggests that many claudins are not only at the tight junction. This localization and the increasing ways of claudin regulation raise the question of what are the other roles for claudins. Claudins may be involved in cell-matrix interaction, scaffolding functions, cell-cell adhesion, permeability and crosstalk with signaling.

In these previous introductory chapters I have given the background information that supports the model outlined in this thesis which hypothesizes that injury to the lung stimulates a change in the extracellular matrix composition of the lungs. In turn localization and expression of tight junction proteins found in the lungs are significantly altered. These changes in the tight junction are sufficient to alter the barrier function of the lungs, leading to flooding of the alveoli and impaired pulmonary function. The methods used in order to test this hypothesis are described in the next chapter. In Chapter Five, isolated alveolar cells plated on different extracellular matrices will be analyzed using immunofluorescence, imunoblotting and transepithelial resistance to determine if the extracellular matrix composition affects tight junction protein localization, tight junction protein expression and the cell monolayer's barrier function. Chapter Six uses transfection of alveolar cells to overexpress a specific claudin and then tests the barrier function of these cells using transepithelial resistance and solute flux. This thesis ends with a discussion of the work presented here and how it relates to current research and disease models as well as future directions in the field.

Chapter 4. Materials and Methods

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I. Coating Transwell Permeable Supports with Matrix Components

Laminin from Engelbreth-Halm-Swarm murine sarcoma basement membrane, rat tail type I collagen, and matrix fibronectin were dissolved in coating buffer (0.1 M $NaHCO_3$ [pH 8.5]) at a concentration of 20ug/ml [160]. Transwells received 1 mL per well and were incubated at 4^{o}C overnight. The wells were then washed twice with PBS (120mM NaCl, 2.7mM KCl, 10mM sodium phosphate [pH7.4]) and further incubated at 37^{o}C for 2 hours in PBS containing 10 mg/mL BSA. Matrix-coated permeable supports were washed twice with PBS at room temperature before use. All experiments were performed on coated permeable supports except for cell spreading experiments, which were done using glass coverslips in 6-well tissue culture dishes that had been treated using 2mL of matrix coating buffer, and then washed as described above.

II. Isolation and Culture of Rat Type II Alveolar Epithelial Cells

Animal protocols were reviewed and authorized by the Institutional Animal Care and Use Committee of Emory University. Sprague-Dawley rat type II alveolar epithelial cells were isolated from lungs lavaged and perfused with elastase using the method of Dobbs and colleagues [161], with modifications [162]. For routine preparations, cells were biopanned with IgG-coated culture dishes to remove alveolar macrophages and other Fc receptor-expressing cells. Standard preparations routinely contained greater than 90-95% type II alveolar epithelial cells. Freshly isolated cells were cultured in Earle's MEM containing 10% FBS, 25ug/ml gentamicin, and 0.25ug/mL amphotericin B

in coated Transwells at 5 x 10^5 cells/ml under conditions that promote differentiation toward a type I-like phenotype [163, 164].

For some experiments, cells isolated from elastase-digested lungs were purified by fluorescence activated cell sorting. Macrophages were depleted using rat IgG/goat anti-rat magnetic beads and elution through an LC column. Eluted cells were centrifuged and resuspended in flow sort buffer (2%FBS in PBS) at 1 x 10^6 cells/mL. Before sorting the cells were labeled for 5 minutes at 37^{o}C using a 1:1,000-fold dilution of Lysotracker red (Molecular Probes), a vital dye that accumulated into type II cell lamellar bodies. Lysotracker red-positive cells were isolated using a FACSVantage flow cytometer based on their high side scatter profile and far forward scatter profile using the 633-nm argon laser excitation. Using this approach greater than 95% of the sorted cells expressed surfactant protein, based on immunofluorescence microscopy.

III.Immunofluorescence Staining

Immunofluorescence staining was performed as previously described [96, 165]. Unless otherwise stated, antibodies were from Invitrogen. Cy-3 conjugated anti-vimentin and FITC-conjugated anti-pan cytokeratin were from Sigma Chemicals, Inc. After two or five days in culture, the cells were washed with PBS three times, fixed in MeOH/acetone 1:1 for two minutes at room temperature, washed three times with PBS, once with PBS plus 0.5% TX-100, then once with PBS plus 0.5% Triton X-100 plus 2% normal goat serum. Cells were incubated with primary anti-rabbit antibodies in PBS/GS for one hour, washed, incubated with Cy2-conjugated goat anti-rabbit IgG in PBS/GS, washed, and then mounted in MOWIOL under a glass coverslip. Cells were imaged by phase contrast

and fluorescence microscopy using an Olympus IX70 with a U-MWIBA filter pack (BP40-490, DM505, BA515-550) or U-MNG filter pack (BP530-550, DM570, BA590-800+). Images were acquired using ImagePro software. Minimum and maximum intensity were adjusted for images in parallel so that the intensity scale remained linear to maximize dynamic range.

IV.Immunoblot

After two or five days in culture, cells on permeable supports were harvested and lysed in triple-detergent lysis buffer (50mM Tris-HCL [pH 8.0], 150mM NaCl, 0.1% SDS, 1% noniodet P-40, 0.5% sodium deoxycholate, protease inhibitor cocktail tablets). Protein concentration was determined for each sample using the Bio-Rad protein assay kit. Total protein (50ug) per sample was diluted into SDS-PAGE sample buffer and resolved on a 10% gel. Proteins were transferred to Immobilon membranes using a Bio-Rad semidry transfer apparatus and the membrane blocked with Blotto (nonfat dry milk in TBS-T [25mM Tris, 140mM NaCl, 3mM KCl, 0.05% Tween-20 (pH7.4)]). Blots were incubated overnight in Blotto containing rabbit anti-claudin or anti-occludin antibodies. Blots were washed, then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, washed again and detected using the ECL reagent. Relative protein expression was normalized to parallel blots probed for actin as a measure of total protein and to the amount of protein expressed by Day two cells on laminin (defined as 1.0 for each protein examined).

V.Barrier Function Measurements

Fluorescent Tracers

Fluorescent dyes have been used to assess paracellular permeability to tracers ranging in size from small molecules (e.g. carboxyfluorescein, 370 D) to macromolecules (e.g. Texas Reddextran, 10 kD) as previously described (136). In brief, medium containing fluorescent tracers is added to the top chamber of cells plated in Transwells and the kinetics of dye transfer across the barrier to the lower portion of the chamber is measured using a PE Biosystems multiwell microplate fluorimeter. Comparison of the rate of paracellular transfer for different sized molecules enables the size threshold for paracellular permeability to be determined. This method also offers the ability to measure macromolecule permeability, which reflects paracellular protein permeability. In general, diffusion across the monolayer will be determined using relatively short time periods (e.g. 5-15 min) where the transcytotic component would be expected to be minimal compared to paracellular flux of tracers between the cells.

Basic TER: TER is measured using an EVOM volt-ohm meter and EndOhm resistance measurement chamber (World Precision Instruments, Inc.) as previously described (31, 136). Day 6 primary rat alveolar epithelial cells plated in Transwells form cell monolayers with a TER in the range of 500-1,000 ohm x cm2, depending on culture conditions. This method offers several advantages for assessing alveolar epithelial barrier function since it is rapid, simple and cells can be repeatedly assessed for barrier function over the course of days without sacrificing their viability. However, this method is

limited in the ability to discern the paracellular and transcellular components of TER, which requires Ussing chamber measurements.

VI. Using adenovirus tagged-claudins to overexpress specific claudins in alveolar epithelial cells

Adenovectors were used to overexpress claudins in alveolar epithelial cells. Adenovirus-based vectors are a useful tool for molecular manipulation of primary alveolar epithelial cells. Adenovectors also have the potential to be applied to studies that they may be pursued beyond the scope of this thesis. Both the pAdEasy and Adeno-X-systems were used to produce adenovirus-based expression vectors. This technique takes advantage of bacterial recombination or direct ligation to insert the gene of interest into the attenuated adenovirus, thus eliminating a major pitfall in the production of adenovector by reducing the need to screen plaques obtained by mammalian cell culture systems. In brief, the cDNA construct of interest was inserted into the multicloning site of a shuttle vector, which is then digested with CeuI and SceI, two rare cutting enzymes, which release the cDNA expression cassette. This cassette is ligated into an adenovector backbone predigested with the same enzymes and transfected into HEK 293 cells to enable virus packaging and propagation. For adenovector transductions, cells are incubated with 106pfu per 35mm dish or $5x10^5$pfu per 3.8cm^2 transwell. In each experiment, cells transduced with Adeno-GFP or empty adenovector will be used as a control to rule out effects of the adenovector on alveolar epithelial cell activity.

Adenovirus- YFP-claudin constructs can be used for the transfection of alveolar epithelial cells. The YFP tag is located on the N-terminus so that it will not interfere with

claudin binding to other tight junction proteins. Overexpression of claudin in the alveolar epithelial cells can be determined through protein expression analysis and fluorescent microscopy to see where the transfected claudin-YFP is located. Note in particular that alveolar epithelial cells transduced with Ad-EGFP show little effect on barrier function; thus, any effects on TER would likely be due to the transduced gene of interest, rather than the adenovector.

Chapter 5. Extracellular Matrix Influences Alveolar Epithelial Claudin Expression and Barrier Function

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Chapter 5. Extracellular Matrix Influences Alveolar Epithelial Claudin Expression and Barrier Function

I. Introduction

The alveolar epithelial barrier is critical to maintain optimal lung function. Failure of this barrier after injury contributes to flooding of the alveolar airspaces with protein-rich fluid, which significantly contributes to the severity of acute lung injury [6]. The need for an intact alveolar epithelial barrier is a critical requirement for resolution of acute lung injury. Patient outcomes positively correlate with the ability to maintain a tight alveolar barrier [8, 166]. Despite its importance, the mechanisms regulating alveolar barrier function remain uncharacterized. In particular, little is known about the effect of cell microenvironment on regulating alveolar epithelial permeability.

Permeability between alveolar epithelial cells is controlled by tight junctions, which are located at cell-cell contacts and consist of several different classes of transmembrane and peripheral proteins [43]. Of these proteins tight junction permeability is most directly regulated by claudins [167, 168]. Tight junction permeability is also modulated by other proteins including transmembrane proteins such as occludin, and scaffold proteins including zonula occludens (ZO)-1 and -2 [60, 169]. Depending on the profile of claudin expression, different epithelia will have different paracellular permeability characteristics [170].

Several studies have demonstrated that lung epithelia express multiple claudins, and that type I and type II alveolar epithelial cells express different claudins [77, 96, 171, 172]. The transition of primary alveolar epithelial cells from a type II cell phenotype to a

type I cell phenotype is sensitive to their microenvironment [163, 173-177]. However, even in vivo, the distinction between type II and type I cells is not always clear, and transitional cells can have characteristics of both cell types [178, 179]. The microenvironment can also promote alveolar cells to undergo an epithelial-to-mesenchymal transition which contributes to pulmonary fibrosis [180, 181]. Consistent with this observed plasticity, alveolar epithelial cells cultured under different conditions show differences in expression and function of gap junction proteins [162, 182, 183]. However, it remains to be determined how the cell microenvironment influences tight junction protein expression.

The extracellular matrix is a critical component of the cellular microenvironment. In the unstressed lung, alveolar cells rest on basement membranes composed of several matrix components including laminin, type IV collagen, and nidogen [184, 185]. In response to acute lung injury, lung extracellular matrix composition is dramatically altered [186]. Specifically, the destruction of alveolar basement membranes induced during injury is followed by increased expression and deposition of fibronectin and type I collagen by alveolar epithelial cells and fibroblasts. Through integrin-mediated signaling, this fibronectin-enriched "provisional" matrix is thought to promote the re-epithelialization of denuded alveolar walls, stimulate vascularization, and provide a scaffold for the accumulation and organization of recruited inflammatory cells [187-189].

Previous in vitro studies have demonstrated that fibronectin promotes wound healing [190] and stimulates the ability of alveolar epithelial cells to form high resistance tight junction barriers after four days in culture [191]; however, the effect of extracellular matrix on the molecular composition and ion permeability of tight junctions has not been

determined. Primary alveolar epithelial type II cells were cultured on defined substrata composed of laminin, type I collagen or fibronectin to determine how extracellular matrix composition affects claudin expression and consequently alters alveolar epithelial barrier function.

We hypothesized that cells grown on a provisional fibronectin injury matrix would have altered tight junction protein localization and expression as well as changes in barrier function as compared to cells grown on a laminin matrix that mimics a healthy basement membrane. We found that cells cultured on fibronectin for two days formed high resistance monolayers, which were associated with claudin localization to the plasma membrane. By contrast, cells on either laminin or type I collagen did not exhibit significant barrier function. However, after five days in culture, cells on all three matrices formed high resistance monolayers. Day five cells on laminin had the highest barrier function, whereas cells on type I collagen and fibronectin were equivalent, with respect to transepithelial resistance (TER) and claudin expression. Together, these observations suggest that fibronectin-rich matrices produced during the early stages of lung injury might promote the fast generation of epithelial cell barriers. However, by Day five, these barriers were suboptimal as compared with barriers produced by cells cultured for five days on laminin. This suggests that the rapid formation of a high resistance monolayer by cells after two days on fibronectin was at the expense of producing a more optimal alveolar epithelial barrier.

III. Results

Effects of Extracellular Matrix on Tight Junction Protein Expression

To test the effect of extracellular matrix on tight junction protein expression, we cultured freshly isolated type II alveolar epithelial cells on permeable supports coated with laminin, type I collagen, or fibronectin. Immunofluorescence microscopy revealed that cells cultured for two days on either laminin or type I collagen showed heterogeneous localization of claudin-18, where some cell-cell interfaces contained claudin-18 and several did not (Figure 8A). In contrast, claudin-18 in cells cultured for two days on fibronectin was more uniformly localized to cell-cell interfaces. Claudin-3 showed a similar pattern of continuous localization for cells on fibronectin, as opposed to cells on laminin or type I collagen, where several cell-cell interfaces lacked detectable

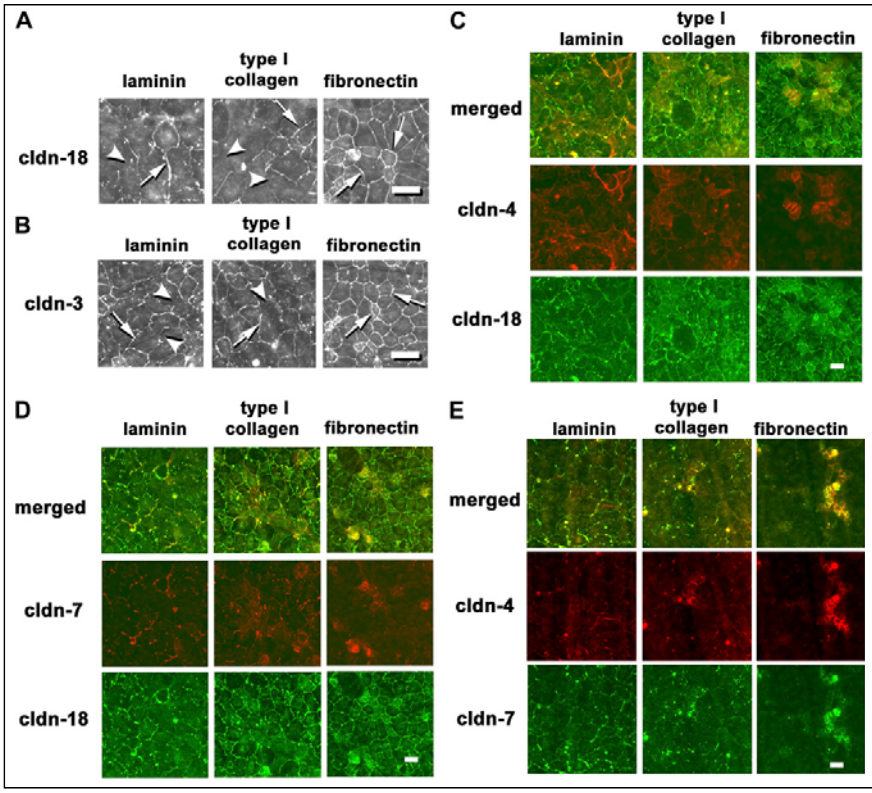

Figure 8. Localization of claudins by cells cultured for 2 days on different extracellular matrices. Freshly isolated type II cells were cultured for 2 days on permeable supports coated with laminin, type I collagen, or fibronectin, and then fiex, permeabilized, and immunostained for claudin-18 (A), claudin-3 (B), claudin -18 and -4 (C), claudin-18 and -7 (D), or claudin-4 and -7 (E). Cells on laminin and type I collagen showed areas of cell-cell contact that lacked claudin-18 (arrowheads), as opposed to cells on fibronectin, which had a more uniform distribution of claudin -18 (arrows). Claudin-3 had a similar distribution. Claudin-4 and -7 colocalized on a subset of cells cultured on fibronectin (E). By contrast, expression of claudin -4 and -7 were more evenly distributed throughout cells cultured on either laminin or collagen. Scale bars, 30um (A and B) and 50um (C-E).

claudin-3 (Figure 8B).

Two other claudins, claudin -4 and -7, showed a different pattern of localization. Cells were cultured for two days on laminin or type I collagen, and showed significant colocalization of claudin-4 and -7 with -18 (Figure 8C and 8D). By contrast, expression of claudin -4 and -7 were detected in a subset of cells cultured for two days on fibronectin. Furthermore, claudin- 4 and -7 colocalized to the same subpopulation of day two cells on fibronectin.

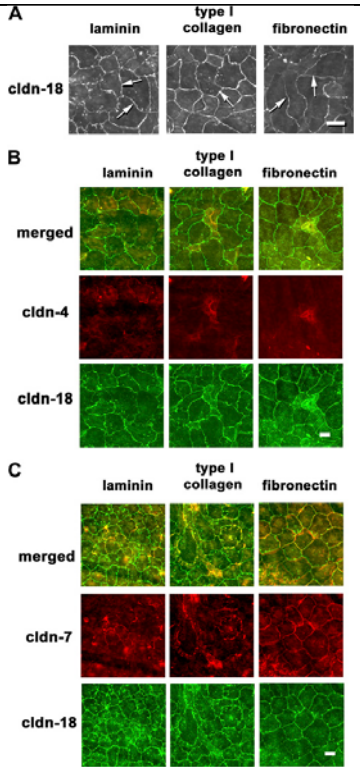

Figure 9. Localization of claudins by cells cultured for 5 days on different extracellular matrices. Freshly isolated type II cells were culltured for 5 days on permeable supports coated with lamini, type I collagen or fibronectin, and then fixed, permeabilized and immunostained for claudin -18 (A), claudin-18 and -4 (B), or claudin -18 and -7 (C). Claudin -18 was continuous and localized to nearly all cell-cell interfaces (arrows), regardless of extracellular matrix. Claudin-4 localization was more limited. By contras, claudin-7 was more uniformly localized throuout the monolayer for all three matrices tested. Scale bar, 30um (A) and 50um (B and C).

We then determined whether these differences persisted during time in culture. After five days in culture, claudin-18 localization was more uniform in cells, regardless of extracellular matrix composition (Figure 9A). Moreover, claudin-4 expression was more limited as compared with Day 3 cells (Figure 9B); however, claudin-7 was more broadly expressed (Figure 9C). In particular, cells cultured for 5 days on fibronectin showed prominent localization of claudin-7 to cell-cell interfaces.

To determine whether matrix-dependent differences in claudin localization were related to differences in total protein, we examined tight junction proteins by immunoblot (Figure 10). In comparing Day 2 cells on different matrices the

protein content of claudin-3, -4, -5, -7,-18, ZO-1, ZO-2 and occludin was equivalent for cells on either laminin or type I collagen. However, cells cultured for two days on fibronectin had significantly lower claudin -3, -4, -5, ZO-1, and ZO-2 than Day 2 cells on laminin. Of note, total claudin-18 protein was comparable, regardless of the extracellular matrix composition, despite the differences in localization observed by immunofluorescence microscopy. In contrast after five days in culture, cells on type I collagen and fibronectin had comparable tight junction protein content, and cells cultured for five days on laminin showed differences in tight junction protein expression. In particular, Day 5 cells on laminin had significantly less claudin-5 and -7, and more ZO-1, as compared with cells cultured for 5 days on either type I collagen or fibronectin. As was the case for Day 2 cells, claudin-18 was comparable for Day 5 cells, regardless of extracellular matrix.

In comparing cells on the same matrix, the main changes in tight junction protein expression that occurred from Day 2 to Day 5 were a decrease in claudin-3 and an increase in ZO-1 (Figure 10). Claudin-4 expression also decreased from Day 2 to Day 5 for cells on laminin or type I collagen to a level comparable to cells cultured for five days on fibronectin. Interestingly, claudin-5 showed the most differences with time in culture in response to matrix composition, as it decreased from Day 2 to Day 5 for cells on laminin, remained unchanged for cells on type I collagen, and increased for cells on fibronectin.

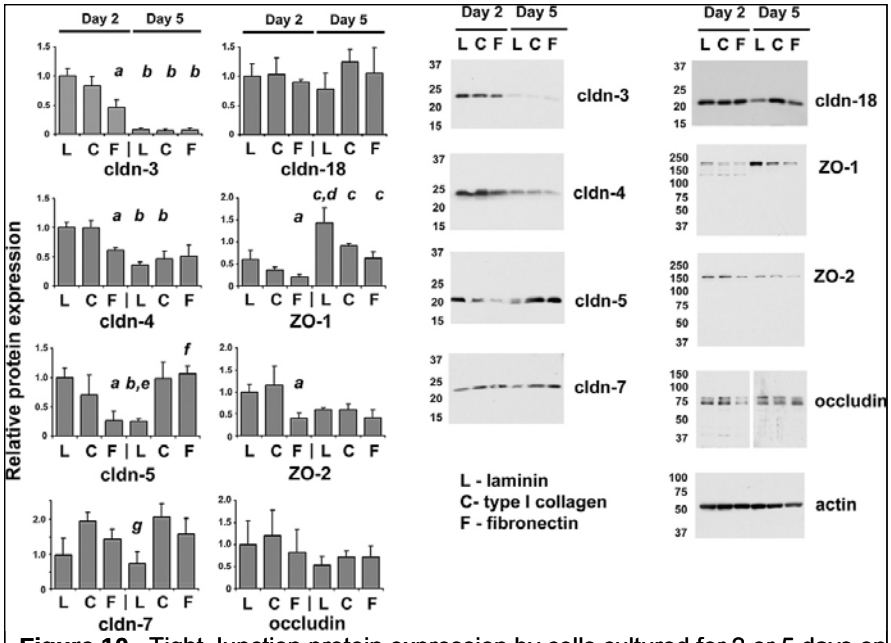

Figure 10. Tight Junction protein expression by cells cultured for 2 or 5 days on different extracellular matrices. Freshly isolated type II cells were cultured on permeable supports coated with laminin (L), type I collagen (C) or fibronectin (F) for 2 or 5 days, and then harvested, resolved by SDS-PAGE, and levels of claudin-3, -4, -5, -7, -18, zonula occludens (ZO)-1, ZO-2, or occludin were determined by immunoblot. A parallel blot was labeled for actin to normalize to total sample protein content. Relative values for each were normalized to cells cultured for 2 days on laminin. Bar graphs reflect the average (+/- SEM) for triplicate independent measurements. [a] Significantly lower than Day 2 cells on laminin ($P<0.04$); [b] significantly lower than Day 2 samples on the same matrix ($P<0.04$); [c] Significantly higher than Day 2 cells on the same matrix ($P<0.04$); [d] significantly higher than Day 5 cells on either collagen or fibronectin ($P<0.03$); [e] Significantly lower than Day 5 cells on either collagen or fibronectin ($P<0.05$); [f] Significantly higher than Day 2 cells on fibronectin ($P<0.05$); [g] Significantly lower than Day 5 cells on collagen ($P<0.05$).

Considering the complex differences in claudin expression exhibited by alveolar epithelial cells as a function of time in culture and extracellular matrix composition, we further characterized the cell cultures. Cells cultured for either two or five days on matrix-coated permeable supports were double labeled with cy3-anti-vimentin (a fibroblast marker) and FITC-anti-pan cytokeratin (an epithelial cell marker) (Figure 11). By this measure, the cells were predominantly epithelial. Some fibroblasts were also present, although they accounted for less than 10% of the cells present in the culture, and they did not significantly proliferate. Note also that the number of cells on fibronectin

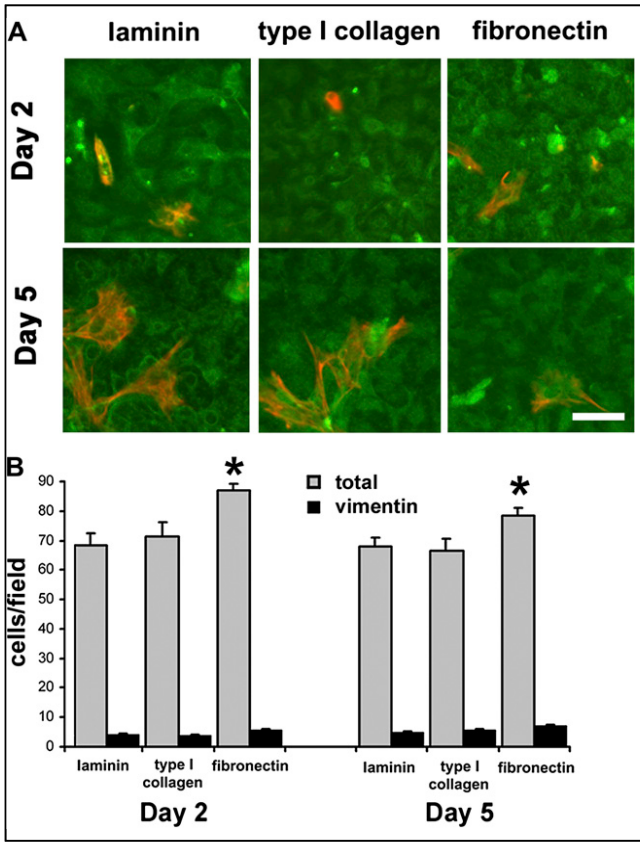

Figure 11. Fibronectin promotes alveolar epithelial cell attachment. (A) freshly isolated type II cells were plated on permeable supports coated with laminin, type I collagen or fibronectin and then cultured for 2 (top row) or 5 (bottom row) days, fixed and then immunolabeled for cytokeratin (green) and vimentin (red) as markers for epithelial cells and fibroblasts, respectively. Note that there were relatively few fibroblasts (red cells) present in the preparations, regardless of matrix composition. (B) This was confirmed by quantitation, which showed that the vast majority of cells in the monolayers were epithelial. Note that there was a significantly higher number of cells attached to the fibronectin-coated wells as compared with the two other matrices (*P < 0.01, Day 2, <0.03, Day 5). Scale Bar, 30um.

was significantly higher than the number of cells on either laminin or type I collagen. Greater cell number on fibronectin is consistent with their ability to spread on the matrix, suggesting a greater affinity for fibronectin as compared with laminin or type I collagen.

Changes in cell phenotype also were assessed using SP-C and connexin32 as type II cell markers and receptor advanced glycosylation end productions and caveolin-1 as type I cell markers. Under the culture conditions tested, neither SP-C nor connexin32 was detectable by immunoblot, although it was present in control purified type II cells. By contrast, receptors for advanced glycosylation end products and caveolin-1 were comparable for cells cultured on all three different matrices. Thus, the differences observed for tight junction protein levels were not tightly linked to other markers for alveolar epithelial cell phenotype.

Effect of Extracellular Matrix on Barrier Function

To determine if the observed changes in tight junction protein content and localization were associated with alteration in alveolar epithelial barrier function, barrier function of alveolar epithelial cells on permeable supports coated with laminin, type I collagen, or fibronectin was measured. Cells cultured for two days on fibronectin formed a monolayer with a TER of 370 (+/- 70) Ohm-cm^2 (n=7); however, cells cultured for two days on either laminin or collagen had TER values of 21 (+/- 9) and 87 (+/- 13) Ohm-cm^2, respectively. Thus, cells cultured on fibronectin were stimulated to form a high resistance barrier as compared with cells on laminin or type I collagen. However, by Day 5, cells cultured on all three different matrices had formed high resistance monolayers with TER values of at least 500 Ohm-cm^2, suggesting that the fibronectin-dependent elevation in TER was a transient phenomenon. In fact, cells cultured on laminin, representative of a healthy basement membrane matrix, had TER on Day 5 of 890 (+/- 31) Ohm-cm^2 (n=4), significantly higher than cells on either type I collagen (530 +/- 63 Ohm-cm^2) or fibronectin (520 +/- 95 Ohm-cm^2) (Figure 12).

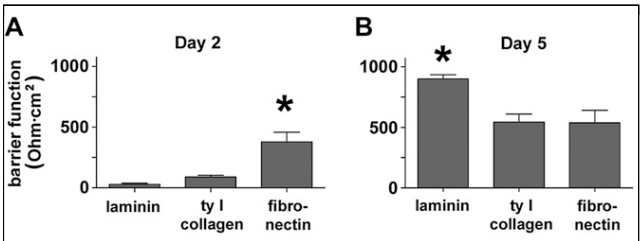

Figure 12. Effect of extracellular matrix on barrier formation by alveolar epithelial cells in vitro. (A and B) Freshly isolated type II cells were plated on laminin, type I collagen, or fibronectin, cultured for 2 (A) or 5 (B) days and then transepithelial resistance (TER) was measured. Shown are means (+/- SEM) for n=6 independent experiments consisting of at least six Transwells. (A) on Day 2, only cells cultured on fibronectin showed a measurable increase in TER (*P<0.05 versus laminin or collagen). (B) By Day 5, cells on all three matrices showed high TER values, suggesting the formation of tight junctions. Cells on laminin showed significantly higher TER than cells on collagen or fibronectin (*P<0.05).

Chapter 6: Targeting Claudin-5 to Improve Alveolar Epithelial Barrier Function

Chapter 6. Targeting Claudin-5 to Improve Alveolar Epithelial Barrier Function

I. Introduction

Acute respiratory distress syndrome (ARDS) leads to flooding of the alveolar space

Acute respiratory distress syndrome (ARDS) is a severe form of lung injury that occurs in response to insults to the lungs [5, 6, 192, 193]. A prominent feature of ARDS is severe damage to the alveolar epithelium [6, 193]. The alveolar epithelium creates a barrier that is necessary for efficient exchange of oxygen and carbon dioxide between the airways and the vasculature [194]. With damage to the alveolar epithelium the alveolar space floods, leading to respiratory failure and the need for mechanical ventilation [6, 193]. The mortality from ARDS is in the range of 40-50% [194] and it is estimated that as many as 150,000 new cases occur each year [194]. I presented data in the previous chapter that cells grown on a fibronectin injury matrix show significant changes in tight junction protein expression and correlate to changes in barrier function.

Tight junctions control paracellular permeability

Lung fluid balance is regulated by the actions of the pulmonary epithelial and endothelial barriers. From the epithelial side there are two major factors that affect the ability of the alveolus to clear fluid from airspaces. One factor is the active clearance of water and ions by specific transporters expressed by alveolar epithelial cells [201]. The second factor is the extent of permeability between alveolar epithelial cells, or whether the alveolus is leaky or not. This paracellular permeability between alveolar epithelial cells is controlled by tight junctions [202]. Intercellular tight junctions carefully regulate the paracellular movement of fluid, proteins and solutes across the alveolar epithelium.

Tight junctions are the sites of cell-cell contact and form an intercellular belt that consists of continuous anastomsing intramembranous strands that form a band between adjacent alveolar epithelial cell membranes [77, 96, 171, 203, 204]. Tight junctions are composed by a number of proteins including the integral membrane proteins and cytoplasmic proteins. Two distinct types of integral membrane proteins, occludin and claudins, have been identified as the constituents of tight junction strands [205]. The integral membrane protein components connect to the cytoskeleton through the cytoplasmic proteins which form plaques and act as adaptor or scaffold proteins. Claudins are the most diverse tight junction protein. There are over 20 claudins with distinctive distribution through various tissues, including the lungs [77, 96, 171, 206]. Claudins are tetraspan transmembrane proteins with two extracellular loop domains where the N- and C- termini are oriented towards the cytoplasm. Claudins form the paracellular ion channels and create the permeability barrier that restricts movement of macromolecules between cells. Different claudins produce different paracellular ion channels with diverse permeability characteristics [75, 207-209].The discovery of claudins is relatively recent, thus there is a lack in understanding the mechanisms cells use to regulate claudins and how claudins regulate permeability. Based on the diversity of claudins and that they are the physical basis of the tight junction barrier, significant disruptions in claudin expression in the alcoholic lung could be an explanation for why alcoholics are more susceptible to ARDS.

We hypothesize that changing the expression level of a specific claudin is sufficient to change the barrier function of alveolar epithelia. The aim of this research is to further elucidate roles for claudins in maintaining the pulmonary barrier and targeting specific claudins to manipulate alveolar epithelial barrier function. This information will

give the context for understanding potential affects of claudin mutations and the potential pharmacologic manipulation of barrier function which may be useful for drug delivery strategies.

II. Results

This data demonstrates the manipulation of claudin expression in primary alveolar epithelial cells. Adenovirus tagged-claudin constructs have been used to overexpress claudins. The data indicates that increasing claudin-5 expression by primary alveolar epithelial cells in vitro recapitulated the leak associated with the alcoholic lung. In overexpression studies, type II alveolar epithelial cells isolated from rats were seeded on transwell permeable supports and incubated for five days. On the fifth day the cells were transfected with adenovirus tagged-claudin constructs, control adenovirus GFP constructs or untreated. Two days after transfection, claudin localization, ion permeability and solute permeability were analyzed.

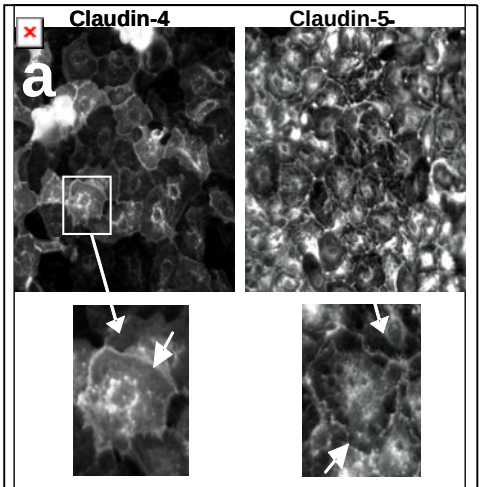

Figure 13. Isolated type II cells plated on transwells at 600,000 cells per well a) transfected with claudin-4-cfp adenovirus b) transfected with claudin-5-yfp adenovirus. Arrows indicate claudin localization at cell periphery.

Localization of transfected claudins in isolated alveolar epithelial cells

Alveolar epithelial cells were transfected with CFP-claudin-4 or YFP-claudin-5 adenovirus constructs. Forty-eight hours after transfection the cells were fixed and permeabilized. By fluorescence microscopy, claudin 4 and 5 were localized primarily at the plasma membranes in the isolated alveolar epithelial cells (Figure 13).

Claudin-5 adenovirus transfection increases ion permeability of alveolar epithelial cells

Alveolar epithelial cells cultured on transwell permeable supports were transfected with adenovirus tagged-claudin constructs or control adenovirus GFP five days after isolation. The ionic permeability of the tight junctions in the alveolar epithelial cells was analyzed through transepithelial electrical resistance (TER) using an EVOM volt-ohmmeter to measure the resistance of the monolayer in Ohms. Measurements were recorded at the time of transfection (t=0), 24 hours after transfection (t=24), and 48 hours after transfection (t=48). These measurements demonstrated that over the 48 hour period following transduction, cell monolayers transfected with claudin-5 had a significantly greater ionic permeability compared to cell monolayers transfected with control adenovirus (Figure 14). This is consistent with the hypothesis that an increase of claudin-5 correlates is sufficient to increase in alveolar epithelial permeability.

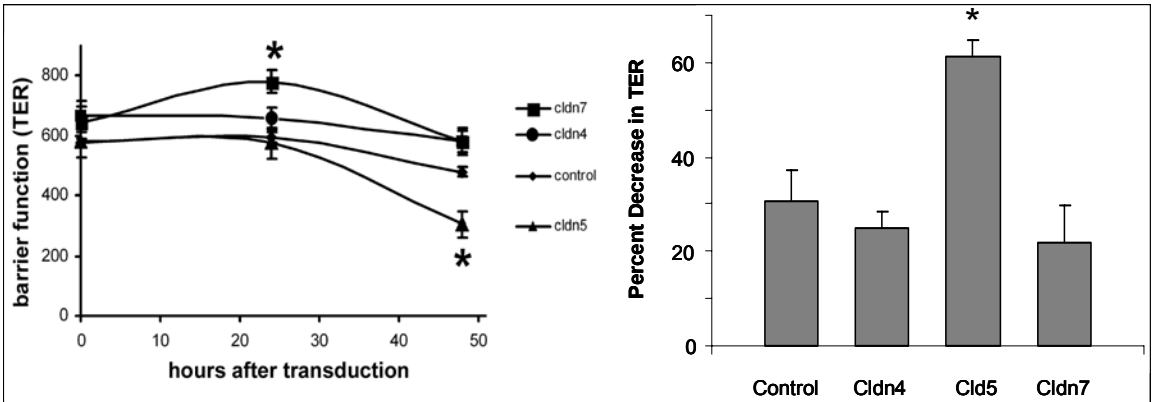

Figure 14. TER. Alveolar epithelial cells cultured on transwell permeable supports were transfected with adenovirus tagged-claudin constructs or control adenovirus GFP five days after isolation. The ionic permeability of the tight junctions in the alveolar epithelial cells was analyzed through transepithelial electrical resistance (TER) using an EVOM volt-ohmmeter to measure the resistance of the monolayer in Ohms. Measurements were recorded at the time of transfection (t=0), 24 hours after transfection (t=24), and 48 hours after transfection (t=48). These measurements demonstrated that over the 48 hour period following transduction, cell monolayers transfected with claudin-5 had a significantly greater ionic permeability compared to cell monolayers transfected with control adenovirus.

Claudin-5 increases paracellular solute flux in isolated alveolar epithelial cell monolayers

Alveolar epithelial cells cultured on transwell permeable supports were transfected with adenovirus tagged-claudin constructs or control adenovirus GFP five days after isolation. In order to determine the molecular permeability of tight junctions in the alveolar epithelial monolayer, 48 hours after transfection PBS was added to the lower well and PBS containing fluorescent tracers (Calcein (0.6 kD) or Texas Red-dextran (10 kD) was added to the top well of each insert. In 30 minute increments over a 2 hour period, the appearance of fluorescent tracer in the lower well was measured using a microplate fluorimeter. These measurements demonstrated that the cell monolayer transfected with claudin-5 had a significantly greater permeability to both Calcein and Texas Red-dextran when compared to cell monolayers transfected with control adenovirus (Figure 15). Again this is consistent with the hypothesis that an increase in claudin-5 is sufficient to induce alveolar epithelial barrier dysfunction.

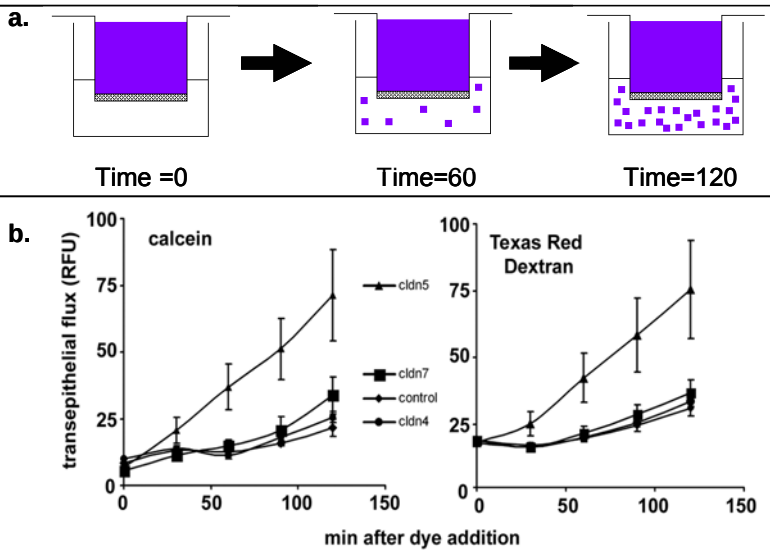

Figure 15. Molecular Permeability. A) Fluorescent dyes can be used to assess paracellular permeability to tracers ranging in size from small molecules to macromolecules. Medium containing fluorescent tracers is added to the top chamber of a transwell and the kinetics of dye transfer across the barrier to the lower portion of the chamber is measured using a multiwell microplate fluoriometer. B) Alveolar epithelial cells cultured on transwell permeable supports were transfected with adenovirus tagged-claudin constructs or control adenovirus GFP five days after isolation. In order to determine the molecular permeability of tight junctions in the alveolar epithelial monolayer, 48 hours after transfection PBS was added to the lower well and PBS containing fluorescent tracers (Calcein (0.6 kD) or Texas Red-dextran (10 kD)) was added to the top well of each insert. In 30 minute increments over a 2 hour period, the appearance of fluorescent tracer in the lower well was measured using a microplate fluorometer. These measurements demonstrated that the cell monolayer transfected with claudin-5 had a significantly greater permeability to both Calcein and Texas Red-dextran when compared to cell monolayers transfected with control adenovirus

Chapter 7. Discussion

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I proposed a model that hypothesizes that injury to the alveolus stimulates a change in the extracellular matrix of the alveolar epithelium. In turn, localization and expression of tight junction proteins found in the alveolar epithelium are significantly altered. These changes in the tight junction are sufficient to alter the barrier function of the alveolus, thus leading to the flooding of the alveolar space.

In this thesis I examined factors that control lung epithelial barrier function. Chapter Five explores the effects of extracellular matrix on tight junction protein expression and function and Chapter Six addresses the manipulation of tight junction protein expression and its affects on barrier function. In looking at the extracellular matrix, we examined cells cultured on fibronectin and type I collagen, matrix components found at low levels in normal lungs, but which increase dramatically after injury. We also used laminin, which mimics a native basement membrane. Primary rat alveolar epithelial type II cells cultured on fibronectin more rapidly developed a high-resistance monolayer as compared with cells on laminin or type I collagen. Previous work by Sugahara and colleagues [191] showed a similar effect that fibronectin had the most rapid barrier function (although the earliest time point they measured was after four days in culture). Here, we found that this effect occurred as early as two days after culture on fibronectin, and correlated with a continuous pattern of claudin-3 and -18 localization to cell-cell contacts, suggesting early formation of tight junctions. The rapid development of a high-resistance barrier was also linked to an enhanced capacity for alveolar epithelia cells to attach to fibronectin as compared with the other two matrices, consistent with a previous study.

Although alveolar epithelial cells on fibronectin quickly formed a tight barrier, their TER only increased an additional 40% by Day 5. Strikingly, the TER of cells on laminin increased nearly 45-fold by Day 5, to a TER that was nearly twice the TER of Day 5 cells on fibronectin. Thus, despite the ability to form a rapid barrier, cells on fibronectin ultimately had suboptimal barrier function as compared with cells on laminin. In contrast to cells on laminin, cells cultured for five days on type I collagen were comparable to Day 5 cells on fibronectin, from the standpoint of both barrier function and tight junction protein expression. This difference in alveolar epithelial cell response is consistent with the concept of laminin as a healthy matrix versus type I collagen and fibronectin as matrix proteins associated with an injured lung.

Cells cultured for 5 days showed increased ZO-1 content as compared with Day 2 cell, regardless of the initial extracellular matrix. In fact, cells on laminin had the highest levels of ZO-1 expression, and formed the most restrictive barriers. This is significant, because ZO-1 is critical for proper insertion of claudins into functional tight junction strands [60]. Also, in an HIV transgenic rat model, we found that impaired alveolar barrier function correlated with decreased ZO-1 expression, consistent with an important role for ZO-1 in alveolar epithelial tight junctions [211]. However, ZO-1 expression is not the sole determinant of epithelial barrier function, as cells cultured for 2 days on fibronectin formed a high-resistance monolayer, yet had lower ZO-1 content than Day 2 cells on laminin or type I collagen.

By Day 5, alveolar epithelial cells on type I collagen had barrier function and tight junction protein expression that were indistinguishable form cells on fibronectin. One possibility is that the cells were either adapting to the culture conditions in general or

were remodeling the extracellular matrix. Consistent with this possibility, alveolar epithelial cells have the capacity to synthesize and degrade several extracellular matrix components, including laminin, collagen and fibronectin [212]. However, in the absence of an ability to remodel the matrix to a more normal composition, it seems likely that the alveolar barrier of cells on fibronectin would remain suboptimal as compared with a barrier formed by cells on a healthy matrix.

If left unopposed, fibronectin-mediated events lead to excessive inflammation and fibroblast proliferation, resulting in permanent disruption and of normal alveolar structure [213]. This is likely to be the case for both acute and chronic forms of lung injury where fibronectin expression is increased [214, 215]. Consistent with this, others have shown that animals deficient in the EDA fibronectin splice variant develop less fibrosis in response to bleomycin-induced lung injury [216].

Following the model hypothesized in this thesis, data is presented here that suggests alveolar epithelial cells cultured on fibronectin had lower claudin-5 content after two days compared with cells cultured on laminin or Type I collagen. This is significant because methandamide has been found to increase paracellular permeability between cultured alveolar epithelial cells, without decreasing cell viability. Methandamide is a nonhydrolyzable analog of the fatty acid amide anandamide, which binds to the cannaboid receptors CB_1, and CB_2 [219]. Type II alveolar epithelial cells express the CB_1 cannaboid receptor, although type I cells do not [220]. Also, CB_1 expression is rapidly lost by alveolar epithelial cells in culture [220]. Methandamide, and the naturally occurring compounds anandamide and oleamide, can also interact with another unknown class of receptors to inhibit gap junctional communication through a signaling cascade

involving $G_{i/o}$ activation [221] or MAP kinases [222]. Whether these signaling pathways are stimulated by methandamide to increase paracellular permeability remains to be determined.

Although methandamide increased paracellular permeability, claudins, occludin, and ZO-1 remained localized to the plasma membrane. However, methandamide-treated cells showed some subtle changes in plasma membrane morphology, consistent with the notion that a gross perturbation of tight junction morphology is not necessary for increased paracellular permeability [223]. In addition, methandamide altered the level of claudin expression, with a fairly dramatic increase in claudin-5 expression. One possible explanation is that expression of claudin-5 and claudin-3 is upregulated by the cells in an attempt to compensate for the methandamide-induced decrease in barrier function. However, we explored a more provocative possibility that claudin-5 might induce a leaky tight junction phenotype.

Consistent with this possibility, studies of retinal pigment epithelial development showed that increased claudin-5 expression is associated with decreased retinal TER in E7 stage chick embryos [224]. The notion of leaky claudins is also suggested by studies where overexpression of claudin-2 by MDCK cells caused a dramatic decrease in TER [225]. This thesis suggests that a comparable effect is induced by claudin-5 expression.

The data presented here suggests that manipulation of claudin-5 expression is sufficient to induce a change in barrier function. We examined both ionic and molecular permeability both of which significantly increased when alveolar epithelial cells were transfected with claudin-5 adenovirus to increase expression of claudin-5. We do not know the mechanism that drives this alteration in permeability.

In studies of the extracellular matrix we found alveolar epithelial cells cultured on fibronectin had lower claudin-5 content after 2 days as compared with cells cultured on laminin or type I collagen. This observation is significant in light of the previous studies showing that increased claudin-5 expression by alveolar epithelial cells induced by methandamide. Consistent with the hypothesis that claudin-5 is sufficient to reduce alveolar epithelial barrier function, cells cultured for 5 days on laminin had higher TER and lower claudin-5 content that cells cultured on either type I collagen or fibronectin. Although several claudins in addition to claudin-5 have been found to reduce rather than promote barrier function in certain cell types [50, 168, 170], the molecular basis for this effect is currently unknown and will be subject to further study.

We have discussed here that changes in extracellular matrix components and tight junction components are associated with decreased barrier function after lung injury, but other factors such as prior chronic alcohol abuse can actually exaggerate this injury. Over the past decade clinical studies have revealed the surprising finding that chronic alcohol abuse is an independent comorbid factor that significantly increases the risk of ARDS by as much as 2-4 fold [195-199]. Based on the two largest studies examining this association, it appears that alcohol abuse contributes to tens of thousands of cases of ARDS [196-199]. A rat model demonstrates that chronic alcohol ingestion causes significant alveolar epithelial dysfunction, including increased protein permeability and a decreased ability to clear a saline challenge in vivo [200]. This is especially relevant because those patients with ARDS who have the greatest inability to remove fluid from the alveolar airspace have a worse prognosis [194]. Further studies in rats show that when alveolar epithelial cells are isolated and cultured from alcohol fed rats they fail to form

tight monolayers and have increased paracellular leak of larger molecules. This holds true even when the cells are cultured up to eight days in the absence of alcohol [200]. The precise molecular mechanisms by which chronic alcohol ingestion increases paracellular permeability in the alveolar epithelium are unknown but are thought to be linked to tight junctions.

In addition to these effects of fibronectin during injury, there is emerging data that subtle remodeling of the alveolar basement membrane can also occur before acute lung injury. For instance, we have demonstrated that chronic alcohol abuse promotes fibronectin expression in lung both in vitro and in vivo, and is associated with increased severity of acute lung injury [215, 217]. In the alcoholic lung, there is little or no histological disruption, despite a significant increase in fibronectin content and deficiency in alveolar barrier function [18, 218]. Thus an impaired alveolar barrier does not necessarily require the alveolus to be significantly damaged, because remodeling of the extracellular matrix and other factors may contribute to an exaggerated injury response [166].

The precise molecular mechanisms by which chronic alcohol ingestion increases paracellular permeability in the alveolar epithelium are unknown but are thought to be linked to tight junctions. A recent study by Fernandez et al. analyzed the effect of chronic alcohol ingestion on the expression claudins within the lung and particularly the alveolar epithelium. They examined gene and protein expression of multiple claudin proteins in the whole lungs and in isolated alveolar epithelial cells in alcohol-fed versus control-fed rats. It was found that alcohol ingestion in rats significantly decreased the protein expression of claudin-1, claudin-3 and claudin-7 and increased the protein

expression of claudin-5 in whole lung samples. In isolated alveolar epithelial cells the trend towards increased claudin-5 expression was also observed [210]. These claudins are critical components of alveolar epithelial tight junctions. This study provided new evidence that alcohol-induced alveolar epithelial barrier dysfunction is associated with changes in claudin protein expression and suggested chronic alcohol ingestion impairs the expression and formation of tight junctions in the alveolar epithelium.

Chronic alcohol abuse not only induces change in the extracellular matrix but is also associated with changes in tight junction expression. In fitting with our model, chronic alcohol abuse induces expression of fibronectin and changes in tight junction protein expression. These alterations lead to a decrease in barrier function that is usually compensated for under healthy conditions. However, the lung is more susceptible to injury such as ARDS and the injury response is exaggerated. It is difficult for the lung that was already injured to recover from a second hit and often leads to an increase in mortality.

Future studies on this project will examine the mechanism that facilitates the extracellular matrix substrata in changing tight junction protein localization and expression, as well as the mechanism that facilitates a claudin's ability to induce changes in barrier function. These mechanism could be linked to a variety of cellular processes such as post translational modification to the organization and oligomerization of claudins. With knowledge of these mechanisms, it is tempting to speculate that manipulation of claudin expression could potentially be used for pharmacologic manipulation of barrier function and possibly be used in treatments of lung injury and even in drug delivery strategies.

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