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Doctor of Philosophy

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ABSTRACT

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The tight junctions (TJ), which are located in the apical region between epithelial and endothelial cells, regulate the paracellular diffusion of ions and small molecules and play an important role in maintaining cell polarity, cell-cell integrity, and permeability. In the lung, epithelial cells are attached by TJ structures. They provide a permeable barrier and cell communication. The loss of barrier integrity, which is maintained by the expression of claudins (Cldn), results in cellular permibilization and leads to paracellular diffusion of solutes and harmful molecules. There are 27 known Cldn homologous members in mice and human. Cldn6 is mostly expressed in embryonic stem cells and associated with the programing of epithelial cells during embryo development and lung morphogenesis. In order to test the hypothesis that Cldn6 expression affects lung morphogenesis, we analyzed the expression pattern of Cldn6 during lung ontogenesis to examine cell-specific expression pattern of Cldn6 during each embryonic period in the mouse lung. Also, we assessed transcriptional regulators and control mechanisms that precisely influence Cldn6 expression in pulmonary cells. We discovered that Cldn6 is an important tight junctional component expressed by pulmonary epithelium during lung organogenesis. We found that normal down-regulation of Cldn6 as development proceeds influences differentiation associated with the transition between the embryonic to the alveolar stage. Conditional gain-of-function and loss-of-function experiments in animal models prove to be the most beneficial tool in deciphering the impact of Cldn in organ formation and maintenance. We generated a conditional transgenic mouse that provides the opportunity to genetically up-regulate Cldn6 in distal lung. Our transgenic mouse showed a delay in lung development and down-regulation of transcriptional factors. Cldn6 is both temporally and spatially controlled in the developing lung and its regulation is maintained by critical transcriptional control networks managed by TTF-1. In lung diseases, altered Cldn expression leads to diseases such as COPD, asthma, and ARDS. The tight junctional proteins are differentially regulated by tobacco smoke exposure and Cldn6 is potentially involved as neighboring epithelial cells respond to tobacco smoke. We exposed adult mice to controlled doses of second hand smoke during four days and A-549 cells to 10% CSE for 6 hours. We discovered that mice lungs respond by down-regulating Cldn6 basal levels and impair barrier function. These results reveal that midgestational up-regulation of Cldn6 and its marked down-regulation as development proceeds illustrate the notion that Cldn6 function is important during early programming stages of lung morphogenesis.

Keywords: claudin 6, mice, transgenic, murine, lung

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CHAPTER 1: Introduction

The lungs are endodermal derived structures that result from complex branching of specialized cells during embryogenesis. Lung development begins with an invagination in the ventral wall of the foregut, which is called the respiratory diverticulum. Embryonic lung development follows four main morphogenetic fetal stages called: embryonic, pseudoglandular, canalicular, and saccular; and in addition there is a pre- and post-natal stage called alveolar. These stages are highly controlled by transcriptional factors and other developmentally essential lung-related proteins (Maeda, Dave et al. 2007).

The embryonic period begins when the lung primordium is generated from the laryngotracheal groove through mesenchymal-epithelial interactions, particularly between the splanchnic mesoderm and epithelial cells (derived from foregut endoderm), which induce cell proliferation, migration and differentiation. During the pseudoglandular stage, through dichotomous branching and morphogenetic processes, the bronchial tree develops. This bronchial tree consists of undifferentiated epithelial cells juxtaposed to the splanchnic mesoderm. In the canalicular stage, the terminal alveolar regions begin to develop the acinus and vascularization begins. The future air-blood tissue barrier begins to form by differentiation of pulmonary epithelium, and in addition to this, surfactants begin to be synthesized. In the saccular stage, which is approximately from embryonic day 17.5 to post-natal day 5, the number of terminal sacs and blood vessels increases considerably. Fibroblastic cells undergo proliferation and differentiation to develop the extracellular matrix, specifically elastin and collagen. Gas exchange regions expand considerably. Finally during the alveolar stage, lung development is characterized by bronchioles and air sacs proliferation, resulting in the development of alveoli.

The secondary alveolar septa are then formed to create mature alveoli (Ten Have-Opbroek, 1981; Warburton et al., 2000).

The formation of the primitive respiratory airways involves the deposit of epithelium for the purpose of protection against invasion and infection by potential pathogens (Breeze and Wheeldon 1977). In the proximal lung, the epithelium is a dense sheet of cells that is composed of four main specialized cell types: ciliated cells, goblet cells, non-ciliated Clara cells, and basal cells (Breeze and Wheeldon 1977, Gaillard, Lallement et al. 1989). Distal airspaces are areas where epithelial cell differentiation leads to surfactant-secreting type II cells and type I cells which are required for efficient gas exchange through diffusion. In mammals, epithelial cells form a sheet of continuous cells united by tight and adherent junctions.

The tight junctions (TJ) are unique membrane domain modifications in the plasma membrane that restrict paracellular transport, mediate cell-cell adhesion, and cell-matrix interactions. Its function is preventing molecular diffusion of lipids and proteins between two different components (Schneeberger and Lynch 1992). TJs are composed of membrane bound proteins such as claudins (Cldn), occludins, junctional adhesion molecules (JAM), and scaffolding proteins like the zonula occludens proteins (ZOs) (Balda and Matter 2000, Chiba, Osanai et al. 2008).

The Cldn family is present exclusively in the TJ and plays an important role in its architecture. This family consists of about 27 homologous members. Cldn expression and distribution during embryonic development and between different tissues is highly controlled and regulated by transcriptional factors and regulatory proteins (Tsukita and Furuse 1998, Morita, Furuse et al. 1999). They have a molecular mass that ranges between 22 to 33 kDa (Morita, Sasaki et al. 1999). Cldn members are divided into two different categories:

housekeeping and tissue specific proteins (Van Itallie and Anderson, 2006). Hydrophobic analysis suggests that Cldn members share common areas in the transmembrane domain, but different C- termini. Each member has four hydrophobic transmembrane domains, two extracellular loops, and two intracellular domains with C- and N-termini. The N-terminis comprises 4 to 10 amino acids and C-terminal has between 21 to 63 amino acids, while the extracellular portion (loops) has between 24 to 53 amino acids (Morita, Furuse et al. 1999).

Cldn proteins are structurally and functionally important in TJ because they interact via cytoplasmic domains with the membrane and scaffolding proteins to maintain and regulate cellular functions (Van Itallie and Anderson 2006). Several proteins interact with Cldn members to form complexes and structures. For example, zona occludens protein (ZO)-1, -2, and -3 interact directly with Cldn to anchor the cell, whereas afadin- 6 (AF-6) interacts indirectly via a signaling cascade (Itoh, Furuse et al. 1999). Cldn proteins have complex interactions between other family members and isoforms. These interactions provide a link to the actin cytoskeleton and form the architectural backbone of the TJ. Another function attributed to Cldn is the recruitment of cytoskeletal and signaling molecules to regulate cell differentiation, proliferation, and other related cell functions (Mitic and Anderson 1998). Among the Cldn family, Claudin 6 (Cldn6) is expressed during epithelial differentiation and epidermal barrier formation (Turksen and Troy 2002).

Cldn6 was primarily identified and isolated in mouse kidney via polymerase chain reaction (PCR) (Morita, Furuse et al. 1999) and subsequently cloned and expressed in transgenic models for the purpose of assessing epidermis biology (Turksen and Troy 2001, Turksen and Troy 2002, Arabzadeh, Troy et al. 2008). Cldn6 encodes a protein of 219 amino acids with a molecular mass of 23.4 kDa (Morita, Furuse et al. 1999). This protein is expressed mainly during

early embryonic development and has been implicated as a critical factor for epidermal differentiation and barrier formation (Turksen and Troy 2001). Anderson et al. (2008) via RT-PCR showed that Cldn6 is highly expressed in the olfactory epithelium and other tissues including lungs (Hong, Hishikawa et al. 2005), but they failed to detect Cldn6 by immunohistochemistry (Morita, Furuse et al. 1999, Turksen and Troy 2004).

Current studies have shown that Cldn6 is mostly expressed in mammary epithelial cells (Quan and Lu 2003) and signals through estrogen receptor alpha (ERα) in MCF-7 cells (Yafang, Qiong et al. 2011). Down-regulation of Cldn6 increases tumor cell formation whereas Cldn6 over-expression reduces breast cancer cell formation (Osanai, Murata et al. 2007). These data indicate that Cldn6 may act as a tumor suppressor in breast cancer (Kramer, White et al. 2000, Resnick, Konkin et al. 2005). Microarray analysis showed that Cldn6 is highly expressed in atypical teratoid and rhomboid tumors (Birks, Kleinschmidt-DeMasters et al. 2010, Antonelli, Hasselblatt et al. 2011). Cldn6 expression regulates blastocyst formation during embryo preimplantation, possibly via TJ enhancement in the trophoblast (Moriwaki, Tsukita et al. 2007).

Although the functional role of Cldn6 in the epidermal permeability barrier is likely significant (Turksen and Troy 2002), recent studies using Cldn6 knockout mice indicate that Cldn6 may also contribute to the function of cellular and molecular principles underlying the control and regulation of branching morphogenesis. The aim of my study is to determine the functional role of Cldn6 regarding permeability, directional cell migration, cell rearrangement, and cell shape changes during lung development. The direct effect of Cldn6 on lung cell differentiation, proliferation, and gene expression has not been clearly demonstrated, nor have the associated key molecular pathway components been identified.

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CHAPTER 2: Research Summary and Workflow

The specific objective of this dissertation was to demonstrate roles for Cldn6 in the maintenance of lung tissue integrity and lung protection mechanisms during disease. We analyzed the following parameters: the expression of Cldn6 in pulmonary cells and genes associated with the formation and functions of tight junction complexes, the transcriptional regulators and co-regulators that control Cldn6, Cldn6 responses to oxidative damage related to enzymatic antioxidant defense mechanisms during disease and recovery, genetic alterations in markers of lung development and cell turnover, and a histological assessment in order to characterize possible impairment of normal lung architecture when Cldn6 expression is increased.

To experimentally address these objectives, this project was divided into three sections. The first describes the expression pattern of Cldn6 during lung morphogenesis; the second involves the analysis of Cldn6 perturbation in cells and adult lungs after smoke exposure; and the final study focuses on the effects of conditional pulmonary Cldn6 overexpression during late-lung embryogenesis.

As outlined in Chapter 3, we morphologically analyzed the expression pattern of Cldn6 during lung morphogenesis. This aim examined cell-specific expression patterns of Cldn6 during each period in the embryonic mouse lung (E11.5 to PN1). We found that normal lung development involved the expression of Cldn6 during the embryonic period and its down-regulation by the late canalicular period (E15.5). Adult lungs conserve basal Cldn6 expression in normal conditions and transcriptional factors such as TTF-1, FoxA2 and Gata-6 regulate the expression of Cldn6 during lung development.

In Chapter 4, we tested the hypothesis that Cldn6 is misregulated in pulmonary epithelial cells and adult mouse lungs after exposure to secondhand cigarette smoke. We discovered that Cldn6 is down-regulation in cells and tissues following exposure. Because cigarette smoke is associated with a general lack of oxygen (hypoxia), we assessed the transcriptional control of Cldn6 in hypoxic environments and considered the functions of Hif-1α, a hypoxia-sensitive regulator, in the mediation of potential responses. Cldn6 transcription was down-regulated by Hif-1α and mutations of Hif-1α response elements in the Cldn6 promoter returned Cldn6 expression to basal levels.

Finally in Chapter 5, we investigated the effects of genetically increased Cldn6 during lung development and maturation. This aim implicated Cldn6 as an important factor that mediate, at least in part, progression through periods of lung morphogenesis. We showed that over-expression of Cldn6 was associated with clear deviations in lung architecture accompanied by altered expression of genes encoding critical lung-specific transcription factors, their important downstream targets critical in lung physiology, and mechanisms of cell proliferation and apoptosis.

CHAPTER 3: Developmental Lung Expression and Transcriptional Regulation of Claudin-6 by TTF-1, Gata-6, and Foxa2

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Abstract

Background: Claudins are transmembrane proteins expressed in tight junctions that prevent paracellular transport of extracellular fluid and a variety of other substances. However, the expression profile of Claudin-6 (Cldn6) in the developing lung has not been characterized.

Methods and Results: Cldn6 expression was determined during important periods of lung organogenesis by microarray analysis, qPCR and immunofluorescence. Expression patterns were confirmed to peak at E12.5 and diminish as lung development progressed. Immunofluorescence revealed that Cldn6 was detected in cells that also express TTF-1 and FoxA2, two critical transcriptional regulators of pulmonary branching morphogenesis. Cldn6 was also observed in cells that express Sox2 and Sox9, factors that influence cell differentiation in the proximal and distal lung, respectively. In order to assess transcriptional control of *Cldn6*, 0.5, 1.0, and 2.0-kb of the proximal murine Cldn6 promoter was ligated into a luciferase reporter and co-transfected with expression vectors for TTF-1 or two of its important transcriptional co-regulators, FoxA2 and Gata-6. In almost every instance, TTF-1, FoxA2, and Gata-6 activated gene transcription in cell lines characteristic of proximal airway epithelium (Beas2B) and distal alveolar epithelium (A-549).

Conclusions: These data revealed for the first time that Cldn6 might be an important tight junctional component expressed by pulmonary epithelium during lung organogenesis. Furthermore, Cldn6-mediated aspects of cell differentiation may describe mechanisms of lung perturbation coincident with impaired cell junctions and abnormal membrane permeability.

Key words lung, claudin-6, transcription, immunofluorescence

Background

Lung development is a complex and coordinated process that requires cellular differentiation and interaction between respiratory epithelial cells and the surrounding mesenchymal environment (Burri 2006). As lung development proceeds, compartmentalization is orchestrated in large part by tight junctions (TJs) between neighboring epithelial cells (Schneeberger and Lynch 2004). Accordingly, developing pulmonary epithelium obtains a polarized conformation and it refines mechanisms that regulate ion and molecular transport between apical and basolateral compartments (Balkovetz 2006). TJs are protein complexes comprising several components including transmembrane proteins such as Claudins (Cldn), Occludins, and junctional adhesion molecules (JAMs) stabilized by various cytoplasmic and cytoskeletal proteins (Harhaj and Antonetti 2004; Aijaz, Balda et al. 2006; Chiba, Osanai et al. 2008).

The Claudin (Cldn) family of proteins plays a critical role in TJs by establishing a junction complex (cellular pore) that controls extracellular ion movement at cell-cell apposition (Will, Fromm et al. 2008; Findley and Koval 2009). The Cldn family is comprised of 27 highly conserved membrane proteins that, similar to other tetraspanins, traverse the cellular membrane four times and contain two EL loops where interdigital interactions with other Cldns occur (Turksen and Troy 2004; Lal-Nag and Morin 2009). While Cldn expression is both temporally and spatially controlled, altered expression of Cldn members may contribute to the modification of intracellular permeability and molecular transport selectivity by specific epithelial cell types (Van Itallie and Anderson 2006; Findley and Koval 2009).

Among Cldn proteins, Claudin-6 (Cldn6) plays a fundamental role in epithelial differentiation and permeability. Embryonic expression of Cldn6 has been identified during

epidermal morphogenesis and is critical for epidermal differentiation and epithelial barrier formation (Turksen and Troy 2001). For instance, specific studies have shown that sufficient expression of Cldn6 correlated with the establishment of the permeability barrier's integrity and function, and overexpression of Cldn6 is associated with defects in epidermal permeability (Arabzadeh, Troy et al. 2006). Complimentary studies employing the overexpression of Cldn6 in transgenic mice resulted in lethal epidermis-related defects including poor temperature stabilization, infection by microorganisms through the skin, and uncontrolled water loss (Turksen and Troy 2002; Turksen and Troy 2004). While there is a general consensus that Cldn6 participates in TJs that maintain homeostasis, abnormalities in its expression or function may also lead to tumorigenesis. For example, Cldn6 expressed by mammary epithelial cells functions as a tumor suppressor (Quan and Lu 2003; Morin 2005; Kominsky 2006) and its downregulation has been implicated in neoplasticity and metastatic disease development (Morin 2005; Wu, Liu et al. 2010).

In the current study, we assessed the precise temporal and spatial distribution of Cldn6 in the embryonic mouse lung. Through immunofluorescent assessment, we discovered precise expression of Cldn6 and co-expression with the critical pulmonary transcription factors thyroid transcription factor 1 (TTF-1), forkhead box A2 (FoxA2), and Gata-6 in Cldn6 expressing cells. Additional experiments that test the hypotheses that these same factors transcriptionally regulate *Cldn6* were also performed. Collectively, data presented suggest controlled pulmonary Cldn6 expression and the likelihood that Cldn6 functions in distinct developmental roles. While such roles have remained largely undiscerned to this point, ongoing research may clarify important Cldn6 functions in differentiating pulmonary epithelium.

Methods

Mice

C57Bl/6 mice were housed and maintained in a conventional animal facility in accordance with institutional guidelines and approved Institutional Animal Care and Use Committee (IACUC) protocols. Embryonic mice were obtained from dams on the days specified following the formation of a vaginal plug that identified embryonic (E) day 0 (Reynolds, Mucenski et al. 2003).

Antibodies and Immunofluorescence

An anti-Cldn6 goat polyclonal antibody (C-20, Santa Cruz Biotechnologies, Santa Cruz, CA) was used at a dilution of 1:20 to identify Cldn6 expression in lung cells at different stages of lung development from E11.5 to post natal day (PN) 1. Co-localizing experiments were also conducted with the following antibodies: TTF-1 (1:100 from Seven Hills BioReagents, Cincinnati, OH), FoxA2 (1:100 from Seven Hills BioReagents), Sox2 (1:100 from Seven Hills BioReagents), and Sox9 (1:100 from Santa Cruz Biotechnologies).

Immunofluorescent staining for Cldn6, TTF-1, FoxA2, Sox2, and Sox9, were performed using standard techniques. Briefly, 5-µm paraffin sections from E11.5 to PN1 were deparaffinized and rehydrated by incubation in decreasing ethanol concentrations. Antigen retrieval was then performed as already outlined (Reynolds, Mucenski et al. 2004). Following antigen retrieval, sections were blocked in 5% donkey serum in PBS for 2 hours at room temperature, followed by incubation with primary antibodies at 4°C overnight. Control sections were incubated in blocking serum alone. After overnight incubation, all sections (including the controls) were washed using PBS/triton prior to the application of fluorescent-conjugated secondary antibodies for 1 hour at room temperature. Specifically, Alexa Fluor® 488 Rabbit

Anti-Goat IgG was used for Cldn6 and Alexa Fluor 633 goat anti-rabbit IgG secondary antibodies were used against all other primaries (Invitrogen, Carlsbad, CA). For dual label immunofluorescence, secondary antibodies were initially applied to the strongest primary in order to minimize decreasing intensity resulting from subsequent washes. All sections were mounted using VectaShield containing DAPI (Vector Laboratories, Burlingame, CA). .

Microarray Analysis and qRT-PCR

Microarray experiments were designed and mRNA analysis was conducted as already described (Xu, Wang et al. 2012). In order to specifically assess *Cldn6* mRNA expression throughout development, total RNA was isolated from whole mouse lungs at various time points with an Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) and treated with DNase. Reverse transcription of RNA was performed using the Invitrogen Superscript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) in order to obtain cDNA for PCR. The following primers were synthesized and HPLC purified by Invitrogen Life Technologies: Cldn6 (For-GCA GTC TCT TTT GCA GGC TC and Rev-CCC AAG ATT TGC AGA CCA GT) and GAPDH (For-TAT GTC GTG GAG TCT ACT GGT and Rev-GAG TTG TCA TAT TTC TCG TGG). cDNA amplification and data analysis were performed using Bio Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and a Bio Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories). Primers were used at a concentration of 75 nM each in 25-µl reactions. Cycle parameters were as follows: 3 min at 95°C for initial denaturation, followed by 40 cycles composed of 1 min at 95°C, 15 sec at 55°C and 15 s at 72°C. Control wells lacking template or RT were included to identify primer-dimer products and to exclude possible contaminants.

Plasmid Construction and Reporter Gene Assays

0.5-kb, 1.0-kb and 2.0-kb of the proximal mouse *Cldn6* promoter were obtained by polymerase chain reaction (PCR) using the Expand High Fidelity PCR System (Roche, Indianapolis, IN). The amplified *Cldn6* promoter fragments were cloned and directionally ligated into the pGL4.10-basic luciferase reporter plasmid (Promega, Madison, WI) and verified by sequencing as described previously (Reynolds, Allisson et al. 2010).

Functional assays of reporter gene constructs were performed by transient transfection of Beas2B and A-549 cells (American Tissue Culture Collection, ATCC, Manassas, VA) using FuGENE-6 HD reagent (Promega) (Porter, Bukey et al. 2011). Beas2B is a transformed human bronchiolar epithelial cell line and A-549 is a human pulmonary adenocarcinoma cell line characteristic of alveolar type II cells (Sporty, Horalkova et al. 2008). Transfections included 100ng of pGL4.10-0.5kb-Cldn6, pGL4.10-1.0kb-Cldn6 or pGL4.10-2.0kb-Cldn6, 100ng pRSV-βgal in order to assess transfection efficiency, and 100ng of expression vectors for key transcription factors including TTF1 (pCMV-TTF-1), FoxA2 (pCMV-FoxA2), or Gata-6 (pCMV-Gata-6). In the place of expression vectors, 100 ng of pcDNA control vector was added to equilibrate total DNA concentration at 300ng. After 48 hours, plates were scraped and centrifuged, and the cleared supernatant was screened for total enzymatic β-gal expression to evaluate efficiency and luciferase activity (Reynolds and Hoidal 2005). Luciferase activity was determined in 20 µl of extract at room temperature with 80µl of luciferase substrates (Promega, Madison, WI) for 10 sec after a 2-sec delay in a Moonlight™ 3010 luminometer (BD Biosciences, San Jose, CA).

Statistical Analysis

Reporter values are expressed as mean ± SD obtained from at least three separate

experiments in each group. Data were assessed by one- or two-way analysis of variance (ANOVA). When ANOVA indicated significant differences, the Student *t*-test was used with Bonferroni correction for multiple comparisons. Results presented are representative, and those with P values < 0.05 were considered significant. Messenger RNA microarray data was normalized using the Robust Multichip Average model and analyzed using three different statistical methods, including Bayesian Analysis of Time Series (BATS), Extraction of Differential Gene Expression (EDGE), and two-way ANOVA (Xu, Wang et al. 2012).

Results

Cldn6 Expression During Mid to Late Prenatal Lung Development

Cldn6 mRNA was initially evaluated by mRNA microarray analysis throughout a developmental time course in order to determine changes in its expression during periods of lung development and maturation. Lung samples from C57Bl6 mice were obtained daily from E12 through PN0 and were hybridized to the Mouse Gene 1.0ST Array (n=3 per time point). Dynamic changes in the expression of *Cldn6* were detected as development progressed and the data suggested a precipitous decline in expression from E15 to PN0 (Figure 1A). Quantitative RT-PCR of total C57Bl6 lung RNA was used to validate the expression profile of *Cldn6* over the same period. There was a strong correlation between mRNA expression detected by microarray analysis (Figure 1A) and assessment by quantitative RT-PCR despite notable differences in magnitude (Figure 1B).

Immunofluorescence was next used in order to correlate protein availability and localization with mRNA expression identified by microarray analysis and quantitative RT-PCR. Expression of Cldn6 was pronounced in the primitive pulmonary tubules of the developing lung from E12.5-E13.5 and the expression was intensely specific to resident epithelial cells (Figure

2A and C). In particular, Cldn6 was detected at not only the apical domain classically associated with tight junctions, but along the basolateral areas as well. The detection of fluorescence diminished as development continued; however, notable expression of Cldn6 was still detected in the primitive conducting and respiratory tubules from E14.5 through E17.5 (Figure 2 E, G, I, and K).

Cldn6 Expression Coincided with TTF-1 Expressing Primitive Pulmonary Epithelium

Thyroid transcription factor (TTF)-1 is a member of the Nkx2 transcription factor family of homeodomain-containing proteins expressed by the lung, thyroid, ventral forebrain, and pituitary (Guazzi, Price et al. 1990). TTF-1 has been consistently implicated in lung development and its functions include the activation of critical gene programs that control pulmonary epithelial cell differentiation during lung morphogenesis (Bohinski, Di Lauro et al. 1994). Because Cldn6 expression was localized to developing pulmonary epithelium, we sought to determine whether Cldn6 and TTF-1 were co-expressed. Co-immunofluorescence revealed that Cldn-6 and TTF-1 expression were both detected in developing epithelial cells at E12.5 and E13.5 (Figure 3A and E). Although TTF-1 expression was nuclear and Cldn6 remained primarily localized to the plasma membrane, concomitant expression was clear through E13.5. While expression patterns continued to overlap, co-expression diminished through PN1 (not shown).

Because Cldn6 expression coincided with the manifestation of TTF-1, we next sought to determine whether Forkhead box A2 (FoxA2) and Gata-6 were observed in Cldn6-expressing cells. FoxA2 is an important nuclear lung transcription factor that contains a winged helix DNA binding domain and Gata-6 is a zinc finger factor that critically influences endoderm formation via the activation of target genes (Costa, Kalinichenko et al. 2001). FoxA2 is required for

foregut formation during early periods of embryogenesis (Ang and Rossant 1994) and it often partners with TTF-1 in the genetic orchestration of respiratory epithelial cell differentiation (Besnard, Wert et al. 2004). Despite weaker co-expression, membranous Cldn6 expression overlapped with FoxA2 at E13.5 (Figure 4A). Gata-6 expression at E13.5 was also highly consistent with the expression of Cldn6 (Figure 4D).

Cldn6 Was Observed in Sox2- and Sox9-Expressing Cells

The molecular phenotypes of developing proximal and distal lung epithelial cell lineages have been associated with the differential expression of the transcription factors Sox2 and Sox9—sex-determining region Y (SRY)-box 2 and 9 (Hashimoto, Chen et al. 2012). Sox genes are highly conserved throughout the animal kingdom (Bowles, Schepers et al. 2000) and Sox2 has been implicated as an early marker for proximal lung cell differentiation (Okubo, Knoepfler et al. 2005) whereas Sox9 has been increasingly connected with distal respiratory trajectories (Perl, Kist et al. 2005). Due to plausible contributions to lung cell delineation, Cldn6 immunofluorescence was used to test whether Cldn6 was expressed with Sox2 and Sox9 during early periods of lung development. Our data demonstrated that Sox2 and Sox9 were both co-expressed with Cldn6 in developing pulmonary epithelium at E12.5 (not shown) and E13.5 (Figure 4G and J).

TTF-1, FoxA2, and Gata-6 Transcriptionally Regulated Cldn6

Due to the observation that Cldn6 was detected in TTF-1 expressing pulmonary epithelial cells as well as in cells that express FoxA2, one of its known transcriptional partners, we determined whether these factors directly influenced the transcription of the *Cldn6* gene. Direct regulation by transcription factors was assessed in Beas2B (human Bronchiolar epithelial cells) and A-549 cells (an immortalized cell line characteristic of alveolar type II cells). Our data

supported the concept that TTF-1 transcriptionally upregulated luciferase reporter plasmids that contained 0.5, 1.0, or 2.0-kb of the *Cldn6* promoter (Figure 5). Transcription of *Cldn6* mediated by TTF-1 was observed in both proximal Beas2B cells and distal A-549 cells. These experiments were repeated with expression vectors for FoxA2 and Gata-6. Gata-6 is a zinc finger containing transcription factor that like FoxA2, is expressed by respiratory epithelial cells where it plays a critical role in endoderm formation (Morrisey, Tang et al. 1998). Gata-6 is a genetic target of TTF-1 that is essential in the viability of bronchiolar epithelial cells during morphogenesis (Morrisey, Tang et al. 1998) and is also a central player in alveologenesis and secondary septation of the immature alveolus (Liu, Morrisey et al. 2002). Our data demonstrated that FoxA2 was sufficient to upregulate the three reporter plasmids containing increasing lengths of the *Cldn6* promoter (Figure 6A and B). Furthermore, Gata-6 was also effective in transcriptionally elevating *Cldn6* expression in both Beas2B and A-549 cells (Figure 6C and D). However, *Cldn6* transcription was only increased by Gata-6 in the 1.0 and 2.0-kb reporters and Gata-6 did not significantly activate the reporter that contained the 0.5-kb *Cldn6* promoter (Figure 6C).

Discussion and Conclusions

Cldn6 Expression in the Developing Lung

Cldns have dynamic, multimodal patterns of expression that for some family members, commence at the earliest stages of *mammalian* embryogenesis. An example of functional Cldn6 at the commencement of embryogenesis is observed when it cooperates with Cldn4 in the stabilization of trophectoderm located at the periphery of the blastocyst (Moriwaki, Tsukita et al. 2007). In fact, removal of Cldn6 and Cldn4 from the trophectoderm elicited hydrostatic pressure imbalances that caused an arrest in development due to a collapse of the blastocyst.

The current research demonstrated that Cldn6 is expressed by developing respiratory epithelium at very early periods of morphogenesis and that its apical and basolateral expression is precisely controlled. Our discoveries relating to the notably high midgestational expression of Cldn6 and its marked decrease as development continues illustrates the notion that Cldn6 functions in the early programming stages of lung development. This concept is supported by previous research that revealed sporadic peaks in the expression of Cldns in diverse tissues during organogenesis followed by periods of diminished expression. For example, Cldns are associated with brain ventricle morphogenesis, particularly in relation to the derivation of the blood brain barrier (Zhang, Liss et al. 2012). Lei *et al.* demonstrated that cell adhesion proteins specific to the developing intestine recruit Cldn family members necessary for the initial formation of the intestinal barrier before their expression detectibly decreases (Lei, Maeda et al. 2012). Westmoreland *et al.* discovered that Cldn6, and to a lesser degree Cldn4 and Cldn12, were each highly expressed in the developing pancreas that like the lung, undergoes a programmed set of branching events during morphogenesis (Westmoreland, Drosos et al. 2012). Their research revealed a distinctive, dynamic distribution pattern of Cldns6, 4, and 12 that related to elevated expression during pancreatic morphogenesis and altered expression during neoplastic disease (Westmoreland, Drosos et al. 2012). Lastly, a theme of augmented Cldn expression during organogenesis and tapered expression following organ formation was detailed in research that centered on nephrogenesis (Haddad, El Andalousi et al. 2011). Research revealed that nephric ducts, ureteric buds, and their derivatives robustly expressed Cldn3 during renal tubule formation and branching. Even though Cldn3 expression normally diminishes following organogenesis, the reintroduction of Cldn3 constructs caused *de novo* tubule branching to occur (Haddad, El Andalousi et al. 2011). Our observation that Cldn6 is specifically

expressed by apical and basolateral areas of branching pulmonary epithelial cells suggests roles central to their development. Because distinct boundaries of Cldn expression are observed in sites that correspond to inductive interactions during embryogenesis, further research may clarify whether Cldns are required in the translation of external signals into morphogenetic outcomes.

Our discovery that Cldn6 was co-expressed with Sox2 and Sox9 suggested plausible roles in the fate determination of developing airway and respiratory epithelium. Sox2 influences proximal airway epithelial cell differentiation and it has been recently implicated in canonical Wnt-β-catenin signaling (Volckaert, Campbell et al. 2013). Gain-of-function experiments showed that ectopically activated Wnt signaling negatively regulated Sox2 signaling required for bronchiolar lineage determination (Hashimoto, Chen et al. 2012). While additional research that seeks to identify links between Cldn6 and Sox2 is needed, the basis for such a link has been established by studies that show Wnt signaling orchestrates Cldn-mediated branching morphogenesis and angiogenesis (Lu, Zhang et al. 2013). While Sox9 is not essential for distal epithelial cell expansion and differentiation, it is considered a common marker for distal cell commitment. In addition to delineating such commitment, Sox9 cooperates with a host of other factors in the fine-tuning of distal cell phenotypes (Maeda, Davé et al. 2007).

Transcriptional Control of Cldn6

Early in lung development, Cldn6 was expressed in the primordial tubules at sites also expressing TTF-1 (Perl and Whitsett 1999). TTF-1 regulates cytodifferentiation and formation of the respiratory epithelium (Kimura, Hara et al. 1996). Later in development (E13.5–E15.5), TTF-1, Gata-6, and FoxA2 are co-expressed by differentiating pulmonary epithelium (Zhou, Lim et al. 1996; Keijzer, van Tuyl et al. 2001). The transcription factors TTF-1, Gata-6, and FoxA2 also significantly influence the transcription of other genes critical to lung function, including

Clara Cell Secretory Protein (CCSP), and surfactant protein (SP)-A, SP-B, and SP-C. Our data revealed that *Cldn6* is also a transcriptional target of TTF-1, Gata-6, and FoxA2; therefore, the functions of *Cldn6* in lung organogenesis may relate to fundamental processes including cell population expansion, differentiation, and function. Furthermore, because TTF-1 regulates target gene expression in concert with other regulatory factors including CBP, PAX8, NFAT, NF-1, RAR, and AP-1, it is likely that the temporal-spatial distribution of Cldn6 expression is influenced in a highly complex fashion (Maeda, Davé et al. 2007).

Conclusions

The present study revealed that Cldn6 is both temporally and spatially controlled in the developing lung and that its regulation is maintained by critical transcriptional control networks managed by TTF-1. While Cldns are central to the coordination of barrier function and signaling, many questions specific to the roles of Cldn6 in the developing lung remain. Further studies are necessary to address uncertainties such as lung-specific redundancies, possible functions of Cldns in tissues that lack TJs, and whether these proteins have a future as therapeutic targets. Conditional gain-of-function and loss-of-function experiments in animal models may prove to be the most beneficial in deciphering the impact of Cldns on organ formation and maintenance.

Competing Interests

The authors declare that they have no competing interests

Author Contributions

FRJ, JBL, and TTW performed immunofluorescence and JBL and TTW completed the quantitative RT-PCR. FRJ and STB were responsible for the cell culture and reporter analyses. PRR conceived of the studies and with the assistance of FRJ, supervised in the implementation,

interpretation, and writing. All authors assisted in manuscript preparation and approved of the final submitted version.

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Disclosures

The authors declare that they have no actual or potential competing financial interests. .

Figures

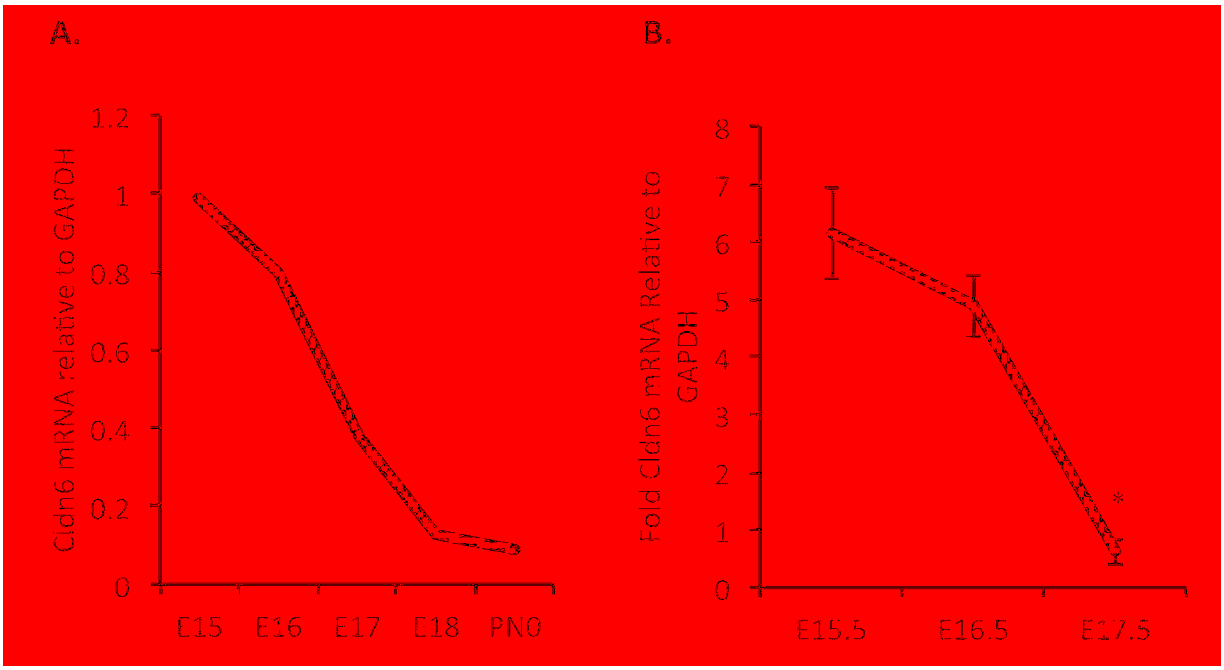

Figure 3.1 Control C57Bl6 Mice Were Screened by Microarray Analysis and Cldn6 Expression Levels Were Derived Relative to GAPDH from E15-PN0 (A). Confirmatory quantitative RT-PCR was conducted using total RNA from embryonic C57Bl6 mice and results are presented relative to GAPDH (B). Representative data from experiments performed in triplicate are shown. *$P \leq 0.05$ when comparisons were made between E15.5 and E16.5 or E15.5 and E17.5.

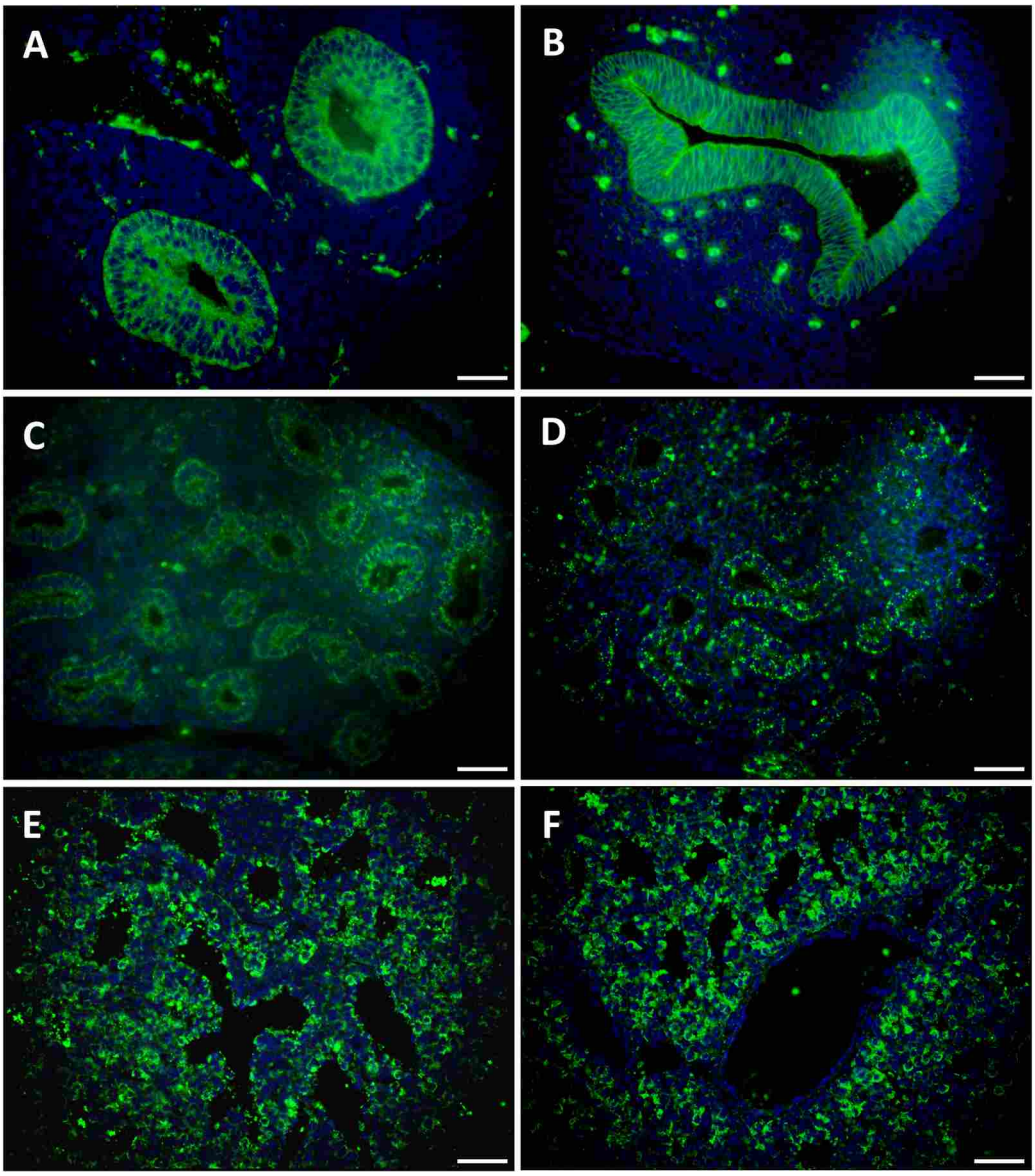

Figure 3.2 Cldn6 Was Immunolocalized During Periods of Murine Lung Morphogenesis. Cldn6 was initially detected in primitive respiratory epithelium at E12.5 (A) and expression persisted in differentiating epithelial cells at E13.5 (B), E14.4 (C), E15.5 (D), E16.5 (E), and E17.5 (F); however, expression diminished as development proceeded. Cldn6 immunoflorescence was revealed by Alexa Fluor® 488 secondary antibodies and DAPI staining was performed for cellular perspective. No immunoreactivity was observed in lung sections incubated without primary antibodies (not shown) and all images are at 400x original magnification. Scale bars represent 20 mm.

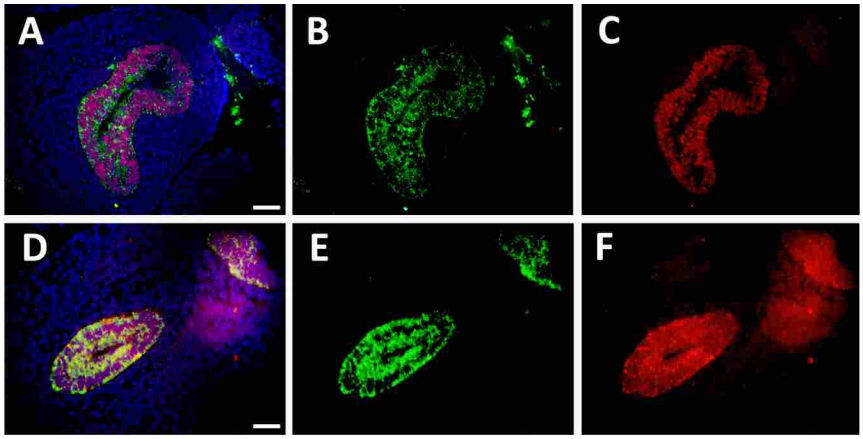

Figure 3.3 Cldn6 Was Co-Localized With TTF-1 at E12.5 (A-C) and E13.5 (D-F). Merged images are shown (A and D) that include Cldn6 immunofluorescence (B and F), TTF-1 immunofluorescence (C and F) and DAPI staining for cellular perspective. No immunoreactivity was observed in lung sections incubated without primary antibodies (not shown) and all images are at 400x original magnification. Scale bars represent 20 mm.

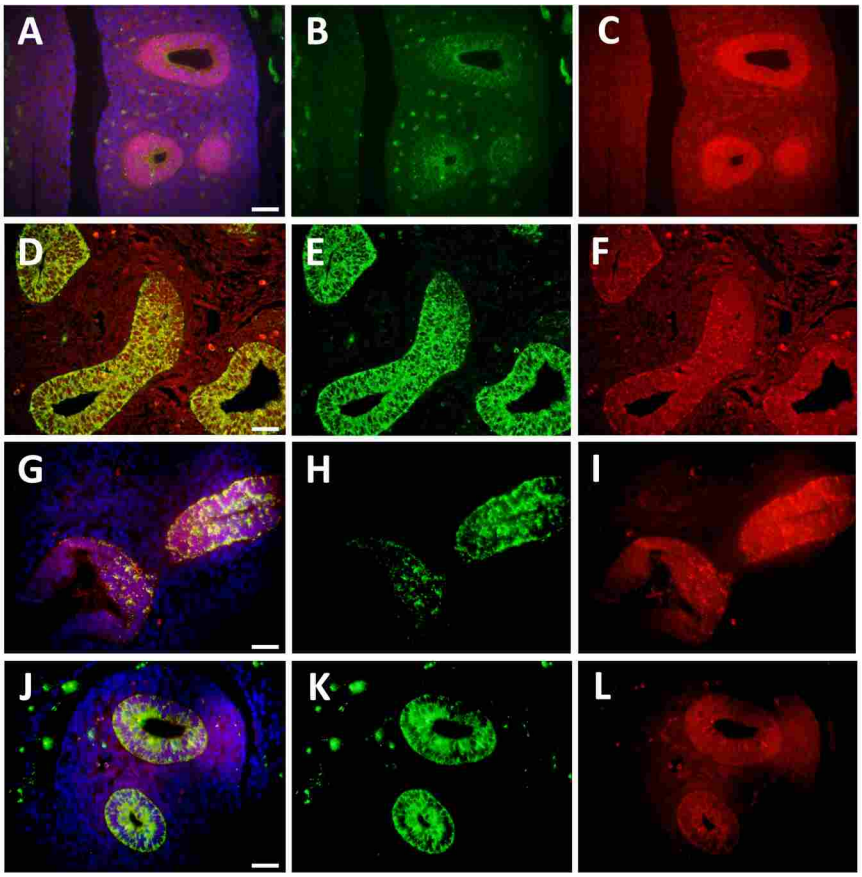

Figure 3.4 At E13.5, Cldn6 Immunofluorescence Was Co-Localized With Foxa2 (A-C), Gata-6 (D-F), Sox2 (G-I), and Sox9 (J-L). Merged images are shown (A, D, G and J) that include Cldn6 (B, E, H, and K), and FoxA2 (C), Gata-6 (F), Sox2 (I), or Sox9 (L). DAPI staining was performed for cellular perspective. No immunoreactivity was observed in lung sections incubated without primary antibodies (not shown) and all images are at 400x original magnification. Scale bars represent 20 mm.

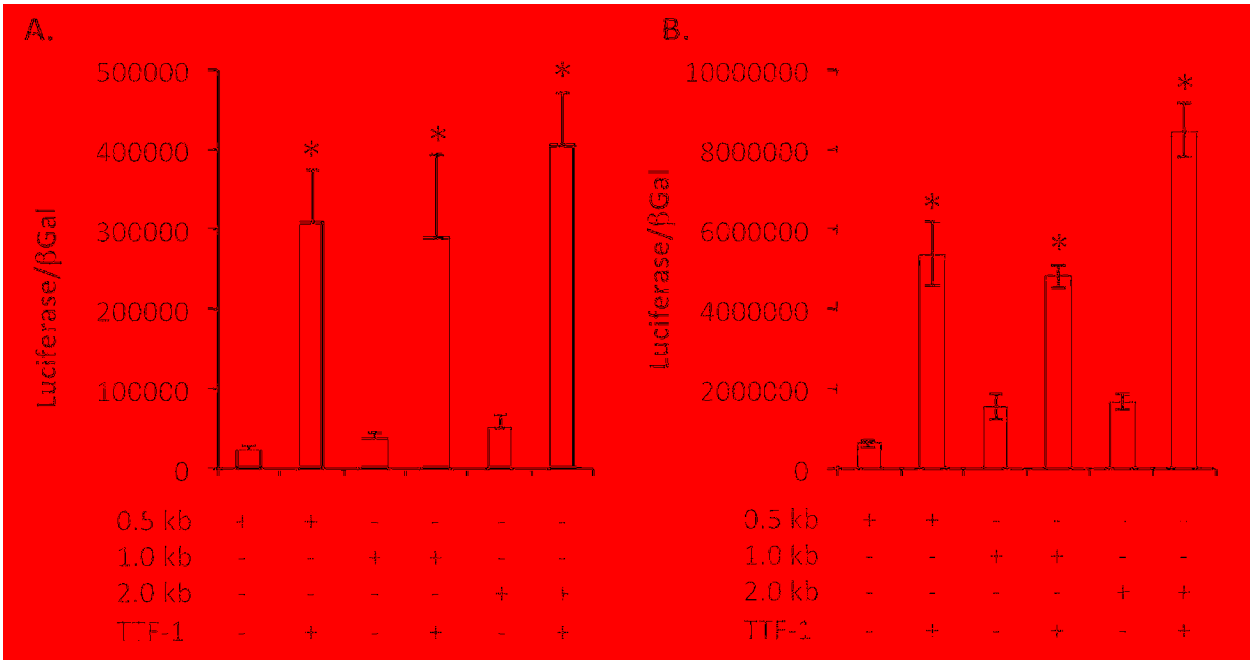

Figure 3.5 TTF-1 Induced Cldn6 Transcription in Bronchiolar Beas2B Cells (A) and A-549 Alveolar Type II-Like Epithelial Cells (B). TTF-1 induced transcription by acting on the 0.5-kb, 1.0-kb, and 2.0-kb proximal mouse *Cldn6* promoters ligated into luciferase reporter vectors. In each case, TTF-1 significantly induced the transcription of the *Cldn6* gene. Significant differences in luciferase activity, normalized to β-galactosidase used to assess transfection efficiency, are noted at *P ≤ 0.05 when compared to non TTF-1 transfected cells. The data shown are representative of experiments performed in triplicate.

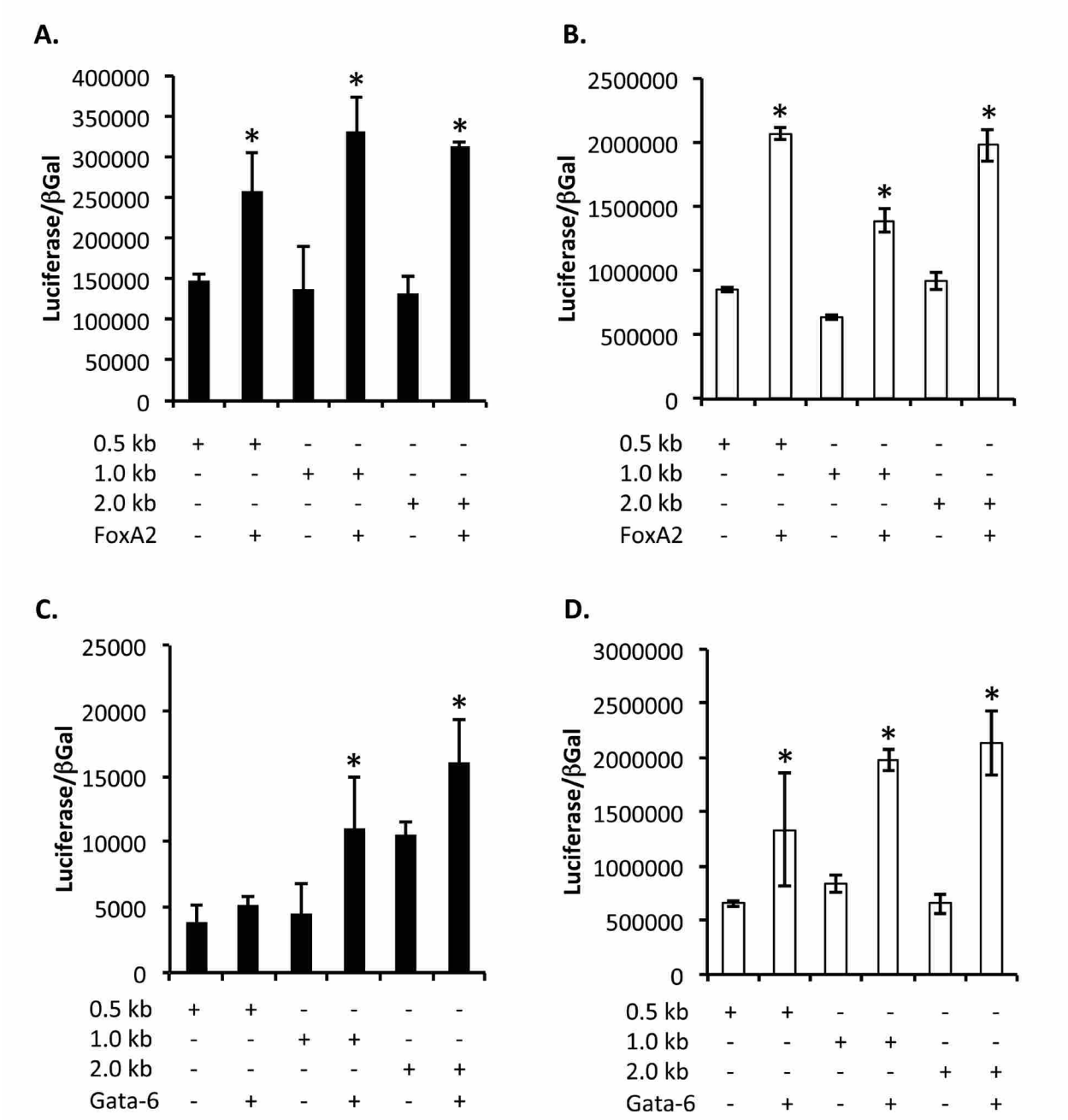

Figure 3.6 Foxa2 (A and B) and Gata-6 (C and D) Induced Cldn6 Transcription in Bronchiolar Beas2B Cells (A and C) and A-549 Alveolar Type II-Like Epithelial Cells (B and D). FoxA2 significantly increased transcription in both cell types by acting on the 0.5-kb, 1.0-kb, and 2.0-kb proximal mouse *Cldn6* promoters ligated into luciferase reporter vectors. Gata-6 did not significantly increase *Cldn6* transcription in Beas2B cells transfected with the 0.5-kb construct (C); however, Gata-6 significantly increased transcription in Beas2B cells when the 1.0-kb and 2.0-kb constructs were available and in all reporter experiments performed in A-549 cells (D). Significant differences in luciferase activity normalized to β-galactosidase are noted at *$P \leq 0.05$ when compared to non-transfected cells. The data shown are representative of experiments performed in triplicate.

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CHAPTER 4: Cigarette Smoke and Hif-1α Inhibit Pulmonary Claudin-6 Expression

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Running Title: Cigarette Smoke Inhibits Pulmonary Claudin-6 Expression

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Abstract

Background: Smoking is considered a major risk factor for several chronic diseases including chronic obstructive pulmonary disease (COPD), a condition involving both emphysema and inflammation of the airways. Claudins contribute to tight junctions by preventing paracellular transport of extracellular fluid and diverse substances, and Claudin-6 (Cldn6) is a tight junction protein expressed prominently in the lung.

Methods and Results: To determine whether Cldn6 was differentially influenced by tobacco smoke, Cldn6 was evaluated in cells and tissues by q-PCR, immunoblotting, and immunohistochemistry following exposure. Q-PCR and immunoblotting revealed that Cldn6 expression was decreased in alveolar type II-like epithelial cells (A-549). Cldn6 was also markedly decreased in the lungs of Balb/C mice exposed to tobacco smoke delivered by a nose-only automated smoke machine (InExpose, Scireq, Canada) compared to animals exposed to room air. Luciferase reporter assays incorporating 0.5-kb, 1.0-kb, or 2.0-kb of the Cldn6 promoter also revealed decreased transcription of Cldn6 when cells were exposed to 10% cigarette smoke extract (CSE). Cldn6 transcriptional regulation was next assessed in hypoxic conditions due to low oxygen tension observed during smoke exposure. Hypoxia and hypoxia inducible factor-1 alpha (Hif-1α) caused decreased transcription of the Cldn6 gene.

Conclusions: These data reveal that tight junctional proteins are differentially regulated by tobacco smoke exposure and that claudins are potentially targeted when barrier epithelial cells respond to tobacco smoke. Further research may show that claudins expressed in tight junctions between parenchymal cells contribute to impaired structural integrity of the lung coincident with smoking.

Key words: claudin-6, lung, tobacco, Hif-1α, transcription

Background

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality (Mannino, Homa et al. 2002; Rabe, Hurd et al. 2007). The World Health Organization noted COPD as one of the four leading causes of death worldwide, which is expected to increase in the coming decade (Organization 2012). COPD manifests itself as one of two different disorders: emphysema and chronic bronchitis. Emphysema is an enlargement of the alveoli and the destruction of the alveolar walls (Filley, Dart et al. 1967; Kinsman, Fernandez et al. 1983). Chronic bronchitis is associated with airway obstruction coincident with chronic persistent cough and the production of excessive mucus (Filley, Dart et al. 1967). Smoking is the main cause of COPD, but long-term exposure to other lung irritants such as air-pollution, particulates, pathogens, and dust may also be contributors (Decramer, Janssens et al. 2012).

The respiratory epithelium is a highly regulated transportation barrier that also serves as a first line of defense against harmful irritant exposure (Knight and Holgate 2003; Vareille, Kieninger et al. 2011). Tight junctions (TJ) are constructed at boundaries between neighboring epithelial cells and are specifically composed of integral transmembrane proteins such as Claudin, occludin, and junctional adhesion molecules (JAMs). Diverse anchoring proteins link these vital transmembrane proteins to the actin cytoskeleton during TJ complex assembly (Tam, Wadsworth et al. 2011).

TJs maintain homeostasis and cellular polarity by regulating the paracellular transport of ions, small molecules, and inflammatory proteins (Godfrey 1997; Kojima, Go et al. 2012). Furthermore, TJs also provide a site for intercellular signaling involved in the assembly, disassembly, and maintenance of other TJs (Matter, Aijaz et al. 2005; Shin, Fogg et al. 2006). TJ disruption increases lung permeability by altering fluid and ion transport, impairs cellular

polarity, and enhances susceptibility to infection or inflammation (Godfrey 1997; Fu, Ko et al. 2009). As such, TJs are classically considered to protect barrier epithelia from inflammation and infection (Guttman and Finlay 2009; Soini 2011; Beeman, Webb et al. 2012). When TJ impairment occurs in the airways, various pulmonary diseases present including chronic bronchitis, asthma, and pneumonia (Wilson, Dowling et al. 1996; Hogg and Timens 2009), (Asgrimsson, Gudjonsson et al. 2006; Holgate 2007; Gorska, Maskey-Warzechowska et al. 2010).

The Claudin (Cldn) family is composed of 27 members that are central components of the TJ complex (Furuse, Fujita et al. 1998; Morita, Furuse et al. 1999) with variable tissue-specific expression patterns (Krause, Winkler et al. 2008; Mineta, Yamamoto et al. 2011). Cldn functions in the formation of TJ sealing (Krause, Winkler et al. 2008; Krause, Winkler et al. 2009), but interestingly, they also form pores that may increase barrier permeability (Krause, Winkler et al. 2008) and enhance solute permeability (Coyne, Gambling et al. 2003). Current evidence suggests that cigarette smoke extract (CSE) induces structural changes in the TJ barriers (Godfrey 1997) and its components like occludins and zonula occludens (ZOs) (Petecchia, Sabatini et al. 2009; Shaykhiev, Otaki et al. 2011), which may involve down regulation of junctional genes including Claudins (Soini 2011). While specific Cldns have not been thoroughly evaluated to date, these structural changes in the epithelial barrier are primarily attributed to cytotoxic effects of cigarette smoke (Merikallio, Kaarteenaho et al. 2011). Claudin 6 (Cldn6) is an important component of undifferentiated stem cells (Wang, Xue et al. 2012), and they assist in determining permeability of airway epithelium (Coyne, Gambling et al. 2003). We sought to elucidate the expression and regulation of Cldn6 due to previous work that described

potential dysregulation in cells exposed to cigarette smoke and the high likelihood of resultant metastasis (Osanai, Murata et al. 2007; Wu, Liu et al. 2010; Xu, Jin et al. 2012).

Cigarette smoke contains a mixture of 4000 substances (Hoffmann, Djordjevic et al. 1997; Henry, Oldfield et al. 2003; Talhout, Schulz et al. 2011), among which are nicotine, carbon monoxide, and carcinogens like benzopyrene, cadmium, and nickel (Stedman 1968; Talhout, Schulz et al. 2011). Thus, it is difficult to determine the cellular mechanism that leads to junctional changes or injuries in the epithelial lining. *In vitro* studies involving CSE have shown increased cell permeability and DNA damage in bronchial epithelial cells (Pierson, Learmonth-Pierson et al. 2013). Research using the pulmonary adenocarcinoma cell line (A-549) has also shown potential TJ targeting (Hoshino, Mio et al. 2001) and cellular apoptosis and necrosis after CSE exposure (Hoshino, Mio et al. 2001; Gál, Cseh et al. 2011). In primitive alveolar epithelial cells, CSE induced irreversible cell arrest (Thorley and Tetley 2007) and parenchymal damage (Esechie, Kiss et al. 2008), but implication of specific TJ components was not determined.

This research tested the hypothesis that TJs comprised of Cldn6 are decreased by tobacco smoke. Loss in the expression of Cldn members is interpreted as a loss in adhesion, which is an important step that leads to smoke-induced lung disease. Collectively, the data presented suggested specific down-regulation of Cldn6 and the plausibility that impaired TJs in the exposed lung correlated with Cldn6 inhibition. While the mechanisms that lead to abnormal cellular permeability and cell turnover in the normal and smoke-exposed lung remain poorly understood, ongoing research may clarify important cell responses mediated by Cldn6 in the compromised lung.

Materials and Methods

Cigarette Smokes Extract (CSE) Preparation

Cigarette smoke extract (CSE) was prepared as described previously (Robinson, Johnson et al. 2012). In short, two Kentucky 2R1 research-reference cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY, USA) were bubbled through 20mL of DMEM using a vacuum pump (100% CSE). The extract was purified using a 0.22mm filter (Pall, Ann Arbor, MI) and diluted to 10% CSE. Each 2R1 cigarette contains total particulate matter (11.7mg.), tar (9.7mg.), and nicotine (0.85 mg).

Cell Culture

A human lung epithelial cell line (A-549), obtained from the Human Science Research Bank (JCRB0076; Osaka, Japan) were plated and grown in Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamine, 10% FCS, and antibiotics. Cells were split into six-well plates and grown to between 50 to 60% confluence. Cultures were exposed to 10% CSE or media alone for 6 hours. RNA was isolated from cells for RT-PCR or lysed to quantify proteins by immunoblotting. As outlined in select experiments, A-549 cells were also transfected with reporter constructs containing 2.0-Kb, 1.0-Kb, or 0.5-Kb of the proximal Cldn6 promoter explained below and exposed to hypoxia (2% O2) conditions for 18 hours.

RNA Isolation and RT-PCR Analysis

In order to assess Cldn-6 mRNA expression after CSE exposure, total RNA was isolated from A-549 cells using an Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA). Reverse transcription of total mRNA and PCR conditions were as previously summarized (29). Reverse transcription of RNA was performed using the Invitrogen Superscript III First-Strand

Synthesis System (Life Technologies, Grand Island, NY). The following primers were synthesized and HPLC purified by Invitrogen Life Technologies: Cldn6 (For-GCA GTC TCT TTT GCA GGC TC and Rev-CCC AAG ATT TGC AGA CCA GT) and GAPDH (For-TAT GTC GTG GAG TCT ACT GGT and Rev-GAG TTG TCA TAT TTC TCG TGG). cDNA amplification and data analysis were performed using Bio Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and a Bio Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories). Conditions included 40 cycles at 95°C for 15 s and 60°C for 60 s. Gene expression was assessed in three replicate pools, and representative data are shown.

Protein Analysis

Whole cell or lung lysates were screened for Cldn6 protein via immunoblotting with a goat polyclonal Cldn6 antibody (C-20, Santa Cruz Biotechnologies, Santa Cruz, CA). Briefly, equivalent amounts of total protein were evaluated by SDS-PAGE, blocked with 5% nonfat milk, and exposed to the primary antibody diluted at 1:200 at 4°C overnight. Exposure to horseradish peroxidase-conjugated secondary antibodies was followed by development with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). To determine loading consistencies, membranes were stripped and reprobed with an antibody against mouse beta-actin (dilution 1:1000; Sigma Aldrich, St. Louis, MO, A1978). Images presented are representative. Blots were densitometrically evaluated using Un-Scan-It software as already described (Reynolds, Kasteler et al. 2011).

Immunohistochemistry

A goat Cldn6 polyclonal antibody (Santa Cruz Biotechnologies) was used at a dilution of 1:20. Lungs were inflation fixed overnight with 4% paraformaldehyde, processed, and sectioned

prior to assessing the expression of Cldn6 using standard immunohistochemical techniques (Reynolds, Kasteler et al. 2011). Control samples were incubated in blocking serum alone.

Mouse Lung Samples

Adult Balb-C mice were exposed to secondhand tobacco smoke or room air for four days as already outlined (Winden, Ferguson et al. 2013). Briefly, mice were randomly assigned to room air- and smoke-exposure groups and treated using an in-house nose-only smoke exposure system (InExpose System, Scireq, Canada). Treated mice were restrained daily and connected to an exposure tower for 10 minutes where they were nasally exposed to secondhand cigarette smoke from two standard research cigarettes (Wood, Winden et al. 2014). The smoke challenge for these studies was chosen according to previously published literature (Reynolds, Kasteler et al. 2008; Rinaldi, Maes et al. 2012). The challenge was associated with a good tolerance of mice to the smoke sessions and an acceptable level of particulate density concentration literature (Reynolds, Kasteler et al. 2008; Rinaldi, Maes et al. 2012). Control animals were similarly handled and restrained but kept under a smoke-free environment. At the conclusion of the exposure experiment, mice were sacrificed, and lungs were inflation fixed with 4% paraformaldehyde for immunohistochemical studies outlined above. Animal use and husbandry was approved by the institutional guidelines and approved Institutional Animal Care and Use Committee (IACUC) protocols.

Plasmid Construction and Mutagenesis

Primers were developed to amplify 0.5-Kb, 1.0-Kb, and 2.0-Kb of the Cldn6 promoter by using the Expand High Fidelity System (Roche, Indianapolis, IN). The Cldn6 promoters amplified were directionally cloned and ligated into the pGL4.10-basic luciferase reporter plasmid (Promega, Madison, WI) and sequenced to confirm fidelity. Site-directed mutagenesis

of potential Hif1-α response elements (GCGTG→GGGGG (HRE1) or TGCTA→GGGGG (HRE2)) in 0.5Kb Cldn6 promoter was performed by using the reporter construct (pGL4.10-0.5kb-Cldn6) and following the manufacturer's instructions for the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA).

Transfection and Reporter Gene Assays

Functional assays of reporter gene constructs were performed by transient transfection of A-549 cells using FuGENE-6 HD reagent (Promega) (Porter, Bukey et al. 2011). A-549 is a human pulmonary adenocarcinoma cell line characteristic of alveolar type II cells (Sporty, Horalkova et al. 2008). Cells were allowed to reach 50 to 60% confluence and transfected with 100 ng pRSV-βgal, 100 ng pGL4-0.5-Cldn6, 100 ng pGL4-1.0-Cldn6, or 100 ng pGL4-2.0-Cldn6, and /or pCMV-Egr-1, or pCMV-TTF-1, and pCDNA control vector to bring total DNA concentration 1.0 μg. Cells were grown for 48 hours, washed, lysed, and frozen for 6 hours. Plates were scraped and centrifuge to obtain a supernatant and used for luciferase and β-gal assays. Assays-reporter were normalized to determine transfection efficiency (Schagat, Paguio et al. 2007). Luciferase activity was determined in 20 μl of extract at room temperature with 80μl of luciferase substrates (Promega) for 10 sec after a 2-sec delay in a Moonlight™ 3010 luminometer (BD Biosciences, San Jose, CA).

Statistical Analysis

Values are expressed as mean ± SD obtained from at least three separate experiments in each group. Data were assessed by one-way analysis of variance (ANOVA). When ANOVA indicated significant differences, the Student's t-test was used with Bonferroni correction for multiple comparisons. Results presented are representative and those with P values <0.05 were considered significant.

Results

Cigarette Smoke Extract Down-Regulated Cldn6 in A-549 Cells

The effect of CSE exposure on Cldn6 expression was examined in A-549 cells. CSE was added to culture medium of A-549 cells at a concentration of 10% for 6 hours and q-PCR was used to analyze differences in mRNA expression. Cldn6 mRNA expression was significantly down regulated in A-549 cells exposed to CSE compared to controls (Figure 1A). Immunoblotting was next used in order to correlate message and protein expression patterns. Similarly, Cldn6 protein expression was significantly decreased in CSE-exposed cells when compared to non smoked (NS) controls (Figure 1B).

Secondhand Smoke Exposure Decreased Cldn6 Expression in the Mouse Lung

Animal experiments were designed in order to assess whether CSE-mediated decreases in Cldn6 expression observed *in vitro* also occur *in vivo*. Immunohistochemistry of control room-air exposed lungs revealed precise paracellular expression of Cldn6 in the airway epithelium (Figure 2A, arrow) and diffuse expression at boundaries between distal respiratory epithelial cells (Figure 2A, arrowhead). Staining for Cldn6 in the lungs of smoke-exposed animals revealed a significant loss of Cldn6 expression (Figure 2B). Immunoblotting for Cldn6 was also performed using whole lung lysates. Intense Cldn6 expression in lungs from non-smoked animals (NS) decreased following smoke exposure (Figure 2C).

CSE Transcriptionally Repressed Cldn6

To assess the impact of CSE exposure on the transcriptional control of Cldn6, 2.0-Kb, 1.0-Kb, or 0.5-Kb proximal promoter sequences were amplified and luciferase reporter constructs were generated. Reporter assays revealed a highly significant decrease in Cldn6

transcription by A-549 cells exposed to CSE when compared to normal media controls (Figure 3).

Hypoxia and Hypoxia Inducible Factor-1 A (Hif-1α) Transcriptionally Repressed Cldn6

Tobacco smoke exposure has long been known to induce periodic exposure to hypoxia (Slotkin 1998). In order to elucidate the possible relationship between hypoxia and smoke-induced inhibition of Cldn6 expression, experiments were designed to test whether hypoxia alone is sufficient to inhibit Cldn6 transcription. A-549 cells were transfected with reporter constructs containing 2.0-Kb, 1.0-Kb, or 0.5-Kb of the proximal Cldn6 promoter and exposed to hypoxia (2% O2) conditions for 18 hours. We discovered that Cldn6 promoter activity during hypoxia conditions was significantly down-regulated in A-549 cells transfected with 1.0-Kb and 2.0-Kb promoter constructs (Figure 4); however, the transcriptional activity of the 0.5-kb promoter constructs during hypoxia was not altered (Figure 4A). Hif-1α is a protein that translocates to the nucleus when oxygen tension decreases in order to regulate target genes involved in cellular responses to hypoxia. To test whether Hif-1α regulated Cldn6 transcription, A-549 cells were co-transfected with Cldn6 reporter constructs and expression vectors that contain constitutively active Hif-1α. Hif-1α transcriptionally inhibited all three Cldn6 constructs (Figure 4B). Sequence analysis of the Cldn6 promoter revealed the presence of two Hif-1α response elements (HRE1 and HRE2, respectively) in the 0.5-Kb promoter sequence. To determine if HRE1 and HRE2 control Hif-1α-mediated Cldn6 transcription, reporter constructs were generated in which HRE1 or HRE2 sequences were mutated prior to co-transfection of the mutant constructs with Hif-1α. Reporter assays involving either HRE1 or HRE2 revealed that Hif-1α had no transcriptional effect on the 0.5-kb Cldn6 promoter when HRE1 was mutated (Figure 5). Experiments involving mutant HRE2 revealed Hif-1α-mediated transcriptional

inhibition that was similar to non-mutant controls (Figure 5). Thus, HRE1 is a key Hif-1α response element involved in the transcriptional inhibition of Cldn6 during hypoxia.

Discussion

Diverse yet anticipated responses occur when cells and tissues are exposed to tobacco smoke. Even with far reaching public health campaigns discouraging smoking, 1,000 American children every day become smokers, potentiating tobacco-related health complications among the general population (Morse and Rosas 2014). Unequal distribution of disease and inconsistent histopathology among smokers suggest the likelihood that genetic determinants centrally influence the impact of tobacco smoke exposure at the cellular level. In the current set of experiments, we sought to understand the integrity of respiratory epithelium, a highly sensitive membrane that is at the forefront of smoke disease pathogenesis.

Our data detailed selective inhibition of Cldn6 following *in vitro* and *in vivo* smoke exposure. Accordingly, the destabilization of cell junctions anatomically interposed between airway and respiratory epithelial cells likely contributes to the early stages of tobacco smoke-induced cell stress responses. Pulmonary epithelial cells function as important immunological and cytoprotective barriers against insults and TJs assist in the maintenance of the system's integrity. Carson et al. employed freeze-fracture techniques to ultrastructurally assess large airway epithelium following lifestyle exposure to cigarette smoke (Carson, Brighton et al. 2013). Discoveries included structural changes to tight junctional complexes and coincident leukocyte infiltration in exposed animals. These findings are further supported by research performed by Schamberger et al. that demonstrated smoke-induced disruption of TJs and impaired barrier permeability (Schamberger, Mise et al. 2014). Specifically, CSE decreased transepithelial electrical resistance (TEER) and inhibited the expression of TJ linker proteins in primary human

bronchial epithelial cells and in bronchi *ex vivo*.

Our discovery that Cldn6 is specifically inhibited by tobacco smoke potentially provides an important step in characterizing specific tetraspanins involved in the destabilization of epithelial cells. Shaykhiev et al. utilized transcriptome analysis to reveal global down-regulation of physiological apical junctional complex components in the airways of healthy human smokers compared to non-smokers and discovered further decreases in smokers with COPD (Shaykhiev, Otaki et al. 2011). While more work is needed in this area, compensation and redundancies among TJ proteins may reveal that transcriptomic/proteomic reprogramming of lung epithelial cell TJs orchestrates a transition from normal barrier physiology toward disease phenotypes. The concept that Cldns influence epithelial barrier integrity was further demonstrated by investigators that evaluated intestinal barrier disruption in cigarette smoke-exposed rats (Li, Wu et al. 2015). While clearly removed from the blood-gas interface maintained by the lungs, intestinal epithelial cells responded to worsening COPD by loosening TJs through the inhibition of Cldns (Coyne, Gambling et al. 2003; Li, Wu et al. 2015).

Our research revealed that Cldn6 is sensitive to decreased oxygen tension and that Hif-1α is a potential regulator of Cldn6 expression. Data are accumulating that suggests a clear role for Hif-1α in the evolution and propagation of diverse inflammatory processes. Li et al. showed that Hif-1α expression is elevated during the development of COPD and that elevated Hif-1α may be associated with increased oxidative stress and apoptosis (Li, Wu et al. 2015). It was proposed that Hif-1α-mediated cell death coincided with destabilization of epithelial cell TJs. Jiang et al. further demonstrated that NF-κB regulates the Hif-1α pathway during smoke exposure and in cases of advancing COPD (Jiang, Zhu et al. 2010). They specifically assessed NF-κB activation following smoke exposure and indicated that Hif-1α activation via NF-κB contributed to cell loss

and inflammation coincident with COPD development. The present research further implicates Hif-1α as having a more robust role in managing cellular responses to tobacco smoke. More work is needed, but adding Cldn6 and other TJ components to the list of Hif-1α targets should add clarity to diseases involving inflammation and cell death of barrier epithelial cells.

In summary, cells and lungs exposed to tobacco smoke elicit a host of programmed responses that may culminate in cell loss and barrier perturbation. Our research clearly identifies specific targeting of Cldn6 expression when tobacco smoke is encountered. Due to diminished oxygen availability in smokers, lung epithelial cells likely disorganize TJs by inhibiting Cldn6 via Hif-1α-mediated control mechanisms. In addition to orchestrating cytokine elaboration during the histopathological remodeling of the chronically exposed lung, Hif-1α may also destabilize barrier cell TJs observed in progressing lung disease.

Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

FRJ, JBL, DRM, and DRW performed the smoke exposure experiments and CMJ conducted the qPCR. FRJ, JBL and STB completed the immunohistochemistry and DCM and DRM assisted with immunoblotting. FRJ, STB, and DCM generated constructs and completed the cell culture experiments. PRR conceived of the study and with the assistance of JAA and FRJ, supervised in its implementation, interpretation, and writing. All authors assisted in manuscript preparation and approved of the final submitted version.

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Disclosures

The authors declare that they have no actual or potential competing financial interests. This work was supported by grants from the Flight Attendant's Medical Research Institute (FAMRI, PRR) and a BYU Mentoring Environment Grant Award (PRR).

Figures

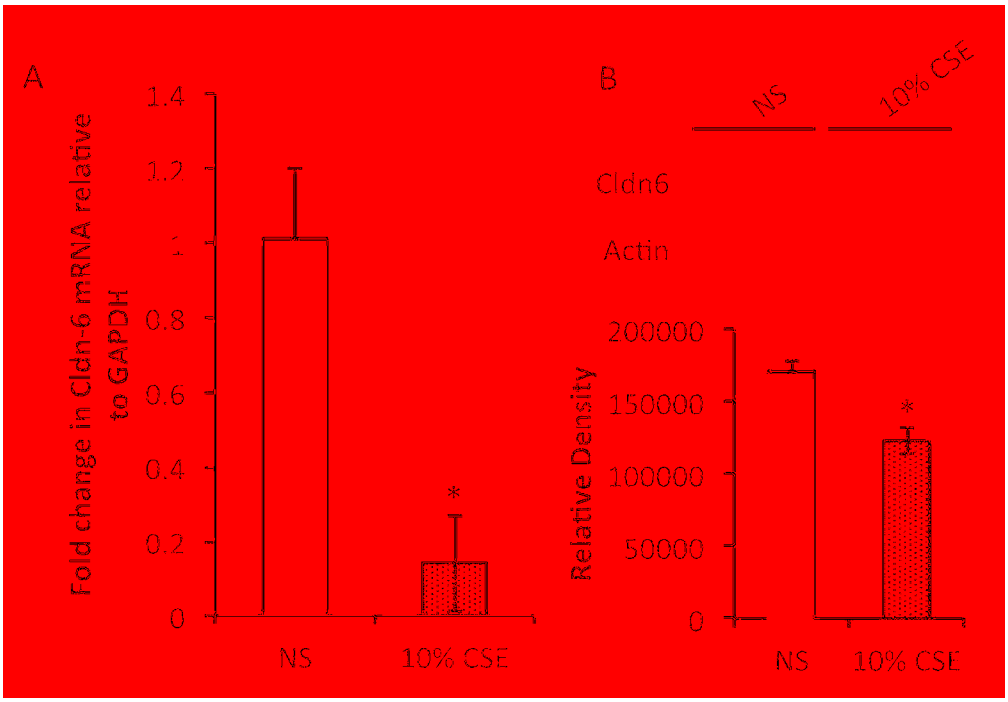

Figure 4.1 CSE Increases Cldn6 Expression *In vitro*. Q-PCR analysis revealed that A-549 pulmonary adenocarcinoma cells exposed to 10% CSE expressed significantly less Cldn6 mRNA compared to normal media controls (A). Immunoblotting demonstrated significantly less Cldn6 protein in CSE-exposed A-549 cells compared to controls. Data are representative of experiments performed in triplicate and $*p \leq 0.05$.

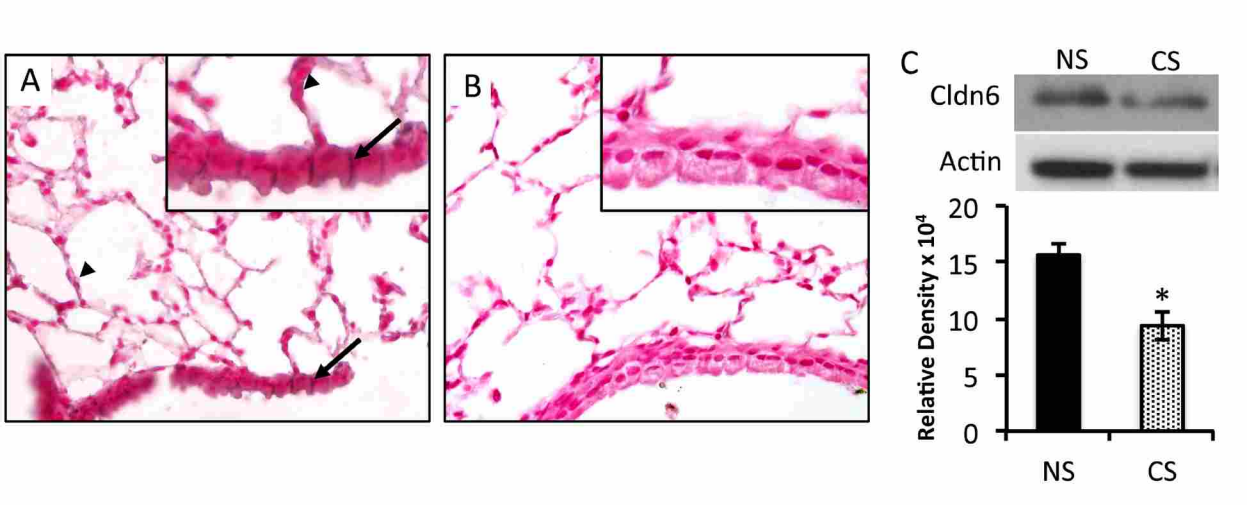

Figure 4.2 Mice Exposed to Secondhand Smoke Expressed Less Cldn6 Compared to Controls. Normal room air-exposed C57BL/6 mice expressed detectible Cldn6 expression on lateral edges of conducting airway epithelial cells (A, Arrow) and distal respiratory epithelial cells (A, Arrowhead). Immunohistochemistry performed on mouse lung sections following four days of secondhand smoke exposure resulted in undetectable levels of Cldn6 expression (B). Immunoblotting for Cldn6 revealed significantly decreased Cldn6 expression following secondhand smoke exposure compared to non-smoked (NS) controls (C). Blotting was performed in triplicate and significant differences from corresponding controls are noted at $P \leq 0.05$ (*).

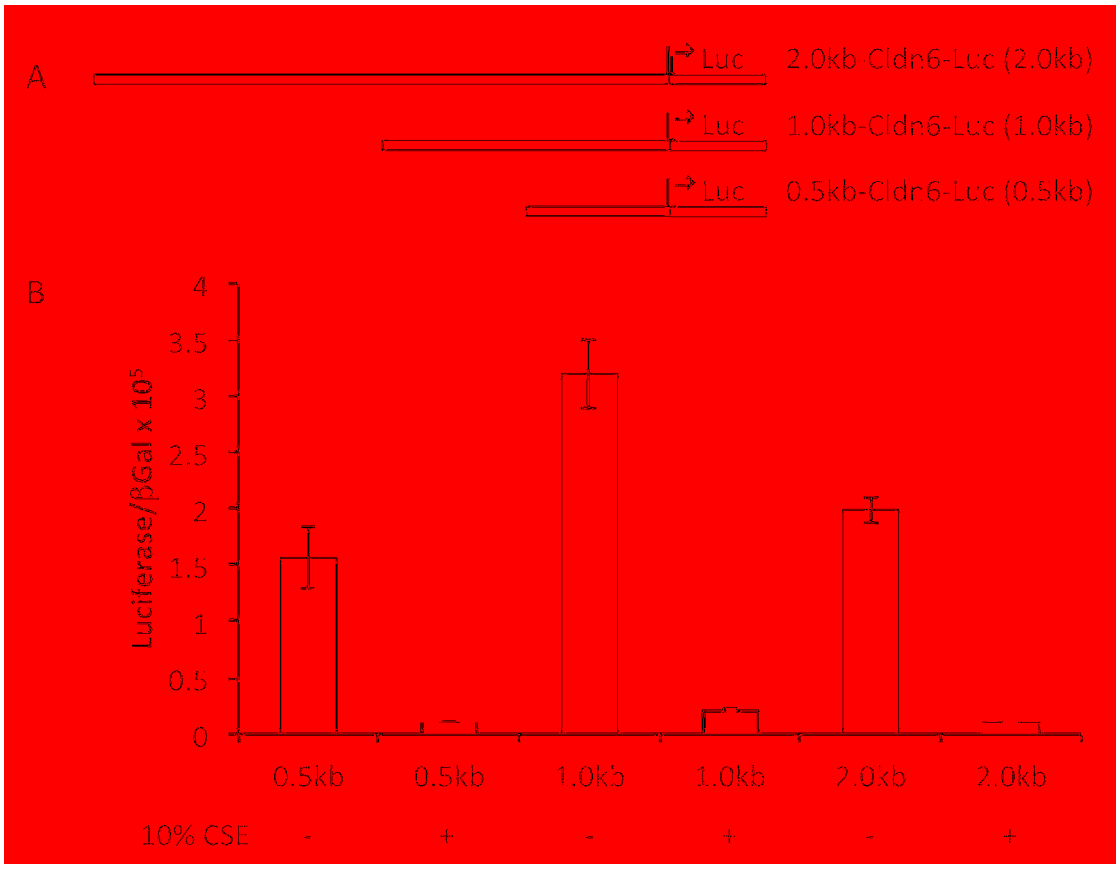

Figure 4.3 2.0-Kb, 1.0-Kb, and 0.5-Kb of the Mouse Cldn6 Promoter Was Amplified, Sequence Verified, and Ligated Into a Luciferase-Reporting Vector (A). 10% CSE significantly inhibited Cldn6 transcription in A-549 cells separately transfected with the three reporter constructs. Experiments were performed in triplicate and significant differences in luciferase levels compared to reporter alone are noted at $P \leq 0.05$ (*).

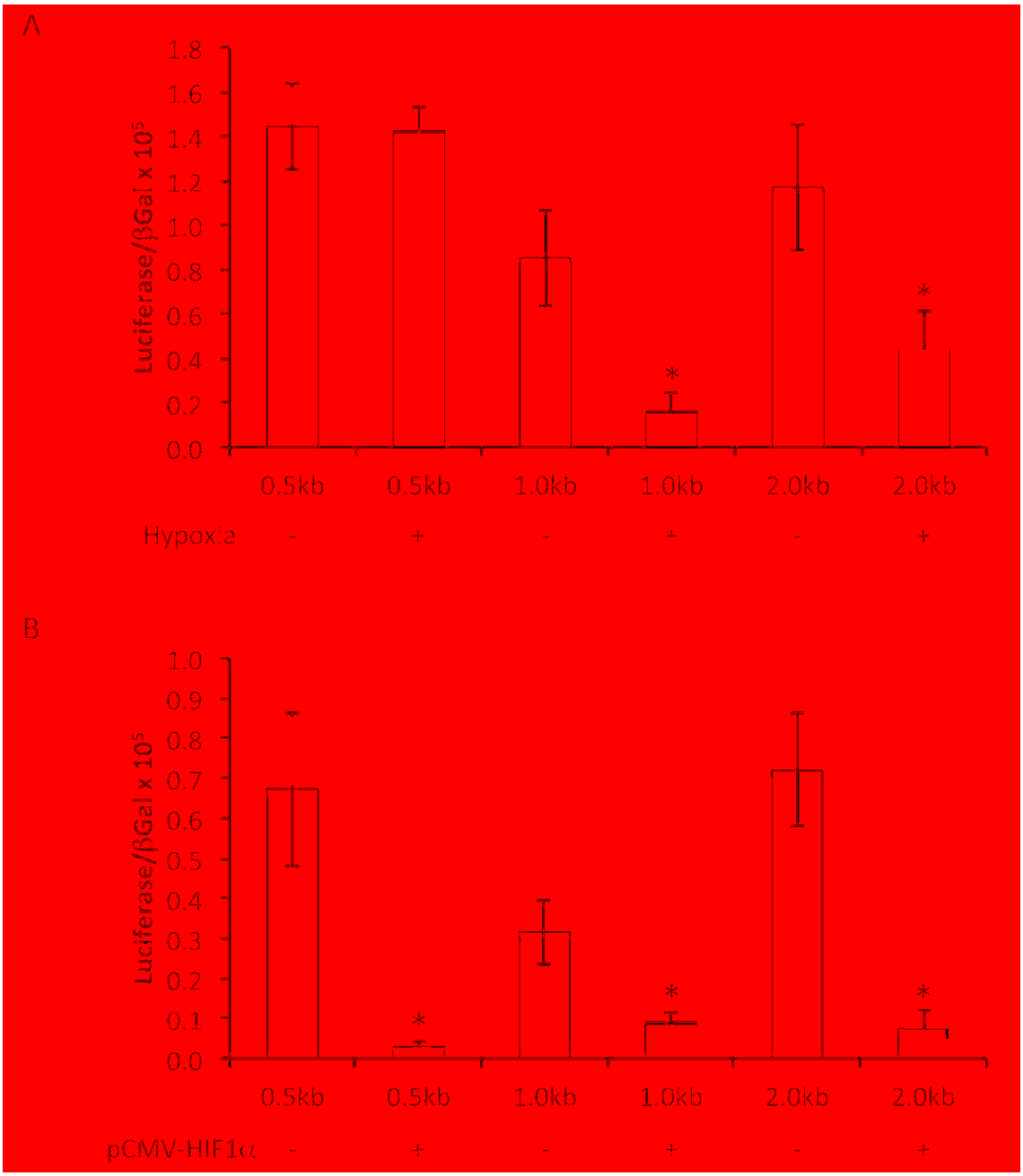

Figure 4.4 2.0-Kb, 1.0-Kb, and 0.5-Kb of the Mouse Cldn6 Promoter Was Amplified, Sequence Verified, and Ligated Into a Luciferase-Reporting Vector. Experiments utilizing each of the three vectors were transfected into A-549 cells and exposed to hypoxia for 18 hours. Hypoxia significantly decreased Cldn6 transcription by the 1.0-kb and 2.0-kb vectors (A). Co-transfection of A-549 cells with one of the three reporters and expression vectors for Hif-1α was conducted to assess Cldn6 regulation by Hif-1α. Hif-1α transcriptionally inhibited the 2.0-kb, 1.0-kb, and 0.5-kb reporters. Experiments were performed in triplicate and significant differences in luciferase levels compared to reporter alone are noted at $P \leq 0.05$ (*).

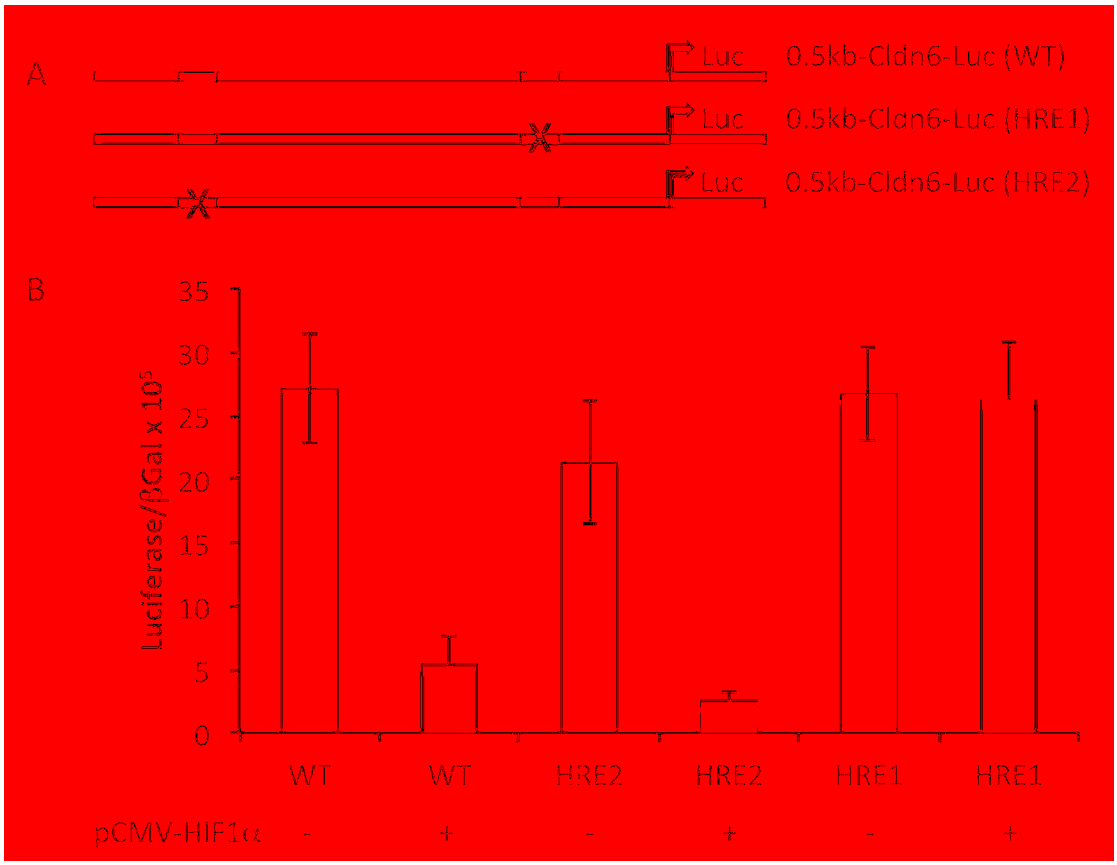

Figure 4.5 Because Hif-1α Inhibited Cldn6 Transcription, Site-Directed Mutagenesis of Potential Hif-1α Response Elements (HRES) Was Conducted. Two HREs were identified in the 0.5-kb Cldn6 reporter (A). Co-transfection experiments involving reporters and Hif-1α were conducted and luciferase levels were obtained when the proximal HRE, 0.5kb-Cldn6-Luc (HRE1), or distal HRE, 0.5kb-Cldn6-Luc (HRE2), was mutated. Comparisons with luciferase from the non-mutant promoter, 0.5kb-Cldn6-Luc (WT), revealed a loss of Hif-1α mediated transcription when HRE1 was mutated (B). Loss of HRE2 resulted in no change in Hif-1α mediated transcriptional inhibition (B). Experiments were performed in triplicate and significant differences in luciferase levels compared to reporter alone are noted at $P \leq 0.05$ (*).

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CHAPTER 5: Conditional Pulmonary Over-Expression of Claudin 6 (Cldn6) During Embryogenesis Delays Lung Morphogenesis

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Abstract

Claudin 6 (Cldn6), a tetraspanin expressed by barrier epithelial cells, is a participant in cell junction assembly. Compromised membrane permeability results when Cldn6 expression is perturbed. In order to assess the effects of persistent tight junctions involving Cldn6 during lung development *in vivo*, a doxycycline (dox)-inducible conditional transgenic mouse was generated that up-regulated Cldn6 in the distal lung. Pups had unlimited dietary access to dox from conception until sacrifice date at embryonic day (E) 18.5. Transgenic pups that expressed Cldn6 were removed by C-section and compared to non-transgenic littermates. Quantitative real-time PCR, immunoblotting, and Cldn6 immunohistochemistry revealed significantly elevated Cldn6 expression in transgenic mice compared to controls. There were no differences between transgenic mice and controls in terms of lung size, lung weight, or whole body weight. Histological evaluations led to the discovery that E 18.5 Cldn6 transgenic pups appeared to be in the early canalicular stage of development wherein respiratory airspaces were thickened and fewer in number. In contrast, controls appropriately appeared to have entered the saccular stage coincident with thin airspace walls and spherical architecture. Immunostaining for critical lung transcription factors including TTF-1 and FoxA2 was conducted to assess regulators of cell differentiation and specific lung cell types were identified via staining for pro-surfactant protein C (Type II cells) or Clara Cell Secretory Protein (Clara cells). Lastly, cell turnover was qualitatively measured via staining for cell proliferation (Proliferating Cell Nuclear Antigen, PCNA) or apoptosis (cleaved Caspase-3). These data suggest that Cldn6 is an important junctional protein potentially involved in the programming of epithelial cells during lung development. Furthermore, genetic down-regulation of Cldn6 as development proceeds may influence differentiation observed in the transition from the canalicular to the saccular lung.

Introduction

Lung development is a complex process of highly organized and dynamic events, which require respiratory epithelium and the surrounding splanchnic mesenchyme (Burri 2006). Developmental events can be divided into structural and functional stages (Potter and Loosli 1951) that commence with the formation of a groove in the foregut at the outset of the embryonic stage of lung development (Hilfer 1983; Ten Have-Opbroek 1991; deMello, Sawyer et al. 1997; Cardoso 2001). During the pseudoglandular stage, tubular branching of the lung airways continues and the derivation of the respiratory parenchyma is initiated (Burri 1984; Copland and Post 2004). In the canalicular stage, lung morphogenesis coincides with pulmonary epithelial cell differentiation that results in the formation of the future air-blood barrier (Gluck 1978; Burri 1984; Torday 1992). Additionally, during the canalicular stage, surfactant proteins become increasingly expressed and respiratory pneumocytes undergo pronounced differentiation (Ballard 1980). A subset of Type II pneumocytes transition to squamous Type I pneumocytes (Hilfer 1983; Leary, Finkelstein et al. 1986), which is essential in the development of definitive alveoli. The saccular stage is characterized by increased vascularization of alveoli and elevated surfactant protein expression. Finally, the alveolar stage is characterized by postnatal alveolar remodeling via septation events that significantly increase alveolar counts and restrict mesenchyme during final microvascular maturation (Adams 1966; Burri 1984; Abe, Yamamoto et al. 2014).

A diversity of signaling and transcriptional control pathways influence processes that regulate the precise deposition of specialized cell types along the proximal-distal pulmonary axis. Such pathways include those associated with fibroblast growth factors (Fgfs), sonic hedgehog (Shh), bone morphogenetic protein 4 (Bmp4), vascular endothelial growth factors (Vegfs), thyroid transcription factor 1 (TTF-1), and Wnts (Potter and Loosli 1951; Gluck 1978;

Ballard 1980; Hilfer 1983; Burri 1984; Ten Have-Opbroek 1991; Torday 1992; deMello, Sawyer et al. 1997; Cardoso 2001; Copland and Post 2004; Stogsdill, Stogsdill et al. 2012). When these or other important genetic pathways are defective or delayed, pulmonary hypoplasia or pulmonary agenesis may occur resulting in abnormally low or absent bronchopulmonary segments and terminal alveoli (Ballard 1980; Stogsdill, Stogsdill et al. 2013).

Tight junctions (TJs) begin to form at cell-cell contacts as the respiratory epithelium develops into a complex monolayer (Crapo, Young et al. 1983; Ward and Nicholas 1984). TJs are critical in the developing lung as they provide the means of compartmentalization required for barrier derivation. TJs are an assembly of resident integral proteins in the membranes of neighboring cells, a collection of diverse accessory proteins, and the cytoskeleton (Balda and Matter 2000; Aijaz, Balda et al. 2006). These junctional structures rely on transmembrane proteins such as occludin, junctional adhesion molecules (JAMs), and a family of tetraspanin molecules called Claudins (Cldns) primarily responsible for establishing robust cell-cell contact (Aijaz, Balda et al. 2006; Chiba, Osanai et al. 2008; Krause, Winkler et al. 2008). This highly conserved family of proteins is composed of 27 members (Mineta, Yamamoto et al. 2011) that produce a variety of tight junctions and thus influence barrier epithelium and characteristic permeability (Morita, Furuse et al. 1999; Anderson 2006). Of this family, Claudin-6 (Cldn6) is identified to be crucial for epithelial cell differentiation and permeability during early embryonic development (Turksen and Troy 2002; Troy and Turksen 2007). In addition to occlusion, a key function for Cldn6 is to also regulate sodium and chloride permeability via the specific geometry of extracellular loops (EL) in the protein's secondary and tertiary structures (Van Itallie and Anderson 2006; Yu, Cheng et al. 2009). During murine lung morphogenesis, Cldn6 is highly expressed between embryonic day (E) 10.5 to E16.5. A general theme exists wherein higher

expression occurs during early stages of lung development with diminished expression as lung morphogenesis proceeds (Turksen and Troy 2002; Troy, Arabzadeh et al. 2009; Jimenez, Lewis et al. 2014).

Given the diversity and complexity of Cldn family members, conditional transgenic over-expression and knock-down models seem to be suitable for testing hypotheses related to tissue-specific roles of individual Cldn members *in vivo*. Indeed, such approaches have been demonstrated when determining the contributions of Cldn6 in deriving models of epidermal morphogenesis and when dissecting trophectoderm formation (Moriwaki, Tsukita et al. 2007). In this research, we evaluated lung morphogenesis in transgenic mice that utilized the human promoter for surfactant protein-C (hSP-C) to conditionally over-express Cldn6 in the respiratory compartment (Perl and Whitsett 1999). The data demonstrate remarkable delays in lung morphogenesis coincident with impaired cell turnover. The data further suggest a role for Cldn6 in the orchestration of perinatal cell differentiation characteristic of maturing pulmonary airspaces. Understanding the role of Cldn6 should shed light on the functions of TJs during lung development and in conditions where compromised lung development predisposes individuals to hypoplastic lung complications.

Materials and Methods

Mice

Two transgenic lines in a C57Bl/6 background were generated and mated to create conditional doxycycline (dox)-inducible mice that over-express Cldn6 (Figure 1A). Dams were fed dox (625 mg/kg; Harlan Teklad, Madison, WI) from before conception until E 18.5. *En block* lungs were harvested and fixed in 4% paraformaldehyde for histological analysis. Tail biopsies were genotyped by PCR for existence of transgenes using the following primers: SP-C-

rtTA forward (5'-GAC ACA TAT AAG ACC CTG GTC A-3') and reverse (5'-AAA ATC TTG CCA GCT TTC CCC-3') and TetO-Cldn6 forward (5'-GAA TTC ATG GCC TCT ACT GGT CTG CA-3') and reverse (5'-TCT AGA TCA CAC ATA ATT CTT GGT GGG A-3'). PCR conditions included 95ºC for 2 minutes and 35 cycles at 95ºC for 30 s, 62ºC (SP-C) or 56ºC (Cldn6) for 30 s, and 72ºC for 45 s. Mice were housed and utilized in accordance with protocols approved by the IACUC at Brigham Young University and at least six mice were included in each group.

Histology and Immunohistochemistry

Cldn6 TG and non-transgenic control lungs (E 18.5) were fixed in 4% paraformaldehyde, processed, embedded and sectioned at 5 μm thickness (Reynolds, Mucenski et al. 2004). Hematoxylin and eosin (H&E) staining was performed to observe general lung morphology. To perform immunostaining for specific markers, slide samples were dehydrated, deparaffinized, processed with antigen retrieval by citrate buffer, and incubated with primary and secondary antisera that utilize HRP conjugation with Vectors Elite Kit (Vector Laboratories; Burlingame, CA) (Reynolds, Mucenski et al. 2003; Reynolds, Stogsdill et al. 2011; Stogsdill, Stogsdill et al. 2012). Antibodies that were used include: Anti-Cldn6 goat polyclonal antibody (C-20, 1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), proliferating cell nuclear antigen (PCNA, SC-7907, 1:500; Santa Cruz Biotechnology), thyroid transcription factor 1 (TTF-1, WRAB-1231, 1:100; Seven Hills BioReagents, Cincinnati, OH), forkhead box A2 (FoxA2, WRAB-1200, 1:100; Seven Hills BioReagents), Clara cell secretory protein (CCSP, WRAB-3950, 1:100; Seven Hills BioReagents), and Surfactant Protein- C (SP-C, WRAB-76694, 1:100; Seven Hills BioReagents).

Immunofluorescence

Immunofluorescent detection of cleaved Caspase-3 (Casp-3, PA5-16335, 1:100; Cell Signaling, Beverly, MA) was performed on 5 μm thick lung sections. Slides were incubated overnight with a rabbit cleaved Casp-3 primary antibody. Sectios were then incubated with anti-rabbit fluorescein conjugated secondary antibodies for 1 hour. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used for nuclear counterstaining. Slides were viewed on a fluorescence microscope with the appropriate excitation and emission filter.

Immunoblotting

Tissues were homogenized in protein lysis buffer (RIPA, Fisher Scientific, Pittsburg, PA). Protein lysates (20 mg) were separated on Mini-PROTEAN® TGX™ Precast gel (Bio-Rad Laboratories, Inc) by electrophoresis and transferred to nitrolcellulose membranes. Membranes were blocked and incubated with a goat polyclonal antibody against Cldn6 (at a dilution of 1:200; Santa Cruz Biotechnology). A secondary donkey anti-goat immunoglobin (Ig)-horseradish peroxidase antibody (1:10,000, Santa Cruz Biotechnology) was incubated in 5% milk for one hour at room temperature. The membranes were incubated with chemiluminescent substrate (Pierce, Rockford, IL) for 5 minutes and the emission of light was digitally recorded by using a C-DiGit® Blot Scanner (LI-COR, Inc, Lincoln, Nebraska). Quantification of Cldn6 was performed by densitometry and normalization with actin provided comparisons between Cldn6 TG and control lung samples.

qRT-PCR

Total RNA was isolated from mouse lungs using an RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). Reverse transcription of RNA was performed using the Invitrogen Superscript III First-Strand Synthesis System (Life Technologies, Grand Island, NY) in order to obtain cDNA

for qRT-PCR. The following primers were synthesized by Invitrogen Life Technologies (Grand Island, NY): Cldn6 (For-GCA GTC TCT TTT GCA GGC TC and Rev-CCC AAG ATT TGC AGA CCA GT) and GAPDH (For-TAT GTC GTG GAG TCT ACT GGT and Rev-GAG TTG TCA TAT TTC TCG TGG). The cDNA amplification and data analysis were performed using Bio Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and a Bio Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories)(Robinson, Johnson et al. 2012). Control wells lacking template or RT were included to identify primer-dimer products and to exclude possible contaminants.

Statistical Analysis

Data were assessed by one- or two-way analysis of variance (ANOVA). When ANOVA indicated significant differences, the Student's t-test was used with the Bonferroni correction for multiple comparisons. The results presented are representative, and P values ≤ 0.05 were considered significant.

Results

Cldn6 Expression Was Up-regulated in the Lungs of Cldn6 TG Mice.

Cldn6 is steadily down-regulated as later stages of lung morphogenesis are encountered (Jimenez, Lewis et al. 2014). Therefore, an inducible, lung specific Cldn6 TG mouse was generated in order to test the hypothesis that persistent Cldn6 impairs normal lung epithelial cell biology (Reynolds, Stogsdill et al. 2011). A human surfactant protein C (hSP-C) promoter was used to express a reverse tetracycline transactivator (rtTA) in alveolar epithelial cells. Double transgenic mice contained the SP-C-rtTA transgene as well as a second transgene that included TetO response elements upstream of the Cldn6 gene (Cldn6 TG, Figure 1A). Dox administration

from conception until sacrifice date at E 18.5 revealed a significant increase in the transcription of the Cldn6 gene, as revealed by quantitative RT-PCR of RNA isolates from Cldn6 TG mouse lungs and age-matched controls (Figure 1B). Protein translation of significantly increased Cldn6 mRNA expression was confirmed by immunoblotting. Lung lysates from Cldn6 TG mice expressed significantly more Cldn6 protein when compared to controls (Figure 1C).

Immunohistochemical analysis was next performed in order to spatially assess elevated Cldn6 in the lungs of Cldn6 TG mice. Cldn6 was only minimally detected in the lungs of E 18.5 control mice (Figure 2). Immunostaining performed with Cldn6 TG mouse lung sections revealed robust, intense staining for Cldn6 in the developing epithelial lining of primitive airways throughout the distal lung (Figure 2). Specifically, Cldn6 expression tended to dominate mid sized airways (Figure 2, arrow) as opposed to the most proximal airways (Figure 2, arrowhead). These data demonstrated that Cldn6 is qualitatively and quantitatively increased in the lungs of Cldn6 TG mice.

Cldn6 Up-regulation Delayed Lung Development in Cldn6 TG Mice

In comparison to controls, morphological alterations in the proximal-distal patterning of the Cldn6 TG mouse lung were discovered following classic hematoxylin and eosin (H&E) staining (Figure 3). Specifically, the numerous distal airspaces observed in 200x and 400x lung images from control lungs (Figure 3, *) were lacking in images obtained from Cldn6 TG mouse lungs. Control mouse lungs displayed stark differences in ciliated columnar epithelium (Figure 3, arrow) and cuboidal/squamous epithelium in the distal lung (Figure 3, arrowhead). Conversely, lungs from Cldn6 TG mice contained a mostly tall cuboidal/columnar epithelial cell phenotype and significantly thickened mesenchymal deposition (Figure 3, #). In summary, control lungs contained thinner alveolar septa, flattened epithelium, and expanding saccules;

however, Cldn6 TG mice exhibited fewer and larger lung spaces with distinctly dense intersaccular mesenchyme. These morphological data suggested delays in the maturation of Cldn6 TG mouse lungs and a hypoplastic canalicular phenotype at a period normally characteristic of the late saccular lung.

To further investigate pulmonary maturation, lung-specific transcriptional regulators were evaluated by immunohistochemistry in the lungs of Cldn6 TG and control mice. Thyroid transcription factor (TTF)-1, also known as Nkx2.1, is a homeodomain-containing transcription factor that is critical for normal lung development (Rojas, Gonzalez et al. 1995). TTF-1 expression was intensely observed in both Cldn6 TG and control mice (Figure 4). However, TTF-1 tended to be diffusely expressed in both the proximal and distal lung compartments in control lungs, but more intensely expressed by airway epithelium in the lungs of Cldn6 TG mice (Figure 4). TTF-1 is known to partner with FoxA2 in the coordination of target gene expression during cell differentiation (Maeda, Davé et al. 2007). FoxA2, which is expressed during lung cell commitment and maturation, was expressed sporadically by epithelium in both the proximal (Figure 5, arrow) and distal lung (Figure 5, arrowhead). Interestingly, FoxA2, a key TTF-1 co-regulator, was almost absent in the Cldn6 TG mouse lung (Figure 5). These findings related to perturbed expression of critical transcription factors support the notion that Cldn6 TG mice are developmentally delayed.

Cldn6 Up-regulation Impaired Proximal and Distal Lung Cell Differentiation

To further phenotypically characterize potential delays in the Cldn6 TG mouse lung, two pulmonary epithelial cell-specific markers were assessed by immunohistochemistry. Clara Cell Secretory Protein (CCSP) and surfactant protein C (SP-C) are targets of TTF-1/FoxA2 transcriptional control programs and they are key proteins expressed by proximal and distal lung

epithelial cells, respectively. CCSP is specifically expressed in non-ciliated epithelial cells of the airway while SP-C is expressed by differentiated alveolar type II cells. As anticipated, marked CCSP expression was detected in the larger airways of controls mice (Figure 6, arrow); however, CCSP was significantly diminished in the airways of Cldn6 TG mice (Figure 6). SP-C was assessed by staining for its proprotein (proSP-C). ProSP-C was detected in numerous differentiating pulmonary epithelial cells in the distal mouse lung of control mice (Figure 6, arrowhead), but highly restricted to persistently larger airways in the Cldn6 TG mouse lung (Figure 6). Abnormal expression of key epithelial cell markers provide additional evidence for delayed pulmonary morphogenesis in Cldn6 TG mice.

Cldn6 Up-regulation Caused a Cell Proliferation/Apoptosis Imbalance in Cldn6 TG Mice

Because the Cldn6 TG mouse lung appeared morphologically delayed (Figure 3) and cell differentiation was potentially impaired (Figures 4-6), we next assessed cell turnover by screening markers for proliferation and apoptosis. Immunohistochemistry was used to assess Proliferating Cell Nuclear Antigen (PCNA), which is a marker of DNA synthesis detectible during the S-phase of a mitotically active cell. As expected, proliferation coincident with PCNA expression was observed in the control mouse lung (Figure 7), but only minimally detected in pulmonary epithelial cell populations in the Cldn6 TG mouse (Figure 7). Interestingly, PCNA activity was not detected in the robust mesenchyme that persisted in the E 18.5 Cldn6 TG mouse lung (Figure 7). Active Casp-3 was also evaluated by immunofluorescence in order to evaluate Casp-3-mediated cell death. Apoptosis controlled by active Casp-3 was observed in the lungs of control mice (Figure 8), but diminished in the lungs of Cldn6 TG mice. No active Casp-3 was observed in control mouse lungs incubated in serum lacking primary antibody (Figure 7, Neg control). Taken together, these data demonstrate decreased cell proliferation and apoptosis in

Cldn6 TG mice as potential causes for delayed morphogenesis observed in Cldn6 TG mice when compared to controls.

Discussion

A key discovery in the current investigation is that persistent, up-regulated Cldn6 positioned between pulmonary epithelial cells causes a developmentally delayed phenotype. These data offer intriguing insight into the potential roles Cldn6 has in the orchestration of branching morphogenesis and cell commitment observed during lung formation. In fact, Cldn6 expression may not only provide barrier integrity during lung organogenesis, but it may also influence the trajectory of terminal cell type differentiation and thus permanently impair normal lung formation.

Defects in lung development that present perinatally are often caused by delayed maturation of the alveolar compartment. A common neonatal condition associated with alveolar hindrance is bronchopulmonary dyplasia (BDP). Specifically, BPD has been characterized by alveolar growth disorders (Chambers and van Velzen 1989; Rojas, Gonzalez et al. 1995) and growth impairment leads to long-term diffuse reduction in the number of alveoli and gas-exchange surface area (Husain, Siddiqui et al. 1998; Jobe 1999). Adult lung injury often results in a growth-arrested lung; however, BPD occurs in growing lungs that have unresolved morphogenetic events. Our research involving highly expressed Cldn6 during lung formation implies a role for TJs in primitive barrier cell populations that transition from one lung developmental period to the next. The formation of definitive alveoli through the process of secondary septation and mesenchymal thinning is fundamentally a postnatal event; however, uncompleted development prior to alveologenesis may be a key factor in compromising later developmental periods (Burri 1997). Neonates susceptible to BPD are generally born in the

early saccular phase, or even in the canalicular phase of lung development for those that are most premature (Burri 1997). Several points during development have been implicated in terms of the pathophysiological mechanisms that lead to BPD including hindered cell proliferation (Jankov and Tanswell 2004), inflammation and fibrosis (Speer 2003), or oxidative stress (Saugstad 2003). Of particular relevance is the work reviewed by Jankov *et al.* that details diminished cellular availability as a causal aspect of lung prematurity and BPD. Our research suggests Cldn6 may influence hindered cell abundance via reductions in both proliferation and apoptosis that ensure cell stagnation in the transgenic lung.

A review of the literature identifies possible *in vivo* models that reproduce lung prematurity similar to BPD. The most relevant model related to the human condition is the baboon that has been prematurely delivered and ventilated (Coalson, Winter et al. 1999). Rodents experience alveologenesis from post-natal days 4-20 and neonatal exposure to hyperoxia inhibits septal formation in a fashion similarly observed in BPD (Warner, Stuart et al. 1998). The present investigation does not purport to establish a novel, working model of BPD, rather our research uncovers previously unknown contributions of Cldn6 in the temporal progression of lung development and maturation.

Diminished staining for TTF-1 and FoxA2, factors implicit in pulmonary epithelial cell refinement, suggested that elevated Cldn6 influences the transcriptional regulation of lung cell differentiation. We have already published *in vitro* research that specifies genetic regulation of Cldn6 mediated by TTF-1, Gata-6, and FoxA2 (Jimenez, Lewis et al. 2014). The current research expands the impact of previous *in vitro* work by demonstrating potential feedback between these factors and Cldn6 availability *in vivo*. TTF-1 regulates target gene expression in concert with other gene regulatory factors to fine tune lung branching morphogenesis and to

coordinate the expression of homeostatic proteins such as surfactant proteins (Bohinski, Di Lauro et al. 1994; Hösgör, Ijzendoorn et al. 2002; Boggaram 2009). In addition, FoxA2 is required for normal airway epithelial differentiation and lung organogenesis (Wan, Kaestner et al. 2004). Thus, Cldn6 augmentation during lung organogenesis may directly or indirectly impact fundamental processes including cell population expansion and differentiation (Jimenez, Lewis et al. 2014).

In summary, susceptibility to impaired branching morphogenesis and lung maturation are features of premature lung disease and other causes of respiratory distress. Our ability to imitate aspects of premature lungs through the genetic up-regulation of Cldn6, a tetraspanin protein involved in TJ assembly, significantly elevates the roles for TJs in the developing lung. Although the results presented in the current publication detail ramifications of Cldn6 over-expression during highly plastic periods of lung embryogenesis, Cldn6 up-regulation should also be carefully screened in the adult mouse. Additional studies in the adult lung that center on increased Cldn6 expression and mechanisms of inflammatory disease, epithelial permeability, and repair processes that rely on proliferation may provide promising targets for intervention.

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Disclosures

The authors declare that they have no actual or potential competing financial interests.

Figures

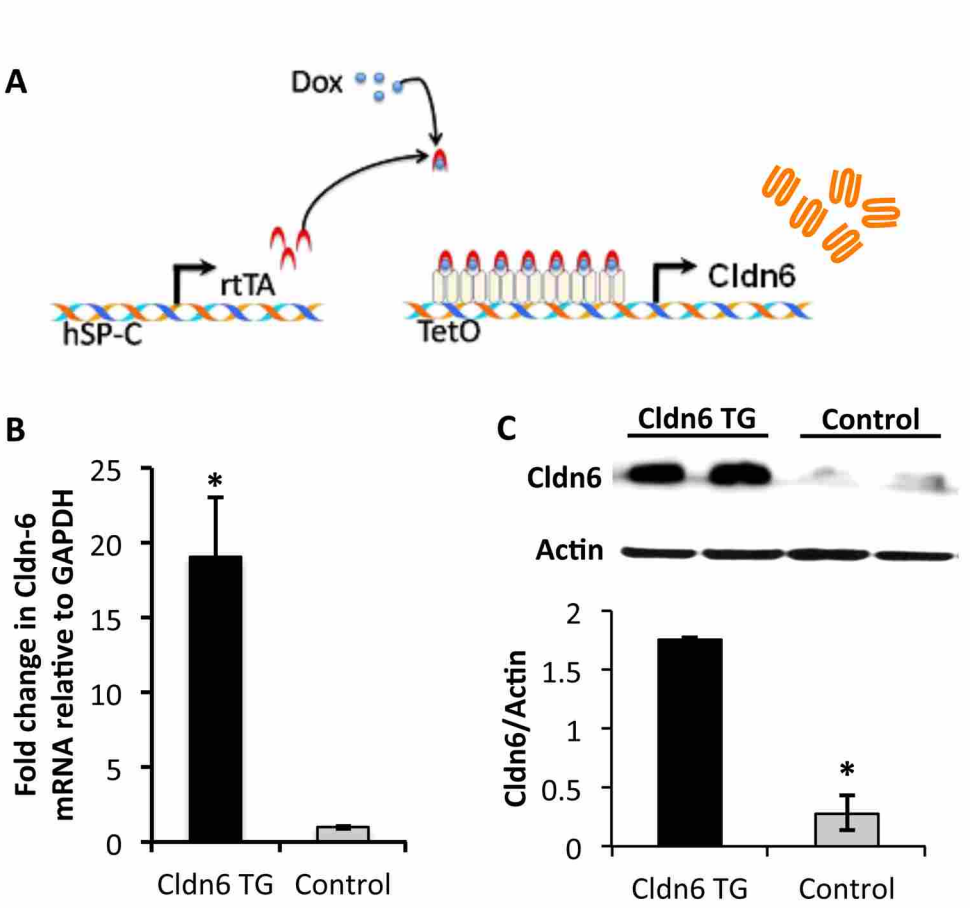

Figure 5.1 Cldn6 TG Mice Up-Regulated Cldn6. A. Doxycycline (dox)-inducible expression of Cldn6 in double transgenic mice. The rtTA protein was expressed using the human SP-C (hSP-C) promoter active in respiratory epithelium. In the presence of dox, rtTA induced the expression of Cldn6 in lung epithelium. B. qPCR for Cldn6 mRNA revealed significantly increased expression in Cldn6 TG mice compaed to controls. Fold changes are presented relative to GAPDH expression. C. Immunoblotting demonstrated significantly increased Cldn6 protein expression in Cldn6 TG mice. Densitometry is presented as ratios of Cldn6 protein intensity divided by actin used as a loading control. Immunoblotting and qPCR data are representative of experiments performed in triplicate and statistical differences are noted (*$P \leq 0.05$).

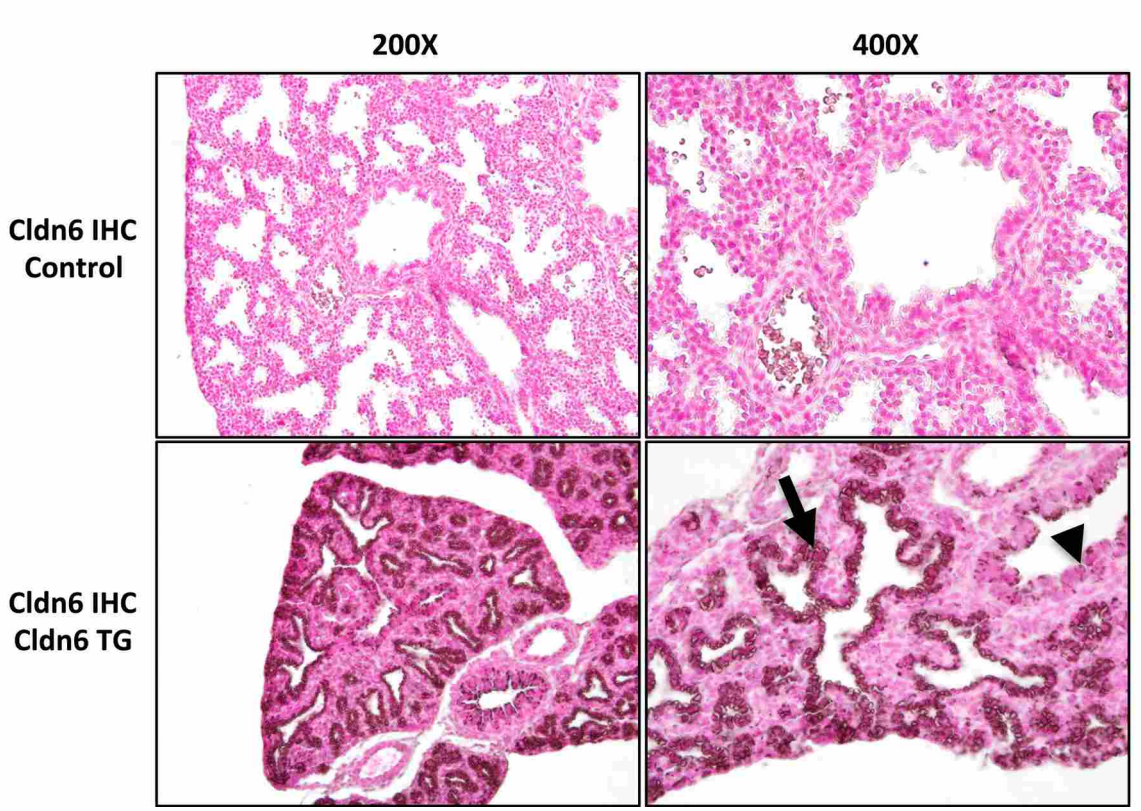

Figure 5.2 Cldn6 TG Mice Expressed Increased Cldn6. Cldn6 was detected by immunohistochemistry in epithelial cells of Cldn6 TG mice, but only minimally detected in the lungs of non-transgenic control littermates. Qualitative staining for Cldn6 revealed staining in the mid sized airways (arrow) and only minimal expression in the larger airways (arrowhead). Images are representative of at least 3 different animals per group and the original magnification of images was at 200x or 400x as indicated.

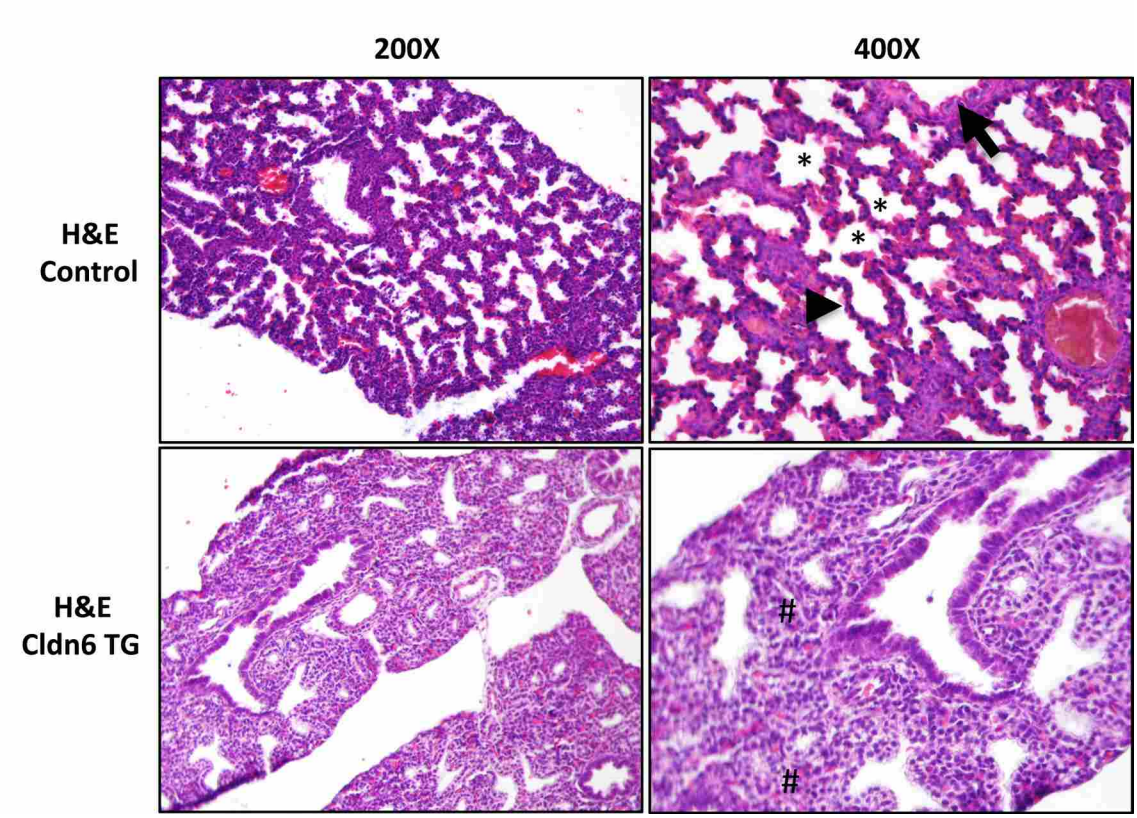

Figure 5.3 Cldn6 TG Mice Expressed Perturbed Lung Morphology. Hematoxylin-eosin staining of lungs from Cldn6 TG mice showed persistence of the cannalicular stage. In particular, prolific distal airspaces in control mouse lungs (*) were lacking in the Cldn6 TG mouse. Controls expressed clear ciliated columnar cells (arrow) and thinning epithelium in the maturing distal lung (arrowhead). A key characteristic of the Cldn6 TG muse lung was persistent thickened mesenchyme (#). Images are representative of at least 3 different animals per group and the original magnification of images was at 200x or 400x as indicated.

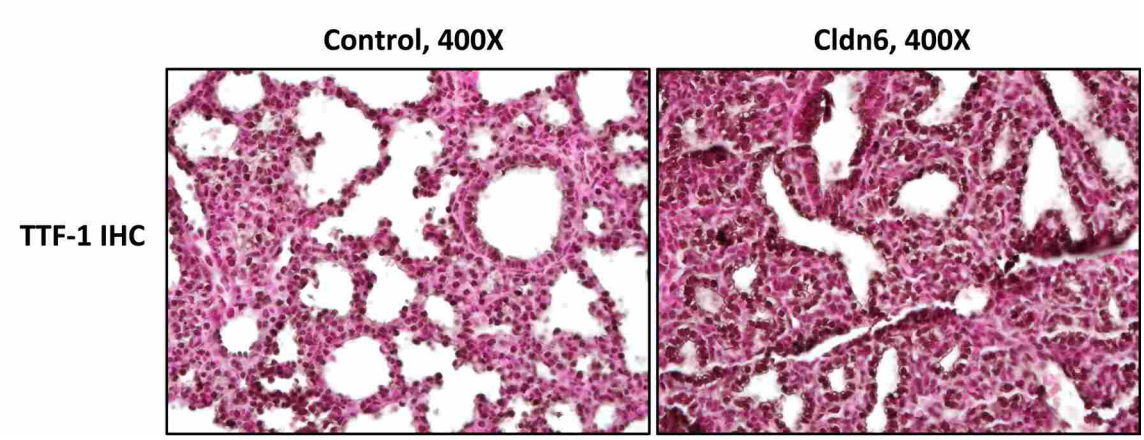

Figure 5.4 TTF-1 Was Expressed by Epithelium in Both the Cldn6 TG and Control Mouse Lung. TTF-1 positive cells were found diffusely throughout the respiratory compartment of control mouse lungs; however, TTF-1 was primarily expressed by airway epithelial cells in the Cldn6 TG mouse. Images are representative of at least 3 different animals per group and the original magnification of images was at 400x as indicated.

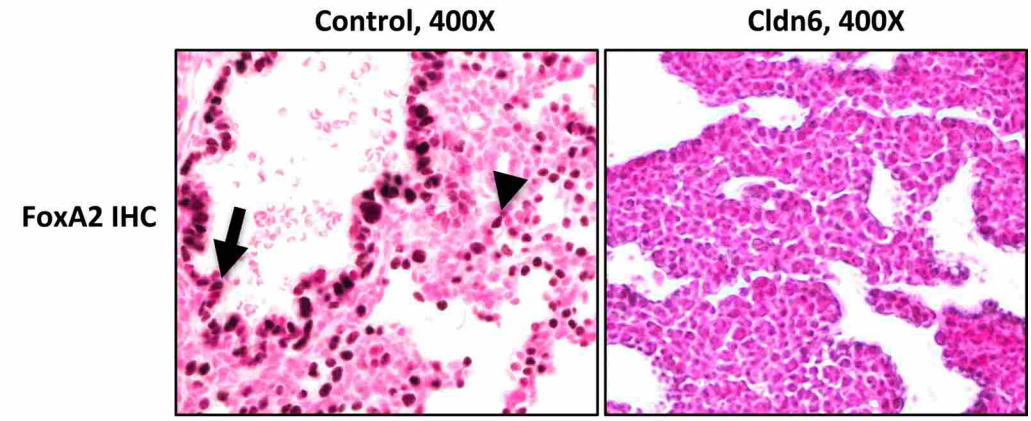

Figure 5.5 Foxa2 Was Not Detected in the Lungs of Cldn6 TG Mice. FoxA2 partners with TTF-1 in order to orchestrate specific gene expression programs necessary for differentiating pulmonrya epithelial cell types. FoxA2 was observed in larger airways of control mice (arrow) and distal lung epithelial cells (arrowhead) in the control mice. However, FoxA2 was nearly absent in the Cldn6 TG mouse lung. Images are representative of at least 3 different animals per group and the original magnification of images was at 400x as indicated.

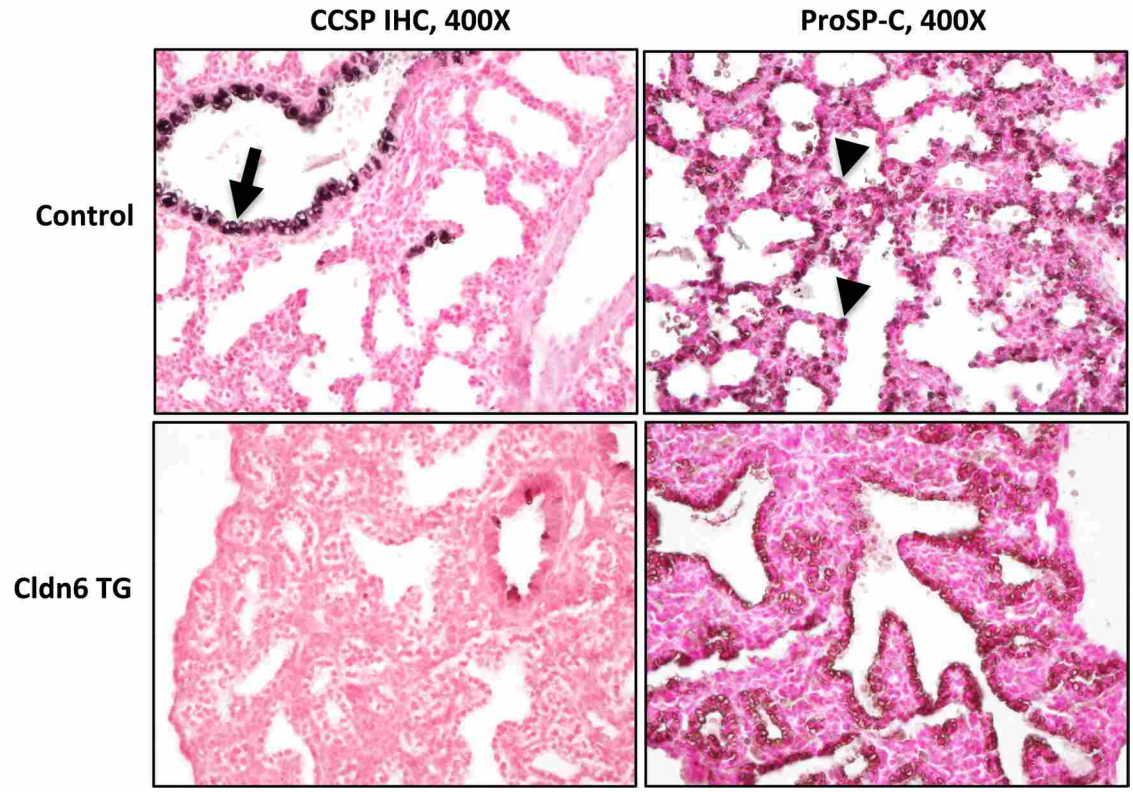

Figure 5.6 Cldn6 TG Mice Had Altered Expression of Proximal and Distal Lung Cell Markers. Clara Cell Secretory Protein (CCSP) was detected in the proximal airways of control mice (arrow), but only a paucity of staining was observed in the airways of Cldn6 TG mice. Staining for the proprotein of surfactant protein C (proSP-C) revealed diffuse staining in the parenchyma of control mice (arrowhead), but proSP-C localization in Cldn6 TG mice was primarily observed in the lining of respiratory airways. Images are representative of at least 3 different animals per group and the original magnification of images was at 400x as indicated.

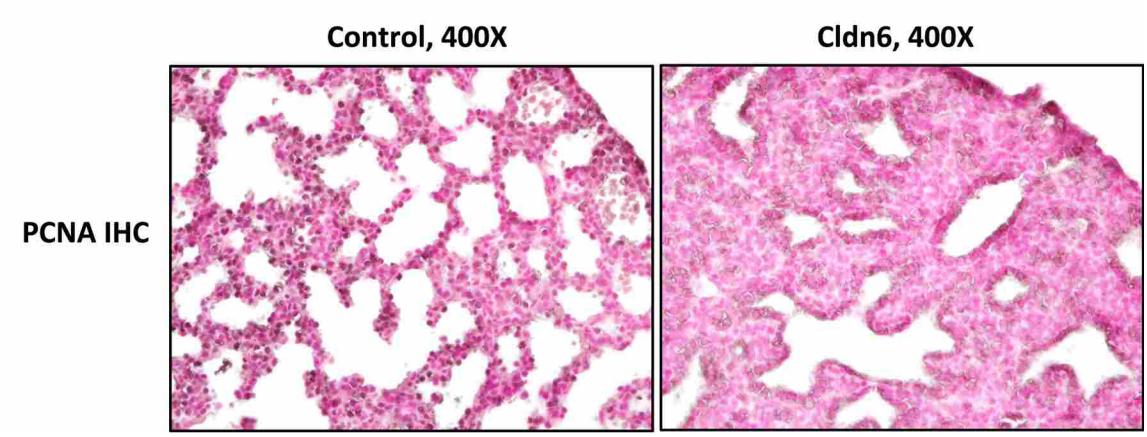

Figure 5.7 Lung Cell Proliferation Was Diminished in Cldn6 TG Mice. Staining for Proliferating Cell Nuclear Antigen (PCNA) revealed highly distributed proliferating cells in control lungs and a near absence of proliferation in the lungs of Cldn6 TG mice. Images are representative of at least 3 different animals per group and the original magnification of images was at 400x as indicated.

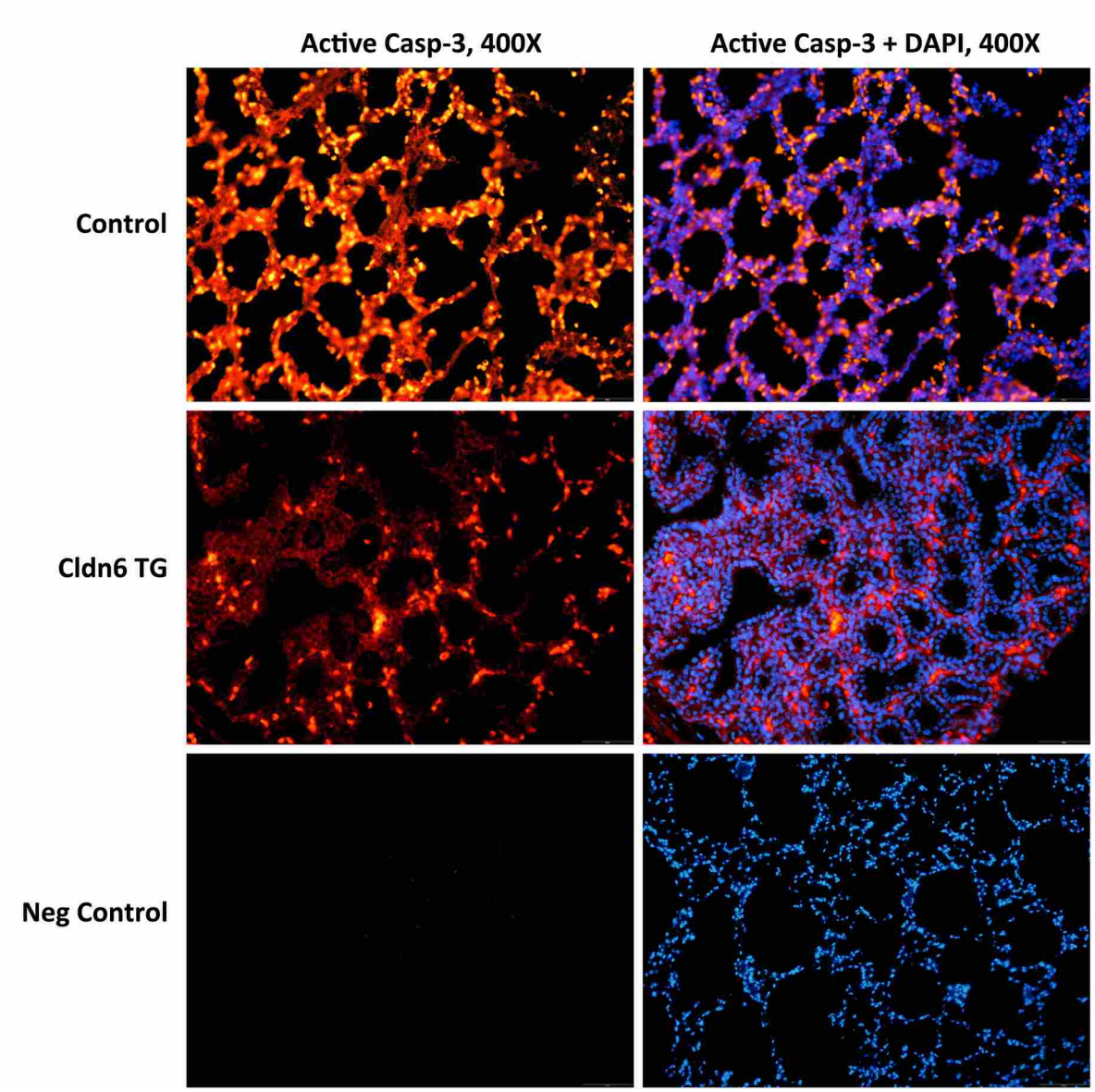

Figure 5.8 Lung Cell Apoptosis Was Diminished in Cldn6 TG Mice. Active caspase-3 (Casp-3) expression in control mouse lungs was detected by immunofluorescence and observed to be highly expressed in the control mouse lung. In comparison to controls, active Casp-3 was decreased in the lungs of Cldn6 TG mice. No expression was observed in control lung sections incubated without primary anti-Casp-3 IgG (Neg control). Images are representative of at least 3 different animals per group and the original magnification of images was at 400x as indicated.

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CHAPTER 6: General Conclusion

The present work analyzed the functional implication of Cldn6 expression during lung organogenesis and its effects during disease onset and development. To our knowledge, this is the first investigation that seeks to elucidate the biology of Cldn6 in the context of lung development and morphogenesis. Pulmonary development involves organized and controlled events of growth and morphogenetic process. These events are regulated by complex interactions among tissue specific genes, signaling molecules, and transcriptional factors (Perl and Whitsett 1999; Warburton, Schwarz et al. 2000; Cardoso 2001).

First, we evaluated the molecular function and expression of Cldn6 in embryonic lung development. Cldn6, a tight junction protein, is up-regulated during the onset of lung development (Turksen and Troy 2001; Troy, Rahbar et al. 2005) and down-regulated as development proceeds (Turksen and Troy 2002). Thus, Cldn6 down regulation, which normally occurs during late gestation, is required for transitioning between the stage of lung growth and morphogenesis. Cldn6 is controlled by transcriptional factors such as TTF-1 (Stahlman, Gray et al. 1996; Silberschmidt, DiLauro et al. 2003). TTF-1 is a nuclear transcription protein expressed by the gene family NKx2, which is selectively expressed in lung cells and the thyroid gland (Boggaram 2009). The expression of TTF-1, although not necessary for the formation of the primitive lung buds, is known to influence development and morphogenesis (Silberschmidt, DiLauro et al. 2003). From a functional standpoint, TTF-1 is involved in the activation and control of lung junctional genes such as Cldns and Occludins which primarily contribute to the development of junctional structures between pulmonary epithelial cells. Furthermore, co-transcriptional factors like Gata-6 (Liu, Morrisey et al. 2002) and SoxA2 (Ganesan and Sajjan 2013) supports TTF-1 by regulating gene expression of surfactant proteins (SP-A, -B, and –C), Clara cell secretory protein (CCSP) (Bingle 1997), and junctional proteins (Niimi, Nagashima et

al. 2001). Our study shows that Cldn6 is involved in prenatal lung organogenesis, is necessary for cell differentiation and proliferation, and is regulated by transcriptional factors.

The lung epithelium is an integral part of the physical barrier against pathogens and hazardous molecules (Matthay, Fukuda et al. 2000), and maintains the balance of gas exchange (Neuhaus, Samwer et al. 2012). In the lungs, junctional structures such as tight junctions and gap junctions are important in holding epithelia sheets. The tight junctions are not a fixed and impermeable barrier; instead, they are dynamic structures and ion selective barriers that actively improve and refresh the lung lining fluid (Schneeberger and Lynch 1992; Anderson 2001). The paracellular permeability is determined by the expression of different Cldns in the epithelial lining (Coyne, Gambling et al. 2003; Krause, Winkler et al. 2008), but it can be altered by harmful gases and pathogens.

Currently, it is unclear how tight junction proteins interact between Cldn members or how they influence the paracellular influx of molecules and ions between the apical to the basal side. Our research revealed that the expression of Cldn6 between epithelial cells is reduced in adult mice exposed to tobacco smoke. This adverse condition causes disruption of tight junctions and cell permiabilization (Amasheh, Rosenthal et al. ; Koval 2013). Furthermore, hypoxia, a condition of O2 deprivation, is another factor that inhibits Cldn expression (Koto, Takubo et al. 2007). Hypoxic condition triggers a set of genes regulated by Hif1a, a transcriptional master regulator. Our data showed that Hif1a down regulates Cldn6 expression in A-549 cells exposed to hypoxia, but mutations on Hif1a response elements (HRE) in the promoter of Cldn6 (0.5Kb) rescue the expression of Cldn6. Our data reveals that Cldns are differentially regulated by tobacco smoke exposure and thus Cldns are potentially involved as neighboring epithelial cell

respond to tobacco smoke. Cell-cell interaction is crucial in lung development and ontogenesis as well as the function and integrity of adult lungs.

Alveolarization, which is an intricate and complex developmental function between late-embryonic and early-postnatal lung development, can be modified by abnormal gene expression. Previously, it was demonstrated that Cldn6 down regulation is necessary to develop normal lungs (Troy, Rahbar et al. 2005; Jimenez, Lewis et al. 2014). Our data supported the conclusion that up-regulation of Cldn6 during late periods of lung development resulted in failure of airspaces formation and delayed lung morphogenesis. Also, up-regulation of Cldn6 during late periods of lung embryo development (E18.5) did not alter embryonic lung formation, but transitorily impaired and delayed branching and alveolar development. Temporal impairment of lung development may be associated to the reduction of cell apoptosis and proliferation.

In summary, this research investigates the function of Cldn6 expression and transcriptional factors not only to understand how the lung develops, but also to comprehend how lungs are repaired from mechanical damage and diseases. Despise of the notable advancements in Cldn6 research provided, additional work is necessary that focuses on the effects over expressing Cldn6 in adult mice lungs that have been exposed to tobacco smoke.

Relevance of Research

About 15 million people in the United States suffer from chronic obstructive pulmonary disease (COPD). Not only is this number of COPD cases increasing, but COPD is also the third leading cause of death in the United States. COPD is due to a combination of disease of the small airways (obstructive bronchiolitis) and parenchymal destruction (emphysema). The chronic inflammation causes lung remodeling and narrowing of the airway. Additionally, the parenchymal destruction triggered by inflammation causes loss of alveolar junctions to the small

airways, decreasing pulmonary elasticity. The first line of defense in combating lung disease is to identify factors that may help to recover junctional structures. We developed a genetically engineered mouse model in which the Cldn6 gene is over-expressed conditionally in the lung. This will allow us to identify possible benefits of persistent tight junctions so that elasticity can persist in the lungs and may recover after damage. This will help us to identify and manipulate signaling cascades that drive the pathological cell behavior producing abnormal responses in lung disease involving edema. This research could provide further evidence supporting the role of Cldn6 in the progression of pulmonary diseases. This research will also improve our knowledge of Cldn6 contributions to normal pulmonary development and diseases possibly mediated by abnormal tight junctions. These experiments will bridge developmental findings with potential disease relevance in both the neonate and the adult.

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Concentration: Lung development, measurement of gene expression, research analysis, and evaluation.

M.S. of Science in Genetics and Biotechnology: August 2011

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Dissertation: Assessment of genetic diversity in Peruvian amaranth (Amaranthus caudatus L. and A. hybridus L.) germplasm using SNP markers. Developing SNP and SSR markers for orphan Andean crops (Ullucus tuberosus, Caldas) using 454 pyrosequencing.

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Concentration: Orphan Andean crops, Pyrosequencing, Flow cytometry techniques to determine genome size, chromosome counting, and molecular techniques.

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Dissertation: Determination of the variability and performance of two species of uncucha tuber (Xanthosoma sagittifolium, L. Schott) under ecological conditions of K'ayra, Cusco-Peru.

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Project: This study was designed to assess gene adaptation, genetic segregation, and population genetics of two Andean crops using SNP and SSR arrays. I used genome reduction and next-generation sequencing to develop SNP and SSR arrays, which later was validated by Fluidigm® technology and evaluated by software-packages for inferring genetic population and phylogenetics.

Duties:

• Design and conduct research using SNP and SSR marker in Andean crops.

• Data collection, management, and analysis

• Operate, maintain and train undergraduate students on laboratory equipment and protocols

• Present results at national scientific meetings

• Prepare and submit data for publication in peer reviewed journals

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Project: This project focuses on Claudin-6, a tight junctional protein that is highly expressed in the lung during critical periods of lung organogenesis. I was the first to characterize a precise temporal-spatial pattern of Claudin-6 expression during embryogenesis and discovered that Cldn-6 is transcriptionally inhibited by tobacco smoke exposure. This work was innovative because the generation of a conditional transgenic mouse that up-regulates Cldn-6 expression using the rtTA-TetOn system.

Duties:

• Design and conduct research using Claudin 6 transgenic mice model.
• Data collection, management and analysis
• Operate, maintain, and train on laboratory equipment
• Present results at national scientific meetings
• Prepare and submit data for publication in peer reviewed journals

Undergrad Research Mentor/Lab Coordinator Jan 2012 to present

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• Prepare students for poster or oral presentation at national scientific meetings

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OTHER RESEARCH EXPERIENCE

Laboratory Manager and Research Jan 1997 to Mar 2002

CICA, Peru I Worked the Centre of Research in the Andean Crops (CICA). I was responsible for the full development and management of the CICA laboratories.

Laboratory Assistant Jan 1996 to Jan 1997

UNSAAC, Peru San Antonio Abad of Cusco State University (UNSAAC). Work as a laboratory assistant.

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PUBLICATIONS AND PRESENTATIONS AT PROFESSIONAL MEETINGS

Peer-reviewed manuscripts

In Print:

- Jimenez F, Maugham P, Alvarez A, Keitlinski K, Smith S, Pratt D, Elzinga D, and Jellen E. 2012. Assessment of genetic diversity in Peruvian amaranth (Amaranthus caudatus L. and A. hybridus L.) germplasm using SNP markers. Crop Sci 2012 -07-0413-ORA.R1
- Kietlinski KD, Jimenez F, Jellen EN, Maughan PJ, Smith SM, Pratt DB. Relationships between the Weedy (Amaranthaceae) and the Grain Amaranths. Crop Science. 2013 Dec;54(1):220-228.
- Felix R Jimenez, Joshua B Lewis, Samuel T Belgique, Tyler T Wood and Paul R Reynolds. Developmental Lung Expression and Transcriptional Regulation of Claudin-6 by TTF-1, Gata-6, and FoxA2. Respiratory Research.
- Chatwin, Warren B., Kyrie K. Carpenter, Felix R. Jimenez, Dave B. Elzinga, Leigh A. Johnson, and Peter J. Maughan. Microsatellite primer development for post oak, Quercus stellata (Fagaceae). Applications in plant sciences 2, no. 10 (2014).
- Duane R Winden, David B Barton, Bryce C Betteridge, Jared S Bodine, Cameron M Jones, Geraldine D Rogers, Michael Chavarria, Alex J Wright, Zac R Jergensen, Felix R Jimenez, and Paul R Reynolds. Antenatal exposure of maternal secondhand smoke (SHS) increases fetal lung expression of RAGE and induces RAGE-mediated pulmonary inflammation. Respiratory research 15, no. 1 (2014): 129.
- Bodine, B. Garrett, Brock G. Bennion, Emma Leatham, Felix R. Jimenez, Alex J. Wright, Zac R. Jergensen, Connor J. Erickson et al. Conditionally induced RAGE expression by proximal airway epithelial cells in transgenic mice causes lung inflammation. Respiratory research 15, no. 1 (2014): 1-10.

In Review:

- Jimenez FR, Maughan PJ, Patton KC, Arano I, Nielsen K, Meza G, and Jellen EN. SNP Discovery and Validation Using Genome Reduction and Next-Generation Sequencing in the Orphaned Andean Tuber Crop Papalisa, Ullucus tuberosus Caldas (2n = 2x = 24). Crop science 2014.
- Felix R. Jimenez, Samuel T. Belgique, Scott A. Albright, Cameron M. Jones, Jason R. Gassman, and Paul R. Reynolds. Conditional pulmonary overexpression of Claudin 6 (Cldn6) during embryogenesis delays lung morphogenesis. Respiratory Research 2014.
- Felix R. Jimenez, Josh B. Lewis, Samuel T. Belgique, Dallin C. Milner, Derek R. Marlor, and Paul R. Reynolds. Cigarette Smoke Induces permeability disruption of Airway Epithelial Tight Junctions. Respiratory Research 2014.

Meeting Abstracts:

- Jimenez FR, Maughan PJ, Meza G, Probst OS, Ames NC, Patton KC, Udall JA, and Jellen EN.. SNP Discovery and Validation Using Genome Reduction and Next-Generation Sequencing in the Orphaned Andean Tuber Crop Papalisa, Ullucus tuberosum. PAG Conference, 2010

- Jimenez FR, Maughan JP, Alvarez A, Kietlinski KD, Smith SM, Pratt DB, Elzinga DB, and Jellen EN. Assessment of genetic diversity in Peruvian amaranth (Amaranthus caudatus L. and A. hybridus L.) germplasm using SNP markers. PAG Conference, 2011
- Jimenez F, Maughan PJ, Alvarez A, Pratt DB, Kietlinski K, Smith S, Udall JA, Patton K, Jellen EN. Application of Markers Developed from Genome Reduction-454 Sequencing in Orphaned Andean Crops. Plant and Animal Genome XIX Conference. San Diego, CA. 2012 Jan.
- Jimenez FR, Lewis JB, Wood TT, Reynolds PR. Developmental expression and transcriptional regulation of claudin-6 in the murine lung. FASEB Journal. 2013 Apr; 27:256.4.
- Jimenez FR, Lewis JB, Wood TT, Belgique ST, and Reynolds PR. Pulmonary Expresion and regulation of Claudin-6 by Tabaco smoke. FASEB Journal. 2014 Apr; 18:834.3

Academic Conference Presentations:

- Jimenez FR, Lewis JB, Wood TT, Reynolds PR. Developmental expression and transcriptional regulation of claudin-6 in the murine lung. FASEB Journal. 2013 Apr; 27:256
- Jimenez FR, Lewis JB, Wood TT, Reynolds PR. developmental expression of Claudin 6 and its contribution to respiratory membrane stability. University of Utah, 2014

INSTITUTIONAL SERVICE

Medical Interpretation — Aug 2009 to Jan 2010
Utah Valley Regional Medical Center, Provo, Utah 84604, USA
Interpreted English to Spanish for doctors and patients for 100 hours

Volunteer Teaching — Jan 2008 to May 2008
West high School, SLC, Utah, 84103, USA
Helping Latino students Math and related courses for 50 hours

AWARDS AND RECOGNITIONS

- BYU Teaching Assistantship: 5
- BYU Research Assistantship: 3
- 2014 American Physiology Society (APS) Minority Travel Award: 2
- 2010 The Marlow C. Woodward Trust Scholarship

PROFESSIONAL AFFILIATIONS

- School of Engineers in Peru (CIP)
- The American Physiology Society (APS), 2011 - present
- Society for Developmental Biology, 2011- present

SKILLS

- Fluent verbal and written proficiency in Spanish and English.

- Strong computer skills including Microsoft office, S10, C+, R program, Auto Cat, Photoshop. Software for population genetics inference such as Past, Arlequin, Structure, and Phylip.

- Laboratory Skills:
  Lab Equipment
    • Light microscopes, incubators, tissue processing machine, tissue embedding machine, microtome, pH reader, spectrophotometer
  Mouse Work and Surgery
    • Mice modeling, Husbandry, Handling
    • Mice embryonic pup removal, Thoracic surgery,
    • Lung resection from embryonic pups
    • Mouse lung lavage
  Molecular Techniques
    • Cell culture, Tissue characterization, transient transfection
    • Mammalian vector cloning and transgenic constructions
    • Immunohistochemistry, Immunofluorescence
    • Western blot, Protein isolation, immunoprecipitation , SDS-PAGE protein analysis
    • Immunohistochemistry
    • DNA isolation, PCR, Gel Electrophoresis, Fluidigm®, SNP genetyping
    • ELISA
    • Flow Cytometry
    • RNA isolation, cDNA Plate Array, qRT-PCR
