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Alterations in Tight Junctional Proteins and Their Effects on Pulmonary Inflammation

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Alterations in Tight Junctional Proteins and Their
Effects on Pulmonary Inflammation

Joshua B. Lewis

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

Alterations in Tight Junctional Proteins and Their Effects on Pulmonary Inflammation.

Joshua B. Lewis

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Doctor of Philosophy

The lungs represent one of the earliest interfaces for pathogens and noxious stimuli to interact with the body. As such, careful maintenance of the permeability barrier is vital in providing homeostasis within the lung. Essential to maintaining this barrier is the tight junction, which primarily acts as a paracellular seal and regulator of ionic transport, but also contributes to establishing cell polarity, cell-to-cell integrity, and regulating cell proliferation and differentiation. The loss of these tight junctions has been documented to result in alterations in inflammation, and ultimately the development of many respiratory disorders such as COPD, Asthma, ARDS, and pulmonary fibrosis. One critical contributor that creates this permeability barrier is the tight junctional protein Claudin. While studies have begun to elucidate the various functions and roles of various Claudins, our understanding is still limited. To initially investigate these proteins, we looked at both temporal and spatial expression patterns for family members during development. A consistent pattern was demonstrated in mRNA expression for the majority of Claudin members. In general, Claudin expression underwent rapid increase during time periods that correlate with the pseudoglandular/canalicular periods. One notable exception was Claudin 6 (Cldn6), which demonstrated decreasing levels of mRNA expression throughout gestation. We also sought to understand expression dynamics during the addition of maternal secondhand smoke (SHS) which resulted in an almost universal decrease in Claudin proteins. To more fully explore expression mechanisms that affect Claudin-6 (Cldn6), we exposed pulmonary alveolar type II (A549) cells to cigarette smoke extract (CSE) and found that it transcriptionally regulated Cldn6 expression. Using a luciferase reporter, we determined that transcription was negatively regulated at multiple promoter response elements by CSE, and transcription was equally hindered by hypoxic conditions. These findings identified Cldn6 as a potential target of SHS and other respiratory irritants such as diesel particulate matter (DPM). We next sought to assess whether an increase in Cldn6 was sufficient to provide a protective advantage against harmful exogenous exposure. To test this, we utilized a doxycycline induced Cldn6 over-expressing mouse, and subjected it to SHS for 30 days to stimulate an inflammatory state. Our findings demonstrated that Cldn6 transgenic animals have decreased inflammation as evidence by decreased total cell infiltration into the airways, decreased polymorphonuclear (PMNs) extravasation, total protein in bronchoalveolar lavage fluid (BALF), and decreased cytokine secretion. Anti-inflammatory advantages were also discovered during experiments involving acute exposure to DPM. In both cases, while stimulation of transgenic mice with SHS or DPM diminished Cldn6 expression, anti-inflammatory evidence emerged suggesting that genetic up-regulation of Cldn6 likely causes the recruitment of other tight junctional components during an organism's response to environmental assault.

Key Words: claudin 6, secondhand smoke, diesel particulate matter, hypoxia, lungs

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Sir Isaac Newton is attributed with the quote, “If I have seen further than others, it is by standing on the backs of giants.” I echo his sentiments, but feel that my own personal success is more of a product of the love and support from the personal giants in my own life rather than my efforts. Indeed, I would be truly ungrateful not to acknowledge the many individuals who have helped me succeed. At this time I would like to formally extend my sincerest gratitude to my advisor and mentor, Dr. Paul Reynolds. In the time that I have known him, he has taught me valuable lessons in both science and research. More importantly however, his friendship, kindness, support, and example have given me the education, tools, and motivation to be the best I can. I also wish to thank Dr. Juan Arroyo for his kind friendship and continual guidance while at BYU. I consider Dr. Arroyo my second mentor, as he has given countless advice that has aided me not only in my scholastic endeavors but also in life. Additionally, I would like to thank Dr. Benjamin Bikman, who I was able to regularly interact with and learn from through various collaborations. To all the students in the Reynolds/Arroyo lab (and there has been many), I am indebted to you for your tremendous aid. I can honestly say I would not have been half as successful without your efforts. Lastly, I feel a profound sense of obligation to express my gratitude towards my family, and specifically my parents. My parents have always supported and encouraged my ambitions and aspirations, and have encouraged me in both my highs and my lows. Their example of perseverance and resiliency despite adversity has been inspiration for me in times of struggle. They may never fully understand the effects of their love and support, but nevertheless, I am eternally grateful.

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LIST OF SYMBOLS AND ABBREVIATIONS

A549	Alveolar Type II like Epithelial Cells
AGE	Advanced Glycated End-Products
ANOVA	Analysis of Variance
ARDS	Acute Respiratory Distress Syndrome
ATI	Alveolar Type I Cell
ATII	Alveolar Type II Cell
BALF	Bronchoalveolar Lavage Fluid
BPD	Brochopulmonary Dysplasia
BYU	Brigham Young University
CLDN	Club Cell Secretory Protein (Clara Cell Secreting Protein)
COPD	Chronic Obstructive Pulmonary Disease
CSE	Cigarette Smoke Extract
DPM	Diesel Particulate Matter
Dox	Doxycycline
E	Embryonic
FAMRI	Flight Attendants Medical Research Institute
FGF	Fibroblast Growth Factor
FoxJ1	Forkhead Box Transcription Factor J1
H&E	Hematoxylin and Eosin
HIF-1 α	Hypoxia Induced Factor 1 alpha
HMGB1	High Mobility Group Box Protein 1
HRE	HIF-1 α Response Element

IACUC	Institutional Animal Care and Use Committee
IL-1 β	Interleukin 1 β
IUGR	Intrauterine Growth Restriction
kDa	kiloDalton
MEG	Mentoring Environment Grant
MMP	Matrix Metalloproteinase
mTOR	Mechanistic Target of Rapamycin
NF- κ B	Nuclear Factor kappa B
PCNA	Proliferative Cell Nuclear Antigen
PE	Preeclampsia
PN	Post Natal
RA	Room Air
RAGE	Receptor for Advanced Glycation End-Products
rtTA	Reverse Tetracycline Transactivator
SAEC	Primary Small Airway Epithelial Cells
SHS	Secondhand Smoke
SP-C	Surfactant Protein C
SAGE	Semi-synthetic glycosaminoglycan ethers
TetO	TetOn
TG	Transgenic
TJ	Tight Junctions
TNF α	Tumor Necrosis Factor alpha
TTF-1	Thyroid Transcription Factor 1

TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type
ZO	Zona Occludins

CHAPTER 1: Introduction

COPD is one of the leading causes of mortality and morbidity and currently is estimated to affect roughly 5% of the world's population or about 329 million individuals (WHO, The Top 10 causes of Death Factsheet 2012). Globally it is projected to be the third leading cause of death by 2020, and it was estimated that the economic burden resulting from COPD was \$2.1 trillion dollars in 2010 (Lomborg, 2013a). Research continues to seek clarity on the mechanisms, trends, or factors that lead to the development of COPD, none of which are completely understood. However, research has shown that cigarette smoke exposure is the most considerable risk factor in developing COPD (D. Morse & I. O. Rosas, 2014). In addition to tobacco cigarette smoke, noxious particulate matter or harmful gases such as found in normal air pollution are also implicated in the development of COPD. Other factors that may play a role in the development of COPD are gender (Aryal, Diaz-Guzman, & Mannino, 2013), occupation (Ulrik et al., 2013) and environmental factors (Gordon et al., 2014; S. Salvi, 2014).

In terms of the pathophysiology, COPD is typically characterized by airflow obstruction that is minimally reversible. This airflow obstruction is due to chronic inflammation and permanent pulmonary airspace enlargement as well as the loss of elastic recoil caused by the destruction of alveolar walls (emphysema). Persistent inflammation in COPD patients is found not only in the airways but additionally in the respiratory parenchyma and the pulmonary vasculature, and results in disruption of normal lung function specifically through remodeling of the distal pulmonary airspaces.

In tandem with this finding, research has begun to demonstrate an insidious correlation between lung cancer and COPD. There have been many studies, including epidemiological studies, population-based cohort studies, lung cancer screening, and interventional trials that

have documented the close relationship between COPD and lung cancer (de-Torres et al., 2015; Kazerouni, Alverson, Redd, Mott, & Mannino, 2004; Young & Hopkins, 2010). Moreover it has been shown that the presence of COPD in the lower airways increases a long-term smoker's risk for developing lung cancer by 4.5 fold, (Z. Wang, 2013; Robert P Young et al., 2009; R. P. Young et al., 2009). Of particular interest is the estimation that worldwide, approximately 90% of lung cancer cases are associated with tobacco smoking (Cokkinides et al., 2009). Thus, it is easy to imagine that similar mechanisms may be activated in the development of these two diseases.

Although these mechanisms are only incompletely understood, tight junctions within lung epithelial have been recognized as critical components in the development of these and many other respiratory diseases. It has long been understood that increased epithelial permeability is characteristic of mucosal inflammation, such as found in many respiratory diseases like COPD, emphysema, acute respiratory disease (ARDS) and acute lung injury (ALI). Confirming their importance, recent evidence has revealed that environmental exposures to noxious material such as air pollution, allergens, and cigarette smoke reduces tight junction barrier integrity thus contributing to the accumulation of persistent inflammatory conditions found in the aforementioned diseases (Rotoli et al., 2008; Andrea C Schamberger, Nikica Mise, Jie Jia, et al., 2014; Thevenot et al., 2013). The evidence unequivocally demonstrates the necessity for maintenance of the permeability barrier, and also alludes to the possibility that mechanisms that strengthen the epithelial barrier may provide possible therapeutic targets by providing protection from harmful materials and preventing inflammatory conditions. Consequently, more research on the individual components within epithelial tight junctions is merited to help illuminate the mechanisms that contribute to these processes.

Tight Junctions

Tight junctions were first observed in 1963 (Farquhar & Palade, 1963) however it was not until 1986 when the first tight junctional protein Zona Occludins-1 (ZO1) was discovered (Stevenson, Siliciano, Mooseker, & Goodenough, 1986). Since then there has been increased research documenting the variety of proteins located at the tight junction, and with each new protein there is additional insight as to the various roles that tight junctions play in maintaining proper physiological functions throughout the body. Initially, tight junctions were believed to be simple paracellular seals. However, it is understood now that tight junctions not only participate in maintaining the barrier function, but are also implicated in regulation of cell polarity (Kirschner & Brandner, 2012), and in proliferation and differentiation (K. Matter & Balda, 2007). As tight junctions are responsible for maintaining the permeability barrier, they are therefore critical in keeping harmful exogenous material and pathogens from penetrating into lung tissue, and as such can be considered part of the innate immune system.

Tight junctions are composed of a range of proteins (Figure 1), such as Junctional Adhesion Molecules (JAMS), Occludins (Occludin), Claudins (Claudin), and members of the Tight-Junctional Associated Marvel Protein (TAMP) family. These tight junctional proteins interact with neighboring cytoplasmic scaffold proteins such as Zona Occludins (Umeda et al., 2006), and Cingulin (Guillemot, Spadaro, & Citi, 2013). This interaction allows for anchoring to the cytoskeleton, as well as providing a possible mechanism to regulate cellular signaling pathways, and thus influence paracellular flux of ions. Due to the fact that tight junctions play such vital roles in maintaining cellular polarity and proper barrier functions in epithelial cells, they have been identified as potential therapeutic targets for drug delivery, in lung disease, and in the

progression of cancer (L. Ding, Lu, Lu, & Chen, 2013; Holgate, Roberts, Arshad, Howarth, & Davies, 2009; Kominsky, 2006; Takahashi, Kondoh, Suzuki, & Yagi, 2011).

Claudins

Claudins were originally first discovered in 1998 by researchers Mikio Furuse and Shoichiro Tsukita while investigating tight junctional proteins (M. Furuse, Fujita, Hiiragi, Fujimoto, & Tsukita, 1998). These transmembrane proteins are similar to Occludin in that they are composed of four transmembrane domains, including two extracellular loops, and two intracellular loops (Figure 2). Despite their physical similarities, it has been shown that Claudin proteins have no sequence similarities with the Occludin family of proteins (M. Furuse et al., 1998). Research has additionally demonstrated that the Claudin family (not Occludin nor Junctional Adhesion Molecules) is primarily responsible for forming selective channels that regulate paracellular transport (Milatz et al., 2017) and constitute the tight junctional component that is critical in maintaining proper physiological conditions. Using knockout mice, it has been shown that Occludin null mice are viable (Saitou et al., 2000) although infertile. In contrast, research has shown that the loss of Cldn1 results in excessive water loss and lethality (M. Furuse et al., 2002), and that the loss of Cldn5 results in severe brain hemorrhaging (Nitta et al., 2003). Additional studies have shown that the loss of Cldn11 and Cldn14 results in the inability to properly maintain mechanisms critical for hearing (Ben-Yosef et al., 2003; Kitajiri et al., 2004) and that the loss of Cldn18 results in increased inflammation in the lung (G. Li et al., 2014). In conclusion, this and other data overwhelming implicates Claudin as the critical tight junctional protein in maintaining normal homeostasis.

At least 24 different Claudins have been identified among mammals and are typically separated into two different groups: the classical (which are composed of Claudins 1-10, 14, 15,

17, and 19), and the non-classical, which constitute the rest. The distinction between the two groups is that within the classical groups, sequence homology has been shown to be very similar. It is currently believed that the larger extracellular loop is responsible for determining the paracellular tightness and the selective ion permeability abilities of the respective Claudin protein (Amasheh et al., 2002; Colegio, Van Itallie, Rahner, & Anderson, 2003; Hou, Paul, & Goodenough, 2005). The second and shorter extracellular loop is believed to cause narrowing of the paracellular cleft (Piontek et al., 2008) and additionally acts as an anchoring point between opposing cell membranes (Blasig et al., 2006).

In terms of functions, certain Claudins have been characterized and grouped according to specific functions and roles. Claudins-2, -10b, and -15 have been identified as selective cation channels (Mikio Furuse, Furuse, Sasaki, & Tsukita, 2001; Günzel et al., 2009; Van Itallie, Fanning, & Anderson, 2003; Yu et al., 2009) and Claudin-10a, and -17 have been identified as selective anion channels (Günzel et al., 2009; Susanne M Krug et al., 2012). Additional functions include water selectivity (Cldn2) and charge selectivity (Cldn-4, -8, -14) Additionally, certain Claudins have specific expression patterns and associated with certain areas of the body. For example, Claudin-1 is essential for maintaining the epidermal barrier (M. Furuse et al., 2002), whereas Claudin-5 is critical in regulating the blood-brain barrier (Nitta et al., 2003). However, in general Claudin proteins are broadly expressed and are not restricted to certain areas in the body.

In regards to Claudin expression within the lungs, Claudins 1, 3, 4, 5, 7, 8, and 18 have been documented in human bronchi and bronchioles (C. B. Coyne, T. M. Gambling, R. C. Boucher, J. L. Carson, & L. G. Johnson, 2003; Brandy L Daugherty et al., 2004; Riitta Kaarteenaho, Heta Merikallio, Siri Lehtonen, Terttu Harju, & Ylermi Soini, 2010; Mazzon &

Cuzzocrea, 2007) and there have been discrepant reports as to expression of Claudin-2 (Lappi-Blanco et al., 2013; Sun, Minshall, & Hu, 2011; R. Xu, Li, Zhou, Perelman, & Kolosov, 2013). Immunohistochemical stains have revealed that Claudins 3, 4, 7, were detected in type 2 alveolar cells, and were not found in type 1 cells (R. Kaarteenaho, H. Merikallio, S. Lehtonen, T. Harju, & Y. Soini, 2010). Additionally, it has been reported that Claudin-1, -3, and -7 are localized between goblet cells and ciliated airway epithelial cells (Flynn, Itani, Moninger, & Welsh, 2009). Our own research has additionally revealed that Claudin-6 is expressed during early embryonic development and that expression diminishes after peaking early in gestation in murine models (F. R. Jimenez, Lewis, Belgique, Wood, & Reynolds, 2014).

Increasing evidence has emerged implicating Claudins as critical players in the innate immune system found in the lungs. Subsequently, many claudins have been targeted in relation to their roles in lung disease and lung cancer, specifically in conjunction with cigarette smoking. For example, one study recently showed that exposure of BEAS-2B cells and cancer cell lines to cigarette smoke for 2 hours initially increased expression in Claudins 2-4 and 7, but after 6 hours of exposure to cigarettes, Claudin 1-5, and 7 decreased in expression (Merikallio et al., 2011b). Another recent study showed through microarray analysis that in a comparison of lung tissue in healthy smokers vs. smokers with COPD Claudin 1, 3, 4, 8, 9, expression was significantly down-regulated, whereas Claudin-7, and 10 expression was significantly up-regulated (R. Shaykhiev et al., 2011). Using knockout studies, it has been additionally shown that removal of Claudin-4 increases susceptibility to lung injury (Kage et al., 2014). Moreover, a subsequent study showed using analysis of bronchioalveolar lavage fluid, that protein expression of Claudins 3, 4, and 18 were increased in severe lung injuries (Jin et al., 2013). Although this evidence demonstrates that Claudin may play a key role in the mechanisms that contribute to lung injury,

more research is needed to further elucidate this possible relationship in order to provide therapeutic value.

Claudin 6

Research surrounding Claudin-6 (Cldn6) is both broad and diverse. Early studies identified Cldn6 as one of the earliest molecules to be expressed in embryonic stem cells that are committed to epithelial cell types (Turksen & Troy, 2001). Due to high expression of Cldn6 in undifferentiated cells (Ben-David, Nudel, & Benvenisty, 2013), Cldn6 has been targeted in many studies surrounding stem cell research. Recently it was shown that conditional expression of Cldn6 in the mouse F9 stem cells triggered epithelial morphogenesis. This study additionally identified Cldn6 as a marker for pluripotent stem cells (L. Wang et al., 2012) which implicates Cldn6 as a key mediator in many critical developmental processes. In a subsequent study by Turksen et. al., it was revealed that when Cldn6 was over-expressed (using a Involucrin- Cldn6 transgenic mouse) that the offspring die shortly after birth, due to the development of a defective epidermal permeability barrier (Turksen & Troy, 2002). Again, this data suggests that Cldn6 plays a critical role during development, especially in contributing to the barrier function found in tissues.

Another area of research surrounding Cldn6 is in the field of cancer. Research has shown that Cldn6 is implicated in breast cancer (Heerma van Voss et al., 2014), ovarian cancer (L. Wang et al., 2013), gastric neoplasms (Z. Lin et al., 2013) and adenocarcinomas (Zavala-Zendejas et al., 2011). Low Cldn6 expression in non-small cell lung cancer has additionally been identified as a prognostic biomarker and correlates with poor survival rates for patients with non-small cell lung cancer (Q. Wang, Zhang, Zhang, Han, & Shan, 2015).

Preliminary Data

Our published data revealed for the first time that Claudin-6 is expressed during early to mid-late embryonic periods in mice (F. R. Jimenez et al., 2014). Using Microarray analysis, mRNA expression levels of Claudin-6 were shown to decrease at around E15.5 continuously until PN0, at which point expression levels were minimal (Figure 3). We further confirmed these findings using immunohistochemistry and created an expression profile to qualitatively assess Claudin-6 during murine lung morphogenesis. As a follow-up, we co-localized Cldn6 with TTF-1 a critical transcription factor in lung development, as well as Gata6, and FoxA2 which acts as regulatory co-factors. Our data demonstrated that Cldn6 was co-expressed with each of these transcription factors. Furthermore, to further elucidate the relationship between Cldn6, TTF-1, Gata6 and FoxA2 we transfected Beas2B cells (Human bronchiolar epithelial cells) and A549 cells (an immortalized cell line that is characteristic of alveolar type II cells) with the aforementioned transcription factors to assess whether they induced Cldn6 expression. We found that each transcription factor induced transcription of Claudin-6.

Having discovered that Cldn6 is transcriptionally regulated by TTF-1, Gata-6, and FoxA2, we suspected that Cldn6 was likely involved in the developmental program. Furthermore, as claudins are responsible for maintaining the epithelial permeability barrier, we sought to investigate additional possible roles and functions of Cldn6. To test our hypothesis, we developed a conditional over-expresser transgenic mouse for the Cldn6 gene. Our mouse utilized the TetO rTTA over-expression model (Figure 4), so that expression of our target gene is up-regulated with the addition of a doxycycline feed diet. In our published results, we showed that our mouse was able to induce Cldn6 over-expression. Moreover, we found that over-expression of Cldn6 resulted in delayed lung morphogenesis. Cldn6 transgenic mice at E18.5

showed a phenotype that would typically be seen at E15.5 in wild type mice. In an examination of TTF-1 and FoxA2, we showed that our transgenic mouse had restricted expression of TTF-1 (as compared to the wild type which had expression throughout the lungs), and that FoxA2 expression was limited (Wild type showed high expression). To further confirm that over-expression of Cldn6 resulted in delayed lung morphogenesis, we stained for PCNA (stain for proliferation), which revealed that in wild type mice PCNA was high, and in our transgenic mouse PCNA was restricted to the tubules. Finally, because normal lung development requires both proliferation as well as apoptosis to occur, we stained for Caspace 3 (a critical mediator of apoptosis). We found that in our transgenic mouse that Caspace 3 was decreased when compared to our controls.

Altogether this data illustrates the potential impact that Cldn6 plays in development. With these insights in mind, combined with the idea that claudin proteins are instrumental in maintaining the permeability barrier, I decided to more fully investigate the role of Cldn6 both during development, and later in adulthood. My preliminary hypothesis in relation to the increase in Cldn6 protein in lung epithelial includes protection against harmful exogenous material and a decreased inflammatory response.

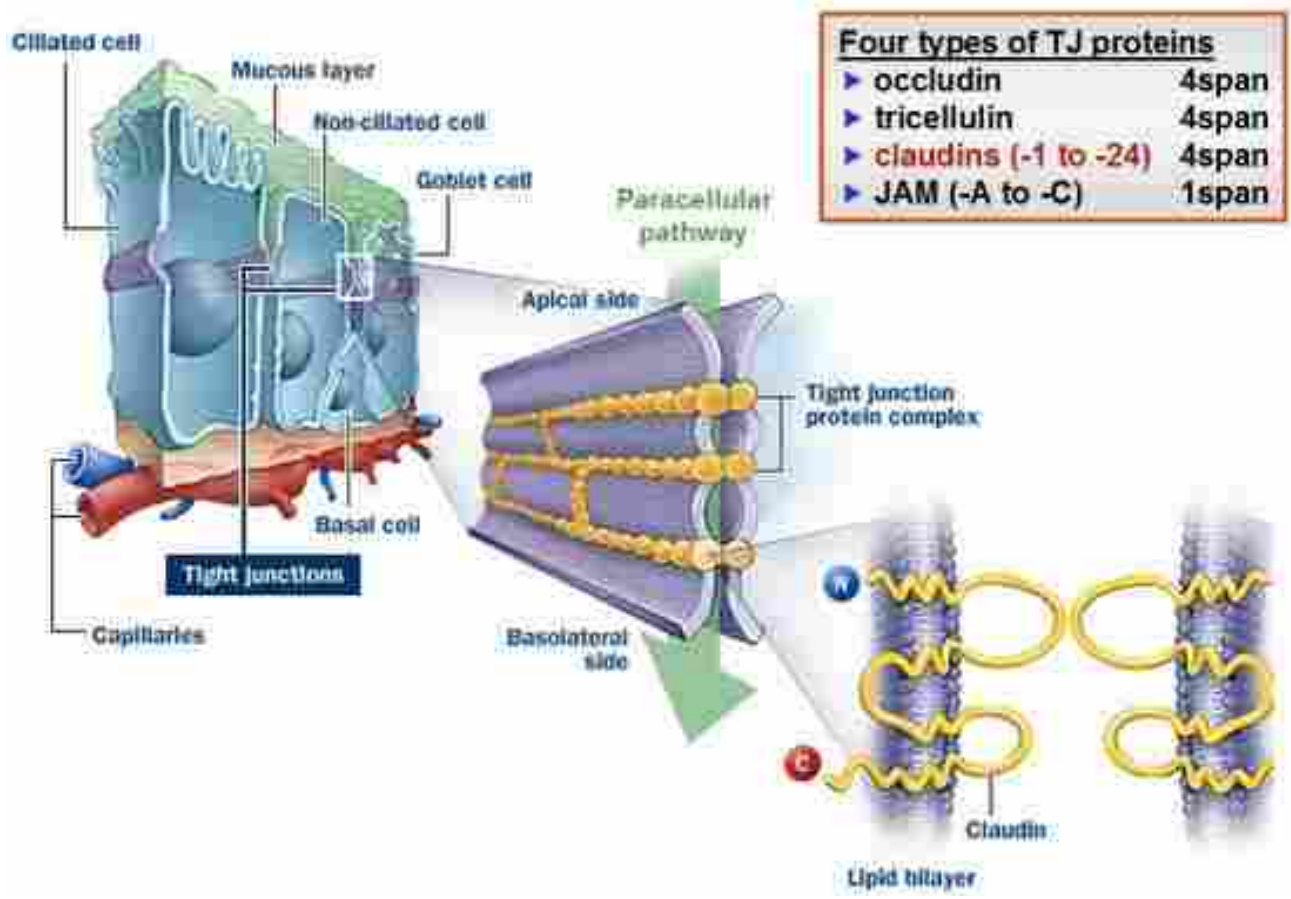


Figure 1.1: Basic Diagram of the Tight Junctional Complex.

Image regenerated from “https://klinphys.charite.de/for721/f_summary_e.htm”

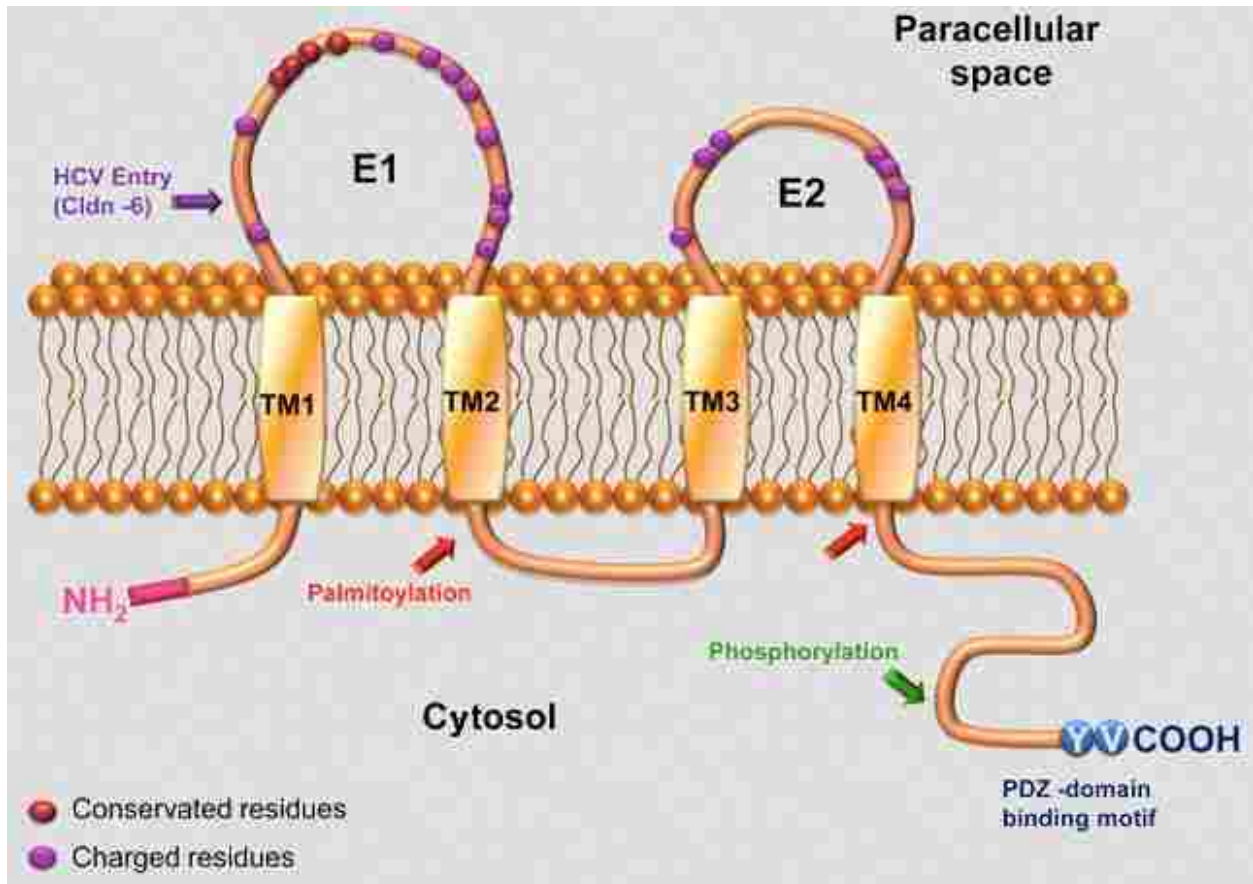


Figure 1.2: Structure of Claudin-6.

Claudins have four transmembrane domains with two extracellular loops. Currently it is believed that the larger extracellular loop is responsible for determining the paracellular tightness and the selective ion permeability abilities of the respective Claudin protein. The second and shorter extracellular loop is believed to cause narrowing of the paracellular cleft and additionally acts as an anchoring point between opposing cell membranes. Image regenerated from the Atlas of Genetics and Cytogenetics in Oncology and Haematology.

“<http://atlasgeneticsoncology.org/Genes/CLDN6ID50974ch16p13.html>”

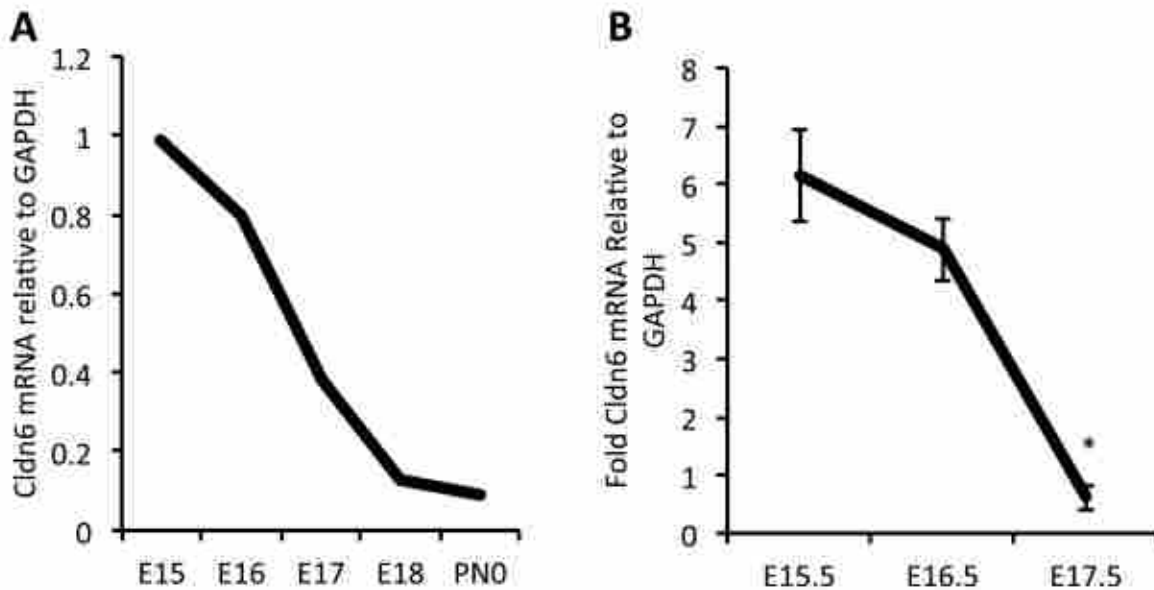


Figure 1.3: Cldn6 Microarray Analysis and RT-PCR.

Control C57B16 mice were screened by microarray analysis and Cldn6 expression levels were derived to GAPDH from E15-PN0. (A) Confirmatory quantitative RT-PCR was conducted using total RNA from C57B16 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. Image was regenerated from “Developmental lung expression and transcriptional regulation of claudin-6 by TTF-1, Gata-6, and FoxA2. June 2014.

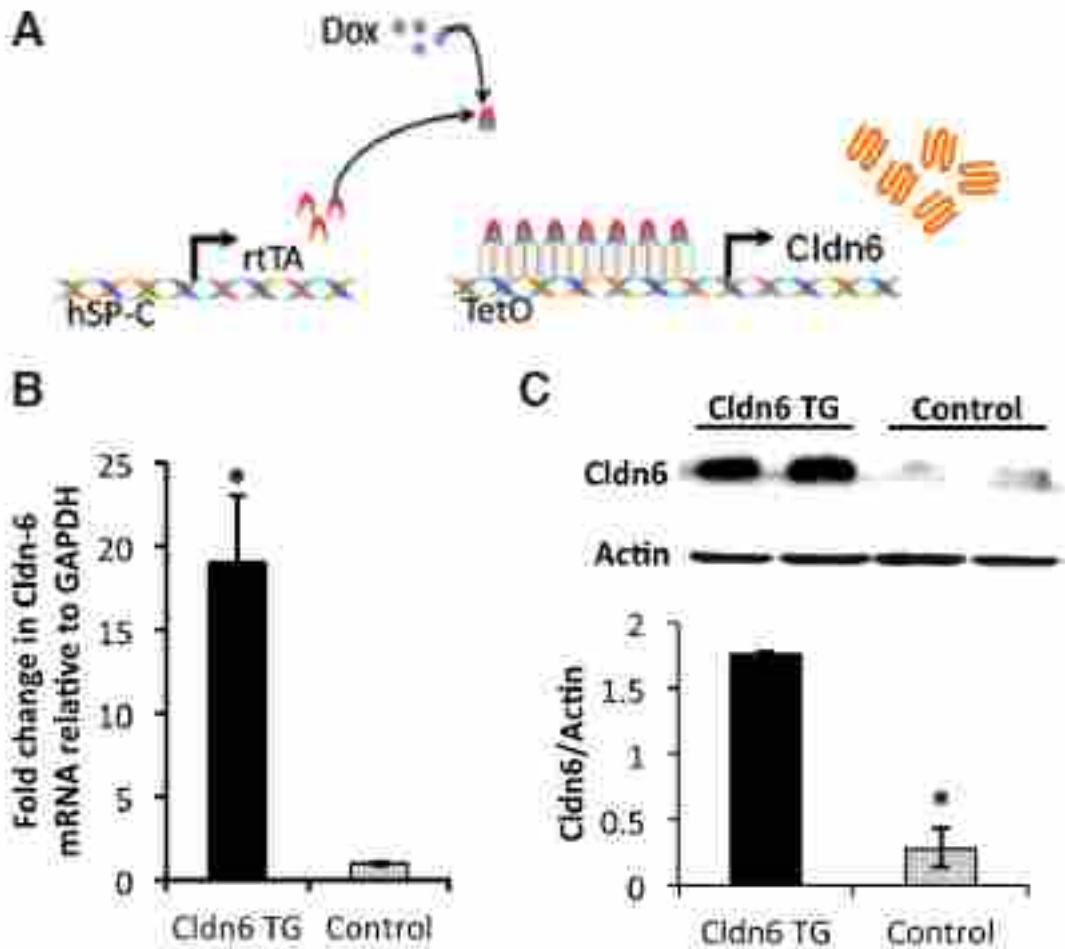


Figure 1.4: Cldn6 Transgenic Model.

(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 compared 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 q PCR data are representative of experiments performed in triplicate and statistical differences are noted (* $P \leq 0.05$). Image is regenerated from “Conditional pulmonary over-expression of Claudin 6 during embryogenesis delays lung morphogenesis.” June 2015

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CHAPTER 2: The Expression Profile of Claudin Family Members in the Developing Mouse Lung and Expression Alterations Resulting from Exposure to Secondhand Smoke (SHS).

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Key words: claudins, lung, tobacco,

Running Title: Lung expression profiling of Claudin

Abstract

Claudins are tight junctional proteins that are implicated in cell polarity and establishing and maintaining epithelial barriers. Our published research has revealed that claudin misregulation adversely impacts cell differentiation and proliferation. Impairment of such critical functions has been linked to anomalous barriers in abnormal lung development and disease states such as acute lung injury, chronic obstructive pulmonary disease, pulmonary fibrosis, and cancer. The current research evaluated the transcriptional expression profiles for claudins 1-11 and 18 in the developing murine lung at embryonic days (E) 14.5, 16.5, and 18.5 and at post-natal day (PN) 2 and PN15. Wild type mice from E12.5, E14.5, E16.5, E18.5, PN2 and PN15 were also sacrificed and assessed by immunohistochemical analysis to qualitatively evaluate claudin protein expression patterns. For the immunostaining, Claudins 1-8 and 18 were selected based on their phylogenetic similarity and published reports describing influence on pulmonary biology. Select dams were also exposed to secondhand smoke (SHS) from embryonic day (E)15.5 to 18.5 which was immediately followed by screening claudin mRNA. Other than Claudin-6, mRNA expression patterns for claudin family members were conserved such that expression decreased at E16.5, increased at E18.5, decreased again at PN1 before reaching a height of expression at PN15. Claudin-6 mRNA expression decreased through gestation and into post-natal periods. Immunohistochemically, expression profiling for individual claudins implicated a subset as plausible orchestrators of proximal vs. distal lung barrier establishment. Assessment of claudin mRNA expression profiles at E18.5 following SHS exposure from E15.5-E18.5 revealed a significant reduction in transcription for all claudins except Claudin-18 (no change). These data support the need for further studies using gene targeted mice that knock-in/out specific claudins so that precise functions in the normal and diseased lung can be

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Introduction

Lung organogenesis is a complex process involving precise regulation of genes, which facilitate mechanisms that mediate both temporal and spatial development, ultimately giving rise to the various lung compartments (Perl & Whitsett, 1999). Implicated in this delicate developmental process are the tight junctional proteins, claudins (Cldn). Mounting evidence suggests the importance of the Cldn family of proteins as critical mediators in normal lung physiology, and that the loss of these barrier proteins contributes to diverse pathologies. For example, recent research has revealed that deficiency of Cldn-18 in lung samples from fetal and postnatal human infants resulted in barrier dysfunction that may lead to the development of bronchopulmonary dysplasia (LaFemina et al., 2014). Reports of divergent expression profiles for Cldn-1, Cldn-3, Cldn-4, Cldn-5 and Cldn-7 have been linked to the development of airways and the differentiation of human alveolar epithelial cells (B. L. Daugherty et al., 2004; R. Kaarteenaho et al., 2010). Cldn-5 has additionally been associated with the alveolar capillary system during angiogenesis (Favre et al., 2003). Research has revealed that overexpression of Cldn-6 during development results in a delay in lung morphogenesis which is characterized by decreased differentiation and apoptosis (F. R. Jimenez et al., 2015b). Altogether, these data further strengthen the idea that Cldn proteins may play a greater role in development than what is currently understood.

In terms of the adult lung, Cldns function as paracellular seals and are therefore associated with abnormal lung function and the development of many respiratory diseases. In addition to preventing the penetrating movement of harmful exogenous material, they contribute

to the formation of the permeability barrier by forming paracellular channels or pores (Gunzel & Yu, 2013; Krause, Protze, & Piontek, 2015). The loss of these basic functions have been documented to contribute to a variety of pathologies such as chronic obstructive pulmonary disease (COPD) (Cuzic et al., 2012), bronchopulmonary dysplasia (BPD) (LaFemina et al., 2014), pulmonary fibrosis (PF) (Lappi-Blanco et al., 2013), Acute Lung Injury (ALI) (Kage et al., 2014), and asthma (Moon et al., 2015). Furthermore, misregulation of Cldn proteins have been linked to both the development and metastatic behavior of lung cancer (Lu et al., 2015; Sharma et al., 2015; Q. Wang et al., 2015). Interestingly, one common factor known to contribute to the development of these abnormalities is cigarette smoke, which has more recently been connected to the misregulation of Cldn family proteins (Cuzic et al., 2012; H. Li et al., 2015; Merikallio et al., 2011a).

Growing evidence continues to demonstrate the insidious risk associated with prenatal exposure to maternal smoking and the development of many respiratory diseases both perinatally and later in life. Studies have revealed that prenatal exposure to environmental tobacco smoke alters DNA methylation and may increase the risk for pulmonary inflammation (J. W. Lee et al., 2015). Epigenetic changes in the lungs as a result of maternal or even grandmaternal exposure have been shown to impact early lung organogenesis and lead later in life to the development of many adult lung diseases (Harding & Maritz, 2012). Furthermore, it has been long understood that exposure to nicotine from SHS has many deleterious effects, most notably the development of fewer and larger alveoli (Collins et al., 1985). Despite the ongoing research that continues to examine the physiological mechanisms that may be targeted in these processes, to our knowledge no research has examined the effects of maternal exposure to SHS on fetal Cldn expression.

Although research has revealed many insights into the various expression patterns of Cldn family members, additional research is needed to better address precise spatial and temporal expression patterns and how expression influences development. The focus of this study was to assess expression patterns of Cldn family members with structural homology and pulmonary characteristics. Screening occurred within the lung during various developmental periods of pre- and post-natal organogenesis and maturation. Furthermore, we examined to what extent maternal exposure to SHS impacts perinatal Cldn expression patterns.

Material and Methods

Mice

Wild Type (WT) mice from a C57B1/6 background were mated and then euthanized at embryonic days (E) 14.5, E16.5, E18.5 as well as post-natal (PN) day 3 and PN15. In addition, a subset of mice was exposed to secondhand smoke (SHS) via a nose only inhalation system (InExpose System, Scireq, Montreal Canada) beginning at E13.5 until E18.5 at which point mice were euthanized (Lewis et al., 2016; Nelson et al., 2015; D. R. Winden et al., 2014; T. T. Wood et al., 2014). On the date of euthanasia, pup lung tissue was harvested and fixed in 4% paraformaldehyde for histological analysis, or snap frozen for mRNA quantification and assessment via qRT-PCR. Mice were housed and utilized in accordance with protocols approved by the IACUC at Brigham Young University.

qRT-PCR

Total RNA was isolated from mouse lungs using an RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). Reverse transcription of RNA in order to obtain cDNA for qRT-PCR, and cDNA amplification was performed using Bio Rad iTaq Universal SYBR® Green One-Step Kit. Data

analysis were performed using a Bio Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories) (Cokkinides et al., 2009). The following primers were synthesized by Invitrogen Life Technologies (Grand Island, NY): Cldn1 (For- TCT ACG AGG GAC TGT GGA TG and Rev- TCA GAT TCA GCT AGG AGT CG), Cldn2 (For- GGC TGT TAG GCT CAT CCA T and Rev- TGG CAC CAA CAT AGG AAC TC), Cldn3 (For- AAG CCG AAT GGA CAA AGA A and Rev- CTG GCA AGT AGC TGC AGT G), Cldn4 (For- CGC TAC TCT TGC CAT TAC G and Rev- ACT CAG CAC ACC ATG ACT TG), Cldn5 (For- GTG GAA CGC TCA GAT TTC AT and Rev- TGG ACA TTA AGG CAG CAT CT), Cldn6 (For- CAT TAC ATG GCC TGC TAT TC and Rev- CAC ATA ATT CTT GGT GGG ATA TT), Cldn7 (For- AGG GTC TGC TCT GGT CCT T and Rev- GTA CGC AGC TTT GCT TTC A) Cldn8 (For- GCC GGC ATC ATC TTC TTC AT and Rev- CAT CCA CCA GTG GGT TGT AG), Cldn9 (For- GCT ACA CTT TGA GCG TCC C and Rev- CCT CTT ATC CAG TCC CGA AG), Cldn10 (For- CCC AGA ATG GGC TAC ACA TA and Rev- CCT TCT CCG CCT TGA TAC TT), Cldn11 (For- TCT TGG TTC CTG TAT GTG CC and Rev- CGT ACA GCG AGT AGC CAA AG), Cldn18 (For- GAC CGT TCA GAC CAG GTA CA and Rev- GCG ATG CAC ATC ATC ACT C) and β -actin (For-ACA GGA TGC AGA AGG AGA TTA C and Rev- CAC AGA GTA CTT GCG CTC AGG A).

Histology and Immunohistochemistry

Lungs from WT mice were isolated at E12.5 E14.5, E16.5, E18.5, PN3, and PN15 before being fixed in 4% paraformaldehyde, processed, embedded and sectioned at 4 μ m thickness. To perform immunostaining for specific Claudin family members, 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) (Z. L. Wang, 2013) Antibodies used included Cldn1 (C0142 1:200, One World Lab Inc., San Diego, CA), Cldn2 (C0143 1:200, One World Lab Inc.), Cldn3 (C0144 1:200, One World Lab Inc.), Cldn4 (1:200, One World Lab Inc.), Cldn5 (C0145 1:200, One World Lab Inc.), Cldn6 (C-20, 1:100; Santa Cruz Biotechnologies, Santa Cruz, CA), Cldn7 (C15163, 1:200, One World Lab Inc.), Cldn8 (25559 1:200, One World Lab Inc.), Cldn18 (ab203563 1:200, Abcam, Cambridge, MA).

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

Cldn family members were selected based on phylogenetic similarity and previous reports that implicate the family member with lung physiology. Quantitative RT-PCR analyses revealed a general trend of mRNA expression for all screened Cldn proteins except Claudin-6 that included a decrease in expression from E14.5 to E16.5 followed by increased expression from E16.5 until E18.5 (Figures 1 and 2). Three days after birth (PN3) was a period associated with decreased Cldn mRNA expression and the height of transcription was detected at PN15 (Figures 1 and 2). Deviations from this trend were observed in Cldn-6 expression wherein intense expression detected at E14.5 notably decreased until E18.5, at which point basal expression generally plateaued (Figure 1). Cldn-18 mRNA expression was similar to the overall trend observed with all other Cldns except Cldn6; however, Cldn-18 mRNA expression was not initially decreased from E14.5 to E16.5 (Figure 2).

Spatial and Temporal Visualization of Cldn Protein Expression

We employed immunohistochemistry to qualitatively visualize Cldn protein expression so that we could assess whether transcription largely correlated with translation. Immunostaining revealed expression of Cldn1 at E16.5 and a peak of expression at PN3 (Figure 3). No notable visualization of Cldn2 was demonstrated (Figure 3). Cldn3 IHC revealed consistent staining throughout the screened time periods (Figure 4), although intensity diminished during E18.5 and PN3 when compared to earlier developmental time periods (Figure 4). Cldn4 images revealed no indication of staining (Figure 4). Cldn5 IHC showed little to know expression during E12.5 and E14.5, but increased expression was detectible from E16.5 to PN15 (Figure 5). Cldn6 IHC revealed elevated expression at the earliest points of development, but expression gradually decreased as development proceeded (Figure 5). Histochemical analysis of Cldn7 showed expression throughout development, but expression was localized to the most proximal airways (Figure 6). Cldn8 immunostaining showed increased staining only at PN3 and was localized to the distal airways and parenchyma (Figure 6). Lastly, Cldn18 expression was most notably detected in the distal lung at E18.5 with marked expression continuing through PN15 (Figure 7).

Transcriptional Impact of Secondhand Smoke

An examination of the effects of maternal SHS treatment on Cldn mRNA expression resulted in the discovery that SHS transcriptionally inhibited all screened Cldn family members except Cldn18, which was unaffected by SHS exposure (Figures 8 and 9). Compared to animals that received room air (RA) treatment only, mice exposed to SHS had significantly decreased mRNA expression for Claudin-1, -2, -3, -4, -5, -6, -7, -8, -9, -10, and -11 (Figures 8 and 9).

Discussion

Our data support the hypothesis that at least in part, developmental programs may be influenced by the deposition of Cldn family members along the proximal-distal lung axis due to the discovery that Cldn proteins increase during key periods of organogenesis. Most Cldn proteins displayed similar patterns of mRNA expression, showing significant increases during the mid to late canalicular stage and continuing into the saccular stage of lung development. During the canalicular stage, final generations of the bronchial tree are formed and early acini begin to appear while capillaries emerge as a meshwork in the mesenchymal compartment. Conserved expression patterns support the concept that functional redundancies among Cldn family members likely exist. Despite such redundancies, specific Cldns are known to regulate key cellular effects. For example, our discovery that Cldn5 expression elevates during this developmental period likely correlates with the establishment of capillary networks as Cldn5 has been identified in endothelial cells and is responsible for maintaining the endothelial permeability barrier (L. Y. Huang, Stuart, Takeda, D'Agnillo, & Golding, 2016). During the late canalicular period, the lungs are also in a consistent transitory stage that is influenced by intraluminal fluid secretion. Indeed some reports suggest that by term, the fetus is actively producing essential lung fluid that has a higher concentration of chloride ions than plasma at a rate of 5 ml/kg/h (O'Brodovich, 1991). In regards to Cldns that serve as chloride ion regulators, Cldn2 (S. M. Krug et al., 2012), Cldn4 (Gong & Hou, 2017), Cldn6 (Sas, Hu, Moe, & Baum, 2008), Cldn8 (Gong & Hou, 2017), Cldn9 (Sas et al., 2008), Cldn 10 (S. M. Krug et al., 2012), Cldn17 (S. M. Krug et al., 2012) have all been identified to influence paracellular secretion of chloride ions. Furthermore, it appears that the flux of NaCl itself can affect the recruitment of specific Cldns such as Cldn1 (Tokuda, Miyazaki, Nakajima, Yamada, & Marunaka, 2010). Thus,

precisely regulated expression of tight junctional proteins such as Cldns is likely critical for normal development programs involving diverse, intersecting mechanisms. Without question, each deserves pointed research focus in the immediate future.

One notable exception to the multimodal expression pattern is Cldn6, which decreased as development continued. A plausible explanation for diminishing Cldn6 expression is that it correlates closely with branching morphogenesis. Published research revealed that consistent Cldn6 expression obtained through the utilization of lung-specific transgenic mice causes notable delays in pulmonary branching morphogenesis (F. R. Jimenez et al., 2015a). Moreover, we have illustrated that Cldn6 is transcriptionally regulated by TTF-1, Gata-6, and FoxA2, critical transcription factors that modulate lung organogenesis. Coupled with the finding from Sugimoto et al., which found that Cldn6 not only induces epithelial differentiation in embryonic stem cells, but also induces the expression of other tight junctional components, it is likely that Cldn6 serves an important role in cell differentiation and the spatial distribution of lung cell types.

As lungs represent one of the earliest interfaces for pathogens and noxious stimuli to interact with the body, careful maintenance of the permeability barrier is vital in maintaining normal health. Current research has demonstrated that exposure to deleterious compounds such as tobacco smoke results in leakier tight junctional complexes (Godfrey, 1997), a characteristic that leads to the progression of diseases such as COPD, PF, ARDS, and asthma (Gonzales, Lucas, & Verin, 2015; Wawrzyniak et al., 2017; Wittekindt, 2017; Yanagi, Tsubouchi, Miura, Matsumoto, & Nakazato, 2015). Additionally, examination of benzo(a)pyrene and 4-(mehylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK), two carcinogenic substances found in tobacco smoke, confirmed notable influence over Cldn synthesis, as they upregulate transcription factors such as Twist, Snail, and ZEB1 known to participate in transcriptional control

mechanisms (Wei et al., 2009; Yoshino et al., 2007). We observed that maternal exposure to tobacco smoke resulted almost universally in a decrease in Cldn mRNA expression. While these data are the first to suggest maternal tobacco smoke exposure influences fetal tight junctional expression, corroborative studies show that bronchial cells sampled from patients with smoke-induced COPD have significantly less Cldns and other tight junctional components when compared to healthy patients (R. Shaykhiev et al., 2011). As tight junctions play a critical role in mediating the innate immune system, it is likely that the loss of these proteins during tobacco exposure contributes to decreased pulmonary function associated with maternal smoking.

Even though these data provide substantial evidence that tight junctional components may well be targeted by tobacco products, it gives little insight into the possible maternal-fetal alterations that may also be in play. For some time now, studies have demonstrated that tobacco smoke exposure *in utero* influences lung function, often predisposing individuals to enhanced disease states later in life (Lannero, Wickman, Pershagen, & Nordvall, 2006; Lodrup Carlsen, Jaakkola, Nafstad, & Carlsen, 1997; McEvoy & Spindel, 2017; Zacharasiewicz, 2016). In fact, substantial evidence exists that shows prenatal exposure is an independent risk factor and perhaps more critical than postnatal exposure (Gilliland, Li, & Peters, 2001; Lannero et al., 2006; Pattenden et al., 2006). New research has revealed that *in utero* exposure to tobacco smoke and even nicotine alone results in alterations of DNA methylation (J. W. Lee et al., 2015), structure (Suter et al., 2015; Wongtrakool, Wang, Hyde, Roman, & Spindel, 2012), and pulmonary function (Wongtrakool et al., 2012), but it remains unclear to what extent Cldn proteins function during such processes. The current data highlights two significant findings. First, the spatial and temporal expression patterns of Cldn family members in the lung during development are precise

and redundant. Secondly, our findings suggest that maternal tobacco smoke likely disrupts tight junctional integrity by decreasing Cldn expression during smoke-mediated cellular responses.

Conclusion

This research provides clarity in the evaluation of developmental Cldn expression. Despite the widespread understanding that maternal smoking is detrimental to fetal health, approximately 12% of pregnant women in the United States smoke (Filion et al., 2011). These data accordingly also provide insight into smoke-mediated lung compromise because tight junctions have emerged as important molecular complexes at the forefront of immunity. Ongoing research of Cldn protein expression profiles and functional contributions they make to lung development and physiology may in due course identify potential therapeutic targets, and yield critical information relative to immunity at the epithelial barrier.

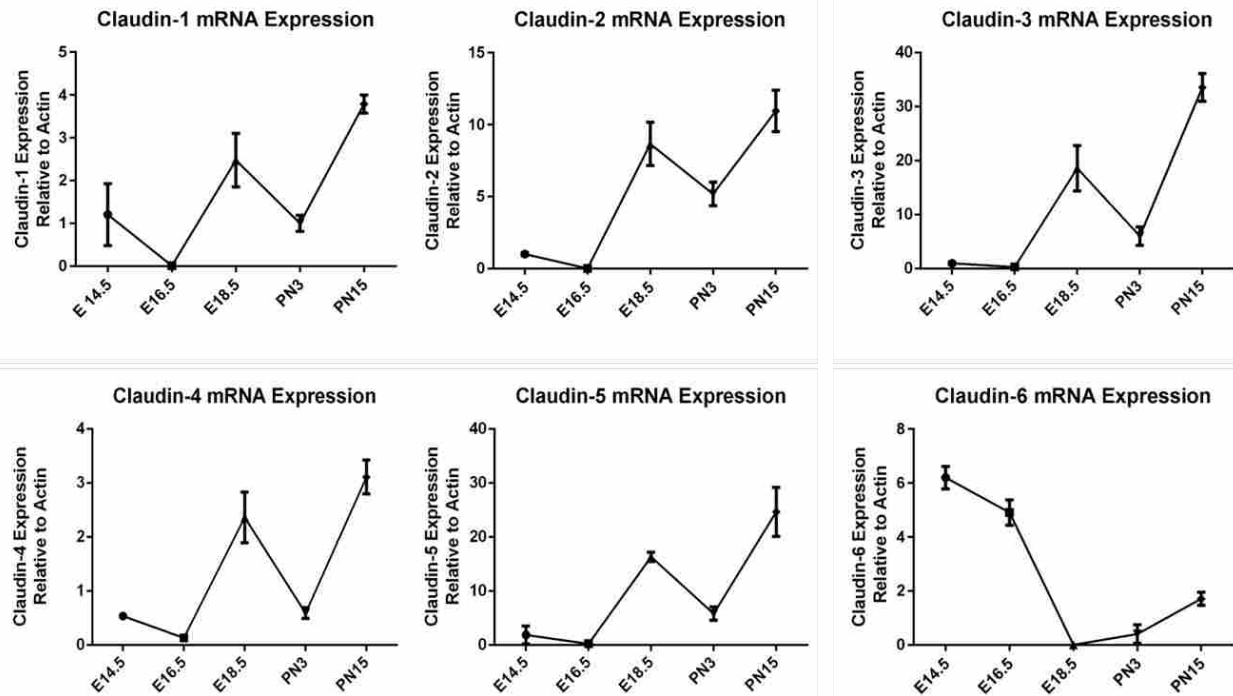


Figure 2.1: Temporal Expression of Cldn-1, -2, -3, -4, -5, and -6.

Messenger RNA expression was screened for Cldn1-6. In the case of Cldn1-5, expression decreased from E14.5 to E16.5 prior to a significant increase just before birth at E18.5. Decreased mRNA expression at PN3 was preceded by a marked increase observed at PN15. Cldn6 mRNA expression significantly decreased as developmental time lapsed. The mRNA concentration was normalized to β -actin and representative data are show as $*p \leq 0.05$. Experiments were performed in triplicate at least three times.

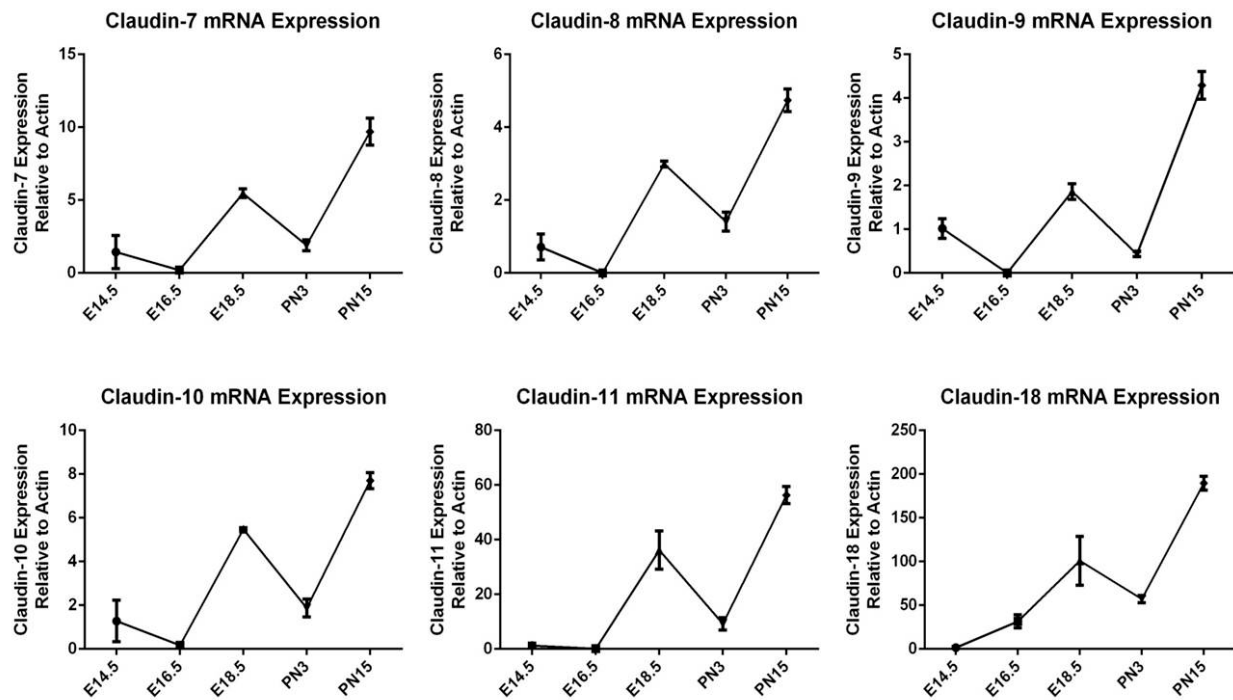


Figure 2.2: Temporal Expression of Cldn-7, -8, -9, -10, -11, and -18.

Messenger RNA expression was screened for Cldn7-11 and 18. In the case of Cldn7-11, expression decreased from E14.5 to E16.5 prior to a significant increase just before birth at E18.5. Decreased mRNA expression at PN3 was preceded by a marked increase observed at PN15. Cldn18 mRNA expression significantly increased throughout development with the only exception being a depression in expression at PN3. The mRNA concentration was normalized to β -actin and representative data are shown as $*p \leq 0.05$. Experiments were performed in triplicate at least three times.

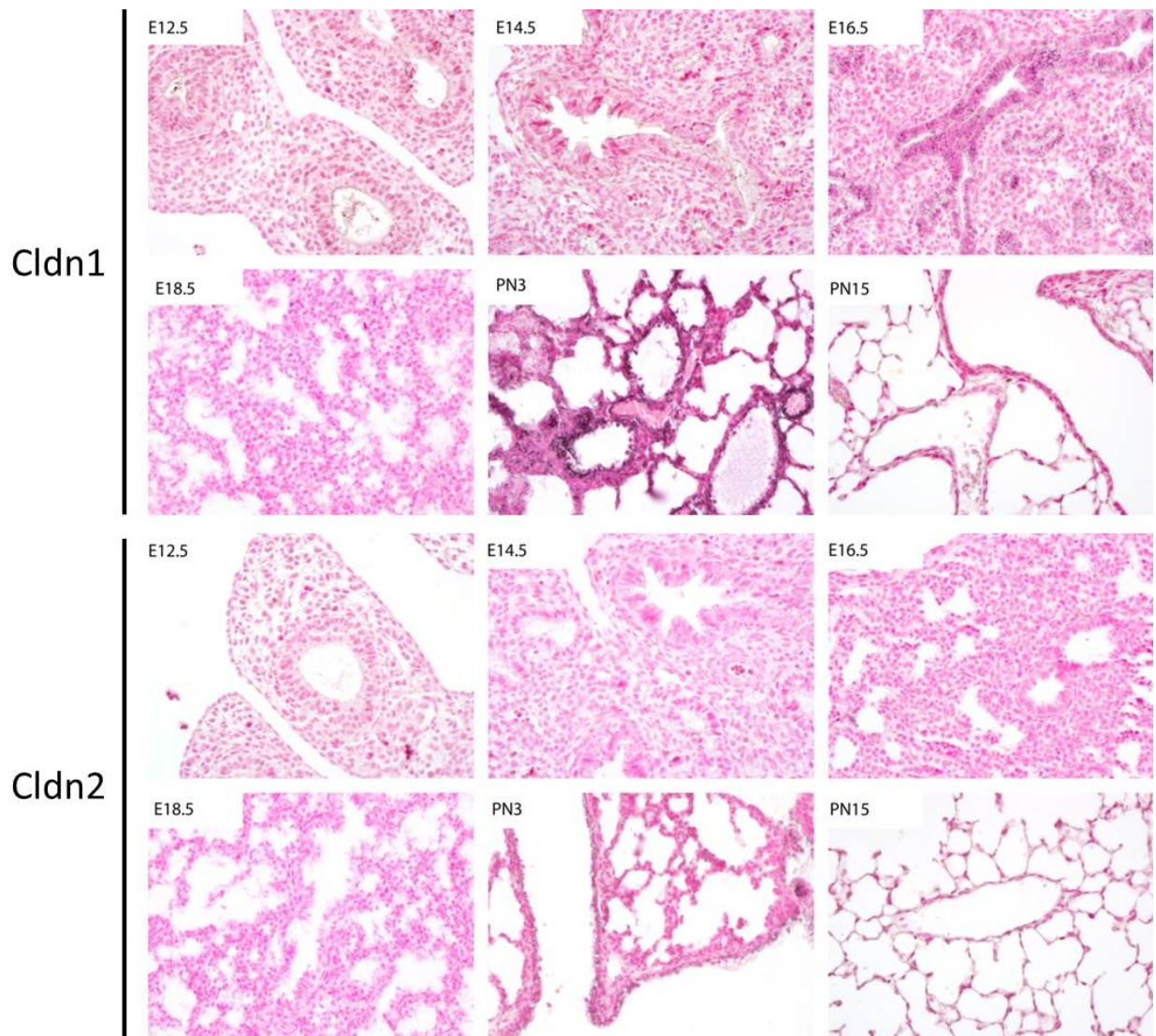


Figure 2.3: Spatial Expression of Cldn1 and Cldn2.

Representative immunostaining for Cldn1 revealed expression at E16.5 and a peak of expression at PN3. No discernable immunostaining for Cldn2 was observed. Images (200x magnification) are representative of experiments involving at least four animals from each group.

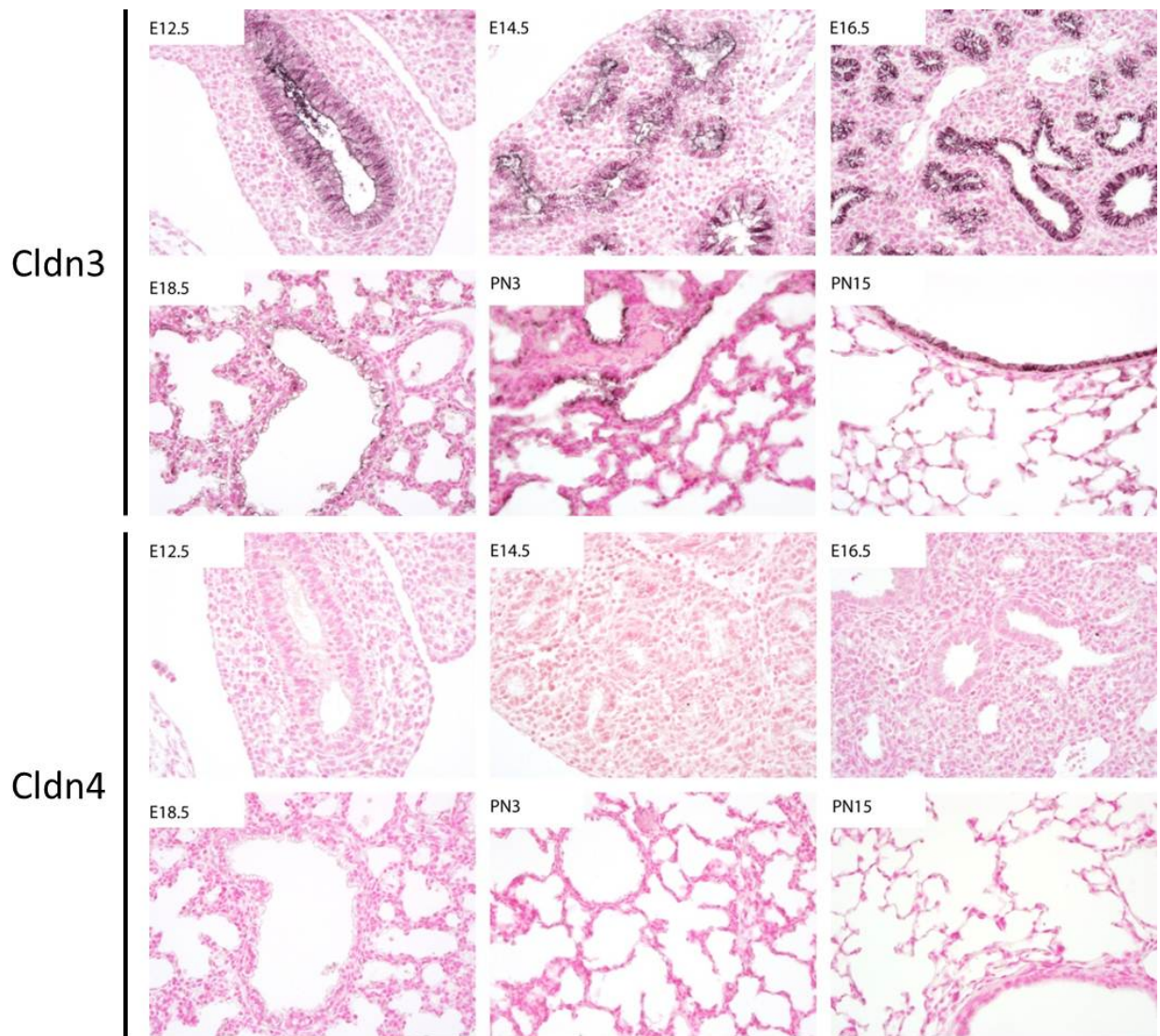


Figure 2.4: Spatial Expression of Cldn3 and Cldn4.

Representative immunostaining for Cldn3 revealed consistent staining throughout the screened time periods, although intensity diminished during E18.5 and PN3 when compared to earlier developmental time periods. Cldn4 assessment resulted in no indication of staining. Images (200x magnification) are representative of experiments involving at least four animals from each group.

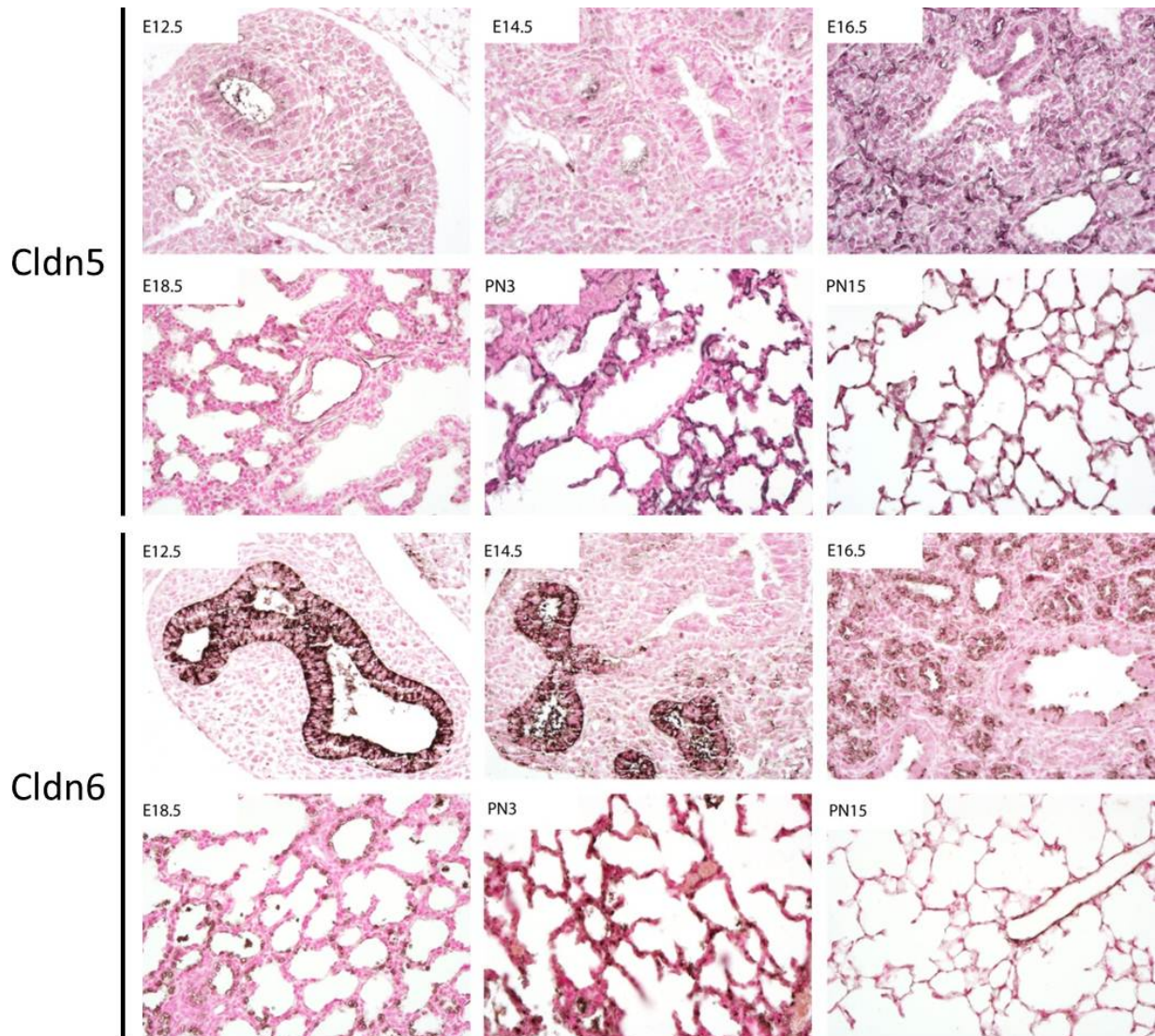


Figure 2.5: Spatial Expression of Cldn5 and Cldn6.

Representative immunostaining for Cldn5 showed little to no expression during E12.5 and E14.5, but increased expression was detectable from E16.5 to PN15. Cldn6 IHC revealed elevated expression at the earliest points of development, but expression gradually decreased as development proceeded. Images (200x magnification) are representative of experiments involving at least four animals from each group.

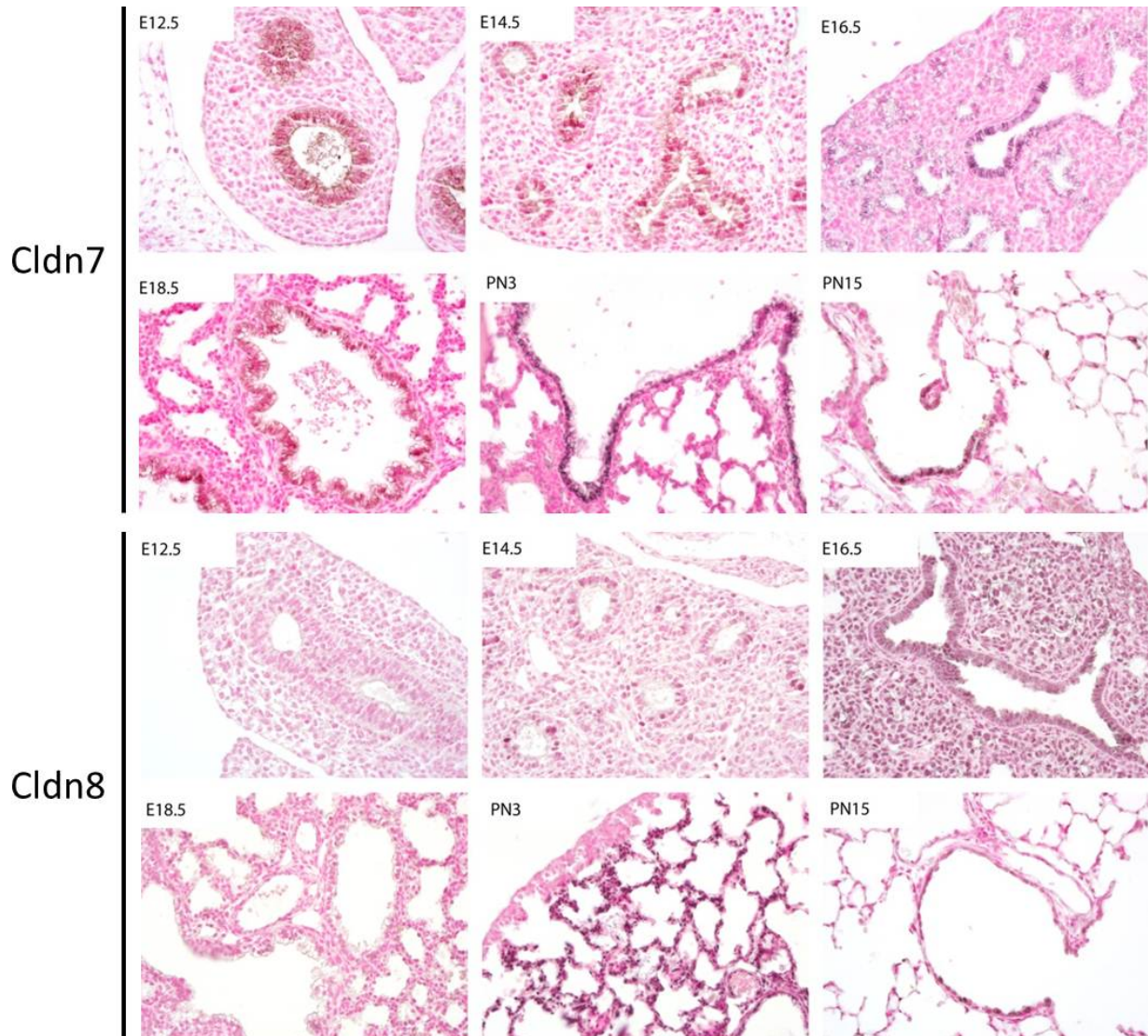


Figure 2.6: Spatial Expression of Cldn7 and Cldn8.

Representative immunostaining for Cldn7 showed expression throughout development, but expression was localized to the most proximal airways. Cldn8 immunostaining showed increased staining only at PN3 and was expression was localized to the distal airways and parenchyma. Images (200x magnification) are representative of experiments involving at least four animals from each group.

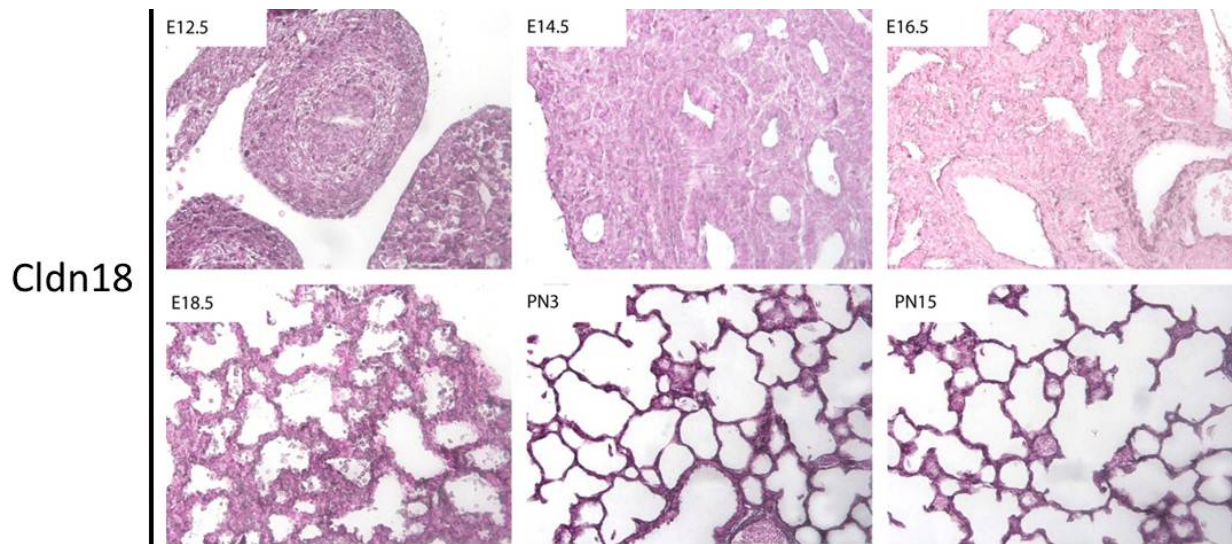


Figure 2.7: Spatial Expression of Cldn18.

Representative immunostaining for Cldn18 demonstrated notable detection in the distal lung at E18.5 with marked expression continuing through PN15. Images (200x magnification) are representative of experiments involving at least four animals from each group.

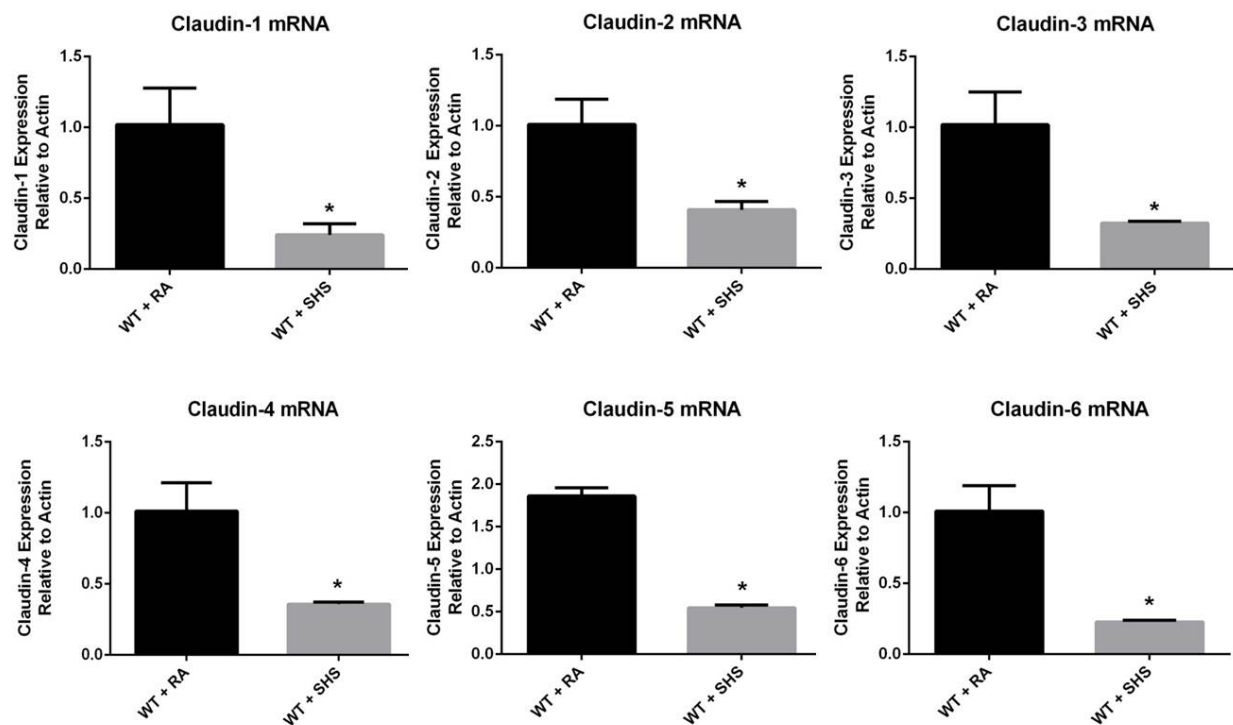


Figure 2.8: Effect of SHS on Claudin(1-6) mRNA Expression.

Messenger RNA expression was screened for Cldn1-6 in E18.5 mouse lungs following maternal exposure to SHS from E15.5-E18.5. Compared to room air exposed controls, Cldn1-6 transcription was significantly inhibited following SHS exposure. The mRNA concentration was normalized to β -actin and representative data are shown as $*p \leq 0.05$. Experiments were performed in triplicate at least three times.

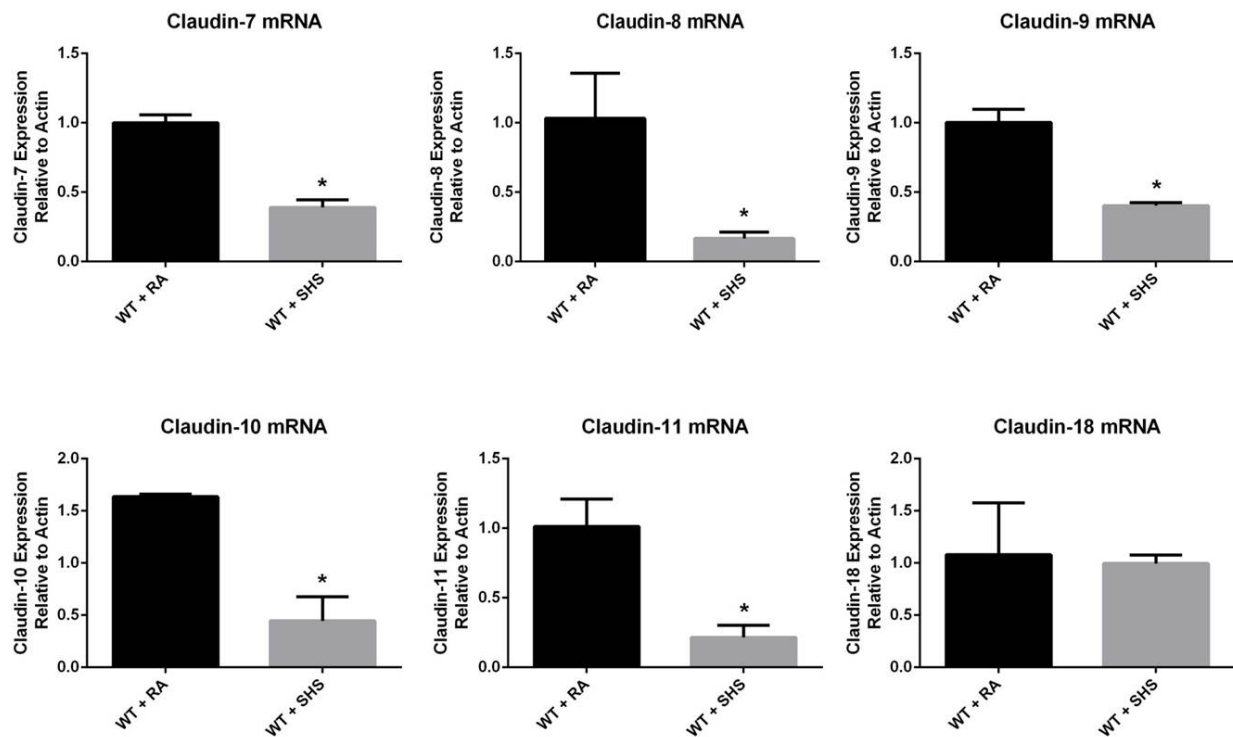


Figure 2.9: Effect of Secondhand Smoke on Claudin (7-11 and 18) mRNA Expression.

Messenger RNA expression was screened for Cldn7-11 and 18 in E18.5 mouse lungs following maternal exposure to SHS from E15.5-E18.5. Compared to room air exposed controls, Cldn7-11 transcription was significantly inhibited following SHS exposure. There was no significant difference in the expression of Cldn18 mRNA following exposure. The mRNA concentration was normalized to β -actin and representative data are show as $*p \leq 0.05$. Experiments were performed in triplicate at least three times.

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CHAPTER 3: Cigarette Smoke and Decreased Oxygen Tension Inhibit Pulmonary
Claudin-6 Expression.

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Abstract

Purpose: COPD is a condition involving perturbed barrier integrity coincident with both emphysema and inflammation of the airways and smoking is considered a major risk factor. Claudins stabilize barriers and contribute to tight junctions by preventing paracellular transport of extracellular fluid constituents. **Methods:** 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. Cldn6 transcriptional regulation was also assessed using luciferase reporter constructs. **Results:** Q-PCR and immunoblotting revealed that Cldn6 was decreased in alveolar type II-like epithelial cells (A549) and primary small airway epithelial cells (SAECs) when exposed to cigarette smoke extract (CSE). Cldn6 was also markedly decreased in the lungs of mice exposed to acute tobacco smoke delivered by a nose-only automated smoke machine compared to controls. Luciferase reporter assays incorporating 0.5-kb, 1.0-kb, or 2.0-kb of the Cldn6 promoter revealed decreased transcription of Cldn6 following exposure to CSE. Cldn6 transcriptional regulation was also assessed in hypoxic conditions due to low oxygen tension observed during smoking. Hypoxia and hypoxia inducible factor-1 alpha (HIF-1 α) caused decreased transcription of the Cldn6 gene via interactions with putative response elements in the proximal promoter sequence.

Conclusions: These data reveal that tight junctional proteins such as Cldn6 are differentially regulated by tobacco smoke exposure and that claudins are potentially targeted when 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.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality (Mannino, Homa, Akinbami, Ford, & Redd, 2002; Rabe 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 (Ehteshami-Afshar, FitzGerald, Doyle-Waters, & Sadatsafavi, 2016). COPD manifests itself as two different disorders: emphysema and chronic bronchitis. Smoking is the most significant 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, & Miravittles, 2012). Respiratory epithelium in the parenchyma and small airways is a highly regulated barrier that also serves as a first line of defense against harmful irritant exposure (Knight & Holgate, 2003; Vareille, Kieninger, Edwards, & Regamey, 2011). Tight junctions (TJ) are constructed at boundaries between neighboring epithelial cells and are specifically composed of integral transmembrane proteins such as claudins, occludins, and junctional adhesion molecules (JAMs) (Schlingmann, Molina, & Koval, 2015). TJs maintain homeostasis and cellular polarity by regulating the paracellular transport of ions, small molecules, and inflammatory proteins (Godfrey, 1997; Kojima et al., 2013). Furthermore, TJs provide a site for intercellular signaling involved in the assembly, disassembly, and maintenance of other TJs (Karl Matter, Aijaz, Tsapara, & Balda, 2005; Shin, Fogg, & Margolis, 2006). As such, TJs are classically considered to be protective entities against epithelial inflammation and infection (Beeman, Webb, & Baumgartner, 2012; Guttman & Finlay, 2009; Ylermi Soini, 2011). When TJ impairment occurs in the airways, various pulmonary diseases characterized by cell death and inflammation present including bronchitis, asthma, and pneumonia (Valthor Asgrimsson, Thorarinn Gudjonsson, Gudmundur Hrafn Gudmundsson, & Olafur Baldursson,

2006; Gorska, Maskey-Warzechowska, & Krenke, 2010; Hogg & Timens, 2009; Holgate, 2007; Wilson, Dowling, & Jackson, 1996). (V. Asgrimsson, T. Gudjonsson, G. H. Gudmundsson, & O. Baldursson, 2006)The claudin (Cldn) family is composed of 27 members that are central components of the TJ complex with variable tissue-specific expression patterns (Krause et al., 2008; Markov, Aschenbach, & Amasheh, 2015; Mineta et al., 2011). Cldn functions in the formation of TJ sealing (Krause et al., 2008; Krause et al., 2009), but interestingly, they also form pores that may increase barrier permeability and enhance solute trafficking (C. B. Coyne et al., 2003; Krause et al., 2015). Recent *in vitro* evidence suggests that cigarette smoke extract (CSE) induces structural changes in TJ barriers (Heijink, Brandenburg, Postma, & van Oosterhout, 2012; C. Huang et al., 2015; Milara, Peiro, Serrano, & Cortijo, 2013; Tharakan, Halderman, Lane, Biswal, & Ramanathan, 2016; Xiao et al., 2011) via impairing individual components like occludins, zonula occludens (ZOs) (M. Chen, Yang, Meng, & Sun, 2015; Petecchia et al., 2009), and claudins (Merikallio et al., 2011a; Ylermi Soini, 2011). While specific Cldns have not been thoroughly evaluated to date, structural changes in the epithelial barrier were primarily attributed to cytotoxic effects of cigarette smoke (Merikallio et al., 2011a). Additional *in vivo* studies that employ first and secondhand smoke have similarly suggested altered tight junctional structures (J. L. Carson, L. E. Brighton, A. M. Collier, & P. A. Bromberg, 2013; R. Shaykhiev et al., 2011) and modification of tight junctional components (Liang et al., 2015; H. Wang et al., 2012).

Both the temporal and spatial expression of Cldn proteins are important in establishing normal lung physiology. As one example, Cldn-1 is found primarily in lower airway epithelium, which contrasts sharply with the expression patterns of Cldn-4 and Cldn-7, which are found throughout the airway (R. Kaarteenaho et al., 2010). Until recently, data related to Claudin 6

(Cldn6) in normal lung tissue were largely absent from the literature. However, recent findings suggest that it may play a significant role during lung embryogenesis. Cldn6 has been shown to be an important component of undifferentiated stem cells (Linlin Wang et al., 2012).

Additionally, it was shown that Cldn6 mediates epithelial morphogenesis in embryonic stem cells, but also induces expression of other tight junctional components such as Cldn7, Occludin, and ZO-1 (Sugimoto et al., 2013). Our own research has confirmed that Cldn6 is involved in epithelial cell differentiation during lung development (F. R. Jimenez et al., 2015a), and that Cldn6 expression in the lung peaks during early embryogenesis and gradually diminishes prior to birth (F. R. Jimenez et al., 2014). These findings combined with the data from Sugimoto et al. (Sugimoto et al., 2013) suggest that Cldn6 may act as an early and essential mediator in establishing airway epithelium permeability. Lastly, research reveals Cldn6 participation in apoptotic and metastatic behaviors (Stadler et al., 2016; Q. Wang et al., 2015; Q. Wu et al., 2010). While the downregulation of other claudins is known to occur in the intestine (H. Li et al., 2015), epigenetic research implicates Cldn6 dysregulation in cells following cigarette smoke exposure (Oka et al., 2009). Because of its early role in airway commitment and its potential perturbation by tobacco smoke (Oka et al., 2009), we chose to assess Cldn6 alterations in the smoke exposed adult lung.

Cigarette smoke contains a mixture of 4000 substances (Henry, Oldfield, & Kon, 2003; Dietrich Hoffmann, Djordjevic, & Hoffmann, 1997; Talhout et al., 2011), among which are nicotine, carbon monoxide, and carcinogens like benzopyrene, cadmium, and nickel (only cite current #53 and #56). (Stedman, 1968; Talhout et al., 2011). Thus, it is difficult to chemically 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, Pinto, & van Hoek, 2013). Research using the pulmonary adenocarcinoma cell line (A549) has also shown potential TJ targeting (Hoshino et al., 2001) and cellular apoptosis and necrosis after CSE exposure (Gál et al., 2011; Hoshino et al., 2001). In primary alveolar epithelial cells, CSE induced irreversible cell arrest (Thorley & Tetley, 2007) and parenchymal damage (Esechie et al., 2008), but implication of specific TJ components was not determined.

This research tested the hypothesis that TJs comprised of Cldn6 are markedly decreased by tobacco smoke. Loss of the expression of Cldn members is interpreted as a loss in adhesion and barrier function, which are important steps that lead 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 Cldn 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.

Material and Methods

Cigarette Smokes Extract (CSE) Preparation

Cigarette smoke extract (CSE) was prepared as described previously (Adam B Robinson, KacyAnn D Johnson, Brock G Bennion, & Paul R Reynolds, 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 5-20% CSE (Paul R Reynolds, Manuel G Cosio, & John R Hoidal, 2006). 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 (A549), 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. Small airway epithelial cells (SAECs, Lonza Inc., Walkersville, MD) are human primary pulmonary epithelial cells isolated from distal airspaces and they were maintained in SAGM supplemented according to the manufacturer's instructions. Cells were split into six-well plates and grown to between 50 to 60% confluence before media was changed. At near total confluence, cultures were exposed to CSE ranging from 5-20% or media alone over a time course that included 2, 4, 6, 8, or 10 hours. In accordance with this initial time and dose curve, most studies described involved 10% CSE and exposure for 6 hours. After 6 hours of exposure, trypan blue exclusion assays were performed and they revealed no significant decrease in A-549 or SAEC cell viability after exposure to 0% (99.2 ± 0.6 and 99.1 ± 1.9), 5% (97.4 ± 2.1 and 96.0 ± 2.9), or 10% (97.1 ± 3.1 and 96.3 ± 2.8) CSE. Trypan blue exclusion assays also revealed insignificant changes in viability in cell cultures exposed to 15 % or 20% CSE for 6 hours (not shown). RNA was isolated from cells for RT-PCR or lysed to quantify proteins by immunoblotting.

RNA Isolation and RT-PCR Analysis

In order to assess Cldn-6 mRNA expression after CSE exposure, total RNA was isolated from A549 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 (P. R. Reynolds et al., 2008). Reverse transcription of RNA was performed using the Invitrogen Superscript III First-Strand Synthesis System (Life Technologies, Grand Island, NY). The following verified 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) and used as outlined previously (Abuazza et al., 2006).

Complementary DNA 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) (Winden DR, 2014). Conditions included 40 cycles at 95°C for 15 s and 60°C for 60 s. Gene expression was assessed in three separate experiments, each conducted in triplicate, 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 (anti-cldn6 C-20, #sc-17669, 1:200, 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 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). Three separate blotting experiments were conducted, each in triplicate, and images presented are representative. Blots were densitometrically evaluated using Un-Scan-It software as already described (Paul R Reynolds, Stephen D Kasteler, Robert E Schmitt, & John R Hoidal, 2011).

Immunohistochemistry

A goat Cldn6 polyclonal antibody (anti-cldn6 C-20, #sc-17669, 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 that have been highly detailed previously (Paul R Reynolds et al., 2011). Briefly, sections were incubated with primary and secondary antisera that utilize HRP conjugation with Vectors Elite Kit (Vector Laboratories; Burlingame, CA). No-primary control samples were incubated in blocking serum alone prior to the utilization of the Vector Elite Kit.

Mouse Lung Samples

Adult Balb-C mice were exposed to secondhand tobacco smoke or room air for four days as already outlined (Duane R Winden 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 (Tyler T. Wood et al., 2014). The smoke challenge for these studies was chosen according to previously published literature (Paul R. Reynolds et al., 2008; Manuela Rinaldi 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 (Paul R. Reynolds et al., 2008; Manuela Rinaldi et al., 2012). Control animals were similarly handled and restrained but kept in 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 as outlined. The Institutional Animal Care and Use Committee (IACUC) at Brigham Young University approved animal use.

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 HIF-1 α response elements (GCGTG→GGGGG (HRE1) or TGCTA→GGGGG (HRE2)) in the 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 A549 cells using FuGENE-6 HD reagent (Promega) (Porter, Bukey, Geyer, Willnauer, & Reynolds, 2011). A549 is a human pulmonary adenocarcinoma cell line characteristic of alveolar type II cells (Sporty, Horalkova, & Ehrhardt, 2008) and SAECs are human primary cells from the small airways and respiratory compartment. Cells were allowed to reach 50 to 60% confluence and transfected with 100 ng pGL4-0.5-Cldn6, 100 ng pGL4-1.0-Cldn6, or 100 ng pGL4-2.0-Cldn6, 100 ng pRSV- β gal, and 100ng pCMV- HIF-1 α as indicated, and pcDNA control vector to bring total DNA concentration 1.0 μ g. 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 (Reynolds, Mucenski, Le Cras, Nichols, & Whitsett, 2004). Assays-reporter were normalized to determine transfection efficiency (Schagat, Paguio, & Kopish, 2007). Luciferase activity was determined in 20 μ l 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 A549 and SAEC Cell Lines

The effect of CSE exposure on Cldn6 expression was examined in A549 cells and SAECs. Cells were grown in fresh media (Figure 1A and 1B) or culture medium supplemented with 5% CSE (Figure 1A and 1B), 10% CSE (Figure 1A and 1B), 15% CSE (not shown), or 20% (not shown) for 2-8 hours. Immunoblotting was used to assess Cldn6 protein expression in order to determine the optimal CSE dose and period of exposure for these cell types. No changes in Cldn6 expression were noted when cells were grown in culture without CSE (Figure 1A and 1B). There was a marked decrease in protein expression when cells were exposed to 10% CSE for 4-8 hours (Figure 1A and 1B). Q-PCR was next used to analyze differences in mRNA expression. Cldn6 mRNA expression was significantly down regulated in A549 and SAEC cells exposed to 10% CSE compared to controls (Figure 1C and 1D). Immunoblotting and q-PCR revealed a correlation between message and protein expression patterns when CSE-exposed cells were compared to non-smoked (NS) controls.

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*. Immunoblotting for Cldn6 was performed using whole lung lysates. Intense Cldn6 expression in lungs from non-smoked animals (NS) decreased following smoke exposure (Figure 2A). Immunohistochemistry was then utilized to qualitatively ascertain relative Cldn6 expression patterns. Staining of control room-air exposed lungs revealed precise paracellular expression of Cldn6 in the airway epithelium (Figure 2B, arrow) and diffuse expression at boundaries between distal respiratory epithelial cells (Figure 2B, arrowhead). Staining for Cldn6 in the lungs of smoke-exposed animals revealed a detectible loss of Cldn6 expression (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 (Figure 3A). Reporter assays revealed a highly significant decrease in Cldn6 transcription by A549 cells exposed to 10% CSE when compared to normal media controls (Figure 3B).

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

Tobacco smoke exposure elicits tissue damage during periodic bouts of HIF-dependent and HIF-independent responses to altered oxygen tension (Eurlings et al., 2014; Nie et al., 2016; Theodore A Slotkin, 1998). Because tobacco smoke contains approximately 4% (40,000 ppm) CO by volume (Longo, 1977), CO's high affinity for hemoglobin leads to the production of elevated carboxyhemoglobin in smokers and contributes to tissue hypoxia (Underner & Peiffer, 2010). In fact, exhaled CO concentration, which is elevated during hypoxia, provides a non-

invasive assessment for determining smoker status (Bailey, 2013). 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. A549 cells were transfected with reporter constructs containing 2.0-Kb, 1.0-Kb, or 0.5-Kb of the proximal Cldn6 promoter (Figure 3A) and exposed to hypoxia (2% O₂) conditions for 18 hours. We discovered that Cldn6 promoter activity during hypoxia exposure was significantly down-regulated in A549 cells transfected with each of the promoter constructs (Figure 4A). HIF-1 α 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-1 α regulated Cldn6 transcription, A549 cells were co-transfected with Cldn6 reporter constructs and expression vectors that contain constitutively active HIF-1 α . HIF-1 α transcriptionally inhibited all three Cldn6 constructs (Figure 4B). Sequence analysis of the Cldn6 promoter revealed the presence of two HIF-1 α response elements (HRE1 and HRE2, respectively) in the 0.5-Kb promoter sequence. To determine if these HREs control HIF-1 α -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-1 α (Figure 5A). Reporter assays involving either HRE1 or HRE2 revealed that HIF-1 α had no transcriptional effect on the 0.5-kb Cldn6 promoter when HRE1 was mutated (Figure 5B). Experiments involving mutant HRE2 revealed HIF-1 α -mediated transcriptional inhibition that was similar to non-mutant controls (Figure 5B). Additional analyses involving HRE mutant constructs and oxygen tension revealed hypoxia regulation of Cldn6 transcription via HRE1 (Figure 5C). Thus, HRE1 is a key 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 (Danielle Morse & Ivan O 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 (Johnny L Carson, Luisa E Brighton, Albert M Collier, & Philip A Bromberg, 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 that smoke induced disruption of TJs and impaired barrier permeability (Schamberger, Mise, Meiners, & Eickelberg, 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*. Studies such as these clearly show that the

architecture of the epithelial barrier, organized by TJs and other molecular components, require careful characterization. A thorough assessment of morphological disturbances of TJs and likely collateral impact on epithelial permeability and resistance should appropriately expand the scope of tobacco research in the lung.

Our discovery that Cldn6 is specifically inhibited by tobacco smoke 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 (Renat Shaykhiev et al., 2011). The work by Shaykhiev and coworkers revealed low, yet detectible Cldn6 expression in the adult lung; however, others have demonstrated more robust levels of Cldn6 in normal adult pulmonary tissue (Micke et al., 2014; Q. Wang et al., 2015). 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 (Hongwei Li 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 (Carolyn B Coyne, Todd M Gambling, Richard C Boucher, Johnny L Carson, & Larry G Johnson, 2003; Hongwei Li et al., 2015). It remains likely that TJs are similarly targeted in pulmonary tissues intimately exposed to tobacco products.

Our research revealed that Cldn6 is sensitive to decreased oxygen tension and that HIF-

1 α is a potential regulator of Cldn6 expression. Data are accumulating that suggests a clear role for HIF-1 α in the evolution and propagation of diverse inflammatory processes. Li et al. showed that HIF-1 α expression is elevated during the development of COPD and that elevated HIF-1 α may be associated with increased oxidative stress and apoptosis (Hongwei Li et al., 2015). It was proposed that HIF-1 α -mediated cell death coincided with destabilization of epithelial cell TJs. Formative work has just been published that demonstrates profound concentration- and time-dependent accumulation of HIF-1 α protein and significant changes in the expression of its targets in A549 human lung epithelial cells (Daijo et al., 2016). It's therefore probable that HIF-1 α manages an important role in tobacco-induced gene expression changes. Jiang et al. additionally demonstrated that NF- κ B regulates the HIF-1 α pathway during smoke exposure and in cases of advancing COPD (Jiang, Zhu, Xu, Sun, & Li, 2010). They specifically assessed NF- κ B activation following smoke exposure and indicated that HIF-1 α activation via NF- κ B contributed to cell loss and inflammation coincident with COPD progression. The present research further implicates HIF-1 α as having a more robust role in managing cellular responses to tobacco smoke, possibly at the interface between neighboring barrier epithelial cells. While CSE inhibits Cldn6 expression (Figures 1-3) by transcriptional amelioration (Figure 4) via HIF-1 α responsive elements in the Cldn6 promoter (Figure 5), the evidence clearly warrants further investigation into the precise connection between HIF-1 α and specific Cldns.

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-1 α -mediated control mechanisms. In addition to orchestrating cytokine elaboration

during the histopathological remodeling of the chronically exposed lung, HIF-1 α may also destabilize barrier cell TJs observed in progressing lung disease.

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Declaration of Interests

The authors declare that they have no conflict of interest.

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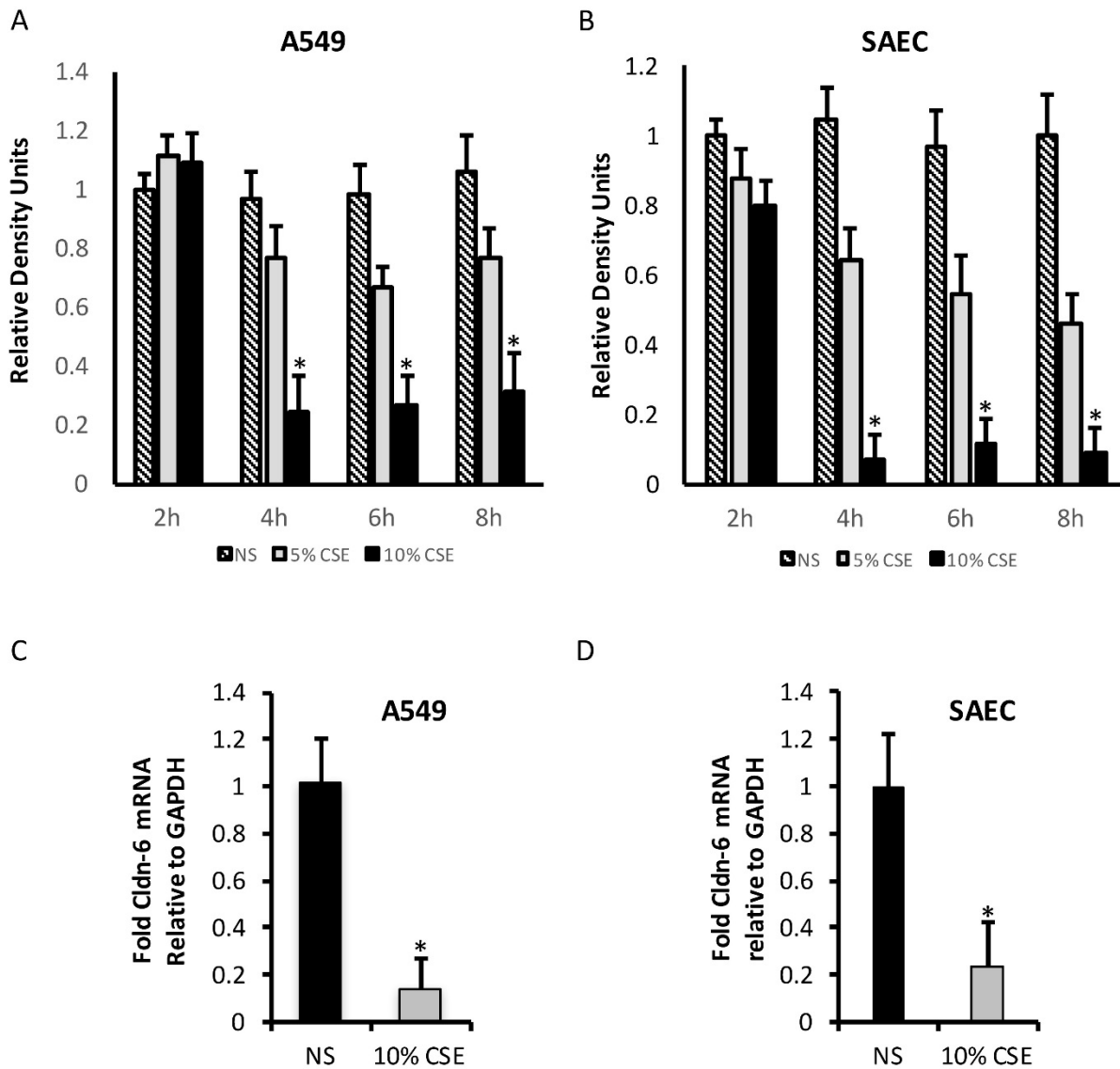


Figure 3.1: CSE Decreased Cldn6 Expression In Vitro.

Immunoblotting for Cldn6 was conducted in A549 cells (A) and SAECs (B) following exposure to no smoke (NS), 5% or 10% CSE for 2-8 hours. A marked decrease in Cldn6 protein expression was detected when cells were exposed to 10% CSE. Q-PCR analyses revealed that A549 cells and SAECs exposed to 10% CSE for 6 hours expressed significantly less Cldn6 mRNA compared to no smoke (NS) controls (C and D). Blotting and q-PCR data are representative of three different experiments each performed in triplicate and $*p \leq 0.05$.

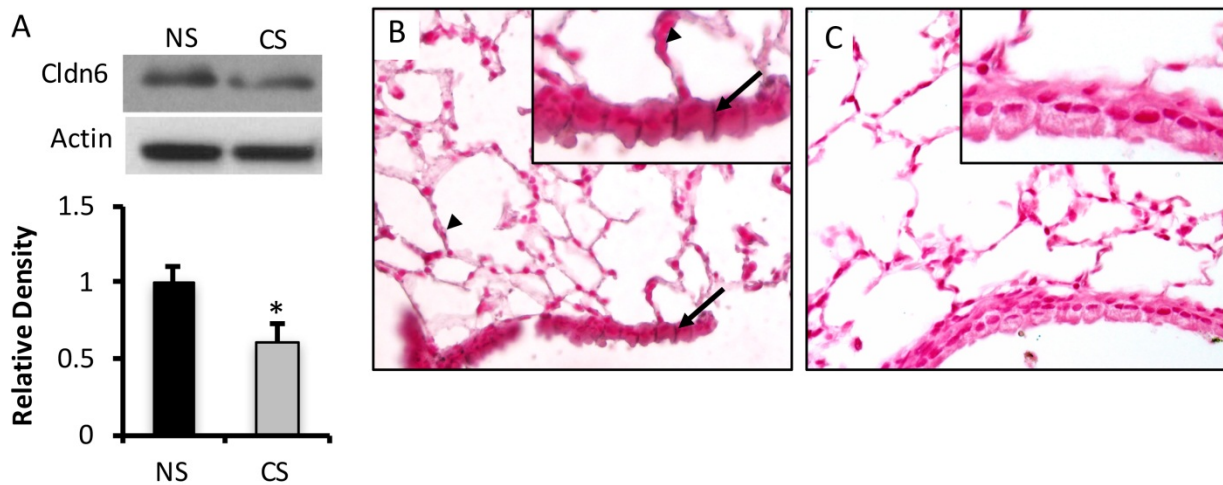


Figure 3.2: Mice Exposed to Secondhand Smoke Expressed Less Cldn6 Compared to Controls.

Immunoblotting for Cldn6 revealed significantly decreased Cldn6 expression in Balb/C mouse lungs following secondhand smoke exposure compared to non-smoked (NS) controls (A, n=6 for each group). Normal room air-exposed mice expressed detectible Cldn6 expression on lateral edges of conducting airway epithelial cells (B, Arrow) and distal respiratory epithelial cells (B, Arrowhead). Qualitative immunohistochemistry performed on mouse lung sections following four days of secondhand smoke exposure resulted in undetectable levels of Cldn6 expression (C). Significant differences in the immunoblotting experiments when compared to controls are noted at $p \leq 0.05$ (*). Images were taken at 40X (or 100X for insets) original magnification.

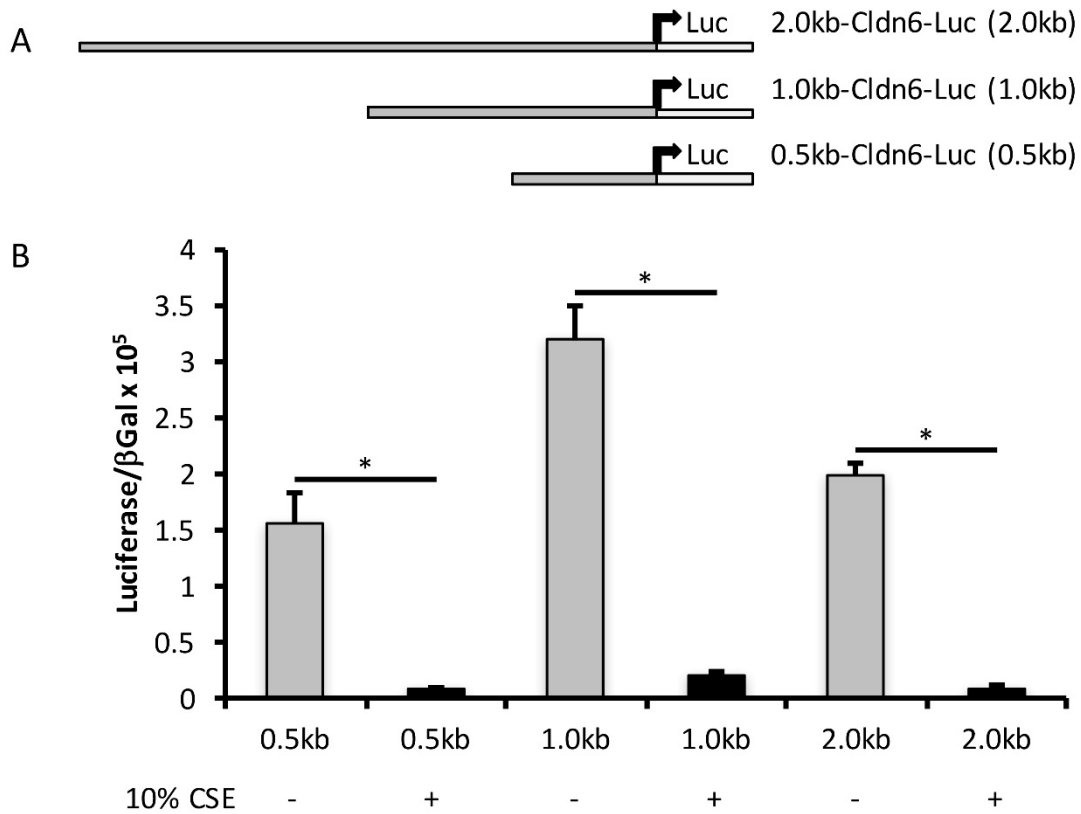


Figure 3.3: CSE Decreased Cldn6 Expression.

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 A549 cells transfected with one of the three reporter constructs. Three different experiments were performed in triplicate and significant differences in luciferase levels compared to reporter alone are noted at $p \leq 0.05$ (*).

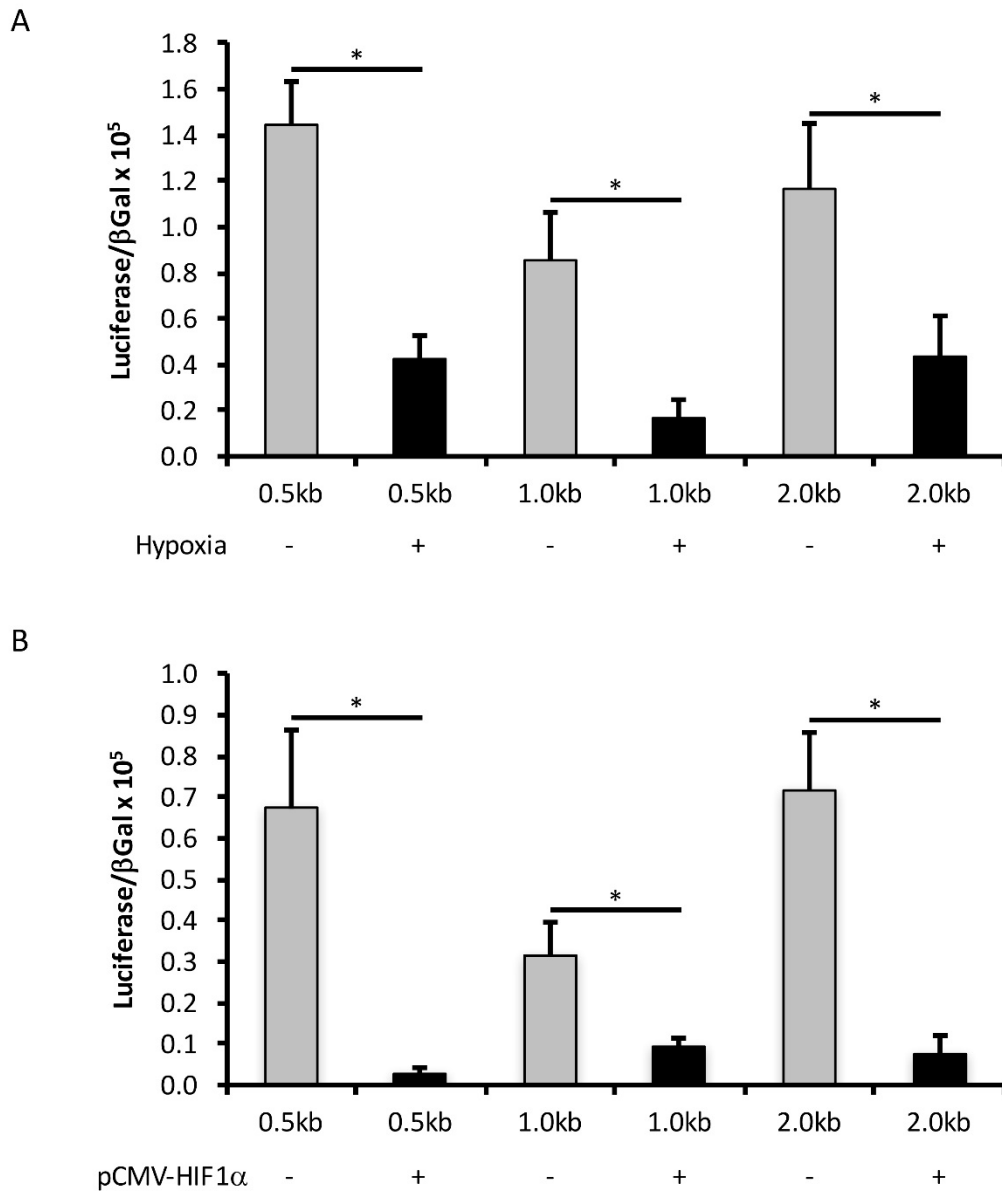


Figure 3.4: Hypoxia Decreases Cldn6 Expression.

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 A549 cells and exposed to hypoxia for 18 hours. Hypoxia significantly decreased Cldn6 transcription via acting on the three reporter constructs (A). Co-transfection of A549 cells with one of the three reporters and expression vectors for HIF-1 α was conducted to assess Cldn6 regulation by HIF-1 α . HIF-1 α transcriptionally inhibited the each of the reporters. Three different experiments were performed in triplicate and significant differences in luciferase levels compared to reporter alone are noted at $p \leq 0.05$ (*).

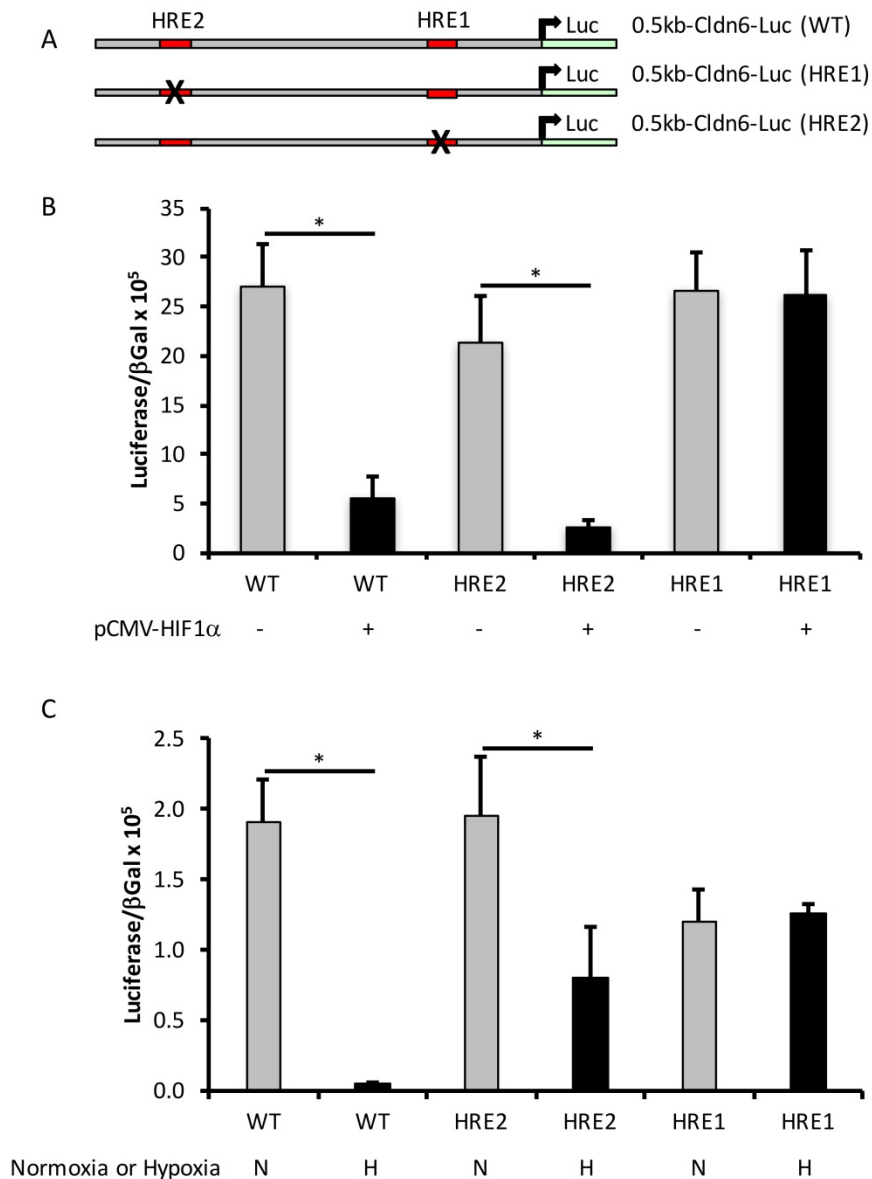


Figure 3.5: HIF-1 α Response Elements (HREs) Influence Cldn6 Expression.

Because HIF-1 α inhibited Cldn6 transcription, site-directed mutagenesis of potential HIF-1 α response elements (HREs) was conducted. Two HREs were identified in the 0.5-kb Cldn6 reporter (A). Co-transfection experiments involving reporters and HIF-1 α expression vectors 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 levels from the non-mutant promoter, 0.5kb-Cldn6-Luc (WT), revealed a loss of HIF-1 α mediated transcription when HRE2 was mutated (B). Loss of HRE1 resulted in no change in HIF-1 α mediated transcriptional inhibition (B). Mutant constructs exposed to 18 hours of normoxia (N) or hypoxia (H) further demonstrated the importance of HRE1 in regulating Cldn6 transcription. Three different 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 4: Up-Regulation of Claudin-6 in the Distal Lung Diminishes Secondhand Smoke-Induced Inflammation Despite Divergent Cytokine Expression

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Abstract

It has long been understood that increased epithelial permeability contributes to inflammation observed in many respiratory diseases. Recently, evidence has revealed that environmental exposure to noxious material such as cigarette smoke reduces tight junction barrier integrity, thus enhancing inflammatory conditions. Claudin-6 (Cldn6) is a tetraspanin transmembrane protein found within the tight junctional complex and is implicated in maintaining lung epithelial barriers. To test the hypothesis that increased Cldn6 ameliorates inflammation at the respiratory barrier, we utilized the Tet-On inducible transgenic system to conditionally over-express Cldn6 in the distal lung. Cldn6 transgenic (TG) and control mice were continuously provided doxycycline from postnatal day (PN) 30 until euthanization date at PN90. A subset of Cldn6 TG and control mice were also subjected to daily secondhand tobacco smoke (SHS) via a nose only inhalation system from PN30-90 and compared to room air (RA) controls. Animals were euthanized on PN90 and lungs were harvested for histological and molecular characterization. Bronchoalveolar lavage fluid (BALF) was procured for the assessment of inflammatory cells and molecules. Quantitative RT-PCR and immunoblotting revealed increased Cldn6 expression in TG vs. control animals and SHS decreased Cldn6 expression regardless of genetic up-regulation. Histological evaluations revealed no adverse pulmonary remodeling via H&E staining or any qualitative alterations in the abundance of type II pneumocytes or proximal non-ciliated epithelial cells via staining for cell specific proSP-C or CCSP, respectively. Immunoblotting and qRT-PCR confirmed the differential expression of Cldn6 and the pro-inflammatory cytokines TNF- α and IL-1 β . As a general theme, inflammation induced by SHS exposure was influenced by the availability of Cldn6. These data reveal captivating information suggesting a role for Cldn6 in lungs exposed to tobacco smoke. Further research is critically

necessary in order to fully explain roles for tight junctional components such as Cldn6 and other related molecules in lungs coping with exposure.

Keywords: claudin-6; lung; tobacco; transgenic

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Running Title: Claudin-6 and secondhand smoke in the mouse lung

Introduction

In recent decades, research surrounding tight junctional proteins has increased and novel insights have revealed much about the function of tight junctions in health and disease. Initially, tight junctions were believed to be simple paracellular seals; however, it is now understood that tight junctions additionally act as key mediators in regulating cell polarity (Kirschner & Brandner, 2012) and in moderating cellular proliferation and differentiation (K. Matter & Balda, 2007). Tight junctions are responsible for maintaining the epithelial permeability barrier and thus they also contribute to the innate immune system. In regards to the lung, tight junctions function in the respiratory defense system by protecting tissues from harmful exogenous materials such as allergens, air pollution, and cigarette smoke constituents (Georas & Rezaee, 2014; Strengert & Knaus, 2011). In addition, tight junctions help regulate the availability of healthy endogenous material and fluids across epithelial barriers (Szaszi & Amoozadeh, 2014). The loss of such critical functions have been implicated in the pathogenesis of many debilitating conditions such as chronic obstructive pulmonary disease (COPD) (A. C. Schamberger et al., 2014; R. Shaykhiev et al., 2011), Emphysema (Bodas, Min, & Vij, 2011; Cuzic et al., 2012), Pulmonary Fibrosis (Lappi-Blanco et al., 2013; Ohta, Chiba, Ebina, Furuse, & Nukiwa, 2012), (Ohta et al., 2012), and acute respiratory distress syndrome (ARDS) (Gu, Liu, Zhao, Wang, & Wang, 2015; Overgaard et al., 2015; Ward, Schlingmann, Stecenko, Guidot, & Koval, 2015). Together, these conditions represent an enormous burden on the healthcare system. As one example, COPD is currently the fourth leading cause of death worldwide and is predicted to become the third leading cause of death by 2030 (Ehteshami-Afshar et al., 2016). The economic burden of COPD alone is in the range of \$18 billion annually (Lopez-Campos, Tan, & Soriano, 2016), and is only expected to increase as death rates similarly rise (Mannino & Buist, 2007). While the

mechanisms surrounding the development of COPD are incompletely characterized, it is known that cigarette smoke is a major risk factor in the development of the condition (Zuo et al., 2014). Thus, as current COPD trends are estimated to rise in the coming years, more research surrounding the possible contributions of tight junctions and the effects on maintaining epithelial barrier integrity may provide novel insights into therapeutic avenues that may aid in combating the disease.

Claudin (Cldn) proteins are critical participants that aid in the establishment of barriers that contain tight junctions (Tsukita & Furuse, 2002). Claudins are tetraspanin transmembrane proteins that interact with structural proteins to form the barrier that impacts the selective permeability observed between epithelial cells (Kielgast et al., 2015; Krause et al., 2008). Cldn-3 and Cldn-18 (B. L. Daugherty et al., 2004; Ohta et al., 2012), are family members abundantly expressed in the lung; however, additional Cldn proteins have been implicated in lung development and in the maintenance of normal lung physiology (Kaarteenaho-Wiik & Soini, 2009; R. Kaarteenaho et al., 2010; Kielgast et al., 2015; Q. Wang et al., 2015).

Cldn6 is one such member of this family of proteins whose role in lung physiology has only recently begun to be elucidated. The expression pattern of Cldn6 peaks during early embryogenesis (E14.5 in mice) and gradually decrease to lower yet detectible levels prior to birth (F. R. Jimenez et al., 2014). TTF-1, Gata-6, and FoxA2, are all transcription factors that are known to regulate critical gene programs that control pulmonary epithelial cell differentiation during lung morphogenesis (Bohinski, Di Lauro, & Whitsett, 1994; Costa, Kalinichenko, & Lim, 2001). Interestingly, these same transcription factors have been shown to regulate Cldn6 expression during pulmonary development (F. R. Jimenez et al., 2014). Precise transcriptional control of Cldn6 is implicated due to the recent discovery that the overexpression of Cldn6

results in a notable delay of lung morphogenesis (F. R. Jimenez et al., 2015b). Furthermore, Cldn6 seems to be implicated in the development of many types of cancers (Liu et al., 2016; Stadler et al., 2016; X. Zhang et al., 2015). In regards to cancers of the lung, current evidence agrees that Cldn6 is implicated, however reports disagree as to whether Cldn6 effect is positive or negative (Micke et al., 2014; Q. Wang et al., 2015). Altogether, these data suggest that Cldn6 may mediate critical functions during development and throughout normal adult life. However, the data confirms that our understanding of Cldn6 and its effects is incompletely understood. Indeed, the evidence supports the idea that deviations from its normal adult pattern of expression may have consequences related to tight junctional effects in compromised adult lungs, however whether those consequences are protective advantages or deleterious in nature remain to be discovered.

The aim of this investigation was to determine the effects of overexpressed Cldn6 in the adult lung, and more specifically determine its potential to protect against inflammation resulting from the exposure to destructive noxious material associated with cigarette smoke.

Material and Methods

Mice

Two transgenic lines of mice were developed from a C57Bl/6 background (Jackson Laboratories, Bar Harbor ME) and used to create conditional doxycycline (dox)-inducible mice that overexpress Cldn6 in the distal lung. Briefly, one line of mice was generated in our laboratory that harbored a single transgene that contained the Cldn6 cDNA sequence downstream of seven concatomerized Tet-on response elements (J. A. Stogsdill et al., 2012). These mice were mated to mice of a second transgenic line that express a reverse tetracycline transactivator (rtTA) downstream of the human surfactant protein C (SP-C) promoter (Reynolds,

Stogsdill, Stogsdill, & Heimann, 2011). Double transgenic SPC-rtTA/TetO-Cldn6 mice (Cldn6 TG) were weaned and genotyped at PN20 (M. P. Stogsdill et al., 2013) and dox (625 mg/kg; Harlan Teklad, Madison, WI) was continuously available until euathanization on PN90. Single or non-transgenic mice were used as controls and were given the same regimen of dox administration. At time of necropsy, *en bloc* lungs were inflation fixed with 4% paraformaldehyde for histology, lavaged for procurement of bronchoalveolar lavage fluid (BALF) (Rao et al., 2010), or resected prior to the isolation of total protein or RNA (Rao et al., 2010). Mice were housed and utilized in accordance with protocols approved by the IACUC at Brigham Young University.

Secondhand Smoke Exposure

As noted, select mice were exposed to SHS generated from 3R4F research cigarettes from Kentucky Tobacco Research and Development Center, University of Kentucky, via a nose-only exposure system (InExpose System, Scireq, Montreal Canada) as outlined previously (T. T. Wood et al., 2014). Briefly, Cldn6 TG and control mice were exposed to daily SHS from PN30 until necropsy on PN90. For comparison purposes, Cldn6 TG and control mice were similarly restrained for the same duration and were exposed to room air (RA). The SHS challenge was determined to be an acceptable level of particulate density concentration according to (M. Rinaldi et al., 2012; Vlahos et al., 2010) and was tolerated without evidence of toxicity. The specific total particulate density concentration was measured weekly and an average of 132.6 mg total particulate matter per cubic meter in the tower was detected. Furthermore, this nose only model of smoke exposure yielded chronic blood carboxyhemoglobin levels of ~5%, a value similarly observed in human smokers (J. L. Wright, Cosio, & Churg, 2008).

Histology and Immunohistochemistry

Cldn6 TG and non-transgenic control lungs were fixed in 4% paraformaldehyde, processed, embedded and sectioned at 4 μ m thickness (Reynolds et al., 2004). Classic hematoxylin and eosin (H&E) staining was performed to observe general lung morphology. Immunostaining for cell-specific markers followed slide dehydration, deparaffinization, and processing with antigen retrieval by citrate buffer. Antibodies that were used include: anti-Cldn6 goat polyclonal antibody (C-20, 1:100; Santa Cruz Biotechnologies, Santa Cruz, CA) CCSP (CCSP, WRAB-3950, 1:100 Seven Hills Bio Reagents, Cincinnati, OH), and proSP-C (proSP-C, WRAB-76694, 1:100 Seven Hills Bio Reagents).

Immunoblotting

Immunoblotting was performed as previously outlined by our laboratory (D. R. Winden et al., 2014). Briefly, tissues were homogenized in protein lysis buffer (RIPA, Fisher Scientific, Pittsburg, PA). 20 μ g of protein lysates were separated on Mini-PROTEAN® TGX™ Precast gel (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose membranes. Membranes were blocked and incubated with polyclonal antibodies against Cldn6 (at a dilution of 1:200; Santa Cruz Biotechnology), TNF- α (sc-52746, Santa Cruz Biotechnology, Dallas, TX, 1:200,) or IL-1 β (ab9722, Abcam, Cambridge, MA; dilution 1:200). Secondary (Ig)-horeseradish peroxidase antibodies were added 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 using a C-DiGit® Blot Scanner (LI-COR, Inc, Lincoln, Nebraska). Immunoblotting was conducted at least twice in triplicate and average band densities were normalized to β -actin densities prior to performing statistical tests.

qRT-PCR

Total RNA was isolated from mouse lungs using an RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). Reverse transcription of RNA in order to obtain cDNA for qRT-PCR and cDNA amplification was performed using Bio Rad iTaq Universal SYBR® Green One-Step Kit. Data analysis was performed using a Bio Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories) (K. Matter & Balda, 2007). The following primers were synthesized by Invitrogen Life Technologies (Grand Island, NY): IL-1 β (For-TGT AAT GAA AGA CGG CAC ACC and Rev-TCT TCT TTG GGT ATT GCT TGG), TNF- α For-TGC CTA TGT CTC AGC CTC TTC and Rev-GAG GCC ATT TGG GAA CTT CT), Cldn6 (For-CAT TAC ATG GCC TGC TAT TC and Rev-CAC ATA ATT CTT GGT GGG ATA TT), and β -actin (For-ACA GGA TGC AGA AGG AGA TTA C and Rev- CAC AGA GTA CTT GCG CTC AGG A).

ELISAs

Molecule-specific ELISA kits that screen for TNF- α or IL-1 β (Ray BioTech, Norcross, GA) were used to assess secretion of these two inflammatory cytokines in lung BALF samples. Briefly, lung BALF samples were isolated from control and treatment groups and screened as outlined in the manufacturer's instructions.

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 representatives and P-values ≤ 0.05 were considered significant.

Results

Cldn6 Expression

Utilizing qRT-PCR, we discovered that *Cldn6* mRNA expression was elevated by approximately ~13 fold in *Cldn6* TG mice (Figure 1A). To confirm that transcriptional mechanisms corresponded with protein expression, we also analyzed protein levels of *Cldn6* and found that although statistically significant, a less robust increase in *Cldn6* protein levels occurred (Figure 1B). These data suggest both mRNA and protein are elevated in our genetic models of *Cldn6* up-regulation. We next sought to assess *Cldn6* expression profiles in the context of SHS exposure. *Cldn6* mRNA expression was profoundly diminished in the lungs of *Cldn6* TG mice following 60 days of SHS exposure (Figure 2A) and blotting for *Cldn6* revealed that SHS similarly decreased protein expression to room air (RA) baselines (Figure 2B).

Lung Morphology

Histological analysis via H&E staining revealed no evidence of tissue simplification or any discernable changes in general lung morphology (Figure 3A). Immunohistochemical staining was next employed using cell-specific markers in order to assess whether there were any cellular changes in the proximal or distal lung compartments. Staining for the propeptide of Surfactant Protein-C (proSP-C) revealed no alterations in the qualitative abundance of alveolar type II cells in the distal lung (Figure 3B). In fact, there was no discernable differences in the relative abundance of alveolar type II cells in *Cldn6* TG or controls regardless of SHS exposure. Immunohistochemical assessment of proximal lung tissue was completed using a marker for Club Cell Secretory Protein (CCSP) elaborated by non-ciliated Club cells in the conducting airways. As was similarly observed relative to proSP-C staining, CCSP immunostaining was

consistent in each of the experimental groups and controls, suggesting no significant alterations in proximal cell quantity (Figure 3C).

Pro-inflammatory Conditions in the Lung

A battery of assessments aimed at discerning inflammatory profiles was next conducted in order to assess whether *Cldn6* expression impacted inflammatory responses to SHS exposure. Quantification of total cells in bronchoalveolar lavage fluid (BALF) resulted in an expected increase in total cells following exposure of control animals to SHS (Figure 4A). While SHS also significantly increased cellular abundance in BALF obtained from *Cldn6* TG animals, significantly less total cells were observed in BALF from *Cldn6* TG animals exposed to SHS when compared to control SHS-exposed mice (Figure 4A). Differential cell analyses were conducted in order to determine the abundance of polymorphonuclear cells (PMNs). These assessments revealed significant inhibition of PMN extravasation into airways of *Cldn6* TG mice exposed to SHS compared to SHS-exposed controls (Figure 4B). In addition to the differential elaboration of cells, there was also a significant difference in the quantity of total protein detected in BALF (Figure 5). As a commonly accepted indirect measure of vascular permeability, the assessment of total protein in BALF revealed a marked increase of protein expression in the BALF of control animals exposed to SHS. Interestingly, *Cldn6* TG mice experienced no change in BALF protein abundance following SHS exposure (Figure 5).

Our attention next focused on the elaboration of pro-inflammatory cytokines. As expected, there was a significant increase in the expression of IL-1 β mRNA and protein in whole lung homogenates from control mice after SHS exposure (Figure 6A and B). There was also a significant increase in the elaboration of IL-1 β by control mice exposed to SHS into BALF (Figure 6C). These assessments also demonstrated that there was no increase in the expression of

IL-1 β message or protein when total mRNA or protein were screened in lysates from Cldn6 TG mice exposed to SHS (Figure 6A and B). While there was a significant increase in the secretion of IL-1 β by Cldn6 TG mice exposed to SHS compared to Cldn6 TG mice exposed to RA, SHS-induced IL-1 β secretion was significantly decreased in Cldn6 TG mice compared to controls (Figure 6C) suggesting that although incompletely, enhancement of tight junctional components may help alleviate certain pro-inflammatory responses. Currently, the literature has demonstrated that Claudins and IL-1 β have significant interactions, however the mechanism by which they affect expression is unclear (Haines, Beard, Chen, Eitnier, & Wu, 2016; Hayashi et al., 2012; Tsujiwaki et al., 2015). TNF- α is another pro-inflammatory cytokine synthesized and secreted following exposure to tobacco smoke. Research has demonstrated that TNF- α interacts with claudin proteins, however more information is needed to elucidate their interactions (Abdullah, Rakkar, Bath, & Bayraktutan, 2015; Mei et al., 2015). Our data demonstrate a significant increase in the expression of TNF- α mRNA from lysates obtained from SHS-exposed Cldn6 TG and controls (Figure 7A). Interestingly, TNF- α protein expression in whole lung lysates was elevated, yet not differentially increased in relation to transgenic control programs (Figure 7B). Lastly, despite a higher baseline of secreted TNF- α into BALF by Cldn6 TG mice exposed to RA (Figure 7C), TNF- α secretion by Cldn6 TG mice was not significantly increased following SHS exposure whereas such secretion was markedly increased following SHS exposure of controls (Figure 7C).

Discussion

The anatomic apposition of the lungs and the environment accounts for the exposure to myriad constituents, each with varying effects on the integrity of pulmonary tissue and the inflammatory status of the individual. Environmental tobacco smoke, or secondhand smoke

(SHS), is a key risk factor for pulmonary disease in all studied demographics (Feigin et al., 2016); however, children are particularly vulnerable due to their lack of control over their living environment. For example, data collected between 2009-2013 from 21 countries in the Global Adult Tobacco Survey (GATS) supported the concept that an estimated 507 million of the 1 billion children residing in the identified countries are exposed to SHS in the home (Mbulo et al., 2016). Without question, identifying molecular mechanisms are necessary so that disease attenuation can be approached in a multifaceted manner. Such an approach should involve cessation programs, direct cellular perturbation, and the exploration into indirect anti-inflammatory modalities. In the current set of experiments, we sought to explore the potential contributions of tight junctions during SHS exposure and hypothesized that more abundant junctions confer measurable anti-inflammatory protection.

Tobacco smoke exposure has been shown to disrupt pulmonary tight junctions. As one example, work conducted by Schamberger et al. (A. C. Schamberger et al., 2014) demonstrated that bronchial tight junctional components were reduced following exposure. Olivera et al. further showed that lung epithelial cells undergo multicellular junctional reorganization during smoke-induced permeability abnormalities (Olivera, Boggs, Beenhouwer, Aden, & Knall, 2007). These and other data suggest that reversing the targeting of tight junctional components during exposure may prove advantageous in lessening smoke-induced cell stress responses and compromised cellular integrity. We recently discovered that pulmonary Cldn6 expression is significantly inhibited by tobacco smoke (F. Jimenez, Lewis, Wood, & Reynolds, 2014). Therefore, the construction of lung-specific transgenic mice that up regulate Cldn6 was undertaken in an effort to counteract Cldn6 targeting by smoke exposure. Intriguingly, Cldn6 mRNA and protein were also significantly diminished in our Cldn6 overexpressing transgenic

mice exposed to SHS (Figure 2). It remains probable that additional barrier constituents are differentially impacted by SHS in the lungs of Cldn6 TG mice.

Our research using bronchoalveolar lavage fluid (BALF) demonstrated that inflammatory characteristics are differentially observed in Cldn6 TG vs control mice. Elevated protein abundance in BALF suggested increased vascular permeability, a finding that coincided with previous research showing cigarette smoke induces BALF protein augmentation (van der Toorn et al., 2013). Our discovery that the induction of total cell and PMN diapedesis were decreased in SHS-exposed Cldn6 TG mice reinforced prior research that demonstrated increased PMN admission into the airways and bronchial tissue of smokers diagnosed with COPD (Stockley, 2002). Pro-inflammatory mediators that mechanistically control pulmonary inflammation were also differentially expressed in the lungs of animals exposed to SHS. We assessed the pro-inflammatory effectors TNF- α and IL-1 β and discovered that IL-1 β was decreased in Cldn6 TG mice exposed to SHS. Known for perpetuating inflammatory axes, TNF- α and IL-1 β induce the release of numerous inflammatory cytokines and enhance leukocyte adhesion during chemotactic transmigration. TNF- α can also induce the elaboration of diverse inflammatory and cytotoxic mediators including IL-1 β , IL-6, platelet activating factor (PAF), and reactive oxygen species (Vlahos et al., 2010). Confirmatory studies revealed that TNF- α and IL-1 β have been detected in lung cells, BALF, and sputum from COPD patients (Caramori, Adcock, Di Stefano, & Chung, 2014). Importantly, the detection of these cytokines in BALF or tissues would have been insufficient because apical and basal elaboration are both possible. Therefore, our data intimate that message and protein expression profiles orchestrate wide reaching effects. It is clear that the synthesis and secretion of these and other inflammatory molecules in the context of Cldn6 may specialize the inflammation programs observed in lungs exposed to SHS.

In summary, this research provides a glimpse of the potential impact of Cldn6 in inflammation that follows SHS exposure. It is well understood that smoking is harmful to health; however, debate continues regarding the link between SHS exposure and disease progression. Because Cldn6, even in the TG animal, is inhibited by SHS, much more research is needed that exhaustively considers other tight junctional components. Such research may reveal that other molecular constituents in the Cldn6 TG animal are necessary for the anti-inflammatory profiles we have observed. We have just recently conducted preliminary research that revealed *increased* expression of key tight junctional proteins including occludin (Runkle et al., 2012), ZO-1 (You et al., 2012), tricellulin (Raleigh et al., 2010) and JAM-a (Ward et al., 2015) in the lungs of Cldn6 TG mice exposed to SHS compared to smoked control mice. Accordingly, Cldn6 TG mice likely up-regulate compensatory components while diminishing Cldn6 availability during the abrogation of SHS-induced inflammation.

Conclusion

This work demonstrates that secondhand smoke (SHS) inhibits Cldn6 expression in lung-specific conditional transgenic mice designed to increase its expression. SHS-induced augmentation of total BALF protein, cells and secreted pro-inflammatory cytokines were all diminished in Cldn6 TG mice exposed to SHS compared to SHS-exposed controls. Intriguing information is provided that suggests roles for Cldn6 in mouse lungs exposed to tobacco smoke. However, further research is needed to fully elucidate discrete roles for this tight junctional protein and other family members in hopes of discovering therapeutically beneficial targets in exposed pulmonary epithelial barriers.

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Declaration of Interests

The authors declare that they have no conflict of interest.

Author Contributions

JBL, DCM, SCA, BJM, TDM, DSB, JRG, and DBT each participated in the several experiments that involved immunoblotting, qPCR, histology and ELISAs. ALL, TMD, and KME conducted smoke exposure experiments. PRR and JAA designed the experiments and provided important intellectual input. JBL, JAA, and PRR generated the manuscript. PRR and JAA conceived of the research project and managed its conduct.

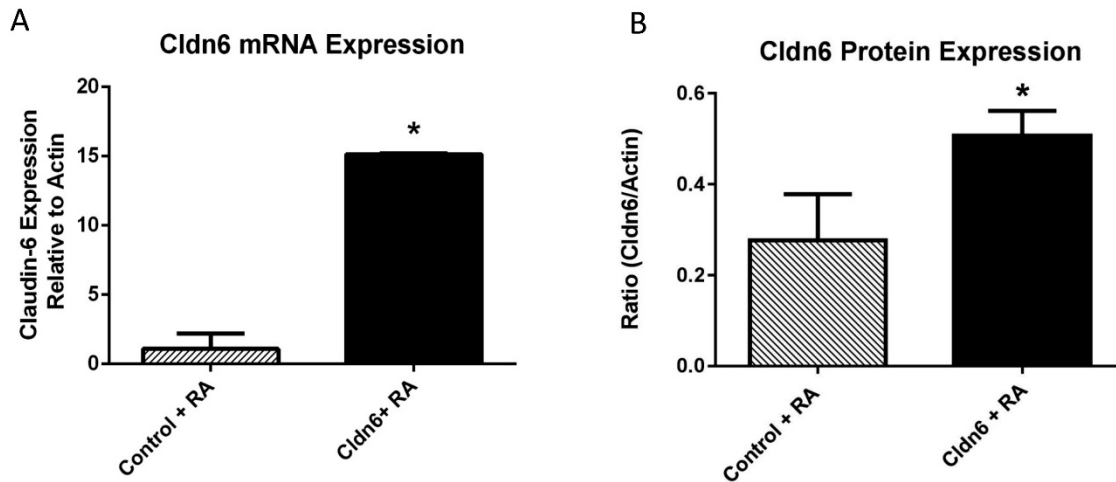


Figure 4.1: Up-regulation of Cldn6.

A, Cldn6 mRNA expression was significantly increased in the lungs of Cldn6 TG mice exposed to room air (RA). The mRNA from each group (n = 6) was normalized to β -actin and representative data are shown with $*p \leq 0.05$. B, immunoblotting revealed that Cldn6 protein is significantly increased in RA-exposed Cldn6 TG mice compared to controls. Representative blots (n = 6 per group) were densitometrically normalized to β -actin and ratios of Cldn6/ β -actin are presented with $*p \leq 0.05$.

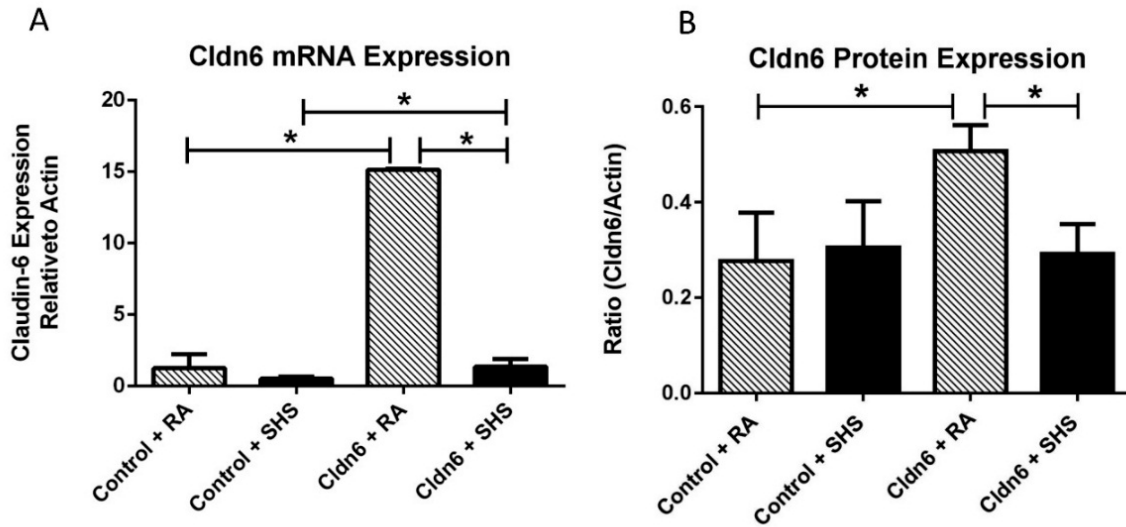


Figure 4.2: Secondhand Smoke Diminishes Cldn6 Expression.

A, Cldn6 mRNA expression was significantly decreased in the lungs of control mice following SHS exposure and up-regulation of Cldn6 in Cldn6 TG mice was inhibited in TG mice exposed to SHS ($n = 6$ per group). The mRNA from each group was normalized to β -actin and representative data are shown with $*p \leq 0.05$. B, immunoblotting revealed that Cldn6 protein was unchanged in control mice following SHS exposure and that protein levels were significantly inhibited in Cldn6 TG mice exposed to SHS. Representative blots ($n = 6$ per group) were densitometrically normalized to β -actin and ratios of Cldn6/ β -actin are presented with $*p \leq 0.05$.

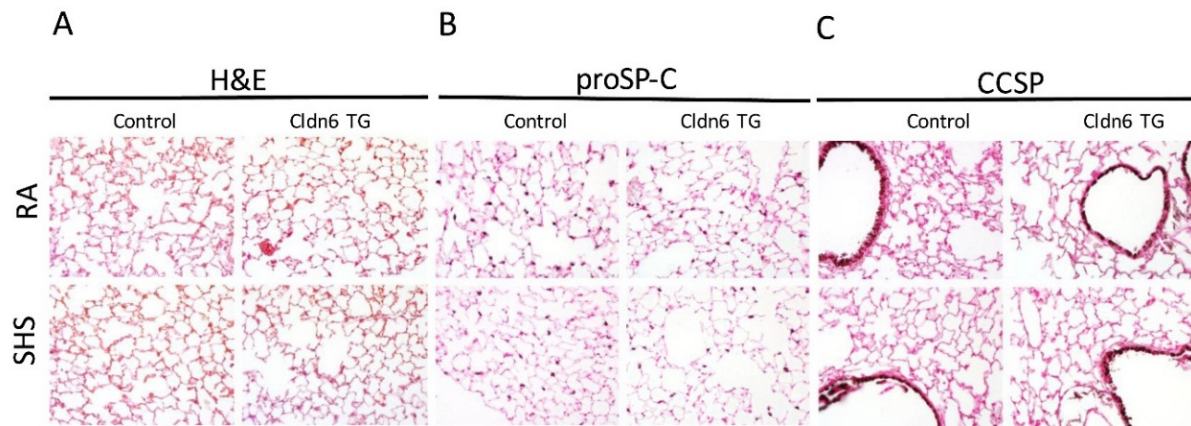


Figure 4.3: Immunohistochemical Analysis of Cldn6 TG Mice.

Representative sections of lung tissue were stained with H&E (A), proSP-C (B), or CCSP (C). No observable anatomical disturbances were observed in the respiratory regions or conducting airways (A). Furthermore, no qualitative differences were detected in lung sections when alveolar type II cells (B) or non-ciliated proximal epithelial cells were counted (C). No staining was observed in controls that lacked primary antibody (not shown). Images (200X magnification) are representative of experiments involving four animals from each group.

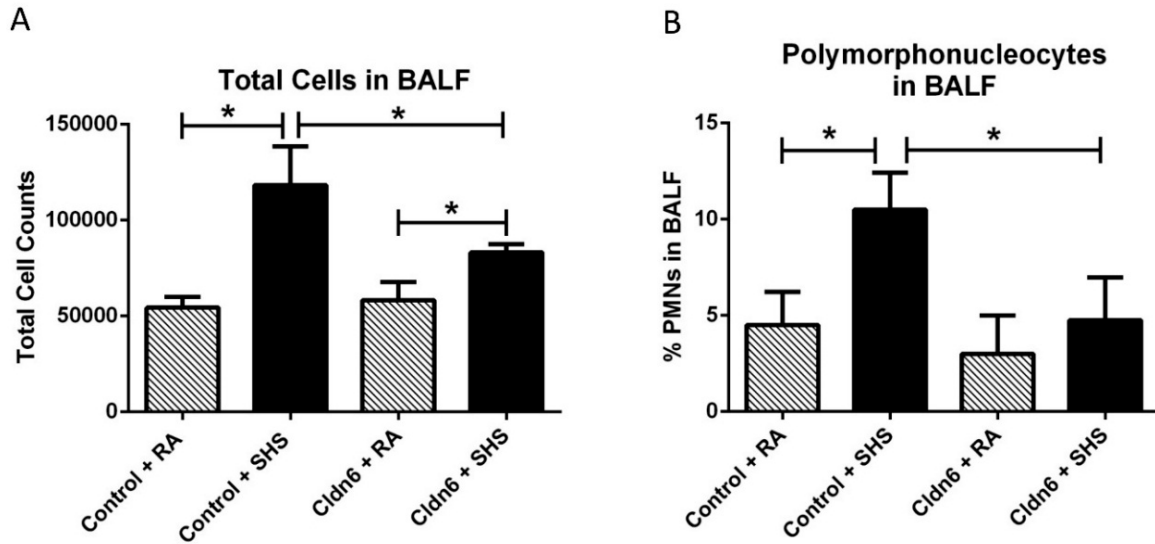


Figure 4.4: Bronchoalveolar Lavage Fluid Analysis.

A, Total BALF cells were significantly increased in control mice + SHS when RA-exposed controls. There was significantly less total cellularity in BALF from Cldn6 TG mice exposed to SHS when compared to SHS-exposed controls. B, The percentage of PMNs was significantly higher in control mice + SHS compared to control mice + RA and PMN quantity was unchanged in Cldn6 TG mice following SHS exposure. Data are representative of experiments involving six mouse lung samples per group and $*p \leq 0.05$.

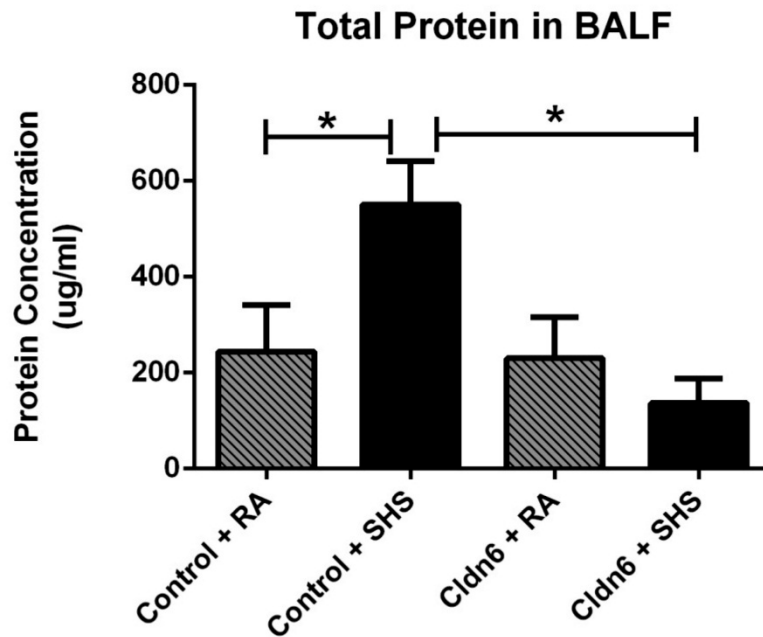


Figure 4.5: Total Protein in BALF.

Total BALF protein was assayed using the BCA technique to demonstrate vascular permeability. Protein was significantly elevated in control mice following SHS exposure and there was no change in total BALF protein from Cldn6 TG mice + SHS when compared to RA groups. Data are representative of experiments involving six mouse lung samples per group and $*p \leq 0.05$.

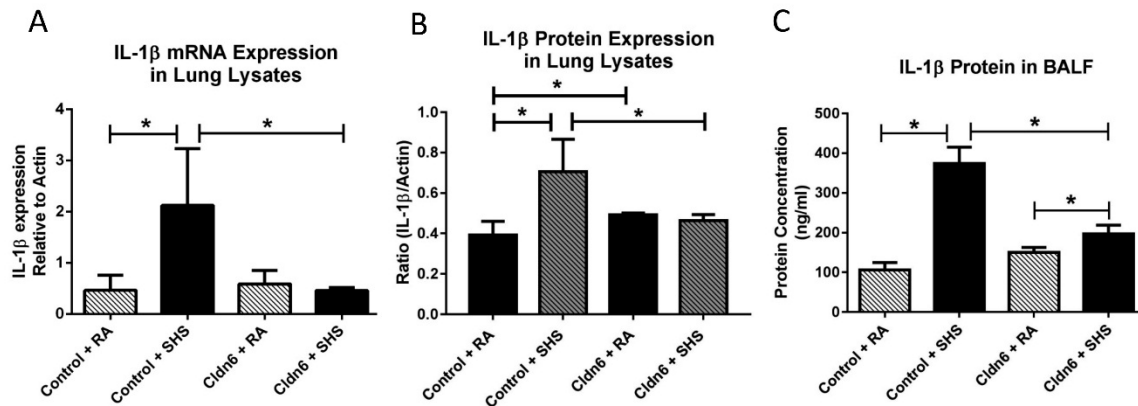


Figure 4.6: IL-1β Expression.

IL-1β mRNA (A) and protein (B) were assayed in whole lung lysates by qPCR and immunoblotting, respectively. SHS increased IL-1β transcription and translation in control animals, but expression of both message and protein were unchanged in SHS-exposed Cldn6 TG mice. C, ELISAs were used to detect secreted IL-1β in BALF. Despite significant increases in the secretion of IL-1β, there was significant inhibition of IL-1β in Cldn6 TG mice exposed to SHS when compared to SHS-exposed controls. Data are representative of experiments involving six mouse lung samples per group and * $p \leq 0.05$.

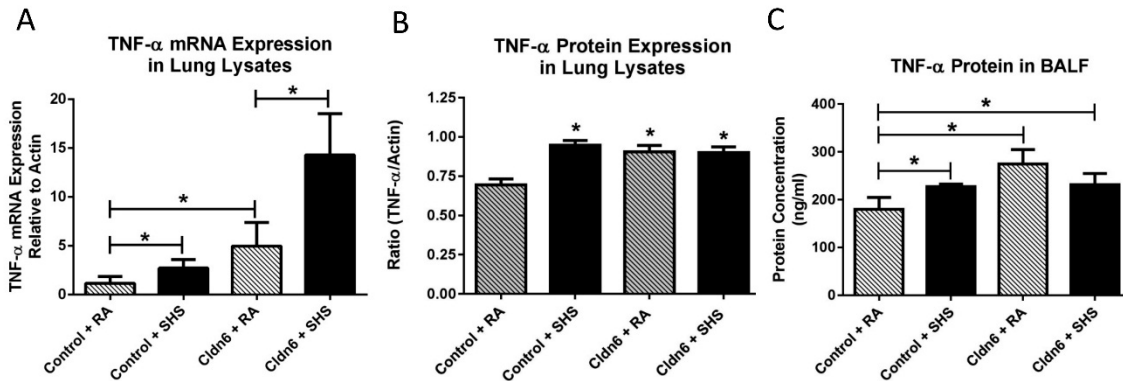


Figure 4.7: TNF- α Expression.

TNF- α mRNA (A) and protein (B) were assayed in whole lung lysates by qPCR and immunoblotting, respectively. SHS increased TNF- α transcription in both control and Cldn6 TG animals. TNF- α protein in lung lysates was significantly increased in SHS-exposed controls, but no increase was observed in Cldn6 TG mice following SHS exposure. C, ELISAs were used to detect secreted TNF- α in BALF. Secreted TNF- α was increased in control mice following SHS exposure. Despite a slightly higher basal level of secretion by Cldn6 TG mice, SHS did not significantly increase TNF- α secretion. Data are representative of experiments involving six mouse lung samples per group and $*p \leq 0.05$.

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CHAPTER 5: Transgenic Up-regulation of Claudin-6 Decreases Fine Diesel Particulate Matter (DPM)-Induced Pulmonary Inflammation.

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Running Title: Claudin-6 and DPM-induced lung inflammation

Abstract

Background: Claudin-6 (Cldn6) is a tetraspanin transmembrane protein that contributes to tight junctional complexes and has been implicated in the maintenance of lung epithelial barriers.

Objective: In the present study, we tested the hypothesis that genetic up-regulation of Cldn-6 influences inflammation in mice exposed to acute environmental diesel particulate matter

(DPM). **Methods and Results:** Mice were subjected to ten exposures of nebulized DPM (PM=2.5) over a period of 20 days via a nose only inhalation system (Sireq, Montreal, Canada).

Using real time RT-PCR, we discovered that the Cldn6 gene was up-regulated in control mice exposed to DPM but DPM significantly decreased Cldn-6 expression in lung-specific transgenic mice that up-regulate Cldn-6 (Cldn-6 TG). DPM caused increased cell diapedesis into

bronchoalveolar lavage fluid (BALF) obtained from control mice, however, Cldn-6 TG mice had less total cells in BALF following DPM exposure. Because Cldn-6 TG mice diminished cell

diapedesis, other inflammatory intermediates were screened in order to characterize the impact of increased Cldn-6 on inflammation. Cytokines that mediate inflammatory responses including

TNF- α and IL-1 β were differentially regulated in Cldn6 TG mice and controls following DPM exposure. **Conclusions:** These results demonstrate that epithelial barriers organized by Cldn-6

mediate, at least in part, diesel-induced inflammation. Further work may show that Cldn-6 is a key target in understanding pulmonary epithelial gateways exacerbated by environmental pollution.

Introduction

In 2012, the World Health Organization estimated that the number of premature deaths associated with outdoor air pollution in urban areas was 3.7 million worldwide (Organization, 2012). This increased mortality is due to exposure to particulate matter of 10 microns (PM 10 μm) or less in diameter, which enables these harmful materials to penetrate deep into the small airways and parenchymal compartments of the lung. Exposure to diesel particulate matter (DPM) has been associated with cardiorespiratory disorders leading to elevated mortality and morbidity, including asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis (Hosseini et al., 2016), (Bates et al., 2015), (Londahl et al., 2012), (Weinmann et al., 2008) (Ma et al., 2014), (Pope et al., 1995). Research has additionally revealed that exposure to DPM significantly increases cytotoxicity, oxidative stress, and pro-inflammatory responses of lung epithelia and macrophages (Michael, Montag, & Dott, 2013), (Lasagni Vitar et al., 2015), (P. R. Reynolds, K. M. Wasley, & C. H. Allison, 2011), (Barton et al., 2014). Our understanding of the possible mechanisms that are implicated in the progression of many of these respiratory diseases are limited and incomplete. In order to combat these devastating health disparities, more research examining the mechanisms that contribute and regulate to the immune response to DPM is required.

In recent decades, researchers have begun to focus on the tight junction as a potential target implicated in the pathogenesis of many respiratory disorders. Initially, tight junctions were believed to be simple paracellular seals, however our current understanding of the functions of tight junctions now include the regulation of cell polarity and moderating cellular proliferation and differentiation (Gonzalez-Mariscal et al., 2014), (Y. Soini, 2012), (Findley & Koval, 2009). Tight junctions have long been viewed as critical in maintaining the permeability barrier;

however, they are now also considered an essential component of the overall innate immune system. Within the lung, tight junctions confer protection by barring harmful penetration of exogenous material such as allergens, cigarette smoke, and air pollution beyond barrier boundaries (Georas & Rezaee, 2014), (Strengert & Knaus, 2011). Additionally, tight junctions act as pores for the flux of endogenous material via cellular fluid between epithelia (Szaszi & Amoozadeh, 2014). The loss of these occluding functions have been demonstrated to be involved in the pathogenesis of many respiratory diseases such as COPD (R. Shaykhiev et al., 2011), (A. C. Schamberger et al., 2014), Pulmonary Fibrosis (Lappi-Blanco et al., 2013), and Acute Respiratory Disease (Ohta et al., 2012).

Claudin-6 (Cldn6) is a tetraspanin protein expressed within the tight-junctional complex that is critical in maintaining the permeability barrier between epithelial cells (Tsukita & Furuse, 2002), (Enikanolaiye et al., 2010). Our own research has revealed that Cldn6 expression peaks during early embryogenesis and gradually decreases prior to birth (F. R. Jimenez et al., 2014). Additionally, critical transcription factors implicit in lung branching morphogenesis (TTF-1, FoxA2 and GATA6) activate transcription of the Cldn6 gene, suggesting that Cldn6 may be an important genetic target during lung organogenesis. Furthermore, succeeding studies have demonstrated that overexpression of Cldn6 during early embryogenesis results in a delay of lung morphogenesis, which was mediated by decreased differentiation and apoptotic signaling (F. R. Jimenez et al., 2015b). These data suggest that Cldn6 is important during early embryonic periods but reveals little as to its function in normal adult lung physiology. Moreover, these data provide no insight into the role of Cldn6 in adverse pathologic conditions. Accordingly, the aim of this undertaking was to address the effects Cldn6 in the adult mouse following acute exposure to DPM.

Material and Methods

Mice

Transgenic mice were developed from a C57B1/6 background (Jackson Laboratories) and were used to create conditional doxycycline (dox) – inducible mice that overexpress Cldn6. Mice that harbor a Tet-On Cldn6 sequence were mated with mice that possess a reverse tetracycline transactivator sequence (rtTA) downstream of the human surfactant protein C (SP-C) promoter in order to generate the Cldn6-SPC TG animals required for these experiments. At PN20, double transgenic mice (Cldn6 TG) were weaned and genotyped (M. P. Stogsdill et al., 2013), and administered dox (625 mg/kg; Harlan Teklad, Madison, WI) until time of euthanasia. Single or non-transgenic mice were used as controls, and were given the same dox regimen. At time of necropsy, *en bloc* lungs were inflation fixed with 4% paraformaldehyde for histology, lavaged for assessment of bronchoalveolar lavage fluid (BALF) (Rao et al., 2010), or resected prior to the isolation of total protein/RNA (D. Morse & I. O. Rosas, 2014). Mice were housed and utilized in accordance with protocols approved by the IACUC at Brigham Young University.

Diesel Particulate Matter (DPM) Exposure

DPM used in the current investigation is the Standard Reference Material (SRM-2975) maintained by the National Institute of Standards & Technology (NIST). This reference DPM was selected because it is widely available and previous research suggested its size permits penetration into the alveolar compartment (Miller et al., 2013). The authors wish to specifically thank A. Ghio (U.S. Environmental Protection Agency, EPA, University of North Carolina, Chapel Hill, NC) for graciously providing SRM-2975. SRM-2975 was obtained from M.E. Wright of the Donaldson Company, Inc., Minneapolis, MN as outlined previously (P. Reynolds, K. Wasley, & C. Allison, 2011). Briefly, the DPM was generated by heavy-duty forklift engines

and collected from a filtering system specifically designed for diesel-powered forklifts (M. Wright, Klein, & Stesniak, 1991). The reported mean diameter of course SRM 2975, determined by area distribution light scattering, was $11.2 \pm 0.1 \mu\text{m}$. However, the mean particle size computed by number distribution is $1.62 \pm 0.01 \mu\text{m}$. To our knowledge, information regarding load, fuel, and other run conditions are not available. Control mice were separated into two groups, vehicle or DPM exposed. Treatment was administered via a nose-only nebulizing system (Scireq, Montreal, Canada). DPM was generated so that animals received a nebulized dose of 15 ng DPM per exposure. Control animals were given the equivalent dosage, but instead only received nebulized PBS. Mice received treatment over a two-week period, with a total of ten exposures. Mice were housed and utilized in accordance with protocols approved by the IACUC at Brigham Young University.

Histology and Immunohistochemistry

Cldn6 TG and non-transgenic control lungs were fixed in 4% paraformaldehyde, processed, embedded and sectioned at $4\mu\text{m}$ thickness (Reynolds et al., 2004). Hematoxylin and eosin (H&E) staining was performed to observe general lung morphology. Immunostaining for cell-specific markers involved tissue sections that 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). Antibodies that were used include CCSP, (WRAB-3950, 1:100 Seven Hills Bio Reagents, Cincinnati, OH) and pro-surfactant protein-C (ProSP-C, WRAB-76694, 1:100 Seven Hills Bio Reagents).

Immunoblotting

Immunoblotting was performed as previously outlined by our laboratory (D. R. Winden et al., 2014). Briefly, tissues were homogenized in protein lysis buffer (RIPA, Fisher Scientific, Pittsburg, PA). Protein lysates (20 µg) were separated on Mini-PROTEAN® TGX™ Precast gel (Bio-Rad Laboratories, Inc) by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked and incubated with a goat polyclonal antibody against Cldn6 (at a dilution of 1:200; Santa Cruz Biotechnology), TNF-α (sc-52746, Santa Cruz Biotechnology, Dallas, TX, 1:200,) or IL-1β (ab9722, Abcam, Cambridge, MA; dilution 1:200). Fluorescent Secondary immunoglobulin (Ig)-horeseradish peroxidase antibodies were added for one hour at room temperature. Membranes were then analyzed and digitally recorded using a Odyssey® CLx Blot Scanner (LI-COR, Inc, Lincoln, Nebraska). Quantification of Cldn6 was performed by densitometry and normalization with actin provided comparisons between Cldn6-SPC TG and control lung samples.

Quantitative Real-time PCR

Total RNA was isolated from mouse lungs using an RT-PCR Miniprep Kit (Stratagene, La Jolla, CA). Reverse transcription of RNA in order to obtain cDNA for qRT-PCR, and cDNA amplification was performed using Bio Rad iTaq Universal SYBR® Green One-Step Kit. Data analysis was performed using a Bio Rad Single Color Real Time PCR detection system (Bio-Rad Laboratories) (Cokkinides et al., 2009). The following primers were synthesized by Invitrogen Life Technologies (Grand Island, NY): Claudin-6 (For) – CAT TAC ATG GCC TGC TAT TC, Claudin-6 (Rev) – CAC ATA ATT CTT GGT GGG ATA TT, TNF-α (For) – TGC CTA TGT CTC AGC CTC TTC, TNF-α (Rev) – GAG GCC ATT TGG GAA CTT CT, IL-1β (For) – TGT AAT GAA AGA CGG CAC ACC, IL-1β (Rev) – TCT TCT TTG GGT ATT GCT TGG, β-actin

(For-ACA GGA TGC AGA AGG AGA TTA C and Rev- CAC AGA GTA CTT GCG CTC AGG A).

ELISAs

Molecule-specific ELISA kits that screen for TNF- α or IL-1 β (Ray BioTech, Norcross, GA) were used to assess secretion of these two inflammatory cytokines in lung BALF samples. Briefly, lung BALF samples were isolated from control and treatment groups and screened as outlined in the manufacturer's instructions.

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 representatives and P-values ≤ 0.05 were considered significant.

Results

Cldn6 Overexpression

qRT-PCR analysis revealed elevated Cldn6 expression (approximately ~4 fold) in Cldn6 TG mice compared to controls (Fig 1A). Protein expression was assessed by immunoblotting in order to confirm correspondence between transcriptional and translational control. There was a less robust, yet statistically significant increase in Cldn6 protein expression observed in Cldn6 TG mice compared to controls (Fig 1B). These data suggested the possibility that translational regulatory mechanisms may influence Cldn6 protein expression despite genetic modification at the transcriptional level. Having confirmed up-regulation of Cldn6, we next sought to assess the effect of acute DPM exposure on Cldn6 expression (Fig 2). Control mice subjected to DPM

exposure showed an approximately 4 fold increase in mRNA levels of Cldn6 (Fig 2A). Elevated Cldn6 protein was also observed in control animals following exposure to DPM (Fig 2B). Intriguingly, when Cldn6 TG animals were exposed to DPM, Cldn6 mRNA and protein expression was significantly diminished.

Lung Morphology

No evidence of tissue simplification or changes to general lung morphology was revealed when H&E histological analyses were completed (Fig 3). General alterations in proximal and distal lung compartments were screened using immunohistochemical staining for cell-specific markers. Staining for the propeptide of surfactant protein-C (ProSP-C) indicated that the qualitative abundance of alveolar type II cells in the distal lung was unchanged (not shown). Furthermore, Club Cell Secretory Protein (CCSP) assessment demonstrated no changes in non-ciliated club cell abundance or distribution in the conducting airways (not shown).

Inflammatory Cell Abundance

To assess inflammatory profiles, we conducted a series of experiments to evaluate whether Cldn6 up-regulation influenced inflammatory responses to DPM. Quantification of total cells in bronchoalveolar lavage fluid (BALF) revealed an expected increase in total cell abundance following exposure of control animals to DPM (Fig 4A). Non-exposed Cldn6 TG animals revealed similar total cell counts as observed in the control + PBS group, but a significant decrease in cell extravasation was detected in the Cldn6 TG + DPM group (Fig 4A). Differential assessments of leukocytes revealed that the PMN population in control and Cldn6 TG mice exposed to DPM was significantly elevated (Fig 4B); however, PMN abundance was significantly attenuated in Cldn6 TG + DPM compared to control mice + DPM (Fig 4B). Evaluation of total protein abundance in BALF samples was undertaken in order to indirectly

assess vascular permeability commonly observed in vessels that are active sites of leukocyte diapedesis. As expected, the addition of DPM resulted in increased BALF protein in control animals (Fig 4C). While BALF protein in Cldn6 TG + PBS was diminished when compared to the control PBS group, a significant reduction in BALF protein was observed in Cldn6 TG + DPM when compared to DPM-exposed controls (Fig 4C).

Pro-Inflammatory Mediators

Having established differential inflammatory cell conditions, we next considered specific pro-inflammatory cytokines. Quantitative RT-PCR analysis confirmed a marked increase in mRNA expression of TNF- α when control animals were exposed to DPM. Interestingly, mRNA expression in lungs from Cldn6 TG + PBS and Cldn6 TG + DPM were indistinguishable from unexposed controls (Fig 5A). To confirm whether mRNA in lung lysates corresponded with secreted protein, we screened BALF fluid for secreted TNF- α using specific ELISAs. Our analysis revealed no significant differences between all groups (Fig 5B). We next sought to assess IL-1 β due to its implication as a partner of TNF- α that also drives cell diapedesis (Damera et al., 2016). Evaluation of IL-1 β showed increased mRNA expression (Fig 6A) and increased BALF protein (Fig 6B) in samples from control mice following DPM exposure. In contrast, Cldn6 TG animals (both PBS and DPM exposed) did not increase IL-1 β transcription or protein secretion when exposed to DPM (Fig 6A and B).

Discussion

According to the World Health Organization (WHO), approximately 92% of the world's population live in locations where the WHO air quality guidelines are not met (WHO). Accordingly, a large majority will live in conditions that may adversely affect their health, while simultaneously increasing the global economic burden. Indeed, current projections suggest that

in 2020, approximately 68 million deaths will occur worldwide and at least 11.9 million of these will be caused by respiratory diseases (Naghavi et al., 2015). With such galling data, it is undeniably clear that the need for identifying molecular mechanisms is essential in combating respiratory disease development and progression. Such insights would likely allow healthcare providers comprehensive and exhaustive approaches in treatment, and may provide improvement in prevention strategies, new diagnostic tools for screening, and new developments in anti-inflammatory modalities. The current set of experiments was designed to assess the potential influence of acute DPM exposure on tight junctions during inflammation. Our hypothesis was that the up-regulation of tight junction constituents may result in an enhanced barrier integrity that lessens the penetration of harmful exogenous material such as DPM and ultimately results in measurable anti-inflammatory protection.

It has been well documented that particulate matter with a diameter range of 2.5-10 μm (PM_{2.5-10}) is associated with pulmonary function loss (Provost et al., 2014; Yang et al., 2017; Zarcone et al., 2016) and inflammation (S. S. Salvi et al., 2000). However, data are less conclusive in examining the direct effects of DPM on the integrity of the alveolar epithelial barrier. One study by Caraballo et al. demonstrated that human alveolar epithelial cells treated with DPM for three hours developed increased permeability, as well as tight junctional disruption evidenced by dissociation of occludin and ZO-1 (Caraballo, Yshii, Westphal, Moninger, & Comellas, 2011). Similarly, Lehman et al. treated a triple co-culture of human epithelial cells (16HBE14o-), monocyte derived macrophages, and dendritic cells with DPM and found that that expression of occludin was modulated. However, the authors of this study found increased permeability only in mono-cultures, not the triple co-cultures, suggesting that additional factors may be important in regulating the permeability barrier (Lehmann, Blank,

Baum, Gehr, & Rothen-Rutishauser, 2009). Despite these studies, conclusive evidence has yet to emerge linking the effects of DPM and Claudin, which has been demonstrated to play a major role in the maintenance of the permeability barrier (Gong & Hou, 2017; Overgaard, Daugherty, Mitchell, & Koval, 2011; Schlingmann et al., 2015). The limited data shows that tight junctions are impacted by DPM exposure and that the buttressing or enhancement of tight junctional components may prove advantageous in suppressing exogenous material-induced cellular responses.

Recent research from our lab has identified Cldn6 as a tight junctional component that is targeted by secondhand smoke (SHS) and hypoxic conditions (F. R. Jimenez et al., 2016). A study conducted by Mavrofrydi et al. examining hypoxia in the context of benzo[a]pyrene showed that the addition of DPM enhanced HIF-1 α activation, a pivotal regulator involved in hypoxic responses (Mavrofrydi & Papazafiri, 2012). To assess whether increasing the abundance of Cldn6 provides similar protective advantages against DPM exposure as observed during SHS and hypoxia, we utilized a lung specific Cldn6 overexpressing transgenic mouse (F. R. Jimenez et al., 2015a; Lewis et al., 2016). Surprisingly, the exposure of DPM to control animals increased Cldn6 expression both transcriptionally and translationally. In the transgenic mouse, DPM decreased Cldn6 mRNA and protein. These data suggest that feedback mechanisms possibly regulate Cldn6 expression during acute DPM exposure. It is plausible that the alveolar barrier's natural response is to express additional tight junctional components such as Cldn6 to overcome acute exogenous challenges and thus maintain normal homeostasis. However, Cldn6 is already elevated in the transgenic mouse and other junctional constituents may be favored during responses to DPM. Some possibilities have emerged in pilot projects wherein we've discovered *increased* expression of key tight junctional proteins including

occludin (Runkle et al., 2012), ZO-1 (You et al., 2012), tricellulin (Raleigh et al., 2010) and JAM-a (Ward et al., 2015) in the lungs of Cldn6 TG mice exposed to particulates when compared to particulate-exposed control mice. Further exhaustive studies are needed to corroborate these discoveries.

Our research using bronchoalveolar lavage fluid (BALF) demonstrated characteristics that have been observed during lung inflammatory lung disease. Elevated BALF protein intimates augmented vascular permeability, which has been associated with allergic airway inflammation and acute lung injury (Ogino et al., 2014). Our discovery that the induction of PMN diapedesis was increased in DPM-exposed control mice, yet significantly diminished in Cldn6 TG mice reinforced prior research that demonstrated increased PMN recruitment into the airways mice and patients exposed to diesel particulates (Bosson et al., 2007; Ogino et al., 2014). This research implicates Cldn6 up-regulation as a means to slowing PMN extravasation in DPM-exposed lungs and thus these potent reservoirs of elastolytic enzymes, leukocyte chemoattractants, and other mediators of inflammation and remodeling are hindered when Cldn6 expression increases.

Molecules involved in the control of lung inflammation were also differentially expressed in the lungs of animals exposed to DPM. Transcription of TNF- α and IL-1 β was significantly increased in DPM-exposed control mice, but not increased in the lungs of exposed Cldn6 TG mice. TNF- α is expressed by macrophages and additional mediators are known to be elaborated following its secretion including IL-1 β , IL-6, platelet activating factor (PAF), eicosanoids, and ROS (Laskin, Sunil, et al., 2010). Secretion of TNF α is increased in mouse models of tissue injury (Pendino, Shuler, Laskin, & Laskin, 1994), by macrophages exposed to diesel exhaust (Kafoury & Madden, 2005), and in the lungs of mouse models following inhalation of diesel

exhaust particles (Laskin, Mainelis, Turpin, Patel, & Sunil, 2010). TNF- α also regulates the expression of ICAM-1, an adhesion molecule expressed by endothelial cells that assists in leukocyte diapedesis (Kyan-Aung, Haskard, Poston, Thornhill, & Lee, 1991; McCrea, Ensor, Nall, Bleecker, & Hasday, 1994). Because TNF- α and IL-1 β cooperate in the modulation of leukocyte migration, such cytokine quantities plausibly explain the decreased total cells counts and PMN diapedesis observed in the exposed TG mice. Elevated synthesis and secretion of these and other inflammatory molecules support the discoveries of Stevens et al., that implicate these perpetuators of the inflammatory axis following DPM exposure (Stevens, Krantz, Linak, Hester, & Gilmour, 2008).

Conclusion

This research adds insight into the potential role of Cldn6 in the development and progression of inflammation mediated through DPM exposure. Negative effects of environmental pollution on pulmonary function is understood; however, mechanisms that lead to deleterious effects are not adequately characterized. As our data demonstrate, increased Cldn6 expression occurs in control animals exposed to DPM, but such was not observed in TG animals. Additional research should be undertaken that seeks to discern mechanisms that cause DPM-induced inhibition of Cldn6 in over-expressing mice yet leave expression unaffected in exposed controls. A recent study published by Sugimoto et al. revealed that Cldn6 trigger epithelial morphogenesis in mouse F9 stem cells, but more importantly found that Cldn6 was able to induce the expression of other tight junctional components including Cldn7, Occludin, and ZO-1 (Sugimoto et al., 2013). Thus, compensatory junctional components are likely up-regulated by Cldn6 TG mice during the abrogation of DPM-mediated increases in total BALF protein, cells and secreted pro-inflammatory cytokines. Further research is required to fully elucidate discrete

roles for this tight junctional protein and other family members in hopes of discovering therapeutically beneficial targets.

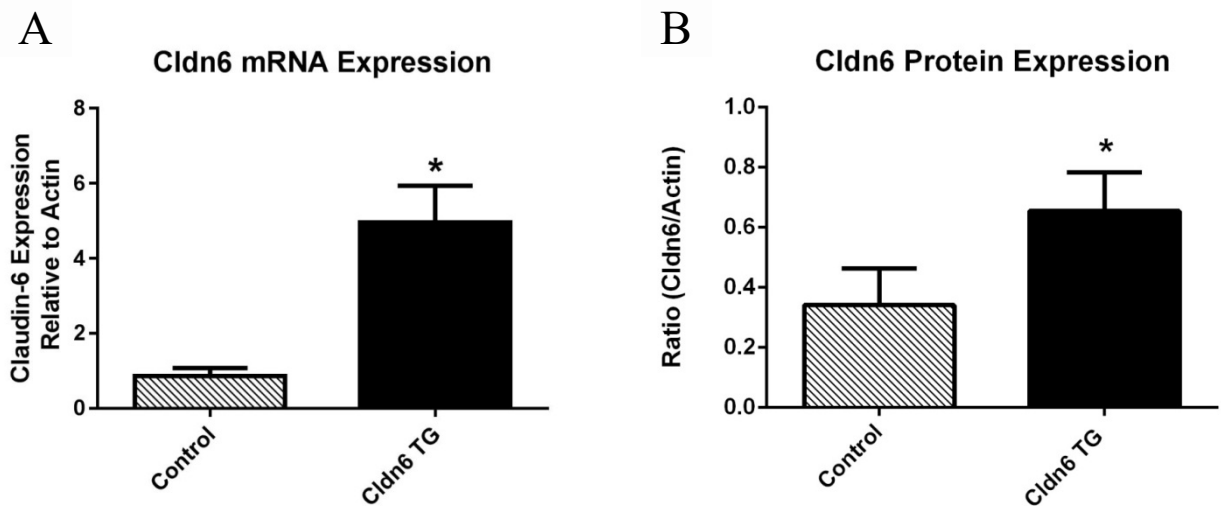


Figure 5.1: Cldn6 Expression in the Transgenic Model.

(A) Cldn6 mRNA expression was significantly increased in the lungs of CLdn6 TG mice. The mRNA was normalized to β -actin and representative data are show with $*p \leq 0.05$. (B) immunoblotting revealed that Cldn6 protein is significantly increased in Cldn6 TG mice compared to controls. Representative blots were densitometrically normalized to β -actin and ratios of Cldn6/ β -actin are presented with $*p \leq 0.05$.

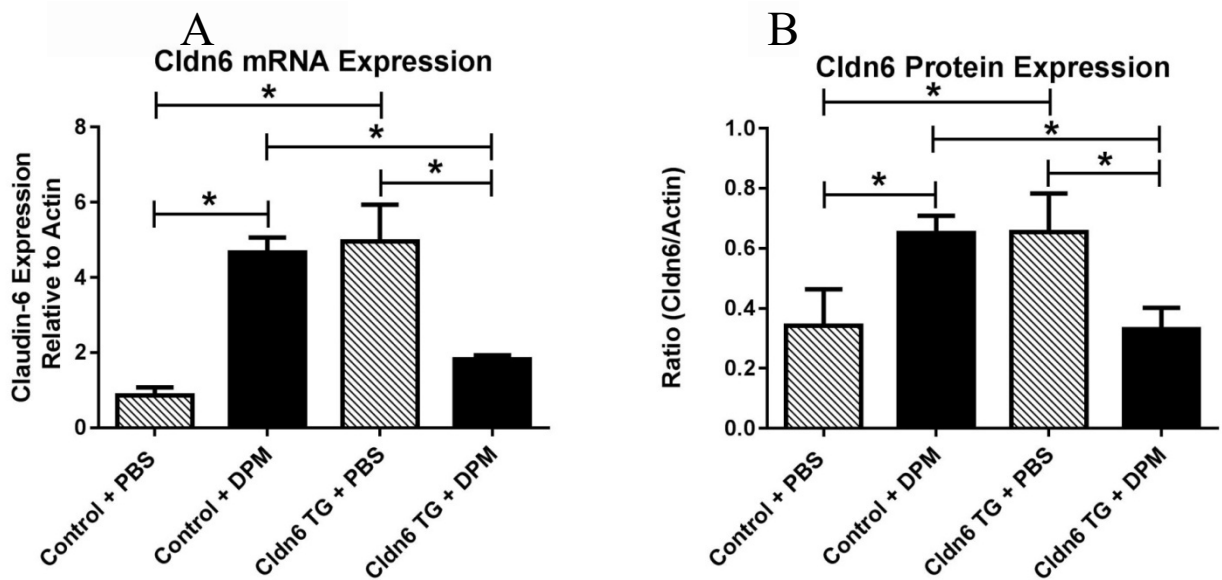


Figure 5.2: Divergent Effects of DPM.

(A) Cldn6 mRNA expression was elevated with the addition of diesel particulate matter (DPM) in control animals. In Cldn6 TG animals the addition of DPM resulted in a decrease in Cldn6 mRNA. The mRNA was normalized to β -actin and representative data are shown with $*p \leq 0.05$. (B) Analysis of Cldn6 protein demonstrated that DPM in control animals increased Cldn6 protein levels. Cldn6 TG animals exposed to DPM showed decreased levels of DPM. Representative blots were densitometrically normalized to β -actin and ratios of Cldn6/ β -actin are presented with $*p \leq 0.05$.

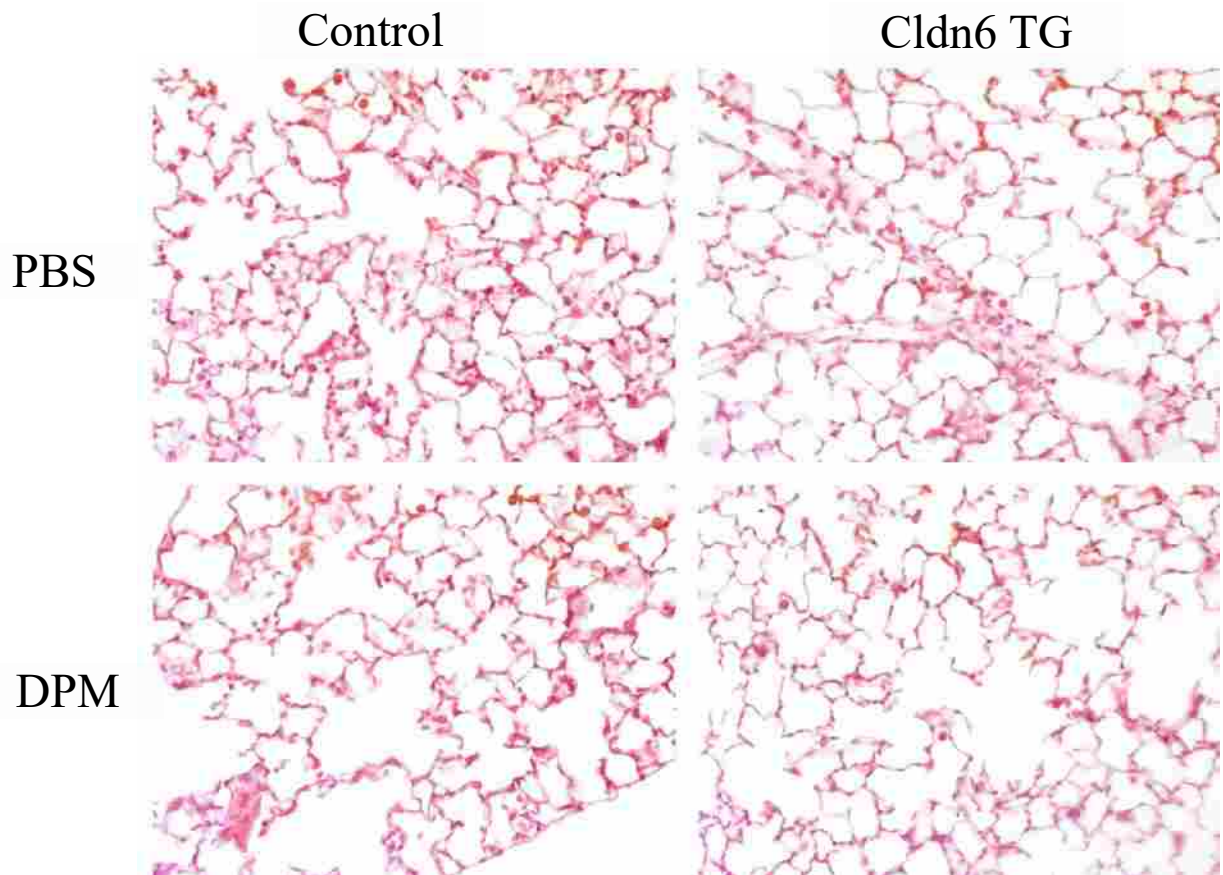


Figure 5.3: Lung Morphology in WT and Cldn6 TG Animals.

Representative sections of lung tissue were stained with H&E. No observable anatomical disturbances were observed in the respiratory regions or conducting airways. No staining was observed in controls that lacked primary antibody (not shown). Images (200x magnification) are representative of experiments involving four animals from each group.

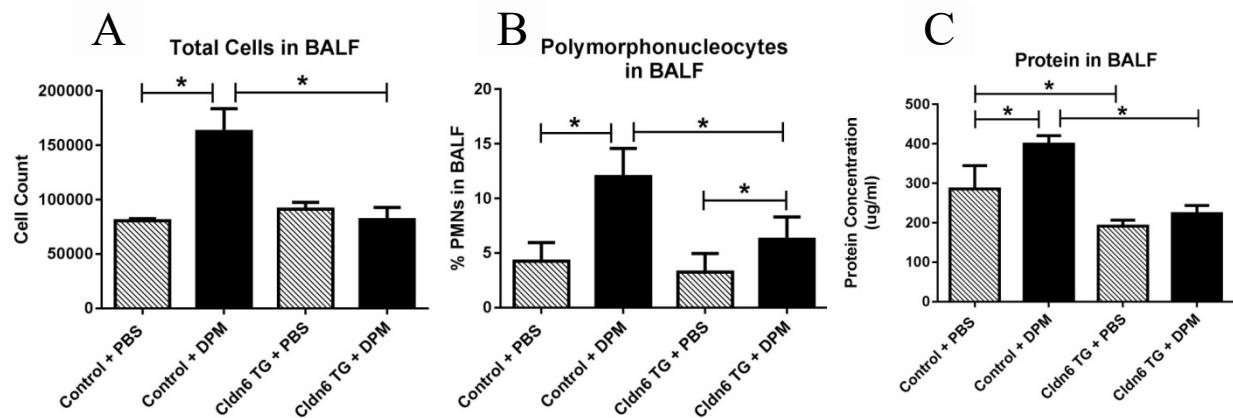


Figure 5.4: Overexpression of Cldn6 Protects Against Inflammation.

(A) Total bronchoalveolar lavage fluid (BALF) cells were significantly increased in control mice + DPM when compared to PBS exposed control animals. There was significantly less total cellularity in BALF from Cldn6 TG mice exposed to DPM when compared to DPM exposed controls. (B) the percentage of PMNs was significantly higher in control mice + DPM compared to control mice and PBS. PMN quantity was slightly elevated in Cldn6 TG mice following DPM exposure, but was significantly decreased when compared to control animals + DPM. (C) Total BALF protein was assayed using the BCA technique to demonstrate vascular permeability. Protein was significantly elevated in mice following DPM exposure. Cldn6 TG animals (both control and DPM exposed) showed decreased BALF protein.

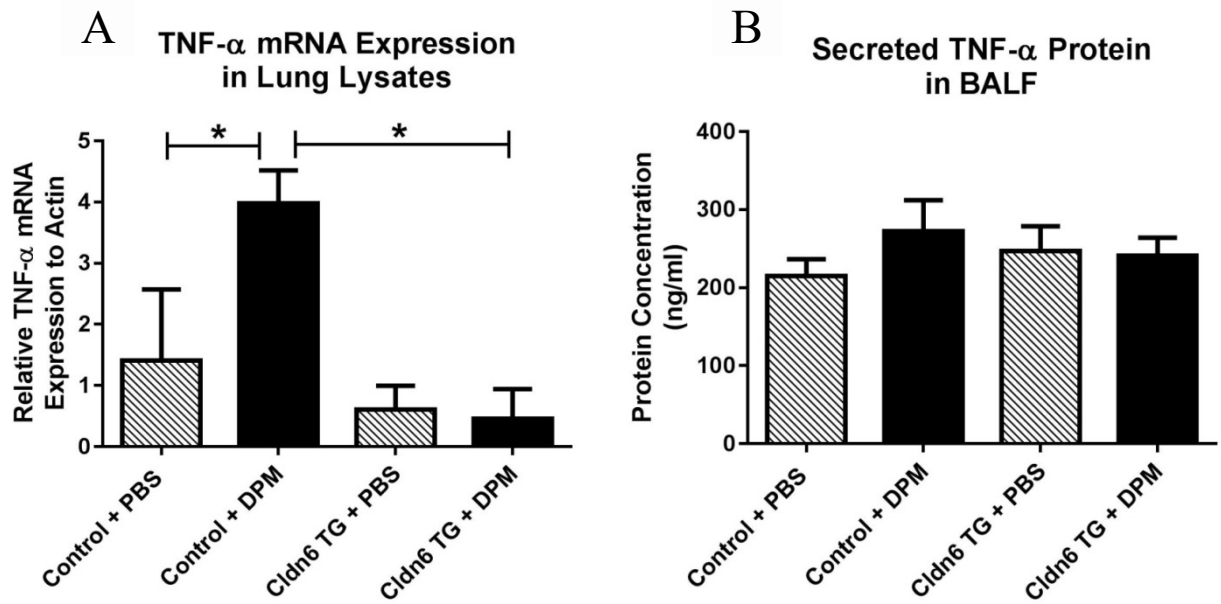


Figure 5.5: Overexpression of Cldn6 Diminishes TNF- α mRNA.

TNF- α mRNA from lung lysates (A) and secreted protein from BALF (B) were assayed by qPCR and ELISA assay respectively. DPM increased TNF- α mRNA in control animals, but showed no effect in mRNA in Cldn6 TG animals. B, Analysis of BALF protein showed no differences in secreted TNF- α among all groups.

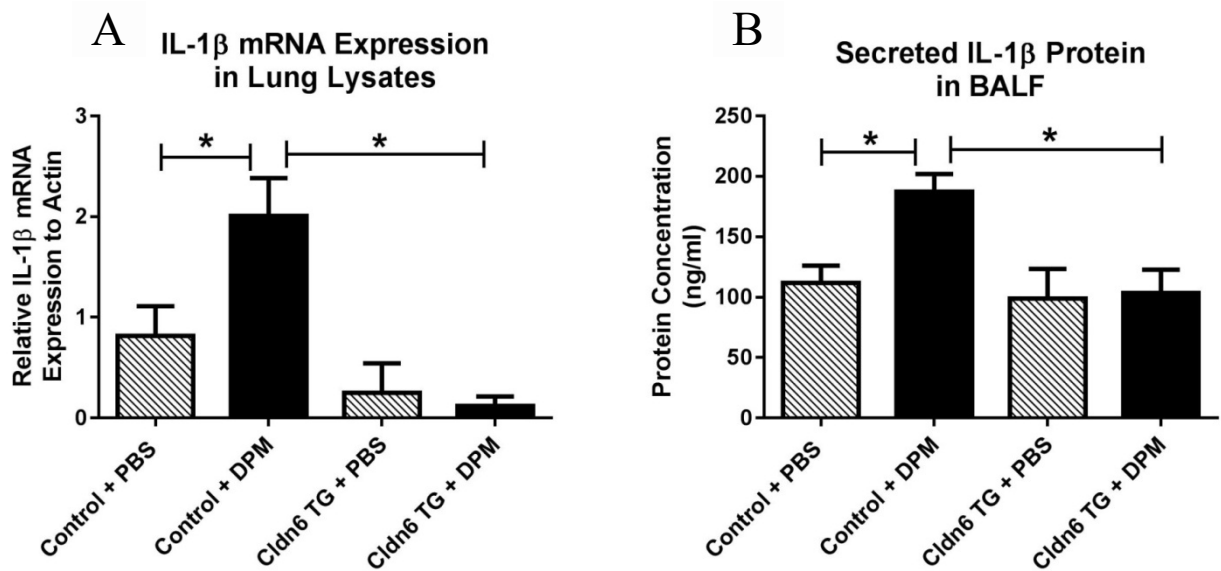


Figure 5.6: Overexpression of Cldn6 Diminishes IL-1 β .

IL-1 β mRNA from lung lysates (A) and secreted protein from BALF (B) were assayed by qPCR and ELISA assay respectively. DPM significantly increased IL-1 β mRNA in control animals but showed no effect in mRNA in Cldn6 TG animals. B, Analysis of BALF protein showed elevated IL-1 β secreted protein when control animals were exposed to DPM. Cldn6 TG animals showed no change in IL-1 β protein levels.

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CHAPTER 6: General Discussion

The current work sought to investigate the possible interactions between environmental irritants such as secondhand smoke and combusted diesel particulates and Claudin proteins. Claudins are tight junctional proteins that are fundamental in maintaining the lung permeability barrier, and the loss of these proteins has been associated with COPD (Cuzic et al., 2012; R. Suzuki et al., 2016), ARDS (Overgaard et al., 2015; Ward et al., 2015), Pulmonary Fibrosis (Lappi-Blanco et al., 2013; G. Li et al., 2014; Ohta et al., 2012). Utilizing a comprehensive and exhaustive approach, we developed experiments to give insights into both the effects of secondhand smoke (SHS) on claudin expression, and the effect of modulating claudin expression to counteract the harmful inflammatory effects of secondhand smoke and diesel particulate matter (DPM).

A Developmental and Secondhand Smoke Context

Critical to our understanding of these various proteins was to first analyze the developmental context in which these proteins are expressed. Previous studies from Kaarteenaho's research group (R. Kaarteenaho et al., 2010) had examined the developmental expression profile of Claudin -1, -3, -4, -5, -7 in the human lung and had demonstrated divergent expression patterns in pseudoglandular, canicular, saccular, and alveolar periods. However to our knowledge, no studies had exhaustively looked at all claudin member expression. Furthermore, the majority of these studies used human samples, but neglected to compare claudin expression in the murine model. Our data established that the murine model mirrors human claudin expression both spatially and temporally. In addition to providing the developmental framework for understanding claudin proteins, this study provided additional insights into the deleterious effects of secondhand smoke on claudin expression. Dams exposed to SHS demonstrated in

their offspring a decrease in almost all claudin mRNA, the exception being Claudin-18. This data further adds to the body of growing evidence that maternal exposure to SHS has long-term negative outcomes. In the context of claudin expression, maternal exposure to SHS decreases claudin expression, thus increasing the likelihood of predisposing offspring to an impaired permeability barrier. Although the entire claudin profile has yet to be fully examined, current literature has demonstrated that a modulation of claudin-3 (Mitchell, Overgaard, Ward, Margulies, & Koval, 2011), claudin-4 (Kage et al., 2014; Mitchell et al., 2011; Wray et al., 2009), claudin-5 (C. B. Coyne et al., 2003; Koval, 2013; Ward et al., 2015), claudin-7, and claudin-18 (LaFemina et al., 2014; G. Li et al., 2014) are associated with decreased alveolar epithelial barrier function. Although our data demonstrates the harmful effect of maternal smoking, more studies are needed to examine the mechanisms and the pathologies associated with these smoke induced deficiencies.

Secondhand Smoke's Negative Effects on Claudin-6 Expression

Having identified SHS as an antagonist of claudin proteins, we then focused our attention on Claudin-6 (Cldn6). As maternal SHS exposure had demonstrated the ability to decrease Cldn6 expression, we then sought to assess the effect of SHS on cells directly. Using a human epithelial lung cell line (A549) and small airway epithelial cells (SAECs), we administered cigarette smoke extract, and noted a significant decrease in both transcriptional as well as translational regulation. Our findings were then further confirmed as we exposed wild type animals to SHS and found decreased levels of both mRNA and protein. To elucidate the mechanism by which SHS was modulating Cldn6 expression, we transfected the Cldn6 promoter region with a luciferase reporter. Administration of CSE significantly inhibited Cldn6 expression at the 0.5 kb, 1.0 kb, and 2.0 kb region of the promoter sequence, demonstrating

CSE's ability to diminish Cldn6 expression. As cigarette smoking is closely associated with hypoxia, we then sought to assess whether key promoter elements known to be activated in hypoxic conditions were similarly involved in regulating Cldn6 expression. Again, utilizing a luciferase reporter, we exposed cells to hypoxic conditions and found that Cldn6 transcription was diminished. HIF-1 α is a protein that translocates to the nucleus when oxygen tension decreases in order to regulate target genes involved in cellular responses to hypoxia. As our findings had demonstrated that hypoxic conditions diminished Cldn6 expression, we then sought to see the effect of constitutively expressing HIF-1 α on Cldn6 transcription. The results revealed that HIF-1 α transcriptionally inhibited Cldn6 expression. Further experiments then demonstrated through mutagenesis of two HIF-1 α response elements within the 0.5 kb promoter (HRE1, HRE2) that loss of function from either HRE1 or HRE2 resulted in Cldn6 expression being unaffected by hypoxic conditions. Altogether, our findings give valuable insights into the mechanisms by which CSE interacts with tight junctional complexes. Moreover, it implicates HIF-1 α as having a more robust role in managing cellular responses to tobacco smoke, possibly at the interface between neighboring barrier epithelial cells, thus demonstrating the greater need for more studies investigating the tight junctional complex.

Claudin-6 Overexpression Protects against Exogenous Material-mediated inflammation

Next, having looked at the effect of tobacco smoke on claudin expression, we sought to determine what effect claudin expression had on protecting against the harmful effects of SHS. To accomplish this we utilized a conditional Cldn6 overexpressing mouse, developed by our lab for prior experimentation. Our previous studies had demonstrated that normal Cldn6 expression was high during periods that correlated with the pseudoglandular/canalicular period (F. R. Jimenez et al., 2014). However these levels diminished to marginal levels just prior to birth.

Additionally, we demonstrated that this decrease in Cldn6 is important for normal development, as animals that continually overexpress Cldn6 undergo abnormal lung branching morphogenesis (F. R. Jimenez et al., 2015a). Using this transgenic animal, we allowed mice to undergo the normal developmental program, but then when mice had reached adulthood, we reinitiated overexpression of Cldn6. These mice were then subjected to SHS exposure for an extended period in order to achieve a chronic state of inflammation. Our hypothesis was that by increasing Cldn6 expression, we might be able to enhance the permeability barrier, thus giving a protective advantage to these animals against harmful exogenous material such as cigarette smoke. The data that we were able to obtain gave us intriguing results. Analyzation of bronchoalveolar lavage fluid (BALF) demonstrated a decrease in vascular leakage, a decrease in polymorphonucleocytes (PMNS), and decreased total cell counts, suggesting that indeed these transgenic animals had decreased inflammation in response to the SHS. We further characterized pro-inflammatory cytokines via enzyme-linked immunosorbent assay (ELISA) and found that overexpression of Cldn6 decreased IL-1 β , thus explaining the decrease in inflammation. Yet, when we analyzed protein expression of Cldn6, our data showed that although our transgenic animal induced overexpression in the room air (RA) animals, Cldn6 overexpression was overcome by the addition of SHS.

While conducting the aforementioned research we had simultaneously begun experiments where we exposed Cldn6 overexpressing animals to nebulized diesel particulate matter (DPM) for an acute exposure period. We once more utilized BALF analysis, and found that again vascular leakage was diminished, polymorphonucleocytes numbers had fallen, and total cell counts were reduced, confirming that these animals had decreased inflammation. Using an ELISA we were able to measure cytokine secretion. Analysis of cytokines revealed decreased

IL-1 β and TNF- α expression, again explaining the possible reason for decreased inflammation. Yet again, examination of Cldn6 levels showed that although room air transgenic animals had elevated Cldn6 levels in both the mRNA and protein, transgenic animals that were treated with DPM showed Cldn6 levels comparable to WT animals.

A Revised Hypothesis and Future Directions

With data from two studies now confirming decreased inflammation in our Cldn6 overexpressing animals, we have reevaluated our hypothesis to explain these intriguing effects. One possibility that might be occurring is the recruitment of other tight junctional components as a result of Cldn6 overexpression. In regards to normal physiology, Claudins are never isolated and are instead found as dimers or tetramers (Schlingmann et al., 2015). Often it is the heteromeric interactions of various claudin proteins that result in the unique physical characteristics (cationic/anionic channels) of individual pores (Schlingmann et al., 2015). As a result, it is plausible, that by increasing Cldn6 expression we may have inadvertently recruited the expression of other claudins such as Cldn3, Cldn4, Cldn7 or Cldn18. Additionally, it is equally probably that by increasing Cldn6 expression, other peripheral tight junctional components such as Occludin, Zona Occludin (ZO), and Junctional Adhesion Molecules (JAMs) are subsequently increased. Currently the literature supports this idea as Cldn6 has been implicated as a pluripotent stem cell marker and has been demonstrated to play a significant role in epithelialization (L. Wang et al., 2012). One study by Sugimoto et.al revealed that Cldn6 was able to induce the expression of other tight junctional components such as Cldn7, Occludin, and Zona Occludin (Sugimoto et al., 2013). Currently, our data is limited and yet to be published, but preliminary studies examining mRNA expression of many of these tight junctional constituents has revealed elevated mRNA expression levels. These findings coupled with the

discovery that Cldn6 overexpression during development delayed lung morphogenesis, suggests that the Cldn6 may play an even greater role in the developmental process, as it may act as a key mediator for tight junction recruitment.

Relevance of Research

Just this year, Goodchild et.al released a shocking report that suggested that the total economic cost of smoking in 2012 totaled \$1852 Billion dollars, or approximately 1.8% of the entire world's annual GDP (Goodchild, Nargis, & Tursan d'Espaignet, 2017). Although these numbers are appalling, they should not be surprising when one considers that COPD is projected to be the third leading cause of death worldwide by 2020 (WHO, The Top 10 causes of Death Factsheet 2012). In regards to the pathophysiology, COPD is characterized by airflow obstruction due to chronic inflammation in the airways and respiratory parenchyma, elastic recoil loss, and destruction of the alveolar walls. One aspect that may be associated with the development of this and many other respiratory diseases, is the loss of the permeability barrier. Implicit in maintaining this barrier, is the tight junctional component Claudin. Individual studies have confirmed that misregulation of claudin proteins is often associated with a host of respiratory diseases (Kaarteenaho-Wiik & Soini, 2009; R. Shaykhiev et al., 2011; Wray et al., 2009). As these proteins are understood to participate in the development and progression of disease, research surrounding these molecules may lead to new modalities and therapies that help ameliorate inflammation.

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GENERAL SUMMARY OF PART II

During the tenure of my dissertation research in the Lung and Placenta Research Laboratory, my perspectives related to inflammation and organ homeostasis were expanded. Key aspects of my dissertation research that focused on the biology of Claudin-6 clarified the concept that cells and tissues are organized so that structural integrity is maintained while also providing defensive properties. For example, tight junctions are established to enhance barrier functionality, yet inherent benefits also emerge related to inflammation modulation. Accordingly, my interests were expanded relative to inflammatory profiling of the lung and placenta.

In Part II of this dissertation, I outline parallel projects I've undertaken that were natural extensions of my Claudin-6 project. Generally speaking, I have sought to understand direct and indirect links related to inflammation that may exist between tight junctional Claudins and RAGE, a transmembrane pattern recognition receptor involved in mechanistically controlling lung inflammation. The chapters that follow represent published projects that provide valuable insight to the field of RAGE signaling and inflammation. Ongoing research will assist in 'closing the loop' that exists between the biology of pulmonary Claudins and inflammatory RAGE signaling.

CHAPTER 7: Plausible Roles for RAGE in Conditions Exacerbated by
Secondhand Smoke Exposure.

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Abstract

Approximately one billion people smoke worldwide and the burden placed on society by primary and secondhand smokers is expected to increase. Smoking is the leading risk factor for myriad health complications stemming from diverse pathogenic programs. First and secondhand cigarette smoke contain thousands of constituents, including several carcinogens and cytotoxic chemicals that orchestrate chronic inflammatory responses and destructive remodeling events. In the current review, we outline details related to compromised pulmonary and systemic conditions related to smoke exposure. Specifically, data are discussed relative to impaired lung physiology, cancer mechanisms, maternal-fetal complications, cardiometabolic, and joint disorders in the context of smoke exposure exacerbations. As a general unifying mechanism, the receptor for advanced glycation end-products (RAGE) and its signaling axis is increasingly considered central to smoke-related pathogenesis. RAGE is a multi-ligand cell surface receptor whose expression increases following cigarette smoke exposure. RAGE is responsible in part for inducing pro-inflammatory signaling pathways that culminate in the expression and secretion of several cytokines, chemokines, enzymes, and other mediators. Understanding the biological contributions of RAGE during cigarette smoke-induced inflammation may provide critically important insight into the pathology of lung disease and systemic complications that combine during the demise of those exposed.

Introduction

Global Burden

Currently, it is estimated that there are nearly 1 billion smokers worldwide (WHO Fact Sheet N° 339). Of this enormous number, approximately 80% live in either low or middle income countries where the effects or burdens of tobacco related illness and death are the most substantial. Furthermore, while the current worldwide population of smokers is estimated at 1 billion, current projections predict that this number will rise to 1.6 billion in the next twenty-five years (J. Mackay, 2006). With such an inordinate number of smokers world-wide, roughly 6 million people are expected to die each year because of tobacco exposure (WHO Fact Sheet N° 339). Of this number, over 600,000 people will die prematurely as a result of exposure to secondhand smoke (SHS). Unfortunately, while these numbers themselves are galling, rampant tobacco use throughout the world has also had societal ramifications as exposure is believed to contribute to over \$500 billion dollars in damages annually (Ekpu & Brown, 2015).

Because it appears that smoking prevalence will continue to rise despite its inherent dangers and costs, research is expanding in order to better understand the consequences of these trends. The intent of this review is to highlight significant health outcomes that result from SHS exposure and suggest a generally unifying mechanistic theme underlying the biological consequences of exposure. Mounting evidence suggests that the signaling effects of Receptors for Advanced Glycation End-Products (RAGE) during exposure to primary and SHS may contribute to inflammatory disease establishment and progression. The biochemical assessments performed to date have linked many tobacco related substances with negative health consequences (Moritsugu, 2007); however, much still remains to be discovered.

Tobacco Smoke

Tobacco smoke contains over 4000 chemical substances (Moritsugu, 2007) and a large portion of these entities have been correlated with damaging health outcomes. The combustion of tobacco smoke produces numerous compounds observed in both gaseous and particulate fractions. Many of these compounds are toxic components that have been demonstrated to induce inflammation, cause irritation, asphyxiation, and even carcinogenesis. Recent studies have suggested that at least 45 of these substances are known carcinogens (Fowles & Dybing, 2003). Some of the key toxins produced by tobacco smoke include benzene (leukemogen) (Protano, Andreoli, Manini, Guidotti, & Vitali, 2012), formaldehyde (an irritant and carcinogen) (Szumska, Damasiewicz-Bodzek, & Tyrpien-Golder, 2015), benzo[a]pyrene (carcinogen) (Abedin, Louis-Juste, Stangl, & Field, 2013), carbon monoxide and cyanide (asphyxiants) (Leone, 2015), acrolein (an irritant) (Noya et al., 2013) and polonium (a radioactive carcinogen) (Kilthau, 1996; Yuan et al., 2016). Additionally, combustion of tobacco products creates a non-enzymatic reaction of reducing sugars and amino groups to create compounds known as advanced glycation end-products (AGEs) (Nicholl & Bucala, 1998). AGEs bind RAGE and have been implicated in a large and diverse group of diseases including: respiratory inflammatory diseases (Robinson, Stogsdill, Lewis, Wood, & Reynolds, 2012), cardiovascular disease (Prasad, Dhar, & Caspar-Bell, 2015), cancer (Malik, Chaudhry, Mittal, & Mukherjee, 2015), diabetes (Nowotny, Jung, Hohn, Weber, & Grune, 2015), neurodegenerative disorders (Espinet, Gonzalo, Fleitas, Menal, & Egea, 2015), placental dysfunction (Alexander et al., 2016; Guedes-Martins, Matos, Soares, Silva, & Almeida, 2013), osteoarthritis (Y. J. Chen et al., 2015), and general inflammation (Van Puyvelde, Mets, Njemini, Beyer, & Bautmans, 2014). In mechanistic terms, AGE-RAGE interaction initiates a cascade of events that result in the

induction of chronic inflammation and impaired cell survival (Ibrahim, Armour, Phipps, & Sukkar, 2013; Ray, Juranek, & Rai, 2016).

While significant damage is induced by active smoking, research has demonstrated that individuals exposed to passive smoking (or secondhand smoke, SHS) are at risk for developing significant health problems (*The Health Consequences of Involuntary Exposure to Tobacco Smoke: A Report of the Surgeon General*, 2006; Moritsugu, 2007). Indeed, literature suggests that SHS may even expose individuals to higher levels of certain deleterious compounds than those observed in mainstream smoke. SHS for example is shown to have higher levels of PAHs (Evans, Thomas, Boardman, & Nash, 1993; Grimmer, Naujack, & Dettbarn, 1987), tobacco-specific nitrosamines (TSNA) (K. D. Brunnemann, Yu, & Hoffmann, 1977; D. Hoffmann, Adams, Brunnemann, & Hecht, 1979; Ruhl, Adams, & Hoffmann, 1980), aromatic amines (Patrianakos & Hoffmann, 1979), aza-arenes (Dong, Schmeltz, Jacobs, & Hoffmann, 1978; Grimmer et al., 1987), carbon monoxide (Daher et al., 2010; Dietrich Hoffmann, Adams, & Wynder, 1979; Krzych-Falta, Modzelewska, & Samolinski, 2015), nicotine (Pakhale & Maru, 1998; Rickert, Robinson, & Collishaw, 1984), ammonia (Klaus D Brunnemann & Hoffmann, 1975), pyridine (K. Brunnemann & Hoffmann, 1977; Johnson, Hale, Clough, & Chen, 1973) as well as the gas phase components of acrolein, benzene, toluene, and isoprene 1,3-butadiene (Klaus D Brunnemann, Kagan, Cox, & Hoffmann, 1990). Recently, thirdhand smoke has also been implicated as a potent source of exposure to the toxins found in cigarettes (Ganjre & Sarode, 2016). Thirdhand smoke is obtained when tobacco smoke constituents become deposited on surfaces and such deposits may undergo oxidation and other diverse chemical processes that result in the synthesis of carcinogenic species including TSNA (Sleiman et al., 2010). In fact, it has been speculated that the dangers associated with thirdhand smoke may be

even more profound than active smoking (Ferrante et al., 2013) due to the process in which thirdhand smoke is generated. As these deposited substances are a product of time and isolation, thirdhand smoke poses real dangers for both active smokers and nonsmokers alike.

Health Outcomes and Morbidities

Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is one of the leading causes of mortality and morbidity and currently estimated to affect roughly 5% of the world's population or about 329 million individuals (WHO, The top 10 causes of Death Fact Sheet 2012). The data overwhelmingly implicate primary or active smoking as the greatest risk factor for developing COPD (Casey, 2016); however, exposure to environmental tobacco smoke is also highly associated with increased risk for COPD in individuals who have never smoked (Hagstad et al., 2014). Globally, COPD is projected to be the third leading cause of death by 2020. Economists have estimated that the economic burden (including both direct and indirect costs) resulting from COPD was \$2.1 trillion dollars in 2010, but believe that this will rise to \$4.3 trillion by 2030 (Lomborg, 2013b). Direct costs alone have been estimated to be in the \$49.9 billion dollar range (Dalal, Christensen, Liu, & Riedel, 2010), suggesting a greater need for preventative measures, as well as improvements in earlier diagnosis and more cost effective treatment.

There is no doubt that management of COPD has improved with more effective treatments and the use of nonpharmacological interventions, such as rehabilitation and noninvasive ventilation (Rennard, 2004; Sutherland & Cherniack, 2004). However, as the worldwide prevalence of COPD is predicted to increase, so will the urgency of improved comprehensive therapy. Inflammation intensifies as COPD progresses (Hogg et al., 2004) and does not "burn out" as with many other chronic inflammatory diseases (Barnes, 2004).

Therefore, there is a pressing need for the development of new molecular targets and associated therapies, particularly as no existing treatment has been shown to reduce disease progression. New therapies for COPD may arise from improvements in existing drugs (for example, longer acting β_2 -agonists and anticholinergics) or from the development of novel therapies when underlying disease processes are better understood. Despite recent advances in the understanding of COPD molecular pathogenesis (Barnes, Shapiro, & Pauwels, 2003), there is clearly a need for more research into its basic mechanisms. Even so, there are still several reasons why drug development in COPD has been difficult. Animal models of COPD for early drug testing are not very satisfactory (Dawkins & Stockley, 2001; Shapiro, 2000). There are uncertainties about testing drugs for COPD, which may require long-term studies (3+ yrs) in relatively large numbers of patients. Many patients with COPD have comorbidities, such as ischemic heart disease and diabetes, which may exclude them from clinical trials of new therapies. There is also little information about surrogate biomarkers in blood, sputum or breath to monitor the short-term efficacy and predict the long-term potential of new treatments. Despite these limitations, a lucid understanding of disease progression during primary and SHS exposure is essential in refining patient care.

In terms of the pathophysiology, COPD is typically characterized by airflow obstruction that is minimally reversible. This airflow obstruction is due to chronic inflammation and permanent pulmonary airspace enlargement as well as the loss of elastic recoil caused by the destruction of alveolar walls observed in emphysema. Persistent inflammation in COPD patients is characteristic not only in the airways, but in the respiratory parenchyma and pulmonary vasculature as well, and results in disruption of normal lung function specifically through remodeling of the distal pulmonary airspaces. Because chronic inflammation is a major defining

characteristic of the disease, extensive research surrounding pro-inflammatory molecular mechanisms have been conducted. The key aim of such research focuses on the attenuation or removal of chronic inflammation that overcomes natural protective mechanisms and the resulting tissue damage seen with COPD. Contributors to this inflammation-related process include imbalances between proteases/antiproteases, oxidative stress, elevated apoptotic indexes, and enhanced neutrophil, macrophage, and T lymphocyte extravasation.

Recent reports have corroborated previous findings that neutrophils are increased in sputum of patients with COPD along with increased interleukin-6 (IL-6) signaling (Ravi et al., 2014). Substantial evidence has implicated primary or active smoking as a major contributor to the recruitment of these neutrophils, however mounting evidence now suggests that secondhand smoke may have a similar effect on neutrophils (Menzies et al., 2006) that is likely mediated through similar interleukin signaling (H. Wu et al., 2014) (Hubeau, Kubera, Masek-Hammerman, & Williams, 2013). Under normal physiological conditions, neutrophils employ proteases and small cationic peptides to attack invading bacteria, viruses, and harmful exogenous material such as particulates found in tobacco smoke. Yet, in chronic inflammatory conditions, neutrophils become major destructors of the alveolar elastic matrix. These neutrophils also release enzymes and other mediators that cleave collagen into fragments that may further activate inflammatory cells (Overbeek et al., 2013). One potent signaling factor that has been demonstrated to drive neutrophilic infiltration is the chemoattractant interleukin-8 (IL-8) which is produced by exposed and damaged epithelium and endothelium (Kobayashi & DeLeo, 2009; Kobayashi, Voyich, Burlak, & DeLeo, 2005). Additionally, other chemoattractants that have been shown to induce neutrophil migration include chemokine CXC motif ligands 1, 2, 5, 8 (CXCL-1, 2, 5, 8) (Beeh et al., 2003; Keatings, Collins, Scott, & Barnes, 1996; Tanino et al.,

2002), leukotriene B4 (LTB4) (Beeh et al., 2003), IFN- γ (Hodge, Hodge, Holmes, & Reynolds, 2005), IL-1 β (Churg, Zhou, Wang, Wang, & Wright, 2009; Thacker, 2006), and TNF- α (Barnes & Karin, 1997). Current data increasingly suggest that these potent inflammatory chemoattractants are elevated with exposure to SHS (Flouris et al., 2012; Merghani, Saeed, & Alawad, 2012). While neutrophils are predominant mediators of chronic inflammation, they are not the only important pro-inflammatory mediator. Macrophages have also been shown to participate in the propagation of inflammation through the release of chemoattractants, and are elevated in the airways, parenchymal bronchoalveolar lavage fluid (BALF), and sputum (Aldonyte, Jansson, Piitulainen, & Janciauskiene, 2003; Finkelstein, Fraser, Ghezzi, & Cosio, 1995; Shapiro, 1999) from affected patients. Studies involving the exposure of mice to SHS have demonstrated increased macrophages in response to SHS (Tsuji et al., 2015). Furthermore, as these adaptive immunity cells play such a vital role in chemoattraction, it is unsurprising that research suggests macrophage recruitment closely corresponds with the severity of the disease (Di Stefano et al., 1998). Like neutrophils, macrophages migrate to injured lung tissue and enhance the release of TNF- α , IL-8, CXC chemokines, monocyte chemoattractant peptide-1 (MCP-1), LTB4 as well as other pro-inflammatory molecules (Barnes et al., 2003). Finally, it should be noted that research indicates that T-cells may act as important intermediaries in the development of emphysema (Majo, Ghezzi, & Cosio, 2001). In a comparison of normal patients and those with smoke induced COPD, diseased patients demonstrated elevated levels of CD3 and CD8 (Majo et al., 2001), two cytotoxic t-cell subgroups that kill infected or damaged cells. CD8 in particular, was shown to be highly correlated with increasing severity in emphysema patients (Majo et al., 2001). Recent analysis of mice subjected to SHS resulted in increased levels of CD4 and CD8, and conversely, inhibition of these cells prevented airspace enlargement, inhibited

cytokine release, and reduced apoptotic signaling (Podolin et al., 2013). Mechanistically, it is likely that CD8 subtly interacts in conjunction with CD4, a T-helper cell whose activation releases cytokines and helps orchestrate the migration and activity of other inflammatory cells. These T-cell mediated processes seemingly disrupt autoimmune regulation, thus enhancing perpetual inflammation.

Cancer

It is estimated that cigarette smoking contributes to 30% of all cancer deaths in developed countries (Pfeifer et al., 2002). Tobacco smoke is believed to be responsible for 70% of lung cancers deaths (Danaei, Vander Hoorn, Lopez, Murray, & Ezzati, 2005) (approximately 1.3 million deaths each year (Siegel, Ma, Zou, & Jemal, 2014)) and 42% of esophageal and oral cavity cancer deaths (Lim et al., 2012). Furthermore, tobacco smoke is believed to contribute significantly to the development of cancers of the larynx, urinary bladder, and pancreas, and to a lesser extent to cancers of the kidney, stomach, cervix, and myeloid leukemia (Agudo et al., 2012). Current evidence largely implicates active smoking as a major risk factor in cancer development, however mounting evidence now suggests that SHS may equally participate. SHS has been shown to increase the risk of developing lung (Hori, Tanaka, Wakai, Sasazuki, & Katanoda, 2016), oropharyngeal (A. Malik et al., 2015), colorectal (S. H. Lee, Hong, Lee, & Lee, 2016), breast (A. Malik et al., 2015), cervical (Shekari et al., 2012), bladder (Shekari et al., 2012), and pancreatic cancer (Vrieling et al., 2010). Moreover studies investigating nitrosamines, some of the most potent carcinogens in tobacco smoke, have demonstrated that high levels are present in both mainstream and SHS (Sleiman, Maddalena, Gundel, & Destailats, 2009). As nitrosamines are readily absorbed through the alveoli and then rapidly distributed through the

blood, it is unsurprising that they are found to play a major role in the induction of many cancers (Balbo et al., 2013; Church et al., 2009).

Compounds such as PAHs, aromatic amines, aza-arenes, carbon monoxide, TSNA, nicotine, ammonia, pyridine, and the gas compounds of acrolein, toluene, isopentene-1,3,-butadiene, and benzene are common in SHS. Overwhelmingly, the data implicate these substances in a host of cancers, although the mechanisms by which this takes place are broad and diverse. For example, recent data has demonstrated that benzene (Gonzalez-Jasso et al., 2003), toluene (Gonzalez-Jasso et al., 2003) and nicotine (Joshi & Tyndale, 2006) have the ability to induce up-regulation of CYP2E1, an enzyme that activates many foreign chemical compounds to become ultimate toxicants (Guengerich & Shimada, 1991). Aside from the ability to produce toxicants, the induction of CYP2E1 has been suggested to be the first step in leading to chemical induced carcinogenesis (Lieber, 1997). Alternatively, PAHs and TSNA has been shown to increase epithelial to mesenchymal transition (EMT), which is closely associated with an invasive or metastatic phenotype. Increased EMT is characterized by a down regulation of genes encoding for epithelial junction (claudins, occludins, e-cadherin) as well as an activation of protein products that promote mesenchymal adhesion. As these epithelial junctions are crucial in regulating cell differentiation, proliferation and polarity, it is unsurprising that the loss of these proteins is often associated with an invasive phenotype (Kase et al., 2000). As these tissues transition from an epithelial to mesenchymal state, the epithelial barrier is disrupted and thus a key initial line of defense in the innate immune system is compromised.

In general, tobacco smoke seems to broadly influence carcinogenesis in three ways. First, the gas and particulate phase of tobacco smoke includes at least 20 substances that can induce lung tumors in rodents (Hecht, 1999, 2003). These compounds directly contribute to

carcinogenesis. Second, tobacco smoke includes substances that are not directly carcinogenic alone, but enhance the activity of carcinogens when co-administered. These substances include tumor promoters, co-carcinogens, and toxicants such as catechol, methyl catechols, and PAHs (Hecht, 2012). One potent example of such a compound is acrolein, which is not strongly carcinogenic when in isolation. However, acrolein expressed by ciliated epithelium can be highly toxic due to the hindrance of clearing tobacco smoke compounds from the lung, resulting in profound exposure to other carcinogens. Furthermore, acrolein reacts directly with DNA and protein, thus triggering genomic silencing of gene targets (Thompson & Burcham, 2008) that may enhance the likelihood of carcinogenesis. Finally, tobacco smoke substantially influences the chronic inflammatory microenvironment. Tobacco smoke causes the recruitment of inflammatory cells, cytokine and chemokines that can act as drivers for cancer development and progression (Cho et al., 2011). It is well documented that infiltration of tumor associated macrophages in tumor lesions is common to a host of cancer types, and is associated with tumor angiogenesis, invasion, and metastasis (Erreni, Mantovani, & Allavena, 2011; Ho et al., 2008; Jang, Lee, Jeon, & Kim, 2013; Mano et al., 2013).

Developmental Complications

As cigarettes are known to be one of the most common teratogens (Ozturk et al., 2016), a number of serious obstetric complications arise with cigarette smoke exposure during pregnancy (F. Arffin, F. H. Al-Bayaty, & J. Hassan, 2012). Approximately 10% of pregnant women in the US smoke, thereby exposing nearly 400,000 fetuses yearly to tobacco specific toxins (Health & Services, 2014). Exposure to smoke during pregnancy has been demonstrated to increase the likelihood of congenital limb deficiencies (Caspers et al., 2013), congenital heart defects (Gianicolo, Cresci, Ait-Ali, Foffa, & Andreassi, 2010), orofacial clefting (Ozturk et al., 2016)

and many other developmental abnormalities. Active smoking has long been considered a teratogenic agent that increases the risk of premature birth, however recent data shows that 22 to 30 % of nonsmoking pregnant women exposed to SHS are also at risk (Salmasi, Grady, Jones, & McDonald, 2010). Developmental defects in the fetus represent substantial pregnancy complications, but perinatally, smoke exposure further enhances mortality via increased risk of sudden infant death syndrome (SIDS) and preterm birth (Dietz et al., 2010). Altogether, it not surprising that many researchers have suggested that cigarette exposure may be the single most important avoidable cause of adverse pregnancy outcomes (Akkar et al., 2015; Bickerstaff, Beckmann, Gibbons, & Flenady, 2012; Higgins et al., 2012).

Nicotine, one of the primary addictive compounds in tobacco smoke, is a key substance that contributes significantly to many of these health problems as even minute levels induce detectible transcriptomic modifications in small airway epithelium (Strulovici-Barel et al., 2010). Nicotine readily crosses the placenta (Hellstrom-Lindahl, Gorbounova, Seiger, Mousavi, & Nordberg, 1998) and binds to nicotinic acetylcholine receptors (nAChRs) which regulate fetal brain development (Dwyer, McQuown, & Leslie, 2009). Interestingly, research has demonstrated that nicotine levels are higher in the amniotic fluid, fetal serum, and placenta, than in the corresponding maternal serum (Luck, Nau, Hansen, & Steldinger, 1985). Studies demonstrating the adverse effects of tobacco smoke on neurodevelopment have provided compelling evidence that nicotine increases cellular damage, reduces the overall cell number, impairs synaptic activity as well as influencing processes such as cell replication to differentiation and apoptosis (Dwyer, Broide, & Leslie, 2008; T. A. Slotkin, 1998; Slotkin, Cho, & Whitmore, 1987). Furthermore, nicotine has been associated with adverse neurocognitive outcomes such as behavioral disorders (Palmer et al., 2016), cognitive dysfunction (Niqlasen,

Obel, Homoe, Korvel-Hanquist, & Dammeyer, 2016), and attention deficit hyperactivity disorder (De Alwis, Tandon, Tillman, & Luby, 2015; Knopik et al., 2016).

While a significant portion of the literature implicates tobacco smoke in neurodevelopmental pathologies, such effects are not limited to the nervous system. Prenatal tobacco smoke exposure has been demonstrated to have striking effects on respiratory development in that it reduces respiratory compliance in infants and impairs lung function in school aged children (Hanrahan et al., 1992; *The Health Consequences of Smoking-50 Years of Progress: A Report of the Surgeon General*, 2014; Health & Services, 2014). Possibly contributing to impaired lung function are data that suggest that maternal smoke exposure may alter Clara cell secretory protein (CCSP) expression in fetal lungs (Stillerman, Mattison, Giudice, & Woodruff, 2008). Indeed, evidence currently suggests that maternal smoke exposure (including SHS) during pregnancy leads to deregulation of gene expression (Votavova et al., 2012). Confirmatory primate studies have shown that *in utero* nicotine exposure adversely affects overall lung development by decreasing lung size and volume, elastin, while increasing type I and type III collagen, alveolar volume and airway wall area (Lodrup Carlsen et al., 1997; Harmanjatinder S Sekhon et al., 1999; H. S. Sekhon, Keller, Proskocil, Martin, & Spindel, 2002; Stocks & Dezateux, 2003). While the immediate ramifications are apparent, researchers have shown that nicotine exposure not only predisposes the fetus to lung dysfunction, but also has the ability to influence asthma in second and third generation offspring likely through epigenetic modulation of the fetal program (Leslie, 2013; Y. F. Li, Langholz, Salam, & Gilliland, 2005; Rehan et al., 2012). Aside from respiratory disorders, nicotine has further been shown to affect endocrine function (Duskova, Hruskovicova, Simunkova, Starka, & Parizek, 2014; Steptoe & Ussher, 2006), increase the likelihood of the fetus to develop chronic kidney disease (CKD)

through increased mitochondrial dysfunction (Stangenberg, Chen, Wong, Pollock, & Saad, 2015), and decrease auditory response and auditory development (Kable, Coles, Lynch, & Carroll, 2009; Katbamna, Klutz, Pudrith, Lavery, & Ide, 2013; Weitzman, Govil, Liu, & Lalwani, 2013). Overwhelming, the data suggests a particularly insidious role for SHS and its ability to influence development.

One further factor that may have a causal role in many developmental deficiencies is the impact of premature delivery, a risk factor that tobacco smoke has been shown to significantly increase (Wagijo, Sheikh, Duijts, & Been, 2015). As tobacco smoke has been shown to increase preterm birth (PTB), it should be noted that it additionally exacerbates intrauterine growth restriction (IUGR) and preeclampsia (PE), two placental diseases closely associated with PTB (F. Arffin et al., 2012; Horta, Victora, Menezes, Halpern, & Barros, 1997; Suter, Anders, & Aagaard, 2013). IUGR is a complication that stems primarily from uteroplacental vascular insufficiency, which ultimately creates an environment of chronic oxygen and nutrient deficiency, resulting in restricted fetal growth (Bolehovska et al., 2015). PE is another disease that impacts placentation wherein maternal hypertension and proteinuria accounts for around 20% of induced PTB (Goldenberg & Rouse, 1998). Because complications such as perinatal hypoxia and asphyxia, cerebral palsy, and persistent pulmonary hypertension of the newborn have been associated with both IUGR and SHS exposure (Brar & Rutherford, 1988; P. H. Gray, M. J. O'Callaghan, J. M. Harvey, C. J. Burke, & D. J. Payton, 1999), it is likely that SHS modulates IUGR and PE symptoms that may culminate in diverse developmental pathologies.

Cardiometabolic Disorders

The intimate connection, both in etiology and outcome, of cardiovascular and metabolic processes has resulted in the term, cardiometabolic diseases. The relevance of this is highlighted

in the numbers: heart disease is the leading cause of death (Roger et al., 2012) and insulin resistance is the most common disorder in the US, affecting half of all adults (Menke, Casagrande, Geiss, & Cowie, 2015). Because of these startling statistics, considerable effort has been devoted over recent decades to elucidate effective strategies to reverse the trends. Overwhelmingly, these efforts have focused on the role of lifestyle variables, particularly our diet. However, while diet is clearly relevant (G. M. Reaven, 1997; Roger et al., 2012), it is also clearly not the entire solution, as cardiometabolic diseases continue unabated. Indeed, such a paradigm has left relatively unexplored the possibility that, in addition to what we ingest, more attention needs to be devoted to what we inhale.

Insulin resistance is the “metabolic” in cardiometabolic disorders. Due to the obvious challenges of determining causality of a cigarette smoke-insulin resistance interaction, most of the findings in humans are correlational in nature (Facchini, Hollenbeck, Jeppesen, Chen, & Reaven, 1992; Ronnema, Ronnema, Puukka, Pyorala, & Laakso, 1996), though limited data exist to establish (Attvall, Fowelin, Lager, Von Schenck, & Smith, 1993; Thatcher et al., 2014) that cigarette smoke exposure increases insulin resistance. Typified by a reduced ability of insulin to elicit action at cells throughout the body, as well as general hyperinsulinemia, insulin resistance is at the heart of most cardiometabolic disorders, such as hypertension (DeFronzo & Ferrannini, 1991; Ferrannini et al., 1987), atherosclerosis (DeFronzo & Ferrannini, 1991), dyslipidemia (G. M. Reaven, 1991), cardiomyopathy (Witteles et al., 2004), and more (Olefsky & Glass, 2010; Urakawa et al., 2003).

Unsurprisingly, cigarette smoke exposure similarly increases the risk of myriad cardiovascular complications through diverse mechanisms, though insulin resistance is clearly a dominant factor (G. Reaven & Tsao, 2003). For example, dyslipidemia (i.e., increased

triglycerides, reduced HDL cholesterol), which is a key predictor in cardiovascular mortality with cigarette smoking (Jeppesen, Hein, Suadicani, & Gyntelberg, 2001), is significantly worse in smokers with insulin resistance compared with more insulin-sensitive smokers (Sijbrands et al., 1994; Tahtinen, Vanhala, Oikarinen, & Keinanen-Kiukaanniemi, 1998).

A second instance of the role of insulin resistance in smoke-induced cardiometabolic disorders is abnormal endothelial physiology. Blood vessels from smoke-exposed humans are less dynamic, having a reduced dilatory capacity (Heitzer et al., 1996), and have increased leukocyte adherence (Adams, Jessup, & Celermajer, 1997), increasing the risk of clot formation. In regards to endothelium-dependent vasodilation, current evidence shockingly revealed that after only 15 to 30 minutes of breathing SHS, vasodilation of coronary arteries in non-smokers was impaired almost to the extent of habitual smokers (Otsuka et al., 2001). Intriguingly, both of these pathological processes are associated with endothelial dysfunction and are exacerbated by insulin resistance (N. G. Chen, Holmes, & Reaven, 1999; Stuhlinger et al., 2002).

Data collected over the past few decades suggest that SHS increases the incidence of coronary heart disease approximately 25 to 30% (2010; Barnoya & Glantz, 2005; Faught, Flouris, & Cairney, 2009). Furthermore, although active smokers receive up to 100 times the dose of smoke than individuals exposed to SHS, an active smokers relative risk for coronary heart disease is 1.78 followed closely by a passive smoker at 1.31 (Law, Morris, & Wald, 1997). SHS contributes to cardiovascular disease by activating blood platelets (Rubenstein, Morton, & Yin, 2010) likely through the combined elevation of both fibrinogen (Iso et al., 1996; Law et al., 1997), and thromboxane (Schmid et al., 1996), thus leading to the development of atherosclerosis. SHS has also been demonstrated to decrease levels of NO, the primary substrate that is implicit in the hemodynamic changes in the vascular system (Barua et al., 2001). Research

has even demonstrated that after only 20 minutes of SHS exposure, direct endothelial cell injury is observed. Mechanistically, SHS exposure has been shown to increase free radicals (Burke & Fitzgerald, 2003) while decreasing antioxidants (Tribble, Giuliano, & Fortmann, 1993), decrease mitochondrial function (Tippetts et al., 2014), decrease protective HDL levels (DeFaria Yeh, Freeman, Meigs, & Grant, 2007; Gotto & Brinton, 2004), and increase arterial stiffness (Mack, Islam, Lee, Selzer, & Hodis, 2003). With such staggering data, new meaning to the current warnings from the Surgeon General that state “there is no safe level of exposure to tobacco smoke” is clear (*The Health Consequences of Smoking-50 Years of Progress: A Report of the Surgeon General*, 2014).

Joint and Movement Disorders

Osteoarthritis (OA), characterized by joint pain, effusion, loss of mobility, and deformity that progresses to functional joint failure, is one of the most common chronic diseases. It is reported to be the most common disease associated with the temporomandibular joint (TMJ) (El-Hakim & Elyamani, 2011). There is not currently any treatment to slow or stop its progression. It is not surprising then, that OA has become the most common cause of long-term disability or physical impairment. It is a major life-altering disorder, and its prevalence in the general population is statistically comparable to major end-stage kidney disease and heart failure. For instance, over 20 million Americans are affected by OA and there are over 500,000 joint replacements performed annually in the United States alone (Abramson & Krasnokutsky, 2006). Many studies, including mouse knee destabilization and TMJ misalignment models, have demonstrated a pattern of biomarker expression associated with the progression of OA (Holt et al., 2012; Larkin et al., 2013; Matias et al., 2016; L. Xu, Golshirazian, Asbury, & Li, 2014). The disease appears to be associated with an initial rise in Tgf- β 1 expression, followed by

upregulation of HtrA1, Ddr2 and Mmp13 expression, resulting in OA as assessed by standardized joint scoring methods such as the Mankin and the Osteoarthritis Research Society International (OARSI) scoring systems (Glasson, Chambers, Van Den Berg, & Little, 2010; Mankin, 1974; Mankin, Dorfman, Lippiello, & Zarins, 1971; Polur, Lee, Servais, Xu, & Li, 2010). Curiously, the expression of HtrA1 and the other factors associated with OA are attenuated in a receptor for advanced glycation end-products (RAGE) knockout (KO) mice following surgically induced OA models (Larkin et al., 2013). This suggests that inflammation may be the trigger for the initiation and onset of OA. It follows that OA would be associated with cigarette smoke. It is noted that early on, the interaction of smoking and OA was controversial (Dube et al., 2016; Felson et al., 1989; Harrison, Silman, Wiles, Scott, & Symmons, 2001; Kang et al., 2016; Mnatzaganian, Ryan, Norman, Davidson, & Hiller, 2011). However, it has been reported that the discrepancies between smoking and OA interaction are likely do to study design and metrics (C. Ding, Cicuttini, Blizzard, & Jones, 2007; Dube et al., 2016). A correlation between smoking and OA and/or cartilage defects is now apparent (C. Ding et al., 2007; Villaverde-Garcia et al., 2016). It is interesting to note that one study showed that the harmful effects of smoking associated with OA were due to both cartilage loss as well as the development of cartilage defects in people with a family history of joint disease (C. Ding et al., 2007). Suggesting that a pre-disposition may be exacerbated by smoking through some bone/cartilage development association. Finally, it is noteworthy that one group who reported no association between direct smoking and OA did report a correlation between the joint disease and indirect smoking (Kang et al., 2016). It is unknown if constituents of tobacco smoke have direct deleterious effects on chondrocyte function or if direct and/or indirect cigarette smoke induces cartilage damage through more global means such as inflammation.

RAGE: A Plausible Mechanism

Although many interrelated mechanistic processes potentially contribute to the diversity of diseases stemming from exposure to first and SHS, RAGE signaling is a program that commonly emerges. An underlying mechanistic theme of the smoke-related disease states outlined in this review is chronic inflammation, in which RAGE is a key modulator. Essential to understanding the clear link between RAGE and disease progression is the key concept that RAGE expression is increased by exposure to tobacco smoke (Nelson et al., 2015; P. R. Reynolds, S. D. Kasteler, R. E. Schmitt, & J. R. Hoidal, 2011; A. B. Robinson, K. D. Johnson, B. G. Bennion, & P. R. Reynolds, 2012; D. R. Winden et al., 2014; T. T. Wood et al., 2014) and the induction of RAGE causes inflammatory disease symptoms similar or identical to the ones described herein (Alexander et al., 2016; Bodine et al., 2014; Larkin et al., 2013; P. R. Reynolds, J. A. Stogsdill, et al., 2011; J. A. Stogsdill et al., 2012; M. P. Stogsdill et al., 2013; D. R. Winden et al., 2013).

RAGE is expressed in a variety of cell types including endothelial and vascular smooth muscle cells, fibroblasts, macrophages/monocytes, osteoprogenitor cells, endothelium and epithelium (Brett et al., 1993) [personal communication]. Although RAGE is predominantly expressed in the lung, it is detectable in a variety of tissues including the heart, brain, placenta, liver, kidney, pancreas, small intestine, and colon (Buckley & Ehrhardt, 2010; Nelson et al., 2015). RAGE is a pattern recognition cell surface receptor that binds many endogenous and exogenous entities such as S100/calgranulins (Reddy et al., 2006), amyloid- β -peptide (Cai et al., 2016), HMGB-1 (W. Huang et al., 2016), and AGEs (Khodeer, Zaitone, Farag, & Moustafa, 2016). Following RAGE-ligand interaction, a cascade of signaling events elicit gene expression modulation via divergent signal transduction pathways (Hudson et al., 2008; Kim, Rogers, &

Criner, 2008; Toure et al., 2008). Because RAGE expression can also increase when ligands accumulate (Schmidt, Yan, Yan, & Stern, 2001) RAGE-ligand interactions may not only induce the defects described in this review, but contribute to the chronicity of inflammatory tobacco smoke exposure observed in these pathological states as well. RAGE activation exacerbates a host of pro-inflammatory responses via MAP kinases (ERK, JNK and P38), NF- κ B, reactive oxidative species (ROS), and other chemokine mediators including TNF- α IL1- β and others (Bianchi, Giambanco, & Donato, 2010). While redundancies exist within the pathway, RAGE signaling generally culminates in the activation of NF- κ B, a transcriptional regulator which not only promotes pro-inflammatory mediator elaboration, but also *de novo* RAGE expression. Thus, RAGE signaling via NF- κ B represents a vicious positive feedback loop that orchestrates chronic inflammation. In contrast to short-lived cellular activation mediated by LPS, engagement of RAGE by its ligands results in prolonged inflammation (L. Lin, Park, & Lakatta, 2009) that if left unchecked, causes severe tissue injury.

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Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 8: Decreased Activation of Placental mTOR Family Members is Associated with the Induction of Intrauterine Growth Restriction (IUGR) by Secondhand Smoke (SHS) in the Mouse.

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Abstract

Cigarette smoke is known to be a risk for the development of intrauterine growth restriction (IUGR). Our objective was to assess the effects of secondhand smoke (SHS) during pregnancy and to what extent it regulates the activation of mTOR family members and murine trophoblast invasion. Mice were treated to SHS for four days. Placental and fetal weights were recorded at the time of necropsy. Immunohistochemistry was used to determine the level of placental trophoblast invasion. Western blots were utilized to assess the activation of caspase 3, XIAP, mTOR, p70, and 4EBP1 in treated and control placental lysates. As compared to controls, treated animals showed: 1) decreased placental (1.4-fold) and fetal (2.3-fold) weights ($p < 0.05$); 2) decreased trophoblast invasion; 3) significantly decreased active caspase 3 (1.3-fold; $p < 0.02$) and increased active XIAP (3.6-fold; $p < 0.05$) in the placenta; and 4) a significant decrease in the activation of placental mTOR (2.1-fold; $p < 0.05$), p70 (1.9-fold; $p < 0.05$) and 4EBP1 (1.3-fold $p < 0.05$). Confirmatory in vitro experiments revealed decreased trophoblast invasion when SW71 cells were treated with 0.5, or 1.0% cigarette smoke extract (CSE). Similar to primary smoking, SHS may induce IUGR via decreased activation of the mTOR family of proteins in the placenta. Increased activation of the placental XIAP protein could be a survival mechanism for abnormal trophoblast cells during SHS exposure. Further, CSE reduced trophoblast invasion, suggesting a direct causative effect of smoke on susceptible trophoblast cells involved in IUGR progression. These results provide important insight into the physiological consequences of SHS exposure and smoke-mediated placental disease.

Keywords: secondhand smoke, IUGR, mTOR, placenta, trophoblast invasion

Introduction

Intrauterine Growth Restriction (IUGR) is a significant complication of pregnancy that affects up to 10% of all pregnancies and significantly increases risks of fetal and neonatal morbidity and mortality (Brar & Rutherford, 1988; Peter H Gray, Michael J O'Callaghan, Jacqueline M Harvey, Christopher J Burke, & Diane J Payton, 1999; Raphael N Pollack & Michael Y Divon, 1992). Complications observed in IUGR patients include perinatal hypoxia and asphyxia, cerebral palsy, and persistent pulmonary hypertension of the newborn (Galan, 2011; Galan et al., 2005; Jacobsson & Hagberg, 2004; Rosenberg, 2008). In addition, several studies have reported a long-term sequelae of IUGR complications including adult hypertension, heart disease, stroke and diabetes (D. Barker, 1993; David JP Barker et al., 1993; Kathleen Holemans, Rita Van Bree, Johan Verhaeghe, Leona Aerts, & F Andre Van Assche, 1993; K Phipps et al., 1993; B. Reusens-Billen, C. Remacle, & J. Hoet, 1989). Placentae from growth-restricted pregnancies are characterized by a number of pathologic findings such as reduced syncytiotrophoblast surface area, decreased trophoblast invasion possibly due to elevated apoptosis, and increased mTOR protein (T.-H. Hung, J. N. Skepper, D. S. Charnock-Jones, & G. J. Burton, 2002; Naonori Ishihara et al., 2002; Levy & Nelson, 2000; T. Mayhew et al., 2003; S. C. Smith, Baker, & Symonds, 1997).

Cigarette smoking during pregnancy is associated with a number of serious obstetric complications including increased rates of spontaneous abortion, premature delivery and IUGR (Farha Arffin, Fouad H AL-Bayat, & Jamiyah Hassan, 2012). In fact, research suggests that prenatal nicotine exposure could affect the fetal central nervous system, brain development and increase infant mortality rates (Junchang Guan et al., 2009; Hayfaa A Wahabi et al., 2013). Exogenous antenatal exposure is also associated with the adult onset of diabetes and

hypertension (Hayfaa A Wahabi et al., 2013). In contrast to studies of direct smoking during pregnancy, studies detailing effects of secondhand smoke during pregnancy are limited. Currently there is conflicting information about the induction of IUGR by secondhand smoke. A correlation was demonstrated between SHS and the induction of IUGR in a recent study conducted in 2013 by Whabi et al., linkages between IUGR and tobacco exposure likely exist despite earlier studies like the one conducted by Subramoney and colleagues (2010) that did not detect a correlation between IUGR and pregnant women exposed to smoke (Sreevidya Subramoney, Tursan d'Espaignet, & Chandra Gupta, 2010; Hayfaa A Wahabi et al., 2013).

While the exact correlation between SHS and IUGR remains to be determined, research has revealed that many harmful constituents found in SHS readily exchange between the mother and the developing fetus. Nicotine's ability to cross the placenta is a well-known phenomenon, as is the induction of localized hypoxia in fetuses exposed to tobacco smoke (Delia A Dempsey & Neal L Benowitz, 2001). In the sheep model, studies showed that infusion of low dosage nicotine leads to fetal hypoxia and elevated fetal blood pressure without affecting maternal blood gases or cardiovascular systems (Junchang Guan et al., 2009). Studies further suggested that SHS significantly increases the risk for spontaneous abortion, preterm delivery, and sudden infant death syndrome (SIDS) and still birth (Delia A Dempsey & Neal L Benowitz, 2001; Yousef S Khader, Nemeh Al-Akour, Ibrahim M AlZubi, & Isam Lataifeh, 2011; Sreevidya Subramoney et al., 2010). Commonality exists such that these studies and others suggest a vulnerability of the fetus to the effects of tobacco smoke.

The mammalian target of rapamycin (mTOR) protein is a phosphatidylinositol kinase-regulated protein kinase that regulates cell growth in response to nutritive insults and growth factors (Blume-Jensen & Hunter, 2001; N. Jansson et al., 2006; Volarević & Thomas, 2000;

Wullschleger, Loewith, & Hall, 2006). Downstream effectors in the mTOR pathway include the 70-kDa ribosomal protein S6kinase 1 (p70S6K) and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) (Sara Roos et al., 2007; Volarević & Thomas, 2000). Activation through the phosphorylation of these intermediate proteins by mTOR is known to regulate translation initiation and protein synthesis of numerous targets (Blume-Jensen & Hunter, 2001; Patti, Brambilla, Luzi, Landaker, & Kahn, 1998). Regulation of mTOR and p70S6K activity is mediated by phosphatidylinositol-3 kinase/AKT signaling (Chung, Crilly, Anderson, Mukherjee, & Kiss, 1997; Ming et al., 1994; Wen, Abbasi, Kellems, & Xia, 2005). Under physiologic conditions, the activation of p70S6K is also mediated by the induction of the extracellular regulated kinase (ERK) pathway (Eguchi et al., 1999; Iijima et al., 2002). In the human placenta, mTOR has been localized to syncytiotrophoblast cells, suggesting a role for this protein in nutrient sensing during pregnancy (Aiko et al., 2002).

Studies have shown that total mTOR protein in the placenta is increased in human IUGR, whereas placental phospho (p)-p70S6K protein is down-regulated during the progression of IUGR. Despite a clear necessity for further research, these data suggest diverse mechanistic effects potentially required for the regulation of target proteins during IUGR.

An increase of placental apoptosis is observed during IUGR (Naonori Ishihara et al., 2002). The X-linked inhibitor of apoptosis protein (XIAP) is an factor that knocks down proteins that regulate cell death (Holcik & Korneluk, 2001; J. Li et al., 2000; Y. Suzuki, Nakabayashi, Nakata, Reed, & Takahashi, 2001). This protein is present in trophoblast throughout placental development, but its expression is significantly decreased near term when apoptosis is maximal highlighting a role for this protein in the temporal regulation of trophoblast apoptosis (Gruslin, Qiu, & Tsang, 2001). Abrogation of apoptosis via XIAP signaling relies on the inhibition of

caspases 3, 7 and 9 (Y. Suzuki et al., 2001)(Suzuki, Nakabayashi, Nakata, Reed and Takahashi, 2001). Thus, controlling the activity of pro-apoptotic caspases appears to be essential for precisely balancing cell death and survival. Studies have recently shown that XIAP expression is decreased near term during hyperthermia induced IUGR suggesting elevated apoptosis is likely to occur through the mediation of XIAP (Arroyo, Anthony, & Galan, 2008). However, the expression profile of XIAP in both the placenta and mesometrial compartment during IUGR has yet to be elucidated.

This study sought to better understand the effects of SHS during hemochorial placentation. Utilization of the murine model provided an essential tool to better evaluate trophoblast biology in the uterine mesometrial compartment, the potential impact of mTOR signaling and apoptotic profiles in the context of SHS exposure.

Material and Methods

Animals and Tissue Preparation

Animal use was approved by the Institutional Animal Care and Use Committee at Brigham Young University. C57 Black 6 (C57BL/6) mice were purchased from Charles River laboratories, Wilmington, MA. To obtain timed pregnancies, females were caged with males overnight. Placentae and mesometrial compartments were dissected from pregnant mice at 18.5 days of gestation (dGA). Placentae and fetuses were weighed and tissues were snap frozen in liquid Nitrogen for RNA and protein analysis. Whole conceptuses were frozen in dry ice-cooled heptane for immunohistochemistry analysis. All tissue samples were stored at -80°C until used.

Secondhand Smoke Treatment (SHS)

The generation of the IUGR pregnancy occurred following exposure of pregnant mice (n=7) to SHS conditions as previously shown by Winden et al (Duane R Winden et al., 2014). Pregnant mice were placed in the nose-only Scireq InExpose cigarette-smoking robot starting at day 14.5 dGA and exposed for four days with necropsy at 18.5 dGA. To induce IUGR, pregnant mice were placed in soft restraints and connected to an exposure tower, wherein a computer-controlled puff of smoke generated every minute resulted in 10 s of SHS exposure (from six cigarettes; 2R1, University of Kentucky, Lexington, KY) followed by 50 s of room air (fresh air) daily to induce IUGR. This procedure was done daily for 10 min during the time of treatment. Control animals (n=7) were placed in soft restraints and exposed only to room air daily for 10 min.

Immunofluorescence (IF)

IF was performed on frozen whole conceptus sections. In summary, slides were washed in a 1x Tris buffer solution (TBS), and blocked with Background Sniper (Biocare Medical, Concord, CA) for one hour. This was followed by incubation overnight with a primary antibody for Cytokeratin 7 (Dako, Carpinteria, CA). Slides were then incubated for 1 hr with donkey anti-mouse TR (Biocare Medical, Concord, CA). 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) was used for counterstaining prior to mounting with glass coverslips. Slides were viewed using a Texas Red excitation and emission filter.

Western Blot Analysis

Western blot analysis was used to determine expression levels of active caspase 3, active XIAP and the mTOR family of proteins in the placenta of control and SHS animals as previously described (Arroyo, Brown, & Galan, 2009). Cell lysates (50 µg) were separated on 4–12% Bis-

Tris gels and transferred to nitrocellulose membranes. Membranes were incubated with antibodies against cleaved caspase 3, phospho- XIAP, phospho-mTOR (Ser2448), total mTOR, phospho- p70 S6 kinase (SK6) (Thr389), total p70^{SK6}, phospho-4EBP1 (Thr37/46), and total 4EBP1 (all from Cell Signaling Technology, Danvers, MA, excluding total p70 from Epitomics, Burlingame, CA and phospho XIAP from Abcam, Cambridge, MA). Membranes were then incubated with a secondary horseradish peroxidase (HRP)-conjugated antibody for one hour at room temperature. The membranes were incubated with ECL substrate, and the emission of light was detected using x-ray film. To determine loading consistencies, each membrane was stripped and reprobed with an antibody against mouse β actin (Sigma Aldrich, St. Louis, MO). Expression levels of the proteins were quantified by densitometry normalized to β actin expression and changes in expression were reported by comparing to the untreated controls.

Invasive Trophoblast Cell Culture and Cigarette Smoke Extract (CSE)

A first-trimester cytotrophoblast cell line Swan71 (SW71; n=9) was used for invasive cytotrophoblast studies. SW71 cells were maintained and cultured in RPMI medium (Mediatech, Manassas, VA). Cell medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. CSE was generated as previously described by Reynolds et al., 2006 (Paul R Reynolds et al., 2006). The solution was generated as follows: two 2RF4 research cigarettes (University of Kentucky, Lexington, KY) were continuously smoked with a vacuum pump into 20 ml of DMEM medium (Mediatech, Manassas, VA). The smoke-bubbled medium was filtered through a 0.22- μ m filter to remove large particles. The resulting medium was defined as 100% CSE. The total particulate matter content of 2RF4 cigarettes is 11.7 mg/cigarette, tar is 9.7 mg/cigarette, and nicotine is 0.85 mg/cigarette. Dilutions were made using DMEM medium to a concentration of 10% CSE.

Cell Treatments

SW71 cells were detached and 20,000 cells/ml were incubated in 2% FBS medium alone or medium supplemented with 0.5%, or 1.0 % CSE for 24 hours (Paul R Reynolds et al., 2006). Real time cell invasion was determined following these treatments.

Real Time Cell Invasion Determination

An xCELLigence was utilized to determine real time invasion of trophoblast cells (Keogh, 2010; Rahim & Üren, 2011). Invasion was assessed in 16 well CIM-Plates (n=9) composed of an upper and lower chamber, each containing 16 wells. The top wells were coated with a 1:40 matrigel concentration and incubated for 4 hr. Trophoblast cells were plated in the top chamber at a concentration of 20,000 cells/well in 2% FBS RPMI in a total volume of 100 μ L in the presence or absence CSE (0.5%, or 1.0%). The bottom chamber wells were filled with 160 μ L of 10% FBS RPMI. The cells were then place in the RTCA DP instrument and invasion readings were done every 15 minutes for 5 hr.

Statistical Analysis

Results were checked for normality and data were shown as means \pm SE. Mann-Whitney tests were used to compare weights, protein expression and invasion indexes. Significant differences between groups were noted at $p < 0.05$.

Results

Fetal and Placental Weights

Decreased fetal and placental weights are characteristics of IUGR. We initially investigated the effects of SHS on placental and fetal weights after SHS treatment of near-term mice (18 days of gestation; dGA) for 4 days. There was a significant 1.4-fold reduction in

placental weight ($p < 0.0003$) and a 2.3-fold reduction in fetal weight ($p < 0.0002$) in SHS exposed mice compared to room air controls (Figures 1a and b). These data implicate SHS in fetal and placental weight deviations coincident with IUGR.

Trophoblast Invasion and Apoptosis

We next investigated trophoblast invasion and placental apoptosis following antenatal SHS treatment. Immunofluorescence (IF) for Cytokeratin 7 (CK7) was utilized to localize trophoblast cells within the mesometrial compartment of pregnant mice. There was decreased invasion of trophoblast cells into the mesometrial compartment in the SHS treated animals (Figure 2b middle panel) when compared to controls (Figure 2a top panel). The no-primary negative staining control is also presented in the bottom panel of Figure 2c. Immunoblotting for active caspase 3 was next performed in order to determine apoptosis in the placenta. We found a 1.3-fold ($p < 0.02$) decrease in placental active Caspase 3 in SHS animals when compared to controls (Figure 2d). In contrast, we observed a significant increase (3.6-fold; $p < 0.05$) in phospho (active) XIAP, an inhibitor of caspase 3 activation (Figure 2e). Our results suggest that SHS is likely involved in decreased trophoblast invasion and elevated XIAP-mediated apoptosis observed in this model of IUGR.

mTOR Family of Proteins in the Placenta

The mTOR family of proteins has been shown to regulate cell growth during altered availability of nutrients (Roos, Jansson, Palmberg, Saljo, Powell and Jansson, 2007). Previous results revealed increased placental mTOR expression during IUGR (Knuth, et al., 2014). Accordingly, we next investigated the expression of active mTOR family of proteins in the placenta in the context of SHS exposure. Placental expression of active mTOR (2.1-fold; $p < 0.05$), active p70 (1.9-fold; $p < 0.05$) and active 4EBP1 (1.3-fold; $p < 0.05$) were significantly

decreased in SHS exposed animals compared to controls (Figure 3a-c). These data suggested a correlation exists between SHS-induced IUGR and decreased activation of placental mTOR family members.

Trophoblast Invasion and CSE

Nicotine is known to be a risk factor for IUGR (Detmar et al., 2008) . A hallmark of IUGR is decreased trophoblast invasion (Juan A Arroyo & Virginia D Winn, 2008). Published reports have already demonstrated enhanced cell invasion in both cancer and trophoblast cells following CSE exposure (Dasgupta et al., 2009; Kraus et al., 2014). We therefore wanted to quantitatively determine the direct effects of CSE on trophoblast invasion in culture. As expected, trophoblast invasion was significantly decreased when cells were treated with 0.5% (5.9-fold; $p < 0.02$), or 1.0% (6.0-fold; $p < 0.02$) CSE when compared to cells incubated in fresh media alone (Figure 4).

Discussion

IUGR remains one of the leading causes of fetal mortality. Research in the recent past has focused on diverse factors including maternal exposure to primary tobacco smoke; however, very little is known in relation to the effects of secondhand smoke during pregnancy. Pointing to the robustness of involuntary smoke exposure, we discovered that a secondhand smoke model of IUGR in the mouse resulted in significant reductions in both fetal and placental weights that coincided with decreased trophoblast invasion. These data were supported by recent work by Vivarigou et al. that highlighted birth size deviations while the number of conceptuses were not affected (Mund, Louwen, Klingelhofer, & Gerber, 2013; Varvarigou, Asimakopoulou, & Beratis, 2008). In fact, our work involving secondhand smoke exposure suggests that like primary smoking, exposure to SHS is also capable of inducing IUGR (Esposito, Horn, Greene, &

Pisano, 2008).

An interesting discovery related to the decreased activation of the pro-apoptotic molecule caspase 3, a finding that potentially sheds light on a plausible mechanistic driver of the IUGR phenotypes we observed. Increased activity of caspase 3 was expected as activation of placental caspase 3 has already been shown to be involved in the development of IUGR (Arroyo et al., 2008; Arroyo et al., 2010; Kimball et al., 2015). Further, XIAP protein was also augmented in our exposed animals, which was also anticipated due to its effects in the regulation of caspase 3. Reports have shown that XIAP protein is differentially regulated in diverse models of placental disease including hypoxia and exposure to polycyclic aromatic hydrocarbons (Detmar et al., 2008; Jeon et al., 2013). Even more interesting is the recent discovery that nicotine treatment of cultured cells confers protection from excessive apoptosis, suggesting a complex mechanism of cellular apoptosis downstream of the addictive nicotine and the wide array of other noxious agents that destroy tissue integrity (Dasgupta et al., 2009). In the current model of IUGR, we observed increased XIAP protein in the placenta of SHS treated animals when compared to controls. This suggested that perhaps increased XIAP is involved in the negative modulation of active caspase 3, potentially as a trophoblast survival mechanism during treatment with SHS.

Decreased nutrient availability and diminished expression of mTOR family members in the placenta are observed during the development of IUGR (Arroyo et al., 2009; N. Jansson et al., 2006; T. Jansson, Aye, & Gberdhan, 2012; Knuth et al., 2015; Sara Roos et al., 2007; Roos, Powell, & Jansson, 2009; Ross, Fennessey, Wilkening, Battaglia, & Meschia, 1996). In the current report, SHS decreased the activation of mTOR protein and the secondary messengers p70 and 4EBP1. This suggests that mTOR signaling may be a common pathway, among others, that drives the nutritional aspect of the IUGR phenotype. Recently published work by our laboratory

demonstrated a role for mTOR in trophoblast invasion (Knuth et al., 2015). Since SHS caused decreased trophoblast invasion and decreased mTOR activity in vivo, confirmatory studies in vitro were undertaken in order to dissect the invasive properties of trophoblast cells in culture. We observed that trophoblasts treated with CSE had decreased invasion in culture. These data implicated tobacco smoke in the control of trophoblast invasion and confirmed the work of others that demonstrated CSE pathways in the orchestration of pre-eclampsia and fetal growth restriction (Ahmed, 2014). Additional work in the near future should aim to test the hypothesis that mechanistic control of decreased invasion is due in part to decreased mTOR activation during SHS treatment.

SHS exposure is a very common occurrence but the consequences of SHS during pregnancy are not well established. In fact, this is the first report that establishes an in vivo correlation between SHS and the mTOR pathway as a mechanism for the development of IUGR disease. Accordingly, these data clarify the need to discern mTOR-mediated placental abnormalities that likely confer complications that may reemerge in adulthood (Arroyo and Winn, 2008). For instance, alterations in the expression and activity of nutrient transporters in affected placentae may not only restrict fetal growth but compromise other mTOR regulated processes including postnatal growth, maturation, and aging (Arroyo et al., 2009; Sara Roos et al., 2007; Roos et al., 2009; Ross et al., 1996). As cigarette smoke exposure correlates with the development of IUGR, a more robust evaluation of mTOR and its temporal effects during gestation and after birth is necessary (Milnerowicz-Nabzyk & Bizon, 2014; Triche & Hossain, 2007). For instance, Milnerowicz-Nabzyk and Bizon further speculated that different chemical fractions of smoke may be more centrally involved in the development of general IUGR as compared to idiopathic IUGR (Milnerowicz-Nabzyk & Bizon, 2014). This phenomenon may

explain why differences in mTOR activation are observed in the various models of IUGR present in the literature today (Arroyo et al., 2009; Kimball et al., 2015; Sara Roos et al., 2007; Roos et al., 2009; Ross et al., 1996). In the end, future studies that investigate downstream signaling molecules associated with decreased mTOR and those that seek to elucidate mechanisms of decreased invasion during SHS induced IUGR are critically necessary. These undertakings are of vital importance, as they should provide new therapeutic avenues that could help in the alleviation of IUGR symptoms and thus improve fetal health.

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Conflict of Interest

The authors report no conflict of interest.

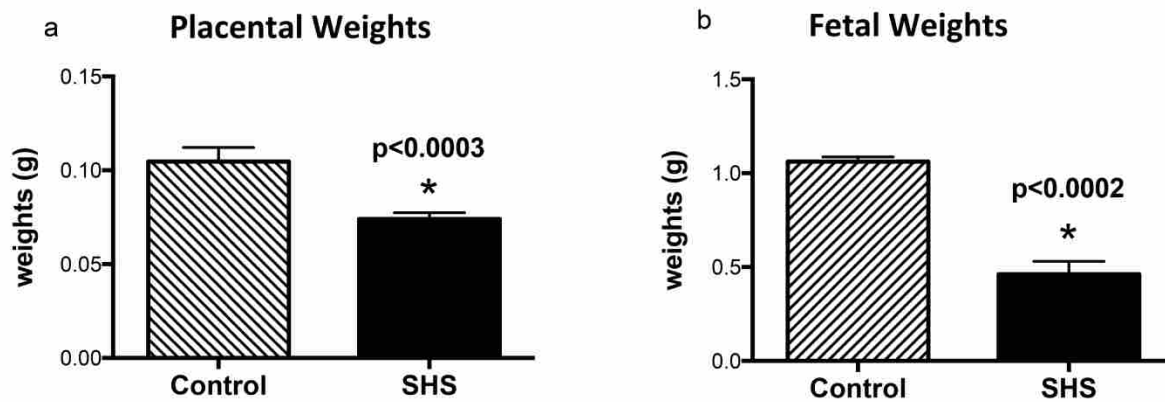


Figure 8.1: Placental and Fetal Weight Differences During Secondhand Smoke Exposure. A significant decrease in placental (a: 1.4-fold; $p < 0.0003$) and fetal weights (b: 2.3-fold; $p < 0.0002$) was observed in SHS-treated animals as compared to controls.

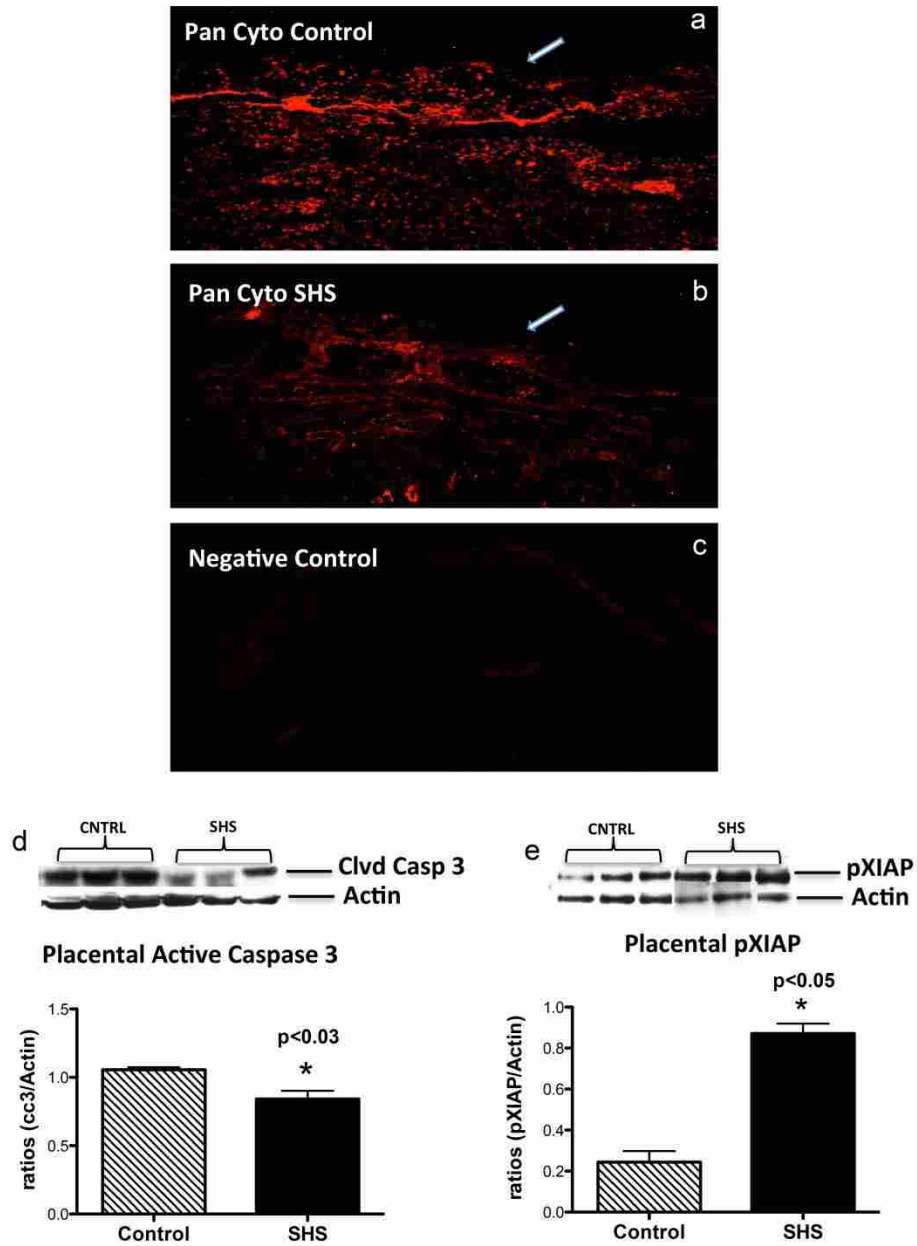


Figure 8.2: Trophoblast Invasion and Apoptosis During SHS Induces IUGR.

(a) CK7 IHC showed decreased trophoblast invasion into the uterine mesometrial compartment of treated animals (a) as compared controls b). Samples were also compared to the negative control (c). (d) Active caspase 3 was increased (1.3-fold; $p < 0.02$) with SHS in the placenta of treated animals as compared to controls. (e) Phospho XIAP was significantly increased (3.6-fold; $p < 0.05$) in the placenta of SHS treated animal as compared to controls.

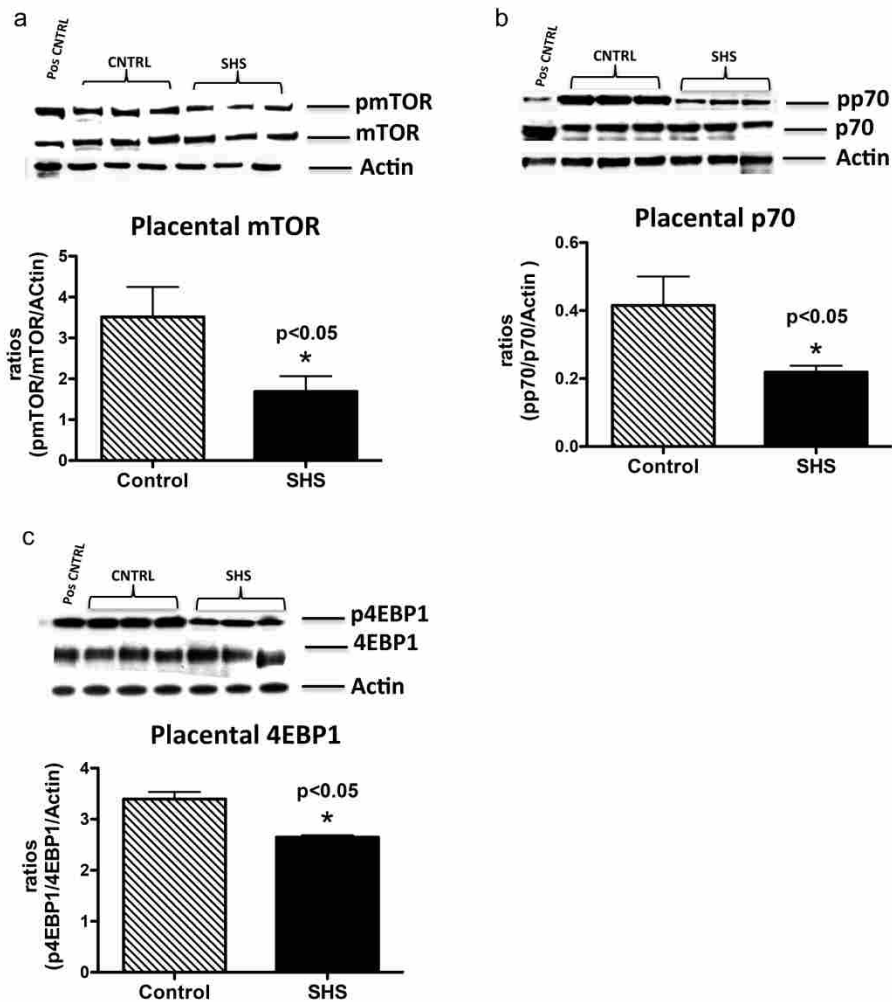


Figure 8.3: Placental mTOR, p70, and 4EBP1 Proteins During SHS Induced IUGR.

Placental mTOR (a), p70 (b), and 4EBP1 (c) proteins during SHS induced IUGR. A characteristic western blot picture is presented for each molecule. There were significant decreases in mTOR, p70 and 4EBP1 activation (2.1-fold, 1.9-fold, 1.3-fold; $p < 0.05$) in the placenta following SHS treatment.

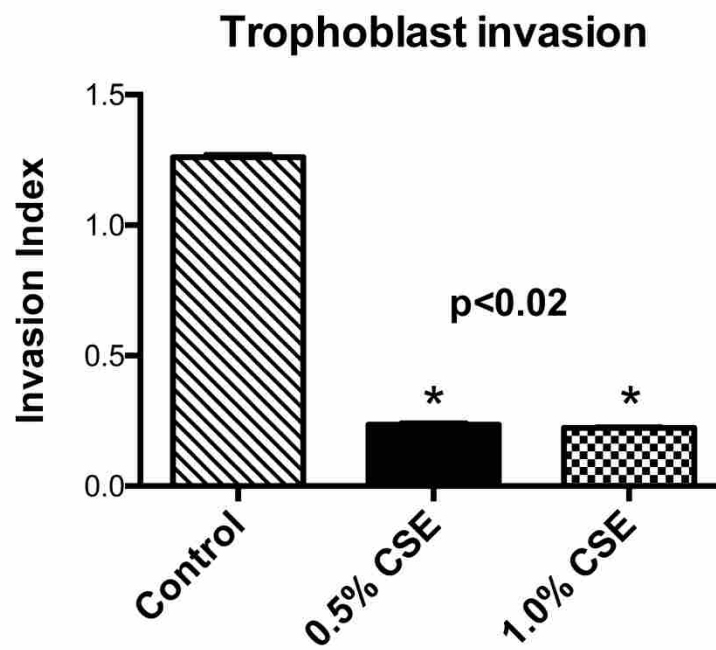


Figure 8.4: CSE Treatment and Trophoblast Invasion.

Cell invasion was decreased with either 0.5% (5.9-fold%; $p < 0.02$), or 1.0% (6.0-fold%; $p < 0.02$) CSE treatment in cultured trophoblast cells.

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CHAPTER 9: Cigarette Smoke Extract (CSE) Induces RAGE-Mediated Inflammation in Ca9-22 Gingival Cells.

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Running Title: Gingival inflammation and RAGE

Abstract

Objective: The oral environment is anatomically positioned as a significant gateway for exposure to environmental toxicants. Cigarette smoke exposure compromises oral health by orchestrating inflammation. The receptor for advanced glycation end-products (RAGE) has been implicated in smoke-induced inflammatory effects; however, its role in the oral cavity is unknown. The purpose of this study was to determine RAGE expression by immortalized gingival cells and the degree to which RAGE-mediated signaling influences inflammation. **Design:** Gingival epithelia cells (Ca9-22) were exposed to 10% cigarette smoke extract (CSE) for six hours and screened for RAGE expression and inflammatory mediators. **Results:** Quantitative PCR and immunoblotting revealed increased RAGE expression following exposure. Furthermore, exposure activated RAGE signaling intermediates including Ras and NF- κ B. IL-6 and IL-1 β were also elevated in cell culture medium from CSE-exposed cells when compared to controls. A family of anionic, partially lipophilic sulfated polysaccharide derivatives known as semi-synthetic glycosaminoglycan ethers (SAGEs) were used in an effort to block RAGE signaling. Co-treatment of CSE and SAGEs ameliorated inflammatory responses. **Conclusions:** These results provide a new perspective on a mechanism of cigarette smoke induced oral inflammation. Further work may show RAGE signaling as a potential target in the treatment of diseases of the oral cavity exacerbated by tobacco smoke exposure.

Keywords: tobacco, CSE, RAGE, gingival, epithelium

Introduction

Gingival epithelial cells comprise an important population of oral epithelium that covers gingival surfaces as well as the delicate lining of gingival sulci and junctional regions (Larjava, Koivisto, Hakkinen, & Heino, 2011). These cells assist in establishing the mucosal tissue critically involved in lining alveolar processes of the mandible and maxilla. In response to insults, including tobacco, gingival epithelium may become disordered and subsequent loss of integrity often impacts periodontal pockets (Larjava et al., 2011). Tobacco smoke exposure is considered the leading etiology of preventable disease in the United States (Jamal et al., 2014). Organs at the interface of exposure, such the respiratory system (P. R. Reynolds et al., 2008; A. B. Robinson, J. A. Stogsdill, et al., 2012) and oral cavity (Gao, Prasad, & Zacharias, 2014), highlight the need to assess molecular disease induction and progression. Periodontitis is a major chronic inflammatory disease in which insults elicit the production of pro-inflammatory cytokines, particularly interleukin IL-1 β , IL-6 and tumor necrosis factor TNF- α (Baeder et al., 2016; Graves, Jiang, & Genco, 2000; Socransky, Haffajee, Cugini, Smith, & Kent, 1998). Molecular signaling that augments the expression of these cytokines leads to destruction of the periodontal tissue and eventually the loss of teeth (Graves et al., 2000).

The receptor for advanced glycation endproducts (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors found in various cell types including smooth muscle cells, fibroblasts, macrophages, and epithelium (P. R. Reynolds, S. D. Kasteler, et al., 2011). Although RAGE was first described as a progression factor in cellular responses induced by advanced glycation end-products (AGEs) that accumulate in hyperglycemia and oxidant stress, studies have also indicated that RAGE binds a myriad of other molecules (Morbini et al., 2006) including pro-inflammatory cytokine-like mediators of the S100/calgranulin family

(S100A12 and S100B), amyloid, high-mobility group box 1 (HMGB1), Mac-1 (Buckley & Ehrhardt, 2010; Sasaki et al., 2000), and specific nucleic acid structures (Schmidt et al., 2001).

RAGE expression increases when cells and tissues are exposed to tobacco smoke, and RAGE-ligand interactions contribute to pathological inflammation (P. R. Reynolds et al., 2008; A. B. Robinson, K. D. Johnson, et al., 2012; Winden DR, 2014; Wood TT, 2014). Specifically, tobacco smoke induces epithelium to increase the expression of RAGE, its ligands, pro-inflammatory signaling intermediates, and various cytokines (P. R. Reynolds, M. G. Cosio, & J. R. Hoidal, 2006; P. R. Reynolds et al., 2008; A. B. Robinson, K. D. Johnson, et al., 2012). Interestingly, RAGE expression has been detected in human gingival tissues from subjects with chronic periodontitis (Katz, Bhattacharyya, et al., 2005) and is overexpressed in gingival tissues of smokers with periodontal diseases compared to nonsmokers with the disease (Katz, Yoon, Mao, Lamont, & Caudle, 2007). Overexpression of RAGE after stimulation by AGEs and nornicotine was also demonstrated in fibroblasts and epithelial cells originated from human gingival tissues (Katz, Caudle, Bhattacharyya, Stewart, & Cohen, 2005). The possibility that gingival epithelium activates RAGE signaling during exposure to tobacco smoke and that smoke-induced inflammation via RAGE contributes to periodontal disease has not yet been evaluated.

Ras oscillates between active GTP-bound and inactive GDP-bound conformations during its role as a molecular switch that regulates the fate of target cells (Donovan, Shannon, & Bollag, 2002). Ras signaling via Raf/MAPK, phosphatidylinositol 3-kinase, JNK/p38, and Rho pathways has been shown to impact development, cellular proliferation, and differentiation (Rao et al., 2010). Furthermore, Ras is a key regulator of normal cell growth and abnormal cellular functions coincident with malignant transformation when signaling occurs through MAPKs (Downward,

2003). We have previously published important research that details precise roles for Ras during RAGE-mediated inflammation (P. R. Reynolds, S. D. Kasteler, et al., 2011; A. B. Robinson, K. D. Johnson, et al., 2012); however, such signaling assessments have not yet been completed in gingival epithelial cells.

In the present study, we tested the hypothesis that gingival epithelial cells exposed to cigarette smoke extract (CSE) induce RAGE expression and activate RAGE-mediated signaling that involves Ras, NF- κ B, and pro-inflammatory cytokine secretion. This work innovates RAGE research due to the utilization of an anionic, partially lipophilic sulfated polysaccharide derivative known as semi-synthetic glycosaminoglycan ethers (SAGEs) that are novel RAGE inhibitors. SAGEs exhibit substantial anti-inflammatory properties at nanomolar concentrations via the inhibition of RAGE binding to its disparate ligands (J. Zhang et al., 2011). A representative SAGE structure is illustrated in Figure 1. We demonstrate that RAGE is increased by gingival epithelium and that RAGE signaling augments cytokine elaboration following CSE exposure. Furthermore, SAGEs were sufficient to significantly decrease the activation of signaling intermediates and cytokine availability. Further research may demonstrate that RAGE and its specific downstream effectors are potential targets in the treatment of tobacco smoke-related periodontal disease.

Material and Methods

Cell Culture and Experimental Conditions

Ca9-22 cells are accepted models of gingival epithelial cells (Imamura et al., 2016) that were maintained in DMEM plus 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cigarette smoke extract (CSE) was generated as previously described with slight modifications (P. R. Reynolds et al., 2006). Briefly, one 2RF4 research cigarette (University of Kentucky, Lexington,

KY) was continuously smoked by connecting the filtered end of the cigarette to a vacuum pump, pulling the particles into 5 ml of DMEM/F12 and the resulting medium was defined as 100% CSE and diluted with culture medium to 10%. The University of Kentucky has determined that the total particulate matter content of 2RF4 cigarettes is 11.7 mg/cigarette, tar is 9.7 mg/cigarette, and nicotine is 0.85 mg/cigarette. Cells were grown to 70-80% confluence before culture media was replaced with 1 mL fresh media or 10% CSE and incubated for an additional 6 hours. Incubation for 6 hours was determined following a time course of exposure that ranged from 2-12 hours. RAGE expression was significantly increased at six hours and no additional increase was significant after that period (not shown). At the conclusion of the exposure period, conditioned cell culture media, total RNA, and total protein was procured. In select experiments, 25 µg/mL SAGEs were added to 1 mL of fresh or 10% CSE prior to 6 hours of exposure. SAGEs were a kind gift from Dr. Glenn Prestwich in the Department of Medicinal Chemistry at the University of Utah, Salt Lake City, Utah.

Protein Quantification and Quantitative Real-time PCR

Proteins were quantified as described previously (Wood TT, 2014). Briefly, cells were homogenized in RIPA buffer supplemented with protease inhibitors (Thermo Fisher Waltham, MA). BCA quantification was performed to ensure equal sample concentrations (Thermo Fisher) and Ponceau S staining of transferred membranes was performed to visualize equal loading (not shown). A RAGE goat polyclonal antibody (#AF1145; RnD Systems, Pittsburg, PA) and an antibody against mouse β actin (A5316, Sigma Aldrich, St. Louis, MO) were used in these studies. Immunoblots shown are representative of three separate experiments that were each conducted in triplicate. Quantitative real-time PCR was performed as previously outlined (Nelson MB, 2015). Briefly, total RNA was isolated from cells, reverse transcribed, and PCR

amplification was conducted using RAGE specific primers (P. R. Reynolds et al., 2008).

Ras and NF- κ B Assessments

Ras activation ELISA kits (Millipore, Temecula, CA) were used to assess active and inactive Ras (P. R. Reynolds et al., 2008). Cell lysates were quantified by BCA protein assay and 20- μ g aliquots were screened for Ras (Santa Cruz Biotechnology, Santa Cruz, CA). In vitro experiments were repeated at least three times, each in triplicate. Total and active NF- κ B levels were assessed through the use of colorimetric high-throughput fast activated cell-based ELISA assays available from Active Motif (Carlsbad, CA). Specifically, cells were screened with antibodies specific to total and active phosphorylated proteins as outlined in the manufacturer's instructions.

Cytokine ELISAs

Culture medium was collected after incubation with control or CSE-containing medium and centrifuged at 2,500 rpm for 5 min. IL-6 and IL-1 β were determined by sandwich ELISA according to the manufacturer's instructions (RnD Systems, Minneapolis, MN).

Statistics

Data are presented as the mean \pm SEM. Data were compared by ANOVA with Tukey's post-hoc analysis (Graphpad Prism; La Jolla, CA). Significance was set at $p < 0.05$.

Results

CSE Increases RAGE Expression

Our initial undertaking was to assess RAGE expression in gingival epithelial cells exposed to CSE. Our data demonstrate that RAGE mRNA expression significantly elevates in

gingival epithelium following six hours of CSE exposure (Figure 2A). In order to correlate message and protein expression relationships, immunoblotting was next conducted using cell lysates. As expected, RAGE protein levels were markedly increased after six hours of CSE exposure (Figure 2B).

CSE-induced Inflammatory Signaling is Abrogated by RAGE Inhibition

Because increased RAGE availability is associated with pro-inflammatory signaling, we next evaluated to what extent RAGE signaling intermediates are elevated when cultured gingival epithelial cells were exposed to CSE. Ras has previously been shown to be an initial intracellular intermediate through which RAGE signaling propagates. As anticipated, CSE exposure caused a significant increase in the expression of the active form of Ras (Figure 3). Concomitant exposure of cells to CSE and SAGEs significantly attenuated the activation of Ras (Figure 3). Despite parallel signaling trajectories within responding cells, RAGE signaling generally culminates in the activation of NF- κ B (Schmidt et al., 2001). When activated, phosphorylation of NF- κ B causes its liberation from cytosolic sequestration allowing it to function as an important nuclear transcriptional activator. There was no difference in total NF- κ B levels (not shown); however, active NF- κ B was significantly increased in the nucleus of gingival epithelial cells exposed to CSE compared to non-exposed controls (Figure 4). Co-incubation of cells with CSE and SAGEs resulted in significant protection from CSE-mediated NF- κ B activation (Figure 4). Combined, these data reveal that inhibition of RAGE through the utilization of SAGEs blocks a cell's normal pro-inflammatory response to cigarette smoke exposure.

To test the hypothesis that CSE-induced RAGE expression leads to pro-inflammatory cytokine production in gingival epithelial cells, we analyzed the levels of IL-1 β and IL-6 in conditioned media from cells with and without CSE exposure. Following exposure to CSE for

six hours, ELISAs revealed a significant increase in the secretion of both cytokines (Figure 5). Secretion of IL-1 β and IL-6 were both significantly diminished when cells were co-incubated with CSE and SAGEs (Figure 5). In each of the preceding experiments, critical controls were included in which cells were exposed to SAGEs in the absence of CSE. Importantly, SAGEs did not alter any end point assay (i.e., signaling intermediate activation or cytokine secretion) when added to cells in the absence of CSE (not shown).

Discussion

Cigarette smoke exposure is the leading cause of preventable deaths worldwide (WHO, 2011) and is among the top ten contributors to the worldwide health burden (M., 2003). Despite concerted social efforts to reduce smoking prevalence, current trends suggest this number will continue to increase (Ng et al., 2014; "Results from the 2010 National Survey on Drug Use and Health: Summary of National Findings. ," 2011). Moreover, cigarette smoke is a pervasive inhaled toxin—almost half of the U.S. population is regularly exposed to cigarette smoke (Pirkle, Bernert, Caudill, Sosnoff, & Pechacek, 2006; Pirkle et al., 1996). Our laboratories have established clear mechanistic roles for RAGE signaling in the smoke-exposed respiratory system, yet to what extent RAGE signaling functions in the oral mucosae during voluntary or involuntary exposure has not been evaluated. The results described in the current manuscript are the first to suggest RAGE functions in inflammatory responses that originate in gingival epithelium and that RAGE abrogation may protect, at least in part, from harmful inflammatory consequences at the dental interface.

Gingival epithelial cells comprise a remarkable cell population intimately charged with the maintenance of dentition and the regulation of immunologic capacities. Gingival cells are capable of a wide array of gene expression programs so that responses to entities at their unique

juxtaposition can be efficient and effective. Our discovery that RAGE, a potent pro-inflammatory feed-forward progression factor (Sims G.P., 2010), is basally expressed by gingival cells (Figure 2) suggests important roles related to stimuli reception and inflammatory propagation. In fact, cells such as gingival epithelial cells function as modulators of inflammation and thus participate in maintaining homeostasis given their role as reservoirs of inflammation effector molecules. However, regulatory mechanisms that control inflammatory mediators in gingival cells can also become dysfunctional when external stimulation, including chronic exposure to tobacco smoke, persists. Such chronic stimulation may culminate in an abundance of pro-inflammatory molecules and cause irreversible remodeling of gingival and periodontal tissues.

Our discovery that the activation of important intermediates implicated in RAGE signaling were diminished following SAGE treatment clearly highlights RAGE as one of the key pathways in the pathophysiology of oral inflammation following smoke exposure. As a promiscuous amplifier of inflammation, RAGE induces the activation of NF- κ B known to modulate profound inflammatory responses (Alexiou P., 2010; Schmidt et al., 2001). Moreover, a prominent NF- κ B-responsive consensus sequence is located in the RAGE promoter, thus activation of RAGE signaling leads to greater RAGE expression (Alexiou P., 2010; Schmidt et al., 2001).

Accordingly, RAGE signaling plausibly functions centrally in the chronic exacerbation of oral impairments associated with tobacco smoke exposure such as those that impact the periodontal apparatus.

A critical point of emphasis in the study of inflammation is the elaboration of pro-inflammatory cytokines. It is well understood that tobacco smoke is a causative agent that stimulates numerous inflammatory pathways throughout the respiratory system. While

pulmonary responses in the proximal airways are generally known to be less robust than those observed distally (H., 1986; Szulakowski P., 2006; van der Vaart H., 2004), events in the most proximal oral and nasal cavities that culminate in cytokine production should not be discounted. We observed marked production of cytokines in response to CSE and significantly reduced cytokine elaboration when cells were simultaneously exposed to both CSE and SAGEs (Figure 5). IL-1 β and IL-6 are both pleiotropic cytokines that are widely expressed by cells in response to inflammatory agents, infections, or microbial endotoxins (Allan, Tyrrell, & Rothwell, 2005). IL-6 generally acts in the acute phase of inflammation and more chronic expression is implicated in sepsis but also metabolic disorders characteristic of obesity and insulin resistance (Khosravi et al., 2013). Our discovery that gingival cells express IL-6 adds notable support to the notion that tobacco exposure not only functions in the progression of periodontal inflammatory disease (Heidari Z., 2016; Shaddox L.M., 2016; Takedachi M., 2016), but also participates in systemic compromise reminiscent of diabetic and other metabolic complications (Morimoto-Yamashita Y., 2012). Like IL-6, IL-1 β has also been linked to the etiopathology of periodontitis and in particular is elevated in gingival crevicular fluid of diabetics with periodontal disease compared to controls (GE, 1997). In light of a connection between IL-1 β availability and degradation of connective tissues in the affected periodontal apparatus (Delima A.J., 2001), our work demonstrating diminished IL-1 β secretion when RAGE signaling is inhibited foreshadows a potential therapeutic modality that targets such deleterious cytokine functions. Surprisingly, RAGE inhibition via SAGE treatment significantly lessened cytokine secretion, but did not completely restore elaboration to control levels. As is the case with these and other pro-inflammatory cytokines and chemokines, alternative signaling pathways such as those mediated by Toll-like receptors (TLRs) likely compensate when redundant pathways are compromised.

In summary, our work demonstrates that RAGE signaling is activated in gingival cells exposed to tobacco smoke and that abrogation of RAGE signaling diminishes inflammation following exposure. While a more robust evaluation that considers a complete battery of pro and anti-inflammatory cytokines is necessary, this initial undertaking provides insight into potential mechanistic pathways that may possess therapeutic value. Furthermore, the assessment of inflammatory amelioration using blockers more specific to RAGE and other parallel pathways is necessary. We have already begun specific receptor blocking experiments so that individual contributions, despite the coalescing of signaling pathways, can be determined. Ultimately, evidentiary support should further prove RAGE targeting as a mechanism that alleviates periodontal distress in individuals unable or unwilling to quit smoking.

Author Contributions

NTS, DJD, JWD, JBL, and SHW each participated in the several experiments. DRW, JAA, BTB, and PRR designed the experiments and provided important intellectual input. NTS and PRR generated the manuscript. PRR and BTB conceived of the research project and managed its conduct.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgements

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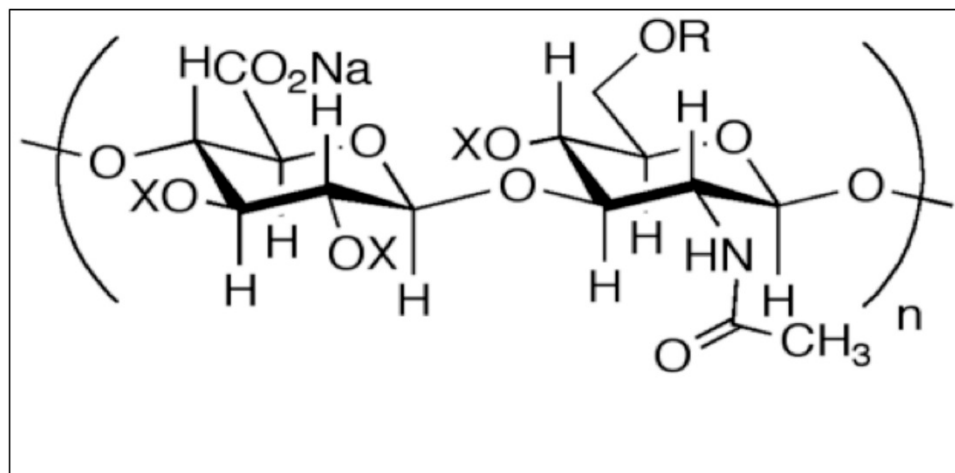


Figure 9.1: Representative Chemical Structure of SAGE.

X = sulfate or H, R = alkyl.

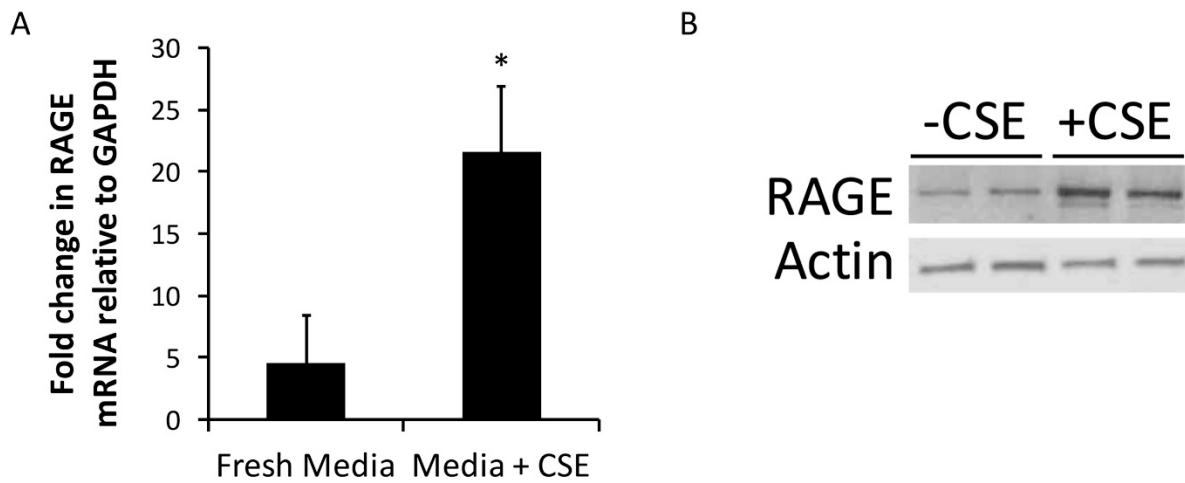


Figure 9.2: CSE Induces RAGE Up-regulation in Ca9-22 Gingival Cells.

qPCR (A) and immunoblotting (B) revealed that Ca9-22 gingival epithelial cells significantly increased RAGE mRNA and protein following 6 hours of CSE exposure. (* $P \leq 0.05$: Fresh Media vs. Media + CSE).

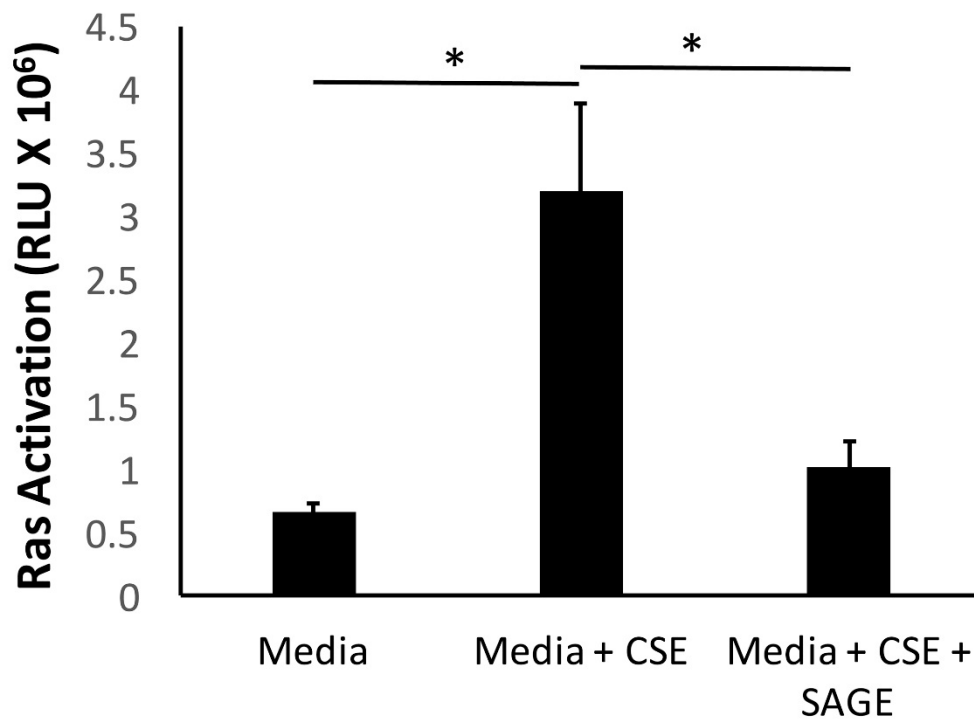


Figure 9.3: Effect of CSE and SAGE on Ras Activation.

Ras activation was augmented by CSE exposure and concomitant treatment with SAGEs decreased Ras activity. (* $P \leq 0.05$: Fresh Media vs. Media + CSE and Media + CSE vs. Media + CSE + SAGE).

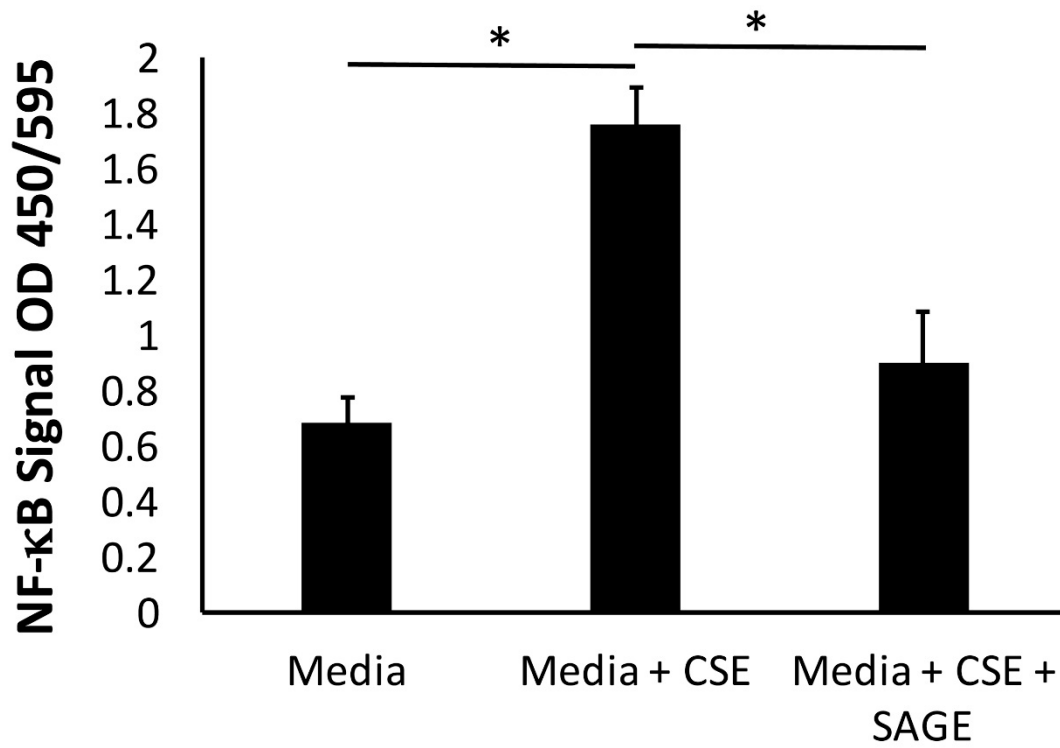


Figure 9.4: Effect of CSE and SAGE on NF-κB.

NF-κB activation was augmented by CSE exposure and concomitant treatment with SAGEs decreased NF-κB activity. (* $P \leq 0.05$: Fresh Media vs. Media + CSE and Media + CSE vs. Media + CSE + SAGE).

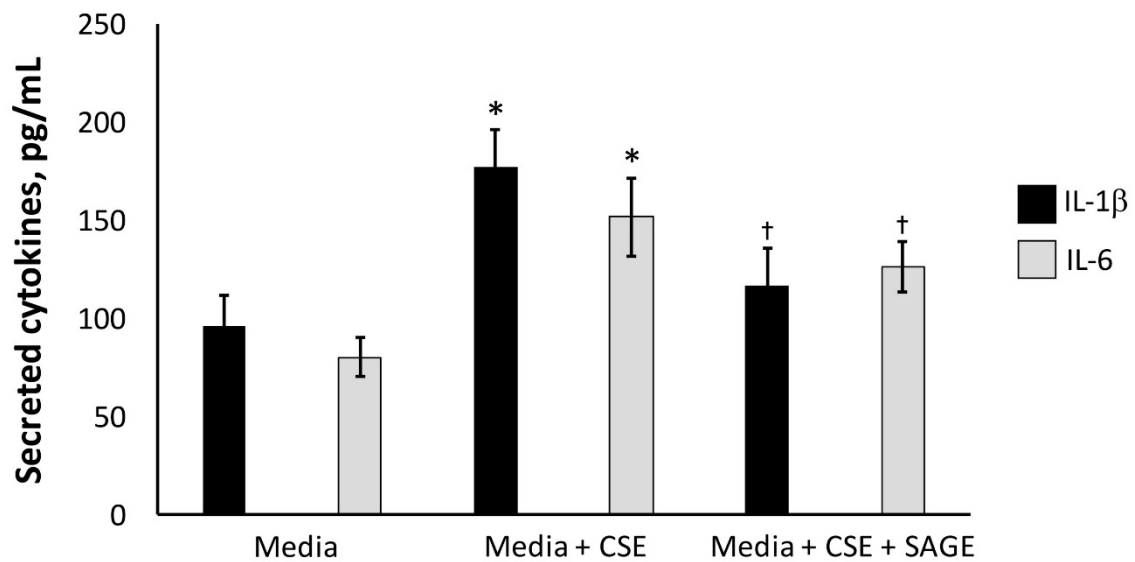


Figure 9.5: Effect of CSE and SAGE on Cytokine Secretion.

Secretion of IL-1 β and IL-6 was increased by CSE exposure and concomitant treatment with SAGEs decreased cytokine secretion. * $P \leq 0.05$ when comparisons were made between Fresh Media vs. Media + CSE and † $P \leq 0.05$ when comparisons were made between Media + CSE vs. Media + CSE + SAGE.

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CHAPTER 10: Inhibition of RAGE Protects from Secondhand Smoke Induced IUGR in Mice.

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Running Title: RAGE inhibition reduces SHS-induced IUGR

Keywords: IUGR, placenta, RAGE, SHS

Abstract

Intrauterine growth restriction (IUGR) is a disease affecting 10% of all pregnancies. IUGR is associated with maternal, fetal, or placental abnormalities. Studies investigating the effects of secondhand smoke (SHS) exposure and IUGR are limited. Receptors for Advanced Glycation End-products (RAGE) are pro-inflammatory transmembrane receptors increased by SHS in the placenta. We tested the hypothesis that inhibition of RAGE during SHS exposure protects from smoke-induced IUGR. C57BL/6 mice were exposed to SHS or SHS + semi-synthetic glycosaminoglycan ethers (SAGEs) known to inhibit RAGE signaling. Trophoblast cells were treated with cigarette smoke extract (CSE) with or without SAGEs in order to address the effects of RAGE inhibition during trophoblast invasion *in vitro*. SHS treated mice demonstrated a significant reduction in fetal weight and placental weight compared with controls. Mice co-treated with SHS and SAGEs were protected from SHS-induced fetal weights decreases. SHS treatment of C57BL/6 mice activated placental ERK, JNK, and p38 and the expression of inflammatory mediators including TNF- α and IL-1 β . SHS-mediated activation of these molecules was reduced to basal levels when SAGE was co-administered. Invasion of trophoblast cells decreased 92% when treated with CSE and CSE-mediated invasion was completely reversed by SAGEs. We conclude that RAGE inhibition protects against fetal weight loss during SHS-induced IUGR. These studies provide insight into tobacco-mediated IUGR development and clarify avenues that may be helpful in the alleviation of placental complications.

Introduction

Human pregnancy is characterized by complex temporal and spatial signaling processes that culminate in the creation of a healthy newborn. Disruption of these carefully orchestrated events has been demonstrated in obstetric complications such as Intrauterine Growth Restriction (IUGR). IUGR has been demonstrated to significantly increase the risks of fetal and neonatal morbidity and mortality. This disease stems primarily from uteroplacental vascular insufficiency which ultimately creates an environment of chronic oxygen and nutrient deficiency, resulting in restricted fetal growth (Bolehovska et al., 2015). Additionally, many complications have been associated with restricted fetal growth such as perinatal hypoxia and asphyxia, cerebral palsy, and persistent pulmonary hypertension of the newborn (Brar & Rutherford, 1988), (P. H. Gray et al., 1999), (R. N. Pollack & M. Y. Divon, 1992). Furthermore, several studies have reported long-term sequelae of IUGR complications including adult hypertension, heart disease, stroke and diabetes (D. J. Barker, 1993), (C. J. Smith et al., 2016), (D. J. Barker et al., 1993), (K. Holemans, R. Van Bree, J. Verhaeghe, L. Aerts, & F. A. Van Assche, 1993), (K. Phipps et al., 1993), (B. Reusens-Billen, C. Remacle, & J. J. Hoet, 1989). Placental dysfunction is common in many pregnancy pathologies including IUGR (Laviola et al., 2005). Abnormalities associated with the IUGR placenta include reduced syncytiotrophoblast surface area (DiFederico, Genbacev, & Fisher, 1999), increased thickness of the exchange barrier formed by the trophoblast and fetal capillary endothelium (N. Ishihara et al., 2002), decreased trophoblast invasion (T. M. Mayhew et al., 2003), (Pijnenborg et al., 1991), increased mTOR protein (S. Roos et al., 2007), and an increase in placental trophoblast apoptosis (Allaire, Ballenger, Wells, McMahon, & Lessey, 2000), (T. H. Hung, J. N. Skepper, D. S. Charnock-Jones, & G. J. Burton, 2002). Although research has discovered many processes that contribute to the development of

these pathologies, much more research is required to fully elucidate the underlying mechanisms associated with placental insufficiency in the context of IUGR.

Cigarette smoking during pregnancy has been a subject of intense study for decades (F. Arffin et al., 2012). In fact, many researchers have posited that smoking cigarettes throughout pregnancy may be the single most important avoidable causes of adverse pregnancy outcomes (Bickerstaff et al., 2012), (Akkar et al., 2015). Indeed, even exposure to low levels of nicotine, the primary addictive chemical compound found in cigarettes, has been demonstrated to affect normal brain development and increase infant mortality rates and lead to the development of IUGR (J. Guan et al., 2009), (H. A. Wahabi et al., 2013). Overall, exposure to cigarettes has been shown to have devastating effects on the developing fetus; however, reports detailing the consequences of passive secondhand smoke (SHS) exposure have yet to produce conclusive findings. Evidence suggests that mothers who are exposed to SHS may experience an increase the risk of newborn orofacial clefting, (Kummet et al., 2016), elevated risks of wheeze development in newborns (Vardavas et al., 2016), and even learning difficulties (Jorge, Botelho, Silva, & Moi, 2016). While data continues to demonstrate the deleterious effects of tobacco smoke, there are conflicting reports as to its correlation with IUGR. A study by Whabi et. al. (2013) found a link between SHS and the induction of IUGR (H. A. Wahabi et al., 2013), yet in contrast, Subramoney et al (2010) reported no correlation between IUGR incidence and pregnant women exposed to smoke (S. Subramoney, d'Espaignet, & Gupta, 2010). Despite the conflicting reports, studies have suggested that SHS significantly increases the risk for spontaneous abortion, preterm delivery, sudden infant death syndrome and stilled birth (D. A. Dempsey & N. L. Benowitz, 2001), (Engel et al., 2013), (Y. S. Khader, N. Al-Akour, I. M. Alzubi, & I. Lataifeh, 2011), (N. L. Lee et al., 2012), (S. L. Lee et al., 2012). Despite ongoing

confusion, more research is needed to fully understand the relationship between placental dysfunction and SHS exposure. Furthermore, an investigation into the possible mechanisms that may be contributing to the development of these adverse outcomes is warranted as it may lead to pioneering insights and possible therapeutic improvements.

The Receptor for Advanced Glycation End-Products (RAGE) is a pattern recognition cell surface receptor increased during SHS exposure (D. R. Winden et al., 2014) (A. B. Robinson, J. A. Stogsdill, et al., 2012). RAGE is capable of binding a host of ligands such as advanced glycation end-products (AGEs), HMGB1, S100/calgranulins, and an additional variety of substances (Morbini et al., 2006). RAGE signaling is implicated in the pathogenesis of many inflammatory diseases such as Alzheimer's (Lubitz et al., 2016), diabetes (Nelson et al., 2015), atherosclerosis (Belmokhtar et al., 2016), COPD (P. R. Reynolds, S. D. Kasteler, et al., 2011), and diverse rheumatological disorders (Q. Wu et al., 2016). Some products generated via RAGE signaling include NF- κ B, Cox-2, IL-1 β and TNF- α , thus RAGE signaling likely influences apoptosis and the mediating of potent pro-inflammatory responses found in many chronic inflammatory pathologies (Bianchi et al., 2010). RAGE has been discovered in many cell types including smooth muscle, endothelium, macrophages, and epithelium, but has its highest expression in the lung and the placenta (Buckley & Ehrhardt, 2010; Holmlund et al., 2007). Because the RAGE signaling axis has been linked with pro-inflammatory cascade events, novel drug therapies that target RAGE or its family of ligands are currently being investigated. One such recent modality with promise is Semi-synthetic Glycosaminoglycan Ethers (SAGEs). SAGEs are partially lipophilic sulfated polysaccharide derivatives that modulate inflammation via the abrogation of RAGE signaling (J. Zhang et al., 2011). In this research endeavor, we examine the potential protective effects of SAGEs, specifically through blocking RAGE

signaling in the lessening of downstream pro-inflammatory targets that contribute to the development of IUGR.

Material and Methods

Animals and Tissue Preparation

C57 Black 6 (C57BL/6) mice were obtained from Charles River laboratories, Wilmington, MA. Mice were housed in a conventional animal facility, supplied with food and water ad libitum, and maintained on a 12 h light-dark cycle. Animals were separated into four groups: Control with PBS injections (Control + PBS), Control with SAGE injections (Control + SAGE). To obtain timed pregnancies, females were caged with males overnight, and confirmed pregnant with evidence of sperm plug. Mice were allowed to undergo normal gestational development until embryonic day 14.5 (E14.5) at which time mice were either restrained and exposed to room air, or restrained and exposed to SHS. On E14.5, pregnant mice were then given either PBS or SAGE injections until E18.5 at which time mice were then euthanized. Placentae were dissected from pregnant mice. Only live birth animals were considered for the study and any mice with absorbed fetuses were noted but excluded from the data sets. The first five conceptuses proximal to the ovaries from each uterine horn were collected and placentae and fetuses were weighed at this stage of pregnancy. Placental and mesometrial compartment tissues were then snap frozen in liquid Nitrogen for RNA and protein analysis. Mice were housed and utilized in accordance with protocols approved by the IACUC at Brigham Young University.

Secondhand Smoke (SHS) Exposure

Mice were exposed to SHS generated from 3R4F research cigarettes from Kentucky Tobacco Research and Development Center, University of Kentucky, via a nose-only exposure

system (InExpose System, Scireq, Montreal Canada). Mice were individually placed in soft restraints and connected to an exposure tower, wherein a computer controlled puff of smoke generated every minute resulted in 10 seconds of SHS followed by 50 seconds of fresh air. Six mice in each group were exposed to SHS from six cigarettes over 10 minutes. This procedure was repeated from E14.5 – E18.5 at which time mice were euthanized. To generate Room Air controls, mice were similarly restrained for a period of 10 minutes but were only exposed to Room Air. The SHS challenge was determined to be an acceptable and was tolerated without evidence of toxicity. Furthermore, this nose only model of smoke exposure yielded chronic blood carboxyhemoglobin levels of ~5%, a value similarly observed in human smokers (J. L. Wright et al., 2008).

SAGE Treatment

SAGEs were a kind gift from Dr. Glenn D. Prestwich from the University of Utah Medical Center. All animal groups were administered an intraperitoneal injection (IP) with 20 µg/day (J. Zhang et al., 2011) of either SAGE or saline (PBS) from E13.5 to E18.5 and exposed to either SHS or room air as previously explained.

Immunoblotting

Western blot analysis was used to assess the expression of a collection of cell signaling proteins in both control, control + SHS exposed, and control + SHS + SAGE treated animals. Briefly, tissues were homogenized in protein lysis buffer (RIPA, Fisher Scientific, Pittsburg, PA). Protein lysates (20 µg) were separated on Mini-PROTEAN® TGX™ Precast gel (Bio-Rad Laboratories, Inc) by electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated overnight with antibodies for RAGE, TNF- α , IL-1 β , phospho ERK, phospho JNK, or phospho p38 (all from Cell Signaling, Danvers MA). A secondary horseradish

peroxidase antibody (1:10,00, 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 using a C-DiGit® Blot Scanner (LI-COR, Inc, Lincoln, Nebraska). Quantification of proteins was performed by densitometry and normalization with actin provided comparisons between treated and control samples.

Invasive Trophoblast Cell culture, Cigarette Smoke Extract (CSE) and SAGEs

A first-trimester cytotrophoblast cell line Swan71 (SW71; n=9) was used for invasive cytotrophoblast studies. SW71 cells were maintained and cultured in RPMI medium (Mediatech, Manassas, VA). Cell medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. CSE was generated as previously described by Reynolds et al., 2006 (P. R. Reynolds et al., 2008). Briefly, two 2RF4 research cigarettes (University of Kentucky, Lexington, KY) were continuously smoked with a vacuum pump into 20 ml of DMEM medium (Mediatech, Manassas, VA). The smoke-bubbled medium was filtered through a 0.22- μ m filter to remove large particles. The resulting medium was defined as 100% CSE. Dilutions were made using DMEM medium to a concentration of 10% CSE.

Cell Treatments

SW71 cells were detached and 20,000 cells/ml were incubated in 2% FBS medium alone, medium supplemented with 0.5% CSE (Reynolds, Cosio and Hoidal, 2006, Reynolds, et al., 2008) or 0.5 CSE and 50 ug/ml SAGEs. All treatments were done for 24 hours. Real time cell invasion was determined following these treatments.

Real Time Cell Invasion Determination

An xCELLigence was utilized to determine real time invasion of trophoblast cells. Protocol was followed as suggested by manufacturer. Briefly Invasion was assessed in 16 well CIM-Plates (n=9). The top wells were coated with a 1:40 matrigel concentration and trophoblast cells were plated in the top chamber at a concentration of 20,000 cells/well in 2% FBS RPMI in a total volume of 100 μ L in the presence or absence CSE or CSE and SAGES. The bottom chamber wells were filled with 160 μ L of 10% FBS RPMI. The cells were then place in the RTCA DP instrument and invasion readings were done every 15 minutes for 24 hr.

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 representatives and P-values ≤ 0.05 were considered significant.

Results

SHS Decreased Placental and Fetal Weights

We first conducted *in vivo* studies in order to investigate the effects of SHS on fetal and placental weights. Compared to room air exposed controls, animals treated with SHS developed significantly lighter placentas (1.13-fold; $p \leq 0.0001$) and fetal weights were significantly less (7.35-fold; $p \leq 0.0001$) following four days of SHS treatment (Figure 1A and B.)

RAGE Expression and Influence of IUGR Following SHS Exposure

After examining placental and fetal weight changes stemming from SHS exposure, RAGE expression was evaluated to determine whether maternal SHS exposure elicited changes

in placental protein expression. RAGE protein levels were elevated (1.13-fold; $p \leq 0.05$) with the addition of SHS when compared to controls (Figure 2).

Inhibition of RAGE by SAGEs and Placental and Fetal Weights.

Because the absence of RAGE signaling may be centrally involved in the protection from SHS-mediated decreases in placental and fetal weights, we next sought to inhibit RAGE in wild-type mice during SHS exposure. This round of *in vivo* studies involved the treatment of mice with SAGEs, a recently developed collection of sulfated polysaccharide derivatives previously shown to significantly inhibit RAGE Signaling. There was no significant recovery in placental weights from animals co-treated with SHS and SAGEs when compared to those exposed to SHS alone (Figure 1A). However, treatment of SHS-exposed mice with SAGEs caused a significant increase in fetal weights (4.22-fold; $p \leq 0.0001$) when compared with animals treated with SHS alone (Figure 1B). Despite significant protection, fetal weights were not completely restored by SAGE treatment when compared to room air exposed controls (1.74-fold; $p \leq 0.0001$, Figure 1B). Importantly, no weight changes were observed when room-air control animals were treated with SAGEs when compared to non-treated controls (not shown).

To further characterize the effects of SAGEs in the placenta, we studied cell-signaling molecules associated with RAGE activation. Increased TNF- α and IL-1 β expression is known to be controlled, at least in part, by RAGE signaling (Xian et al., 2015) (D. Li et al., 2015). SHS treatment increased both TNF- α (1.34-fold $p \leq 0.05$) and IL-1 β (1.03-fold $p \leq 0.05$) in the placenta of SHS-treated animals when compared to controls (Figure 3A and B). Interestingly, treatment with SAGEs decreased both TNF- α (1.84-fold $p \leq 0.05$) and IL-1 β (1.16-fold $p \leq 0.05$) in the SHS exposed animals when compared to SHS-exposed animals (Figure 3A and B). To further investigate RAGE signal transduction in the placenta, we next looked at phosphorylated levels of

ERK, JNK and p38. An increase in p-ERK activation (2.61-fold, $p \leq 0.05$) was observed in the placenta from animals treated with SHS when compared to room air controls (Figure 4A). When SAGEs were added to the SHS animals, activated ERK was significantly diminished (2.26-fold, $p \leq 0.05$) when compared to animals treated with SHS alone (Figure 4A). Similarly, increased activation of JNK (1.79-fold, $p \leq 0.05$) occurred following SHS exposure and treatment with SAGEs was sufficient to significantly inhibit SHS-mediated p-JNK levels (1.45-fold, $p \leq 0.05$) (Figure 4B). In our analysis of p-P38, we observed a significant increase in p-P38 levels in SHS-exposed placentas compared to room air exposed controls (2.44-fold, $p \leq 0.05$) and a significant restoration of p-P38 to unexposed baseline when SHS and SAGEs were co-administered (1.55-fold, $p \leq 0.05$, Figure 4C).

Trophoblast Invasion

A cellular hallmark of diminished placental weights observed in IUGR is decreased trophoblast invasion (J. A. Arroyo & V. D. Winn, 2008). Previous reports from our laboratory have already demonstrated decreased cell invasion by trophoblast cells following exposure to cigarette smoke extract (CSE) (Mejia et al., 2016). We therefore sought to quantitatively determine the plausible protective effects of SAGEs on cultured trophoblast invasion treated with CSE. As expected, CSE treatment decreased trophoblast invasion (7.4-Fold; $p < 0.02$) in culture (Figure 5A). Diminished invasion orchestrated by CSE was completely reversed when SAGEs and CSE were concomitantly added to the media of cultured trophoblasts (Figure 5B).

Discussion

RAGE signaling has been a major focal point in inflammatory research over the past several years. Numerous early studies sought to elucidate roles for RAGE signaling in the pulmonary apparatus (M. P. Stogsdill et al., 2013) (D. R. Winden et al., 2013) (Barton et al.,

2014), however roles are not clearly understood in terms of systemic expression and RAGE's relationship to non-pulmonary related pathologies. In regards to pregnancy, recent research has demonstrated that RAGE activation may play an important role in normal placentation (Konishi et al., 2004) as RAGE is localized to the developing human trophoblast. Furthermore, current research has confirmed that increased RAGE levels are found in pre-eclamptic (PE) placentas (Alexander et al., 2016). Because one of the primary activators of the RAGE signaling axis is tobacco smoke exposure, we sought to examine the possible effects of SHS exposure during pregnancy. Our initial investigation revealed that the addition of SHS during embryogenesis was sufficient to cause decreased placental and fetal weights. Unsurprisingly, RAGE expression was demonstrated to be elevated by SHS. These data corroborate recent findings that the addition of SHS increases RAGE protein in a variety of tissues (D. R. Winden et al., 2014), (Prasad et al., 2015).

To further confirm that RAGE signaling may be implicated in IUGR, we tested a newly developed compound that inhibits RAGE signaling. The addition of SAGEs into SHS-exposed animals during pregnancy resulted in significant protection against fetal weight loss. This positive outcome suggests that more research is needed to further evaluate the possible therapeutic benefits of RAGE targeting in the placenta. Indeed, although our data only revealed significant protection, it remains possible that a more robust study that involves a broad dose curve may reveal complete protection against fetal and placental weight loss in the context of smoke exposure.

Because our *in vivo* studies implicated RAGE signaling as a modulator of IUGR, we sought to characterize additional RAGE-mediated targets. Extracellular signal-regulated Kinase (ERK) is a protein usually associated with proliferation. Previous studies have demonstrated that

a p-ERK is elevated in patients with PE and IUGR (Bahr et al., 2014). Consistent with this data, our research demonstrated increased levels of p-ERK when animals were exposed to SHS. Furthermore, as ERK is at least partially mediated through the RAGE signaling axis, when SAGE was administered in conjunction with SHS exposure, active ERK protein levels were significantly blocked. The p38 MAPK and JNK belong to the MAPK family of intracellular signal transducers involved in mammalian growth. In the RAGE signaling pathway, these downstream mediators activate NF-kB which then in turn influences transcriptional regulation. Studies have demonstrated that p38 and JNK protein levels remain unchanged in IUGR, however phosphorylated p38 and JNK levels are decreased in placental disease (Laviola et al., 2005). Our own data showed increases in overall p-P38 and p-JNK protein when control and SHS groups were compared. When SHS smoke animals were given SAGE treatment, these animals decreased both p-P38 and p-JNK. These data suggest that these potent intermediates of inflammation and their subsequent activation are at least partially enabled through the RAGE signaling pathway. Furthermore this assertion supports previous reports that implicates RAGE signaling in the activation of these proteins in patients with PE, IUGR and gestational diabetes (Alexander et al., 2016).

Endothelial cell dysfunction is believed to largely contribute to the pathogenesis of PE/IUGR. Inflammatory cytokines such as IL-1 β and TNF- α have been shown to induce functional alterations in endothelial cells (Daneva, Hadzi-Lega, & Stefanovic, 2016) and have been found elevated in PE patients (Xian et al., 2015). It has been well documented that SHS increases TNF- α and IL-1B levels. Furthermore, research has demonstrated that RAGE signaling increases both of these pro-inflammatory cytokines, thus propagating RAGEs ability to affect systemic inflammation. Our western blot analyses demonstrated elevated levels of TNF- α

and IL-1 β with the addition of SHS. However, TNF- α and IL-1 β were both diminished to below the control levels when SAGEs were introduced suggesting that this potential therapeutic may be influencing IUGR by decreasing inflammation.

A hallmark of IUGR/PE is shallow invasion of trophoblast cells (Harmon et al., 2016). Diminished invasion ultimately results in a high resistance, low-capacity perfusion system, creating large-scale nutrient and oxygen deficiencies. Using an *in vitro* system we demonstrated that the addition of SHS was sufficient to drastically decrease trophoblast cell invasion and that SAGE treatment notably restored invasion.

We conclude that inhibition of RAGE protects against fetal and placental weight loss during SHS-induced IUGR. This conclusion is notably supported by the discovery that hindered trophoblast invasion was reversed by SAGE treatment. Our results further suggested that there is a correlation between RAGE activation and the development of IUGR during SHS exposure. These studies provide insight into tobacco-mediated IUGR progression and clarify possible avenues for alleviating placental complications during SHS exposure.(Ahmed, 2014)

Declaration of Interests

The authors have nothing which they wish to declare.

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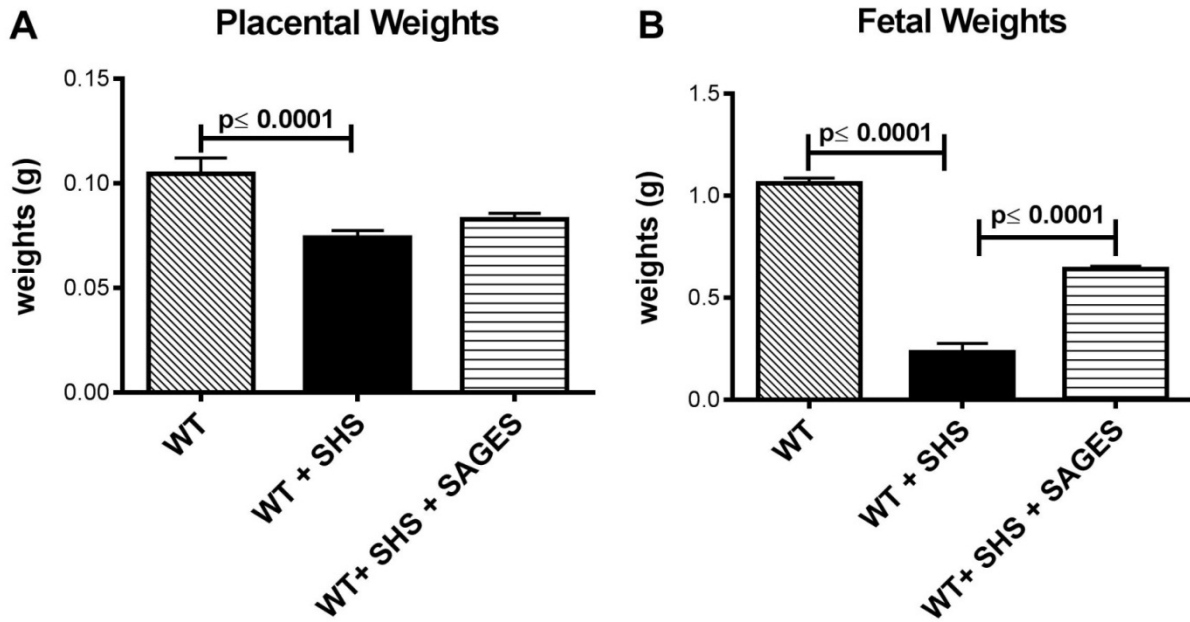


Figure 10.1: Placental and Fetal Weight Differences with Secondhand Smoke (SHS) Exposure and SAGE Treatment.

A significant decrease in placental (A: 1.4-fold; $p \leq 0.0003$) and fetal weights (B: 2.3-fold; $p \leq 0.0002$) was observed in SHS-treated animals. Placental and fetal weight differences during secondhand smoke (SHS) exposure with SAGE treated mice showed no significant recovery in placental weights when comparing placenta from WT animals co-treated with SHS and SAGES to those exposed to SHS alone. A significant increase in fetal weights (4.22-fold; $p \leq 0.0001$) was observed in SHS-exposed mice with SAGES when compared with animals treated with SHS alone.

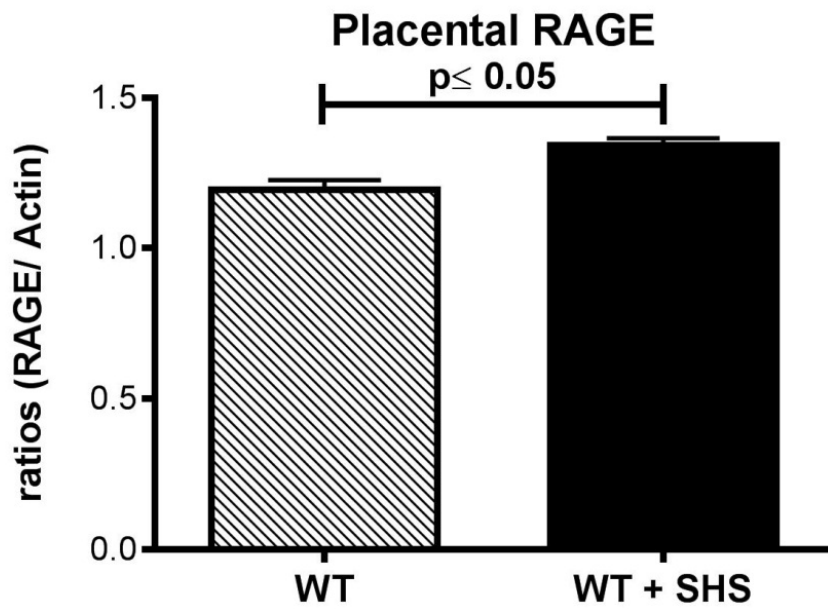


Figure 10.2: Placental RAGE Expression During SHS Induced IUGR.

RAGE was increased (1.13-fold; $p \le 0.05$) with SHS in the placenta of treated animals when compared to controls.

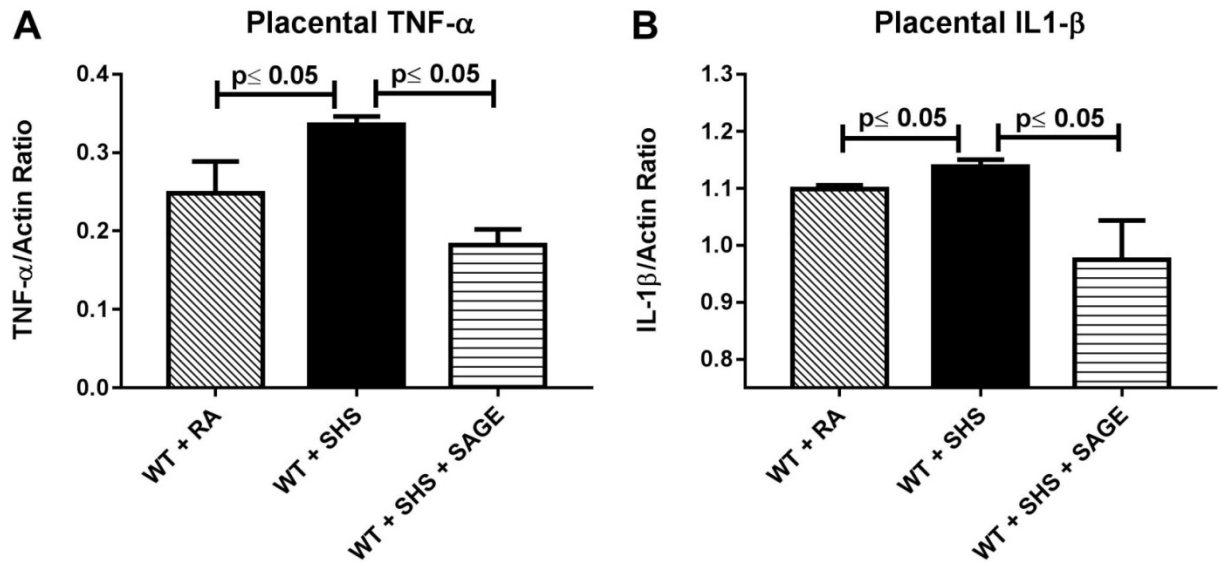


Figure 10.3: Placental TNF- α and IL-1 β Expression During SHS Induced IUGR.

An increase in both TNF- α (1.34-fold; $p \leq 0.05$) and IL-1 β (1.03-fold; $p \leq 0.05$) was observed in placentas from SHS-treated animals when compared to room air controls. In contrast, a significant decrease of both TNF- α (1.84-fold; $p \leq 0.05$) and IL-1 β (1.16-fold; $p \leq 0.05$) was observed when animals were co-treated with both SHS and SAGE when compared to SHS only exposed animals.

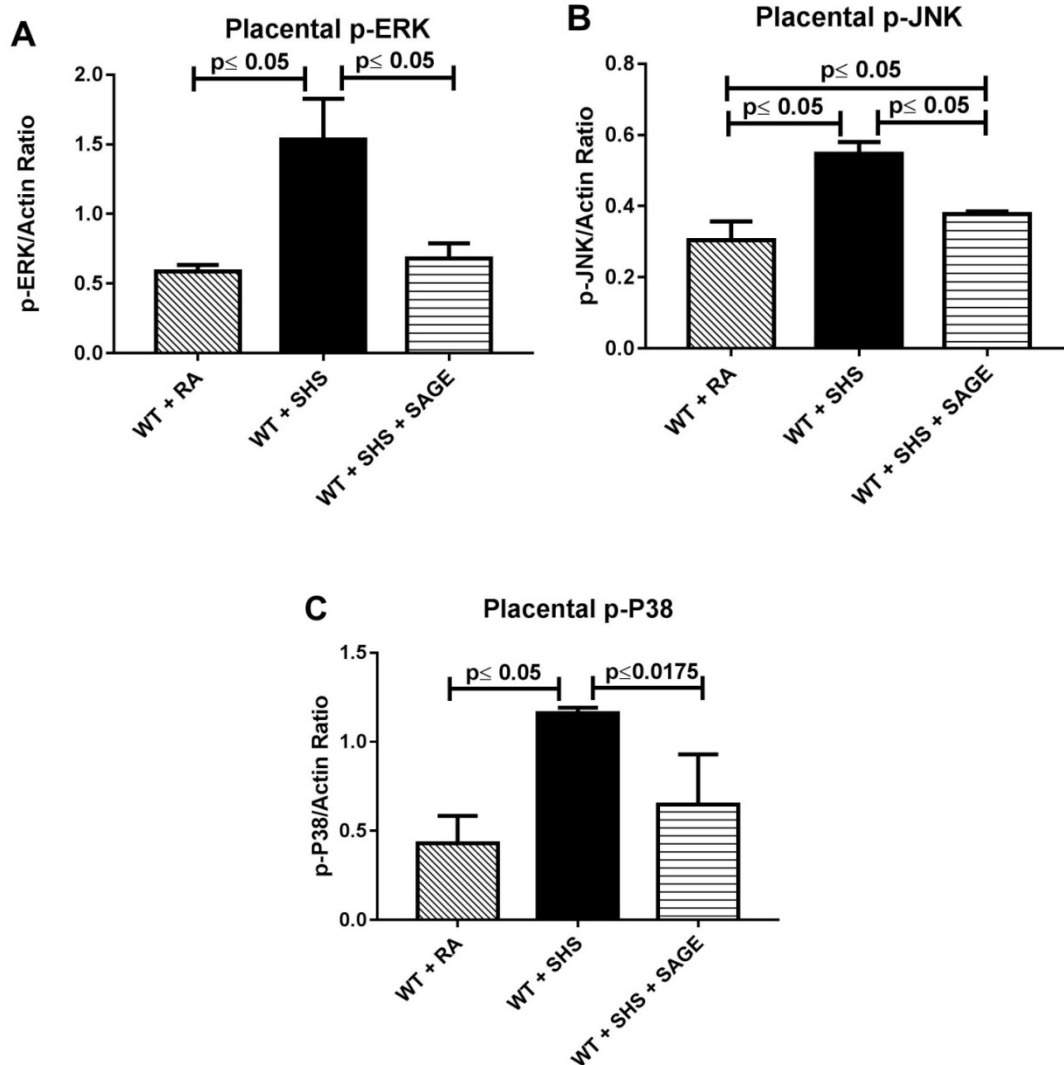


Figure 10.4: Placental pERK, pJNK and pP38 Expression During SHS Induced IUGR.

A significant increase in pERK activation (2.61-fold; $p \leq 0.05$) was observed in placentas from animals treated with SHS when compared to room air controls. In contrast, when Sages were added to the SHS animals, activated ERK was significantly decreased (2.26-fold; $p \leq 0.05$) when compared to animals treated with SHS alone (A). A significant increase in activation of JNK (1.79-fold; $p \leq 0.05$) occurred following SHS as compared to room air controls. When SHS animals were co-treated with SAGES, a significant decrease in pJNK levels (1.45-fold; $p \leq 0.05$) was observed when compared to animals treated with SHS alone (B). Similarly, a significant increase in pP38 levels in SHS exposed placentas was observed compared to room air exposed controls (2.44-fold; $p \leq 0.05$) and a significant restoration of pP38 (1.55-fold; $p \leq 0.05$) was observed in the SHS and SAGE treated animals when compared to animals treated with SHS alone (C).

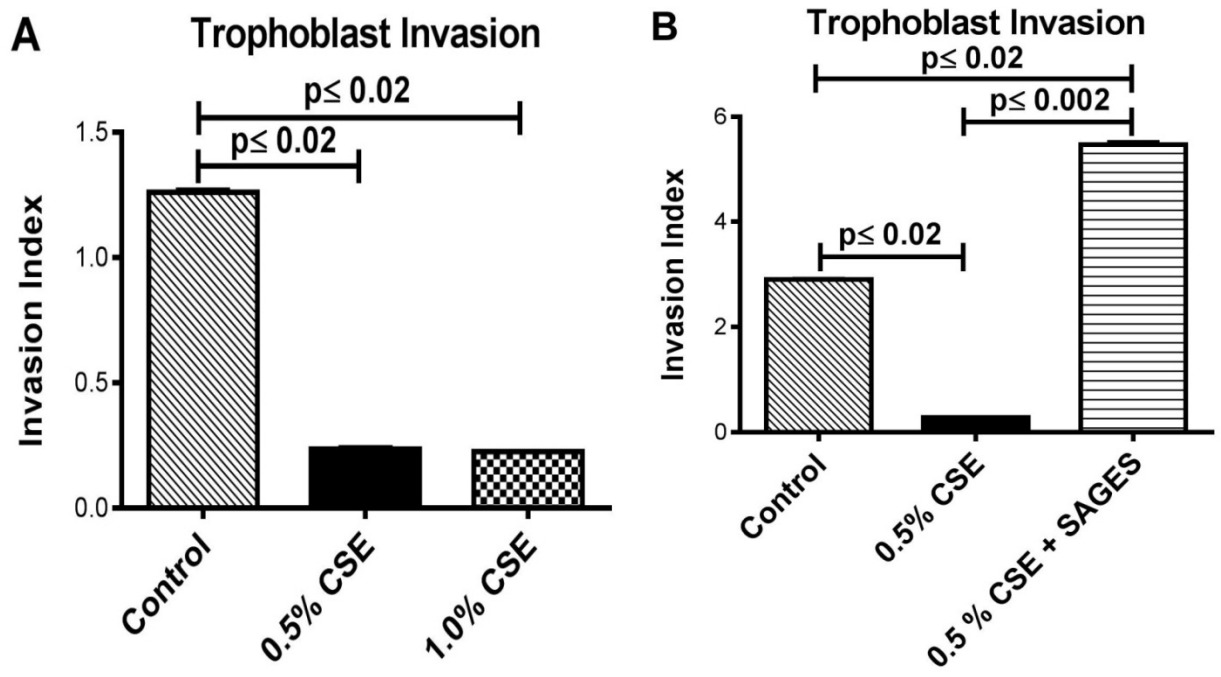


Figure 10.5: CSE and SAGE Treatment and Trophoblast Invasion.

Cell invasion was decreased (7.4-fold; $p \leq 0.02$) with 0.5% CSE treatment in cultured trophoblast cells. Decreased invasion mediated by CSE was completely reversed in SAGE treated trophoblast.

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increases fetal lung expression of RAGE and induces RAGE-mediated pulmonary inflammation. *Respir Res*, 15, 129. doi: 10.1186/s12931-014-0129-7

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Zhang, J., Xu, X., Rao, N. V., Argyle, B., McCoard, L., Rusho, W. J., . . . Krueger, G. (2011). Novel sulfated polysaccharides disrupt cathelicidins, inhibit RAGE and reduce cutaneous inflammation in a mouse model of rosacea. *PLoS One*, 6(2), e16658. doi: 10.1371/journal.pone.0016658

CURRICULUM VITAE

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Minor: German
Brigham Young University
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Dissertation: Alterations in Tight Junctions and Their
Effects on Pulmonary Inflammation.
Advisor: Dr. Paul R. Reynolds

Professional Experience:

Research Assistant (Nov. 2011- Present)
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Supervisor: Dr. Paul R. Reynolds
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Responsibilities:

- Design and conduct hypothesis-based research focused on inflammatory pathways and pathologies, as well as alveolar development.
- Maintain and operate research lab equipment
- Collect and interpret data.
- Present findings at professional research conferences.
- Prepare and submit data for publication in peer reviewed journals.

Undergrad Research Mentor/Lab Coordinator (Aug. 2014-Present)
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Responsibilities:

- Mentor undergraduates working in the combined Reynolds/Arroyo Lung and Placenta Research Lab.
- Train students in mouse surgeries, tissue sample preparation, histology, RNA and protein analysis procedures.
- Coordinate and run lab meetings.
- Track lab materials and order when necessary.
- Assist undergrads in planning and coordinating original experiments.
- Prepare students for poster or oral presentations at national scientific meetings.

Teaching Assistant & Laboratory Instructor (Aug. 2014-Present)
Brigham Young University
Department of Physiology and Developmental Biology
Provo UT, 84602

Physiology Lab (PDBio 305)
Embryology (PDBIO 484)

Responsibilities:

- Teach, tutor, and manage weekly classes
- Perform maintenance work and service lab equipment
- Grade assignments and exams
- Hold regular office hours.

Project Manager and Glass Artists (2002-Present)
Holdman Studios and the Art Institute
3001 Thanksgiving Way
Lehi, UT 84043

Responsibilities:

- Project Manager over the Cordoba, Argentine LDS Temple
- Contribute to the designing, building and installation of large scale stained glass windows for both private and public locations.
- Trained glassblower
- Early achievements include designing the prototype for the Rexburg, ID LDS Temple.

Equipment and Locker Room Attendant (2011-2013)
Brigham Young University
Richards Building and Smith Fieldhouse
Provo, UT 84602

Responsibilities:

- Duties included management of facility equipment, distribution of facility use passes, and assisting students with questions about facilities.

Freelance German Translator (2007-Present)

- Translating, interpreting, and proofreading German/English documents

Service and Outreach:

Missionary (Dec. 2005-Dec. 2007)
Church of Jesus Christ of Latter-day Saints
Hamburg, Germany

- Performed Humanitarian work
- Taught weekly English classes
- Taught tenets of the LDS Faith in German

Volunteer Soccer Coach (2010-2012)
South Franklin Community Center
Provo, UT 84604

- Coached the 5-7 year olds on how to play soccer and develop good sportsmanship

German Tutor (2011-2013)
Utah State Hospital
Provo, UT 84604

- Tutored psychiatric patient in German

Emergency Room Volunteer (2012-2013)
 Utah Valley Regional Medical Center
 Provo, UT

- Assisted hospital staff, patients and patient's family in Emergency Room department.

Student Director (2015-Present)
 Volunteer Care Clinic
 Provo, UT 84606

- Responsible for organizing, and maintaining Student Volunteer Program at local community clinic.

Skills:

- Communication: Good interpersonal skills, able to work in various atmospheres, fluent German speaker.
- Lab Skill: RT-PCR, PCR, Protein Isolation, Immunoblotting, Immunohistochemistry, Immunofluorescence, DNA/RNA isolation, Cell Culture, various other lab techniques, etc.
- Proficient with Microsoft Word, Excel, PowerPoint, Adobe Photoshop, Adobe Illustrator, JMP, Prism.
- Certified as a Phlebotomist (2014).
- Certified as an Emergency Medical Technician (Basic level, 2014).

Publications:

Peer Reviewed Manuscripts

- Robinson AB, Stogsdill JA, Lewis JB, Wood TT, Reynolds PR "RAGE and tobacco smoke: insights into modeling chronic obstructive pulmonary disease." *Front. Physio.* 2012; 3:301
- Jimenez FR, Lewis JB, Belgique ST, Wood TT and Reynolds PR. "Developmental lung expression and transcriptional regulation of Claudin-6 by TTF-1, Gata-6, and FoxA2." *Respiratory Research*, 2014 Jun 26 15:70.
- Jimenez FR, Belgique ST, Lewis JB, Albright SA, Jones CM, Howell BM, Mika AP, Jergensen TR, Gassman JR, Morris RJ, Arroyo JA, and Reynolds PR. Conditional pulmonary overexpression of Claudin-6 (Cldn6) during embryogenesis delays lung morphogenesis. *Int J Dev. Biol*, doi: 10.1387/ijdb.150086pr.

- Mejia C, Lewis J, Jordan C, Mejia J, Ogden C, Monson T, Winden D, Watson M, Reynolds PR, Arroyo JA. Decreased activation of placental mTOR family members is associated with the induction of intrauterine growth restriction by secondhand smoke in the mouse. *Cell Tissue Research*, doi: 10.1007/s00441-016-2496-5
- Lewis J, Milner D, Lewis A, Dunaway T, Egbert K, Albright S, Merrell B, Monson T, Broberg D, Gassman J, Thomas D, Arroyo JA, Reynolds PR. Up-regulation of Claudin-6 in the Distal Lung Impacts Secondhand Smoke-Induced Inflammation. *IJERPH*. doi: 10.3390/ijerpha13101018
- Jimenez FR, Lewis JB, Belgique ST, Milner DC, Lewis AL, Dunaway TM, Egbert KM, Winden DR, Arroyo JA, Reynolds PR. Cigarette Smoke and Decreased oxygen tension inhibit pulmonary claudin-6 expression. *Exp. Lung Research*. doi: 10.1080/01902148.2016.1261309

Submitted Manuscripts

- Inhibition of the Receptor for Advanced Glycation End-products (RAGE) receptor protects against secondhand smoke (SHS) induced intrauterine growth restriction (IUGR) in mice. Lewis JB, Mejia C, Monson TD, Bodine JS, Dunaway TM, Egbert KM, Lewis AL, Broberg DS, Hall PD, Reynolds PR, Arroyo JA. *American Journal of Reproductive Immunology*.
- Transgenic up-regulation of Claudin-6 decreases diesel particulate matter (DPM)-induced pulmonary inflammation. Lewis JB, Bodine JS, Gassman JR, Muñoz SA, Milner DC, Lewis AL, Dunaway TM, Egbert KM, Christiansen CE, Monson TD, Broberg DS, Arroyo JA, Reynolds PR. *American Journal of Respiratory and Critical Care Medicine*.
- The relationship between RAGE and secondhand smoke in disease pathogenesis. Lewis JB, Bikman BT, Arroyo JA, Reynolds PR. *International Journal of Environmental Research and Public Health*.
- Cigarette Smoke Extract (CSE) Induces RAGE-Mediated Inflammation in Gingival Epithelial Cells. Sanders NT, Dutson DJ, Durrant JW, Lewis JB, Albreit SA, Wilcox S, Bikman BT, Arroyo JA, Reynolds PR, Winden DR. *International Journal of Dentistry*.

In-Preparation Manuscripts

- Organic cation transporter novel type-1 (OCTN-1) and pulmonary responses to secondhand tobacco smoke (SHS). Milner DC, Lewis JB, Gassman JR, Monson TD, Broberg DS, Arroyo JA, Reynolds PR, Erhardt K. *In preparation*

- Growth arrest-specific protein 6 (Gas6) is sufficient to induce inflammation in rat model. Dunaway TD, Lewis JB, Edwards MM, Chapster S, Thomas DB, Broberg DS, Monson TD, Hall PD, Egbert KE, Lewis AL, Reynolds PR, Arroyo JA.
- Expression profile of Claudin family members in the developing lung. Lewis JB, Jimenez FR, Chavarria M, Gassman JR, Jergensen TR, Albright S, Morris R, Baker P, Christiansen C, Schrader T, Millner DC, Bodine JS, Arroyo JA, and Reynolds PR. *Journal of Histochemistry and Cytochemistry*
- Spatial expression of Receptor for Advanced Glycation End-products (RAGE) in diverse tissue and organ systems differs following exposure to secondhand cigarette smoke. Gassman JR, Lewis JB, Milner DC, Lewis AL, Bodine JS, Dunaway TM, Monson TD, Broberg DS, Hall PD, Arroyo JA, Reynolds PR. *In preparation*

Abstracts:

- Expression profile of Claudin family members in the developing lung. Lewis JB, Jimenez FR, Chavarria M, Gassman JR, Jergensen TR, Albright S, Morris R, Baker P, Christiansen C, Schrader T, Millner DC, Bodine JS, Arroyo JA, and Reynolds PR. 2015. *Dev Biology* in press.
- Altered Inflammatory responses in tobacco smoke-exposed mice that over-express the tight junctional protein Claudin-6. Lewis JB, Jimenez JR, Milner DC, Lewis AL, Dunaway TM, Egbert KM, Albright SC, Merrill BJ, Monson TD, Gassman JR, Broberg DS, Thomas DB, Arroyo JA, Reynolds PR. *FASEB J* April 2016 30:305.12
- Organic cation transporter novel type-1 (OCTN-1) and pulmonary responses to secondhand tobacco smoke (SHS). Milner DC, Lewis JB, Gassman JR, Monson TD, Broberg DS, Arroyo JA, Reynolds PR, Erhardt K. *FASEB J* April 2016 30:50.4
- Transgenic up-regulation of Claudin-6 decreases diesel particulate matter (DPM)-induced pulmonary inflammation. Lewis JB, Bodine JS, Gassman JR, Muñoz SA, Milner DC, Lewis AL, Dunaway TM, Egbert KM, Christiansen CE, Monson TD, Broberg DS, Arroyo JA, Reynolds PR. *FASEB J* April 2016 30:305.11
- Receptor for Advanced Glycation End-products (RAGE) inhibition protects from Intrauterine Growth Restriction (IUGR) during Secondhand Smoke (SHS) in mice. Jordan C, Lewis JB, Bodine JS, Lewis AL, Dunaway TM, Egbert KM, Monson TD, Ogden KC, Wright TJ, Mejia CA, Reynolds PR, Arroyo JA. *FASEB J* April 2016 30:1033.4
- Spatial expression of Receptor for Advanced Glycation End-products (RAGE) in diverse tissue and organ systems differs following exposure to secondhand cigarette smoke. Gassman JR, Lewis JB, Milner DC, Lewis AL, Bodine JS, Dunaway TM, Monson TD, Broberg DS, Hall PD, Arroyo JA, Reynolds PR. *FASEB J* April 2016 30:1b741

- Protective role of ergothioneine from tobacco smoke-induced oxidative stress in vitro and in vivo. Selo MA, Clerkin CG, Talbot BN, Walsh JJ, Nakamichi N, Kato Y, Lewis JB, Reynolds PR, Ehrhardt C and Nickel S *FASEB J* April 2016 30:982.1
- Differential Placental Expression of the Receptor for Advanced Glycation End-Products (RAGE) in Normal and Complicated Pregnancies. Alexander K, Lewis J, Mejia M, Howell B, Reynolds PR, and Arroyo JA. 2015. *FASEB J* 29:972.2.
- Expression Profile of Claudin Family Members in the Developing Lung. Lewis J, Jimenez F, Chavarria M, Gassman J, Jergensen T, Schrader T, Millner D, Bodine J, Arroyo JA, and Reynolds PR. 2015. *FASEB J* 29:LB747.
- Pulmonary expression and regulation of Cldn6 by tobacco smoke. Jimenez FR, Lewis JB, Belgique ST, Wood TT and Reynolds PR 2014. *FASEB J* 28:834.3
- Developmental expression and transcriptional regulation of claudin-6 in the murine lung. Jimenez FR, Lewis JB, Wood TT, and Reynolds PR 2013. *FASEB J* 27:256.4.
- Nasal Administration of Diesel Exhaust Particles does not Evoke dysfunction or Initiate Autophagy in Murine Femoral Arteries. Cho J, Kim JS, Lewis JB, Reynolds PR, Bikman BT, Symons JD, 2016
- Activation of AXL Receptor via Gas6 induces Preeclampsia in Rats. Dunaway TM, Lewis JB, Hirschi KM, Mejia CA, Mejia JF, Hall PD, Reynolds PR, Arroyo JA. 2016
- Inhibition of the Receptor for Advanced Glycation End-products (RAGE) protects from secondhand smoke (SHS) induced intrauterine growth restriction (IUGR) in mice. Lewis JB, Mejia CA, Clinton J, Monson TD, Bodine JS, Dunaway TM, Egbert KM, Wright TJ, Ogden KC, Broberg DS, Hall PD, Nelson SM, Hirschi KM, Reynolds PR, Arroyo JA, 2016.
- Abrogation of RAGE signaling using semi-synthetic glycosaminoglycan ethers (SAGEs) ameliorates inflammation in mice exposed to secondhand tobacco smoke. Hirschi KM, Lewis JB, Hall PD, Wright TJ, Egbert KM, Ogden KC, Nelson SM, Clark JC, Milner DC, Arroyo JA, Reynolds PR. 2016

Awards and Recognitions:

Tuition Funded: 5
 Teaching Assistantships: 5
 Research Assistantships: 3
 PDBIO/Neuroscience Graduate Retreat Poster Competition: 3rd Place
 BYU Department of Physiology and Developmental Biology Graduate Student
 Scholarship: 2

Professional Affiliations:

The American Physiological Society,	(2014-Present)
Society for Developmental Biology,	(2014-Present)
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