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Characterization of Secondhand Smoke (SHS) and Materno-Fetal Interactions in Receptors for Advanced Glycation End-Products (RAGE)-Targeted Mice

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ABSTRACT

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Receptors for advanced glycation end-products (RAGE) are pattern recognition receptors of the immunoglobulin superfamily highly expressed in the lung. Likely functions include the modulation of pulmonary inflammation during disease. However, the contributions of RAGE in the developing lung in cases where secondhand smoke (SHS) exposure occurs are unknown. In order to test the hypothesis that RAGE misexpression adversely affects lung morphogenesis, we exposed gestating dams to a controlled dose of SHS during the last four critical days of *in utero* lung morphogenesis. We discovered that both maternal and fetal lungs respond to SHS by up-regulating RAGE. Exposed fetuses were markedly smaller compared to controls and lungs were compromised in terms of apoptotic status, collagen abundance necessary in the derivation of respiratory compartments, and the expression of MMP-9, a protease known to target extracellular matrix. Interestingly, RAGE knock out animals similarly exposed to SHS were protected, in part, from the same SHS-mediated pulmonary abnormalities. We next generated a conditional transgenic mouse that provided an opportunity to genetically augment distal lung RAGE expression in the absence of SHS exposure. Our RAGE transgenic mice (RAGE TG) were severely hypoplastic and ultrastructural analysis demonstrated weakened basement membranes in RAGE TG animals compared to controls. Specific observations in RAGE TG mice included diminished type IV collagen required for basement membrane derivation, augmented MMP-9 expression, and inhibition of pulmonary vasculature visualized by Pecam-1 staining, a marker of vascular endothelial cells. The further observation that FoxM1, a critical transcriptional regulator of endothelial cell differentiation, was inhibited in RAGE TG mice suggested a novel potential mechanism of impaired vascularization mediated by RAGE. These data provide evidence that RAGE expression must be tightly regulated during lung organogenesis. Furthermore, additional research into the nuances of RAGE signaling during development may shed needed light on the pathobiochemistry of adult lung diseases that potentially have *in utero* origins.

Key Words: RAGE, transgenic, apoptosis, lung

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CHAPTER 1: Introduction

Lung Development

The vertebrate lung is generated via complex branching programs and the derivation of specialized cell types programmed by tissue specific transcription factors (Maeda, Davé, & Whitsett, 2007). The initial budding of the mouse lung commences near embryonic day (E) 9-9.5 as the endoderm experiences a ventral diverticulum into the surrounding splanchnic mesoderm under the control of thyroid transcription factor-1 (TTF-1) signaling (Maeda et al., 2007), (Serls, Doherty, Parvatiyar, Wells, & Deutsch, 2005). The primordial lung buds branch to form the primary tubules that will dictate proximal conducting airway structures (Maeda et al., 2007). Factors secreted from the splanchnic mesenchyme such as FGF10 direct proper growth and branching of newly formed lung buds (Min et al., 1998) prior to extensive branching and budding that form the bronchial and bronchiolar tubes during the pseudoglandular phase of lung development (E11.5-E16). It is at this period that specialized cell subsets begin to emerge. Alveologenesis progresses through the canalicular (E16.5 to E17.5) and saccular (E17.5 through post natal (PN) 4) stages of lung development by way of dilations of the terminal acinar tubules. Lastly, extensive mesenchymal thinning and angiogenesis occur at post natal day (PN) 4 until PN20 in order to permit the most efficient gas exchange (Figure 1.1).

Critical aspects of pulmonary organogenesis are specific signaling pathways and transcription factor expression patters that require precise regulation and fidelity. In addition to Wnt and FGF signaling, sonic hedgehog (SHH), bone morphogenetic protein 4 (BMP4), forkhead box (FOX) proteins and vascular endothelial growth factors (VEGFs) greatly influence cell proliferation, migration and differentiation (Figure 1.2) (Bellusci, Furuta, et al., 1997;

Bellusci, Grindley, Emoto, Itoh, & Hogan, 1997; Bellusci, Henderson, Winnier, Oikawa, & Hogan, 1996; Schmidt, Yan, Yan, & Stern, 2001; Shiratori et al., 1996; Shu, Jiang, Lu, & Morrisey, 2002; Warburton et al., 2000; Weaver, Dunn, & Hogan, 2000; Wert, Dey, Blair, Kimura, & Whitsett, 2002). Significant alterations in lung formation occur as a result of pathway and transcription factor misregulation (Maeda et al., 2007).

Receptor for Advanced Glycation End-Products (RAGE)

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobin superfamily of cell surface receptors. The receptor contains a V-region-like domain crucial for ligand binding and two C-region like domains, a single-pass hydrophobic transmembrane domain and a short, 43 amino acid, highly charged cytoplasmic domain essential for intracellular signaling (Figure 1.3) (Buckley & Ehrhardt, 2010; Warburton et al., 2005). RAGE is a dynamic receptor capable of binding ligands of various types, being able to distinguish tertiary structures (Morbini et al., 2006). RAGE was initially characterized and named for its ability to bind to non-enzymatically glycoxidized macromolecules, advanced glycation end-products (AGEs). However, RAGE also binds other molecules including pro-inflammatory cytokine-like mediators of the S100/calgranulin family (S100A12 and S100B), amyloid β-fibrils, high mobility group box 1 (HMGB1), Mac-1 (Buckley & Ehrhardt, 2010; Schmidt et al., 2001) and specific DNA and RNA structures (Sims, Rowe, Rietdijk, Herbst, & Coyle, 2010).

Because RAGE binds a handful of ligands it is thus linked to several divergent signaling pathways. These include PI3/Akt (Toure et al., 2008), RhoGTPases (Hudson et al., 2008), Jak/STAT (J. Y. Kim et al., 2008), and Src family kinases (Reddy et al., 2006). Of notable

interest are two pathways stimulated by RAGE activation that are in response to either damage-associated molecular patterns (DAMPs) via NFκB pathways (Bianchi, Giambanco, & Donato, 2010) or tobacco smoke induced pulmonary inflammation that utilizes the Ras pathway (Reynolds, Kasteler, Schmitt, & Hoidal, 2011). Key DAMP molecules are those of the S100/calgranulin family and HMGB1, both of which bind to RAGE and other receptors including toll-like receptor 4 (TLR4) (Halayko & Ghavami, 2009; Sims et al., 2010). These molecular ligands, normally secreted post apoptotic or necrotic events, can serve as trophic factors in low concentrations or enhance the inflammatory/cell death response in high concentrations. Downstream gene products produced through RAGE signaling have included NFκB, Cox-2, IL-1β, and TNF-α (Bianchi et al., 2010). Because the RAGE gene contains NFκB binding sites in its promoter (Li & Schmidt, 1997) and is regulated by Egr-1 in cases of tobacco smoke-related disease (Reynolds, Cosio, & Hoidal, 2006), a possible auto-inflammatory loop may be triggered suggesting RAGE involvement in chronic disease states.

RAGE Isoforms

In addition to the full length RAGE isoform, other variants exist as a byproduct of variable mRNA splicing. These isoforms include dominant negative RAGE (dn-RAGE), a membrane anchored splice variant of RAGE capable of ligand binding but lacking the intracellular domain necessary for signal transduction. RAGE has also been shown to exist in a soluble secreted form sRAGE. The sRAGE isoform yields the same V and C-regions of the full length-RAGE but lacks both the hydrophobic transmembrane and the intracellular domains (Buckley & Ehrhardt, 2010). The altered variants of RAGE are thought to bind and sequester ligands without the consequences of activating signal transduction, thereby inhibiting transcription of gene products proposed as inflammatory targets in various tissues.

Roles of RAGE in Development

RAGE has been detected in many cell types including endothelium, smooth muscle, macrophages and epithelium, however it is most abundant in the lung (Brett et al., 1993) limiting its expression to alveolar type (AT) II cells and to the basolateral membranes of ATI differentiated cells (Schmidt et al., 2001). RAGE is normally observed at low levels in non-pulmonary adult tissues throughout the body and up-regulated in cases of injury and disease. However, RAGE expression is found at high quantities during lung neonatal development and in adult pulmonary tissue suggesting possible contributions to lung organogenesis and homeostasis. Meneghini, Francese, Carraro, and Grilli (2010) showed an additional interesting embryonic role for RAGE when RAGE ligands including HMGB1 and S100B functioned to promote proliferation and neuronal differentiation upon binding and subsequent activation. Numerous studies have also shown RAGE activation and downstream gene activation in tumor progression, further suggesting ties to proliferation (Arumugam, Simeone, Schmidt, & Logsdon, 2004; V. Kim, Rogers, & Criner, 2008; Taguchi et al., 2000). In addition to fostering proliferation, RAGE has been shown to enhance adherence of epithelial cells to collagen coated surfaces and dynamically influence cell spreading, critical behaviors required for the developing alveolus wherein cuboidal ATII cells transition to become squamous ATI cell necessary for efficient gas exchange (Demling et al., 2006). Despite a growing collection of evidence for embryonic functions, there remains little support for actual roles of RAGE in murine lung development.

RAGE-Mediated Pathology

Aside from developmental aspects, RAGE has been linked to numerous pathological states including diabetes (Bohlender, Franke, Stein, & Wolf, 2005), atherosclerosis, sepsis, rheumatoid arthritis and Alzheimer's disease (Sims et al., 2010). Concentrations of RAGE have

found to be up-regulated in the disease states of diabetes (Bohlender et al., 2005), lung fibrosis (He et al., 2007), acute respiratory distress syndrome (ARDS) (Nakamura et al., 2011), polycystic kidney disease (Park, Seo, & Park, 2010) and chronic obstructive pulmonary disease (COPD) (Ferhani et al., 2010; Wu, Ma, Nicholson, & Black, 2011) among many others. Since its identification in response to high AGE concentrations observed in diabetic states, numerous studies have also shown a relationship between inflammation and RAGE (Morbini et al., 2006; Sparvero et al., 2009). Specifically the receptor was found to up-regulate various pro-inflammatory cytokines including IL-6 (Schmidt et al., 1994) TNF-α (Yan et al., 1996), IL-1β (Hofmann et al., 1999) MCP-1 (Reynolds, Kasteler, et al., 2011), IL-8 (Reynolds, Kasteler, et al., 2011), INF-α (Ruan et al., 2010) and other molecules such as matrix metalloproteinase 9 (MMP-9) (Zhang et al., 2011). RAGE has also been shown to promote endothelial progenitor cell apoptosis through oxidant cell stress (Chen et al., 2010).

Destabilization of Respiratory Epithelium by RAGE Over-expression

Previous experiments performed in the Reynolds lab have shown that tobacco smoke increases RAGE expression and the expression of many of its known ligands. We have also demonstrated that transgenic up-regulation of RAGE in the adult lung recapitulates aspects of COPD, a smoke-induced disease with significant healthcare impact (M. P. Stogsdill et al., 2013). Specifically, RAGE transgenic mice have emphysema and elevated pro-inflammatory signaling. Because our data show that RAGE upregulation is sufficient to induce smoke-related abnormalities, I decided to assess the effects of RAGE abrogation in the context of maternal transfer of smoke particulates to the developing conceptus. Preliminary hypotheses in relation to RAGE inhibition in the developing lung include protection from abnormal branching

morphogenesis, cellular differentiation delays, impaired angiogenesis, and remodeling stemming from uncontrolled cellular destabilization and apoptosis.

Figures

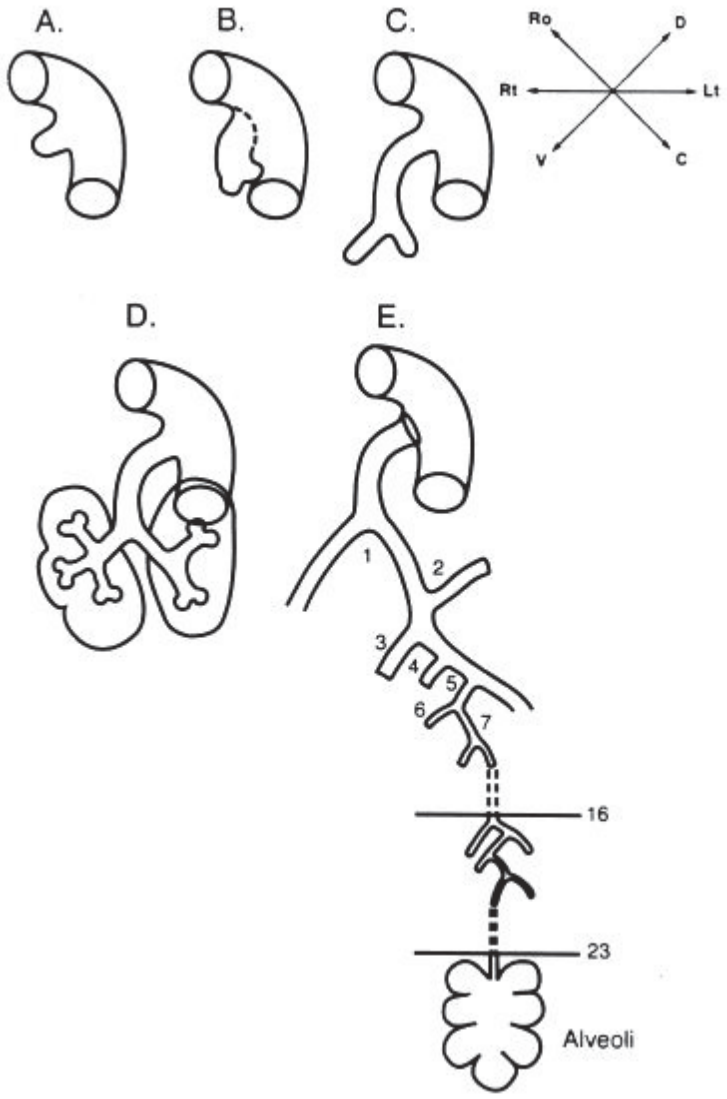

Figure 1.1: Key Morphogenic Events in Human and Mouse Lung
The primitive endoderm evaginates into the splanchnic mesenchyme to form the initial lung bud (A). Through reciprocal signaling between the splanchnic mesenchyme and newly formed lung outgrowths, extensive elongation and branching occur, thereby forming the primary bronchi (B and D). Further generational branching and cellular differentiation occur until well-structured alveoli are present in the distal lung (E). Image regenerated from Warburton et al. (2000).

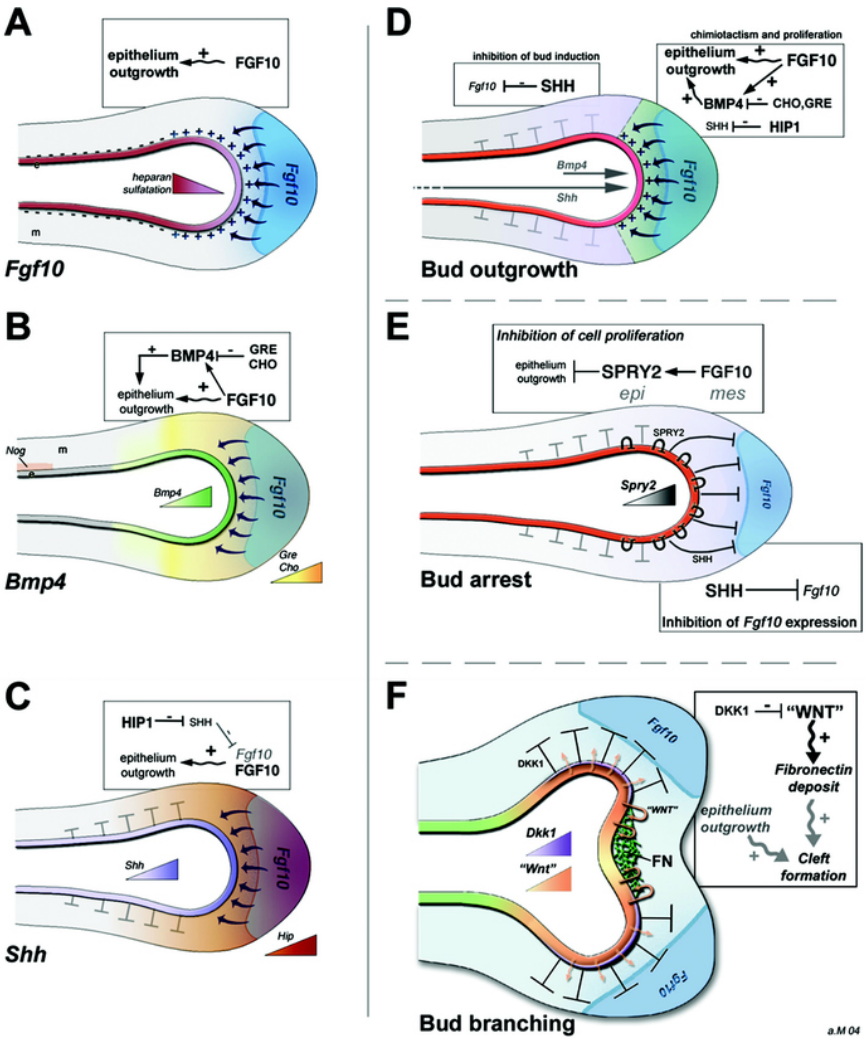

Figure 1.2: Functional Integration of Key Growth Factor Signaling Pathways

The functional integration of key growth factor signaling pathways in lung bud outgrowth, bud arrest, and bud branching is shown. Panel A depicts the function of FGF10 in stimulating bud outgrowth. Panel B depicts the function of BMP4 in stimulating lung branch tip outgrowth together with FGF10. Panel C depicts the functional interaction of SHH and Hip with FGF10. Panel D superimposes the functional integration of Fgf10, Bmp4, and Shh to mediate the delicate balance between chemotaxis and proliferation leading to bud induction versus inhibition of bud outgrowth. Panel E depicts the events that may determine interbranch length by leading to arrest of bud outgrowth. FGF10 induces SPRY2, which in turn inhibits epithelial outgrowth. Panel F depicts a potential mechanism for bud tip splitting in which WNT signaling drives Fibronectin (FN) deposition between the branch tips, leading to epithelial cleft formation. Image adapted Warburton et al. (2005).

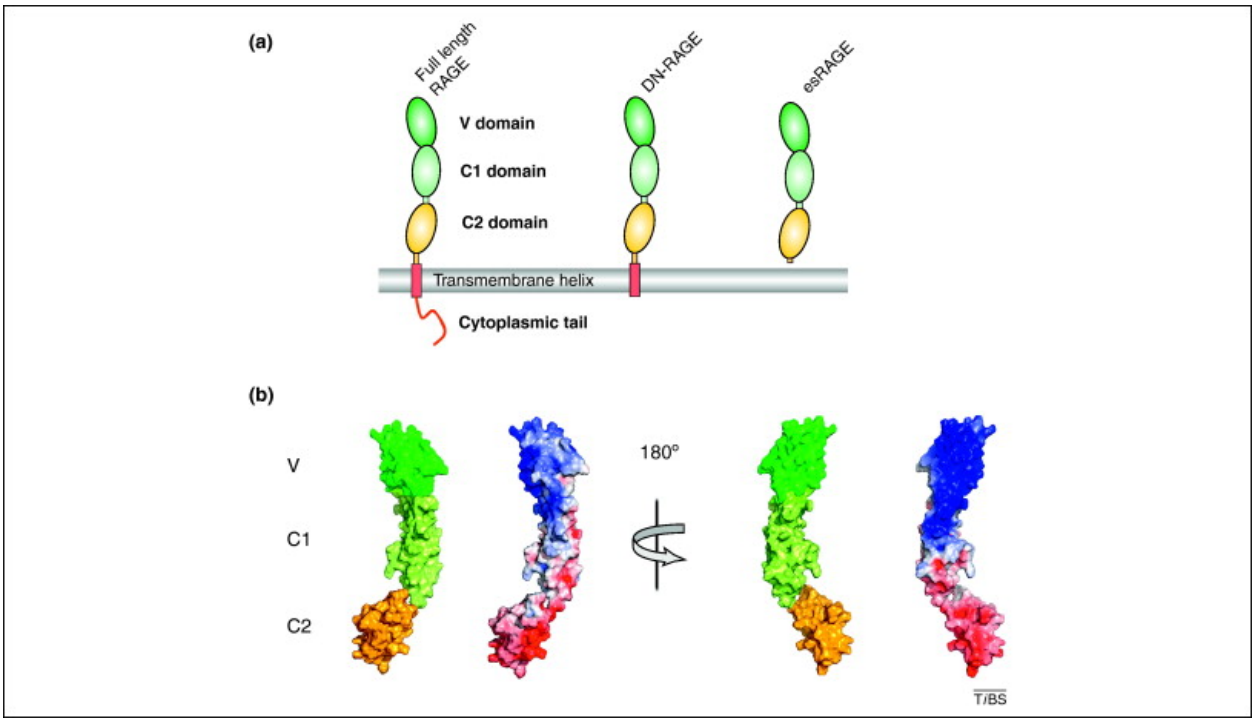

Figure 1.3: Structure of RAGE

A cartoon image showing full-length, dominant negative (DN-RAGE) and esRAGE, with highlighted extracellular (V, C1 and C2), transmembrane and cytoplasmic domains of RAGE (A). The surface representation model of RAGE is depicted (B). The blue, white and red image shows the charge distribution of amino acids within the ectodomain of RAGE. Blue areas represent localized positive charge, whereas red areas represent negatively charged regions. Image regenerated from Fritz (2011).

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CHAPTER 2: Antenatal Exposure of Maternal Secondhand Smoke (SHS) Increases Fetal Lung Expression of RAGE and Induces RAGE-Mediated Pulmonary Inflammation

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Abstract

Background: Receptors for advanced glycation end-products (RAGE) are immunoglobulin-like pattern recognition receptors abundantly localized to lung epithelium. Our research demonstrated that primary tobacco smoke exposure increases RAGE expression and that RAGE in part mediates pro-inflammatory signaling during exposure. However, the degree to which RAGE influences developing lungs when gestating mice are exposed to secondhand smoke (SHS) has not been determined to date.

Methods: Timed pregnant RAGE null and wild type control mice were exposed to 4 consecutive days of SHS from embryonic day (E)14.5 through E18.5 using a state of the art nose-only smoke exposure system (Scireq, Montreal, Canada). RAGE expression was assessed using immunofluorescence, immunoblotting, and quantitative RT-PCR. TUNEL immunostaining was performed to evaluate effects on cell turnover. Matrix abnormalities were discerned by quantifying collagen IV and MMP-9, a matrix metalloprotease capable of degrading basement membranes.

Results: Pulmonary RAGE expression was elevated in both dams exposed to SHS and in fetuses gestating within mothers exposed to SHS. Fetal weight, a measure of organismal health, was decreased in SHS-exposed pups, but unchanged in SHS-exposed RAGE null mice. TUNEL assessments suggested a shift toward pulmonary cell apoptosis. Matrix in SHS-exposed pups was diminished as revealed by diminished collagen IV and increased MMP-9 expression.

Conclusions: RAGE augmentation in developing pups exposed to maternal SHS weakens basement membranes and compromises pulmonary integrity.

Key words: RAGE, tobacco, lung, collagen

Running Title: Maternal smoke exposure compromises the developing fetal lung

Introduction

Lung development involves precisely programmed events wherein communication between endoderm and the surrounding mesoderm coordinates cell commitment and differentiation (Ten Have-Opbroek, 1991). As development concludes, a vast surface area of respiratory epithelium is positioned opposite a dynamic basement membrane through which gases pass to and from a considerable vascular network. An environment conducive to the appropriate spatial and temporal expression of target genes makes the coordination of specific gene programs possible. Such programs result in the deposition of respiratory tissues critically necessary for terrestrial life.

The receptor for advanced glycation end products (RAGE) is a cell-surface membrane protein of the immunoglobulin superfamily composed of three domains: an extracellular ligand binding domain, a domain necessary for membrane docking, and a cytosolic domain essential in the perpetuation of intracellular signaling events (Buckley & Ehrhardt, 2010). RAGE is expressed in several organs, but basal expression is primarily observed in the lung (Reynolds et al., 2008). In fact, most other organs known to highly express RAGE and its signaling intermediates are those in a diseased state (Ott et al., 2014). While its role in development is less understood, RAGE may function in discrete ways during the programming of squamous epithelium that must spread and appropriately adhere to matrix substrates (Demling et al., 2006). For example, RAGE is identified to the baso-lateral membrane of alveolar epithelial cells and localization in this domain enhances the binding of these epithelial cells to collagen in the matrix (Demling et al., 2006). Combined with its role in the establishment of organ architecture, RAGE may also influence organogenesis via its involvement in apoptotic pathways intricately associated with defining cell populations in the mature alveolus (J. A. Stogsdill et al., 2012).

While RAGE expression during lung organogenesis may assist in defining the respiratory compartment, its participation in lung inflammatory signaling may further explain developmental abnormalities. RAGE binds advanced glycation end-products (AGEs) during the orchestration of inflammation and AGEs are commonly detected in tobacco smoke (Yamagishi, Matsui, & Nakamura, 2008); however, the impact of elevated receptor availability during embryogenesis has not been clearly tested. Additional RAGE ligands including cytokine-like mediators of the S100/calgranulin family and high mobility group box 1 (HMGB-1) (Buckley & Ehrhardt, 2010; Taguchi et al., 2000) further implicate downstream signaling pathways potentially involved in mechanisms of abnormal lung derivation. Examples of deleterious effectors to cell turnover and differentiation include Ras and NF-kB (Bianchi et al., 2010; Reynolds, Kasteler, et al., 2011), two factors we've discovered to be RAGE targets. Because these signaling molecules increase in cases of elevated apoptosis and matrix resorption, RAGE may initiate a signaling axis wherein embryonic tissue loss and irreversible parenchymal remodeling occur.

Tobacco smoking and exposure to secondhand smoke (SHS) are widely accepted to be causative factors for childhood asthma and chronic obstructive pulmonary disease (COPD) affecting nearly 3 billion people worldwide (Rycroft, Heyes, Lanza, & Becker, 2012). Seminal research by Tager *et al.* initially showed that SHS affected fetal lung development in a landmark study of the effects of smoke exposure on neonatal pulmonary function (Tager et al., 1993). Subsequent reasoning led to the concept that antenatal factors could affect normal lung development and that chronic diseases have their origins *in utero*. Short and long term effects of fetal exposure to maternal smoking during gestation results in hypoplastic lungs with fewer air saccules coincident with bronchopulmonary dysplasia (BPD) persistently reduced pulmonary function, and increased incidence and lifelong pulmonary disease. In fact, significant suppression

of alveolarization in severe cases of SHS exposure causes neonatal lethality. It is important to emphasize that the main effects of *in utero* SHS exposure on lung growth and differentiation are likely the result of specific alterations in late stages of fetal lung development.

In the current study, the expression dynamics of RAGE was evaluated in the context of SHS exposure and RAGE availability. The current research suggests that RAGE signaling causes deterioration of the alveolar basement membrane through MMP-9 mediated collagen IV destruction, and that RAGE-mediated inflammation observed during SHS exposure may influence the trajectory of pulmonary morphogenesis.

Methods

Animals and SHS Exposure

All mice were in a C57Bl/6 background. Time mated mice were obtained and embryonic (E) day 0 was noted as the day a vaginal plug was discovered. Pregnant mice were exposed to secondhand smoke (SHS) commencing on E14.5 and the fourth consecutive day of SHS was E17.5. Dams were then sacrificed on E18.5, pups were weighed, and lungs were resected for histology, or molecular characterization. Mice were housed in a conventional animal facility supplied with pelleted food and water *ad libitum* and maintained on a 12-hour light–dark cycle. During exposure, mice were placed in soft restraints and connected to the exposure tower. Animals were exposed to SHS generated by six standard research cigarettes (2R1, University of Kentucky, Lexington, KY) through their noses using a nose-only exposure system (InExpose System, Scireq, Canada). A computer-controlled puff was generated every minute and mice were exposed to 10 minutes of secondhand smoke with a ten minute room-air exposure. This was repeated three times. The SHS-exposed group inhaled SHS from six consecutive cigarettes per day for four days. The SHS challenge chosen in the present study was associated with a good

tolerance of mice to the SHS sessions, and an acceptable level of particulate density concentration according to literature (Rinaldi et al., 2012; Vlahos et al., 2010). Control animals were restrained similarly and were exposed to filtered room air for the same duration. Animal use was in accordance with IACUC protocols approved by Brigham Young University.

Lung Morphology and Immunohistochemistry

Lungs were fixed in 4% paraformaldehyde, embedded in paraffin, and 5 μm sections were obtained. Sections were dehydrated, deparaffinized, and antigen retrieval was performed using the citrate buffer method (Reynolds, Mucenski, Le Cras, Nichols, & Whitsett, 2004; Reynolds, Mucenski, & Whitsett, 2003). RAGE immunofluorescence was completed using goat polyclonal IgG (AF1145, 1:500, R&D Systems, Minneapolis, MN). Sections were blocked in 5% donkey serum in PBS for 2 hours at room temperature, followed by incubation with primary antibodies at 4°C overnight. Control sections were incubated in blocking serum alone. After overnight incubation, all sections (including the controls) were washed using PBS/triton prior to the application of Alexa Fluor® 488 Rabbit Anti-Goat IgG (Invitrogen, Carlsbad, CA) secondary antibodies for 1 hour at room temperature. For immunohistochemistry, slides were blocked, incubated with primary and appropriate secondary antibodies that utilize HRP conjugation with the Vector Elite Kit (Vector Laboratories; Burlingame, CA). Antibodies for collagen IV (1:500, Abcam, Cambridge, MA, ab6586), and MMP-9 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, sc-6840) were used in the experiments. No staining was observed in sections without primary or secondary antibody.

Immunoblotting

Lungs from E18.5 mouse embryos were homogenized in RIPA buffer with protease inhibitors (Thermo Fisher). BCA quantification was performed to ensure equal sample loading (Thermo Fisher). Immunoblotting was performed using antibodies against RAGE (AF1145), collagen IV (Abcam, 1:5,000, ab6586) and MMP-9 (Santa Cruz, 1:500, sc-6840) using standard protocols discussed in previous work (Reynolds, Kasteler, et al., 2011; Reynolds, Schmitt, et al., 2010). Goat anti-rabbit (Vector Labs, Burlingame, CA, PI-1000 secondary antibody concentration was 1:10,000 for collagen IV and 1:5,000 for all other blots. Band densities were assessed using UN-SCAN-IT software (Silk Scientific, Orem, UT).

qRT-PCR

Quantitative Real-Time PCR was performed using total RNA from lungs of E18.5 mice, and was performed as previously described [15]. After isolation, total RNA was converted to cDNA, and qRT-PCR was performed using primers specific for *Rage* (5′- ACT ACC GAG TCC GAG TCT ACC -3’ and 5′- GTA GCT TCC CTC AGA CAC ACA -3′) and *GAPDH* (5'- TAT GTC GTG GAG TCT ACT GGT -3' and 5'- GAG TTG TCA TAT TTC TCG TGG -3') synthesized and HPLC purified by Invitrogen Life Technologies (Grand Island, NY).

Statistical Analysis

Results are presented as the means ± S.D. of six replicate pools per group. Means were assessed by one and two-way analysis of variance (ANOVA). When ANOVA indicated significant differences, student t tests were used with Bonferroni correction for multiple comparisons. Results are representative and those with p values <0.05 were considered significant.

Results

Secondhand Smoke (SHS) Exposure During Embryogenesis Induces RAGE Expression

Pregnant dams were nasally exposed to SHS during the last four consecutive days of gestation. Compared to basal pulmonary RAGE expression (Figure 2.1A), immunofluorescence revealed that lungs in wild type pregnant dams respond to SHS exposure by increasing RAGE expression (Figure 2.1B). Confirmatory experiments that utilized immunoblotting were aimed at quantifying RAGE expression. Compared to room air exposed animals, lungs from pregnant wild type mice exposed to SHS markedly increased RAGE protein expression (Figure 2.1E) and densitometry of the bands suggested a significant increase (Figure 2.1F). RAGE expression was not detected in RAGE null mice following room air exposure (Figure 2.1C) or exposure to SHS (Figure 2.1D). Lastly, quantitative RT-PCR was performed using total RNA from whole lungs in order to correlate protein and mRNA expression levels. A significant increase in RAGE mRNA was observed in wild type animals exposed to SHS when compared to room air controls (Figure 2.1G).

Immunofluorescence and immunoblotting for RAGE were next repeated using fetal E18.5 lung samples obtained from pups derived from room air or SHS-exposed pregnant dams. As was the case in pregnant dams, wild type pups experienced a notable increase in RAGE localization following SHS (Figure 2.2B) when compared to room air controls (Figure 2.2A). Immunoblotting (Figure 2.2E) and densitometry of the resulting bands (Figure 2.2F) further confirmed a coordinated increase in RAGE expression as a product of SHS availability. Just as was observed in adults, RAGE was not detected in tissues obtained from RAGE KO animals (Figure 2.2C and D).

RAGE Abrogation Protected Against SHS-induced Fetal Weight Loss and Lung Apoptosis

Fetal weights were obtained due to the observation that SHS exposure correlated with smaller offspring. Average total fetal weights revealed that SHS-exposure of wild type dams resulted in a significant decrease (Figure 2.3). Averages also revealed that RAGE KO mice experienced no significant weight loss following SHS exposure when compared to room air exposed counterparts (Figure 2.3). Because of an established link between RAGE and apoptosis in the developing lung (M. P. Stogsdill et al., 2013), we next assessed apoptotic trends via immunostaining for TdT-FragEL DNA Fragmentation (TUNEL), a commonly accepted marker of cell death. Apoptosis was not detected in lungs from E18.5 mice whose mothers experienced room air throughout gestation (Figure 2.4A). TUNEL labeling revealed sporadic cells actively undergoing apoptosis in lungs obtained from SHS-exposed wild type mice (Figure 2.4B, arrows). While RAGE KO mice also manifested apoptotic cells following SHS exposure (Figure 2.4C), the frequency of TUNEL positive cells was detectibly diminished. Lastly, quantification of proliferating cell nuclear antigen (PCNA) revealed no measurable differences in the proliferation status of pulmonary cells, regardless of mouse background or SHS exposure (not shown).

RAGE Mediates Diminished Collagen and Elevated MMP-9 Following SHS Exposure

An immunohistochemical analysis of type IV collagen was completed on lungs from pups obtained from room air and SHS-exposed dams. The relative abundance of type IV collagen was sought due to it being a plentiful collagen subtype common in basement membranes. Staining for type IV collagen revealed a qualitative decrease in lungs from wild type mice exposed to SHS (Figure 2.5B) compared to room air exposed controls (Figure 2.5A). Lungs from SHS-exposed RAGE KO mice also appeared to have diminished type IV collagen abundance (Figure 2.5C). Quantification of total type IV collagen was obtained by

immunoblotting and densitometry revealed that SHS exposure correlated with decreased expression (Figure 2.5D and E). Interestingly, decreased type IV collagen synthesis following SHS exposure was partially blunted in pups from RAGE KO animals (Figure 2.5D and E).

In order to mechanistically identify molecules that potentially function in the targeting of collagen metabolism, an analysis of matrix metalloprotease 9 (MMP-9) was conducted. MMP-9 is a downstream effector of RAGE and it degrades a host of collagens, including type IV (Parks & Shapiro, 2000; M. P. Stogsdill et al., 2013). Immunostaining for MMP-9 revealed a marked increase in sporadic pulmonary cells obtained from E18.5 wild type pups following SHS (Figure 2.6B) when compared to room air exposed controls (Figure 2.6A). While MMP-9 positive cells were also observed in lung sections from SHS-exposed RAGE KO mice (Figure 2.6C), their frequency was reduced. Immunoblotting was next employed in order to quantitatively assess MMP-9 expression in total lung homogenates. Blotting (Figure 2.6D) and densitometry (Figure 2.6E) suggested profound up-regulation of MMP-9 in SHS-exposed wild type mice. However, the absence of RAGE, at least in part, protected SHS-exposed RAGE KO mice from the high MMP-9 levels observed in SHS-exposed wild type mice (Figure 2.6D and E).

Discussion and Conclusions

Effects of SHS on RAGE Expression and Fetal Weight

The current investigation sought to simultaneously elucidate the expression profiles of pulmonary RAGE in gestating dams and their developing fetuses following SHS exposure. To our knowledge, this is the first study that seeks to delineate the biology of RAGE in the context of SHS exposure. Nicotine and a host of other tobacco related entities are known to traverse the placental barrier (Vaglenova, Birru, Pandiella, & Breese, 2004) and lungs of such exposed

fetuses experience developmental anomalies including branching defects and cell differentiation delays (Sekhon et al., 1999). Our discovery that dams and pups both induce higher RAGE expression suggests potential RAGE-mediated coping mechanisms that clearly warrant future exhaustive studies. Unequivocal support for deleterious inflammation mediated by tobacco smoke-induced RAGE has been provided by our laboratory (P R Reynolds et al., 2006; Reynolds, Kasteler, et al., 2011; Robinson, Johnson, Bennion, & Reynolds, 2012; Robinson, Stogsdill, Lewis, Wood, & Reynolds, 2012). While the intent of the current publication was not to delineate the inflammatory effects of RAGE signaling during SHS exposure or the specific contributions of its participants, its augmentation alone necessitates concern. In fact, a higher level of concern is potentially suggested by this and related work due to the fact that the dams in the current study were exposed to SHS. Research performed by Seller and Bnait (1995) and several others was conducted on the premise that dams were exposed to primary smoking and second hand effects in pups included skeletal abnormalities and neural tube defects. Our research indicates that in addition to primary smokers that are unable or unwilling to quit, even SHS exposure of expecting adults is a concern for the developing fetus.

The observation that fetal weights were decreased following SHS exposure supports the previous work using primary smoking by Esposito, Horn, Greene, and Pisano (2008). A highly intriguing result of our studies was the effect RAGE inhibition had on SHS stimulation. Smaller gestational and term weights are associated with a host of perinatal complications including renal development and sensory processing (Gill, May-Benson, Teasdale, & Munsell, 2013; Zohdi et al., 2012). Because RAGE knock out pups exposed to SHS were not smaller at term, further inspection into potential mechanisms of protection would be highly informative. Such studies at

minimum should consider RAGE effects on placentation, nutrition, and relationships to maturation following early periods of organogenesis.

Effects of SHS and RAGE on Pulmonary Matrix Deposition

SHS-induced alterations in cell turnover, including instances of apoptosis, are complicated by compromised extracellular matrix. Cells necessary for the normal physiology of the lung such as endothelium, respiratory epithelium, and conducting airway epithelium require spatial integrity stabilized by collagen and other matrix molecules. A significant determinant of the architectural matrix between cells is type IV collagen synthesis and secretion (Akashi et al., 2001; Timpl, 1989). In fact, the importance of type IV collagen as a stabilizing molecule is confirmed in research centering on COPD (Atkinson & Senior, 2003) and other adult inflammatory diseases (Ohbayashi & Shimokata, 2005). Our qualitative and quantitative data revealing diminished type IV collagen identifies an important concept relating to SHS exposure. First of all, extracellular matrixes are targeted by smoke exposure and secondarily, RAGE expressed by epithelial cells plausibly functions to signal matrix secreting cells in the developing lung.

RAGE-ligand interactions initiate signaling via the activation of signaling intermediates prior to the activation of NF-κB (Robinson, Stogsdill, et al., 2012). The current research sought to determine to what extent MMP-9, a matrix metalloprotease (MMP) secreted by fibroblasts, alveolar macrophages, and epithelial cells, functions in SHS exposed pups. MMPs are endopeptidases that can destroy components of the extracellular matrix and MMP-9 is an NF-κB target (Soler-Cataluna et al., 2005) that specifically targets type IV collagen (Klein & Bischoff, 2011). Because of their destructive capabilities, MMPs are recognized as not only central players in instances of disease, but effectors during remodeling events observed during

development as well (Andrea Page-McCaw, Andrew J. Ewald, & Zena Werb, 2007). For example, MMPs are also considered potent mediators of normal lung morphogenesis that assist in the orchestration of definitive lung parenchyma (Buckley, Medina, Kasper, & Ehrhardt, 2011; Sipes et al., 2011).

Notably, MMP-9 has been directly implicated in the progression of bronchopulmonary dysplasia (BPD), a developmental lung anomaly characterized by inflammation, lack of alveolar septation, and abnormal pulmonary vascular development (Coalson, 2006). Our work builds upon these discoveries by identifying MMP-9 as a SHS target that likely effectuates end points via RAGE-mediated pathways.

In summary, RAGE expression and matrix destabilization are probable byproducts of pulmonary SHS exposure during embryogenesis. This study suggests that protection from damaging SHS-induced effects such as fetal weight decreases, matrix abundance, and MMP imbalances is possible when RAGE is inhibited. Despite notable advancements in SHS research provided by the current research, additional work is necessary that focuses on RAGE signaling during pulmonary branching morphogenesis and to what extent RAGE alone is capable of inducing SHS related lung phenotypes.

Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

DRW, DBB, BCB, and JSB were responsible for animal husbandry and smoking. DRW, GDR and MC performed immunohistochemistry and AJW and ZRJ assisted with immunoblotting. CMJ was responsible for the qRT-PCR experiments. PRR conceived of the study and with the assistance of DRW, supervised in its implementation, interpretation, and

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

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Figures

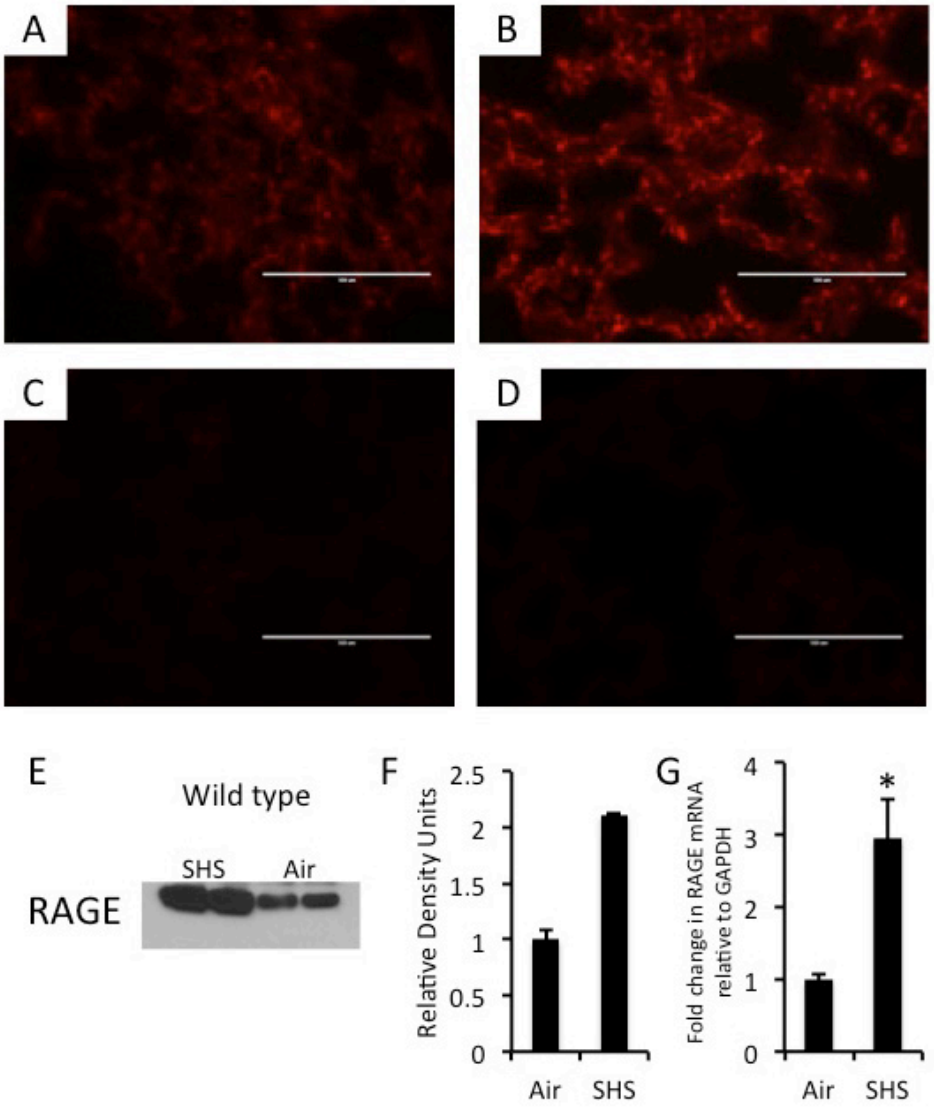

Figure 2.1: RAGE Expression in Adult Lung

Significant up-regulation of RAGE was observed by immunofluorescence using sections of adult mouse lung from wild type dams (A and B) and age-matched RAGE KO animals (C and D). Specifically, red RAGE immunofluorescence was increased in wild type lungs exposed to SHS (B) compared to room air controls (A) and no expression was detected in RAGE KO animals exposed to room air (C) or SHS (D). Images were at 100X original magnification. Immunoblotting using 10 µg of total lung lysates revealed marked up-regulation of RAGE in lungs exposed to SHS compared to room air controls (E). Densitometry of RAGE in the blots revealed an approximate 100% increase in protein expression (F). Quantitative RT-PCR revealed a significant up-regulation of RAGE mRNA.

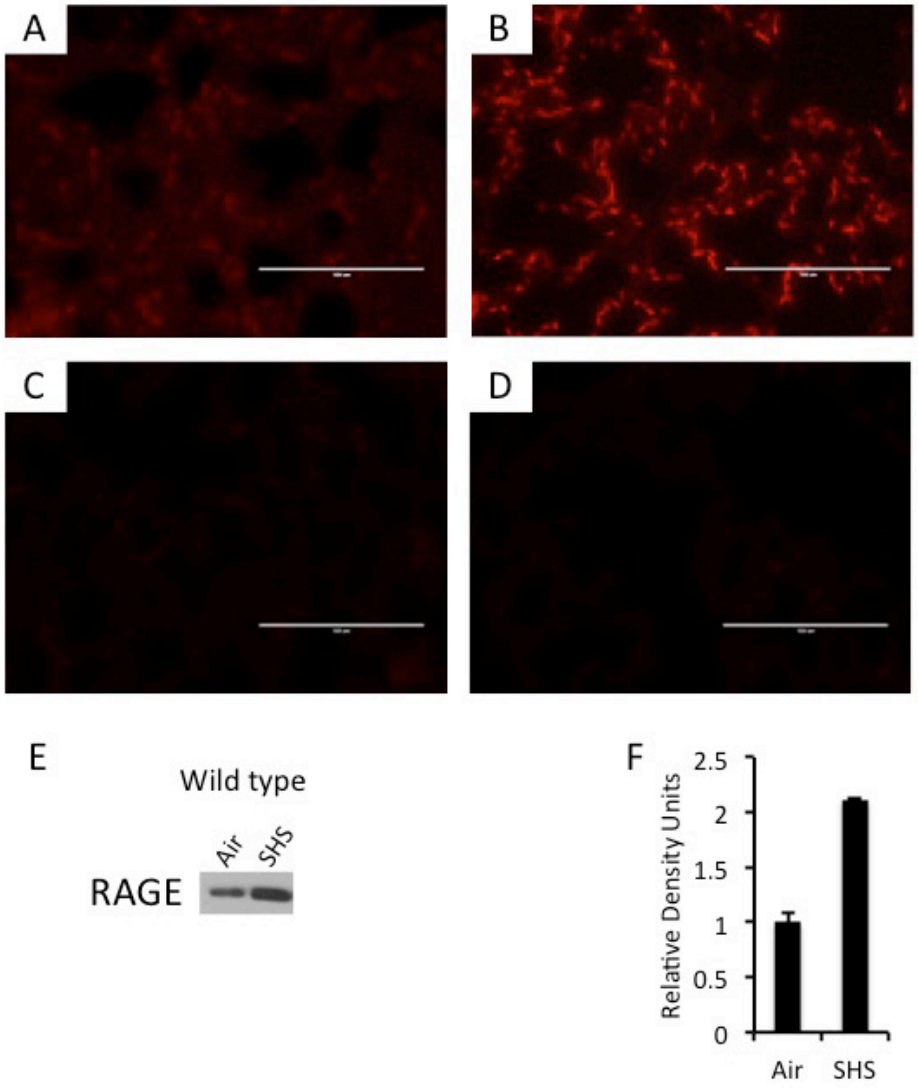

Figure 2.2: RAGE Expression in Fetal Lung

Significant up-regulation of RAGE was observed by immunofluorescence using sections of E18.5 mouse lungs in pups from time mated pregnant wild type dams (A and B) and age-matched RAGE KO animals (C and D). Specifically, red RAGE immunofluorescence was increased in wild type lungs exposed to SHS (B) compared to room air controls (A) and no expression was detected in RAGE KO animals exposed to room air (C) or SHS (D). Images were at 100X original magnification. Immunoblotting using 10 µg of total E18.5 lung lysates revealed marked up-regulation of RAGE in lungs exposed to SHS compared to room air controls (E). Densitometry of RAGE in the blots revealed an approximate 100% increase in protein expression (F).

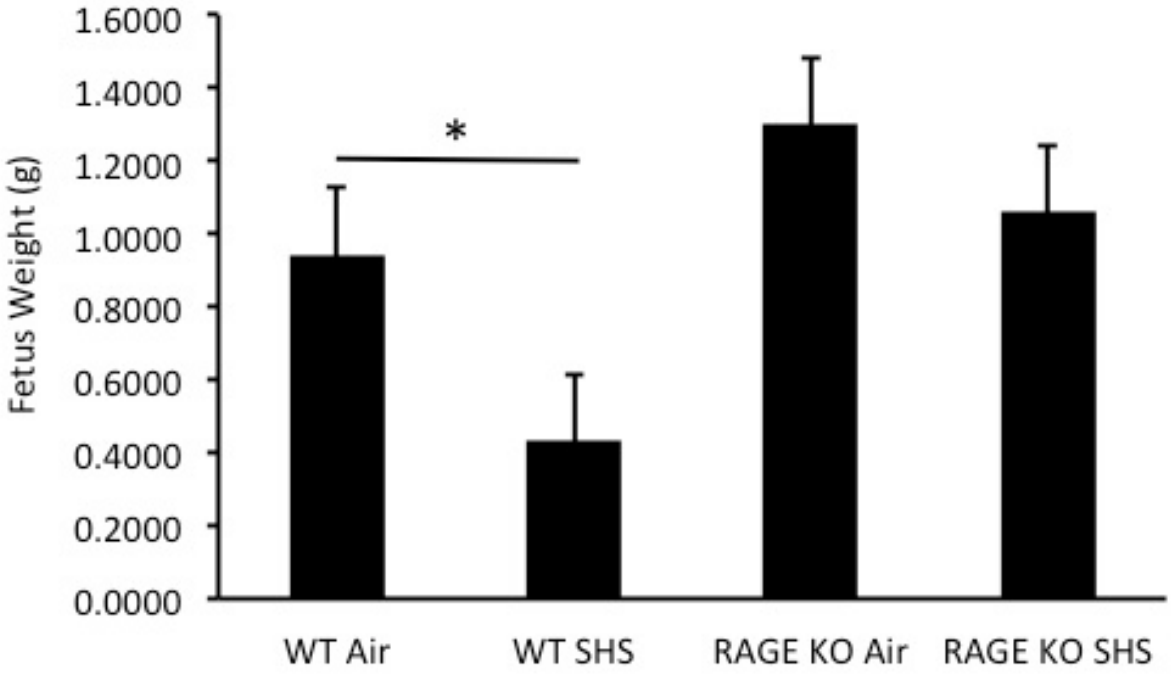

Figure 2.3: Fetal Weight Comparison between Room Air and SHS-exposed Pups
Pups from SHS exposed dams were noticeably smaller. Measurement revealed that SHS-exposed wild type pups were significantly decreased compared to age-matched pups from dams exposed to room air during gestation. SHS-exposed RAGE KO pups were not significantly decreased when compared to age-matched RAGE KO pups exposed to room air. *Statistical difference ($P \leq 0.05$) with at least three replicates per group.

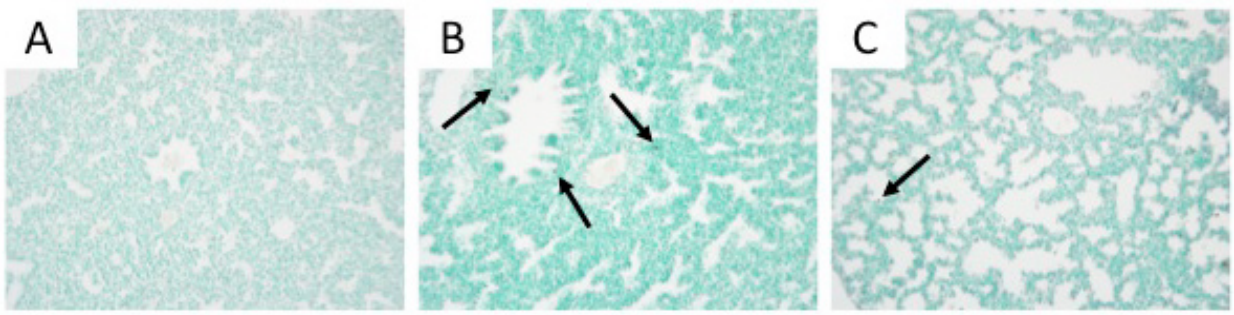

Figure 2.4: TUNEL Staining Showing Active Apoptosis

A detectable increase in the number of actively apoptosing cells by TUNEL staining was observed in wild type pups exposed to SHS (arrows, B) when compared to room air controls (A). There was no statistical difference in the occurrence of TUNEL-positive cells when room air wild type pups (A) and RAGE KO pups (not shown) were compared. Apoptotic cells were observed in RAGE KO pups exposed to SHS (C, arrow); however, the frequency was markedly decreased.

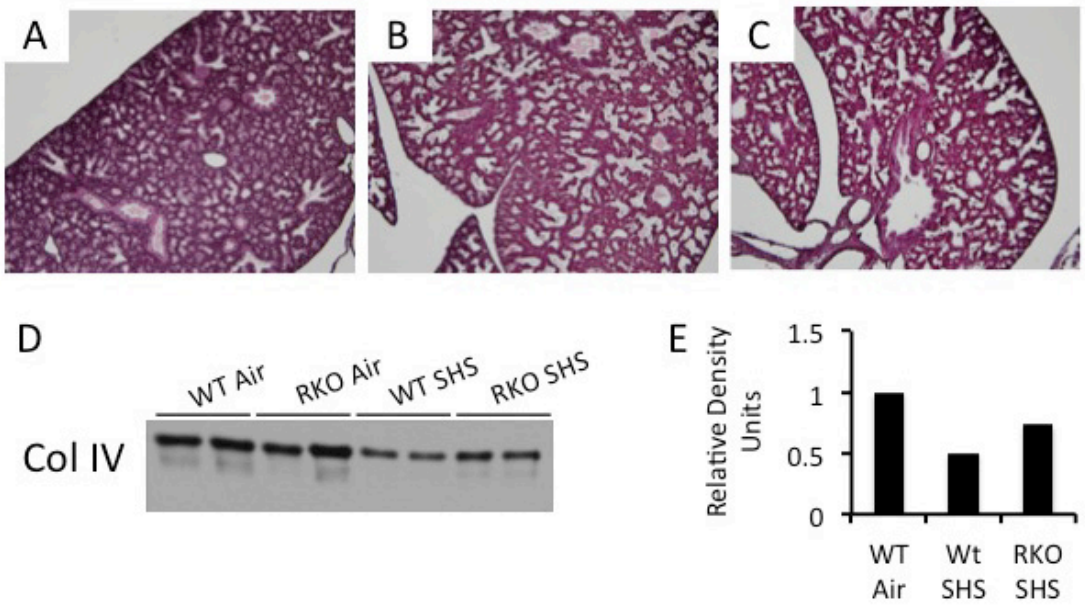

Figure 2.5: Collagen IV Changes Associated with Basement Membranes

Lungs from wild type pups exposed to room air (A) and SHS-exposed wild type (B) and RAGE KO lungs (C) were stained for type IV collagen to qualitatively determine relative changes in type IV collagen associated with basement membranes. Only subtly qualitative decreases in type IV collagen were observed in SHS exposed animals. Immunoblotting for type IV collagen using equal aliquots of 10 µg total lung protein revealed markedly decreased total type IV collagen expression in SHS-exposed pups (D). Immunoblotting (D) and densitometry of the resulting bands (E) demonstrated that SHS-induced decreases in type IV collagen expression in wild type animals is more severe when compared to SHS-exposed RAGE KO mice.

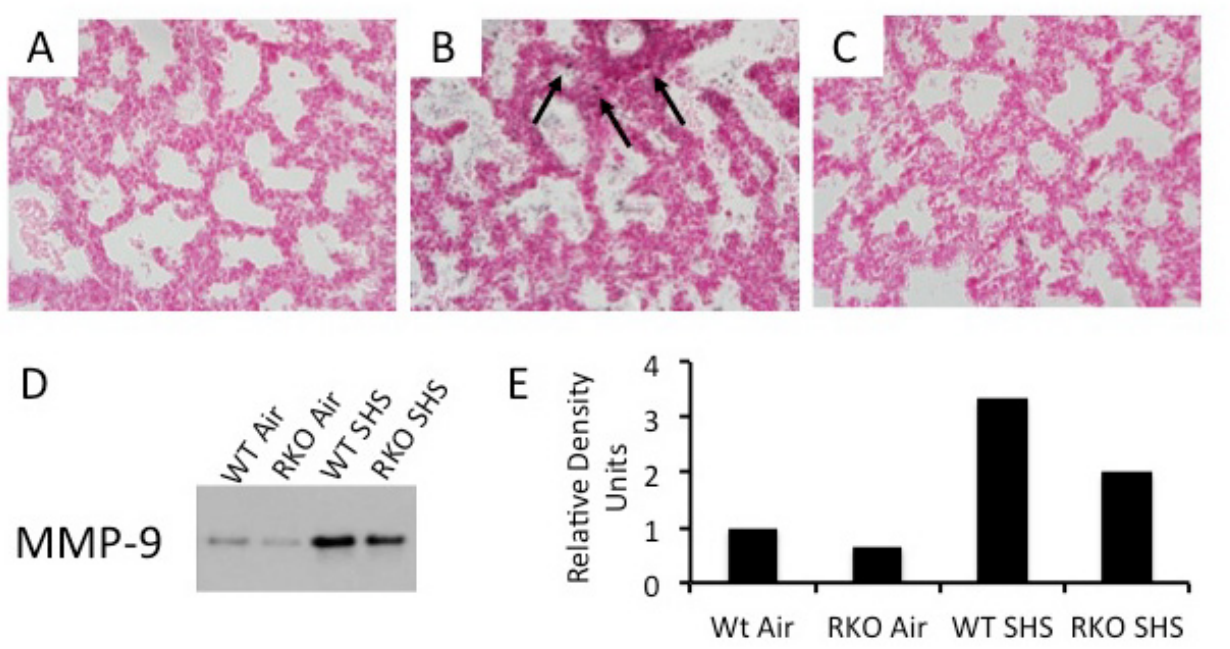

Figure 2.6: MMP-9 Increased Expression in SHS-exposed Pups

Lungs from wild type room air exposed (A) and SHS-exposed wild type (B) and RAKE KO mice (C) were qualitatively immunostained for MMP-9. SHS induced a detectibly higher number of MMP-9 positive cells (B, arrows) compared to SHS-exposed RAGE KO mice (C). Immunoblotting for MMP-9 and related densitometry (E) using standardized aliquots of 10 µg total lung protein revealed markedly increased MMP-9 expression in SHS-exposed wild type animals and a detectible measure of protection in SHS-exposed RAGE KO animals.

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CHAPTER 3: Conditional Over-Expression of RAGE by Embryonic Alveolar Epithelium Compromises the Respiratory Membrane and Impairs Endothelial Cell Differentiation

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Abstract

Background: Receptors for advanced glycation end-products (RAGE) are cell surface receptors prominently expressed by lung epithelium. Previous research demonstrated that over-expression of RAGE by murine alveolar epithelial cells during embryogenesis caused severe lung hypoplasia and neonatal lethality. However, the effects of RAGE over-expression on adjacent matrix and endothelial cells remained unknown.

Methods: RAGE transgenic (TG) mice were generated that conditionally over-expressed RAGE in alveolar type II cells when fed doxycycline (dox) from conception to E18.5. To evaluate effects on the basement membrane, immunostaining and immunoblotting were performed for collagen IV and MMP-9, a matrix metalloprotease capable of degrading basement membranes. To assess changes in vasculature, immunostaining, immunoblotting and qRT-PCR were performed for Pecam-1, a platelet endothelial cell adhesion marker also known as CD31. Lastly, to characterize potential regulatory mechanisms of endothelial cell differentiation, immunoblotting and qRT-PCR for FoxM1, a key endothelium-specific transcription factor of the Forkhead Box (Fox) family, were completed.

Results: Qualitative immunostaining for collagen IV was less in RAGE TG mice compared to controls and immunoblotting revealed decreased collagen IV in the RAGE TG mouse lung. Additionally, elevated MMP-9 detected via immunostaining and immunoblotting implicated MMP-9 as a possible downstream effector in matrix destabilization mediated by RAGE signaling. Lastly, Pecam-1 assessment revealed a decrease in the prevalence of microvascular endothelial cells coincident with FoxM1 abrogation in RAGE TG mice compared to controls.

Conclusions: RAGE over-expression by alveolar epithelium weakened the basement membrane and associated matrix via increased MMP-9 activity. Furthermore, over-expression of RAGE inhibited FoxM1, suggesting that anomalous transcriptional control contributes to decreased endothelial cell prevalence in the TG mouse lung.

Keywords: RAGE, transgenic, endothelium, lung, collagen

Introduction

Lung development results from reciprocal interactions between respiratory epithelium derived from the foregut endoderm and the surrounding splanchnic mesenchyme. A sequential series of developmental events leads to the formation of airways juxtaposed with vast capillary networks necessary for efficient gas exchange in the neonate (Ten Have-Opbroek, 1991). Each of these programmed events reveals that normal pulmonary morphogenesis depends on a very specific program of temporal and spatial control of cellular differentiation, migration, and proliferation.

The receptor for advanced glycation end products (RAGE) is a cell-surface membrane protein of the immunoglobulin superfamily composed of three domains: an extracellular ligand binding domain, a domain necessary for membrane docking, and a cytosolic domain essential in the perpetuation of intracellular signaling events (Buckley & Ehrhardt, 2010). RAGE is basally expressed in diverse locations throughout the body and it is most abundantly expressed by lung alveolar epithelium (Reynolds et al., 2008). While its role in development is less understood, RAGE has been implicated in cell spreading and adherence (Demling et al., 2006). Demling et al. specifically showed that RAGE is localized to the basolateral membrane of human alveolar epithelial cells, and that increased expression of RAGE in these cells leads to increased binding to collagen and a squamous cellular morphology (Demling et al., 2006). Another plausible role for RAGE in development may involve its ability to control apoptosis that refines epithelial cell quantity in definitive alveoli (J. A. Stogsdill et al., 2012).

While the precise control of RAGE expression during lung organogenesis likely assists in controlling lung- specific genetic programs, its participation in lung inflammatory signaling in the adult may explain developmental abnormalities when expression levels rise. RAGE is already widely known to bind advanced glycation end- products (AGEs) during the orchestration

of vascular inflammation related to hyperglycemia (Aronson, 2008); however, the impact of elevated receptor availability on alveolar vasculature during embryogenesis has not been clearly tested. In addition to AGEs, RAGE also binds cytokine-like mediators of the S100/calgranulin family, high mobility group box 1 (HMGB-1) and amyloid β-fibrils (Buckley & Ehrhardt, 2010; Taguchi et al., 2000). These ligands further implicate downstream signaling pathways potentially involved in mechanisms of abnormal lung derivation. For example, S100s and HMGB-1 lead to RAGE-mediated activation of Ras and NF-kB (Bianchi et al., 2010; Reynolds, Kasteler, et al., 2011). Because these signaling molecules increase in cases of elevated apoptosis and matrix resorption, RAGE may initiate a signaling axis wherein embryonic tissue loss and irreversible parenchymal remodeling occur.

Previous research showed that a conditional transgenic (TG) mouse model that enhances RAGE expression exhibited neonatal lethality due to severe pulmonary hypoplasia (Reynolds, Stogsdill, Stogsdill, & Heimann, 2011). In the current study, the effect of RAGE over-expression on the development of the alveolar basement membrane and adjacent vasculature were analyzed. Electron microscopy of TG lungs identified notable separation of ATI cells from fragmented basement membranes. The current research suggests that RAGE over-expression causes deterioration of the alveolar basement membrane through MMP-9 mediated collagen IV destruction, and that RAGE over-expression diminishes vascularization during organogenesis, possibly via inhibition of FoxM1.

Methods

Animals

All mice were in a C57Bl/6 background. RAGE Transgenic (TG) mice that overexpress RAGE were created by mating SP-C-rtTA and TetO-RAGE single transgenic mice and transgene expression was induced by feeding dams doxycycline (dox) from before conception until sacrifice date at E18.5 (Reynolds, Stogsdill, et al., 2011). Animal use was in accordance with IACUC protocols approved by Brigham Young University.

Lung Morphology and Immunohistochemistry

Lungs were fixed in 4% paraformaldehyde, embedded in paraffin, and 5 μm sections were obtained. Standard H&E staining was performed and immunohistochemistry was conducted using standard procedures previously elaborated (Reynolds, Allison, & Willnauer, 2010). Sections were dehydrated, deparaffinized, and antigen retrieval was performed using the citrate buffer method (Reynolds et al., 2004; Reynolds et al., 2003). Slides were blocked, incubated with primary and appropriate secondary antibodies that utilize HRP conjugation with the Vector Elite Kit (Vector Laboratories; Burlingame, CA). Antibodies for the following were used: Pecam-1 (1:500, BD Pharmingen, San Jose, CA, #553370), collagen IV (1:500, Abcam, Cambridge, MA, ab6586), and MMP-9 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, sc-6840). No staining was observed in sections without primary or secondary antibody. Picro-sirius red staining was performed to assess general collagen (Direct Red 80, Sigma-Aldrich, Saint Louis, MO, #365548).

Electron Microscopy

Whole mouse lungs were excised and fixed overnight in a 2% glutaraldehyde/0.06 M sodium cacodylate solution (pH 7.3), rinsed in sodium cacodylate and placed in 1% osmium tetroxide/sodium cacodylate, then uranyl acetate overnight. Tissues were dehydrated in a graded acetone series and embedded in Spurr's resin. 80 nm sections were obtained using a RMC MT-X Ultra Ultra-microtome (Tucson, AZ) and stained using Reynolds' lead citrate. Sections were photographed using a FEI Tecnai T12 electron microscope.

Immunoblotting

Lungs from E18.5 mouse embryos were homogenized in RIPA buffer with protease inhibitors (Thermo Fisher). BCA quantification was performed to ensure equal sample loading (Thermo Fisher). Immunoblotting was performed using antibodies against Pecam-1 (Santa Cruz, 1:1,000, sc-376764), FoxM1, (Santa Cruz, 1:1,000, sc-271746), collagen IV (Abcam, 1:5,000, ab6586) and MMP-9 (Santa Cruz, 1:500, sc-6840) using standard protocols discussed in previous work (Reynolds, Kasteler, et al., 2011; Reynolds, Schmitt, et al., 2010). Goat anti-rabbit (Vector Labs, Burlingame, CA, PI-1000) secondary antibody concentration was 1:10,000 for collagen IV and 1:5,000 for all other blots. Band densities were assessed using UN-SCAN-IT software (Silk Scientific, Orem, UT).

qRT-PCR

Quantitative Real-Time PCR was performed using total RNA from lungs of E18.5 mice, and was performed as previously described (Robinson, Johnson, et al., 2012). After isolation, total RNA was converted to cDNA, and qRT-PCR was performed using primers specific for Pecam-1 (5′-CTC CAA CAG AGC CAG CAG TA-3′ and 5′-GAC CAC TCC AAT GAC AAC C A-3′), FoxM1 (5′-GCG ACT CTC GAG CAT GGA GAA TTG TCA CCT G-3′ and 5′-GCG

CTA CTC GAG TTC GGT TTT GAT GGT-3′), and GAPDH (5′-CAG GGC TGC TTT TAA CTC TGG-3′ and 5′-TGG GTG GAA TCA TAT TGG AAC A-3′) synthesized and HPLC purified by Invitrogen Life Technologies (Grand Island, NY).

Statistical Analysis

Results are presented as the means ± S.D. of six replicate pools per group. Means were assessed by one and two- way analysis of variance (ANOVA). When ANOVA indicated significant differences, student t tests were used with Bonferroni correction for multiple comparisons. Results are representative and those with p values <0.05 were considered significant.

Results

Embryonic Up-regulation of RAGE Destabilizes the Respiratory Membrane

Traditional staining with hematoxylin and eosin revealed a striking phenotype wherein the distal lung architecture in RAGE TG mice lacked sufficient parenchymal tissue (Figure 3.1A and B). Isolated areas of distal lung development were sporadically observed; however, vacuous regions that lacked matrix and associated cells were common. A closer inspection of RAGE TG lung tissues by electron microscopy identified diminished, poorly fused basement membranes that comprise the respiratory membrane (Figure 3.1C and D, arrows). Because of significant lung tissue loss and only diffuse basement membranes in the lungs of E18.5 RAGE TG mice following uninterrupted dox administration, our focus centered on basement membrane stability in the context of RAGE up-regulation.

Collagen is Diminished in RAGE Over-expressing Lungs

A general analysis of collagen was completed using a picro-sirius red stain, and the data revealed little qualitative difference when RAGE TG mice and controls were compared (Figure 3.2A and B). Immunohistochemistry was subsequently performed for collagen IV, a prolific collagen subtype common in basement membrane structure. Qualitatively, staining for collagen IV revealed a discernible decrease in lungs from RAGE TG mice (Figure 3.2D) compared to control lung sections (Figure 3.2C). In order to quantitatively assess collagen IV abundance, immunoblotting using equal concentrations of lung homogenates was performed and collagen IV was significantly diminished in RAGE TG lungs compared to controls (Figure 3.2E).

In order to decipher functional molecules in possible mechanisms of collagen metabolism, an analysis of matrix metalloprotease 9 (MMP-9) was conducted. MMP-9 has recently been identified as a molecule downstream of RAGE signaling in the lung (Parks & Shapiro, 2000; M. P. Stogsdill et al., 2013). Immunostaining for MMP-9 revealed a marked increase in cells from RAGE TG mouse lung that were actively synthesizing the protease (Figure 3.3A and B). While MMP-9 positive cells were observed in all lung sections, cell counts per high powered field (HPF) revealed a significant increase in the lungs of RAGE TG mice compared to controls (Figure 3.3C). In order to quantitatively assess MMP-9 expression in lung homogenates, immunoblotting for MMP-9 was performed and band densities from RAGE TG mouse lungs coincided with the significant elevation of MMP-9 positive cells (Figure 3.3D).

Foxm1 and Endothelial Cell Abundance is Decreased in RAGE Over-expressing Lungs

Because lungs from RAGE TG mice exposed to dox from conception to E18.5 experienced significant epithelial cell death (J. A. Stogsdill et al., 2012) and compromised basement membrane stability, endothelial cell biology was also considered due to its proximity.

It was hypothesized that abnormalities in alveolar epithelial cell turnover might occur in tandem with the contraction of endothelial cells that share the same weakened basement membrane. Immunostaining for Pecam-1, a common endothelial cell marker, revealed indistinguishable differences when comparing RAGE TG and control lungs (Figure 3.4A and B). However, quantitative real-time PCR using equal concentrations of mRNA from both groups revealed a significant decrease in the expression of Pecam-1 mRNA in RAGE TG mice (Figure 3.4C). The decrease in message was corroborated with immunoblotting for Pecam-1 in lung homogenates wherein protein quantification was significantly decreased in the lungs of RAGE TG mice compared to controls (Figure 3.4D).

Because transcription factors that orchestrate epithelial cell differentiation were already known to be impaired in RAGE TG mice (Reynolds, Stogsdill, et al., 2011), an interest in transcriptional regulation and the derivation of endothelial cells was cultivated. FoxM1 was identified based on its effects on cell cycle progression in mesenchymal cells and because FoxM1 null mice exhibit pulmonary microvascular abnormalities associated with diminished Pecam-1 and vascular endothelial growth factor (VEGF) expression (I. M. Kim et al., 2005). Real-time PCR analysis of FoxM1 revealed a nearly three-fold decrease in the expression of the transcription factor in the lungs of RAGE TG mice compared to controls (Figure 3.5A). Furthermore, when comparing control lung samples, immunoblotting identified a significant decrease in the abundance of FoxM1 protein in RAGE TG lungs (Figure 3.5B).

Discussion

Effects of RAGE Over-expression on Basement Membrane

The use of a conditional mouse model involving alveolar epithelium allowed for the assessment of RAGE over- expression on the respiratory membrane that is comprised of alveolar epithelium, endothelium, and a fused basement membrane. A sound respiratory membrane in advanced organisms is critical to terrestrial life and accordingly, its strength and permeability must be maintained. A significant determinant of the basement membrane's interposition between cells in the respiratory membrane is due to intricate collagen IV deposition (Nakano et al., 2001; Timpl, 1989). In fact, the importance of collagen IV as a chief component of the blood-gas barrier is confirmed in research that identifies impaired basement membranes in clinical conditions including COPD (Atkinson & Senior, 2003). Our qualitative and quantitative data revealing diminished collagen IV identifies an intriguing paradigm in which increased expression of pattern recognition receptors such as RAGE by epithelial cells can signal changes in the surrounding matrix.

RAGE-ligand interactions initiate signaling via the activation of signaling intermediates such as Ras, extra- cellular signal regulated kinase (ERK) 1/2, and c-Jun-NH2-terminal kinase (JNK) 1/2 prior to nuclear translocation of active NF-κB (Robinson, Stogsdill, et al., 2012). The current research sought to assess possible roles for MMP-9, a matrix metalloprotease (MMP) secreted by fibroblasts, alveolar macrophages, and epithelial cells through pathways culminating in NF-kB activation (Soler-Cataluna et al., 2005). MMPs comprise a family of 24 related endopeptidases that degrade a variety of substrates, including components of the extracellular matrix, and MMP-9 specifically targets collagen IV (Pardo & Selman, 2006). The destructive functions of MMPs are critical in tissue remodeling observed in development and disease

(Andrea Page-McCaw, Andrew J Ewald, & Zena Werb, 2007). Based on their multiple biological activities, MMPs are considered participants in diverse pathological processes in the lung (Rosas et al., 2008), but also as effectors during lung morphogenesis (Buckley et al., 2011; Lee et al., 2011). Notably, MMP-9 has been directly implicated in the progression of bronchopulmonary dysplasia (BPD), a developmental anomaly characterized by inflammation, lack of alveolar septation, and abnormal pulmonary vascular development (Coalson, 2003). We have shown that the over-expression of RAGE results in increased MMP-9 and decreased collagen IV. As recently demonstrated in adult RAGE TG mice (M. P. Stogsdill et al., 2013), this particular study confirms that MMP-9 is increased via a RAGE-mediated pathway during development as well. While some additional studies in abdominal aortic aneurisms and HaCaT keratinocytes describe MMP-9 induction via RAGE signaling, only in the current study was lung development considered (Zhang et al., 2009; Zhu et al., 2012). Our new evidence points to MMP-9-mediated remodeling in the developing lung under the control of RAGE signaling, a particularly important concept given the abundance of RAGE in the lung.

Effects of RAGE Over-expression on Endothelium

Over-expression of RAGE by alveolar epithelial cells also directly influenced endothelial cell behaviors in the lung. The lung mesenchyme undergoes vasculogenesis (de novo formation of vessels) and angiogenesis (branching of new vessels from preexisting ones) in precisely controlled pathways that require appropriate levels of VEGF and other regulatory molecules (Breier, Albrecht, Sterrer, & Risau, 1992; Risau, 1997). We demonstrated that RAGE over-expression led to decreased development of pulmonary microvasculature associated with diminished levels of Pecam-1 (I. M. Kim et al., 2005). Previous research revealed abnormal thyroid transcription factor-1 (TTF-1) expression in the lung of perinatal RAGE TG mice

(Reynolds, Stogsdill, et al., 2011). TTF-1 is a phosphorylated member of the Nkx2 family of homeodomain-containing transcription factors and TTF-1 phosphorylation mutants have deficits in lung vascularity due to decreased Pecam-1 and VEGF expression (DeFelice et al., 2003). Figure 3.6 demonstrates significantly decreased TTF-1 expression in the lungs of RAGE TG mice compared to controls during periods through E18.5. It is therefore likely that decreased staining for Pecam-1 relates to misregulation of TTF-1 among other important factors.

A central feature likely involved in endothelial cell derivation and the coordination of vessel formation in the RAGE TG mouse lung are the contributions of FoxM1. The Forkhead Box (Fox) proteins consist of extensive transcription factors homologous with the Winged Helix/Forkhead DNA binding factors (Clark, Halay, Lai, & Burley, 1993). Notably, Fox proteins FoxA2, FoxJ1, FoxF1, and FoxP each play critical roles in transcriptionally regulating branching morphogenesis and vasculogenesis in the developing lung (Brody, Yan, Wuerffel, Song, & Shapiro, 2000; Kalin et al., 2008; Wan et al., 2004). In particular, deletion of FoxM1 is lethal during the embryonic period and selective inhibition revealed it is important during pulmonary development where it activates genes involved in G1/S and G2/M progression through its interaction with Cdk-Cyclin complexes and p300/CBP co-activators (Costa, 2005). Pulmonary microvascular abnormalities associated with diminished levels of Pecam-1, transforming growth factor (TGF)-β receptor type II, ADAM-17, VEGF receptors, FLK-1, Aurora B kinase, LAMA4, and the FoxF1 transcription factor were observed in lungs of FoxM1 null mice (I. M. Kim et al., 2005). Conversely, conditional up-regulation of FoxM1 caused increased proliferation of alveolar and bronchial epithelial cells, as well as smooth muscle and endothelial cells via the activation of cell cycle promoting factors (Kalinichenko, Lim, Shin, & Costa, 2001). Because RAGE TG mice express less FoxM1, and VEGF is a direct transcriptional target of FoxM1

(Mirza et al., 2010), the data identify as a precursor to measurable lung destruction in smokers (Gordon et al., 2011). Accordingly, in addition to disease progression observed in mature lungs, this work further implicates RAGE signaling in pathways that regulate lung remodeling during embryogenesis.

Conclusion

In summary, matrix destabilization and FoxM1-mediated effects on respiratory membrane cells are probable determinants of the hypoplastic lung observed in perinatal RAGE TG mice. Our prior research that revealed a fas ligand- mediated shift toward apoptosis in the lung periphery (J. A. Stogsdill et al., 2012) and impaired ATII-ATI transition reveals that both abnormal apoptosis and loss of repair mechanisms mediated by FoxM1 likely result in impaired lung development. Even with these important advancements in the study of RAGE signaling during lung morphogenesis, additional research is still required that centers on precise signaling pathways involved in cell responses to RAGE up-regulation.

Competing Interests

The authors declare that they have no competing interests.

Authors Contributions

NTF, BRB, AJG, ABR, and JAS performed immunohistochemistry and AJW and ZRJ assisted with immunoblotting. NTF and ABR were responsible for the qRT-PCR experiments. PRR conceived of the study and with the assistance of DRW, supervised in its implementation, interpretation, and writing. All authors assisted in manuscript preparation and approved of the final submitted version.

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Figures

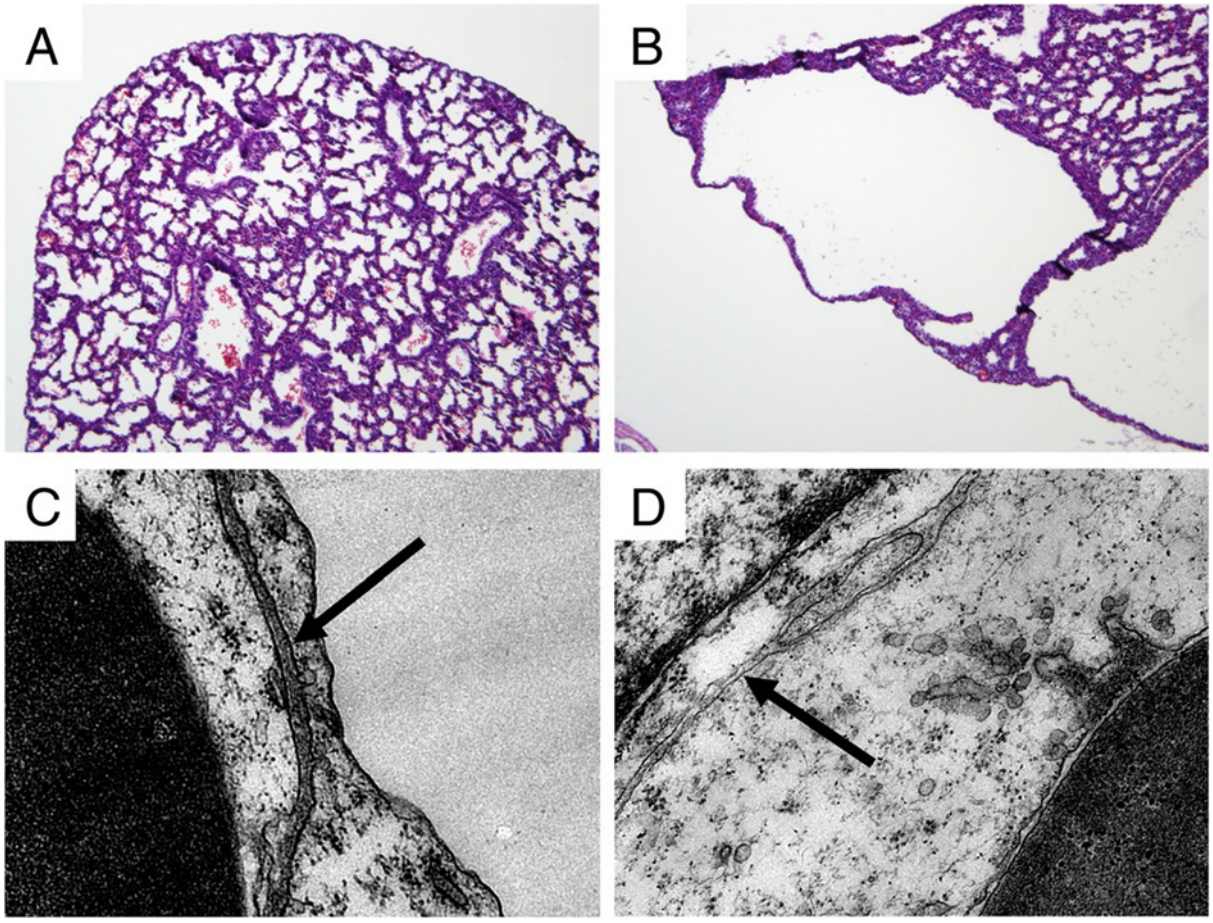

Figure 3.1: Lung Histology and Ultrastructure in RAGE TG Mice Compared to Controls
Significant lung hypoplasia was observed in RAGE TG mice exposed to doxycycline from conception to E18.5 (B) compared to lungs from control littermates (A). Images were at 100X original magnification. Electron microscopy of RAGE TG and control mice fed doxycycline from conception until sacrifice date at E18.5 revealed altered basement membrane (BM) integrity in the alveolar compartment. Wild type BM was distinct and pronounced (C, arrow) compared to RAGE TG BM that appeared diminished and fragmented (D, arrow). Magnification for each image is 6,500X.

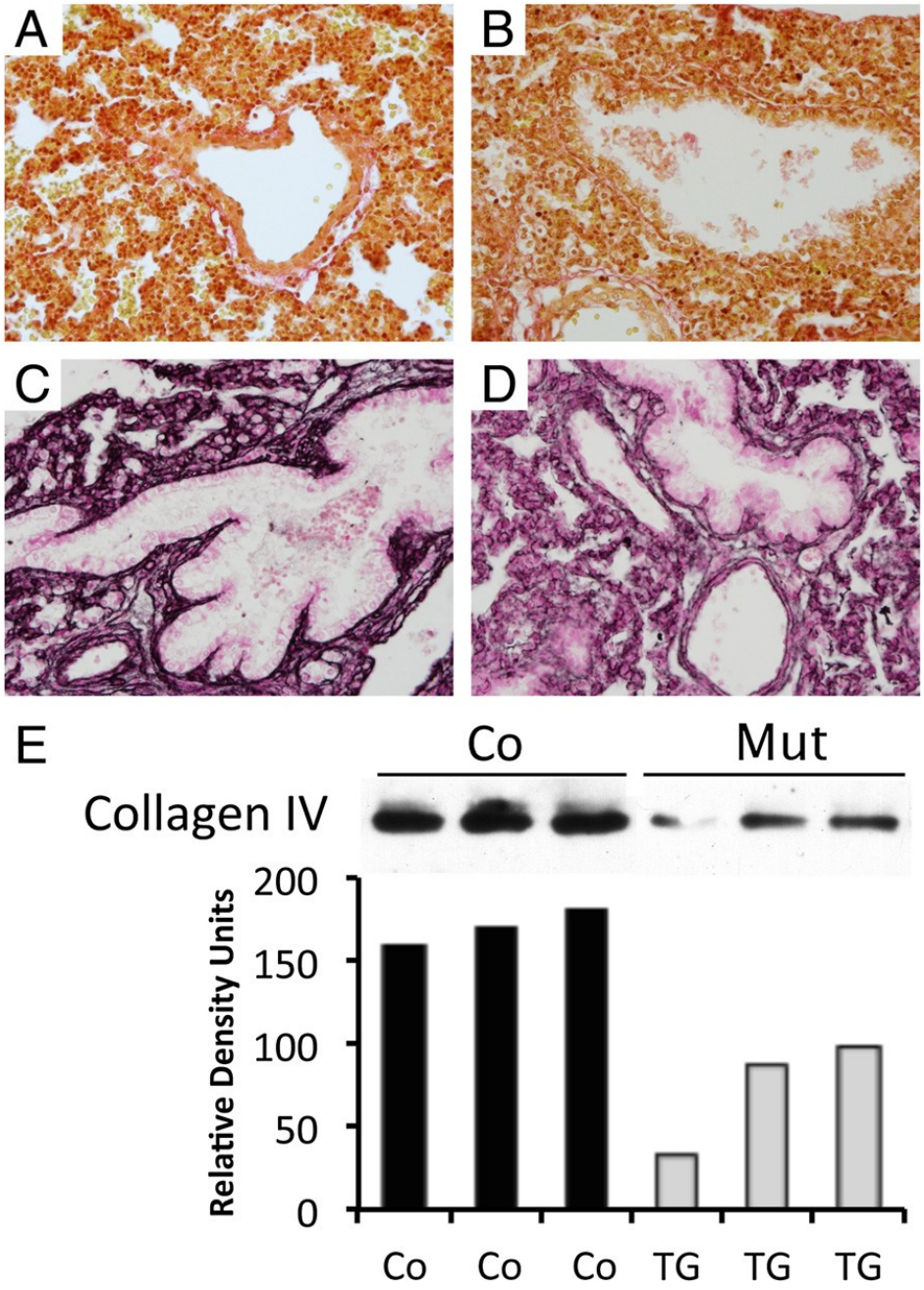

Figure 3.2: Collagen Expression in RAGE TG Mice Compared to Controls

Lungs from Control (Co, A and C) and RAGE TG (B and D) mice were stained for Pico-Red (A and B) to visualize total collagen content and type IV collagen (C and D) and only qualitative decreases were observed. Images were at 100X original magnification. Immunoblotting for type IV collagen using equal aliquots of 10 μg total lung protein revealed markedly decreased total type IV collagen expression in RAGE TG mouse lungs compared to controls (E). *Statistical difference ($P \leq 0.05$) with at least three replicates per group.

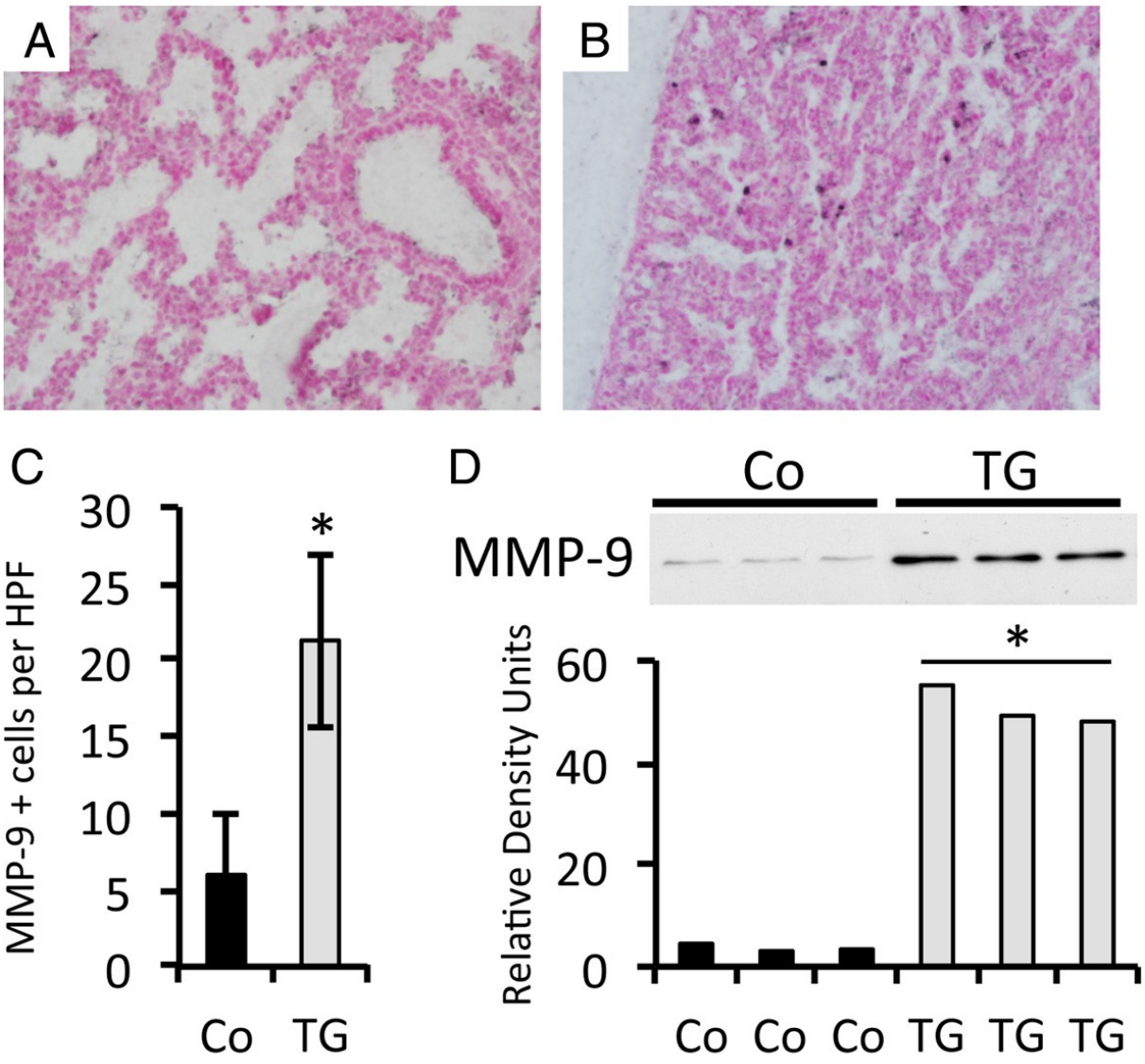

Figure 3.3: MMP-9 Expression in RAGE TG Compared to Controls

Lungs from control (Co, A) and RAGE TG (B) mice were qualitatively immunostained for MMP-9 and blinded counts of MMP-9 positive (+) cells were averaged per high powered field (HPF, C). Images were at 100X original magnification. Immunoblotting for MMP-9 using standardized aliquots of 10 μg total lung protein revealed markedly increased MMP-9 expression in RAGE TG mouse lungs compared to controls (D). *Statistical difference ($P \leq 0.05$) with at least three replicates per group.

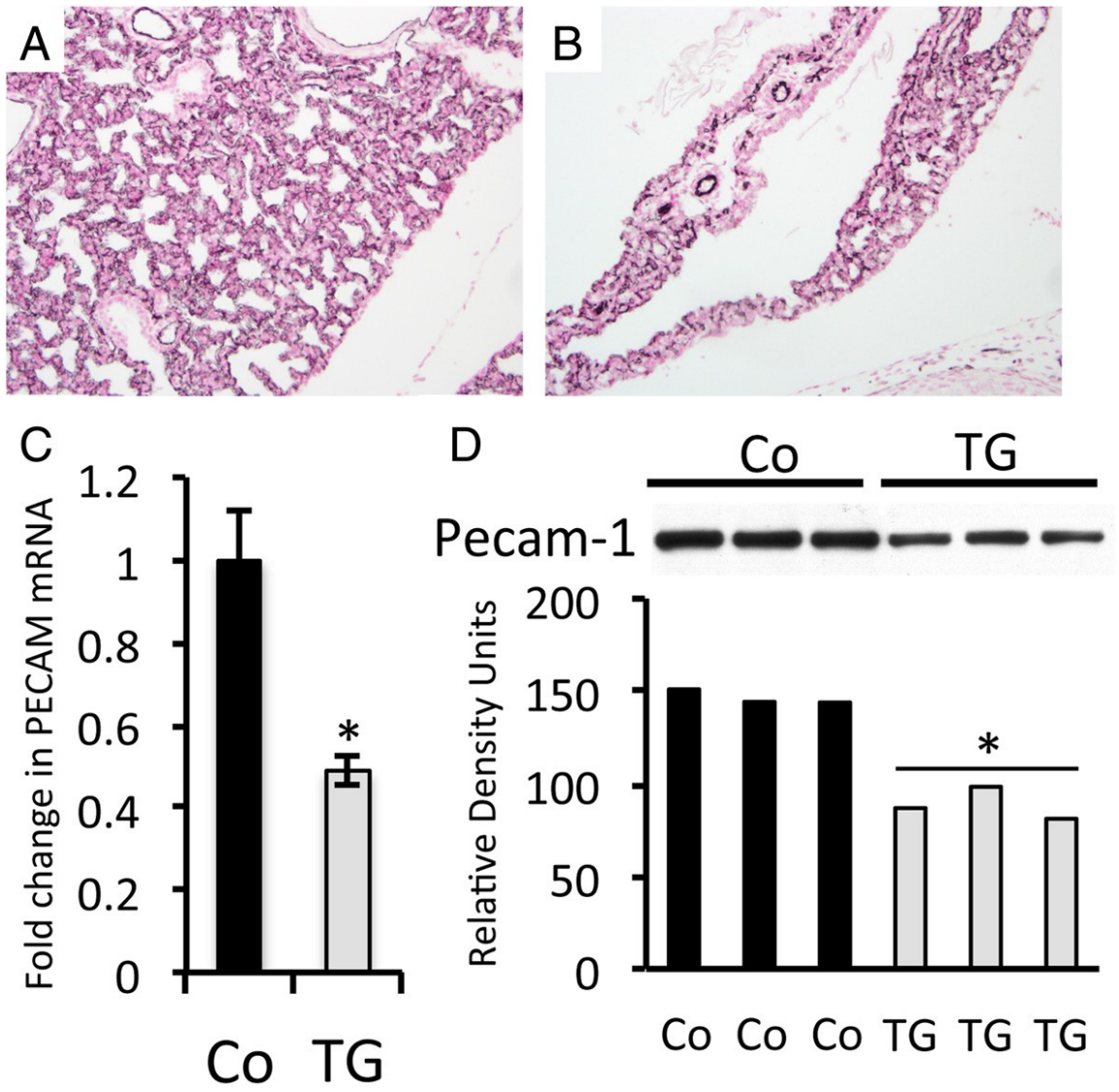

Figure 3.4: Pecam-1 Expression in RAGE TG Compared to Controls

Lungs from control (Co, A) and RAGE TG (B) mice were qualitatively immunostained for Pecam-1, revealing no appreciable differences. Images were at 100X original magnification. Quantitative RT-PCR (C) and immunoblotting (D) for Pecam-1 using standardized aliquots of total lung mRNA or protein revealed markedly decreased Pecam-1 expression in RAGE TG mouse lung compared to controls. *Statistical difference ($P \leq 0.05$) with at least three replicates per group.

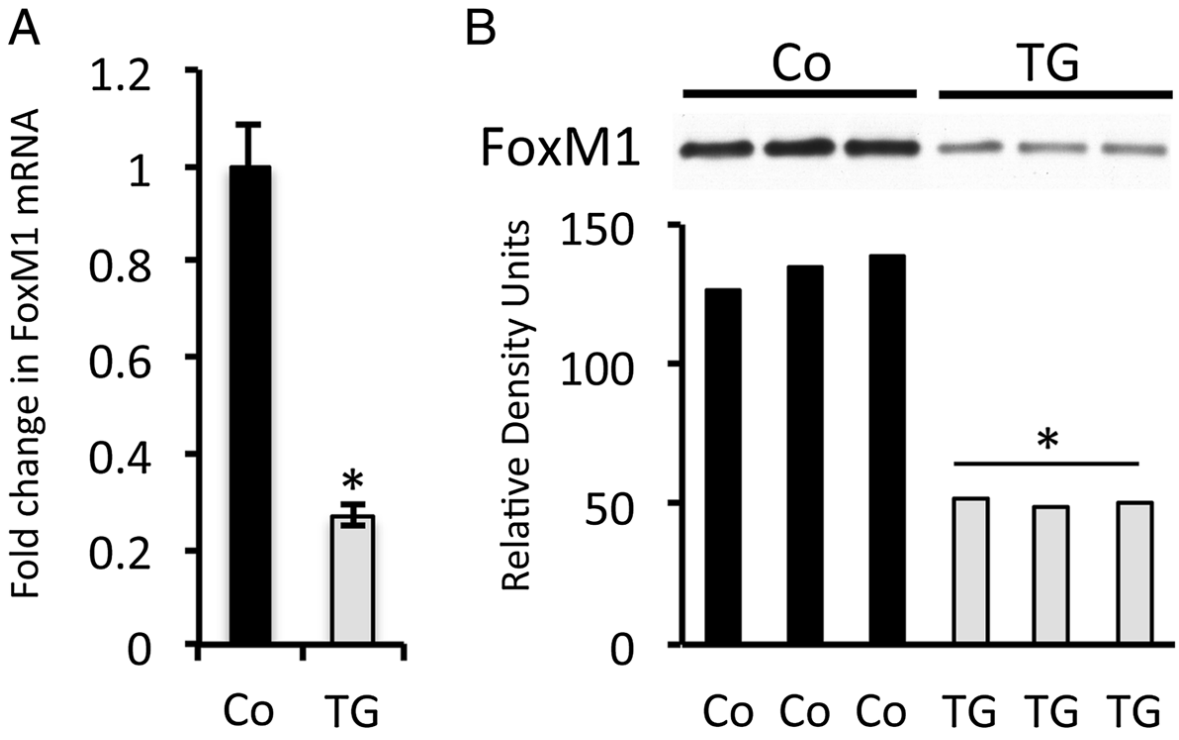

Figure 3.5: FoxM1 Expression in RAGE TG Compared to Controls

Quantitative RT-PCR (A) and immunoblotting (B) for FoxM1 using standardized aliquots of total lung mRNA or protein revealed markedly decreased FoxM1 expression in RAGE TG mouse lung compared to controls (Co). *Statistical difference ($P \leq 0.05$) with at least three replicates per group.

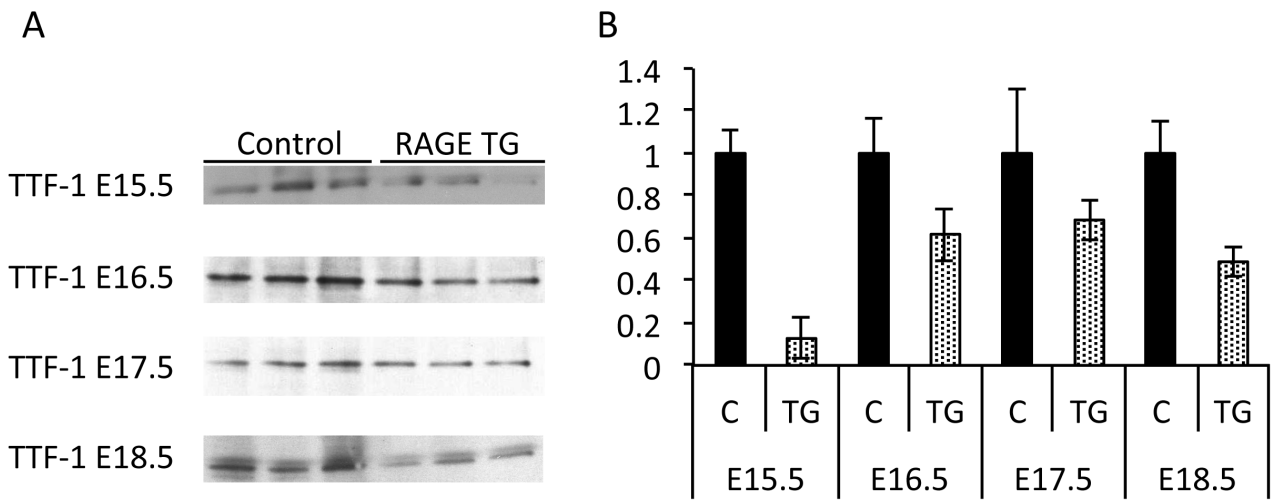

Figure 3.6: Lung Homogenates from E15.5-E18.5 RAGE TG Mice
RAGE TG mice and controls were immunoblotted for TTF-1 using a rabbit polyclonal antibody (WRAB-76694, Seven Hills Bioreagents, Cincinnati, OH) at a dilution of 1:1000. B. Densitometry of resulting bands were quantified as outlined in the Methods.

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CHAPTER 4: GENERAL DISCUSSION

The current investigation sought to simultaneously elucidate the expression profiles of pulmonary RAGE in gestating dams and their developing fetuses following SHS exposure. To our knowledge, this is the first study that seeks to delineate the biology of RAGE in the context of SHS exposure. Our discovery that dams and pups both induce higher RAGE expression suggests potential RAGE-mediated coping mechanisms that clearly warrant future exhaustive studies. Unequivocal support for deleterious inflammation mediated by tobacco smoke-induced RAGE has been provided by our laboratory (P R Reynolds et al., 2006; Reynolds, Kasteler, et al., 2011; Robinson, Johnson, Bennion, & Reynolds, 2012; Robinson, Stogsdill, Lewis, Wood, & Reynolds, 2012). Our research indicates that in addition to primary smokers that are unable or unwilling to quit, even SHS exposure of expecting adults is a concern for the developing fetus.

The observation that fetal weights were decreased following SHS exposure supports the previous work using primary smoking by Esposito, Horn, Greene, and Pisano (2008). A highly intriguing result of our studies was the effect RAGE inhibition had on SHS stimulation. Such studies at minimum should consider RAGE effects on placentation, nutrition, and relationships to maturation following early periods of organogenesis.

SHS-induced alterations in cell turnover, including instances of apoptosis, are complicated by compromised extracellular matrix. Cells necessary for the normal physiology of the lung such as endothelium, respiratory epithelium, and conducting airway epithelium require spatial integrity stabilized by collagen and other matrix molecules. A significant determinant of the architectural matrix between cells is type IV collagen synthesis and secretion (Akashi et al., 2001; Timpl, 1989). Our qualitative and quantitative data revealing diminished type collagen IV identifies an important concept relating to SHS exposure. First of all, extracellular matrixes are

targeted by smoke exposure and secondarily, RAGE expressed by epithelial cells plausibly functions to signal matrix secreting cells in the developing lung. A significant determinant of the basement membrane's interposition between cells in the respiratory membrane is due to intricate collagen IV deposition (Nakano et al., 2001; Timpl, 1989). Our qualitative and quantitative data revealing diminished collagen IV identifies an intriguing paradigm in which increased expression of pattern recognition receptors such as RAGE by epithelial cells can signal changes in the surrounding matrix.

The current research sought to determine to what extent MMP-9, a matrix metalloprotease (MMP) secreted by fibroblasts, alveolar macrophages, and epithelial cells, functions in SHS exposed pups. Because of their destructive capabilities, MMPs are recognized as not only central players in instances of disease, but effectors during remodeling events observed during development as well (Andrea Page-McCaw, Andrew J. Ewald, & Zena Werb, 2007). Our work builds upon these discoveries by identifying MMP-9 as a SHS target that likely effectuates end points via RAGE-mediated pathways. The current research sought to assess possible roles for MMP-9, a matrix metalloprotease (MMP) secreted by fibroblasts, alveolar macrophages, and epithelial cells through pathways culminating in NF-kB activation (Soler-Cataluna et al., 2005). MMPs comprise a family of 24 related endopeptidases that degrade a variety of substrates, including components of the extracellular matrix, and MMP-9 specifically targets collagen IV (Pardo & Selman, 2006). Based on their multiple biological activities, MMPs are considered participants in diverse pathological processes in the lung (Rosas et al., 2008), but also as effectors during lung morphogenesis (Buckley et al., 2011; Lee et al., 2011). We have shown that the over-expression of RAGE results in increased MMP-9 and decreased collagen IV. As recently demonstrated in adult RAGE TG mice (M. P. Stogsdill et al., 2013), this particular

study confirms that MMP-9 is increased via a RAGE- mediated pathway during development as well. Our new evidence points to MMP-9-mediated remodeling in the developing lung under the control of RAGE signaling, a particularly important concept given the abundance of RAGE in the lung.

We demonstrated that RAGE over-expression led to decreased development of pulmonary microvasculature associated with diminished levels of Pecam-1 (I. M. Kim et al., 2005). Previous research revealed abnormal thyroid transcription factor-1 (TTF-1) expression in the lung of perinatal RAGE TG mice (Reynolds, Stogsdill, et al., 2011). TTF-1 is a phosphorylated member of the Nkx2 family of homeodomain-containing transcription factors and TTF-1 phosphorylation mutants have deficits in lung vascularity due to decreased Pecam-1 and VEGF expression (DeFelice et al., 2003). Figure 3.6 demonstrates significantly decreased TTF-1 expression in the lungs of RAGE TG mice compared to controls during periods through E18.5.

A central feature likely involved in endothelial cell derivation and the coordination of vessel formation in the RAGE TG mouse lung are the contributions of FoxM1. Notably, Fox proteins FoxA2, FoxJ1, FoxF1, and FoxP each play critical roles in transcriptionally regulating branching morphogenesis and vasculogenesis in the developing lung (Brody et al., 2000; Kalin et al., 2008; Wan et al., 2004). Conversely, conditional up-regulation of FoxM1 caused increased proliferation of alveolar and bronchial epithelial cells, as well as smooth muscle and endothelial cells via the activation of cell cycle promoting factors (Kalinichenko et al., 2001).

In summary, RAGE expression and matrix destabilization are probable byproducts of pulmonary SHS exposure during embryogenesis. This study suggests that protection from damaging SHS-induced effects such as fetal weight decreases, matrix abundance, and MMP imbalances is

possible when RAGE is inhibited. Despite notable advancements in SHS research provided by the current research, additional work is necessary that focuses on RAGE signaling during pulmonary branching morphogenesis and to what extent RAGE alone is capable of inducing SHS related lung phenotypes.

Relevance of Research

This research will improve our knowledge of the contributions of RAGE during pulmonary development, particularly as it participates in signaling stemming from SHS exposure. The research is notable because it aims to demonstrate for the first time the role of RAGE in developing lungs exposed to maternal SHS. RAGE has been linked to the spreading and branching of lung parenchyma throughout organogenesis (Demling et al., 2006) as well as to an increasing array of smoke related maladies. Because abnormal spreading and branching in tobacco smoke environments may lead to embryonic complications resulting from branching delays, this research may also provide insight into compromised lung function observed in the offspring of smokers.

Disease progression observed in smoke-related pulmonary complications such as COPD in the adult and lung hypoplasia in the neonate has been greatly debated. Numerous models have surfaced for COPD and hypoplasia including the vicious cycle hypotheses, which involve chronic inflammation and alveolar destabilization (Sethi, Mallia, & Johnston, 2009). Consideration of these models naturally leads to various proposed therapeutic interventions. Because RAGE has been shown to be upregulated in human lungs diagnosed with smoke-related lung diseases (Ferhani et al., 2010), this research could also provide further evidence for its role in pulmonary disease progression.

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• Sigma Theta Tau International	2011– Current
• American Dental Education Association (ADEA)	2011– Current
• ADEA – Biomedical Subcommittee	2011– Current
• American Nursing Association	2009 – Current
• National League of Nursing	2009 – Current
• Body Worlds 3 Community Advisory Panel	2008
• Provo Peak – Second Miler	2007 – Current
• Human Anatomy and Physiology Society	2006 – Current
• American Heart Association	2005 – Current
• Favorite Professor BYU Women's Basketball Team	2005
• Unit Commissioner for the Boy Scouts of America	2003 – Current
• Woodbadge member BSA	2002 – Current
• Charter Representative for LDS Church to BSA	2000 – 2003, 2007 – 2010
• Cubmaster Award	1993
• LDS mission to Montreal Canada	1985 – 1987

Professional Experience

2011-Current Roseman University of Health Sciences
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Assistant Professor
Responsibilities: Taught course combining embryology, histology, anatomy, physiology, and pathophysiology for dental students. This course helps to prepare students for first year national board examinations. Block director for eight courses. Coordinate adjunct faculty to teach blocks. Serve on the admissions, nursing faculty search, ADEA abstract review committee, by-laws committee, curriculum committee, and CODA accreditation committee. Co-chair the NBDE Part 1 course review committee. Serve on the Student Professionalism Board.

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Educational Consultant
Responsibilities: Design curriculum for biomedical course combining embryology, histology, anatomy, physiology, and pathophysiology for dental students. This course helps to prepare students for first year national board examinations.

2005 – 2011 Ameritech College (American Institute of Medical and Dental Technology) / Provo and Draper, Utah
Instructor
Responsibilities: Anatomy and Physiology, Clinical Mathematics, Pharmacology, and Chemistry instructor, develop curriculum for nursing and surgical technician courses to ensure students are prepared to pass licensing exam, teach CPR and AED courses. Serve on the curriculum, technology in the classroom, exam, and NLNAC accreditation committees. Developed curriculum for and taught above courses as well as lifespan development. Manage the online grade book for the nursing department. Resolve in-house IT problems.

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2007 Ameritech College / Provo and Draper, Utah
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Responsibilities: Assist Campus Directors and Campus Directors of Education as a corporate resource in the development, implementation, and the continuous improvement of the educational programs and processes at the campus level, while functioning as a resource to and providing guidance, assistance, and oversight to the Campus Academics Department. To carry the vision and philosophy of Ameritech College to all staff, students, faculty, and customers, as well as the public at large.

2006 – 2007 Ameritech College / Provo, Utah
Campus Director – Provo Campus
Responsibilities: Provide leadership to all faculty members, administrative staff and students, maintaining a professional, caring demeanor at all times. Plan, direct, organize and control all School campus operations with the assistance of headquarters administrative staff. Monitor and control the effectiveness of processes intended to meet the needs of students while remaining in compliance with State, federal and accreditation guidelines. Provide guidance to the School's administrative staff on budgeting and strategic planning issues. Approve student payment arrangements and sign checks as required. Organize and supervise the day-to-day functions of the Administration staff. Coordinate and facilitate communication and information flow between the School's departments and with the administrative staff to ensure efficient operations. Maintain compliance with federal and State regulations as well as ABHES and other accrediting

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Responsibilities: Coordinate all instruction for the college, access compliance with accreditation standards, ensuring a high level of instruction through classroom observation and student evaluation of instructors, direct curriculum re-development for all programs, re-structure all programs to fit into a modular format with general education core classes, hold program director meetings, in-service instructors on curriculum development and teaching methodologies, Anatomy and Physiology instructor, teach CPR and AED courses.

2003 – 2004 Healthcare Career College – Academy of Nursing / Salt Lake City, Utah
Academic Dean
Responsibilities: Coordinate all instruction for the college, access compliance with accreditation standards, hiring of faculty and instructors, ensuring a high level of instruction through classroom observation and student evaluation of instructors, coordinate the moving of course material to the internet through the IT department with instructors and faculty, course scheduling and resource allocation, curriculum committee chair.

2002 – 2003 Academy of Nursing / Salt Lake City, Utah
Science Department Chair
Responsibilities: Coordinate instruction of the science courses at the Academy of Nursing, preparation of material for IT department for web.

2002 Academy of Nursing / Salt Lake City, Utah
Full-time Faculty
Responsibilities: Anatomy and Physiology instructor.

2002 Academy of Nursing / Salt Lake City, Utah
Part-time Instructor
Responsibilities: Anatomy and Physiology instructor.

1996 – 2011 Brigham Young University / Provo, Utah
Adjunct Faculty
Responsibilities: Anatomy instructor, Human Dissection instructor, develop curriculum for courses.

1997	Brigham Young University / Provo, Utah Part-time Faculty Responsibilities: Human Biology instructor, develop curriculum for course.
1988 – 1994, 2014	Brigham Young University / Provo, Utah Teaching Assistant Responsibilities: Lecture and laboratory teaching assistant for a variety of courses including: anatomy, dissection, histology, embryology, physiology, zoology.
1991 – 1994, 2014	Brigham Young University / Provo, Utah Research Assistant Responsibilities: Organize research staff and manage colonies of mice for research, write SOPs and reports.
1994	University of Washington / Seattle, Washington Research Assistant Responsibilities: Write SOPs and reports for embryo cultures and DNA testing.

Other Professional Experience

2000 – 2002	ClearCourse Inc. / Provo, Utah Director, Project Engineering Responsibilities: Help project managers to ensure that projects were completed and on track and on budget, allocate resources to different teams, find outside resources.
1998 – 2000	ClearCourse Inc. / Provo, Utah Project Manager Responsibilities: Manage teams of graphic artists, software developers and software engineers to develop educational software.
1995 – 1998	Taras Development Corp. and William M. Bancroft / Provo, Utah Graphic Designer Responsibilities: Design interfaces for educational software. Knowledge of Adobe Photoshop, Premiere, In Design and most Microsoft products.
1995	Taras Development Corp. / Provo, Utah Educational Consultant Responsibilities: Provide content for software training package in the field of Teratology.

Certification

- RN
- LPN
- BLS Instructor
- BLS
- ACLS
- EMT - Basic

Patents

Method and system for computerized authoring, learning, and evaluation
U.S. Patent Number 6,315,572
A method and system for computerizing authoring, learning, and evaluation is provided. The computerized system comprises a central processing unit and related memory and storage capacity to operate an authoring, learning, and evaluation system stored in a computer program. The authoring, learning, and evaluation system comprises an authoring portion and a presentation portion. Through the use of intuitive interface elements, the authoring portion allows an author to quickly and easily construct a lesson from a pool of relevant data. Data can be organized into objects or concepts that are related to one another in a reasoned fashion so that coherent testing of the data, as presented to a user, may be achieved.

Systems and methods for organizing data relationships
U.S. Patent Number 20,020,042,041
Systems and methods for creating and using a cognitive index, selectively providing objects from the index, and organizing information to generate questions contextually relevant to the information associated with the index. A computer device is used to create a cognitive index that includes various objects and/or information that may be selectively related. The index further includes one or more conceptual nodes that may be related to one or more associated nodes through one or more expressions. Each associated node may include objects and/or information relating thereto, which may be selectively and/or automatically provided from the index. The objects and/or information associated with the index may be used to provide information requested and/or to generate questions contextually relevant to the information associated with the index.

Research

Characterization of Secondhand Smoke (SHS) and Materno-Fetal Interactions in Receptors for Advanced Glycation End-Products (RAGE)-Targeted Mice
The presence of Cytochrome p-450 1b1, a liver enzyme present between gestational days 9-12, in mice.
2,4 and 2,5 diaminotoluene as an agent for chemically induced birth defects, fetotoxicity and carcinogenesis in mice.
Carbon black oil as an agent for chemically induced birth defects, fetotoxicity and carcinogenesis in mice.

Research Publications

Peer-Reviewed Publications

Winden D.R., Ferguson N.T., Bukey B.R., Geyer A.J., Wright A.J., Jergensen Z.R., Robinson A.B., Stogsdill J.R., and Reynolds P.R. 2013. Conditional over-expression of RAGE by embryonic alveolar epithelium compromises the respiratory membrane and impairs endothelial cell differentiation. *Respir Res* 14(1):108.

Winden D.R., Barton D.B, Betteridge B.C, Bodine J.S, Jones C.M., Rogers G.D., Chavarria M., Wright A.J., Jergensen Z.R., and Reynolds P.R. 2014. Antenatal Exposure of Maternal Secondhand Smoke (SHS) Increases Fetal Lung Expression of RAGE and Induces RAGE-Mediated Pulmonary Inflammation. In preparation.

Tippetts, Trevor S., Winden, Duane R., Swensen, Adam C., Thatcher, Mikayla O., Saito, Rex X., Condie, Tyler X, Simmons, Kurtis X., Judd, Allan M., Prince, John T., Reynolds, Paul R., Bikman, Benjamin T. 2014. Cigarette smoke increases cardiomyocyte ceramide accumulation and disrupts mitochondrial function. Submitted.

Thatcher, Mikayla O., Tippetts, Trevor S., Nelson, Michael B., Swensen, Adam C., Winden, Duane R., Anderson, Madeline C., Johnson, Ian E., Porter, James P., Prince, John T., Reynolds, Paul R., Bikman, Benjamin T. 2014. Ceramides mediate cigarette smoke-induced metabolic disruption. Submitted.

Non-peer Reviewed Publications

Human Anatomy Guide Robert E. Seegmiller, David Busath and Duane Winden Outernet Publishing LLC ©2009

Human Anatomy: Laboratory Manual 2nd Edition Duane Winden and Robert E. Seegmiller Outernet Publishing LLC ©2009

Human Anatomy: Laboratory Manual 2nd Edition, Companion CD ROM Duane Winden, Robert E. Seegmiller Outernet Publishing LLC ©2002

Human Anatomy: Laboratory Manual 1st Edition Duane Winden and Robert E. Seegmiller Outernet Publishing LLC ©2001

Human Anatomy: Laboratory Manual 1st Edition, Companion CD ROM Duane Winden and Robert E. Seegmiller Outernet Publishing LLC ©2001

Destinos: CD ROM Supplement McGraw-Hill ©1999

Teratology Training Guide Duane R Winden and Robert E Seegmiller Taras Development Corporation ©1996

Developmental Toxicity of Carbazole and Benzo (a) carbazole in Mice Duane Winden Brigham Young University 1994

Abstracts

Nelson M.B., Tippetts T., Winden D.R., Reynolds P.R., and Bikman B. RAGE activation reduces cardiomyocyte mitochondrial function in a ceramide-dependent manner. ADA Meeting 2014.

Tippettes T., Winden D.R., Wagner M., Condie T., Reynolds P.R., and Bikman B. 2014. Ceramide is necessary for cigarette smoke-induced reduced heart mitochondrial function. EB Meeting 2014.

Thatcher M., Tippetts T., Johnson I., Holub Z., Nelson M., Winden D.R., Reynolds P.R., and Bikman B. Ceramide mediates cigarette smoke-induced skeletal muscle metabolic disruption. ADA Meeting 2014.

Wood T.T., Winden D.R., Barton D.B., Betteridge B.C., Marlor D.R., Wright A.J., Jones C.M., Chavarria M., Rogers G.D., and Reynolds P.R. 2014. Targeted mice reveal a role for RAGE in an early inflammatory response to tobacco smoke. *FASEB J*, EB Meeting 2014