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## Characterization of Altered Epithelial Cell Turnover and Differentiation in Embryonic Murine Lungs That Over-Express Receptors for Advanced Glycation End-Products (RAGE)

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Characterization of Altered Epithelial Cell Turnover and Differentiation in  
Embryonic Murine Lungs That Over-Express Receptors for  
Advanced Glycation End-Products (RAGE)

Jeffrey A. Stogsdill

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of  
Master of Science

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June 2012

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## ABSTRACT

### Characterization of Altered Epithelial Cell Turnover and Differentiation in Embryonic Murine Lungs That Over-Express Receptors for Advanced Glycation End-Products (RAGE)

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Masters of Science

Receptors for advanced glycation end-products (RAGE) are multi-ligand cell surface receptors highly expressed in the lung that modulate pulmonary inflammation during disease. However, the contributions of RAGE signaling are unknown during pulmonary organogenesis. In order to test the hypothesis that RAGE misexpression adversely affects lung morphogenesis, conditional transgenic mice were generated that over-express RAGE in alveolar type II cells of the lung. When RAGE is over-expressed throughout embryogenesis, severe lung hypoplasia ensues, culminating in perinatal lethality. Flow cytometry and immunohistochemistry employing cell-specific markers for various distal cell types demonstrated anomalies in key epithelial cell populations resulting from RAGE up-regulation through embryonic (E) 18.5. Electron microscopy also identified significant morphological disturbances to distal cell types including separation from the basement membrane. Possible mechanisms leading to the disappearance of pulmonary tissue by increased RAGE expression were then evaluated. A time course of lung organogenesis commencing at E12.5 demonstrated that increased RAGE expression primarily alters lung morphogenesis beginning at E16.5. TUNEL immunohistochemistry and immunoblotting for active caspase-3 confirm a shift toward apoptosis in lungs from RAGE over-expressing mice when compared to wild type controls. Assaying for NF- $\kappa$ B also revealed elevated nuclear translocation in lungs from transgenic mice compared to controls. An RT-PCR assessment of genes regulated by NF- $\kappa$ B demonstrated elevated expression of Fas ligand, suggesting increased activity of the Fas-mediated signal transduction pathway in which ligand-receptor interaction triggers cell death. These data provide evidence that RAGE expression must be tightly regulated during organogenesis. Furthermore, additional elucidation of RAGE signaling potentially involved in branching morphogenesis and cell cycle abnormalities may provide insight into the progression of RAGE-mediated lung diseases.

Keywords: RAGE, lung, apoptosis, NF- $\kappa$ B, FasL

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## SYMBOLS AND ABBREVIATIONS

AGE	Advanced Glycation End-Product
ANOVA	Analysis of Variance
ATI	Alveolar Type I cell
ATII	Alveolar Type II cell
Bmp4	Bone Morphogenic Protein 4
BPD	Bronchopulmonary Dysplasia
BYU	Brigham Young University
CCSP	Clara Cell Secreting Protein
COPD	Chronic Obstructive Pulmonary Disease
DAMP	Damage Associated Molecular Patterns
Dox	Doxycycline
E	Embryonic day
Egr-1	Early Growth Response 1
FACS	Fluorescent Activated Cell Sorting
FAMRI	Flight Attendants Medical Research Institute
FasL	Fas Ligand
FasR	Fas Receptor
Fgf	Fibroblast Growth Factor
FoxJ1	Forkhead Box Transcription Factor J1
FoxA2	Forkhead Box Transcription Factor A2
H&E	Hematoxylin and Eosin
HMGB1	High Mobility Group Box Protein 1
IACUC	Institutional Animal Care and Use Committee
IL-1 $\beta$	Interleukin 1 $\beta$
kDa	kiloDalton

MEG	Mentoring Environment Grant
MMP	Matrix Metalloproteinase
NF- $\kappa$ B	Nuclear Factor kappa B
PCNA	Proliferative Cell Nuclear Antigen
PN	Post Natal
RAGE	Receptor for Advanced Glycation End-Products
RAR	Retinoic Acid Receptor
rtTA	Reverse Tetracycline Transactivator
Shh	Sonic Hedgehog
SP-C	Surfactant Protein C
sRAGE	soluble Receptor for Advanced Glycation End-Products
TetO	TetOn
TG	Double Transgenic
TNF $\alpha$	Tumor Necrosis Factor $\alpha$
TTF-1	Thyroid Transcription Factor 1
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
VEGF	Vascular Endothelial Growth Factor
Wnt	Wingless related MMTV
WT	Wild Type

## GENERAL INTRODUCTON

The vertebrate lung is a complex branching organ comprised of numerous specialized cell types and tissue specific transcription factors (1), necessary to optimize oxygen intake and release of gaseous byproducts. In total the average human lung encompasses 70 m<sup>2</sup> of surface area, capable of supporting 250 ml/min of oxygen consumption at rest and upwards of 5500 ml/min oxygen consumption during strenuous exercise (2). In order to accomplish the efficiency required of the lung, coordinated and extensive branching events followed by alveolarization take place during embryogenesis and well into post natal life; the four organogenic periods are embryonic, pseudoglandular, canalicular and saccular/alveolar (Figure 1). Each of these stages is identified by differential epithelial and mesenchymal cell expression that contributes to significant changes in organ structure.

### *Lung Morphogenesis*

The mouse lung “tree” as mapped by Metzger et. al. reveals a remarkably stereotypical pattern for development (Figure 2) (3). Through the utilization of three separate modes of branching (domain branching, planar bifurcation and orthogonal bifurcation), each of which is controlled by a subroutine of local patterning signals under the direction of large scale signaling, the entirety of the lung is constructed in a predictable pattern (3).

The initial stage of lung production begins by the budding of the endoderm as a ventral diverticulum between the thyroid and the stomach in an area of tissue expressing thyroid transcription factor-1 (TTF-1) near embryonic day (E) 9-9.5 in the mouse and at 5 weeks of gestation in the human (1, 2, 4). The primordial lung buds evaginate into the surrounding splanchnic mesenchyme as primary tubules are formed (1). Fibroblast growth factor (Fgf) 10 among other factors is secreted from the splanchnic mesenchyme and epithelial branch tips in order to direct proper growth and branching of the newly formed lung buds (5). Upon generation of the rudimentary lung buds, primary bronchi are differentially separated in the human and mouse to give rise to left and right lobes. This process induces a trilobed right and bilobed left lung in humans, and a quadrilobed right and unilobar left lung in the mouse by E12. Although the lobular outcome between mice and humans differs, the processes which generate lobe formation are relatively conserved (2).

Formation of the primary airways initiates the pseudoglandular phase of lung development (E11.5-E16 in the mouse; 5 to 17 weeks in the human) in which extensive branching and budding occur resembling the appearance of pre-acinar airways which form the bronchial and bronchiolar tubes. It is at this stage that specialized cell subsets emerge from undifferentiated tissues and primarily form ciliated, non-ciliated columnar, goblet and neuroendocrine cells that line the conducting airways, and alveolar type (AT) I and II cells begin to encompass the respiratory compartments.

These differentiation events occur in large part via canonical Wnt signaling, among other pathways, and are induced by peripheral lung mesenchymal cells and differentiating lung epithelial cells (1, 2, 6-8).

Alveologensis is characterized by dilations in the terminal acinar tubules and it progresses through the canalicular (murine E16.5 to E17.5 and 16 to 27 weeks of gestation in the human) and saccular/alveolar (E17.5 through postnatal [PN] 20 in the mouse, 27 weeks of gestation through two years and beyond in the human) stages of lung development. Fgf10 signaling events continue throughout the canalicular and saccular/alveolar periods and control continued airway branching and terminal alveolar formation (9). NF- $\kappa$ B nuclear translocation, either by over-expression or inflammation induced mechanisms, can disrupt normal late gestational branching morphogenesis by inhibiting Sp1 mediated Fgf10 expression (10, 11). Extensive mesenchymal thinning and angiogenesis occur from PN4 until PN20 in order to permit proper gas exchange.

At the core of proper pulmonary organogenesis lies specific signaling pathways and transcription factor expression, which require precise spatial and temporal regulation. In addition to those mentioned previously, sonic hedgehog (SHH), bone morphogenetic protein 4 (BMP4), forkhead box (FOX) proteins and vascular endothelial growth factors (VEGFs) greatly influence cell activity including proliferation, migration and differentiation (12-20). Significant alterations in lung formation occur as a result of pathway and transcription factor misregulation (1).

### *The Receptor for Advanced Glycation End-Products*

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors. The receptor contains an extracellular 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 3) (21). The cytoplasmic domain of RAGE contains four possible phosphorylation sites, S391, S399, S400 and T401, of which only S391 is conserved among humans, mice, Guinea pigs, rats, rabbits, dogs and cats (22). Replacement of S391 to alanine was sufficient to abrogate PKC $\zeta$ -dependent phosphorylation and subsequent signal transduction *in vitro* (22). Although not explicitly stated, RAGE acts as a receptor tyrosine kinase (RTK) cell surface receptor, requiring homodimerization to effectively potentiate intracellular signaling cascades (23).

RAGE is a dynamic receptor capable of binding a handful of ligands and it has the ability to distinguish complex tertiary structures (24). Originally characterized and named for its capacity to bind to non-enzymatically glycosylated macromolecules, advanced glycation end-products (AGEs), RAGE can receive other molecules including pro-inflammatory cytokine-like mediators of the S100/calgranulin family (S100A8,9,12,14 and S100B), amyloid  $\beta$ -fibrils, high mobility group box 1 (HMGB1), Mac-1 (21, 25) and specific DNA and RNA tertiary structures (26).



Because RAGE binds a handful of ligands, it is thus linked to several signaling pathways. These include PI3/Akt (27), RhoGTPases (28), Jak/STAT (29), and Src family kinases (30). Of notable interest are two pathways stimulated by RAGE activation that are in response to either damage-associated molecular patterns (DAMPs) (NF- $\kappa$ B pathways) (31) or tobacco smoke-induced pulmonary inflammation (Ras pathway) (32). Key DAMP molecules are those of the S100/calgranulin family and HMGB1, both of which bind to RAGE among other receptors including toll-like receptor 4 (TLR4) (26, 33). These DAMP molecules, 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 as shown by *in vitro* studies (34). A handful of downstream gene products produced through RAGE signaling include NF- $\kappa$ B, Cox-2, IL-1 $\beta$ , and TNF- $\alpha$  (31). Because the RAGE gene contains NF- $\kappa$ B and SP-1 promoter binding sites (35) and is regulated by Egr-1 in cases of tobacco smoke-related disease (36), a possible auto-inflammatory loop may be triggered giving suggestion to RAGE involvement in chronic disease states.

The RAGE molecule described above, also termed full length-RAGE, is one of several isoforms that exist for the receptor due to alternative splicing variants of the RAGE mRNA. Dominant negative RAGE (dn-RAGE) is 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 esRAGE. The esRAGE isoform yields the same V and C-regions of the full length-RAGE but lacks both the hydrophobic transmembrane and the intracellular domains (21). The altered variants of RAGE are thought to bind and mop up ligands without the consequences of activating signal transduction and initiating gene transcription and have been proposed as inflammatory reduction targets for various tissues. Additionally, full-length RAGE can be cleaved by MMPs to render sRAGE, a non-splice variant of RAGE closely resembling esRAGE in structure and function (37).

RAGE has been found to be expressed in many cell types including endothelium, smooth muscle, macrophages and epithelium, however it most abundant in the lung (38) limiting its expression to ATII cells and to the basolateral membranes of ATI differentiated cells (25). Normally observed in low levels throughout the body and up-regulated only in cases of injury and disease, RAGE expression in contrast is found at high quantities during late lung neonatal development and in adult pulmonary tissue suggesting possible contributions to homeostasis and organogenesis. Meneghini et al. (2010) show that ligands including HMGB1 and S100B promote proliferation and neuronal differentiation of neural progenitor cells upon RAGE binding and subsequent activation (39). In addition, RAGE has been shown to enhance adherence of epithelial cells to collagen coated surfaces and dynamically influence cell spreading a possible role in ATII to ATI cell transition (40). However, there is little evidence demonstrating the actual roles of RAGE in murine lung development.

Aside from developmental aspects, RAGE has been linked to numerous pathological states including diabetes (41), atherosclerosis, sepsis, rheumatoid arthritis and Alzheimer's disease (26). Concentrations of RAGE have found to be up-regulated in the disease states of lung fibrosis (42), acute respiratory distress syndrome (ARDS) (43), polycystic kidney disease (44) and chronic obstructive pulmonary disease (COPD) (45) among many others. Since its identification in response to high AGE concentrations as in diabetic states, numerous studies have also shown to find a relation between inflammation and RAGE (24, 46). Specifically the receptor has been found to up-regulate various cytokines including IL-6 (25), TNF- $\alpha$  (47), IL-1 $\beta$  (48), MCP-1 (49), IL-8 (49), INF- $\alpha$  (50) and other molecules such as matrix metalloproteinase 9 (MMP-9). RAGE has also been shown to promote apoptosis through a variety of intrinsic and extrinsic stress pathways in a host of cell and tissue types (51-53).

In light of these data, RAGE has become increasingly studied within the context of disease progression, cancer biology and developmental organogenesis. With respect to lung biology, little is known about the contributions of RAGE to pulmonary development. RAGE knock-out (KO) mice exist and particularly during the first year of life, the lungs of these mice are phenotypically indistinguishable from their wild type counterparts. We therefore sought to test the hypothesis that pulmonary RAGE over-expression would be detrimental to lung biology and development. By obtaining and mating two transgenic lines of mice (SP-C—rtTA and TetO—RAGE), we generated

embryos that over-express RAGE in ATII cells of the lung to study the effects of increased RAGE on branching morphogenesis, pulmonary cellular differentiation and apoptosis. Our findings have been published in the American Journal of Respiratory Cell and Molecular Biology.

Figures

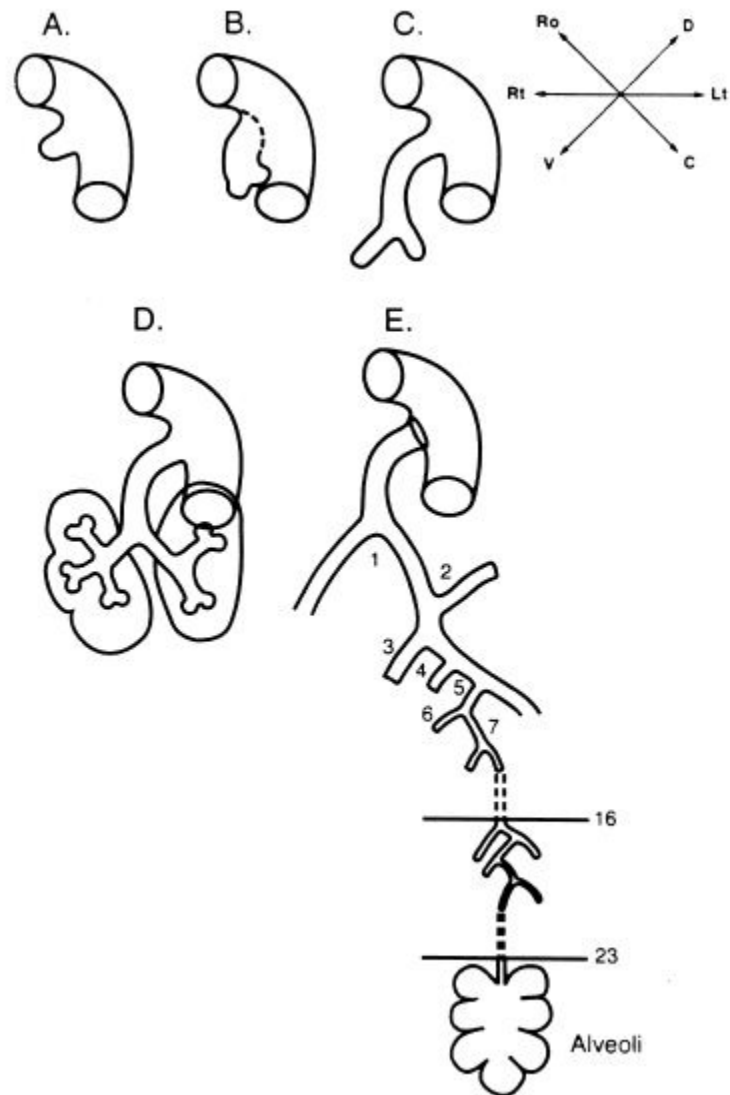


Figure 1 Key morphogenic events in human and mouse lung development. The primitive endoderm evaginates into the splanchnic mesenchyme to form the initial lung bud (A). Through reciprocal signaling between the splanchnic mesenchyme and newly formed lung outgrowth, extensive elongation and branching occur, thereby forming the primary bronchi (B-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 (2).

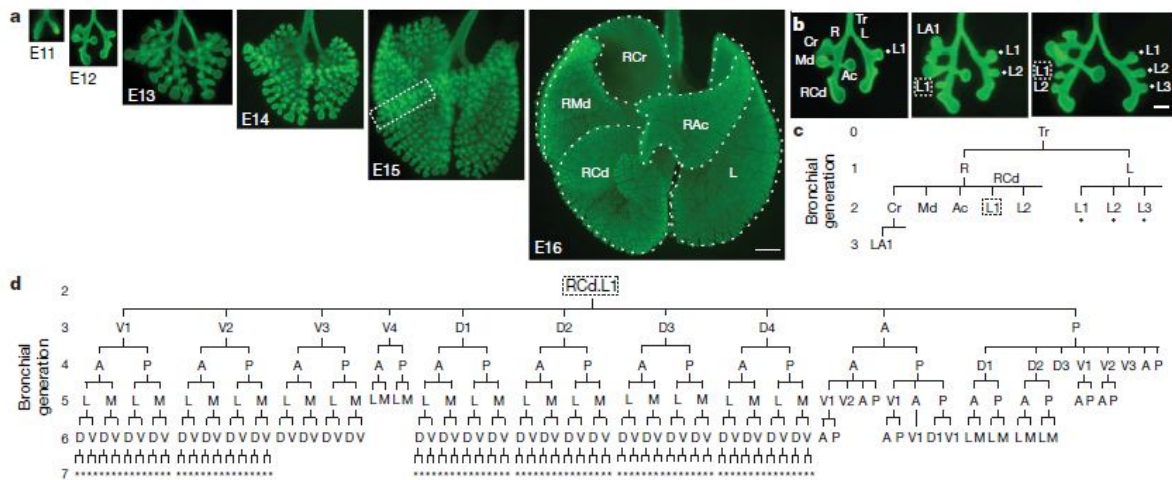


Figure 2 Branching morphogenesis of the murine lung. The stereotypical branching pattern of the mouse lung is visualized through whole mount staining from E11 through E16 (A-B). The specific branching generations in the bronchi and bronchial tubes are diagrammed up to seven generations (C-D). Image regenerated from Metzger et al. 2008 (3).

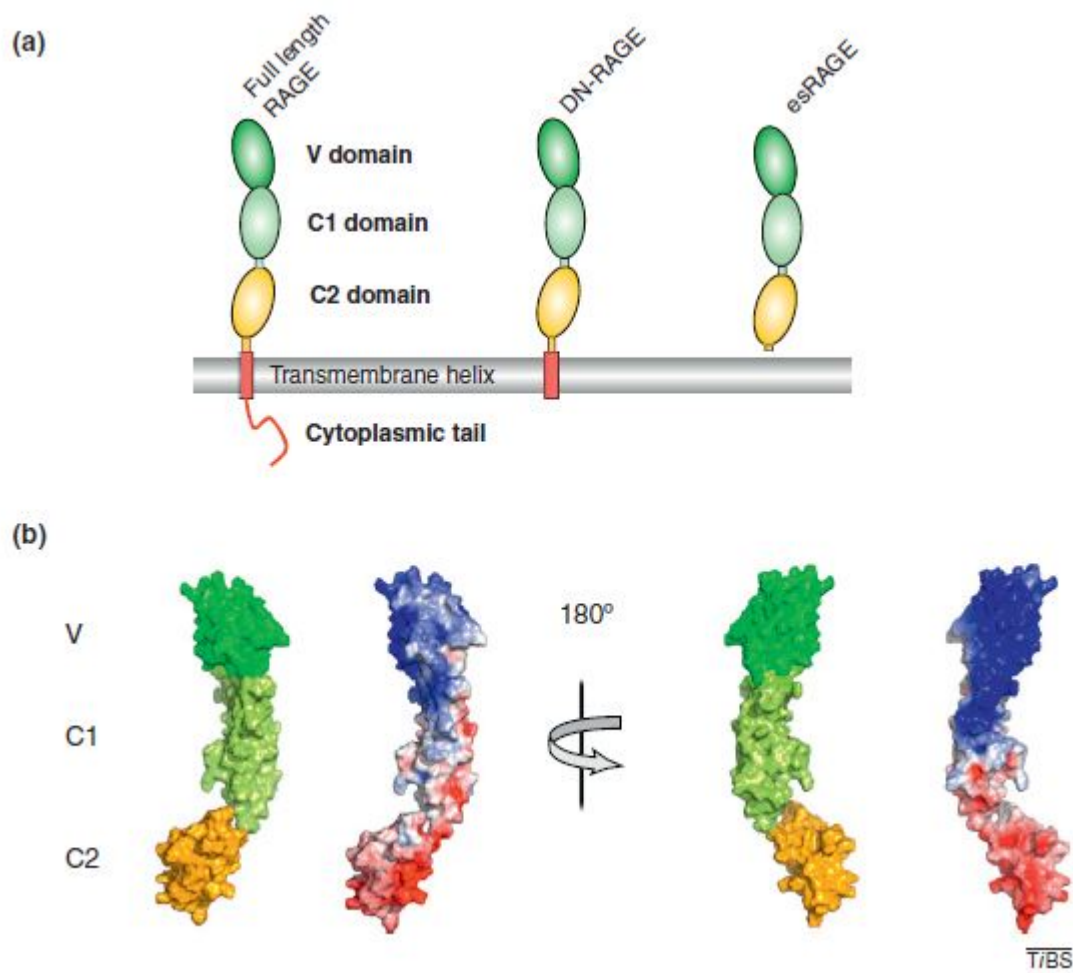


Figure 3 Structure of RAGE. A cartoon image showing full-length, dominant-negative (DN-RAGE) and esRAGE, and highlighting the extracellular (V, C1 and C2), transmembrane and cytoplasmic domains of RAGE (A). The surface representation model of RAGE (B). The blue, white and red image shows the charge distribution 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 (54).

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The following two chapters are copies of published manuscripts that outline the body of research completed while at Brigham Young University. The manuscripts are published in the *American Journal of Respiratory Cell and Molecular Biology*:

### Chapter 1

Reynolds P.R., Stogsdill J.A., Stogsdill M.P., and Heimann N.B. 2011. Up-Regulation of RAGE by Alveolar Epithelium Influences Cytodifferentiation and Causes Severe Lung Hypoplasia. *American Journal of Respiratory Cell and Molecular Biology* 45(6): 1195-202.

### Chapter 2

Stogsdill J.A., Stogsdill M.P., Porter J.L., Hancock J.M., Robinson A.B., and Reynolds P.R. 2011. Embryonic over-expression of RAGE by alveolar epithelium induces an imbalance between proliferation and apoptosis. *American Journal of Respiratory Cell and Molecular Biology*. Epub Feb 16, 2012.

## CHAPTER 1

UP-REGULATION OF RAGE BY ALVEOLAR EPITHELIUM INFLUENCES  
CYTODIFFERENTIATION AND CAUSES SEVERE LUNG  
HYPOPLASIA AND PERINATAL LETHALITY

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*Abstract*

Receptors for advanced glycation end-products (RAGE) are cell-surface receptors expressed by pulmonary tissue that influence alveolar type (AT)II - ATI transition required for normal alveolar formation. However, the precise contribution of RAGE in interactions between pulmonary epithelium and splanchnic mesenchyme during lung organogenesis remains uncertain. In order to test the hypothesis that RAGE misexpression adversely affects lung morphogenesis, conditional transgenic mice were generated that over-express RAGE. Mice that over-express RAGE throughout embryogenesis experienced 100% mortality and significant lung hypoplasia coincident with large, vacuous areas in the periphery when compared to normal airway and alveolar architecture observed in control mouse lungs. Flow cytometry and immunohistochemistry employing cell-specific markers for distal (FoxA2) and respiratory epithelium (TTF-1), ATII cells (proSP-C), and ATI cells (T1- $\alpha$ ) demonstrated anomalies in key epithelial cell populations resulting from RAGE up-regulation. These results reveal that precise regulation of RAGE expression is required during lung formation. Furthermore, abundant RAGE results in profound alterations in epithelial cell differentiation that culminate in severe respiratory distress and perinatal lethality.

Key Words: Hypoplasia / Lung / RAGE / Transgenic / Mouse

## *Introduction*

Pulmonary development is a highly ordered and coordinated process that requires precise reciprocal interactions between differentiating respiratory epithelium derived from endoderm and the surrounding splanchnic mesenchyme. During the initial embryonic stage of murine lung development, spanning from embryonic (E) day 9.5 to E11.0, primordial lung buds undergo branching to form the main lobar bronchi. The subsequent pseudoglandular stage (E11.5 to E16.5) is associated with extensive branching and budding that lead to the formation of the intrapulmonary conducting and peripheral lung airways. As embryonic development progresses, distinct populations of differentiated respiratory epithelial cell types arise, producing a morphologically dynamic arrangement of cells that line the conducting airways (ciliated, non-ciliated columnar, goblet and neuroendocrine cells) and respiratory (alveolar type (AT) I and ATII cells) compartments. During the canalicular (E16.5 to E17.5) and saccular (E17.5 through postnatal (PN) 4) stages of lung development, alveolar saccules form via dilations of terminal acinar tubules. In the latter stages of the saccular period and into the alveolar period (PN5 to PN20), the formation of an extensive capillary network coincides with mesenchymal thinning necessary for efficient gas exchange in the neonate (1). It is clear therefore that lung morphogenesis is dependent upon specific temporal and spatial control of cell proliferation, migration, and differentiation.

Numerous signaling and transcriptional control pathways intimately influence processes that regulate precise deposition of specialized cell types along the proximal-distal pulmonary axis. Such pathways include those associated with fibroblast growth factors (Fgfs), sonic hedgehog (Shh), bone morphogenetic protein 4 (Bmp4), vascular endothelial growth factors (Vegfs), thyroid transcription factor 1 (TTF-1), and Wnts (2-10). When these or other important genetic pathways are misregulated, pulmonary hypoplasia (incomplete lung development), or pulmonary agenesis (complete lack of lung formation) may occur resulting in abnormally low or absent bronchopulmonary segments and terminal alveoli (11).

Receptors for advanced glycation end-products (RAGE) are members of an immunoglobulin superfamily of cell-surface receptors expressed in many cell types including endothelium, smooth muscle, fibroblasts, and epithelium (12). RAGE contains an extracellular ligand-engaging V-region-like domain and two C-region-like domains, a single transmembrane domain, and a short, highly charged cytoplasmic domain essential for signal transduction (13-16). RAGE expression is most abundant in the lung (17,18) and it is prominently detected on membranes of well-differentiated ATI cells (19). Identification in ATI cells during development (20) has implicated RAGE in important developmental processes such as the spreading and adherence that characterize the transitioning of ATII cells into ATI cells necessary for effective gas exchange and alveolar stability (21). Although first described as a progression factor in



cellular responses to hyperglycemia and oxidant stress rich in advanced glycation end-products (AGEs), RAGE has also been distinguished as a pattern recognition receptor that binds S100/calgranulins, amyloid- $\beta$ -peptide, and high mobility group box-1 (HMGB-1) to influence gene expression during disease progression (13,22,23). Despite up-regulation of the receptor in cases of injury and disease, high levels of temporally and spatially regulated RAGE expression during lung development and in adult pulmonary tissue suggest probable functions in lung morphogenesis and homeostasis (24). Additionally, RAGE expression in other tissues that coordinate branching morphogenesis such as the kidney (25) and salivary gland (26) further suggests functions that are likely associated with organogenesis. Despite these compelling data, the full extent of RAGE contribution to lung formation and maintenance has not been adequately evaluated. Understanding the role of RAGE in development, particularly in the early stages of airway generation and patterning, could provide insights that link receptor-mediated signal transduction pathways to critical lung-specific transcriptional control programs of branching morphogenesis and cytodifferentiation.

In the current investigation, we test hypotheses relating to altered RAGE expression. In a developmental context, RAGE knock out mice exhibit normal lung morphogenesis and at the conclusion of the alveolar period, are indistinguishable from wild type mice with basal RAGE expression (27). However with increasing age, lungs from RAGE knock out mice become increasingly fibrotic (28). While other genes may

be able to compensate for the absence of RAGE, in the long term, alveolar homeostasis may be lost by disturbances in alveolar remodeling (29, 30). To date, no research has been completed that attempts to reconcile potential pulmonary effects when the full spectrum of RAGE expression (RAGE knock out, wild type, RAGE over-expression) is evaluated. Utilizing pulmonary epithelial cell-specific transgenic mouse technology, we demonstrate that RAGE over-expression in respiratory epithelium results in significant pulmonary hypoplasia and neonatal lethality. This phenotype demonstrates that while redundant mechanisms may substitute when RAGE is absent, excessive RAGE availability influences pathways that culminate in dysmorphogenesis. Extending the research trajectory of this important mouse model may provide profound insight into precise stages of lung development involving the biology of pulmonary epithelium in the proximal and distal lung.

### *Materials and Methods*

#### Mice

All mice were in a C57Bl/6 background including wild type mice (Jackson Laboratories, Bar Harbor, ME) and RAGE knock out mice (31). Two transgenic lines were generated and mated to create conditional doxycycline (dox)-inducible mice that over-express RAGE (Figure 1). Select Dams were fed dox (625 mg/kg; Harlan Teklad, Madison, WI) from before conception until E18.5 or during various periods where

indicated. *En block* lungs were resected, weighed, and imaged using an Olympus SZX12 camera or fixed in 4% paraformaldehyde for histological analysis. Tail biopsies were genotyped by PCR for existence of transgenes using the following primers: SP-C-rtTA forward (5'-GAC ACA TAT AAG ACC CTG GTC A-3') and reverse (5'-AAA ATC TTG CCA GCT TTC CCC-3') and TetO-RAGE forward (5'-TGT CGA GTT TAC TCC CTA TCA GTG-3') and reverse (5'-GGA GAG AGG ACC TTC CAA GC-3'). PCR conditions included 95°C for 2 minutes and 30 cycles at 95°C for 30 s, 62°C (SP-C) or 67°C (RAGE) for 30 s, and 72°C for 45 s. Mice were housed and utilized in accordance with protocols approved by the IACUC at Brigham Young University and at least six mice were included in each group.

### Histology and Immunohistochemistry

Lungs from double transgenic and age-matched wild type mice were processed, embedded in paraffin, and sectioned (32) then stained with hematoxylin and eosin according to standard procedures. Antibodies included: proSP-C (1:1000), FoxA2 (1:2000), TTF-1 (1:1000, Seven Hills Bioreagents, Cincinnati, OH), and T1- $\alpha$  (1:2000, Hybridoma Bank, University of Iowa). Immunohistochemical staining protocols were discussed previously (32-35).

### RT-PCR and Immunoblotting

Messenger RNA from RAGE knock out, wild type and double transgenic mice were isolated from lungs using the Absolutely RNA<sup>®</sup> Kit (Stratagene, La Jolla, CA). Semi-quantitative RT-PCR analysis of RAGE and GAPDH involved a SuperScript III Synthesis Kit (Invitrogen) as previously described (24). Lungs were removed from RAGE knock out, wild type and transgenic mice and total homogenates were quantified using a BCA Protein Assay kit (Fisher Scientific). Immunoblotting for RAGE and GAPDH in equal concentrations of total lung protein was performed as previously cited (24).

### Flow Cytometry

*En block* embryonic lungs were removed from E18.5 animals and subjected to flow cytometric analysis using a FACS Canto Flow Cytometer (BD Biosciences, San Jose, CA, see Online Supplement for experimental details).

### Electron Microscopy

Whole mouse lungs were excised and fixed overnight in a 2% glutaraldehyde/.06M 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 Ultramicrotome (Tucson, AZ) and stained using Reynolds' lead citrate. Sections were photographed using a FEI Tecnai T12 electron microscope.

### Statistical Analysis

Flow cytometry and lung weights are presented as the means  $\pm$  S.D. The mean percentage of ATI and ATII cells in wild type mouse lung was standardized to 1 and analyzed using the Student's *t* test. Average weights were assessed by one-way ANOVA and when ANOVA indicated significant differences, the Student's *t* test was used. Values of  $p \leq 0.05$  were considered significant.

### *Results*

#### Conditional Over-Expression of RAGE in the Lung

Semi-quantitative RT-PCR and immunoblot analyses were used to evaluate RAGE mRNA and protein levels so that RAGE induction in transgenic mice could be verified. RAGE mRNA was undetectable in E18.5 RAGE knock out mouse lungs and increased expression was observed in lungs from age-matched RAGE transgenic mice when compared to basal RAGE expression in lungs from non-transgenic control littermates (Figure 2A). Immunoblotting was performed in order to determine whether increased RAGE mRNA expression corresponded with augmented protein synthesis.

RAGE protein expression was significantly increased in E18.5 RAGE transgenic mice fed dox when compared to dox-administered wild type littermates (Figure 2B).

### Increased Pulmonary RAGE Expression Alters Lung Size and Induces Perinatal

#### Lethality

Gross imaging of lungs from RAGE transgenic mice (Figure 3C) revealed observable translucency due to tissue loss and weighed significantly less than lungs from age-matched wild type controls (Figure 3B) or RAGE knock out mice (Figure 3A). Assessment of pups allowed to deliver at full term revealed that RAGE transgenic mice fed dox experienced 100% lethality within four hours of birth when compared to 0% lethality observed among dox-fed single and non-transgenic control littermates.

### Over-Expression of RAGE Alters Lung Morphology and Lung-Specific Cell Populations

Hematoxylin and eosin (H&E) staining of lungs from E18.5 RAGE transgenic mice fed dox revealed extensive morphological disturbances. Large vacuous areas lined with squamous, cuboidal and columnar cells were pronounced in RAGE transgenic mice fed dox throughout gestation when compared to dox-exposed control mouse lungs (Figure 4A and B, asterisks). Regions of the RAGE transgenic lung appeared to experience adequate lung parenchymal development potentially capable of eventual gas exchange (B, arrow); however, significant lung hypoplasia was observed in the majority of the lung. E18.5 wild type and RAGE transgenic mice that lacked any

dox administration during embryogenesis were indistinguishable suggesting insertion of the transgenes alone had no deleterious effects (Figure 4C, D). When transgenic mice were fed dox during sequential stages of embryogenesis, critical periods of RAGE-mediated lung malformation were identified (Figure 4E-H). Dox administration from E0-8.5 or E8.5-12.5 resulted in no observable differences between RAGE transgenic and control mouse lung (Figure 4E, F). Dox availability from only E12.5-15.5 (Figure 4G) resulted in significant lung hypoplasia similar to dox administration from E0-18.5 (Figure 4B, asterisk). An intermediate phenotype characterized by enlarged airways resulted when dox was administered from E15.5-18.5 (Figure 4H).

Because lungs from RAGE transgenic mice exposed to dox throughout development experienced significant lung hypoplasia and respiratory failure, potential abnormalities in specific cell populations were assessed. Expansion or contraction of cell populations was qualitatively assessed via immunohistochemistry using antibodies against cell-specific markers. General identification of distal pulmonary epithelium by FoxA2 and respiratory epithelium by TTF-1 revealed a detectible decrease in epithelial cell abundance. Specifically, FoxA2 was detected predominantly in terminal airways and throughout distal tissues in control lungs (Figure 5A) whereas localization was restricted to a subset of terminal airways and only sporadically detected in distal lung tissue of RAGE transgenic mice (Figure 5B). The localization of TTF-1-positive epithelial cells was similar in both wild type and RAGE transgenic lungs; however, the

quantity of positive cells was noticeably diminished in lungs in RAGE transgenic mice (Figure 5D) compared to controls (Figure 5C). Staining for proSP-C, an antibody that recognizes surfactant protein C in ATII cells prior to secretion revealed undetectable changes in ATII cell prevalence but increased intensity of proSP-C in individual ATII cells in RAGE transgenic mouse lungs (Figure 5F, arrow) when compared to controls (Figure 5E, arrow). Immunostaining for T1- $\alpha$ , an ATI cell-specific marker, revealed significant decreases in ATI cell populations in RAGE transgenic mouse lungs. Similar to FoxA2 staining, T1- $\alpha$  localization was limited to the lining of large vacuous areas and infrequent throughout distal lung tissue of RAGE transgenic mice (Figure 5H), whereas abundant T1- $\alpha$  expression was detected in terminal airways of lungs from control mice (Figure 5G).

In order to obtain quantitative data of the alterations in distal lung cell populations in the lungs from RAGE transgenic mice, flow cytometric analysis was employed using lungs from dox-fed E18.5 wild type and RAGE transgenic mice. Data identify a significant decrease in the percentage of ATI and ATII cells in RAGE transgenic mouse lungs when compared to age-matched wild type lungs (Figure 6A, B). When the percentage of each cell type from E18.5 wild type lungs was averaged and normalized to 1, a significantly decreased percentage of both ATI and ATII cells in RAGE transgenic mouse lungs was discovered (Figure 6C).



Because general histological staining of RAGE transgenic mice revealed marked hypoplastic lung formation (Figure 4) and decreased ATI cell abundance (Figure 5), ultrastructural analysis of the alveolar compartment was undertaken so that the effects of RAGE over-expression regarding alveolar epithelium and alveolar integrity could be evaluated. Electron microscopy revealed discernible, fused basement membranes contributing to the respiratory membrane in dox-fed wild type mice (Figure 7A, asterisk) and abnormally arranged and fragmented basement membranes in RAGE transgenic mice fed dox (Figure 7B, asterisk). Furthermore, ATI cells in wild type mice appeared to maintain normal attachment to the basement membrane (Figure 7C, asterisk) whereas remaining ATI cells in RAGE transgenic mice appeared to be in the process of sloughing off from the lung parenchyma resulting in detectible gaps between individual cells and lung proper (Figure 7D, asterisk). Interestingly, sloughing ATI cells appeared to have a bleb-like morphology associated with impaired cell integrity (Figure 7D, arrow) when compared to normal ATI cells in wild type mice (Figure 7C, arrow).

### *Discussion*

A central discovery in the current study is that significant lung hypoplasia and airspace enlargement develops when RAGE expression is increased in respiratory epithelium during embryonic development. These compelling data provide intriguing insight into the role RAGE may have in the orchestration of branching morphogenesis during lung formation. Because differences in lung development between RAGE knock

out and wild type mice are imperceptible, our data suggests that while redundant receptors likely compensate for the absence of RAGE, increased availability of RAGE at critical periods of branching morphogenesis permanently impairs normal lung developmental trajectory by potentially hindering terminal cell type differentiation.

Beginning at approximately E9 and continuing throughout gestation, lung morphogenesis relies on precise interplay between differentiating foregut endoderm and surrounding splanchnic mesenchyme. Numerous cell signaling pathways function during processes of cell differentiation along the proximal-distal pulmonary axis and misregulation of associated genetic programs cause severe developmental abnormalities (6,36-40). For the purposes of considering the effectiveness of the current RAGE transgenic mouse model, SP-C expression is observed at E10.5, and may even be discernible as early as E9 (41). Because there still appears to be distal lung epithelial cell populations in the RAGE transgenic mouse lung exposed to dox throughout gestation, initial branching of the main stem bronchi and resulting lobe formation may not be completely perturbed. This determination is reinforced by data showing no difference between wild type mouse lung and lungs from RAGE transgenic mice fed dox from E0-8.5 or E8.5-12.5 (Figure 4). Common events in branching morphogenesis and budding occurring after E12 normally include expansion of both conducting airways and acinar spaces in the periphery (11), therefore RAGE augmentation appears to limit the formation and differentiation of secondary and tertiary branching. These data are

similar to those involving the deletion of *Titf-1*, *Fgf10*, and *FgfRIIIb*, which each result in absent lung lobes and a near complete arrest of branching (36,38,42,43). Evidence that ATI and ATII cells persist in RAGE transgenic mouse lung reveals an intermediate phenotype; however, and demonstrates that RAGE over-expression throughout gestation leads to impaired commitment and/or viability of cells necessary in the derivation of peripheral lung structures.

Because the data reveal abnormal concentrations of ATI and ATII cells in lungs that over-express RAGE, increased availability of RAGE possibly influences the regulation of terminal cell differentiation involved in the formation of sufficient ATI and ATII cells. Such initial dysregulation therefore likely occurs at a point earlier in lung developmental sequelae than terminal cell differentiation in the respiratory compartment. Previous research has revealed that RAGE may function in stabilizing the alveolus by enhancing cell spreading and adherence of alveolar epithelial cells to collagen-rich surfaces such as those observed in basement membranes (21). Related work also suggests that RAGE functions in mediating cellular transition from ATII to ATI cells commonly observed during pulmonary development (21). The roles of RAGE in ATI cells, which cover more than 95% of the respiratory surface, therefore contributes to the development of an extremely thin and expansive phenotype, presumably necessary to ensure effective bi-directional gas exchange. The data presented in the current investigation show that with only a modest increase in RAGE expression

(Figure 2) during late stages of lung organogenesis, significant lung hypoplasia results. The experiments demonstrate that RAGE up-regulation may alter a homeostatic balance between discrete distal lung cell populations (Figure 6) and instability of the alveolar compartment as shown by electron microscopy (Figure 7). In particular, the ultrastructural analysis depicted in this investigation clearly reveal disrupted basement membrane and cell integrity suggesting possible increased synthesis and activation of matrix metalloproteinases (MMPs) and pro-apoptotic molecules (44-46). Because pro-inflammatory mediators can influence apoptosis of structural cells in the lung and secretion is considered an upstream event in the pathogenesis of lung hypoplasia and bronchopulmonary dysplasia (47-50), elucidation of potential RAGE-mediated imbalances between apoptosis and proliferation in the distal lung is necessary.

The current mouse model induces the expression of full length RAGE capable of RAGE signaling, therefore RAGE transgenic mice likely distort the ratio between RAGE and its decoy receptor, soluble RAGE (sRAGE), formed by alternative splicing (51). Increased availability of RAGE capable of signal transduction therefore pronounces the effects of RAGE signaling. Because RAGE signaling sustains inflammation (52), promotes apoptosis (53), and may stagnate terminal cell proliferation and differentiation (21,54), persistent RAGE expression may exacerbate developmental events that contribute to hypoplastic conditions leading to respiratory distress.

In summary, susceptibility to impaired branching morphogenesis is a feature of premature lung disease such as bronchopulmonary dysplasia and persistent airspace enlargement that causes respiratory distress. Our ability to imitate this deleterious phenotype via the expression of RAGE, a cell surface pattern recognition receptor, significantly elevates the role of RAGE biology in lung development. Additional studies that focus on the role of increased RAGE availability and the mechanisms leading to pulmonary inflammation, diminished alveolar epithelial cell viability, and impairment of extracellular matrix/basement membrane durability are underway and may plausibly lead to the identification of targets for intervention. While the results presented herein relate to the context of RAGE up-regulation during highly plastic developmental milestones, increased RAGE expression must also be characterized in the adult mouse. Ongoing preliminary studies in our lab expand the pathobiological understanding of increased RAGE expression in the adult. Research reveals that RAGE transgenic mice administered dox for 30, 60, and 90 days commencing on PN20 (a period associated with the completion of alveologenesis) results in loss of parenchymal tissue and enlarged respiratory airspaces. It is therefore necessary that additional studies that seek to identify downstream targets of increased RAGE expression and possible endogenous ligand(s) responsible for orchestrating RAGE-mediated alterations in lung physiology continue.

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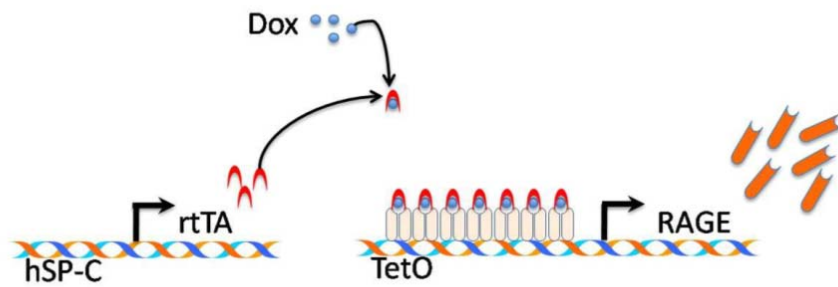
*Figures*

Figure 1 Doxycycline-inducible expression of RAGE in double transgenic mice. The rtTA protein was expressed using the human SP-C (hSP-C) promoter in respiratory epithelium. In the presence of doxycycline (dox), rtTA activates the expression of RAGE by lung epithelium.

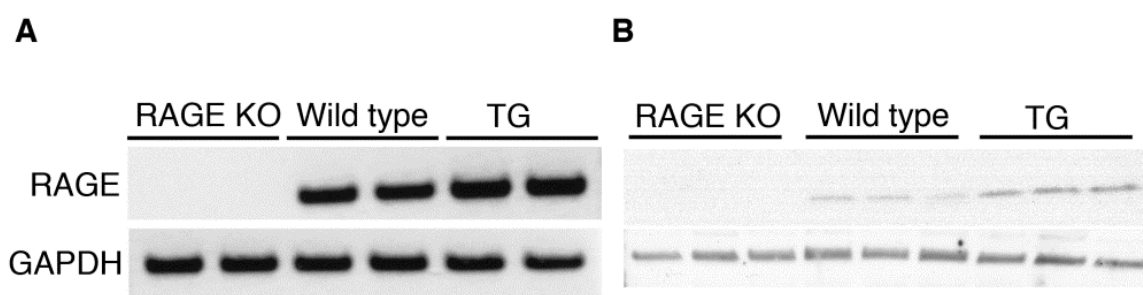


Figure 2 RAGE transgenic mice have increased pulmonary RAGE expression. Representative semi-quantitative RT-PCR (A) and immunoblotting (B) revealed undetectable RAGE expression in RAGE knock out mouse lung and increased expression of RAGE in RAGE transgenic mice (TG) compared to wild type controls. Densitometry revealed a 60 to 70% increase in RAGE mRNA expression and approximately 100% increased RAGE protein expression when band densities from wild type mouse lungs were averaged and normalized to 1. GAPDH was used as a loading control and representative experiments are shown.



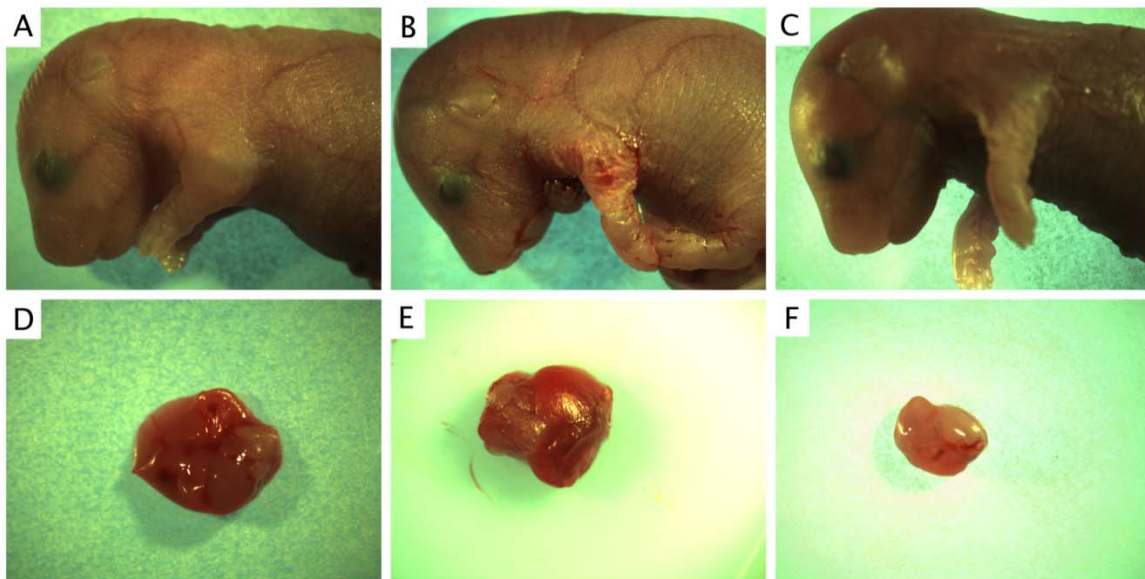


Figure 3                      Decreased lung size from RAGE transgenic mice. Representative imaging of lungs from E18.5 RAGE transgenic mice (C) were smaller and more translucent when compared to lungs from RAGE knock out (A) and wild type (B) mice. Average lung weights  $\pm$  S.D. from at least three mice per group are provided. All images are at 7X magnification and scale bars represent two mm.

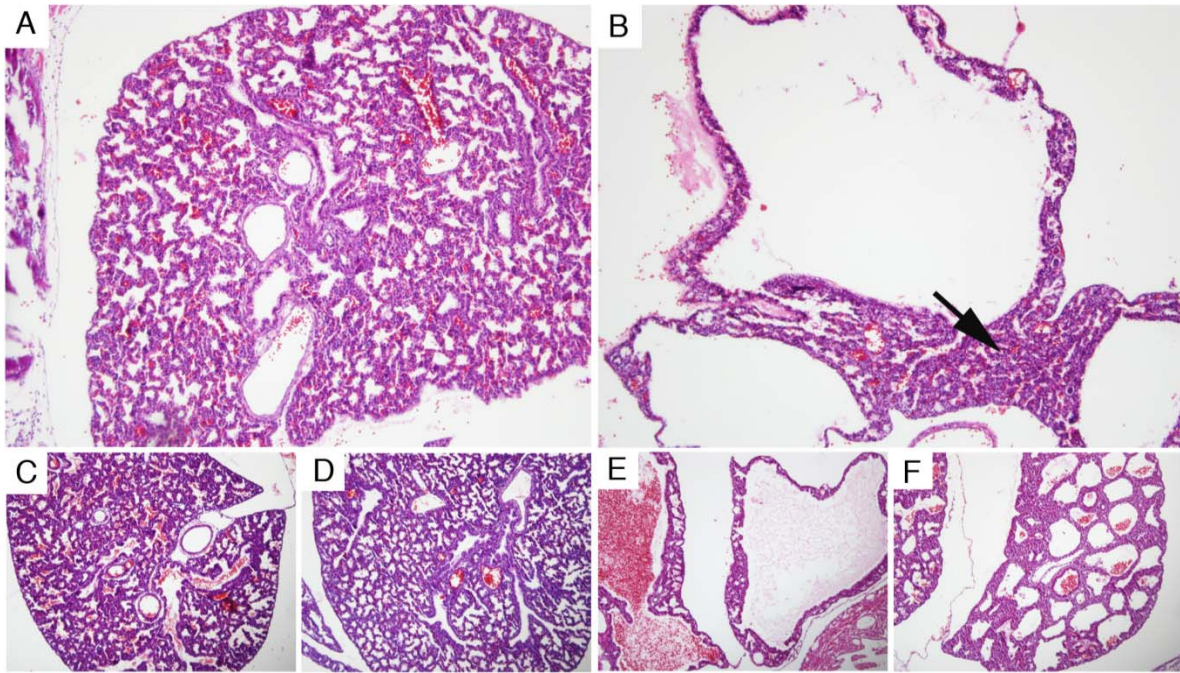


Figure 4 Lung hypoplasia in RAGE transgenic mice. H&E preparations revealed significant lung hypoplasia in RAGE transgenic mice exposed to dox from E0 to sacrifice date at E18.5 (B, asterisks) compared to dox-exposed wild type controls (A). Only sporadic areas of lung parenchyma in RAGE transgenic mice appeared normal (B, arrow). Wild type (C) and RAGE TG mice (D) without dox administration appeared normal. Dox administration of RAGE transgenic mice from E0-8.5 (C), E8.5-12.5 (D), E12.5-15.5 (E), and E15.5-18.5 (F) revealed that RAGE over-expression from E12.5-15.5 is sufficient to induce significant hypoplasia similar to dox exposure throughout embryogenesis (asterisk) and that RAGE over-expression from E15.5-18.5 induces an intermediate phenotype characterized by large conducting airways that extend into the lung periphery (H). All images are at 100X original magnification and scale bars represent 500  $\mu$ m.

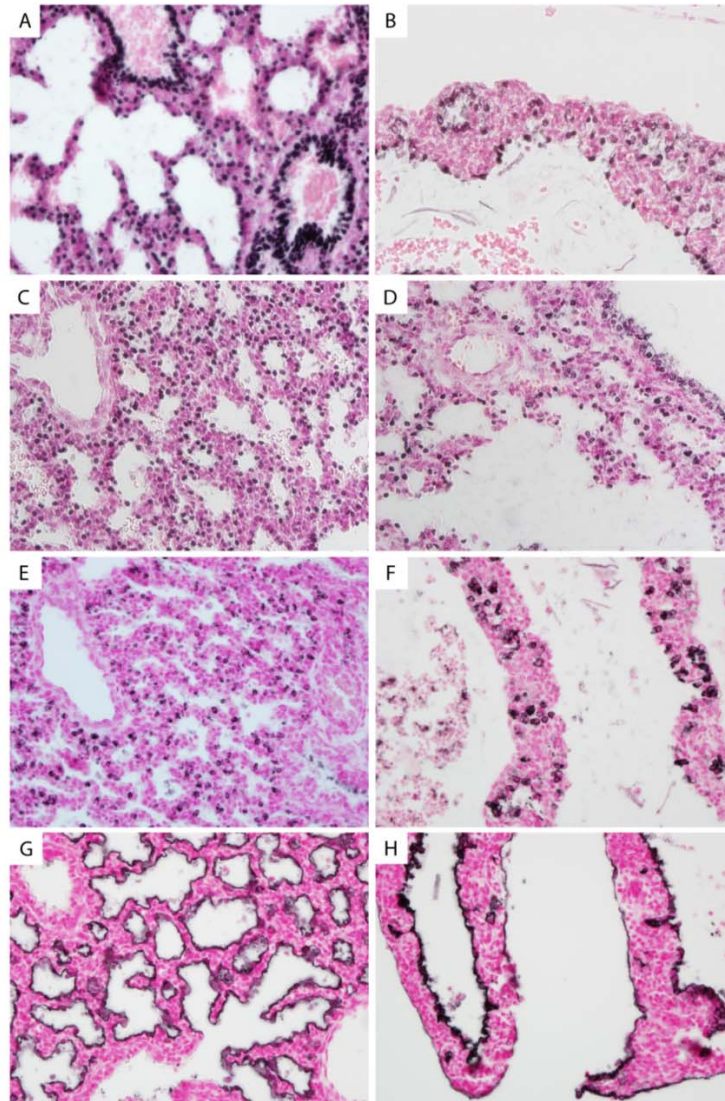


Figure 5 Altered distribution of pulmonary cell types in RAGE transgenic mice. Immunostaining for epithelium-specific markers FoxA2 (A and B), TTF-1 (C and D), proSP-C (E and F), and T1- $\alpha$  (G and H) was performed on sections from E18.5 mouse lung exposed to dox from E0-18.5. FoxA2 intensely identified distal lung epithelium in wild type mouse lung (A) but was detectibly diminished in RAGE transgenic mice (B). TTF-1 staining was diffusely identified in peripheral respiratory epithelium in wild type mice (C) and noticeably reduced in RAGE transgenic mice (D). While the quantity of ATII cells that stain positive for proSP-C were not perceivably different in RAGE transgenic mice (F) compared to wild type mice (E), individual ATII cells appeared to have increased proSP-C immunoreactivity (arrows). ATI cell identification via T1- $\alpha$  staining revealed consistent ATI cell localization throughout the respiratory compartment in wild type mice (G) compared to significant reductions in ATI cell quantity in RAGE transgenic mice (H). All images are at 400X original magnification and scale bars represent 50  $\mu$ m.

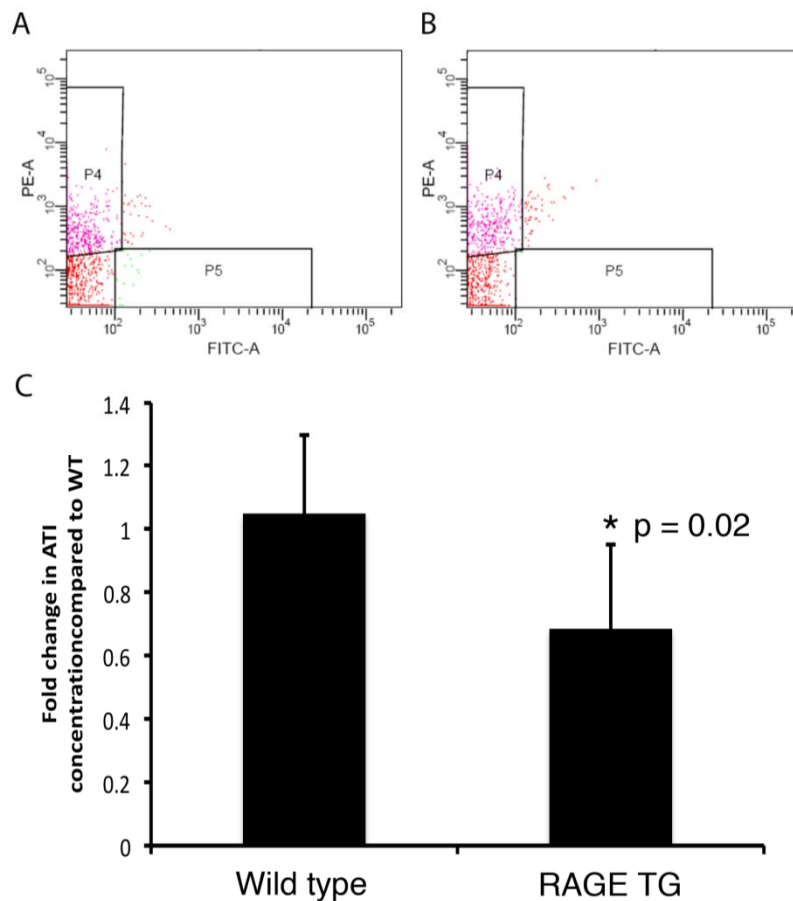


Figure 6 RAGE transgenic mice have significantly diminished alveolar epithelial cell quantities. Cell sorting analysis revealed a significant decrease in ATI and ATII cell populations in whole lungs isolated from RAGE transgenic (B) compared to wild type mice (A). Phycoerythrin-T1- $\alpha$  antibody complexes (PE-ATI) and fluorescein isothiocyanate-proSP-C antibody complexes (FITC-ATII) identify populations of ATI and ATII cells, respectively. When percentages of ATI and ATII cells in dox-exposed control mice were normalized to 1, a significant decrease in cell quantity was observed in RAGE transgenic mice following dox administration (C). Images are representative of six mice per group,  $p = 0.02$  and  $0.05$ .



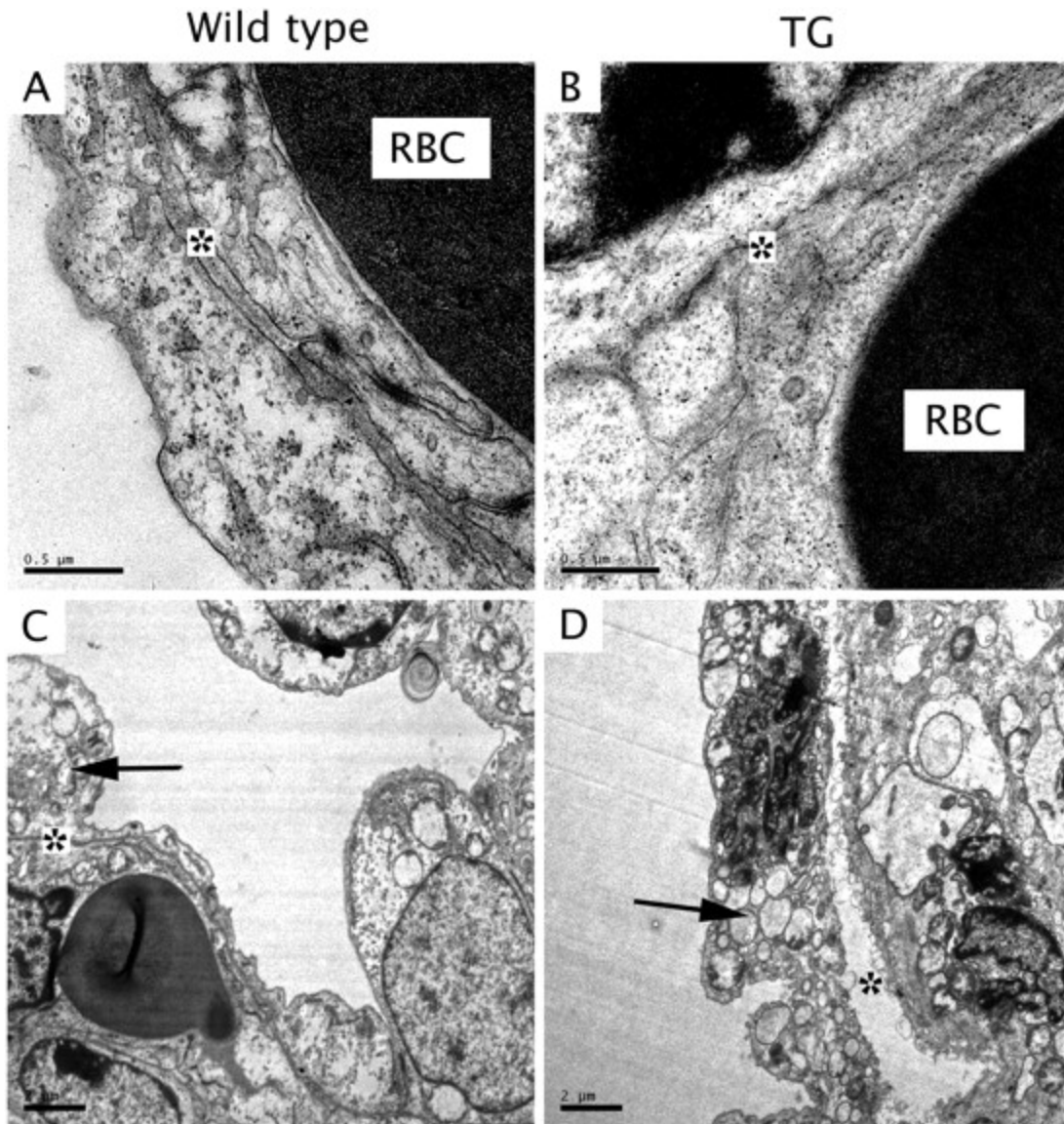


Figure 7 Ultrastructural analysis of the respiratory membrane in RAGE transgenic and wild type mice. Electron microscopy revealed structurally sound respiratory membranes in wild type mice comprising discrete fused basement membranes (A, asterisk) whereas basement membranes in RAGE transgenic mice (TG) were fragmented and disarranged (B, asterisk). ATI cells in wild type mice appropriately adhered to the basement membrane (C, asterisk) and maintained normal cellular morphology (C, arrow); however, ATI cells in RAGE TG mice appeared to be separating from lung parenchyma (D, asterisk) and had a bleb-like intracellular appearance associated with cell destabilization was observed (D, arrow). Images are 6,500X (A, B) or 2,100X (C, D).

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## CHAPTER 2

EMBRYONIC OVER-EXPRESSION OF RAGE BY ALVEOLAR  
EPITHELIUM INDUCES AN IMBALANCE BETWEEN  
PROLIFERATION AND APOPTOSIS

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**Running Title:** Apoptosis in RAGE over-expressing lung

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*Abstract*

Receptors for advanced glycation end-products (RAGE) are multi-ligand cell surface receptors highly expressed in the lung that contribute to alveolar epithelial cell differentiation during embryogenesis and the modulation of pulmonary inflammation during disease. When RAGE is over-expressed throughout embryogenesis, severe lung hypoplasia ensues, culminating in perinatal lethality. However, possible mechanisms that lead to the disappearance of pulmonary tissue are unclear. A time course of lung organogenesis commencing at E12.5 demonstrated that increased RAGE expression primarily alters lung morphogenesis beginning at E16.5. TUNEL immunohistochemistry and immunoblotting for active caspase-3 confirm a shift toward apoptosis in lungs from RAGE over-expressing mice when compared to wild type controls. This observation supports previous work wherein electron microscopy identified cellular blebbing of alveolar epithelium in embryonic RAGE over-expressing mice. Assaying for NF- $\kappa$ B also revealed elevated nuclear translocation in lungs from transgenic mice compared to controls. An RT-PCR assessment of genes regulated by NF- $\kappa$ B demonstrated elevated expression of Fas ligand, suggesting increased activity of the Fas-mediated signal transduction pathway in which ligand-receptor interaction triggers cell death. These data provide evidence that RAGE expression must be tightly regulated during homeostatic organogenesis. Furthermore, additional elucidation of RAGE signaling potentially involved in cell cycle abnormalities may provide insight into the progression of RAGE-mediated lung diseases.

Key Words: RAGE, Apoptosis, Lung, Transgenic

## *Introduction*

The vertebrate lung is a complex branching organ comprised of numerous specialized cell types programmed by a host of intricate signaling and transcriptional control mechanisms. During the pseudoglandular stage, undifferentiated cells become ciliated columnar epithelial cells, non-ciliated Clara cells, goblet cells, and neuroendocrine cells that line the conducting airways. Distally, parenchymal cells in the eventual respiratory compartment differentiate to become alveolar type II (ATII) and I (ATI) cells. Tight regulation of complex interrelated processes that control cell growth and differentiation is therefore critical in order to form a viable lung (1).

The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin 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 (2-4). RAGE is a dynamic receptor capable of binding ligands with variable, yet related tertiary structures (5). Initially characterized and named for its ability to bind non-enzymatically glycosylated macromolecules, advanced glycation end-products (AGEs), RAGE also binds a myriad of other molecules including pro-inflammatory cytokine-like mediators of the S100/calgranulin family, amyloid  $\beta$ -fibrils, and high mobility group box 1 (HMGB-1) (2, 6, 7).

RAGE is expressed in many cell types including endothelium, smooth muscle, macrophages and epithelium, however it is most abundantly expressed by alveolar epithelium (8). While some evidence suggests minimal expression by ATII cells (5, 9), differentiated ATI cells are the predominant RAGE expressing cells in the lung (10). RAGE is clearly detected in pulmonary cells during lung neonatal development (11), suggesting possible contributions to organogenesis and homeostasis. RAGE has been shown to promote epithelial cell adherence and dynamically influence cell spreading, implying a possible role in ATII to ATI cell transition (12). Moreover, recent findings have suggested a role for RAGE in cytoskeletal disruption, revealing possible functions related to cell plasticity and survivability (13). Expression of RAGE is also up-regulated in cases of injury and disease. For example, full-length membrane bound RAGE (mRAGE) is increased in oxidative asthma (14), smoke related chronic obstructive pulmonary disease (15-18), acute respiratory distress syndrome (19), and acute lung injury induced by radon, cytokines, hyperoxia, and lipopolysaccharide (20, 21). Soluble RAGE (sRAGE) capable of binding ligand but unable to transduce intracellular signaling is also increased in a subset of these diseased conditions (19, 21-23). However, precise roles for RAGE during lung development remain enigmatic.

Because RAGE binds specific ligands, it is linked to several downstream signaling pathways. For example, RAGE activation can occur in response to damage-associated molecular patterns (DAMPs) via the NF- $\kappa$ B pathway (24). Research also

identifies pro-inflammatory RAGE signaling involving NF- $\kappa$ B following Ras activation in cells and tissues exposed to tobacco smoke (25). Key DAMP molecules are members of the S100/calgranulin family and HMGB-1, both of which efficiently bind RAGE (26, 27). These molecules, normally secreted following apoptotic or necrotic events, can serve as trophic factors in low concentrations or enhance inflammatory/cell death responses at higher concentrations. Downstream gene products produced through RAGE signaling include NF- $\kappa$ B, Cox-2, IL-1 $\beta$ , and TNF- $\alpha$  (28). Because the promoter for the RAGE gene contains NF- $\kappa$ B binding sites (29) and is regulated by Egr-1 in cases of tobacco-related disease (30), a possible auto-inflammatory loop may be triggered suggesting a central role for RAGE in tissue loss observed in chronic disease states.

In the current study, lung developmental progression was evaluated in lungs that use the promoter for surfactant protein C (SP-C) to conditionally over-express RAGE in the respiratory compartment. Data reveal that the SP-C promoter is active as early as E10 in primitive pulmonary epithelial cells in both the proximal and distal lung (31 and Supplemental Figure 1). However, use of the SP-C promoter to drive RAGE expression did not induce anomalies until E16.5, which progressively worsened resulting in severely diminished lung formation at E18.5. Previously published data revealed that distal lung structures exhibited marked signs of nuclear fragmentation and cellular blebbing when viewed by electron microscopy (31), both of which are typical hallmarks of apoptosis. Our work involving immunohistochemical TUNEL

procedures validate apoptotic characteristics evident in electron microscopy and revealed significant increases in cells that express blunt DNA fragments. Immunoblotting of lung lysates also confirmed active apoptosis via a caspase-3 mediated pathway. NF- $\kappa$ B, and a potent pro-inflammatory downstream product, Fas ligand (FasL), were both up-regulated in the lungs of RAGE TG mice, indicating that cellular apoptosis was likely activated via extrinsic pathways. Taken together, these data suggest a homeostatic role for RAGE in normal physiological environments and provide mechanistic insights into deleterious processes that are activated when RAGE is over-expressed during development and disease.

### *Methods and Materials*

#### Mice

Mice were in a C57Bl/6 background (Jackson Laboratories, Bar Harbor, ME). Two transgenic lines (hSP-C-rtTA and TetO-RAGE) were crossed to create doxycycline (dox, 625 mg/kg; Harlan Teklad, Madison, WI)-inducible mice that up-regulate RAGE in alveolar epithelium. RAGE transgenic (TG) mice had both transgenes and controls were age-matched single or non-transgenic littermates. Dams were fed dox before conception until sacrifice date at embryonic day (E) 12.5 to E18.5. Tail biopsies were genotyped as previously described (32). Mice were utilized in accordance with protocols approved by the IACUC at Brigham Young University.

### Histology and Immunostaining

Lungs from E15.5-E18.5 RAGE TG and wild type mice (n = 3 per group) were processed, embedded and sectioned (33). Lungs were stained with hematoxylin and eosin for general lung morphology at 24 hour intervals beginning at E12.5. Immunohistochemistry used antibodies for RAGE (AF1145, 1:500, R&D Systems, Minneapolis, MN) and proliferating cell nuclear antigen (PCNA, SC-7907, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA). The TdT-FragEL DNA Fragmentation Detection Kit (Calbiochem, Rockland, MA) was used to immunohistochemically evaluate apoptosis.

### Immunoblotting

Lungs from E15.5-E18.5 RAGE TG mice and wild type controls were homogenized in RIPA buffer supplemented with protease inhibitors (Thermo Scientific, Pittsburg, PA). To ensure equal loading of protein samples, quantification of protein concentration was obtained using the BCA Protein Assay Kit (Thermo Scientific). Protein samples (10 µg each) were subjected to immunoblotting using anti-PCNA (1:1000, Santa Cruz), total Caspase-3 (#9662, 1:1000, Cell Signaling, Beverly, MA), RAGE (AF1145), and GAPDH (11). Membranes were incubated with appropriate secondary antibodies, detected with ECL-plus (Amersham, Piscataway, NJ) and developed. Band



densitometry used digitized images and the Un-Scan-It software (Silk Scientific, Orem, UT).

#### Assessment of Nuclear NF- $\kappa$ B, FasL and Bcl-2

Nuclear lysates were isolated from lungs from E16.5 RAGE TG and controls (n = 3 per group) using the NE-PER extraction kit (Fisher Scientific). Total nuclear protein was quantified using the BCA technique (32) and total nuclear NF- $\kappa$ B was assessed in nuclear lysates (3  $\mu$ g each) using the ELISA based TransAM p65 NF- $\kappa$ B Transcription Factor Assay Kit (Active Motif, Carlsbad, CA). NF- $\kappa$ B activity in lysates from transgenic mice was reported after normalizing activity in controls to 1. Total RNA from RAGE TG and wild type lungs (n = 3 per group) was isolated from E16.5 animals using the Absolutely RNA Kit (Stratagene, Santa Clara, CA). RNA was quantified and 1  $\mu$ g of each sample was converted to cDNA and assessed using the Mouse NF- $\kappa$ B - Regulated cDNA Plate Array (Signosis, Sunnyvale, CA).

#### Statistical Analysis

For immunohistochemistry, at least eight high-power fields (400X) per slide (n = 3 per group) were randomly imaged and blindly counted PCNA or TUNEL-positive cells were statistically evaluated at each time point using a Student's t test of the means. At least 3 samples were used for immunoblots and cDNA screens per group and a

Student's t test was employed to assess the level of significance at  $\alpha=0.05$ . All values were presented as means  $\pm$  S.D. and values of  $p \leq 0.05$  were considered significant.

## *Results*

### Lung Hypoplasia was Temporally Affected by RAGE Over-Expression During Lung Organogenesis

Immunostaining for RAGE revealed epithelial-specific patterns of RAGE augmentation in the RAGE TG mouse compared to wild type controls (Figure 1). RAGE immunoblotting also confirmed expression in wild type mice as early as E15.5 and significant increases in expression in lung lysates from TG animals (Figure 1I). Previous work demonstrated that embryonic over-expression of RAGE adversely affects lung development, leading to an embryonic lethal phenotype. However, the timing and mechanisms of RAGE-mediated underdevelopment of the lung remained unclear. A progressive time course evaluation of lungs from RAGE TG and wild type control mice indicated that elevated RAGE expression adversely impaired lung morphogenesis beginning at E16.5 (Figure 2I and J), culminating in severely underdeveloped lungs at E18.5 (Figure 2M and N). Histological evaluation revealed that lung development transpired normally from E12.5 through E15.5 in RAGE TG mice (Figure 2B, D, F, and H) when compared to wild type littermates (Figure 2A, C, E, G). After E15.5, a period that coincides with the canalicular stage of pulmonary

development, notable tissue loss occurred and the degree of tissue loss increased as embryogenesis progressed. Wild type and RAGE TG mice in the absence of dox administration histologically appeared similar to dox-fed wild type mice (32) suggesting there were no adverse effects to pulmonary organogenesis due to the transgenes alone or by dox administration.

#### Proliferation was Unchanged in the RAGE Over-Expressing Lung

Because diminished cellular proliferation may contribute to lung hypoplasia, cellular propagation was studied in the context of RAGE over-expression. Immunohistochemistry of lung samples from E15.5 to E18.5 was performed utilizing antibodies against proliferating cell nuclear antigen (PCNA), a marker of cell proliferation that detects cells in the S phase of the cell cycle. As anticipated, numerous proliferating cells were positively stained for PCNA at each time point in both wild type and RAGE TG lungs (Figure 3A). After the percentage of PCNA positive cells at each time point was obtained and evaluated, differences between the groups were not statistically significant (Figure 3B), even though the sum of all PCNA-positive cells trended downward as pulmonary development progressed (Supplemental Table).

In order to further assess qualitative PCNA expression (Figure 3A), immunoblotting for PCNA was performed. Lysates from E15.5-E18.5 lungs resected from RAGE TG mice revealed diminishing PCNA expression when compared to lungs

from age-matched controls (Figure 3C and D); however PCNA expression was not significantly different between the two groups of mice (Figure 3C and D).

#### Apoptosis was Elevated in the RAGE Over-Expressing Lung

In order to assess whether increased apoptosis contributed to severe lung hypoplasia observed in RAGE over-expressing mice, a quantitative TUNEL staining approach was used. The number of positively identified apoptosing cells via TUNEL staining was significantly increased in lungs from RAGE TG mice compared to wild type controls at each day from E15.5 through E18.5 (Figure 4A and B). As revealed by representative immunostains (Figure 4A), cells undergoing apoptosis were sporadically detected in lung parenchyma. In addition to the immunohistochemistry, immunoblotting for caspase-3 was performed in order to detect both extrinsic and intrinsic apoptotic pathways. RAGE TG lungs had a significant increase in the amount of total (35 kDa) and active caspase-3 (17 and 19 kDa forms) compared to wild type littermates (Figure 4C). Densitometry of the bands showed a significant increase in the amount of active caspase-3 in RAGE TG mice at E15.5 compared to wild type E15.5 lungs (Figure 4D). While the amount of active caspase-3 in RAGE TG mouse lungs declined as E18.5 approached, each time point was characterized by significantly increased active caspase-3 expression when compared to controls (Figure 4D).

## Nuclear Translocation of NF- $\kappa$ B and Expression of its Targets were Misregulated in the RAGE Over-Expressing Lung

Total nuclear protein was isolated from whole lung lysates procured from E16.5 mice and assessed for NF- $\kappa$ B activity because of the severity of hypoplasia observed at that time (Figure 2J). After normalizing nuclear NF- $\kappa$ B expression in the wild type animals to one, ELISA revealed a significant increase in the activation and nuclear translocation of NF- $\kappa$ B in lungs from RAGE TG mice (Figure 5A). Because NF- $\kappa$ B activation was elevated in RAGE TG mice, two NF- $\kappa$ B regulated genes that function in apoptotic pathways were screened. Compared to controls, RAGE TG lungs expressed Fas ligand (FasL) at levels approximately two-fold higher at E16.5 (Figure 5B). Significantly increased FasL, known to promote apoptosis following interaction with its receptor (FasR), suggests that extrinsic apoptotic pathways were activated in RAGE TG mice. Interestingly, expression of Bcl-2, an anti-apoptotic factor, was significantly diminished in RAGE TG mouse lungs at E16.5 (Figure 5C). Additional data further suggest that abnormal levels of NF- $\kappa$ B, FasL, and Bcl-2 expression in the RAGE TG mouse after E16.5 continue to adversely affect lung development (not shown).

### *Discussion*

In the current study, we used a gain-of-function methodology to determine mechanisms that underlie lung hypoplasia resulting from RAGE signaling. This

research is a natural extension of our previous work wherein we demonstrated that RAGE up-regulation caused pulmonary underdevelopment via hindered cytodifferentiation and possible impairment of branching morphogenesis (32). Through the utilization of a tetracycline-inducible respiratory epithelial cell-specific RAGE over-expressing mouse, we discovered the precise timing of lung hypoplasia and airspace enlargement directly attributable to RAGE augmentation in the alveolar compartment during embryonic development. Furthermore, in spite of possible compensation via proliferation of respiratory epithelium during the pseudoglandular period of development, elevated apoptosis of differentiating epithelial cells during the late pseudoglandular and canalicular periods was a significant cause of irreversible lung simplification.

Our finding that general lung histology in RAGE TG mice was indistinguishable compared to age-matched controls through E15.5 (Figure 2) suggested that early signaling events transpiring between foregut endoderm and surrounding splanchnic mesoderm were unaffected. In particular, mesenchymal FGF-10 (34), endodermally derived FGF-R2 (35), SHH/GLI 2,3 (36), and retinoic acid receptors (RARs) (37) each play important roles during early tracheal-pulmonary morphogenesis. Because lung organogenesis appeared normal through E15.5, our data reveal that these early signaling molecules are likely unaffected by RAGE over-expression, despite detection of RAGE as early as E13.5 (Figure 1). Despite the lack of an early phenotype, the

expression of thyroid transcription factor 1 (TTF-1), a critical early lung transcriptional regulator, is decreased in RAGE TG mice (32). TTF-1 is known to interact with a host of factors necessary to lung maturation (1), so it is possible that later prenatal events such as surfactant homeostasis, vasculogenesis, host defense, fluid homeostasis, and inflammation mediated by TTF-1 contributed to impaired lung formation in the RAGE TG mouse.

A combination of TUNEL staining and immunoblotting for caspase-3 led to the discovery that apoptosis was significantly elevated in RAGE TG mice in the late pseudoglandular (E15.5), canalicular (E16.5-E17.5), and early sacular periods of lung development (E18.5). In regards to caspase expression, our data supports previous research that focused on lung apoptosis and the correspondence between elevated total and active caspase-3 expression (38). The detection of apoptosis during these periods also corroborated findings by Kresh et al. (39), who studied the ontogeny of apoptosis in embryonic rat lungs. Specifically, epithelial cell apoptosis was detected from the canalicular stage and beyond, suggesting the need to precisely control respiratory epithelial cell deletion during normal lung organogenesis (40). Despite the notion that deletion of pulmonary epithelium by apoptosis may be a physiologically significant event in lung remodeling during late stages of gestation, excessive cell death as observed here (Figure 4) likely contributes to deleterious lung tissue loss.

Clear and predictable patterns of lung and respiratory epithelial cell apoptosis

during fetal development exist (38, 41). Such patterns provide opportunities for the elucidation of molecular regulatory pathways that control mechanisms of cell death. One such well-studied pathway involves the recruitment of activated NF- $\kappa$ B. NF- $\kappa$ B was initially identified as a transcription factor in B cells and has since been ubiquitously detected in the cytoplasm of all cell types (42). When stimulated, NF- $\kappa$ B translocates to the nucleus where it regulates the expression of more than 200 genes that influence cell growth, survival, and inflammation (43). Because a significant increase in nuclear NF- $\kappa$ B activity was observed in RAGE TG mice compared to controls (Figure 5A), it appears likely that NF- $\kappa$ B may be an important signaling intermediate downstream of RAGE. This discovery led to the proposal that the Fas/FasL pathway, a cascade directly influenced by NF- $\kappa$ B signaling, may be a modulator of late-gestational apoptosis mediated by RAGE augmentation. The Fas/FasL pathway is a widely distributed apoptosis signal transduction pathway in which ligand-receptor interaction triggers cell death (44, 45). FasL is a type II transmembrane protein belonging to the tumor necrosis factor family (46, 47) that is cleaved by MMP-7 to elaborate ligand. Binding of FasL to Fas receptor (FasR) activates intracellular caspases 8 and 3 and culminates in apoptosis (47, 48). Studies by others show that ATII cells and ATII-like cell lines express Fas (49) and that apoptosis can be induced in these cells by crosslinking agonistic anti-Fas antibodies both *in vitro* and *in vivo* (50-52). Our discovery that FasL was significantly up-regulated in RAGE TG mouse lungs suggests



that the Fas/FasL pathway may be a pivotal mediator of apoptosis in the airway epithelial compartment during the pericanalicular period. Furthermore, because normal RAGE expression elevates during this pericanalicular period, our data may suggest remodeling of pulmonary airspaces via ATII apoptosis may occur at least in part due to RAGE-mediated mechanisms.

An additional observation was that Bcl-2 was down-regulated in the lungs of RAGE TG mice (Figure 5C). The Bcl-2 gene was originally discovered in a follicular B-cell lymphoma where a chromosomal translocation moves the gene into juxtaposition with transcriptional enhancer elements of the immunoglobulin heavy chain locus (53). Bcl-2 is situated upstream of the apoptotic pathway and it provides an important decisional checkpoint against irreversible cellular damage after delivery of a death stimulus (54, 55). Specifically, Bcl-2 is an anti-apoptotic mitochondrial outer membrane permeabilization protein that functions by extending cellular survival via inhibition of a variety of apoptotic deaths (53-59). We observed that Bcl-2 was decreased in RAGE TG mouse lungs at E16.5, a period that coincided with apoptosis potentially mediated by FasL.

In summary, susceptibility to impaired branching morphogenesis and elevated apoptosis are features of premature lung diseases such as neonatal respiratory distress and bronchopulmonary dysplasia (BPD). Because RAGE signaling increases the secretion of pro-inflammatory cytokines (IL-1 $\beta$  and IL-6) and chemokines (MCP-1)

known to be involved in BPD pathogenesis (22, 25, 60, 61), the possibility exists that RAGE, at least in part, contributes to a premature BPD phenotype. Our ability to imitate and extend postcanalicular apoptosis via up-regulation of FasL, a target of NF- $\kappa$ B, through RAGE signaling reveals important RAGE-mediated functions in normal lung development (Figure 6). Abnormal RAGE signaling that influences Fas/FasL pathways should be further studied so that specific contributions to pulmonary architecture and cellular remodeling in the developing lung can be clarified. While the current results relate to the context of RAGE up-regulation during developmental milestones, the apoptotic index stemming from increased RAGE expression in the adult must also be characterized. It is therefore necessary that additional studies continue, which seek to identify downstream targets of increased RAGE expression and possible endogenous ligand(s) responsible for orchestrating RAGE-mediated alterations in lung formation and physiology.

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Figures

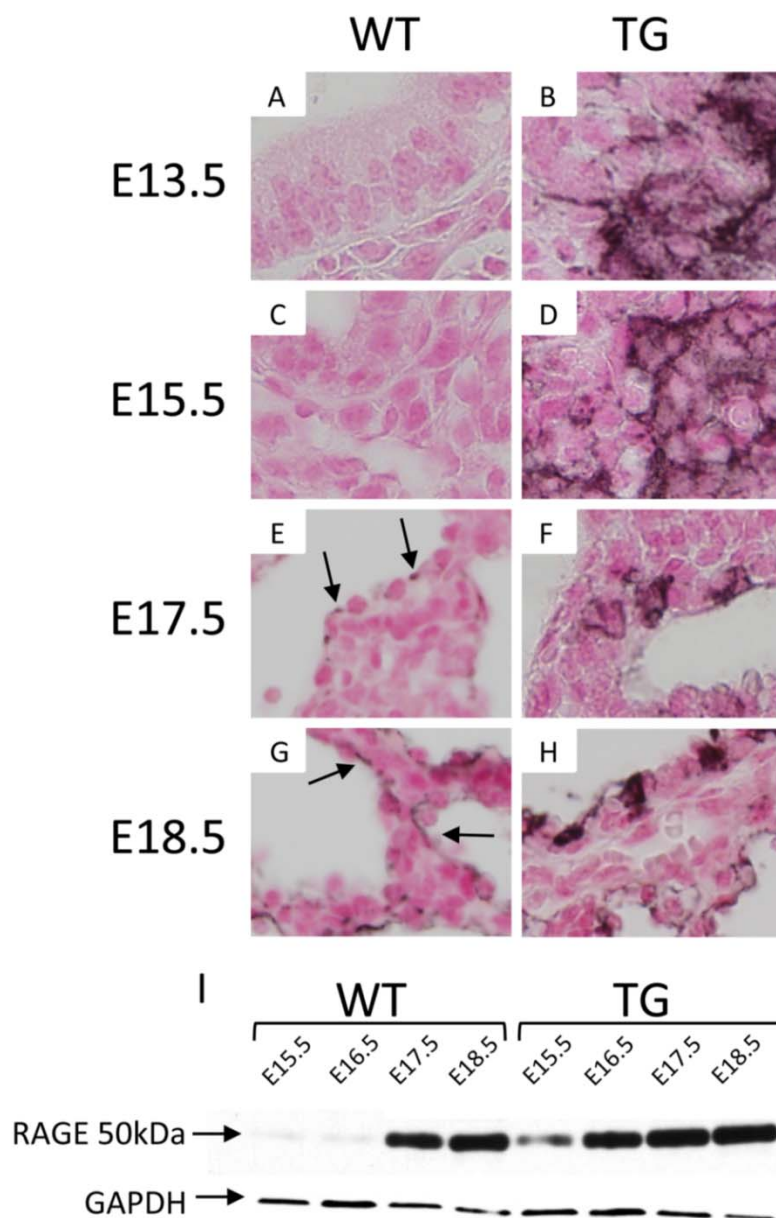
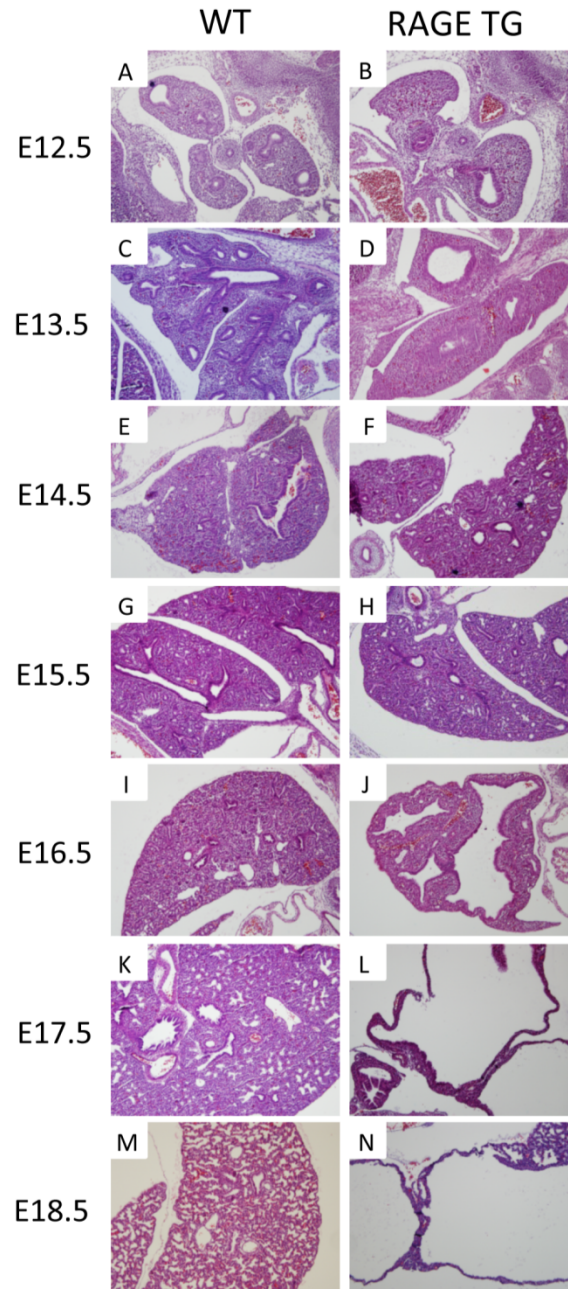
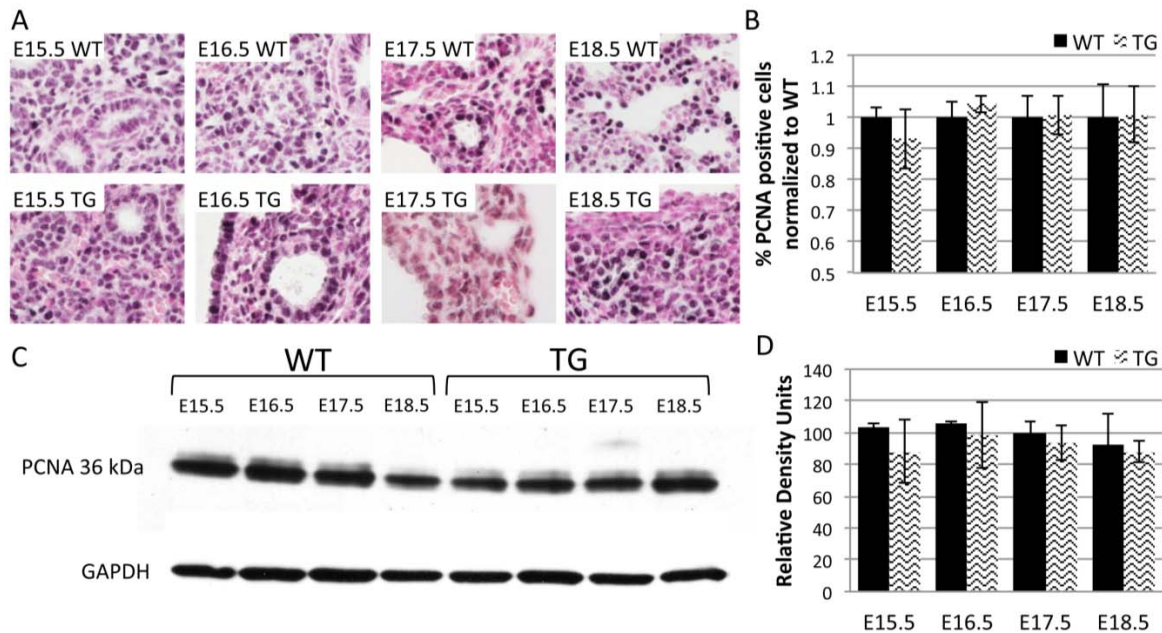


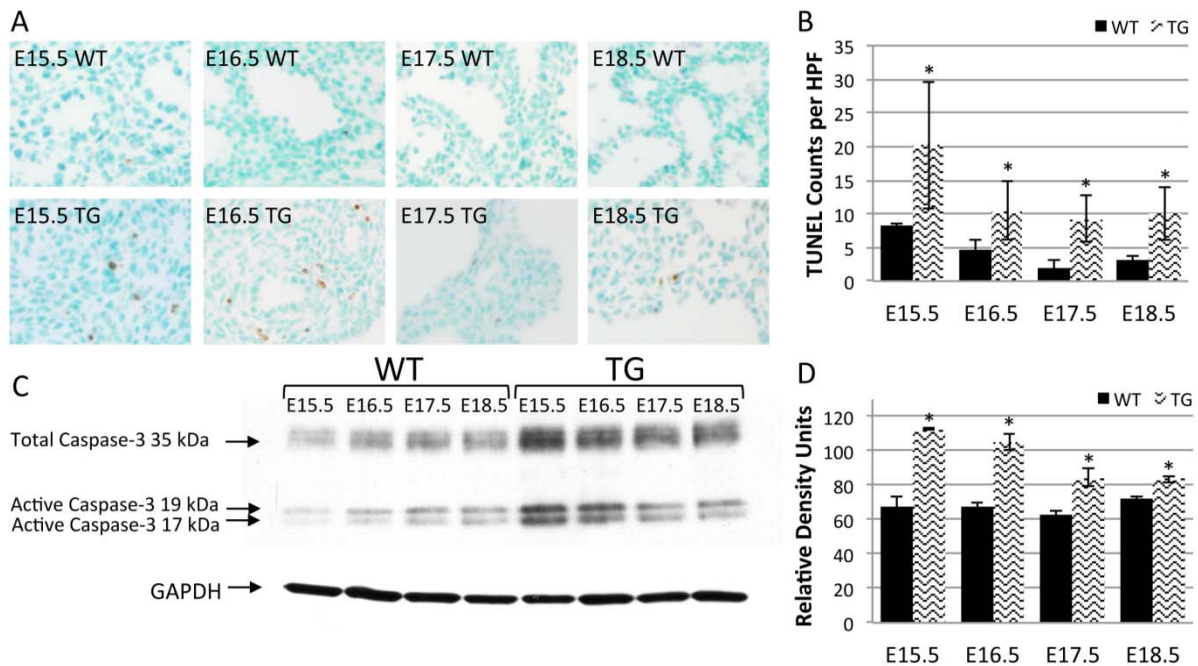
Figure 1 RAGE expression was elevated in embryonic RAGE TG mouse lungs. Compared to wild type mouse lungs with basal RAGE expression (E and G, arrows), immunohistochemistry revealed increased RAGE expression in RAGE TG mouse lungs at E13.5 (B), E15.5 (D), E17.5 (F) and E18.5 (H). Original magnification of representative images was 400X. Immunoblotting for RAGE identified detectable expression as early as E15.5 and elevated expression in lungs from TG mice at from E15.5-E18.5.



**Figure 2** RAGE over-expression altered lung organogenesis beginning at E16.5 and continuing through E18.5. A representative time course of H&E sections revealed standard lung formation through E15.5 in the dox-fed RAGE TG mice (B, D, F, H), compared to dox-exposed wild type counterparts (A, C, E, G). Histology at E16.5 (J) revealed the initial stages of aberrant lung structures, culminating in profound tissue loss at E17.5 and E18.5 in the RAGE TG mouse model (L and N). All images are representative and at 100X original magnification.



**Figure 3** Proliferation was not diminished in RAGE TG lungs. Immunostaining for PCNA from E15.5 to E18.5 demonstrated wide-spread proliferation in both the wild type and RAGE TG lungs (A). Cell counts by blinded individuals were performed to view potential differences in the quantity of proliferating cells. 100 cells were identified and counted in clusters from at least eight random 400X viewing areas. The percentage of positively stained (encompassing both punctate and heavy nuclear staining) cells was determined. The percentage of PCNA-positive cells in RAGE TG lungs was insignificant when compared to the normalized percentage from wild type lungs (B). Immunoblotting for PCNA from whole lung lysates suggested no significant change in PCNA expression from E15.5 in the distal lung (D). A minimum of three animals were evaluated in each experimental group and  $*p \leq 0.05$ .



**Figure 4** Apoptosis was significantly increased in the developing RAGE TG lung. A detectable increase in the number of actively apoptosing cells by TUNEL staining was observed in RAGE TG lungs compared to wild type lungs at all time-points tested (A). TUNEL-positive cells in 400X images were sporadic throughout the distal lung with occasional staining found in cuboidal/columnar cells in the airways of RAGE TG mice. Cell counts performed by blinded individuals demonstrated an increase in the number of positively stained cells at all time-points (B). Immunoblotting confirmed that active apoptosis was occurring in a caspase-3 mediated manner, indicated by a significant increase in the amount of total and cleaved caspase-3 products (17 and 19 kDa) found within lung lysates of RAGE TG lungs (C). Density of the cleaved caspase-3 bands was significantly increased in RAGE TG mouse lungs at all time points (D). A minimum of three animals were evaluated in each experimental group and  $*p \leq 0.05$ .



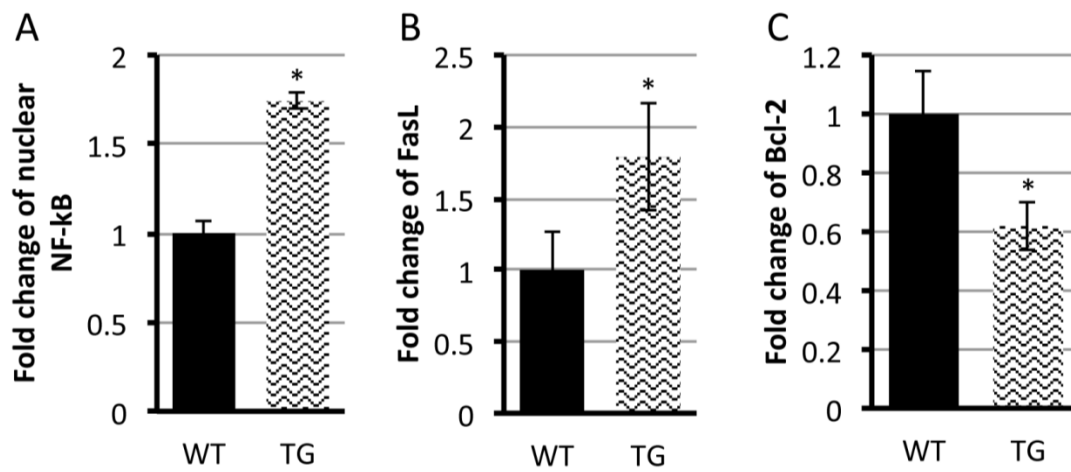


Figure 5 NF- $\kappa$ B and its targets, FasL and Bcl-2, were misregulated in RAGE TG lungs compared to controls. (A) ELISA experiments showed that active NF- $\kappa$ B protein was elevated in nuclear lysates from E16.5 RAGE TG mice compared to age-matched wild type mice. Messenger RNA levels of the pro-apoptotic factor FasL were increased in RAGE TG mice at E16.5 (B) and anti-apoptotic Bcl-2 mRNA was diminished (C) when compared to controls. Measurements for FasL and Bcl-2 were standardized to GAPDH. A minimum of three animals were evaluated in each experimental group and \* $p \leq 0.05$ .

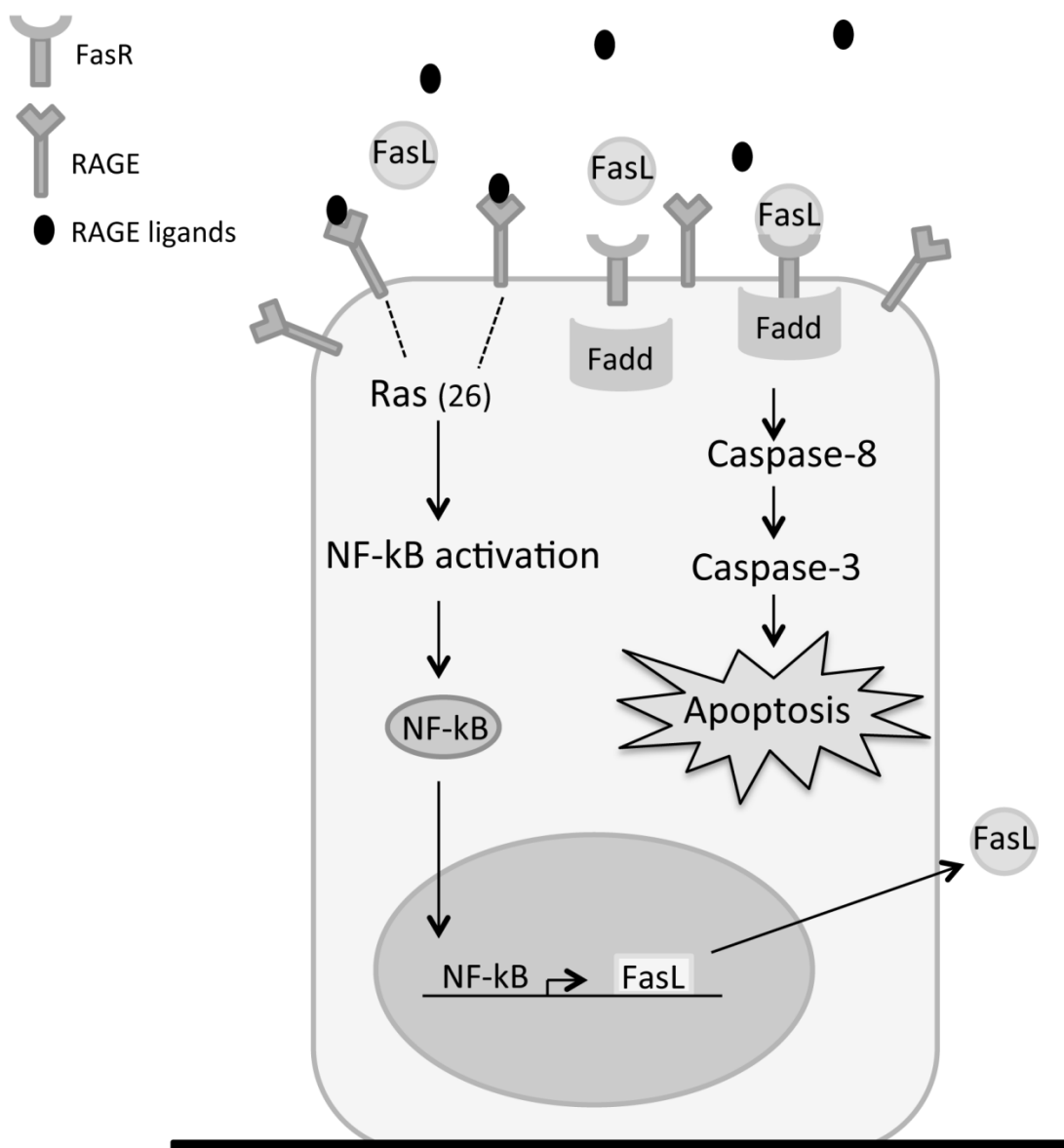
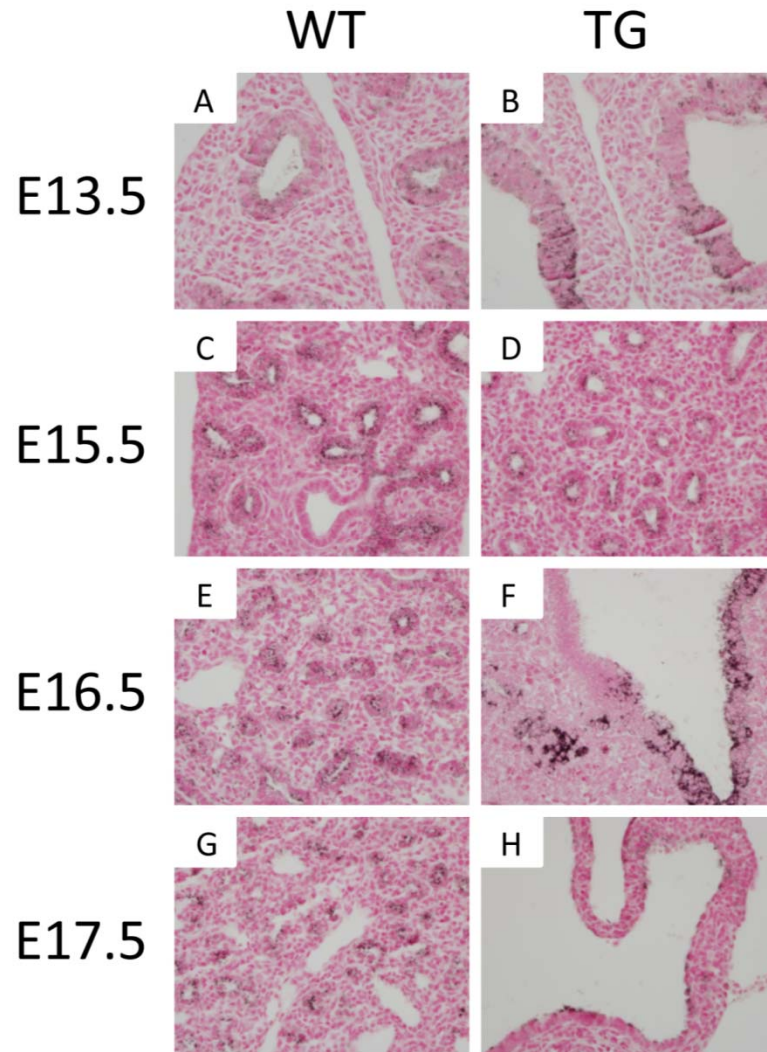


Figure 6 Working model that demonstrates RAGE-mediated pro-apoptotic signaling. RAGE signals through Ras and causes the nuclear translocation of active NF-κB. In the lungs of RAGE TG mice, active caspase-3 and elevated FasL suggest RAGE influences pro-apoptotic signaling involving these molecules during lung formation.



*Supplemental Figure*

Supplemental Figure 1 Lung sections from E13.5-E18.5 RAGE TG mice and wild type controls were immunostained for proSP-C using a rabbit polyclonal antibody (WRAB-76694, Seven Hills Bioreagents, Cincinnati, OH) as already described (33).

*Supplemental Table***Table S1: PCNA-positive cells  
normalized to WT**

	Wild type	TG
E15.5	1.0 ± 0.033	0.932 ± 0.097
E16.5	1.0 ± 0.053	1.044 ± 0.027
E17.5	1.0 ± 0.068	1.007 ± 0.062
E18.5	1.0 ± 0.108	1.01 ± 0.090

Supplementary Table 1

Number of PCNA positive lung cells normalized to WT.

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## GENERAL DISCUSSION

### *RAGE Misexpression Yields Hypoplastic Lungs, Anomalous Cytodifferentiation and Abnormal Branching*

RAGE expression has been identified in the murine lung as early as E17.5 by immunohistochemistry and E16.5 by western blot analysis (1, 2). The work performed herein represents the data when RAGE is temporally and quantitatively misexpressed in the distal developing lung. In these studies, increased RAGE expression in ATII cells led to severely hypoplastic lung at E18.5, a lethal phenotype, when compared to aged-matched littermates. Flow cytometry and immunohistochemistry revealed that distal cell markers were aberrantly concentrated within the lungs at E18.5, namely pro-SP-C (ATII cells) and T1- $\alpha$  (ATI cells). The decrease in these two cell populations hints at the probability that RAGE signaling can affect normal cellular differentiation within the developing lung. Because ATII and ATI cell markers are initially identified during the late pseudoglandular phase and into the canalicular phase (3-5), coincident with pre-RAGE expression in the wild type lung, the data presented here suggest that RAGE expression could limit terminal differentiation of embryonic epithelial cells into distal cell types.

Beyond cellular differentiation, other key milestones required for proper lung formation include branch growth and segmentation of primitive lung buds and branch tips (4, 6). These events give rise adequate numbers of alveoli and eventual surface area

for respiration. Fgf10 is one of the key mesenchymal secreted factors necessary for promoting airway extension and branching (7, 8). Although not explicitly tested here, it is highly probable that RAGE signaling can alter proper branching morphogenesis in the lung. Both direct and indirect activation (through cytokine release) of NF- $\kappa$ B was shown to limit Fgf10 expression in the developing lung by binding to the transcriptional factor Sp1. Due to the inhibition of Fgf10 release from the lung mesenchymal cells, fetal mouse lung explants failed to properly branch as did wild type lungs (9). Because RAGE has been shown to promote NF- $\kappa$ B nuclear localization, it is likely that the RAGE over-expressed lungs demonstrate dilated or improperly branched airways. Ultimately, abnormal RAGE expression underlies the deleterious developmental phenotypes represented here.

#### *Extrinsic Apoptotic Pathways are Activated Via Increased RAGE Expression*

Due to the extreme phenotype witnessed at E18.5, two general possibilities outlining the pathway of altered lung architecture in the RAGE over-expresser were identified: early branching failure extending to dilated airways and/or degeneration of lung tissue following typical pulmonary development. Because of the ties that RAGE has with apoptosis in other organ and cell systems (10-12) and the “sponge-like” phenotype observed in driving RAGE expression from E15.5-E18.5 (13), it was hypothesized that pups exposed to dox throughout embryogenesis would exhibit an increased apoptosis index coincident with late-stage pulmonary degradation. A time-



course viewing of lung formation from E12.5 to E18.5 revealed a pattern congruent with the degeneration model. The data demonstrated here, suggest that RAGE pathways are tied to increased production of FasL, most likely through NF- $\kappa$ B activation, which stimulate extrinsic apoptotic pathways, culminating in cleavage of caspase-3 and eventual cell death.

Proliferation markers were also studied to verify any potential imbalances due RAGE over-expression. Although minimal at best, the RAGE over-expressing lungs showed no significant change to cellular proliferation from wild type lungs. This suggests that a driving force for lung hypoplasticity is due through increased cell death and not decreased cellular expansion. As hinted previously, it is also highly probable that branching morphogenic alterations will be witnessed alongside the increased apoptotic events in these lungs.

#### *Application of the Data and Future Directions*

RAGE detection in the murine lung is highly prevalent peri-natally, post-natally and throughout adulthood (1, 2, 14, 15). However, prior to this study, little was known about the function of RAGE in the developmental context. The data presented here strongly suggest that RAGE signaling is associated with apoptosis in a FasL mediated manner. Previous research has demonstrated that postcanalicular lung development is characterized by extensive thinning of the interstitium and coordinated loss of ATII cells (16-18). The loss of ATII cells has been attributed to both terminal differentiation

into ATI cells and increased ATII cell death through apoptosis (19). Several studies performed by de Paepe et al, highlight the regulative effects on lung development by FasL mediated cellular death. Both rabbit and murine models show timely ATII cell death from E18.5 through PN5. The increase in apoptosis correlated with increased expression of Fas mRNA and Fas protein to the ATII cells of both model lungs. FasL also co-localized with ATII cells during the same peri-natal period (20, 21).

Further studies identified the timely kinetics of FasL expression within the distal lung compartments on ATII behavior. Through an over-expression model, FasL was shown to function pro-apoptotically in the developmental time period between canalicular lung development and just before birth. These mice suffered severe respiratory distress and eventual death. Strikingly, FasL over-expression both before and after birth, resulted in increased ATII cell apoptosis, but also expansion of the ATII population several days post-natally. Limiting FasL over-expression to post-natal periods acted in pro-proliferative fashion (22). These data suggest that that FasL-mediated cell apoptosis acts as a pruning mechanism to diminish ATII cell populations before birth (possible to allow space for ATI cells), but reverses its role post-natally to help re-expand the ATII cell concentration within the lung.

The mechanism by which Fas and FasL are up-regulated post-canalicularly, however, is still not understood. The work provided in this study regarding pulmonary RAGE proposes a hypothesis in which homeostatic RAGE signaling promotes timely

increases in ATII cell Fas and FasL expression in order to finely tune distal lung development. Because RAGE expression in a wild type lung is present shortly before the increase in death signals, and because RAGE signaling has been shown to promote FasL mediated cell death, the proposed hypothesis fits the pruning model described. Isolation and culturing of primary ATII cells from wild type, RAGE knockout and RAGE over-expressing lungs should provide opportunities to study the pathways involved that control FasL release and Fas plasma membrane localization.

The possible control of cell death by RAGE also promotes questions regarding excessive cell death in pathological or abnormal developmental states where RAGE has been found to be up-regulated. Bronchopulmonary dysplasia (BPD) is a chronic lung condition which generally affects preterm babies either through prenatal inflammation and/or mechanical ventilation. Nearly 12.5% of pregnancies in the United States are considered preterm, and of that, 43% are affected by BPD (23). BPD is evident by increased alveolar air space and decreased alveoli number with simultaneous oxidative stress, sustained inflammation, anti-protease imbalance and increased apoptosis (24, 25). BPD pathogenesis also involves the increased secretion of pro-inflammatory cytokines (IL-1 $\beta$  and IL-6) and chemokines (MCP-1) (26-29). The transcription factor NF- $\kappa$ B has also been shown to be a crucial mediator of BPD (23). However the control mechanisms involved in BPD pathogenesis is still unclear.

RAGE pathways have been shown to regulate the control of cytokine/chemokine secretion involved in BPD progression including IL-1 $\beta$ , IFN- $\gamma$ , IL-6, MCP-1 and other known progression factors including MMP-9 (26-29)(unpublished data Reynolds). The data proposed here show that RAGE can regulate NF- $\kappa$ B activation within lung morphogenesis and that its over-expressed deleterious effects are concurrent with “preterm” timing. In addition, targeting of RAGE has been shown to protect against hyperoxia induced lung injury (30), suggesting that its availability may promote hyperoxia induced lung injury and promotion of BPD due to mechanical ventilation for preterm infants. Continued testing using the RAGE transgenic mouse should shed more light on the possible contributions of RAGE to a BPD phenotype.

Similar mechanisms in place that regulate BPD are also implicated in other chronic lung diseases including acute respiratory distress syndrome, asthma and chronic obstructive pulmonary disease (COPD) (31). RAGE has been shown to be drastically up-regulated in these pathologies (32-34). It is likely that the pathways implicated in the developmental work performed here are also employed in adulthood lung diseases. Work utilizing the RAGE transgenic mouse is already in place (unpublished data Reynolds), suggesting chronic RAGE up-regulation in ATII cells of mature lungs can imitate an emphysematous phenotype even in the absence of cigarette smoke, an exogenous ligand of RAGE (27).

While the work performed here shows for the first time a mechanism for pulmonary cell death within the developing lung, further testing is needed to elucidate the precise mechanisms that control apoptosis of ATII cells upon RAGE up-regulation. This model may be of considerable use for study of development and adult pathologies within the lung.

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## CURRICULUM VITAE

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## Education

Master of Science, Physiology and Developmental Biology – Expected June 2012

Brigham Young University, Provo, Utah, USA

Thesis: Characterization of altered epithelial cell turnover and differentiation in embryonic murine lungs that over-express receptors for advanced glycation end-products (RAGE).

Advisor: Dr. Paul R. Reynolds, Assistant Professor

GPA: 3.85

Bachelor of Science, Physiology and Developmental Biology – December 2010

Brigham Young University, Provo, Utah, USA

GPA: 3.61

## Professional Experience

Teaching Assistant: Tissue Biology (PDBio 325) August 2011 to April 2012

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## Duties:

- *Conduct weekly review sessions*
- *Entertain questions from students*
- *Teach class when called upon*
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Research Assistant

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## Duties:

- *Design and conduct research using the RAGE over-expressing mouse model*
- *Data collection, management and analysis*
- *Operate, maintain and train on laboratory equipment*
- *Present results at national scientific meetings*
- *Prepare and submit data for publication in peer reviewed journals*

Undergrad Research Mentor/Lab Coordinator    January 2010 to December 2010  
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Duties:

- *Mentor undergraduates working in the Reynolds pulmonary lab*
- *Train students in mouse surgeries, tissue sample preparation, histology using immunohistochemistry and protein analysis by western blot*
- *Coordinate and run lab meetings*
- *Track lab materials and order when necessary*
- *Assist undergraduates in planning and conducting original experiments.*
- *Prepare students for poster or oral presentation at national scientific meetings*

## Publications

### In print:

Reynolds P.R., Stogsdill J.A., Stogsdill M.P., Heimann N.B. Up-regulation of RAGE by alveolar epithelium influences cytodifferentiation and causes severe lung hypoplasia. *Am. J. Respir. Cell Mol. Biol.* 2011; 45(6): 1195-1202

Stogsdill J.A., Stogsdill M.P., Porter J.L., Hancock J.M., Robinson A.B. Reynolds P.R. Embryonic over-expression of RAGE by alveolar epithelium induces an imbalance between proliferation and apoptosis. *Am. J. Respir. Cell Mol. Biol.* 2012

### In Review:

Stogsdill M.P, Stogsdill J.A., Bodine B.G., Fredrickson A.C., Sefcik T.L, Reynolds P.R. Characterization of a new mouse model of COPD via conditional over-expression of RAGE by alveolar epithelium. In review with *Am. J. Physiol.*

## Published Abstracts Presented at National Scientific Meetings

2011

Stogsdill J.A., Stogsdill M.P., Reynolds P.R. Embryonic over-expression of RAGE in mouse lung causes an imbalance between apoptosis and proliferation leading to severe lung hypoplasia. Poster Presentation. Gordon Research Conference: Lung development, injury and repair. Newport, RI. August 2011

Bukey B.R., Porter J.L., Hancock J.M., Stogsdill J.A., Reynolds P.R. RAGE over-expressing embryonic mice degrades type IV collagen and destabilizes the respiratory membrane. Poster Presentation. Society of Developmental Biology Annual Meeting. Chicago, IL. July 2011

Geyer A.J., Ferguson N.T., Stogsdill J.A., Robinson A.B., Reynolds P.R. Conditional over-expression of RAGE by alveolar epithelium during development diminishes pulmonary endothelium. Poster Presentation. Society of Developmental Biology Annual Meeting. Chicago, IL. July 2011

Stogsdill M.P., Stogsdill J.A., Porter J.L., Bodine B.G., Reynolds P.R. Persistent over-expression of RAGE in adult mouse lung causes airspace enlargement coincident with emphysema. Oral Presentation. Experimental Biology. Washington D.C. April 2011

Stogsdill J.A., Stogsdill M.P., Reynolds P.R. Embryonic over-expression of RAGE in mouse lung causes an imbalance between apoptosis and proliferation leading to severe lung hypoplasia. Poster Presentation. Experimental Biology. Washington D.C. April 2011

2010

Stogsdill J.A., Stogsdill M.P., Reynolds P.R. Conditional up-regulation of receptors for advanced glycation end-products (RAGE) in alveolar epithelium causes respiratory distress and perinatal lethality. Poster Presentation. Experimental Biology. Anaheim, California. April 2010

## Awards and Recognitions

Teaching Assistantships: 2

Research Assistantships: 2

Fall 2011: Research Presentation Award. Graduate Student Society BYU

Summer 2011: Outstanding Poster Presentation Award at the Gordon Research Conference. Newport, RI

Spring 2010: BYU Office of Research and Creative Activities research grant

Spring 2010: Semi-finalist for the David S. Bruce award for the American Physiological Society (APS)

Spring 2010: BYU College of Life Sciences Best Poster/Presentation Award

Fall 2009: BYU College of Life Sciences Annual Fund Scholarship

Winter 2009: BYU Scholarship

Fall 2008: BYU Department of Zoology Scholarship

## Laboratory Skills

### Lab Equipment

- Light microscopes, incubators, tissue processing machine, tissue embedding machine, microtome, pH reader, spectrophotometer

### Mouse Work and Surgery

- Embryonic pup removal as early as E12.5
- Lung resection from embryonic pups
- Mouse lung lavage

### Molecular Techniques

- Western blot
- Immunohistochemistry
- DNA isolation, PCR, Gel Electrophoresis
- ELISA
- Flow Cytometry
- RNA isolation, cDNA Plate Array

## Volunteer and Service Work

Medical Interpretation

August 2008 to August 2009

Utah Valley Regional Medical Center, Provo, Utah 84604, USA

Supervisor: Roger Gonzalez

*Interpreted English to Spanish for doctors and patients for 100 hours. Sat in on consultations and surgeries.*

IMPACT Program Director

September 2008 to August 2009

Center for Service and Learning, Brigham Young University

*Directed a volunteered-based program which provides healthy activities and interactions for at-risk youth in the Provo community. Required mediating cooperative efforts from parents, teachers, guidance counselors and student volunteers.*

Church Mission to Mexico City, Mexico June 2005 to June 2007  
 Mission for the Church of Jesus Christ of Latter-day Saints  
*Learned Spanish, served and taught church principles for two years in Mexico City, Mexico. Helped local church leaders organize church meetings*

#### Current Relevant Coursework

PDBIO 601 – Cell and Molecular Physiology  
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