Genetic and Epigenetic Regulation of *NAMPT/PBEF* Expression in Human Lung Endothelium

BY

VENKATESWARAN RAMAMOORTHI ELANGOVAN
M.S., Illinois Institute of Technology, Chicago, IL, 2009
B.Tech., Bharathidasan University, India, 2005

THESIS

Submitted as partial fulfillment of requirements
for the degree of Doctor of Philosophy in Cellular and Molecular Pharmacology
in the Graduate College of the
University of Illinois at Chicago, 2016
Chicago, Illinois

Defense Committee:

Joe GN 'Skip' Garcia, MD, Advisor, University of Arizona
Chinnaswamy Tiruppathi, PhD, Chair
Jason X-J Yuan, MD, Department of Medicine, University of Arizona
Steven Dudek, MD, Department of Medicine, University of Illinois at Chicago
Subhash Pandey, PhD, Department of Psychiatry, University of Illinois at Chicago.

Acknowledgements

To the god almighty, my father **Mr.Elangovan**, my mother **Umamaheswari Elangovan**, sister **Aarati Priya**, brother in-law **Vijay**, my mentors **Dr.Joe G.N. Garcia, Dr.Vishwanathan Natarajan, Dr.Ting Wang, Dr.Xioaguang Sun** and **Lakshmi Natarajan** to whom I owe all of this.

I would also like to thank **Dr. Steven Dudek, Dr.Jeffery Jacobson** and **Dr. Roberto Machado** for their continued inspiration and valuable suggestions that helped me progress through my thesis work.

I would like to express my gratitude to my committee members **Dr. Jason Yuan**, **Dr. Chinnaswamy Tiruppathi, Dr. Subhash Pandey** for their encouragements and valuable suggestions that was instrumental in the development of this thesis.

Finally, I will also like to thank **Dr.Ayako Makino, Dr.Ankit Desai**, **and Dr.Louise Hecker** and **Dr.Wenli Ma, Dr.Djanybek Adyshev, Dr.Saad Sammani** and my colleagues in Dr.Garcia's lab who have been there for me with their kind words, deeds and support.

Thank you all for the continued support and guidance that made me a better person in life and in science.

CONTRIBUTION OF AUTHORS

Chapter 1 is the introduction that provides the basic understanding of the challenges facing the treatment of ARDS and specifically the lack of mechanistic understanding of this devastating syndrome. Chapter 2 is the literature review that highlights the recent and long term understanding of ARDS and highlights the importance of studying this devastating syndrome in the humans. Chapter 3 describes the materials and methods utilized in the experiments. Chapter 4 and Chapter 5 includes data from a publication in which I was a co-first author and drove all the epigenetic experiments (Sun, X., V. R. Elangovan, B. Mapes, S. M. Camp, S. Sammani, L. Saadat, E. Ceco, S. F. Ma, C. Flores, M. S. MacDougall, H. Quijada, B. Liu, C. L. Kempf, T. Wang, E. T. Chiang & J. G. Garcia (2014) The NAMPT promoter is regulated by mechanical stress, signal transducer and activator of transcription 5, and acute respiratory distress syndrome-associated genetic variants. *Am J Respir Cell Mol Biol,* **51,** 660.). Further Chapter 5 also includes unpublished data that has been submitted for publication where I am the first author. Chapter 6 represents a publication (Adyshev, D. M., V. R. Elangovan, N. Moldobaeva, B. Mapes, X. Sun & J. G. N. Garcia (2013) Mechanical Stress Induces Pre–B-cell Colony-Enhancing Factor/NAMPT Expression via Epigenetic Regulation by miR-374a and miR-568 in Human Lung Endothelium. *American Journal of Respiratory Cell and Molecular Biology,* **50,** 409.) in which I was the second author and contributed extensively to the design and execution of the experiments and generated all the figures with the supervison of the first author Dr.Djanybek Adyshev and my mentor Dr.Joe G.N. Garcia who also oversaw the editing of the manuscript. Chapter 7 has unpublished data and is included in a publication that has been submitted with me as the First author. Chapter 8 summarized my evidences for solving the questions described in Chapter 1 and discusses my conclusions from my data.

TABLE OF CONTENTS

CHAPTER **PAGE NUMBER**

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF FIGURES

FIGURE **PAGE**

LIST OF FIGURES (continued)

List of Abbreviations or Nomenclature

ALI	Acute Lung Injury
ARDS	Acute Respiratory Distress Syndrome
VILI	Ventilator induced Lung Injury
PBEF	Pre-B cell colony-enhancing factor
NAMPT	Nicotinamide Phosphoribosyl transferase
HPAEC	Human Pulmonary Artery Endothelial Cells
HLMVEC	Human Lung Micro Vascular Endothelial Cells
LPS	Lipopolysaccharide
ECIS	Electronic Cell-substrate Impedance Sensing
HDAC	Histone Deacetylase
5-Aza	5-aza-2'-deoxycytidine
S1P	Sphingosine 1 Phosphate
HAT	Histone Acetyl Transferase
TER	Transendothelial Resistance
CS	Cyclical Stretch

Summary

Hypoxemia, pulmonary edema and persistent inflammation are cardinal features of acute respiratory distress syndrome (ARDS) and patients suffer from respiratory failure resulting from flooding of the alveolar space. Mechanical ventilation remains the only therapeutic option available to support respiration. However, excessive mechanical forces and tidal volumes characteristic of mechanical ventilation results in structural and physiological injury to the lung tissue which is exacerbated with prior insults. With effective pharmacotherapies yet to emerge novel strategies are required for alleviation of this morbid syndrome affecting over 200,000 people with high mortality rates. Orthologous gene expression arrays utilizing multispecies pre-clinical models of lung injury identified NAMPT a novel cytozyme significantly upregulated with chemoattractant and inflammation inducing properties and localized to the lung endothelium, epithelium and leukocytes. However, the mechanisms of its upregulation remain less understood. In this thesis, I have studied and elucidated the biochemical, genetic and epigenetic factors that drive NAMPT gene expression in the human lung endothelium. Our results demonstrate that excessive mechanical stress drives NAMPT gene expression further influencing ARDS susceptibiity associated genetic variants in a STAT5 dependent manner. The upregulation occurred via epigenetic mechanisms targeting the NAMPT 5’ UTR and 3’UTR. Further extracellular NAMPT altered the histone H3K9 acetylation profile in a TLR4 dependent manner. These results shed light on the influences of NAMPT transcription in VILI and provide novel targets for development of therapeutic strategies to alleviate ventilator-induced lung injury. Further, the influence of SNP’s on transcription of NAMPT would be a valuable resource for personalized medicine.

1. INTRODUCTION

1.1 Acute Respiratory Distress Syndrome (ARDS) and Ventilator-Induced Lung Injury (VILI)

Acute respiratory distress syndrome (ARDS) is a rapidly progressive devastating syndrome affecting over 200,000 people a year. The prevalence of ARDS in patients is decreasing, yet the mortality rates have been high (~40%) for several decades (Zambon and Vincent 2008, Blank and Napolitano 2011). With effective pharmacotherapeutic strategies yet to emerge, over 80% of all adult patients who die of ARDS have a mortality window of ~3 weeks from the onset of the syndrome (Villar, Blanco, and Kacmarek 2016). Thus, patients with ARDS are often in the ICU for extended periods, increasing the burden on healthcare. The underlying cause of high mortality rates within ARDS patients is unclear and needs much attention.

Despite substantial efforts aimed at understanding the complexity of this syndrome, therapeutic strategies targeted at alleviation of the pathophysiology is severely lacking, resulting in the aforementioned high mortality rates. Progression to ARDS occurs subsequent to a primary insult in the lung, such as inhalation of smoke, drowning in water, infection or sepsis. The primary pathophysiology of ARDS is diffused alveolar damage and microvascular endothelial breakdown leading to flooding of the lungs and subsequently respiratory failure. The importance of respiration as a critical physiological function where the oxygen from inhaled air is exchanged for carbon dioxide is well established and does not warrant more discussion. Gas exchange is thus an important function for survival and occurs in the alveolar sacs of the lung. The alveoli are lined by

thin epithelial cells while the adjoining pulmonary capillaries are lined by endothelial cells. The primary response of the lungs, to inciting stimuli especially in response to infection, is the recruitment of neutrophils and macrophages, critical mediators of host defense and inflammation. Sustained inflammation occurring in the setting of damage to the lung causes breakdown of the alveolar epithelial cells and pulmonary endothelial cells, resulting in the flooding of alveolar space and refractory hypoxemia. These events culminate in respiratory failure and patients with ARDS need to be supported artificially via mechanical ventilation.

1.2 Mechanical Ventilation

Mechanical ventilation is a life-saving intervention used to mechanically assist patients suffering from respiratory failure. Mechanical ventilation, though lifesaving, unfortunately contributes to lung injury, often-aggravating prior inflammatory insults, known as Ventilator-Induced Lung Injury (VILI) (Dreyfuss and Saumon 1998). In a landmark multi-centered study, reduction in ventilator tidal volumes applied to ARDS patients significantly decreased the high mortality rates compared to patients with normal high tidal volumes demonstrating the deleterious impact of VILI. In VILI, extensive tissue distensions caused by necessary but exceeding pressures required to sustain respiration induce structural and physiological damages to the lung. Distensions by mechanical ventilation is, however, heterogeneous inducing higher volutrauma in parts of the lung leading to increased inflammation which results in systemic inflammation. Despite a multitude of potential factors that may be responsible for increased mortality, inflammation leading to multiple organ system failure is a leading cause of death resulting from ARDS and mechanical ventilation.

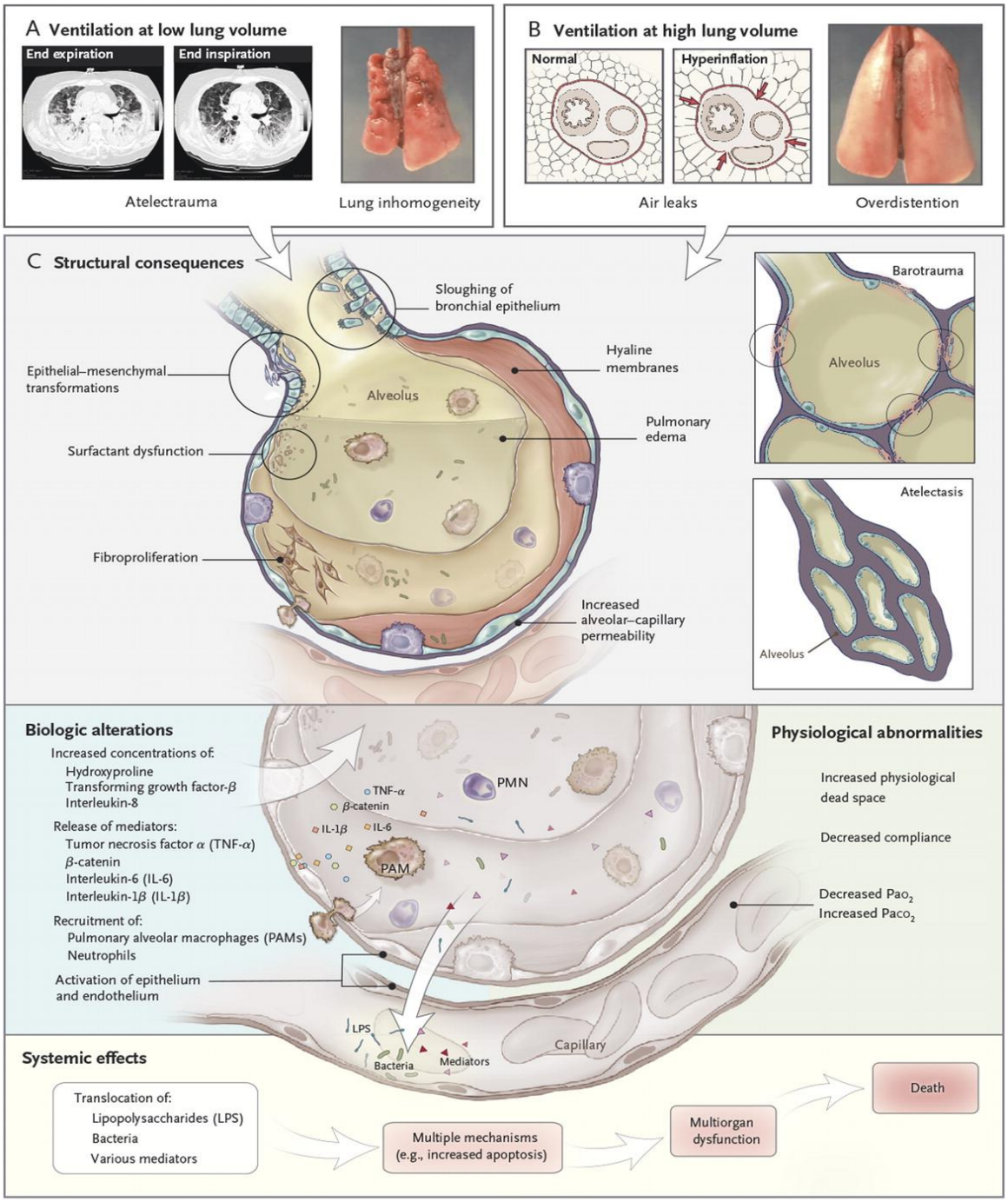

Figure 1: VILI induces a multitude of physiological alterations. "Reproduced with permission from (Slutsky AS, Ranieri VM Ventilator-induced lung injury. N Engl J Med. 2013 Nov 28; 369(22):2126-36), Copyright Massachusetts Medical Society.

1.3 Role of the Lung Endothelium in ARDS and VILI

The vascular endothelium forms a single layer of cells lining the lumen forming a semi-permeable barrier separating circulating blood from the tissues and alveolar space. Endothelial cells under normal conditions are integral to the alveolar-capillary system maintaining gas exchange homeostasis. However, endothelial cells respond to diverse stimuli and are capable of performing metabolic roles (Nitric Oxide, endothelin secretion), stimuli response (activation leading to inflammatory cascade) and signal transduction (Ryan, 1982). Pulmonary vascular permeability, a cardinal feature of ARDS/VILI, is enhanced by ARDS upregulated cytokines, cytoskeletal remodeling or through mechanical stress (Orfanos et al. 2004, Dudek and Garcia 2001) leading to protein-rich fluid leakage into the lungs highlighting their clinical relevance in the setting of ARDS/VILI.

1.4 Genetics and Genomic Approaches to VILI Pathobiology

A complex disorder such as ARDS, plagued by lack of clear stratification strategies to define the disease or challenges in finding appropriate families which may be useful to trace lineages, can benefit by genome-wide association approaches to identify mechanisms of disease. Such association studies comparing affected and unaffected candidates for changes in gene expression or variants in their genome with rigid stratification are well suited to identify genes that contribute to ARDS susceptibility (Garcia 2005). In support of this observation, population-based stratification of ARDS patients revealed increased susceptibility in African-Americans compared with Caucasians which the Garcia lab has shown to be attributable to genetic variations in

genes causative for ARDS(Moss and Mannino 2002). These observations highlighted the feasibility and robustness of studying clinically relevant genes and their genetic variants using genomic approaches. Thus, a “candidate gene approach” combined with orthologous-gene expression arrays and genetic variants contributing to susceptibility targeted to increase the confidence of association is a powerful technique to identify genes linked to a particular trait. Such a candidate gene approach helped identify several novel genes pivotal for the progression of ARDS and VILI severity. Whole genome-wide profiling between VILI patients and case controls identified NAMPT as a novel gene upregulated in VILI (Grigoryev et al. 2004, Ye, Zhang, et al. 2005) whose function and genetic variants confer susceptibility to VILI. (Ye, Simon, et al. 2005)

1.5 Role of NAMPT in VILI Pathobiology

Samal et al screened cDNA libraries from peripheral blood lymphocytes in 1994 and reported a novel gene, critical for pre-b-cell colony-enhancing function that works in synergy with stem cell factor and IL-7 but without any intrinsic enzymatic activity (Samal et al. 1994). Since that initial finding, Nicotinamide Phosphoribosyl transferase’s (NAMPT) role as a pleiotropic molecule intricately involved in multitude of physiological has expanded to processes including NAD-biosynthetic pathway, aging, cancer, obesity, atherosclerosis and lung injury is well established(Revollo, Grimm, and Imai 2004, Zhou, Wang, and Garcia 2014). Attributable to their upregulation in severe chorioamnionitis and induced by pro-inflammatory cytokines in the epithelial cells, NAMPT is a stretch and inflammation induced molecule (Nemeth et al. 2000, Ye, Simon, et al. 2005). NAMPT exerts its influence in two different forms, the secreted extracellular (eNAMPT) form imparting its function as a cytokine and intracellular

(iNAMPT) form that performs anti-apoptotic functions inside the cell(Dahl et al. 2012). Despite several lines of evidence currently recognizing the clinical relevance of this important molecule in multiple pathophysiology's, literature citing *NAMPT* was modest when the significance of NAMPT as a lung-intensive molecule became evident as result of work done in our lab (Ye, Simon, et al. 2005, Hong et al. 2008). Orthologous gene expression arrays utilizing multispecies pre-clinical models of lung injury identified NAMPT gene expression, protein expression and secretion being consistently upregulated in VILI(Ye, Simon, et al. 2005), with extracellular NAMPT exerting its influences on pro-inflammatory ontologies (Grigoryev et al. 2004). Besides increased eNAMPT in the serum can be a useful and reliable biomarker for ARDS and VILI syndrome. Recent path-breaking work accomplished in our lab has also identified the role of eNAMPT in pro-inflammatory cytokine activation in murine lungs and human endothelial cells through TLR4 binding (Camp et al. 2015) which is discussed later in this thesis. iNAMPT the other form of NAMPT is a rate-limiting enzyme in the NAD biosynthetic pathway, influencing cellular NAD levels. NAD is a co-enzyme essential for Sirt-1 deacetylase activity that has important cellular functions regulating transcription factors, chromatin structure and gene transcription. Thus, NAMPT may influence cell metabolism and gene transcription. Dysregulation of NAMPT has been mechanistically linked to VILI and ARDS with increased transcription and secretion, however the mechanisms of *NAMPT* transcription in response to VILI (mechanical stress) and ARDS inducing stimuli (LPS, TNF-alpha and IL-1beta) is very poorly understood. Identification of the mechanisms regulating the transcription of this clinically relevant molecule would expand the therapeutic strategies necessary to modulate the

activity of this gene and thereby NAMPT influenced severity of VILI. In this thesis, I have elucidated the transcriptional regulation of NAMPT in human lung endothelial cells and the regulation of the transcript stability through multiple epigenetic mechanisms.

1.6 Role of Epigenetics in ARDS and VILI

Epigenetics is defined as the heritable changes in gene expression not occurring at the level of the underlying DNA sequence. Established well in the developmental context, epigenetics pathways have also been intricately associated to the mechanisms of several diseases including cancer, inflammation, respiratory and cardiovascular diseases (Weinhold 2006). Epigenetics is broadly classified into three distinct categories that include mechanisms regulating transcription –DNA methylation, histone modifications and mechanisms working through the post-transcriptional alteration of stability mediated by microRNA binding to the 3'UTR of the target mRNA. Even though changes in DNA epigenetic signatures are stable in a developmental context, environmental factors induce epigenetic changes that are more dynamic in nature. Infections and local physiological changes such as hypoxia (Bayarsaihan 2011) induces dynamic yet reversible epigenetic changes affecting gene transcription. While distinct promoter mediated epigenetic changes are positively correlated with genes actively transcribed in inflammation thereby increasing its severity (Medzhitov and Horng 2009), the inhibition of promoter epigenetic mechanisms attenuates severities of inflammation (Thangavel et al. 2014, Thangavel et al. 2015) exemplifying the significance of epigenetic mechanisms in driving inflammation. In addition to promoter epigenetic mechanisms, post-transcriptional mechanisms mediated by microRNAs also mediate and enhance inflammation (O'Connell, Rao, and Baltimore 2012). Dysregulation of

microRNA have been mechanistically linked to several genes causing pulmonary inflammatory diseases including cystic fibrosis, Idiopathic Pulmonary Fibrosis, pulmonary artery hypertension(PAH) and several microRNA's can serve as potential biomarkers (Kishore et al. 2014). Analysis of dysregulated microRNA in animal models of VILI identified microRNA that also regulates genes implicated in VILI induced ontologies like inflammation and immune response (Zhou, Garcia, and Zhang 2011). However, knowledge acquired about epigenetic mechanisms have come from in-vitro experiments involving cells and in-vivo animal models, epigenetic data from patients would bridge the gap between genetics, genomics and epigenetics.

Methylation of CpG dinucleotides is a critical mediator of epigenetic regulation directly inhibiting transcription factor binding or recruiting repressive chromatin modifying complexes. Genetic variations occurring in the genome may alter CpG sites and modify transcript levels. Association studies showing genetic variation correlating with CpG sites have been validated in disorders such as psychosis (van den Oord et al. 2015) and genes associated with thromboembolic arterial diseases (Ye et al. 2015).

Analysis of population-based NAMPT non-coding promoter genetic variants identified significant variations that introduce CpG sites or remove them. Further DNA methylation analysis of NAMPT promoter from PBMC has identified several sites that are differentially methylated between populations, illustrating the potential for integrating genetic and epigenetic information in the regulation of ARDS influential genes further enabling personalized medicine.

1.7 In-vitro Cyclic Stretch

Cells in the human body are rarely in static conditions as represented in cell culture dishes grown in the lab. Rather they are in a dynamic environment especially in the cardiovascular and pulmonary systems, where cells are constantly facing mechanical load. The human lung microvasculature is constantly exposed to shear stress resulting from blood flow and stretch contributed by respiratory cycles (Birukov 2009). Our lab has consistently utilized an apparatus that mimics spontaneous breathing or high tidal volume mechanical ventilation. Changes in cells occur including alterations in signaling, protein synthesis and cellular differentiation. To study cells in a dynamic environment, where they are subjected to increased mechanical stress, cells are seeded on collagen coated flexible bottom membrane plates and loaded onto cylindrical-shaped loading posts. Equibiaxial strain applied to cells generates mechanical load, which gets into intracellular signals via mechanotransduction (Hahn and Schwartz 2009).

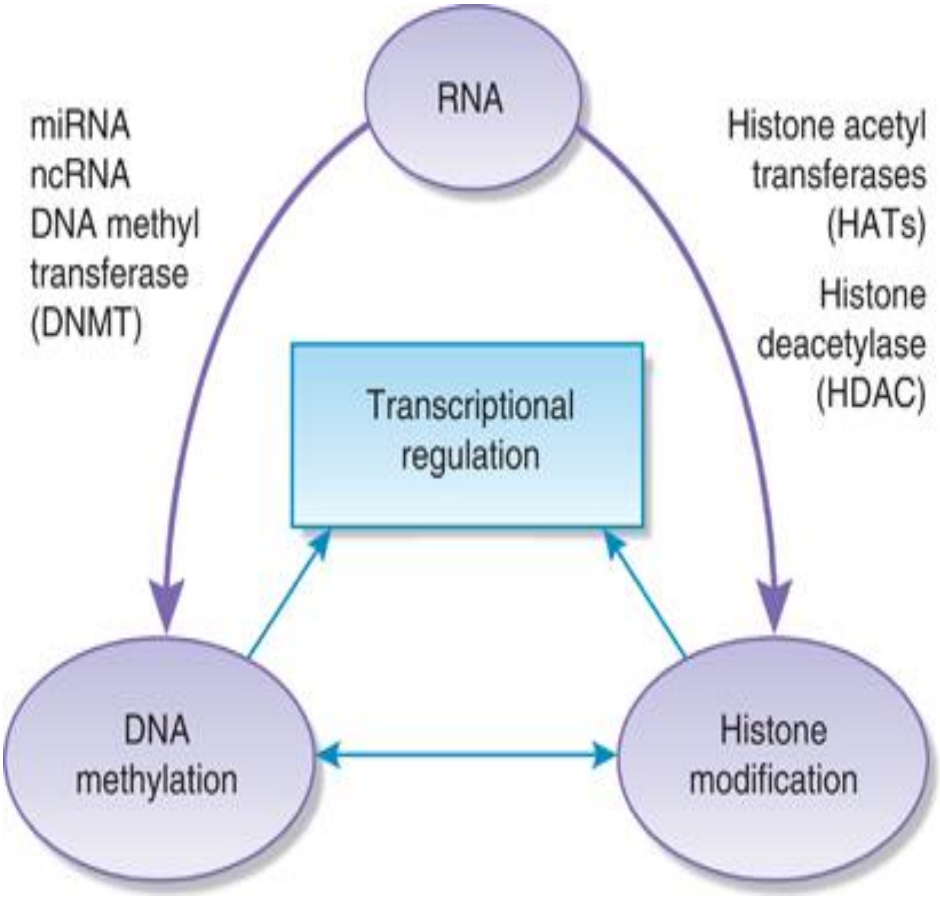

Figure 2: Overview of the various epigenetic mechanisms. Epigenetics include the interplay between DNA methylation, histone modifications and RNA mediated post-transcriptional regulation that alters the generation and stability of transcripts

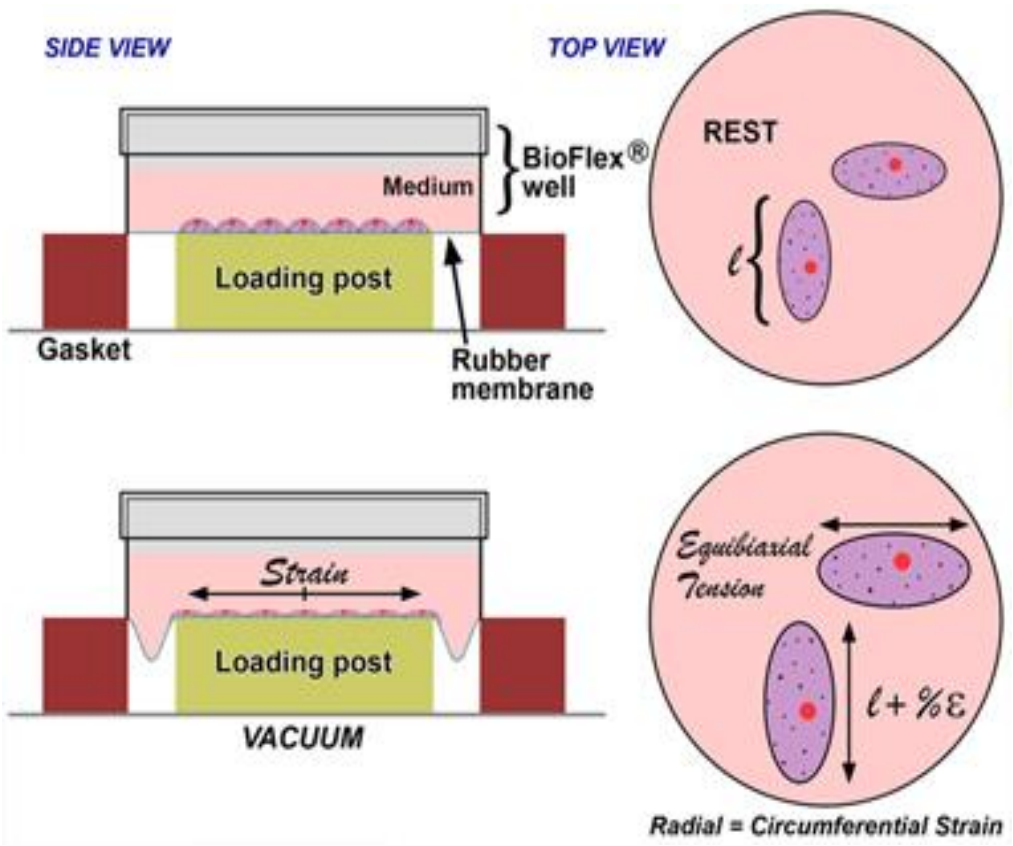

Figure 3: Representation of mechanical strain application to cells. The cells are plated on collagen coated membrane plates and equibiaxially stretched to mimic autonomous breathing or mechanical ventilation. “Reproduced with permission from Flexcell International”

2. REVIEW OF LITERATURE

2.1 ARDS and VILI

Described for the first time in 1967 acute respiratory distress syndrome (ARDS) is complex syndrome characterized by the onset of hypoxemia, non-cardiogenic pulmonary edema induced bilateral infiltrates and persistent inflammation. (Fan, Villar, and Slutsky 2013). Since the primary result of persistent inflammation is breakdown of the epithelial and endothelial barrier, fluid accumulation in the lung leading to respiratory failure. Patients suffering from respiratory failure are treated by mechanical ventilation where an apparatus that can force air into the lungs (positive air pressure) drives the breathing. Mechanical ventilation in diverse situations out of respiratory failure resulting from asthma, COPD, sepsis or neurological disorders that impair respiratory mechanisms support life.

Despite being the only therapy available for the alleviation of respiratory failure, mechanical ventilation is associated with increased mechanical forces applied to the lung tissues, which ultimately lead to structural and physiological damages that can be detrimental for the patients. Since ventilators are often used when respiratory failure occurs because of a primary insult, the removal of underlying factors would be necessary to decrease the use of ventilators for extended periods.

The use of ventilators are often associated with several determinants of VILI including excessive alveolar distention (volutrauma), increased transpulmonary pressures (barotrauma), and collapse of the alveolar sacs attributable to the repeated opening and closing of the alveoli (atelectrauma) (Fan, Villar, and Slutsky 2013).

2.1.1 Volutrauma

Ventilators use excessive mechanical forces to maintain adequate respiration and overdistension of the alveolar sacs resulting from increased lung volumes is a cardinal feature of mechanical ventilation (Albaiceta and Blanch 2011). Overdistension of the cells in the alveolar sac lead to their disruption and cellular apoptosis of the pulmonary epithelial and endothelial cells resulting in the initiation of inflammatory processes. (Vlahakis and Hubmayr 2005). Further overdistension of lung tissues leads to increased cytokine release into the systemic circulation. Sustained release of inflammatory mediators and cytokines into the circulation leads to multiple organ dysfunction syndrome (Halbertsma et al. 2005) which is the major factor contributing to mortality inspite of the causative reason being respiratory failure induced hypoxemia. Thus, volutrauma caused by use of ventilators is a major cause of development of inflammatory processes in the lung.

2.1.2 Barotrauma

Another critical factor that is integral for the severity of ventilators is the pressures utilized for sustaining respiration via ventilation. Barotrauma has been historically referred to as the leakage of air into the extra-alveolar space. The increased pressures imparted to the lungs as with volutrauma leads to increased stress to the cells and causes alveolar rupture and endothelial dysfunction leading to inflammatory mediators being dispersed into the systemic circulation (Halbertsma et al. 2005).

2.1.3 Atelectrauma

Since there exists significant heterogeneity of injury in the lung, the local environment exhibits differences in mechanical stabilities that may cause increased alveolar

instability and localized lung unit collapse leading to atelectrauma. The mechanical ventilation process involves cyclic opening and closing of the alveolar sacs and leads to the collapse of the alveolar sacs resulting in loss of function. This can be effectively prevented by utilizing a sufficiently increased positive end expiratory pressure. Current knowledge is insufficient to understand the impact of atelectrauma in the context of inflammation even though there is evidence that alveolar recruitment is beneficial in ALI and ARDS (Mols, Priebe, and Guttmann 2006) and disruption of this process can contribute to the progression of lung injury in VILI.

2.1.4 Biotrauma

Biotrauma in mechanical ventilation refers to the contribution of increased inflammatory processes occurring in the lungs leading to a systemic inflammation and mortality contributed by multiple organ system dysfunction (Imai et al. 1994). Analysis of lavage fluid in the lungs of patients indicated increased cytokines including TNF α and MIP-2, IL-6 in both experimental animal models and human studies (Halbertsma et al. 2005). The increased inflammatory mediators are leaked into the systemic circulation and cause systemic inflammation leading to the death. Of the various mechanisms that have been explored volutrauma seems to be the most prominent factor leading to increased Biotrauma via their influences on cellular disruption and increased inflammatory processes. Thus mechanical ventilation may lead to the activation of the innate immune system and may lead to increased cytokine activation (Halbertsma et al. 2005, Matthay and Zimmerman 2005).

2.1.5 ARDSnet Study

Large tidal volumes have been traditionally used in mechanical ventilation and have been associated with significantly increased cytokines in the lavage and higher mortality rates. The critical participation of mechanical ventilation in the pathogenesis associated with lung injury and mortality is well demonstrated in the ARDS net trials where lower tidal volumes (6ml/kg) were used in place of the large tidal volumes (12ml/kg) and showed significant decreases in lung inflammatory mediators and reduced the mortality rates from ~40% to ~22%. (Slutsky 1993, Hickling, Henderson, and Jackson 1990, Hickling et al. 1994). However, this study also found that it may cause respiratory acidosis and decreased arterial oxygenation. To understand the regulation of inflammation IL6 levels were studied and the study observed that lowering of tidal volume reduced the amount of IL-6 present in the lavage fluid. This critical study evaluated the importance of tidal volumes used in mechanical ventilation influencing the severity of VILI. The approach of utilizing lower tidal volumes is becoming a standard of care for ARDS patients supported by VILI. These experiments not only highlighted the useful strategy of lowering the tidal volume but also highlighted the importance of high tidal volume ventilation in contributing to the severity of lung injury and systemic inflammation. Decreases in systemic inflammation were also positively correlated with decreased ventilator days for patients.

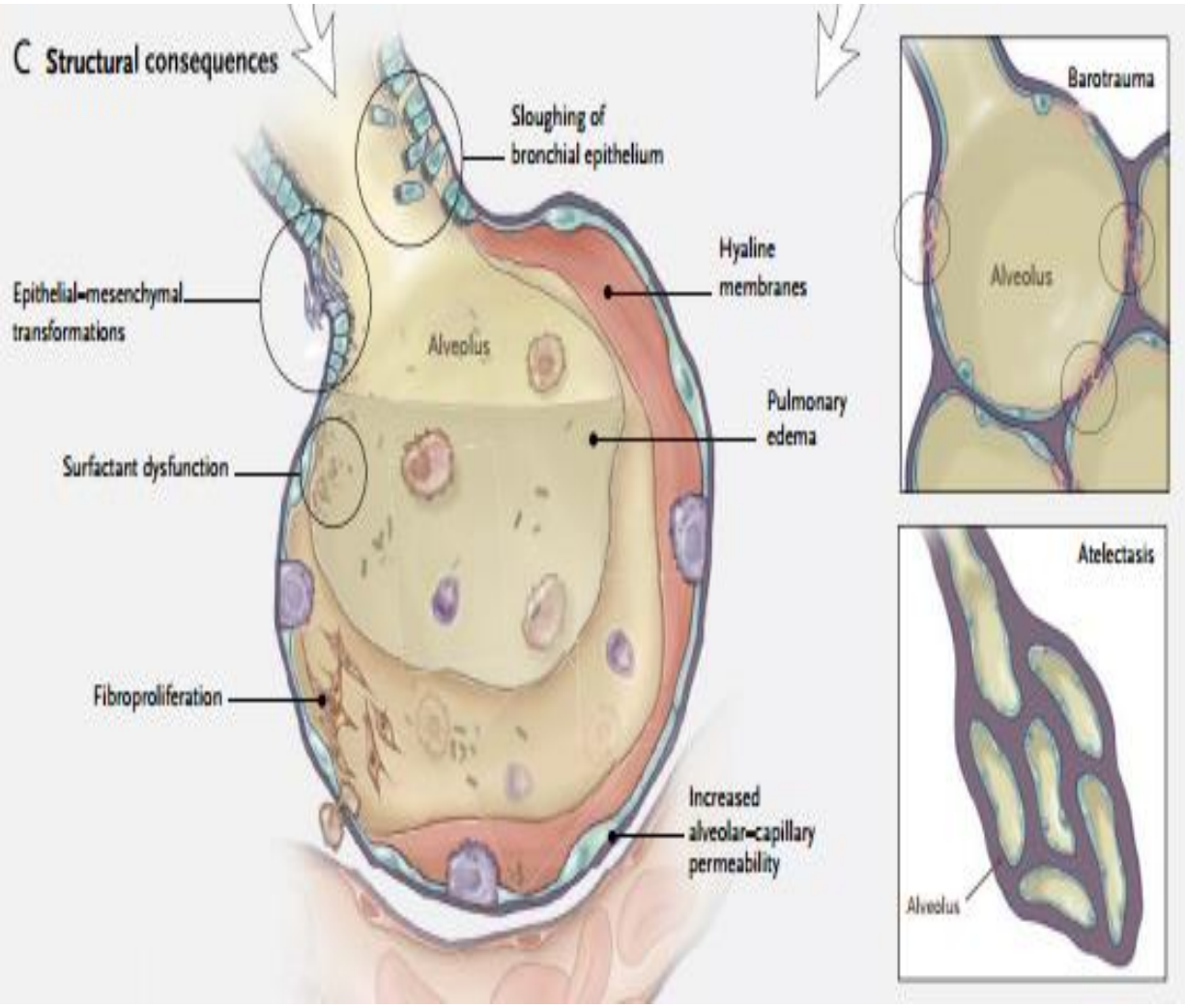

Figure 4: Atelectrauma leads to atelectasis in VILI. “Reproduced with permission from (Slutsky AS, Ranieri VM Ventilator-induced lung injury. N Engl J Med. 2013 Nov 28; 369(22):2126-36), Copyright Massachusetts Medical Society.

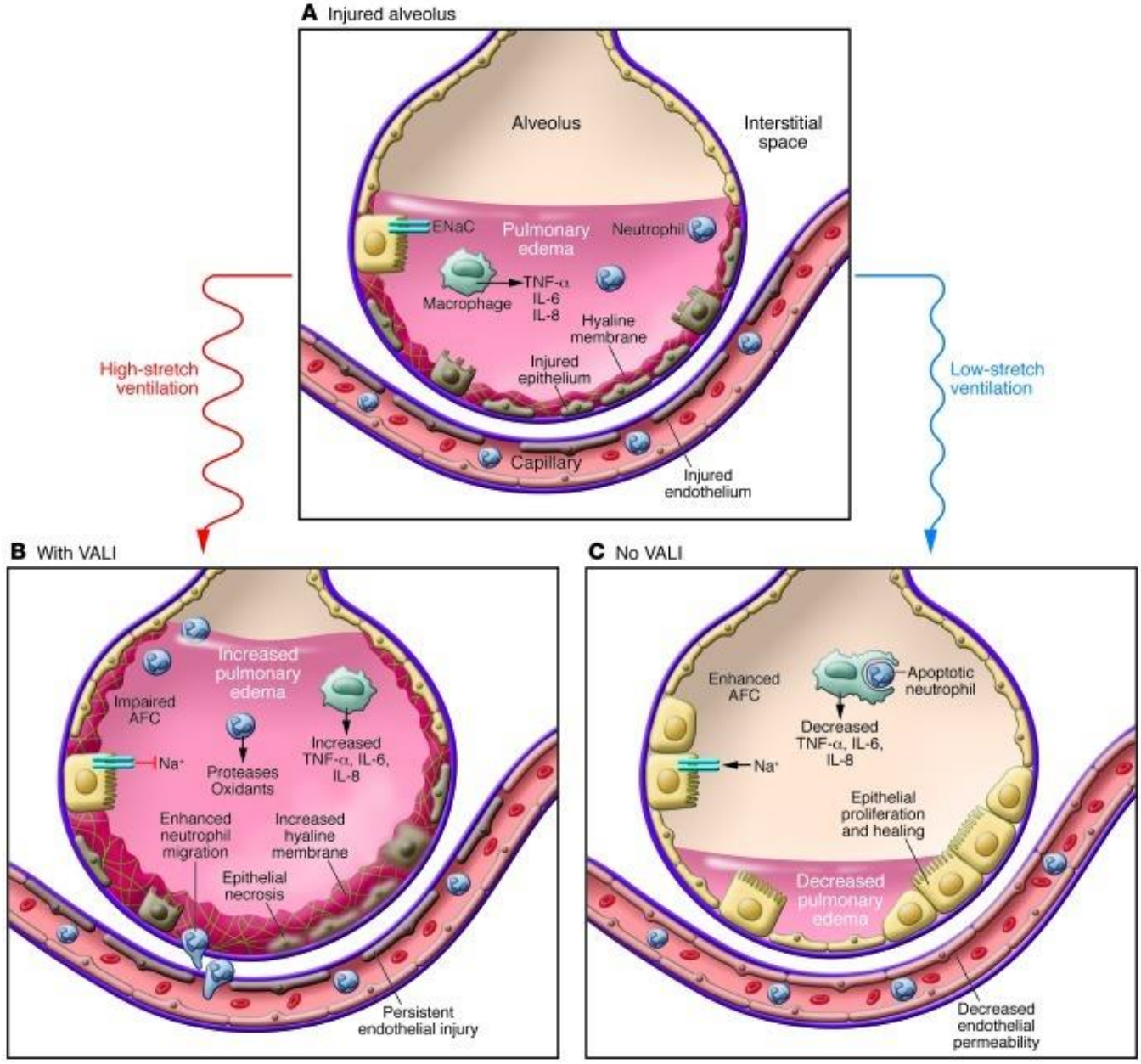

Figure 5: Representation of the impact of VILI on lung pulmonary edema. Reproduced with permission from Michael A. Matthay, Lorraine B. Ware, Guy A. Zimmerman. The acute respiratory distress syndromeJ Clin Invest. 2012;122(8):2731-2740. doi:10.1172/JCI60331.

2.2 The Endothelium

2.2.1 Cytoskeletal Elements of Endothelial Cells

Myosin Light Chain Kinase (MYLK) is a critical molecule that phosphorylates regulatory MLCs which further allows its interaction with actin. In endothelial cells the contraction of the actomyosin complex is a strong physiological event that overcomes the intercellular junction mediated adhesion of adjacent endothelial cells thus allowing for the retraction of cells and hence increased endothelial barrier permeability. The evidence for the importance of myosin light chain kinase gene came from a set of experiments in mice where nmMYLK knockout mice demonstrated reduced susceptibility to lipopolysaccharide and ventilator-induced lung injury. Further, utilizing MLCK inhibitors in wild-type mice significantly reduced the endothelial cell barrier dysfunction and inflammation associated with lipopolysaccharide treatment.

Initially identified as an important molecule that was found in activated T cells, it was aptly named as macrophage migration inhibitor factor (MIF) for its role in inhibiting random macrophage migration. MIF is an important ARDS candidate gene and recognized biomarker. The elevated MIF transcript and protein levels observed in murine and canine models of VILI and in human lung endothelium cells validated the increased gene expression as observed by gene microarray expression datasets.

2.2.2 Barrier Function of Endothelial Cells

Sphingosine 1-phosphate (S1P), a bioactive sphingolipid and a critical lipid mediator, is a known enhancer of endothelial cell barrier function in vivo that exerts in function by binding with the S1P receptor 1 (S1PR1). S1PR1, the receptor for S1P is a pertussis-

toxin-sensitive, Gi-coupled receptor, that substantially increases cortical actin polymerization that is critical for endothelial cell barrier enhancement in a Rac GTPase-dependent manner. Mechanistically, the activation of S1PR1 by the binding of its ligands enhances the interactions of vascular endothelial cadherin and b-catenin in junctional complexes by the phosphorylation of cadherin as well as p120- catenin thus facilitating the formation of cadherin/catenin/actin complexes. The understanding of the mechanistic role of S1P in its endothelial cell barrier enhancing properties emphasizes its importance as a therapeutic target in restoring the endothelial cell barrier integrity. In support of the barrier enhancing functions of S1PR1, while In vivo administration of S1PR1 antagonists induced the disruption of barrier integrity in pulmonary endothelium, administration of S1PR1 agonists such as SEW2871 and FTY720, significantly enhanced the endothelial barrier function.

2.2.3 Inflammatory Signaling in Endothelial Cells

Elevated levels in response to ventilator-associated mechanical stress identified CXCR4 as a novel candidate gene associated with ARDS. CXCR4 was characterized as a strong candidate gene based on stringent evaluation by multiple filtering strategies. The expression of CXCR4 is modulated extensively by the interleukins where interleukin-4, interleukin-13, and granulocyte– macrophage colony-stimulating factor reduces and interleukin-10 and transforming growth factor-b (TGFb) increases CXCR4, giving prominence to the idea that CXCR4 may be critical for the fibrotic response to ARDS via TGFb signaling.

2.3 Epigenetics

2.3.1 DNA Methylation

DNA methylation is an epigenetic mark generated by the covalent addition of a methyl group to a cytosine occurring in CpG dinucleotide context (Robertson and Jones 2000). The presence of 5-methylcytosine was first described in 1948 by Hotchkiss and in general there exists an inverse relationship between CpG methylation and gene transcription. The covalent addition occurs through a series of steps that transfer a methyl group from S-adenosyl-L-methionine to the 5' position of the cytosine. A family of enzymes known as the DNA methyltransferases that include three members DNMT1, DNMT2 and DNMT3 mediates this process(Jeltsch 2002).

An analysis of the distribution of CpG sites in the genome revealed their existence in several regions including the repetitive regions of the genome such as LINE's, transposons (Robertson 2005) and also in gene bodies and gene promoters. Though several lines of study extend the significance of DNA methylation in diverse processes such as X-chromosome inactivation, Rett-syndrome and cancer, the scope of this introductory review has been limited to their regulation of promoters and the 5'UTR of genes. 5-methylcytosine is randomly distributed in the genome except for regions encompassing the immediate 5' regions of genes where a stretch of CpG sites forms CpG islands (Antequera and Bird 1993, Illingworth and Bird 2009). Such CpG sites extend for 1000bp on average and represented in over 70% of the gene promoters (Saxonov, Berg, and Brutlag 2006). Despite the over-representation of CpG sites in the gene promoters, CpG islands appear depleted of CpG methylation, occurring as

hypomethylated regions (Deaton and Bird 2011). CpG methylation can potentially alter the rate of transcription by restricting access to transcription factors or recruitment of repressive factors that condense chromatin. From this perspective, the lack of methylation occurring in the CpG islands result in nucleosome depletion at transcription start sites and enable transcription initiation (Schones et al. 2008, Deaton and Bird 2011). Even though the human genome remains the same across all cell and tissue types, DNA methylation occurring in the promoters of genes contribute towards tissue specific expression. For instance, multiple endothelial specific genes were regulated by differential DNA methylation occurring in their promoter regions when comparing expressing and non-expressing cells (Shirodkar et al. 2013).

Aberrant DNA methylation is a hallmark of several disease pathologies including inflammatory disorders, neurological disorders and cancer. The importance of disease pathology is perhaps evident from studies in cancer where the 5' region of tumor suppressor genes have been shown to be hyper methylated (Ehrlich 2002) and the repetitive elements are hypomethylated (Robertson 2005). From the perspective of endothelium changes in the DNA methylation of participating genes plays pivotal roles in the regulation of EC proliferation through their promoter altered methylation on cyclin A gene (Jamaluddin et al. 2007), apoptosis by modulation of methylation induced by Lox-1 (Mitra, Khaidakov, et al. 2011, Rao et al. 2011) and H2O2 induced apoptosis in brain endothelial cells (Rao et al. 2011) and endothelial cell heterogeneity via modulations of VWF promoter (Yuan et al. 2016). DNMT dependent genome-wide methylation DNA methylation patterns occurred in response D-flow that develops in arterial regions capable of altering gene expression in endothelial cells and 5-aza-2'-

deoxycytidine dramatically reduced endothelial inflammation (Dunn et al. 2014). Further 5-aza-2'-deoxyctidine a potent DNA methyl transferase inhibitor have been shown to decrease inflammation in a wide variety of cells including endothelial cells. These data suggest a significant role for DNA methylation is a critical regulatory mechanism participating in the regulation of endothelial cell physiology. The impact of DNA methylation on inflammation is increasingly evident from several studies that have implicated changes in DNA methylation occurring in the context of cardiovascular diseases, oral infection, colitis and rheumatoid arthritis demonstrating the critical importance of DNA methylation changes in the context of disease development.

In addition to genome-wide methylation changes occurring in the context of inflammation, several critical genes participating in the inflammatory response have been targeted by alterations of DNA methylation including TLR4, TLR2, TNF-α, IL-1beta and IL-6. TLR4 is an important cytokine regulating NF-kB pathway. In intestinal epithelial cells, TLR4 methylation levels directly corresponded with the amount of transcripts and influenced the inflammatory responses (Takahashi et al. 2009). TLR2 promoter hypermethylation was associated with immune dysbiosis (Benakanakere et al. 2015), TNFα was found to be epigenetically modulated by changes in DNA methylation occurring in a developmental context and in activation by stimulation (Sullivan et al. 2007). Further, in human articular chondrocytes the expression of IL1B promoter strongly correlated with demethylation on specific sites on the promoter ((Hashimoto et al. 2009). IL-6 a critical inflammatory mediator was found to be hypomethylated at single CpG site in the IL-6 promoter that was associated with increased IL-6 secretion and hence causing pathogenesis of rheumatoid arthritis((Ishida et al. 2012).

The DNA methylation changes occurring in VILI and in particular NAMPT promoter are lacking and warrants attention due to their influences on modulating the severity of VILI. During placentation in mice DNA, methylation was not significantly changed at the NAMPT promoter (Kim et al. 2011) but was associated with increased H3K9 acetylation.

2.3.2 Histone Modifications, HAT's, HDAC's and Sirt's

Histones are proteins forming the core component of chromatin. There are 4 types of histones H1,H2, H3 and H4 which form a octameric unit consisting of H2A and H2B heterodimers as well as H3 and H4 heterodimers, whose positioning is sequence dependent (Eslami-Mossallam, Schiessel, and van Noort 2016). The histone N-terminal tails are regions where extensive post-transcriptional modifications can occur having influences on the structure of the nucleosome. The post-translational modifications include phosphorylation, acetylation, ubiquitination, methylation and each has its own role in the regulation of chromatin relaxation and condensation (Bannister and Kouzarides 2011). Similar to other post-translational modifications of proteins each of the histone monomers can be modified at several residues, with each residue imparting a specific function depending on the histone. Acetylation of histones and methylation of histones has been the most studied with respect to gene transcription. Acetylation of histones neutralizes the positive charges on histones and relaxes the chromatin allowing access for transcription factors (Dong and Weng 2013). The acetylation's of histones is achieved by enzymes known as histone acetyl transferases (HAT's) and are deacetylated by histone deacetylases (HDAC's). The interplay between these two enzymes determines the status of histone mediated chromatin modifications leading to gene transcription.

The well-studied mechanisms of histone modifications affecting endothelial cells are perhaps the regulation of NOS where chromatin based repression was identified as a regulator of NOS in non-endothelial cells via recruitment of HDAC1 and MeCP2 (Matouk and Marsden 2008). HDAC 7 is a zinc dependent deacetylase and HDAC7 knockout mice was embryonic lethal through the impairment of normal vasculature development (Chang et al. 2006, Matouk and Marsden 2008). Another important deacetylase HDAC1 has also been implicated in angiogenesis (Kim et al. 2001). Hyperacetylation of H3 and H4 is an important mark of activation and are present in the promoters of upregulated genes and laminar shear stress has been found to increase these activation marks in endothelial cells. (Illi et al. 2003). These suggest that histone post-translation modifications are critical regulators of gene expression in endothelial cells.

The histone modifications occurring in the context of inflammation has been studied in systems like atherosclerosis (Wierda et al. 2010), bacterial infection (Schmeck et al. 2005), COPD via their modulation of HDAC activity (Barnes 2006) and in asthma through increases in HAT activity (Barnes, Adcock, and Ito 2005) each impacting the post-translation modifications of histones. Thus, most of the studied mechanisms have indicated changes in the HDAC activity or HAT activity. Decreased HDAC activity and increased HAT activity leading to gene transcription is an obvious conclusion to arrive at with respect to histone modifications. However, it is worthy of note that in some cell types including macrophages and human lung microvascular endothelial cells endotoxin upregulates gene expression, but the relative HDAC activity is increased and an

activating mark H3K9 is globally reduced. Thus, the impact of global HDAC and HAT activities may need to be evaluated separately for each modification and gene locus.

The impact of histone hyperacetylation occurring in the promoter regions would suggest increased gene transcription for most genes. However, HDAC inhibitors have been effectively used for decreasing inflammation and along with DNA methylation inhibitor, have been effective at reducing inflammation via suppression of gene transcription occurring at pro-inflammatory cytokine genes (Adcock 2007, Thangavel et al. 2014). These suggest a deeper understanding of the mechanisms involved with each modification needs to be evaluated for targeted approaches.

VILI and mechanical stress can induce changes in the epigenetic pathways and in particular chromatin modifications. Mechanotransduction refers to the transfer of mechanical stress into intracellular signals activating transcription of genes or physiological changes like actin cytoskeletal remodeling. Mechanotransduction have been implicated in the chromatin remodeling in epithelial cells within the context of autoimmune disorders (González et al. 2011). Mechanical ventilation (volutrauma) in pre-term lamb induced changes in chromatin modifications leading to increased gene expression. The DNA methylation patterns were altered with increased DNMT1 and the histone mark H3K9 was significantly decreased. These conditions were also concomitant with increased HDAC1 activity in the lung. Further treatment with HDAC inhibitor TSA positively affected the epigenetic determinant and decreased the volume trauma induced alveolar dysfunction. While most of the increases in gene transcription occur with increases in acetylation in pulmonary disorders and nasal HFV, mechanical ventilation decreased the acetylation status leading to hypoacetylation in the lungs

(Albertine 2013, Joss-Moore, Albertine, and Lane 2011). Thus, there exist within the same systems different mediators of lung epigenetic changes. VILI seems to be unique in this aspect to induce hypo-acetylation, whose mechanisms and consequences need to be understood.

Sirtuins are a family of NAD dependent proteins that performs deacetylase activity on histone and non-histone targets. There are totally seven family members that share the deacetylase function and are localized either in the cytoplasm, nucleus or in the mitochondria. The seven family members are named Sirt-1 to Sirt-7. Sirt-1 is the most studied with varied impacts on endothelial dysfunction, inflammation, aging, metabolism and influence gene transcription. Mostly present in the nucleus they have nuclear localization signals and nuclear export signals that allow for nucleo-cytoplasmic shuttling.

As with the other classes of HDAC's Sirt's are also critically involved in the maintainence and functions of endothelial cells affecting angiogenesis, fibrosis of vascular senescence and endothelial metabolism. Reports have suggested that the use of resveratrol increases the endothelial barrier function while knockdown or silencing of Sirt-1 in EC decreases the barrier function. Sirt-1 is an important molecule due to their modifications of histone and non-histone targets thereby altering transcription. Sirt-1 has multiple targets in the transcription machinery altering the acetylation status of transcription factors like NF-kB, Foxo1, STAT3/STAT5 and the histone H3 specifically targeting the activation mark H3K9. Sirt-1 through these mechanisms exerts its influences in transcription.

Since Sirt-1 is a critical molecule influencing transcription and aging, efforts have been directed at understating its regulation including transcription, post-transcriptional regulation, translational and post-translational modifications. In addition, the modulation of the activity of Sirt-1 is a critical regulatory mechanism that can be attenuated by the availability of NAD or via its natural antagonist DBC1 or increased by resveratrol or its natural activator AROS. Since Sirt-1 can modulate gene transcription and downregulation of Sirt-1 or inactivation of Sirt-1 is a common mechanism that is co-related with increased histone acetylation and gene transcription.

2.3.3 MicroRNA Regulation

Discovered in 1993 by Ambros, Lee and Feinbaum, microRNA's are a novel class of small non-coding RNA that regulate the stability of mRNA molecules post-transcriptionally (Lee, Feinbaum, and Ambros 1993). They are evolutionarily conserved, sequence specific, single-stranded 18-30bp RNA molecules that form imperfect binding with the 3'UTR of target mRNA regulating the rate of translation (Fabian, Sonenberg, and Filipowicz 2010). The 3' untranslated region is a powerful region containing multiple regulatory modules that decide the spatial and temporal fate of the mRNA (Kuersten and Goodwin 2003) via the regulation by trans-acting factors The biogenesis of microRNA is a multi-step process involving drosha and dicer (Tomankova, Petrek, and Kriegova 2010). The first step is the cleavage of the stem-loop or primary miRNA. This generates a smaller fragment that is called the pre-miRNA (70-110bp) and is exported out of the nucleus by exportin-5. Once the pre-miRNA is in the nucleus, dicer works on it to generate a double stranded molecule that can be separated. The mature miRNA is then assembled onto a RISC complex, that facilitates

the binding with the 3’ UTR of its target mRNA (Pratt and MacRae 2009) and either repressing translation or tagging the RNA for degradation.

Even though the microRNA and siRNA work in similar manner, miRNA form imperfect binding with its target while siRNA forms a complete perfect binding with its target sequence thereby inducing silencing. The mechanisms of binding as stated before is imperfect in nature and is regulated by its seed sequence and its anchor sequence. The seed sequence is a heptametrical sequence starting from the 5’ end of the miRNA that binds perfectly with the canonical sites on the 3’UTR and imperfectly with the rest of the sequences (Hausser and Zavolan 2014). Since the mature miRNA are highly conserved, they have multiple targets and may regulate multiple genes simultaneously, as well as a target 3’ UTR being targeted by multiple microRNA.

MicroRNA’s are vital for the timely expression and stability of mRNA transcripts as they work at a stage just preceding the translation. The importance of microRNA has been well established in several biological processes including stress signaling (Mendell and Olson 2012), cancer, viral diseases, immune diseases, neurodegeneration (Li and Kowdley 2012) and innate immunity (McKiernan and Greene 2015). Dysregulation of microRNA is a common consequence of factors influencing disease progression and the implication of such dysregulation results in altered stability of participating mRNA transcripts and rate of translation. The quantities of microRNA themselves are regulated at several levels that include the transcription of the miRNA, processing of the miRNA through the drosha, dicer or exportin5 and the localization of the miRNA (O'Connell et al. 2010, McKiernan and Greene 2015). Thus, disruption of any of these pathways can

potentially modulate the levels of miRNA available to alter the stoichiometry of translation.

The dysregulation of miRNA in lung pathophysiology is, not surprisingly, strongly illustrated by the studies on lung cancer and the importance of such dysregulated microRNA have been demonstrated well in non-cancerous pulmonary disorders like asthma, cystic fibrosis, COPD and pulmonary artery hypertension (Sessa and Hata 2013). In cystic fibrosis dysregulation of miRNA leads to regulation of CFTR and other inflammatory mediators (McKiernan and Greene 2015). Several microRNA directly regulates the inflammatory process in cystic fibrosis including the decreased expression of miR-31 regulating IRF-1 and miR-93 leading to increases in IL-8. Further miR-126 was downregulated in in-vivo and in-vitro models and these have TLR2 and TLR4 signaling inhibitor translocase of the outer membrane (Sonneville et al. 2015) as their direct targets. Dysregulation of several microRNA including miR-146, miR-155, miR-21 have been implicated in the inflammatory processes mediated by NF-kB (Ma et al. 2011).

In EC biology microRNA's have been implicated in angiogenesis (Wu, Yang, and Li 2009), flow sensitive endothelial dysfunction (Kumar et al. 2014), vascular inflammation and EC barrier dysregulation (Chamorro-Jorganes, Araldi, and Suárez 2013). In a review article relating microRNA's to vascular biology Munekaxu Yamakuchi has stated that EC's have a specific set of miRNA that play a major role in endothelial cells and designated them as endothelial miRNA's. EC-specific dicer knockdown in mice led to dysregulated blood vessel development in-vivo and knockdown of dicer in endothelium leads to impaired proliferation and tube formation (Yamakuchi 2012) suggesting

microRNA perform significant roles in maintaining homeostasis in EC and are vital for endothelium. Further several microRNA have been associated with modulation of endothelial cell permeability including miR-125, miR-126 miR-218 (Chamorro-Jorganes, Araldi, and Suárez 2013). These confirm the essential role of microRNA in the regulation of endothelial cells.

miRNA regulation occurs downstream of diverse signaling pathways and external stimuli exert significant influences on the quantities of specific miRNA present in the cell. Of significance to the work done here is miRNA that can be modulated by TLR4 signaling, LPS and mechanical stress. Several miRNA including miR-146a, miR-155 and miR-21 seem to be regulated by TLR4 signaling and in turn regulate the participants of the TLR4 signaling pathway including myd88 and NF-kB. Further, microRNA also targets the TLR genes themselves (He, Jing, and Cheng 2014, Nahid, Satoh, and Chan 2011). Further miR-155 and miR-125 have been widely cited as vital dysregulated miRNA in response to various inciting stimuli including VILI (Vaporidi et al. 2012), endotoxin (Quinn, Wang, and Redmond 2012)and innate immune pathways(Lodish et al. 2008).

Further NAMPT mRNA has been suggested to be regulated by miRNA in several pathophysiology's including colorectal cancer where they are targeted by miR-26b (Zhang, Tong, and Huang 2013). Increased expression of miR-34 in obesity reduced NAMPT and led to decreases in NAD levels thereby counteracting the effects of Sirt-1 leading to NF-kB signaling (Choi et al. 2013). These data suggest the potential for the regulation of NAMPT via 3'UTR mechanisms.

Therapeutics targeting microRNA provides unique opportunities to modulate gene expression, as they are easy to target in-vitro and in-vivo. MicroRNA's are small RNA nucleotides whose functions by utilizing microRNA mimics that function, as the name suggests, mimicking the functions of endogenous miRNA. Complementary to mimicking the function of miRNA, molecules that are complementary to the miRNA sequences can be utilized as "sponges" to inhibit the microRNA functions. These approaches have been used successfully in several experiments in-vivo ((van Rooij, Purcell, and Levin 2012). Relevant to the upregulation or downregulation of specific miRNA antagomir and mimics can be used respectively to modulate the functions of miRNA. Our prior data in support of this has been published for nmMYLK gene where mimics supplemented for miRNA downregulated by stimuli attenuate the upregulation of nmMYLK gene and hence attenuating endothelial contractility (Adyshev et al. 2013b).

3. EXPERIMENTAL MATERIALS AND METHODS

Portions of the text were reproduced here with permission from "Unique Toll-Like Receptor 4 Activation by NAMPT/PBEF Induces NFκB Signaling and Inflammatory Lung Injury." *Sci Rep* no. 5:13135. doi: 10.1038/srep13135.

3.1 Endothelial Cell Culture

Human pulmonary artery endothelial cells (HPAEC) were obtained from Lonza (Walkersville MD). HPAEC were grown in endothelial growth medium-2 containing hEGF, Hydrocortisone, GA-1000, FBS, VEGF, hEGF-B, R3-IGF-1, Ascorbic acids and Heparin. Endothelial cell cultures were maintained at 37°C in a 5% CO2 incubator. Cells between passages 5 and 7 were used for experiments. Media was changed every alternate day until they were trypsinized and reseeded in appropriate cell culture dishes for experiments.

3.2 Reagents

5-aza-2'-deoxycytidine was purchased from Sigma Aldrich (St Louis, MO), dissolved in DMSO and used at a concentration range between 0.1 uM and 10uM for 72 hours without replacement. LPS was obtained from Sigma Aldrich (St. Louis, MO) and used at 100ng/ml at various time points as indicated in the respective experiments. Trichostatin-A was obtained from cell signaling and used at 1000nM concentration. STAT2, STAT3 and STAT5 antibodies (sc-476, sc-482, and sc-835) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SsoFast EvaGreen super mix was obtained from BioRad (Hercules, CA). Dual Luciferase assay reagents were obtained from Promega (Madison, WI). Chromatin immunoprecipitation was accomplished using a commercially available kit EZ-Magna ChIP obtained from EMD Millipore (Billerica, MA).

3.3 In-Silico 5'UTR Predictions

Genomatix software was used to identify the potential transcription factors binding to the NAMPT promoter. Specifically, several transcription factor modules were identified that included NF-kB, MSRE and STAT transcription factors.

3.4 In-Silico 3'UTR Predictions

The 3' UTR of *NAMPT* (NM_005746.2) was identified using the UCSC genome browser sequence retrieval tool. The microRNA potentially binding to the *NAMPT* 3'UTR was identified using in-silico analysis tools that effectively combined the miRanda prediction algorithm with a score based on experimental evidence for mRNA downregulation. The mirSVR score was used to identify potential conserved microRNA targeting the *NAMPT* 3'UTR. Since the algorithm is an in-silico prediction, several other in-silico identification algorithms like TargetScan, DIANA-microT were used for verifying and increasing the confidence of the predicted mirSVR miRNA. TargetScan considers five additional features of 3′ UTRs beyond seed complementarity and use site inter-specific conservation data to prediction microrna binding. DIANA-microT-CDS is specifically trained on sets of miRNA recognition elements (MREs) located in both the 3' UTR and CDS regions.

3.5 Mechanical Stress

HPAEC were trypsinized from T-75 flasks and seeded onto Collagen-I coated flexible-bottom membrane plates and grown to confluence in EGM-2 media containing 2%FBS. The flexible membraned plates containing cells were loaded onto 25mm loading stations. They were stretched at either 5% physiological stretch (0.5Hz, 30 cycles/min) or 18% pathological stretch (0.5HZ, 30cycles/min) simulating spontaneous breathing

and ventilator mediated breathing for the duration of stretch. The tension to the loading stations was applied using the FX-5000 tension system that provides uniform radial and circumferential strain along all radii. EC cultures grown on BioFlex plates maintained in the same incubator without any stretch was used as static controls. Transfection of luciferase reporter vectors or siRNA/miRNA were done on cells plated on the BioFlex plates and incubated for 24 hours before being subjected to mechanical stress. Cells were lysed either directly in SDS/LDS lysis buffer for western blot or Trizol for RNA extractions. For simultaneous isolation of RNA/DNA, lysis buffer provided in the Zymo Duet Kit was used to lyse cells directly on the BioFlex plate.

3.6 *NAMPT* Promoter-Luciferase Construction

A three kb region (3018 bp) upstream of Exon 1 for the *NAMPT (NM005746.2)* was identified amplified and clone into the PGL3-Basic vector. Promoter deletion fragments were generated by designing primers that amplified fragments serially regressive in length by 300bp. The fragments generated (-3018, -2728, -2428, -2128, -1828, -1528, -1228, -928, -628, -328) were ligated into PGL3 vector containing the ampR gene. An aliquot of the ligation mixture was transformed into DH5-α cell and plated onto LB ampicillin agar plates. Several colonies from each plate were picked and plasmid DNA was isolated using the Qiagen plasmid miniprep kit (Hercules, CA) and sequenced to verify the integrity of the insert. Selected colonies that had the right insert were amplified further and plasmid DNA was extracted using the Endotoxin Qiagen maxiprep kit. The constructs were then resequenced to ascertain the integrity of the inserts before use for transfection into endothelial cells.

3.7 Western Blot

Cells grown on tissue culture plates or on BioFlex flexible membrane plates were lysed directly on the plate using LDS lysis buffer. Cells in the lysis buffer were scraped off the plates transferred to 1.5ml centrifuge tubes and sonicated for 12 cycles of 10 seconds each with 20 seconds of rest on ice using the Qsonica water bath sonicator (Newton, CT). The sonicated samples were then spun at 12000 g for two minutes and supernatant was transferred to fresh tubes. The protein content in the samples was then quantified using the DC reagents from BioRad (Hercules, CA) and equal amounts of protein lysates were resolved on 4-12% Novex Tris-glycine gels from Invitrogen (Carlsbad, California). The electrophoresed samples were transferred onto 0.2 µm pore-size PVDF membranes in Transfer Buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol at pH 8.3) for 1 hour at 100V. Membranes were incubated for 30 minutes at room temperature in a blocking buffer containing 5% nonfat dry milk powder dissolved tris-buffered saline supplemented with 0.1% Tween 20(TBST). The membranes were then incubated with primary antibodies diluted in TBST containing 1% non-fat dry milk, rocking overnight at 4°C. Membranes were washed three times in TBST for 15 min each, followed by incubation in secondary antibody conjugated to horseradish peroxidase for 1 hr at room temperature in TBST containing 1% non-fat dry milk. Membranes were washed 3 times for 15 min each and peroxidase activity was detected with Pierce chemiluminescent substrate (Thermo Fisher Scientific; Waltham, Massachusetts). Band intensity was quantified with ImageJ and intensities of target immunogen were normalized to b-actin or GAPDH control, and expressed as arbitrary units.

3.8 Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assay (ChIP) assays were performed with commercially available EZ-magna ChIP kit (Millipore, Temecula, CA) as per manufacturer's recommendations. For methylation inhibition experiments utilizing 5-aza-2-deoxycytidine EC were seeded on D-150 dishes at $3X10^6$ cells at 30% confluency and allowed to adhere for 24 hours. The media was changed and cells were treated with either vehicle (DMSO) or 5-aza-2'-deoxycytidine for 72 hours before cells without any change in media. For mechanical stress experiments, cells were seeded on multiple BioFlex membrane plates and were either unstretched for static controls or stressed at 18 % CS (pathological stress). For all experiments, cells were cross-linked for 10 min by adding formaldehyde directly to the medium to a final concentration of 1% and quenched with glycine. Cross-linked cells were then washed twice with cold PBS (with protease inhibitors), scraped in PBS and pelleted at 800 X g for 15 minutes. The supernatant was removed and the pellet was suspended in 200 µl SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0), and incubated for 10 min on ice. The lysates were then sonicated for fifteen cycles of 15 s each, resting on ice for 45 s between cycles. After sonication, the samples were centrifuged and the supernatants diluted 10-fold in ChIP dilution buffer with protease inhibitors. Cross-linked chromatin was incubated overnight with the STAT2, STAT3, STAT5 antibodies or control IgG at 4°C. The protein-DNA complexes were then bound to Protein A beads and pulled down using the magnarack. After extensive washing, pellets were eluted by ChIP elution buffer supplemented with proteinase K and incubated at 62C for 2 hours followed by 95C for 15 minutes. The beads were carefully avoided and the supernatant was

removed from the tubes. An aliquot of DNA was used as a template in PCR. Primers specific for the STAT binding region in the MSIR of the NAMPT promoter was used to amplify 200bp fragment in a qPCR reaction with primer pair specific for GAPDH region binding ac-H3K9 used as a positive control.

3.9 Luciferase Assays

In all luciferase experiments, the target gene was fed to firefly luciferase and was co-transfected with phRL-TK, a Renilla luciferase vector. Subsequent to treatment, cells were washed in PBS thrice and lysed directly on the plate with passive lysis buffer. After incubation on the plate for 15 minutes on ice, the cells were scraped and transferred to 1.5 ml centrifuge tubes and freeze thawed thrice to completely lyse the cells. The debris on were then pelleted and the supernatant was transferred to a fresh tube. An aliquot of the lysate was then assayed for changes in the luciferase activity using the Dual Luciferase assay system in a GloMax-Multi + Detection system equipped with automated dual injectors. The raw firefly luciferase units were normalized to Renilla luciferase units and samples were compared with vehicle to represent fold changes.

3.10 Bisulfite Specific Primer Design:

The 3kb region upstream of the *NAMPT* Exon1 was used as a reference sequence and the corresponding bisulfite converted sequence was obtained using the methprimer software. The bisulfite-converted sequences were used for designing primers specific for converted DNA by targeting the primers to regions not including the CpG sites and directing the end of the primer sequence to a converted T i.e. a cytosine in the original sequence that has been converted to T in the bisulfite converted sequence. Two sets of sequences were designed for each region to be utilized in a nested approach. The

primers were validated using commercially available DNA that included unmethylated and unconverted genomic DNA, unmethylated converted DNA and methylated converted DNA. The primers validated and used in the final analysis detected only the unmethylated converted DNA and methylated converted DNA equally but not the unmethylated unconverted DNA.

3.11 DNA Methylation Analysis

Genomic DNA and RNA from EC were isolated simultaneously using the Zymo Duet kit (Irvine, CA). 1ug of isolated genomic DNA was bisulfite converted as recommended using the EZ DNA lightning kit from Zymo Research (Irvine, CA). An aliquot of bisulfite converted DNA was used to amplify the PBEF promoter region utilizing primers specifically designed to discriminate bisulfite converted and unconverted DNA. The PCR products were cloned into PGL4 sequencing vector from Invitrogen (Carlsbad, CA), plated on ampicillin selection LB agar plates, and left overnight at 37C for colony growth. Several colonies were picked from each plate and sequenced directly at Eton Biosciences (San Diego, CA). The sequenced clones were then used for methylation analysis using the BISMA software.

3.12 Real-Time PCR and semi-quantitative PCR

cDNA was generated using high capacity cDNA reverse transcription kit from Life Technologies (Carlsbad, CA) as per manufacturer's recommendation. Real-Time PCR quantified changes in NAMPT/PBEF transcripts in a Bio-Rad CFX96 apparatus using SYBR green reagents obtained from Life technologies (Carlsbad, CA), primers specific for PBEF/NAMPT and an aliquot of cDNA. Primers specific for GAPDH was used for normalization. All reactions were performed in triplicate and the changes were

evaluated by the ΔΔCt method. All primers designed for quantification were designed on exons to eliminate genomic DNA detection and the primer sequences are provided in the table.

3.13 microRNA Real-Time PCR

Total RNA including small RNA from treated EC was isolated using the miRNEasy kit from Qiagen (Valencia. CA). For reverse transcription of miRNA, an aliquot of total RNA was used in a reverse transcription reaction containing miRNA specific reverse transcription primer and reverse transcription reagents provided by Invitrogen (Carlsbad, CA). The reverse transcription was carried out at 16°C for 30 minutes, 42°C for 30 minutes and 85°C for 5 minutes. An aliquot of the reverse transcription mix was further used in a qPCR reaction with the respective microRNA primer mix, Taqman universal PCR mix and qPCR was performed at 50°C for 2 minutes, 95C for 10 minutes, repeated once each and 40 cycles of 95°C for 15s and 60°C for 60S in a BioRad CFX qPCR (Hercules, CA). The reactions were normalized to U6 snoRNA as an internal control. The Ct values were then used for changes in mature miRNA expression using standard ΔΔCt method as recommended by MIQE guidelines.

3.14 TER and ECIS:

Cells were seeded in polycarbonate wells containing evaporated gold microelectrodes (8W10E, Biophysics, Troy, New York) in EBM-2 with 2% FBS for 24 hours until confluent. TER measurements were performed using an electrical cell substrate impedance-sensing system (ECIS) (Applied Biophysics) as previously described (Garcia et al. 2001). Briefly, current was applied across the electrodes by a 4,000-Hz AC voltage source with amplitude of 1 V in series with a 1 MΩ resistance to

approximate a constant current source (~1 μA). The in-phase and out-of-phase voltages between the electrodes were monitored in real time with the lock-in amplifier and subsequently converted to scalar measurements of transendothelial impedance, of which resistance was the primary focus. TER was monitored for 30 min – several hours to establish a baseline resistance (R0). As cells adhere and spread out on the microelectrode, TER increases (maximal at confluence), whereas cell retraction, rounding, or loss of adhesion is reflected by a decrease in TER. These measurements provide a highly sensitive biophysical assay that indicates the state of cell shape and focal adhesion (Giaever and Keese 1993). Values from each microelectrode were pooled at discrete time points and plotted versus time as the mean ± SE of the mean.

3.15 Plasmid Transfection into Endothelial Cells

Both the NAMPT 5'UTR-luciferase constructs and NAMPT luciferase-3'UTR constructs were transfected into endothelial cells using the Fugene HD reagent from Promega (Madison, WI). Briefly, 2ug of DNA to be transfected were suspended in 100ul of opti-MEM without any serum or antibiotics and 6ul of Fugene HD was added (1:3 ratio of DNA: reagent). The complexes were mixed using gentle pipetting and incubated for 15 minutes to form the complexes. The complex was then distributed dropwise onto the cells in 6 well dishes, swirled. The complex containing media was changed next day morning with fresh culture medium.

3.16 RNAi Experiments

Silencing of EC for specific genes was accomplished using either the siPORT amine reagent from Invitrogen (Carlsbad, CA) or Lipojet in-vitro transfection reagent from Signagen (Rockville, MD). siRNA specific for each target gene was obtained from

Dharmacon or from IDT. The targets and specific siRNA used is listed in the table below. All miRNA mimics (final concentration 30nM) and antagomirs (final concentration 50nM) were transfected into EC using the siPORT amine transfection reagents from Invitrogen (Carlsbad, CA). Non-targeting controls at equimolar concentrations were transfected into EC for nonsequence-mediated effects in miRNA experiments.

3.17 Co-Transfection Experiments

For co-transfection experiments utilizing a reporter vector and the microRNA modifiers, the 3'UTR reporter vectors were transfected with Xfect reagent from clontech (Mountainview, CA) for 4 hours and fresh media added. Further, the microRNA modifiers were transfected for additional four hours before media was changed again. 24 hours later cells were treated with LPS (100ng/ml), TNF-α (10ng/ml) or 18% injurious stretch for additional 24 hours before lysing for luciferase activity.

3.18 Statistical Analysis

For all *in vitro* (n of 3 or more) or *in vivo* (n of 3-6) experiments, values are shown as the mean ± SEM and data were analyzed using standard student's t test or two-way ANOVA. Significance in all cases was defined at $p < 0.05$.

4. TRANSCRIPTION OF NAMPT AND IMPACT OF GENETIC VARIANTS IN EXCESSIVE MECHANICAL STRESS

This research was originally published in the American Journal for Respiratory Cell and Molecular Biology. Sun X, Elangovan VR, Mapes B, Camp SM, Sammani S, Saadat L, Ceco E, Ma SF, Flores C, MacDougall MS, Quijada H, Liu B, Kempf CL, Wang T, Chiang ET, Garcia JG. The NAMPT promoter is regulated by mechanical stress, signal transducer and activator of transcription 5, and acute respiratory distress syndrome-associated genetic variants. Am J Respir Cell Mol Biol. 2014 Nov;51(5):660-7. doi: 10.1165/rcmb.2014-0117OC. Portions of the text and all the figures have been reproduced here with permission.

4.1 Overview

To restate, ARDS is a devastating syndrome characterized by rampant inflammation. Patients undergo respiratory failure, because of alveolar flooding caused by endothelial barrier dysfunction. Ventilators providing support through mechanical ventilation are required for respiratory support, but are concomitant with increased pulmonary inflammation even in the absence of prior insult initiated by syndromes like ARDS. Despite intensive efforts to attenuate the severities of VILI, effective therapies are lacking and hence novel strategies are necessary to reduce VILI severity. Candidate gene based approaches, a powerful technique to study complex diseases, had identified several novel molecules significantly modulated in multi-species, pre-clinical models of VILI. Among multiple candidate genes, our lab identified Nicotinamide Phosphoribosyl transferase (NAMPT), a novel “cytozyme” upregulated by stretch and inflammatory cytokines, increased in VILI. Further evidences from our lab indicate that

extracellular NAMPT bind directly to TLR4 receptor, influencing NF-kB pathway, and hence modulating the severity of VILI induced inflammation. Thus, NAMPT is a novel molecule mechanistically linked to severe VILI pathophysiology through upregulation in transcription and subsequent secretion into the plasma (Ye, Simon, et al. 2005, Camp et al. 2015)

Lung immunohistochemistry from murine pre-clinical models revealed that NAMPT localized to the lung endothelium, epithelium and leukocytes. The transcriptional upregulation of this molecule is not understood and the objectives of this part of my thesis has been to elucidate the mechanisms driving transcription of this pivotal molecule, in excessively stressed human lung pulmonary artery endothelial cells a source of extracellular NAMPT secretion. NAMPT promoter activity in response to physiological stretch (5% CS) or excessive stretch (18% CS) was assessed utilizing full-length promoter luciferase constructs. Besides the functional elements of the NAMPT promoter responsive to excessive stretch was delineated by serial deletion constructs.

Identification of transcription factors binding to the NAMPT promoter would align novel targets, directed at attenuating the severity of VILI, through modulation of *NAMPT* transcripts. In-silico analysis of NAMPT promoter identified several potential transcription factor modules including NF-kB, ARE, MSRE and STAT transcription factors. Further, the predicted transcription factors mapped to specific regulatory regions and localized multiple STAT transcription factor binding modules to the NAMPT MSIR. ChIP assays performed with antibodies specific for the members of the STAT family of transcription factors elucidated the influence of STAT5 transcription factor in the mechanical stimulation driven upregulation of NAMPT.

Previous studies from our lab have demonstrated the association of severe sepsis related ARDS susceptibility to genetic variants in the NAMPT promoter. In particular, an identified SNP C-1543T significantly associated with ARDS. NAMPT promoter luciferase constructs harboring either of the SNP revealed that the T-allele had a significantly reduced activation (~ 2 fold) compared to the C-allele. The T-allele presented significantly lowly in the ALI population validating that genetic factors increase transcript levels contributing to the severity of ALI (Ye, Garcia, 2005). In this study, two NAMPT promoter variants rs59744560 (-948G/T) and rs7789066 (-2422A/G) identified in the European descent cohorts significantly co-related with increased ventilator days and mortality. To evaluate the impact of the relevant SNP's the NAMPT full-length promoter luciferase construct mutated to either genotype demonstrated increased NAMPT transcription in excessive stress compared to the wild type. STAT5 transcription factor further influenced the regulation of all three genotypes. These data together demonstrated the regulation of NAMPT promoter through biochemical mechanisms (STAT5 binding) and genetic variants (rs59744560 and rs7789066) in human lung endothelium.

4.2 NAMPT Expression in BAL and Pulmonary Endothelium is Increased

NAMPT consistently increases in the bronchoalveolarlavage (BAL) and serum of pre-clinical models of lung injury and ARDS patients and NAMPT protein expression also increased in the lung endothelium (Ye, Simon, et al. 2005). In this study, NAMPT expression in BAL macrophages and pulmonary endothelium evaluated by immunohistochemistry studies confirmed previous observations that NAMPT expression increased in pre-clinical models of lung injury. These data demonstrated both in-vivo

and in-vitro that NAMPT is a stretch responsive gene increased in ARDS and VILI besides validating the potential for using increased levels of NAMPT as a potential biomarker in ARDS.

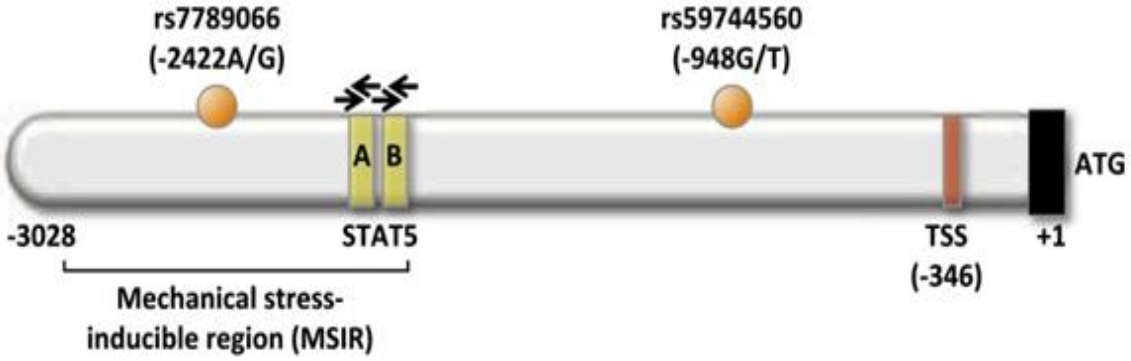

Figure 6: Description of the 3kb region upstream of NAMPT +1 ATG. The NAMPT promoter representation shows the location of two SNP's rs7789066 (-2422 A/G) and rs59744560 (-948 G/T) and STAT5 transcription factor binding modules present in the NAMPT promoter.

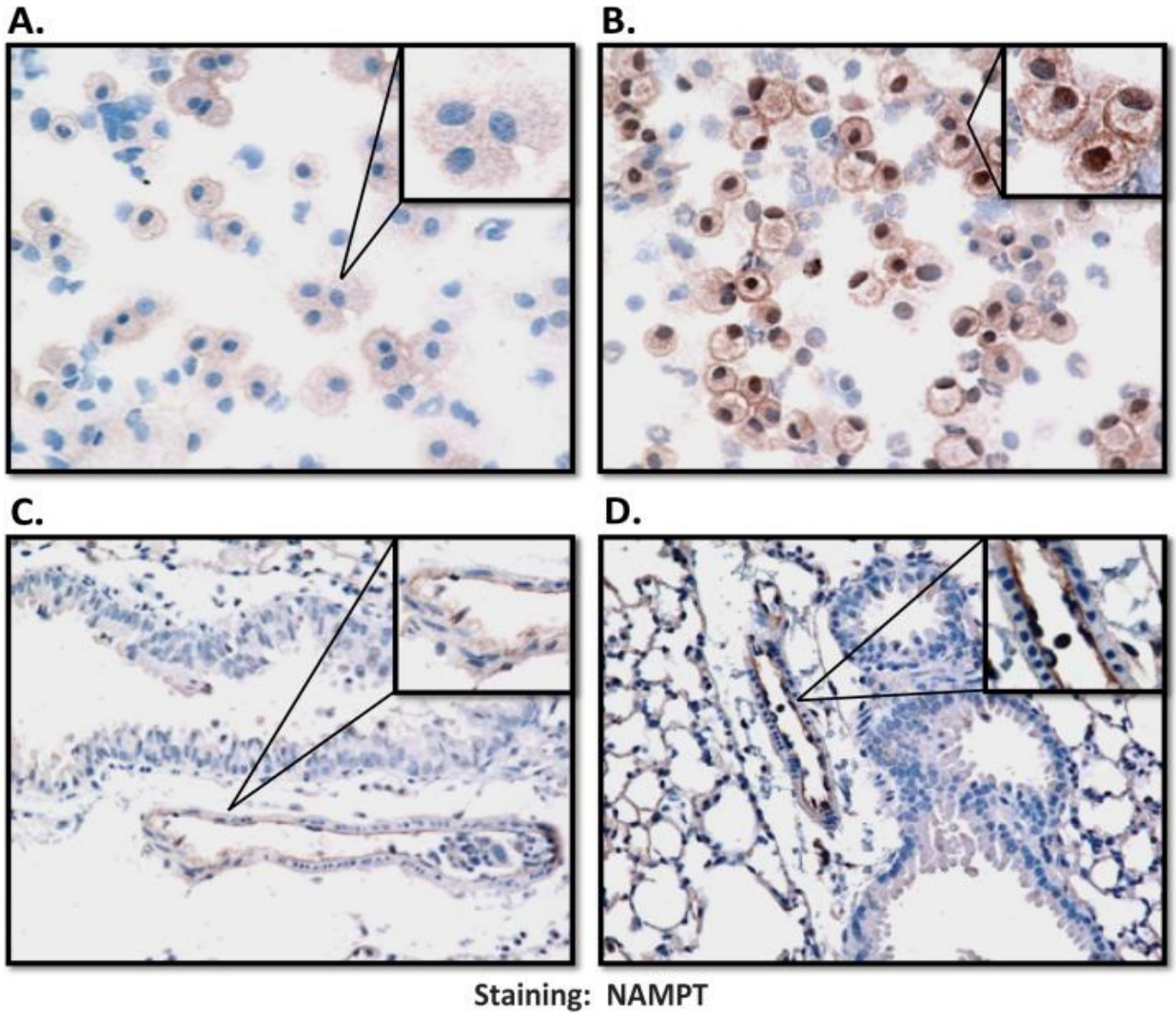

Figure 7: Immunohistochemistry in mice tissue shows increased expression and localization of NAMPT to the leukocytes and lung endothelium. NAMPT protein expression is significantly increased in response to high tidal volume mechanical ventilation in mice and compared to spontaneous breathing (A), NAMPT expression was increased in leukocytes of BAL (B). Similar immunohistochemistry experiments showed that compared to spontaneous breathing mice(C) increased NAMPT expression and localization in the mice lung endothelium (D).

4.3 Excessive mechanical stress of lung EC increases NAMPT gene expression

NAMPT mRNA is increased in ALI patients compared to controls and in pre-clinical animal models of ALI (Ye, Simon, et al. 2005). However, the spatial and temporal expression of *NAMPT* in human lung EC has not been characterized previously despite increased protein expression in the lung endothelium. To evaluate the impact of excessive stretch on NAMPT gene expression, cells seeded on BioFlex membrane plates were stretched at either 5% CS (physiological) or 18% CS (pathological) for several time points ranging from 0.5 hrs to 48 hrs. RNA isolated from these cells were used for cDNA conversion and qPCR analysis was performed using primers specific for NAMPT, with primers targeting GAPDH used as internal controls. As expected NAMPT expression increased in response to excessive mechanical stress (18% CS) in lung EC compared to physiological stretch (5% CS) which did not change significantly. The expression pattern showed a time-dependent modulation, with significant increases occurring at 4hr, 6hr and 48hrs. In complementary approaches to validate the excessive mechanical stress induced NAMPT expression, full-length NAMPT promoter construct (-3028 to +1 ATG) was transfected into lung EC and stretched at 18% magnitude. Compared to unstretched cells (static) cells, excessive stress increased NAMPT promoter luciferase activity between 1 and 4 hours validating the increases of NAMPT mRNA expression. Thus, mechanical stress is a positive contributor to upregulated NAMPT mRNA expression in human lung EC and deciphering the mechanisms of this regulation is of vital importance to generate pharmacotherapies.

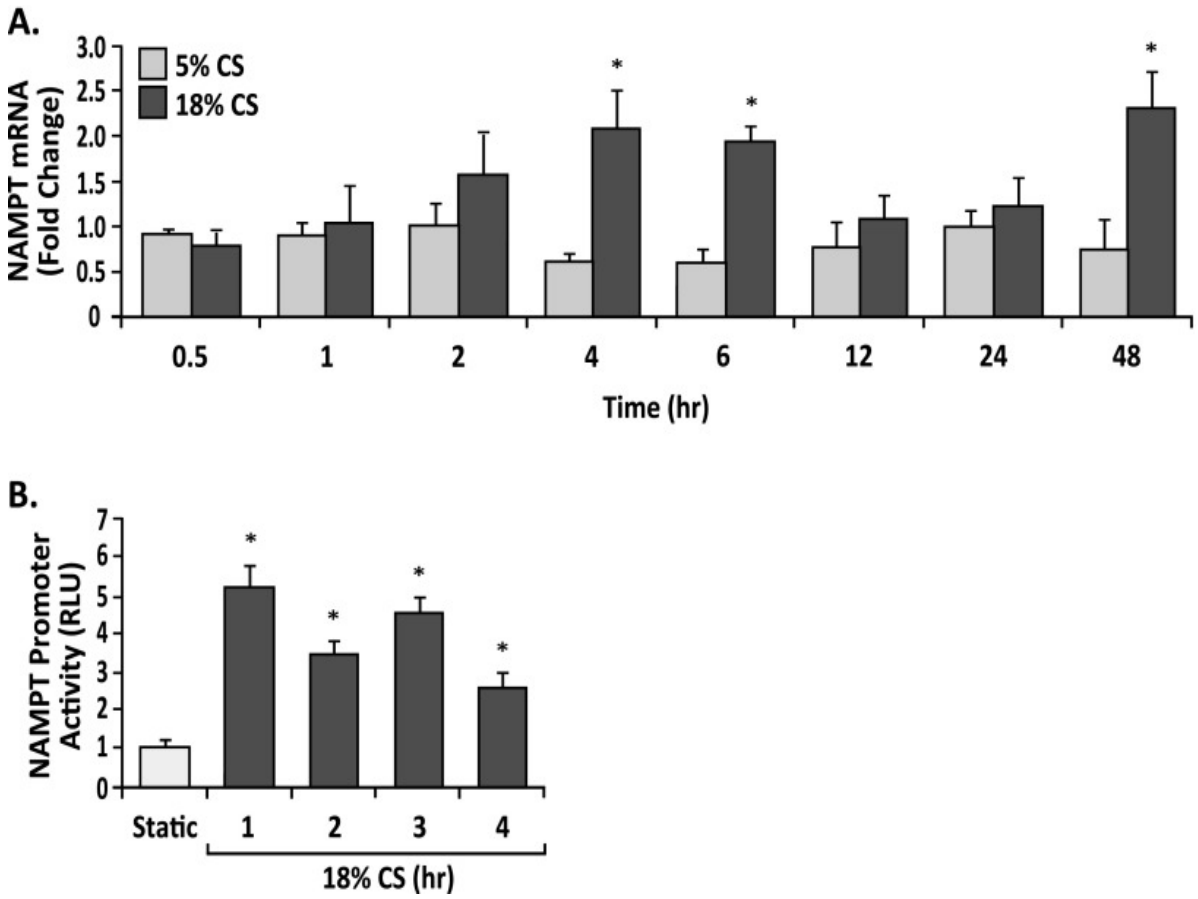

Figure 8: NAMPT mRNA and promoter-luciferase activity is upregulated in human lung EC exposed to 18% CS. NAMPT gene expression assayed by qPCR showed time-dependent changes in response to excessive mechanical stress but not physiological stress (A) with increased expression at 4hrs, 6hrs and 24hrs. Complementary approaches utilizing NAMPT promoter constructs transfected into lung EC and stretched excessively (18% CS) increased promoter luciferase activity (~5 fold).

4.4 Delineating the functional elements of the NAMPT Promoter

To further understand the mechanisms driving NAMPT transcription in response to mechanical stress; it was necessary to determine the functional and regulatory elements of the NAMPT promoter and potential transcription factors binding to the specific regulatory regions. The full- length promoter construct was serially deleted at the 5' end to generate a series of fragments lacking 300bp successively at the 5'end. The serially deleted constructs were then transfected into lung EC and stretched at 18% CS to identify the regulatory regions of the NAMPT promoter. The basal level expression and mechanically induced expression was significantly attenuated between -2428bp and -1228bp suggesting that the region bound repressing factors and thus acting as a negative regulatory region. The repressive effect was not observed in the full-length construct or the -2728bp fragment suggesting an enhancer effect for this region. The fact that basal level and mechanically inducible promoter activities were fully functional between -1228 and -628bp suggested that this region is the proximal promoter/constitutively active region. Further, the data demonstrated that the mechanical-stress inducible region lay between -2428 and -2128bp as the response to 18%CS was attenuated in the absence of this fragment. The serial deletion luciferase assays thus demonstrated that the NAMPT promoter has multiple regulatory regions including a proximal promoter region extending from -628bp to -1228bp, a negative regulatory region from -1228bp to -2428bp and enhancer/distal promoter were ranging from -2428bp to -3028bp. The delineation of the promoter regions in the NAMPT promoter thus led to identifying transcription factors that bound to specific regulatory elements driving the inducible transcription of NAMPT promoter.

4.5 STAT5 drives mechanical stress mediated NAMPT expression.

In-silico analysis of potential transcription factors as described in the materials and methods identified several potential transcription factors binding the NAMPT promoter. Based on the location of binding, STAT family of transcription factors potentially bound the mechanical stress inducible region lying between the -2428bp and -2128bp. Chromatin immunoprecipitation assays utilizing antibodies targeting the STAT family of transcription factors (STAT2, STAT3 and STAT5) revealed that STAT5 transcription factor was increasingly bound to the MSIR of the NAMPT promoter, while STAT3 showed minimal yet increased binding to the same region whereas STAT2 binding was insignificant. These experiments showed that STAT5 transcription factors are important for the increased NAMPT transcription in response to mechanical stress. Since the NAMPT promoter consisted of a proximal and distal promoter with predicted STAT transcription factor binding sites, I also studied the binding of STAT transcription factors to the proximal promoter. The proximal promoter depicted the same trends in binding with STAT5 transcription factor significantly bound higher compared to STAT2 and STAT3 transcription factors. Further silencing of STAT5 significantly attenuated mechanical induced increases of NAMPT promoter activity. These experiments decisively established STAT5 as the critical transcription factor driving NAMPT expression in mechanically induced lung endothelial cells. This is the first report to have implicated STAT5 as an important transcription factor driving NAMPT a critically important cytokine in mechanical stress.

4.6 NAMPT genetic variants confer increased transcription in a STAT-5 dependent manner

Genetic variants of NAMPT promoter have been associated with increased susceptibility to ARDS and increased days on mechanical ventilation. In this study through sequencing of ARDS patients and controls our group identified two SNPS's rs59744560 (-948 G/T) and rs7789066 (-2422 A/G) positively associated with increased susceptibility in two European descent cohorts. In-vitro luciferase assays were performed with a wild-type full-length promoter luciferase constructs or those harboring one of the two ARDS susceptibility associated SNP's. Cells were co-transfected with one of the plasmids and either negative control siRNA or STAT5 silencing RNA and stretched at 18% magnitude. In-vitro luciferase assays revealed that compared to unstretched cells, excessive mechanical stress increased NAMPT promoter activity that was attenuated by silencing STAT5 transcription factor. This data conclusively proved that NAMPT expression is critically modulated by STAT5 transcription factor in mechanical stress. Further study of the two genetic variants built into the NAMPT wild-type plasmid demonstrated that both SNP's increased NAMPT expression higher than the wild-type induction. This data provided in-vitro evidence linking increased NAMPT expression, influenced by genetic factors that are positively associated with susceptibility to ARDS. These data suggest that genetic factors may influence increased transcription of NAMPT leading to higher mortality and days on ventilation. The most interesting finding of these genetic factors was that both the SNP's were regulated in a STAT5 dependent manner, where silencing of STAT5 significantly attenuated mechanical stress induced NAMPT expression for rs59744560 but partially attenuated

similar responses for rs7789066. Taken together genetic factors may influence increased NAMPT expression causing higher mortality, and are influenced by STAT5.

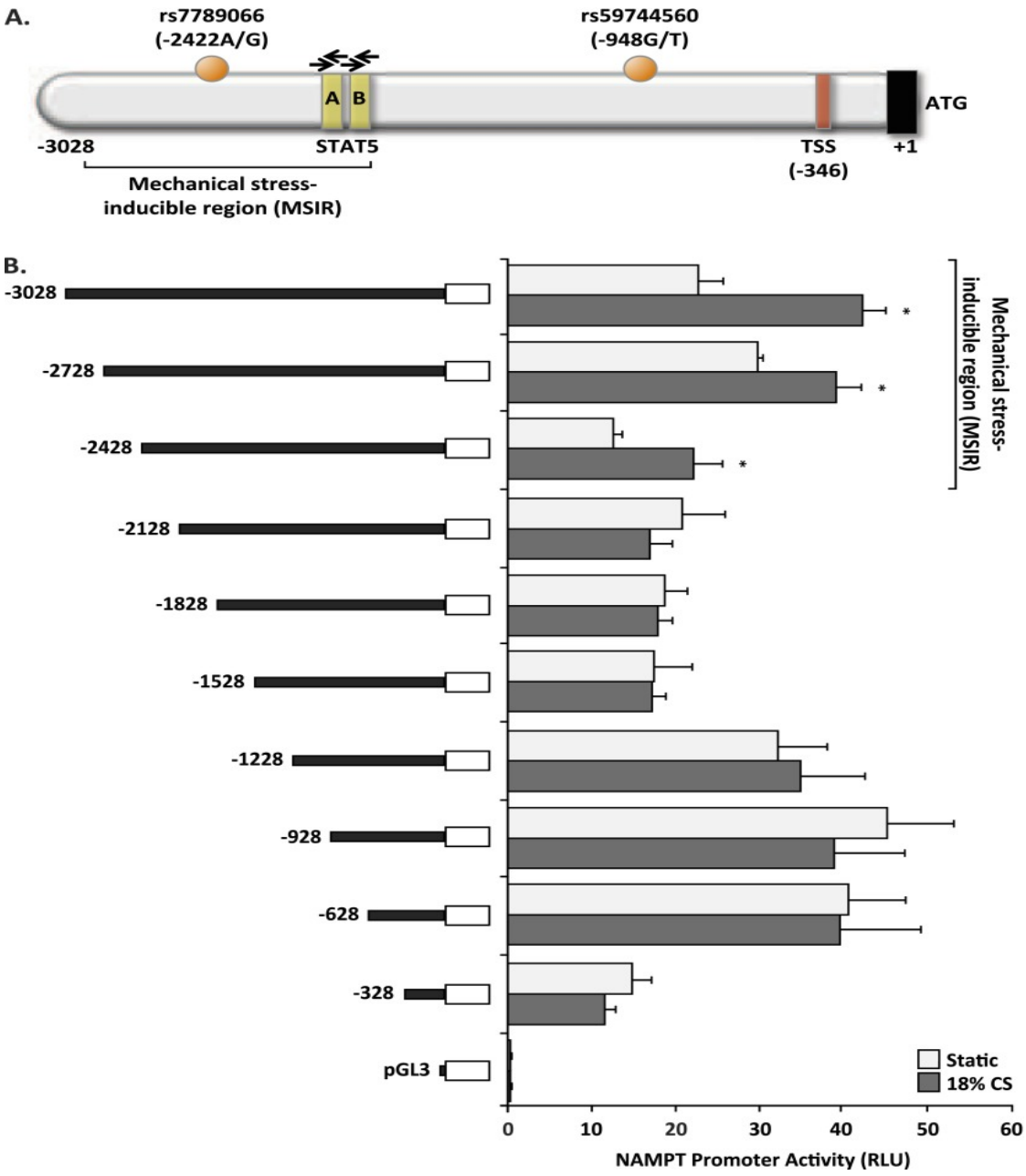

Figure 9: Delineation of the functional elements of the NAMPT promoter. Serial deletion assays utilizing NAMPT promoter constructs transfected into human lung EC and subjected to excessive mechanical stress revealed three different regulatory regions in the NAMPT promoter. -628bp to -1228bp was the proximal promoter, -1228bp to -2428bp was identified as the negative regulatory region and the region from -2128 to -3028 was identified as the mechanical stress inducible region.

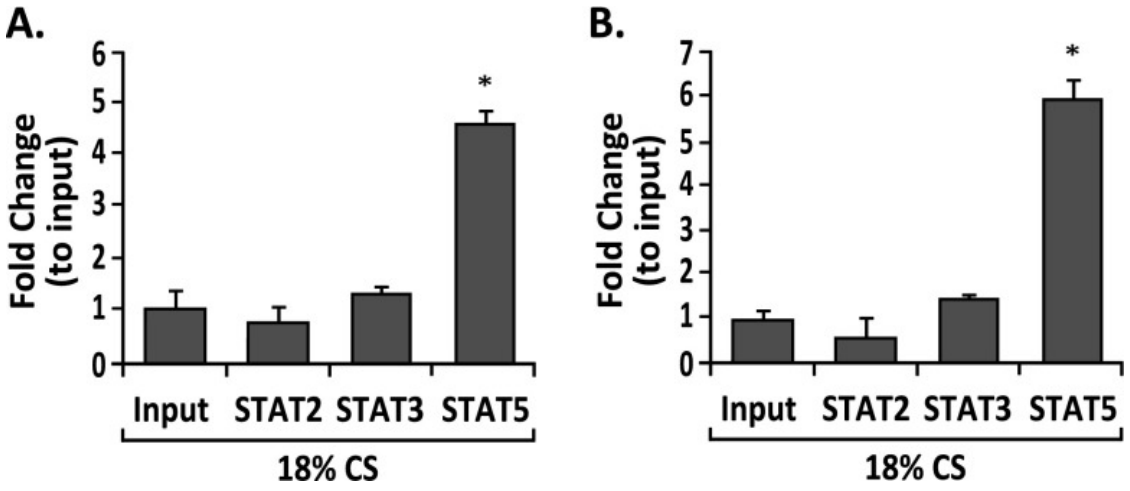

Figure 10: Excessive mechanical stress drives increased STAT5 (and STAT3) to the MSIR and proximal promoter of NAMPT. Chromatin immunoprecipitation assay was performed with antibodies targeted for STAT2, STAT3 and STAT5 followed by qPCR Primers specific for regions containing in-silico predicted NAMPT STAT transcription factor binding modules. The results demonstrated increased STAT5 (and STAT3) transcription factor binding to both the MSIR and proximal promoter modules. This data is evident for STAT5 transcription factor as a critical driver of excessive stress induced NAMPT expression.

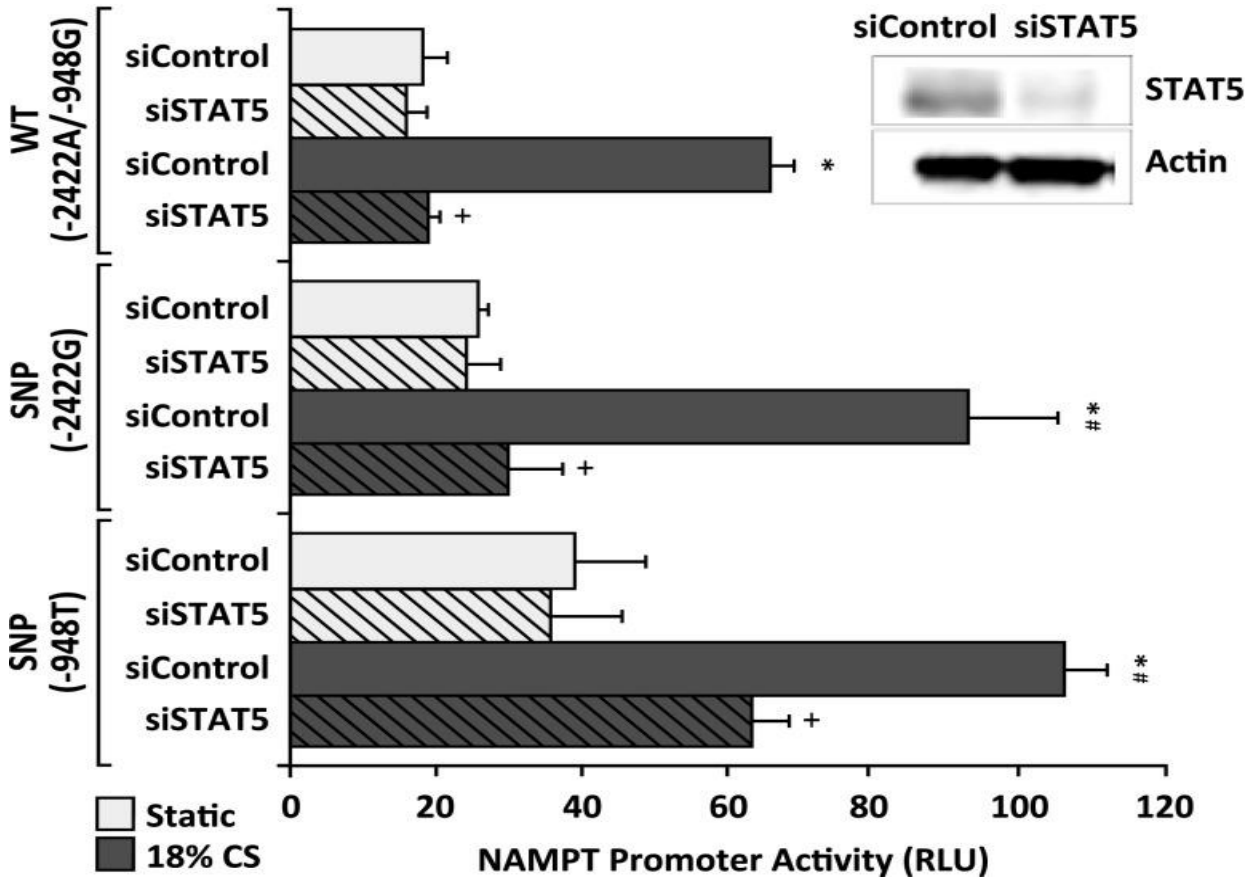

Figure 11: Excessive stress (18% CS) upregulated NAMPT promoter-luciferase promoter activity and SNP's influence higher transcription in a STAT-5 dependent manner. Excessive mechanical stress induced increases in NAMPT promoter activity in human lung EC transfected with full-length promoter luciferase construct harboring WT (-948G/-2422A), -2422G NSP or the -948T SNP. Both the SNP's conferring increased susceptibility in ARDS (-2422G or -948T) conferred increased transcriptional responses compared to the WT induced increases. Further silencing of the STAT5 transcription factor attenuated increases in NAMPT promoter luciferase activity in all genotypes was mechanistically linking transcription regulation to STAT5 binding.

5. EPIGENETIC MODIFIERS ALTER NAMPT TRANSCRIPTION AND STABILITY

5.1 Overview

DNA methylation occurring in CpG dinucleotide context is a critical epigenetic mediator of transcription. Whole genome sequencing analysis demonstrated the significance of altered DNA methylation in lung injury, where LPS induced acute lung injury in rat models associated with aberrant DNA methylation causing the pathophysiology of ARDS (Zhang et al. 2013). Biochemical experiments utilizing 5-aza-2'deoxycytidine a potent DNA methylation inhibitor resulted in resolution of lung injury by increasing T-reg number in LPS induced ALI (Singer et al. 2015) or by rescuing UCHL1 concomitantly decreasing total cell count and BAL PMN in GADD45-/- mice subjected to ventilator-induced lung injury(Mitra, Sammani, et al. 2011). These data implicate aberrant DNA methylation occurring in gene promoters of inflammatory genes as a causative factor for inflammation and the potent use of DNA methyl transferase inhibitor (DAC) in alleviating inflammation in lung injury.

In this study, DNA methyltransferases inhibitors treatment on human lung endothelium, effectively reactivated NAMPT gene promoter activity in a dose dependent manner and subsequently increased STAT5 and STAT3 binding to the MSIR region of the NAMPT promoter. These data suggest that NAMPT promoter is under transcriptional control via DNA methylation and changes occurring in response to mechanical stress may contribute to increased transcription. Histone deacetylases (HDAC's) are a family of proteins that exert influences on the acetylation status of both histone and non-histone proteins, thereby altering transcription of genes and stability of proteins. HDAC inhibition

has been widely utilized to suppress inflammation in diverse tissues and in this study, we sought to understand the role of HDAC inhibition in the regulation of NAMPT promoter activity. The specific role of HDAC class I and II inhibitors(Trichostatin-A) versus the HDAC class III Sirt-1(silencing) in the regulation of NAMPT gene expression occurring in lung EC were compared revealing contrasting roles for the two classes of molecules in regulating NAMPT transcription. While Class I and II HDAC inhibition led to attenuation of induced increases in NAMPT transcription, Class III HDAC silencing led to increased NAMPT transcription, suggesting distinct activation of deacetylase functions in EC.

5.2 In-Silico identification of CpG sites/islands in NAMPT promoter.

3kb region upstream of the NAMPT 1st exon retrieved from UCSC genome browser and in-silico analysis of CpG sites was performed with methyl primer express. In-silico prediction revealed significantly high representation of CpG sites (up to 2 kb) with two distinct CpG islands. Comparative analysis of TNF-α promoter a similar cytokine had significant representation of CpG sites but did not have any CpG islands. The CpG islands covered the constitutively active promoter and the negative regulatory region from -366 TSS to -2428bp. Further, the region corresponding to the mechanical stress inducible region contained four CpG sites. To understand the influence of CpG methylation on STAT transcription factors binding to the NAMPT promoter, the consensus sequence for STAT was probed for the presence of CpG sites. The STAT binding modules on the NAMPT promoter lacked any CpG motif, suggesting any regulation of DNA methylation for STAT transcription factors must occur independently of the STAT binding sites.

5.3 Pharmacological inhibition of methyltransferases increases NAMPT transcripts

NAMPT promoter is significantly represented by dense CpG sites, resulting in two distinct CpG islands. 5-aza-2’ deoxycytidine has been increasingly used to remove methyl groups from DNA thereby reactivating gene expression. In this study 5-aza-2’deoxycytidine as a pharmacological inhibitor of DNA methylation was used to understand the influence of DNA demethylation on NAMPT promoter activity. Compared to vehicle treated cells pharmacological inhibition of DNA methyltransferases in lung EC significantly increased NAMPT expression in dose-dependent manner suggesting DNA methylation is an influential determinant in the regulation of the NAMPT promoter.

5.4 Pharmacological inhibition of methyltransferases induces increases in STAT5 binding

Chromatin immunoprecipitation experiments with STAT3 and STAT5 antibodies in experiments utilizing pharmacological intervention through DNA demethylation agent 5-aza-2’ deoxycytidine revealed that STAT5 and STAT3 were increasingly bound to the STAT modules in the MSIR. The increases in binding of STAT5 transcription factor despite lack of CpG sites in the binding motif, suggests demethylation events occurring in close proximity of the binding site in the MSIR contributes to increased access to STAT5 transcription factors. Demethylation may contribute to increased transcription factor availability by relaxing the chromatin. The acetylation of histone H3K9 is linked to regions of increased transcription and the observation of increased acetyl-H3K9 binding to the same region as STAT5 lends support to the hypothesis that demethylation

induced chromatin relaxation contributes to increased transcription factor binding. This is not further explored in this thesis but used as an indicator for demethylation events occurring in the locus of the STAT binding module located in the MSIR.

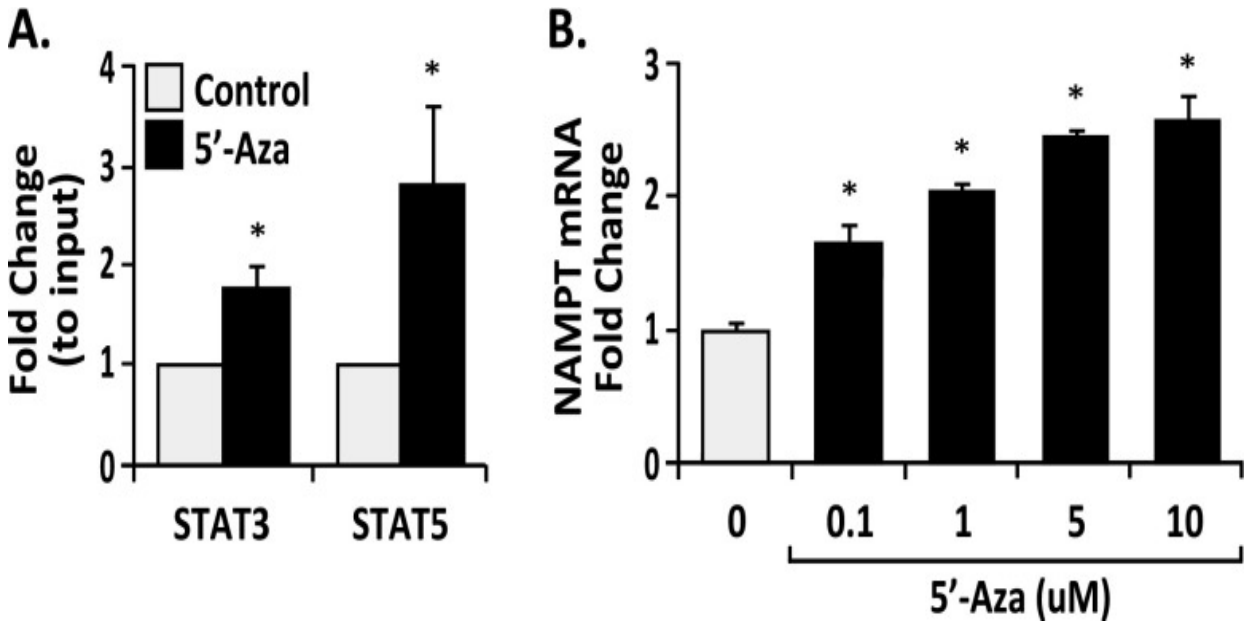

Figure 12: 5-aza-2'-deoxycytidine mediated DNA inhibition increases NAMPT mRNA transcripts and higher binding of STAT5 and STAT3 transcription factors. qPCR performed with primers specific for NAMPT to evaluate mRNA levels and with primers specific for the predicted STAT binding region in the MSIR. Demethylation of DNA increased the STAT5 (~3 fold) and STAT3 (~ 2 fold) transcription factors (A) compared to vehicle. Further mRNA was increased in a dose-dependent manner (B) in response to methylation inhibition.

5.5 Characterization of basal level methylation in the NAMPT promoter.

The NAMPT promoter is significantly represented by dense CpG sites and pharmacological demethylation increased NAMPT gene transcription. The NAMPT promoter can be distinctly classified into several regulatory modules and has been described earlier in this thesis. Surprisingly STAT5 and STAT3 increased binding to the MSIR of the NAMPT promoter despite the lack of CpG sites in the STAT consensus sequence. This led us to hypothesize that demethylation of several CpG sites adjacent to the STAT binding module may influence the increased binding of STAT5 either through synergistic interaction with a transcription factor binding to an adjacent demethylated site or through relaxation of chromatin induced by demethylation of the NAMPT promoter.

However, the methylation status of the NAMPT promoter is lacking and it is necessary to understand the basal level methylation profile occurring in the various regulatory regions. Bisulfite sequencing of a continuous 2kb region upstream of the NAMPT promoter that represents the two CpG islands revealed that the proximal promoter region/constitutively active region was completely demethylated, while the adjacent negative regulatory region was substantially methylated. Since methylation of CpG residues restricts access to transcription factors and suppresses transcription, the observation of CpG methylation in the negative regulatory region and the absence of CpG methylation in the constitutively active promoter was consistent with their regulatory roles.

5.6 Demethylation in the NAMPT promoter upon excessive stress and endotoxin treatment

Genomic DNA from human lung EC treated with LPS or subjected to 18% CS was used in bisulfite sequencing was performed to delineate the methylation changes occurring in the NAMPT promoter. Compared to unstimulated cells methylation levels in the region encompassing -2428bp to -1228bp showed decreased methylation levels in response to both excessive stretch and endotoxin challenges. Specifically, a CpG site located less than 100bp from the STAT module in the MSIR was demethylated. In-silico prediction of transcription factors binding to the demethylated site identified Pax5. These data demonstrated for the first time that distinct changes in the methylation profile occur in the NAMPT promoter in response to stimuli and inversely co-related with gene transcription.

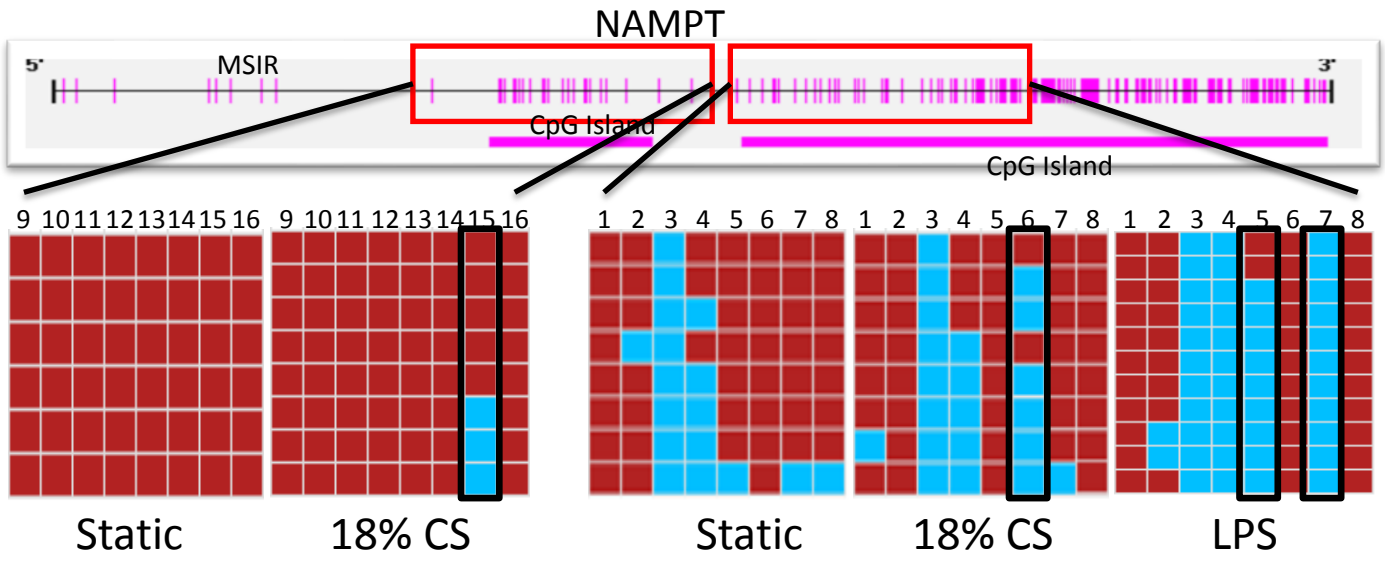

Figure 13: Endotoxin and mechanical stress induces decreases in DNA methylation in the NAMPT promoter. Bisulfite sequencing of DNA from human lung EC demonstrated decreases in DNA methylation occurring in both the CpG islands of the NAMPT promoter. Compared to unstretched or unstimulated cells 18% CS and endotoxin challenges DNA methylation was significantly decreased. Blue bars depict unmethylated alleles, while red bars depict methylated alleles. Each row is representative of a unique allele and column depictive of individual CpG sites.

5.7 Pharmacological HDAC inhibition attenuates LPS induced NAMPT gene expression

From prior studies and recent evidences, LPS has been demonstrated to be a strong agonist upregulating NAMPT gene and protein expression in lung murine tissues and in human lung EC (Ye and Garcia, 2004). Human lung EC was treated in-vitro with two different doses of Trichostatin-A (400nM, 1000nM) in the absence and presence of LPS (100ng/ml). The basal level NAMPT was significantly reduced by ~40% for both doses in the absence of any stimulation. While LPS elevated NAMPT transcripts robustly (~4 fold) in the absence of Trichostatin-A, pre-treatment with Trichostatin-A attenuated the impact of endotoxins (at both doses) in upregulating NAMPT gene transcripts. These results were consistent with the suppression of inflammatory cytokine transcription by Trichostatin-A. Experiments performed with EC and assayed for NAMPT protein expression revealed that endotoxins increased NAMPT protein expression 24 hour's post-treatment. Pre-treatment with 1000nM Trichostatin-A increased attenuated endotoxin induced NAMPT protein expression in human lung EC, however 400nM pre-treatment did not prevent upregulation.

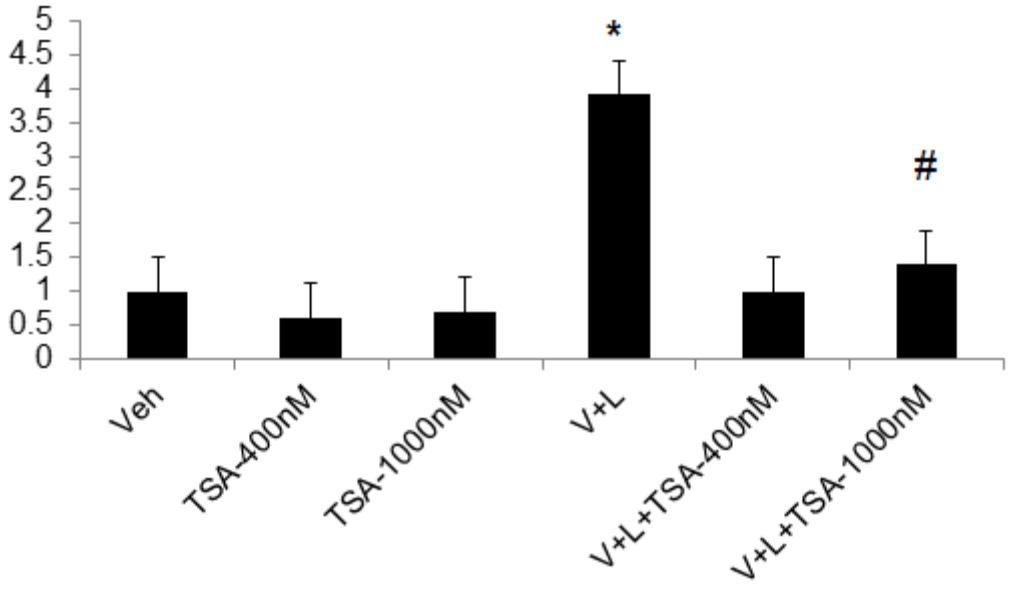

Figure 14: Trichostatin-A attenuated LPS induced NAMPT expression in human lung EC. Human lung EC were either pre-treated with vehicle or HDAC class I and II specific inhibitor Trichostatin-A (100nM and 1000nM) and were further unstimulated or stimulated with LPS (100ng/ml). Trichostatin-A decreased NAMPT transcripts (~40%) at both doses in unstimulated cells. Further pre-treatment with Trichostatin-A attenuated LPS induced increases in NAMPT mRNA.

5.8 Silencing of Sirt-1 increases NAMPT gene expression and protein expression

Sirt-1 is an important NAD-dependent deacetylase that works on histones to deacetylate and hence suppress transcription. Sirt-1 mediated regulatory control of transcription factors like c-myc (Menssen and Hermeking 2012) and Foxo1 (Tao et al. 2011) have been associated with increased NAMPT transcription. Further Sirt-1 and NAMPT form a feedback loop regulating each other. To understand the role of Class III HDAC's which are NAD-dependent deacetylases in the regulation of NAMPT in human lung EC, siRNA specific for Sirt-1 was transfected and the transcriptional responses for NAMPT were evaluated using qPCR and western blot assays. Silencing of Sirt-1 significantly increased NAMPT transcript levels (~ 3 fold) suggesting that Sirt-1 regulates NAMPT transcriptional activity in human lung EC. Further western blot assays showed that depletion of Sirt-1 in human lung EC, significantly increased NAMPT protein expression that paralleled LPS induced NAMPT protein expression in negative control transfected cells. However, there was no synergistic effect of LPS and Sirt-1 depletion in modulating NAMPT, implying that the pathways are synergistic with each other and modulation of Sirt-1 expression or activity may be a mechanism through which NAMPT transcription may be modulated. The expression of Sirt-1 is decreased in several cell lines that have been positively co-related with increased gene transcription of cytokines like TNF-α (Shen et al. 2009) in macrophage cell lines. We further sought to study if Sirt-1 is modulated in response to LPS in the human lung EC and surprisingly Sirt-1 expression is increased. The mechanisms of upregulation and the functional implications of this upregulation are discussed in further chapters.

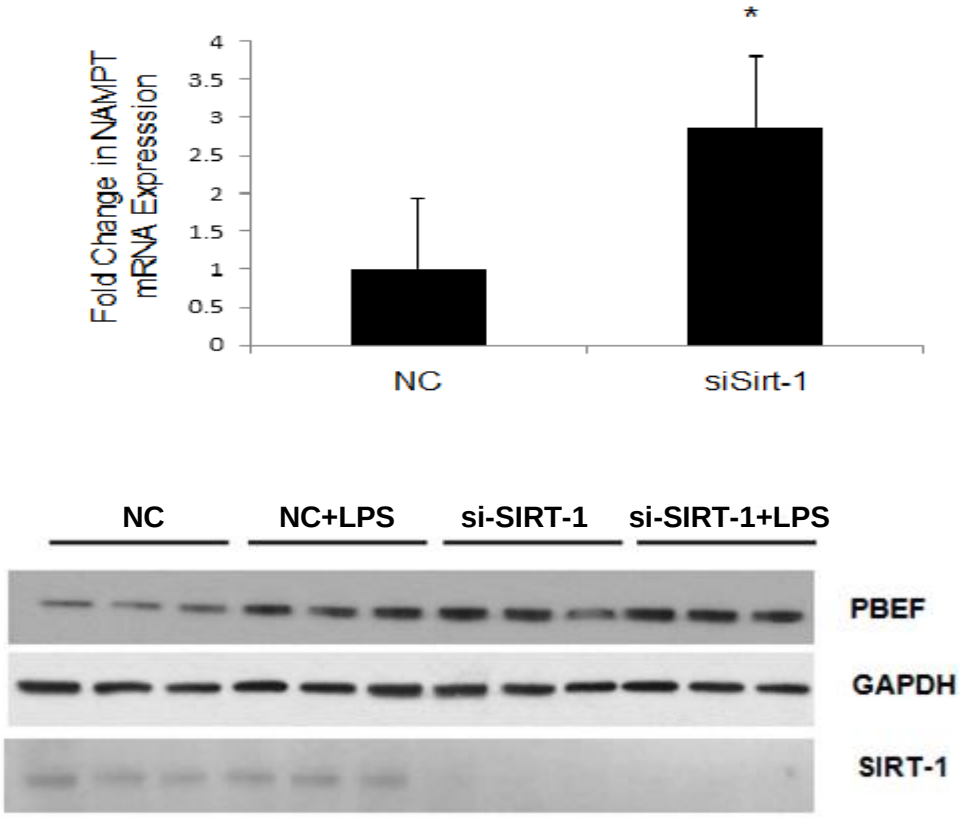

Figure 15: NAMPT gene and protein expression is upregulated by silencing Sirt-1. Human lung EC were transfected with negative control or siRNA specific for Sirt-1 and NAMPT mRNA transcripts assayed by qPCR. Silencing of Sirt-1 significantly increased NAMPT transcripts (A). Further EC were transfected with negative control or siRNA specific for Sirt-1 and were either unstimulated or stimulated with LPS. Cell lysates were electrophoresed and antibodies specific assayed NAMPT levels for NAMPT. The results showed that silencing Sirt-1 increased NAMPT levels to those stimulated by LPS in negative control transfected cells. Further LPS induced increases subsequent to Sirt-1 silencing were not synergistic suggesting an overlap of pathways.

6. POST-TRANSCRIPTIONAL REGULATION OF NAMPT PROMOTER

This research was originally published in the American Journal for Respiratory Cell and Molecular Biology Adyshev DM, Elangovan VR, Moldobaeva N, Mapes B, Sun X, Garcia JG. Mechanical stress induces pre-B-cell colony-enhancing factor/NAMPT expression via epigenetic regulation by miR-374a and miR-568 in human lung endothelium. Am J Respir Cell Mol Biol. 2014 Feb;50(2):409-18. doi: 10.1165/rcmb.2013-0292OC. Portions of the text and all of the figures have been reproduced here with permission.

6.1 Overview:

While promoter epigenetic regulation is an important contributor for transcription, the post-transcriptional regulation of transcripts through the 3'UTR binding of small non-coding RNA known as microRNA (miRNA) plays a vital role in the stability of the transcript and translation into protein. Dysregulation of miRNA have been observed in several mouse models of acute lung injury including LPS mediate acute lung injury(Song et al. 2010) ventilator-induced lung injury(Vaporidi et al. 2012) and sepsis(Sun et al. 2012). Further microRNA modifiers including miRNA mimics and miRNA antagomirs have been effectively utilized to modulate dysregulated miRNA and hence attenuate increased inflammation in-vivo in mouse models and in-vitro in cultured cell lines. These data indicate the importance and feasibility of utilizing microRNA modifiers to attenuate lung injury. The analysis of dysregulated miRNA in mouse models of ALI and VILI further identified several miRNAs that overlapped in the dysregulated ontologies including immune responses and inflammatory pathways

(Zhou, Garcia, and Zhang 2011). The epigenetic mechanisms regulating NAMPT promoter activity has been described in the previous chapters. Dysregulation of NAMPT has been linked to ventilator-induced lung injury. The post-transcriptional regulation of NAMPT can thus provide us vital mechanisms that we can target for generating novel therapeutic strategies aimed at attenuating severe inflammatory syndrome like VILI. Since NAMPT is significantly upregulated by inflammatory cytokines and excessive mechanical stress, we initially tested if the increases in NAMPT transcripts are supported by post-transcriptional mechanisms. Prior publications arising from our lab have successfully utilizing microRNA mimics and antagomirs in modulating nmMYLK mediated inflammatory lung injury (Adyshev et al. 2013a). Human lung EC were transfected with either the NAMPT 3'UTR or the TNF-α 3'UTR fused downstream of a luciferase vector driven by CMV promoter. Transfected cells were then treated with LPS or excessive mechanical stress and luciferase assays were performed. The results indicated that the stability of the transcripts was significantly increased in response to both endotoxin and excessive mechanical stress with augmented 3'UTR luciferase activities. These experiments provided preliminary evidence that inflammatory agonists and mechano-transduction can each increase the stability of the mRNA transcripts through their 3'UTR. Through in-silico analysis, several miRNAs that are potentially dysregulated by inflammatory stimuli potentially contributing to increased stability were identified. Further, we were able to utilize miRNA mimics and antagomirs for selected miRNA to modulate the stability of NAMPT mRNA transcripts as well as reduce the inflammation-induced hyper-permeability, demonstrating the feasibility of developing miRNA modulators based therapeutic strategies to alleviate inflammatory lung injury.

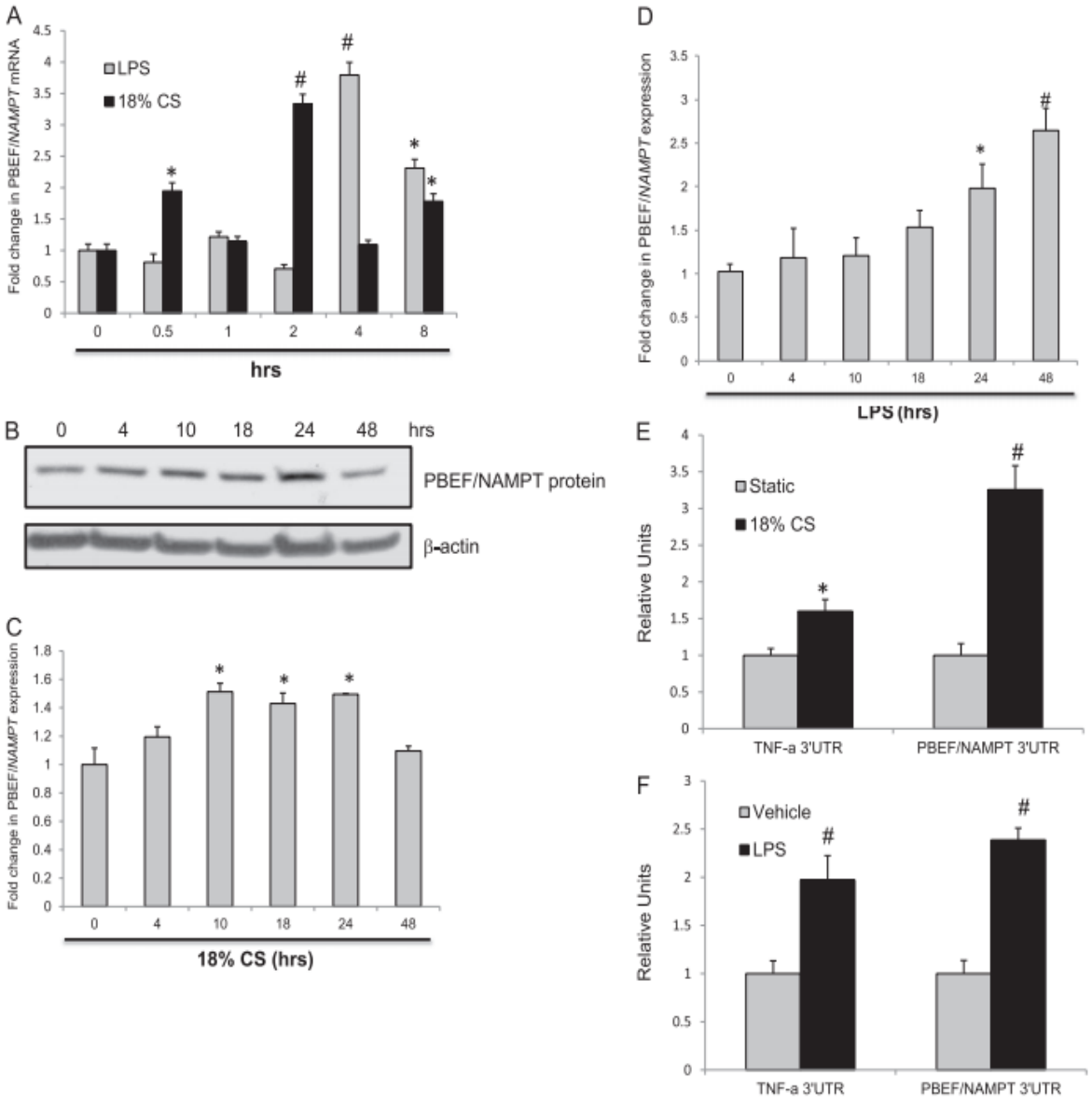

Figure 16: NAMPT mRNA, protein and 3'UTR activity is increased in response to endotoxin challenges and mechanical stress. qPCR assays using primers specific for NAMPT and western blot with lysates from LPS and 18% CS treated human lung EC exemplified increases in NAMPT gene transcripts(A) and protein transcripts (B, C and D). Further NAMPT 3'UTR luciferase constructs transfected into EC and stimulated with LPS or 18% CS (24hrs) showed increases in stability of luciferase transcripts mediated by 3'UTR.

6.2 miR-568 is altered in excessive mechanical stress and endotoxin challenges.

In-silico analysis of the NAMPT 3' UTR identified several microRNA based on their potential to bind and modulate NAMPT transcript stability. hsa-miR-374a and has-miR-568 were selected based on their ability to bind multiple sites on the NAMPT 3'UTR, increased prediction for negative regulation and conservation across mammals. qPCR assays that determine the levels of mature miRNA were used for studying the time-dependent changes occurring in human lung EC exposed to endotoxins or excessive mechanical stress. The results indicated that has-miR-568 was modulated in a time-dependent manner and the expression levels corresponded inversely with NAMPT mRNA transcripts occurring at similar time-points. These data linked has-miR-568 with regulation of NAMPT mRNA transcripts through the 3' UTR. hsa-miR-374a was also significantly downregulated in response to mechanical stress, endotoxins and TNF-α and was also established to be a crucial regulator of nmMYLK transcript stability (Adyshev and Garcia, 2013). The PCR data showing the downregulation of hsa-miR-374a is not included in this thesis. microRNA mimics and antagomirs significantly altered basal level and induced changes in the NAMPT 3'UTR luciferase activity. Several microRNAs are dysregulated in inflammatory disorders and utilization of microRNA mimics and antagomirs against miR-155 have been effectively utilized in LPS induced macrophages to attenuate inflammation. Since miR-374a and miR-568 downregulate NAMPT mRNA and are downregulated in inflammatory conditions contributing to increased NAMPT stability, utilization of microRNA mimics for miR-374a and miR-568 would reduce NAMPT gene and protein induction and alleviate inflammation mediated by NAMPT. The feasibility and experimental evidence for utilizing microRNA mimics in the prevention of NAMPT upregulation are provided in

figures 17 and 19. These data conclusively show the impact of miR-374a and miR-568 mimics in the attenuation of inflammation mediated by NAMPT.

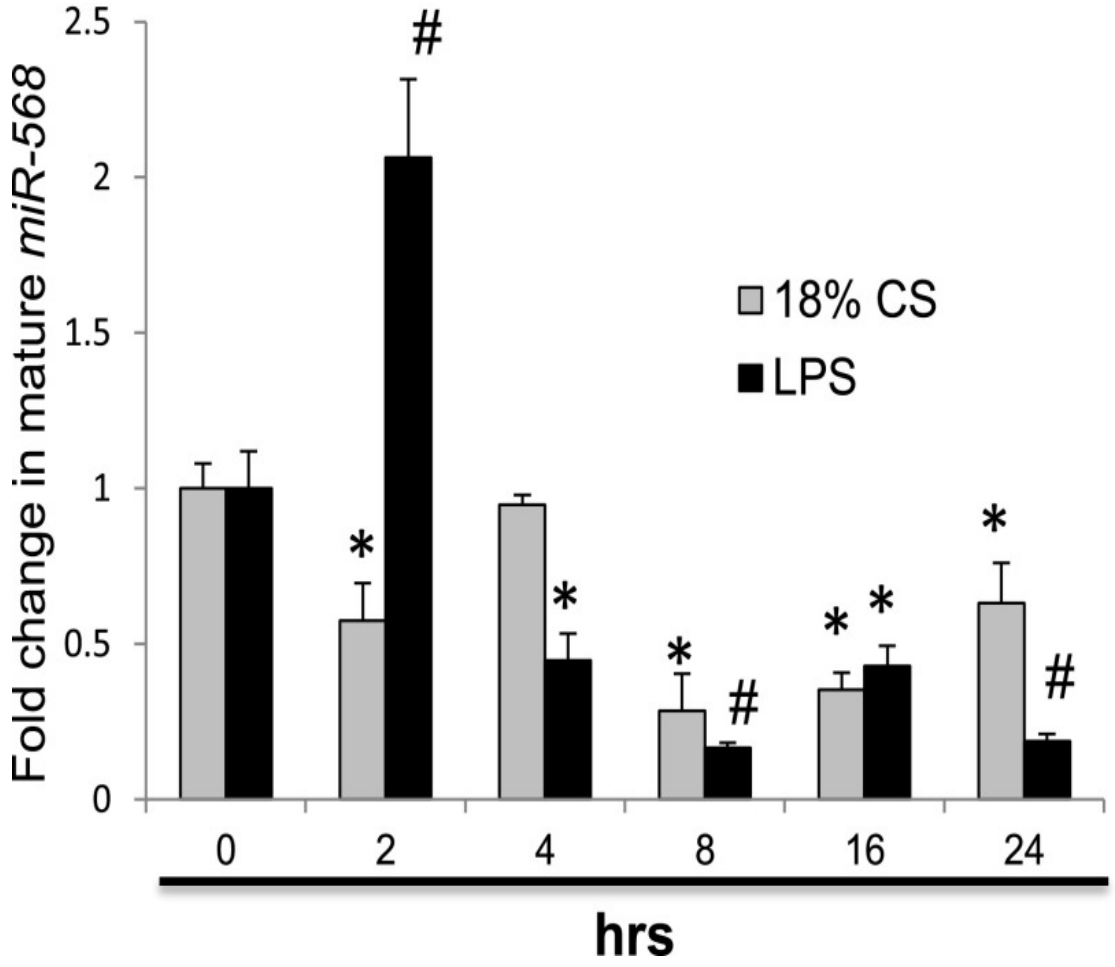

Figure 17: mature miR-568 levels in human lung EC is modulated in response to LPS or excessive mechanical stress. qPCR assays were used to evaluate mature miR-568 transcripts in human lung EC treated with LPS or excessive mechanical stress over various time points. The results demonstrated time-dependent changes in mature miR-568 levels with significant changes occurring at time points that inversely correlated with increased mRNA transcripts (Figure 14a).

6.3 Mimics of miR-568 attenuate LPS mediated endothelial hyperpermeability

Lung endothelial permeability can be studied through electrical cell impedance substrate assay, a sensitive but robust assay where cells are plated on wells containing gold plated electrodes. Measurement of endothelial permeability is measured as a change of resistance occurring in response to environmental stimuli. To evaluate the role of miR modifiers in the regulation of hyperpermeability, human lung EC were transfected with negative control or mimics of miR-568 and were either unstimulated or stimulated with LPS as pro-inflammatory stimuli. Increases in permeability across the endothelial barrier are recorded as a decrease in resistance. A pro-inflammatory agonist like LPS induces hyper-permeability of the endothelial layers thus reducing the resistance measurements. The ECIS experiments revealed that neither negative control nor mimics of miR-568 caused significant changes in the resistance measurements. However, treatment of LPS on cells treated with negative control induced strong decreases in endothelial barrier resistance indicating increased hyperpermeability, while LPS treatment of cells transfected with mimics for miR-568 significantly attenuated endotoxin-induced hyper-permeability. These data indicate the efficacy of utilizing microRNA modifiers like mimics targeting specific mRNA and their associated physiological outcomes. In this case we have successfully utilizing microRNA mimics for miR-568 in decreasing the hyper-permeability induced by LPS in human lung EC. Even though these experiments were conducted in-vitro, prior data from literature provides strong support for utilizing microrna modifiers *in-vivo* in pre-clinical models.

6.4 MiRNA mimics can attenuate NAMPT mRNA and protein upregulation

microRNA mimics and anti-mirs (antagomirs) have been used respectively for decreasing or increasing targeting mRNA transcript stability. miR-374a and miR-568 is decreased in EC subjected to endotoxin or excessive mechanical stress showing an inverse relationship with the mRNA transcripts. Mimics of miR-374a or miR-568 transfected into human lung EC and subjected to mechanical stress or endotoxin stimuli revealed the efficacy of utilizing such microrna modifiers to modulate gene expression. While negative control RNA or NAMPT 3'UTR non-targeting microRNA like miR-1290 did not prevent NAMPT mRNA induced by inflammatory and 18% CS stimuli, NAMPT 3' UTR targeting miR-374a and miR-568 effectively prevented the induced increases. These observations were consistent for both LPS and excessive mechanical stress. Mimics for miR-374a and miR-568 not only prevented induced increases in NAMPT mRNA, but were consistent with decreases at the protein level too. Increases in NAMPT gene transcripts and protein have been linked to higher inflammation and severity of VILI. Attenuation of NAMPT mRNA transcripts and protein through supplementation of mimics for microRNA downregulated by environmental stimuli generates novel strategies to decrease the severity of VILI.

6.5 Mimics and antagomirs alter NAMPT 3'UTR activity

To ascertain that the attenuation of induced increases in NAMPT mRNA was mediated through NAMPT 3'UTR binding of miR-374a and miR-568, NAMPT 3'UTR-luciferase constructs were co-transfected with microRNA mimic specific for miR-374a or miR-568. The data revealed that NAMPT 3'UTR luciferase activity were significantly decreased at

the basal conditions and attenuated induced increases in response to 18% CS or endotoxins. In complimentary approaches, the antagomirs for miR-374a or miR-568 were transfected into human lung EC significantly increased NAMPT mRNA, protein and 3' UTR activity. These data together proves conclusively that microRNA mimics can be utilized to decrease induced increases in NAMPT transcript and these act specifically through their binding to the 3' UTR. Thus, microRNA modifiers can be effectively used as novel therapeutic strategy to attenuate inflammatory lung injuries.

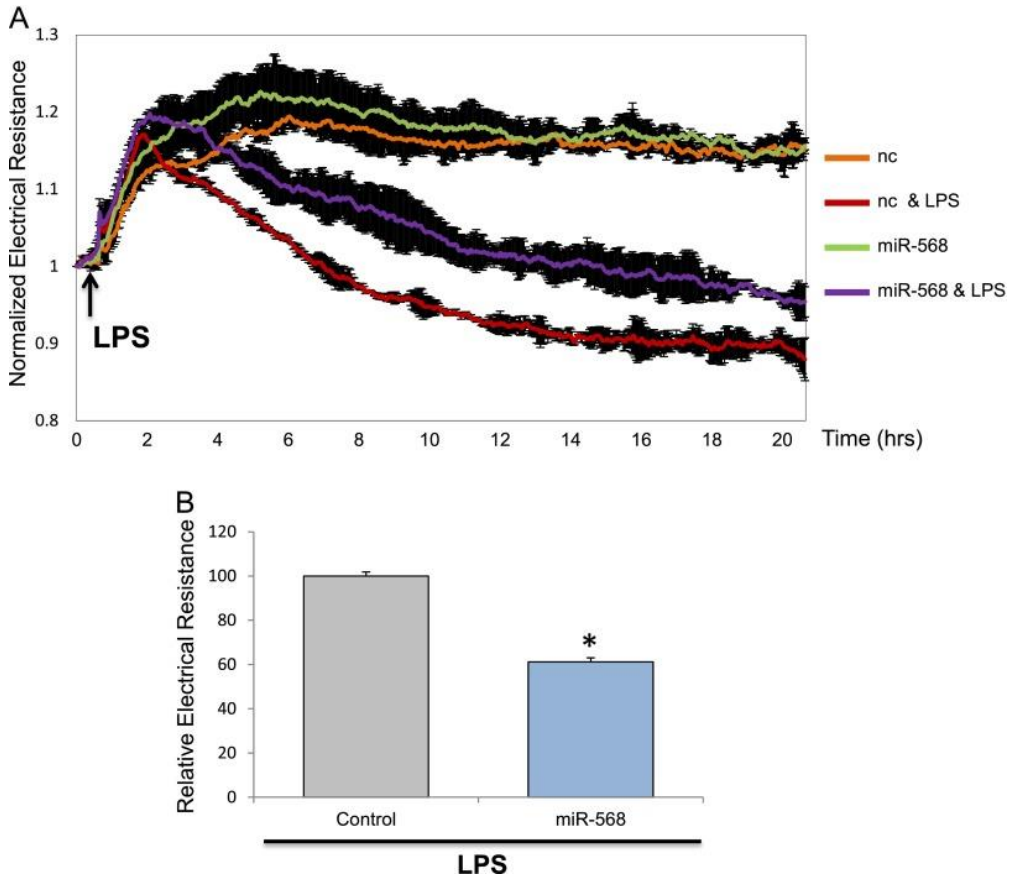

Figure 18: microRNA mimic specific for mature miR-568 attenuated LPS mediated hyperpermeability. Cells were transfected with negative control or mimics specific for miR-568 and were either unstimulated or stimulated with LPS. The changes in permeability were measured as modulations in resistance. While LPS induced hyperpermeability, miR-568 mimics partially attenuated LPS mediated hyperpermeability, but not at the basal level.

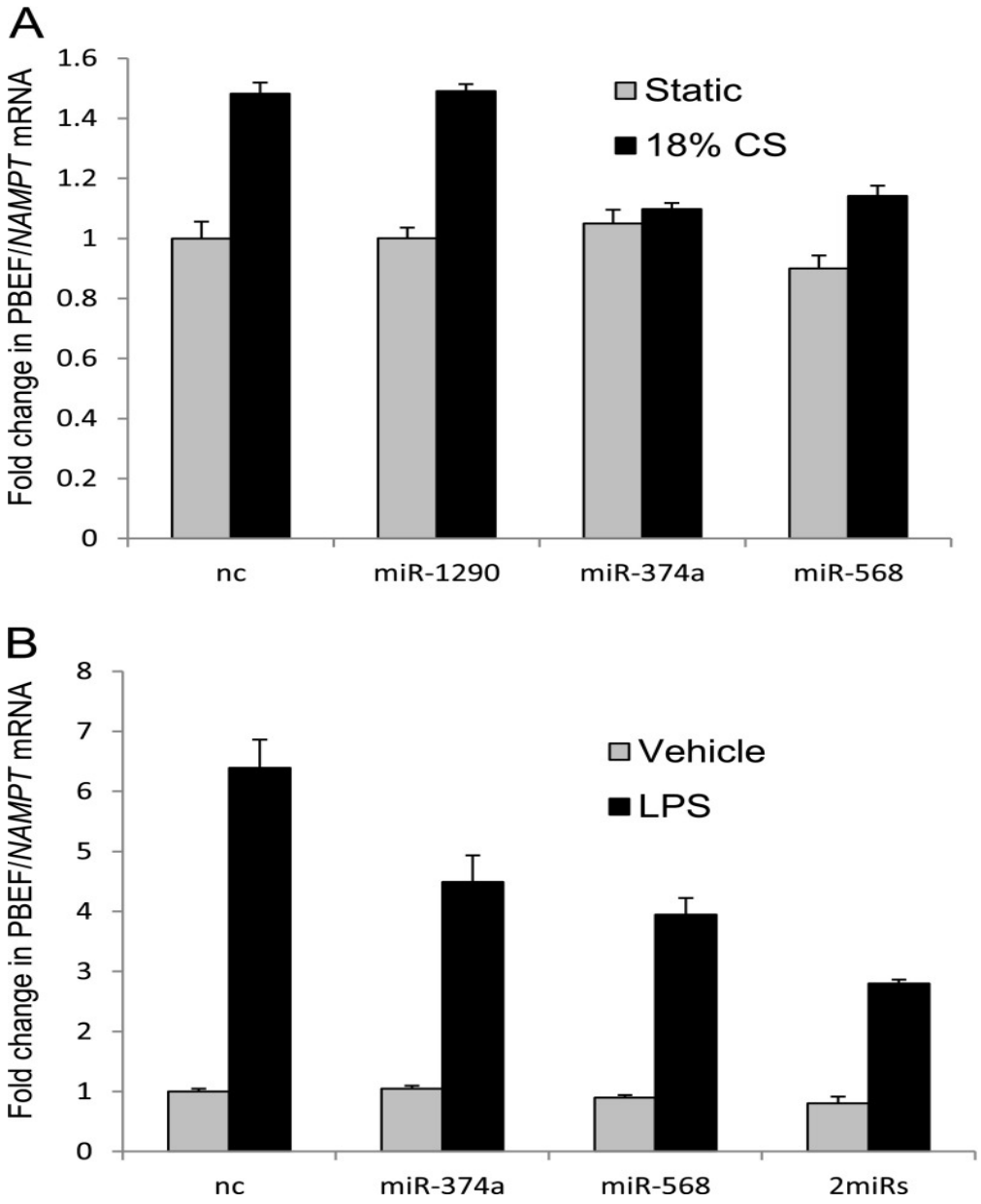

Figure 19: microRNA mimics for miR-374a and miR-568 attenuate induced increases in mRNA. Human lung EC were transfected with negative control, NAMPT 3'UTR non-targeting microRNA mimic miR-1290 or NAMPT 3'UTR targeting microRNA mimics for 374a or 568 and were either unstimulated or stimulated by 18% CS or LPS. Negative control or miR-1290 did not prevent the induced changes in NAMPT mRNA expression; however, miR-374a and miR-568 attenuated the induced increases in NAMPT mRNA in response to 18%CS (A) or LPS (B).

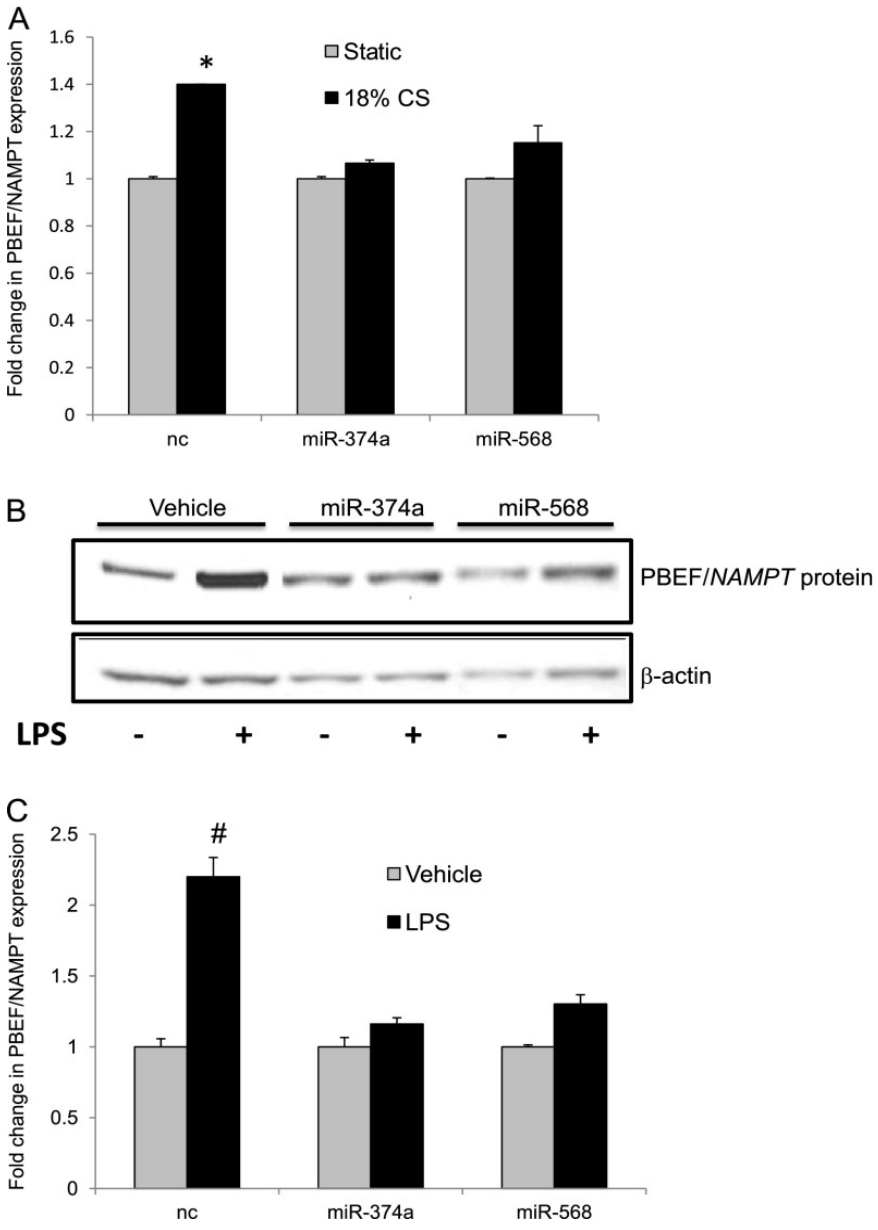

Figure 20: microRNA mimics for miR-374a and miR-568 attenuate induced increases in protein. Human lung EC were transfected with negative control, NAMPT 3'UTR non-targeting microRNA mimic miR-1290 or NAMPT 3'UTR targeting microRNA mimics for 374a or 568 and were either unstimulated or stimulated by 18% CS or LPS. Negative control or miR-1290 did not prevent the induced changes in NAMPT mRNA expression; however, miR-374a and miR-568 attenuated the induced increases in NAMPT mRNA in response to 18%CS (A) or LPS (C).

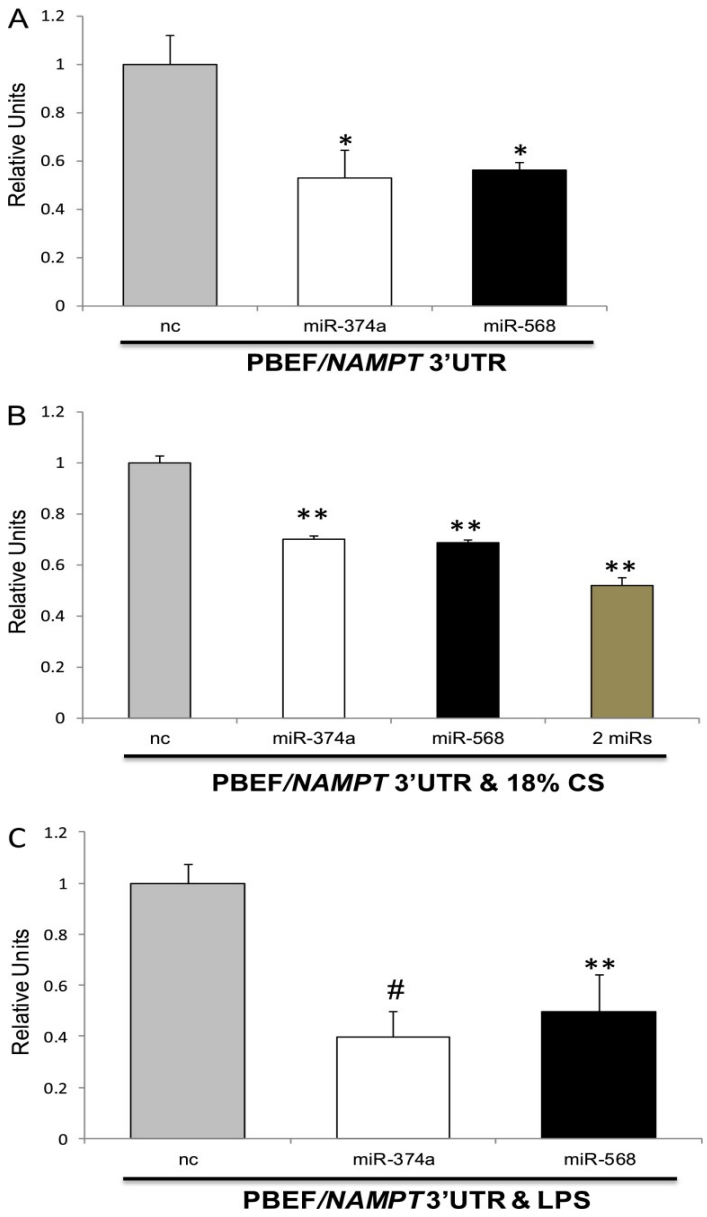

Figure 21: microRNA mimics reduce basal and induced NAMPT 3' UTR activity. Human lungs EC were transfected with NAMPT 3'UTR luciferase vector and co-transfected with either negative control or microRNA mimics for miR-374a or miR-568. Cells were either unstimulated or stimulated by 18%CS or LPS. Compared to negative control mimics for miR-374a or miR-568 reduced basal level (A), 18% CS induced (B) or LPS induced (C) NAMPT 3'UTR activity.

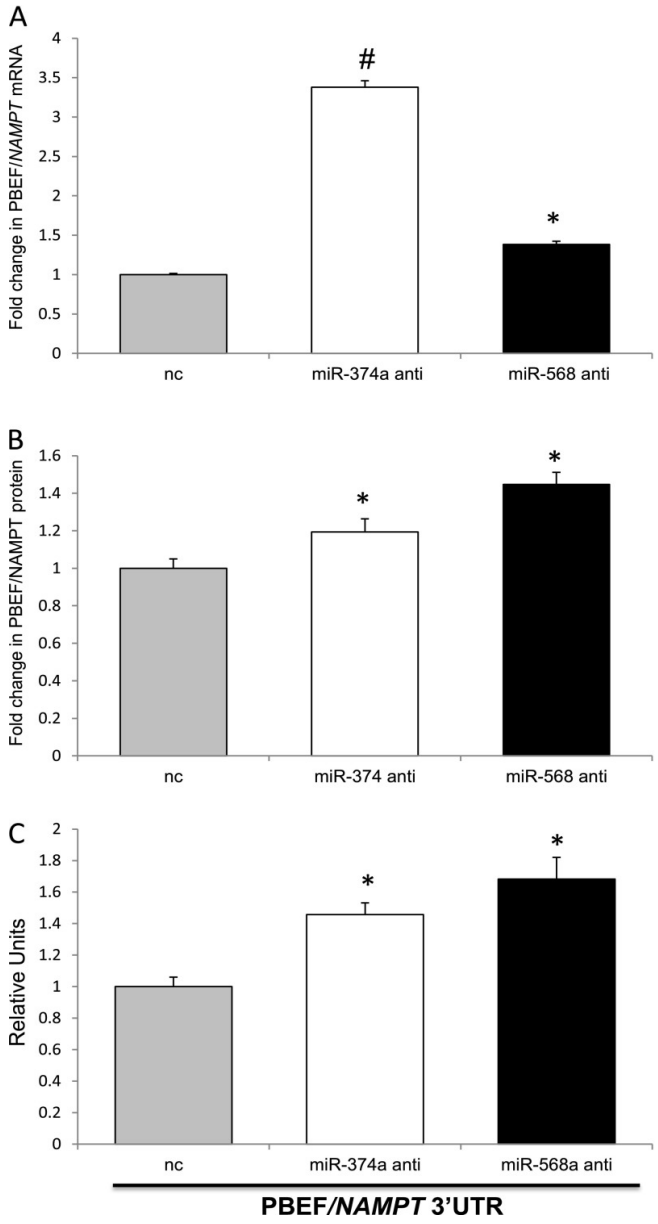

Figure 22: microRNA mimics reduce basal and induced NAMPT 3' UTR activity. Human lungs EC were transfected with NAMPT 3'UTR luciferase vector and co-transfected with either negative control or microRNA antagomirs for miR-374a or miR-568. Compared to negative control mimics for miR-374a or miR-568 increased mRNA (A), protein (B) or NAMPT 3'UTR activity (C).

7. INFLUENCES OF EXTRACELLULAR NAMPT IN EPIGENETIC MODIFICATIONS OF VILI PATHOBIOLOGY

7.1 Overview

NAMPT exists in two forms, an intracellular form and a secreted extracellular form. NAMPT is a potent chemoattractant recruiting neutrophils, and via its extracellular action induces intracellular NAMPT, thereby inhibiting neutrophil apoptosis. The increases in the half-life of neutrophil leads to increased inflammation. Further Biochemical inhibition and genetic ablation of NAMPT confers protection against inflammatory lung injury by attenuating the NF-kB pathway genes suggesting they influence pro-inflammatory ontologies. Recently our lab has identified the direct binding of NAMPT to the TLR4 receptor and activating the NF-kB pathway(Camp et al. 2015). LPS mediates pro-inflammatory cytokine gene expression and induces immune responses through the binding and activation of TLR4(Lu, Yeh, and Ohashi 2008). Few mechanisms exist to explain the role of TLR4 axis in epigenetic pathways. In mouse lung fibroblasts, LPS decreased Thy-1 gene mediated by H3 and H4 hypo acetylation in a TLR-4 dependent manner, which was essentially reversed by silencing TLR4(Xing et al. 2015). Since NAMPT binds the TLR4 receptor, I studied the influence of TLR4 receptor mediated, gene-expression associated, post-translational modification on histone H3. Specifically, NAMPT treatment of human lung EC decreased global H3K9 acetylation, which was reversed by blocking the TLR4 receptor using CLI095. Concomitantly, expression of Sirt-1, a deacetylase targeting H3K9, is upregulated. As stated earlier in the literature review, NAMPT is increased in VILI and bind the TLR4 receptor independent of co-factors or in the absence of LPS. NAMPT decreased

acetylation of H3K9 in a TLR4-dependent manner, potentially through increases in its upstream ligand Sirt-1. These data suggest that NAMPT may influence the epigenetic landscape in human lung EC.

7.2 NAMPT induces global H3K9 hypo acetylation in human lung EC via TLR4

The identification of NAMPT as direct ligand of TLR4, a receptor with significant influences on histone acetylation, led me to studying the impact of extracellular NAMPT in the epigenetic modulation of lung endothelium. Human endothelial cells were treated with extracellular NAMPT in the presence or absence of TLR4 inhibitor (CLI095, 50uM). Cell lysates were prepared and probed with antibodies specific for acetyl H3K9 and normalized to pan H3K9. Extracellular NAMPT treatment of endothelial cells significantly decreased H3K9 acetylation, which was essentially reversed by TLR4 inhibitor. These data suggest that extracellular NAMPT through its binding to the TLR4 receptor may significantly influence the epigenetic landscape of lung endothelium. Since NAMPT can bind TLR4 independently of any other co-factor or LPS and given that extracellular NAMPT is elevated in VILI, NAMPT may potentially regulated the epigenetic modifications associated with VILI.

7.3 LPS in EC increase Sirt-1

NAMPT induced reduction in acetyl H3K9 is a deacetylation event and thus I looked at the gene expression changes associated with the upstream ligand for H3K9 acetylation, the Sirt-1 deacetylase. Because the deacetylation events are increased in a TLR4 dependent manner, a strong agonist like LPS was used to induce TLR4 activation and Sirt-1 gene expression changes were quantified by qPCR. Consistent with decreases in

acetylation of H3K9, Sirt-1 gene expression increased sharply by 2hrs and remained elevated upto 4hrs before reducing to basal levels at 8hrs and 24hrs. Further, protein expression also significantly increased at 24hrs (Data not shown). These data suggest increases in Sirt-1 expression mediate the trend towards deacetylation.

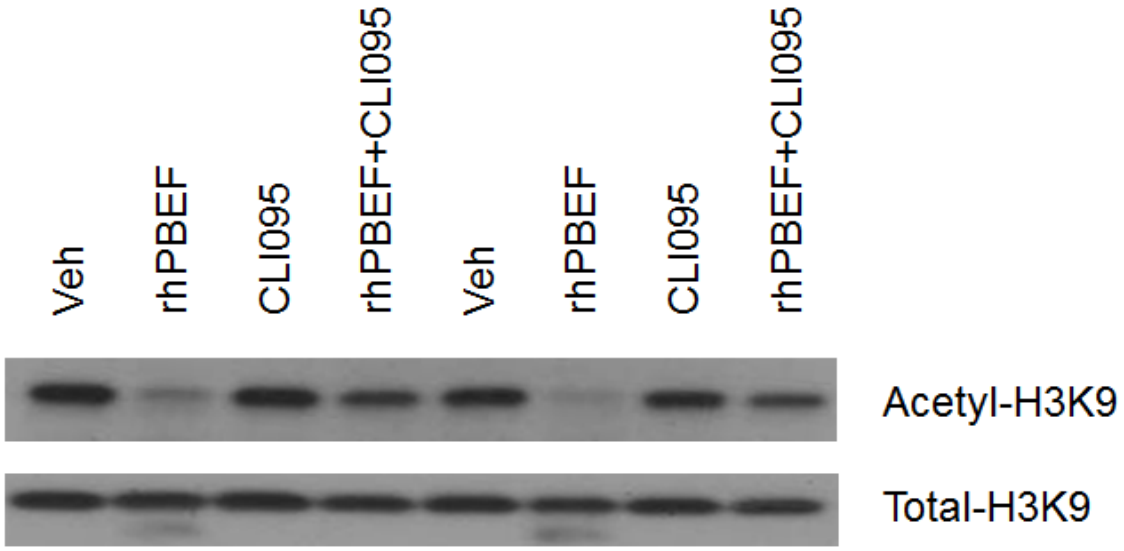

Figure 23: NAMPT reduces the acetylation of H3K9 in TLR4-dependent manner. Human lung EC was treated with rhPBEF with and without the TLR4 inhibitor CLI095. The cells were lysed and western blot was performed with antibodies for acetyl H3K9. Total H3K9 was used as loading control. Results demonstrate extracellular NAMPT reduced the H3K9 acetylation, reversed by TLR4 inhibitor.

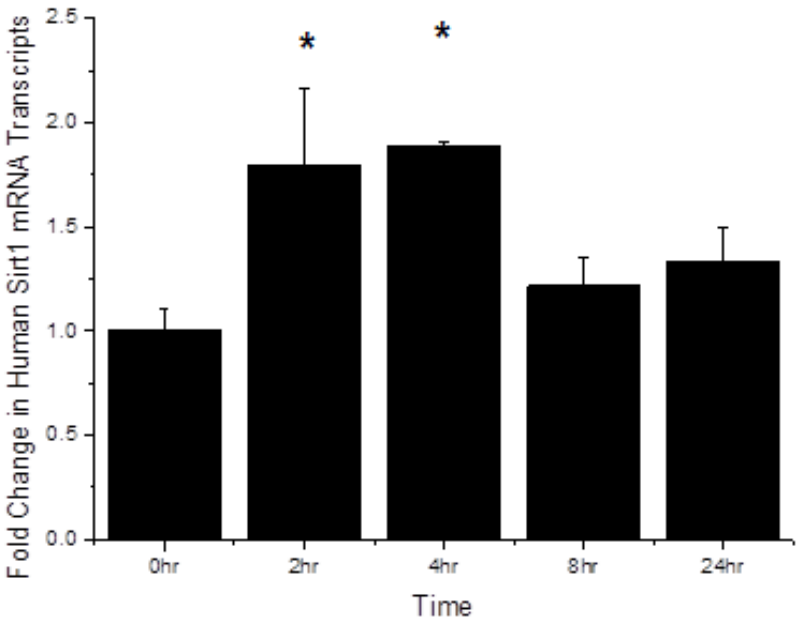

Figure 24: Sirt-1 expression is increased by TLR4 activation. qPCR assays demonstrated increased expression of Sirt-1 mRNA transcripts in LPS mediated TLR4 activation in human lung endothelial cells. RNA from cells activated with LPS was reverse transcribed and probed with primers specific for Sirt-1. The results showed Sirt-1 was increased as early as 2hrs and stayed elevated at 4hrs. The data represented for n=3.

8. DISCUSSION AND CONCLUSION

8.1 Clinical Significance of NAMPT

Our prior studies underline the importance of NAMPT as novel molecule in VILI. The completion of the objectives of this thesis has demonstrated mechanisms activating *NAMPT* transcription via biochemical, genetic and epigenetic mechanisms and participants of whom may be targets for therapeutic strategies. Nicotinamide phosphoribosyl transferase is a novel “cytozyme” functioning as an intracellular dimeric type II nicotinamide phosphoribosyltransferase enzyme and as an extracellular molecule inducing pro-inflammatory gene expression. A stretch and inflammation induced cytokine; NAMPT is significantly increased in cardiac tissues during cardiac arrest and resuscitation (Beiser et al. 2010)in amniotic membranes during gestation (Nichols, Evans, and Smith 1990), and is released from visceral fat during obesity(Simon et al. 2006). As an extracellular cytokine relevant for ARDS and VILI, NAMPT is significantly upregulated via mechanisms whose understanding would help in generating novel therapeutic strategies.

Previous observations from our lab utilizing in-vitro cell endothelial culture models and in-vivo mouse models identified extracellular NAMPT/PBEF as a direct neutrophil chemoattractant. In addition to acting as a chemoattractant, intratracheal administration of NAMPT not only caused significant increases in injury, but also exacerbated VILI (Hong et al. 2008) and the indices of lung injury in animal models. Analysis of the mechanisms driving the influences of NAMPT in VILI through genomic profiling suggested significant induction of lung injury associated modules including the NF-kB

pathway, leukocyte extravasation pathway, apoptosis and TLR4 pathway genes. Complementary approaches utilizing neutralizing antibodies, silencing RNA and heterozygous knockout mice demonstrated a significant decrease in VILI mediated indices of lung injury and associated gene expression signatures. These data confirmed NAMPT as a novel molecule pivotal in VILI. Despite significant progress in understanding the intricate involvement of NAMPT in the pathophysiology of ARDS, the receptor responsible for the extracellular NAMPT mediated induction of NF-kB pathway remained enigmatic until recent experimental evidences implicated TLR4 as a receptor for NAMPT.

Lung gene ontology gene signatures invoked by both NAMPT and TLR4 activation overlapped significantly and extracellular NAMPT augmented genes in the TLR4 signaling, suggesting a role for the intricate involvement of TLR4 signaling in the extracellular NAMPT mediated VILI. Using in-vitro and in-vivo approaches utilizing TLR4 pharmacologic inhibitors, TLR4 neutralizing antibodies, TLR4 siRNAs, NAMPT/PBEF–TLR4 SPR analysis, and $TLR4^{-/-}$ mice, TLR4 was found to be unequivocally required for the NAMPT/PBEF mediated inflammatory responses. NAMPT was found to induce gene signatures that significantly overlapped with TLR4 gene signatures involving the NF-kB pathway (Ye, Simon, et al. 2005, Camp et al. 2015)

NAMPT SNP’s in the promoter region were strongly associated with susceptibility to ARDS (Ye, Simon, et al. 2005) which were independently validated to be associated with increased ARDS mortality (Bajwa et al. 2007).

8.2 Biochemical and Genetic mechanisms drive NAMPT gene transcription

The critical role of NAMPT in the development of VILI necessitates the understanding of its regulation, which is clearly lacking. Our data strongly supports the observation that excessive stress (18% CS) significantly increases NAMPT promoter activity and transcription compared to physiological stretch (5% CS) in human lung endothelium. Serial deletion assays or promoter bashing experiments has been traditionally used to understand the regulatory elements, of a promoter, regulating gene expression under basal or inducible conditions. The NAMPT promoter luciferase constructs were generated by serial deletion in a 5' to 3' unidirectional manner and assayed by luciferase activities. Based on their basal and inducible (excessive stress) activities, the NAMPT promoter can be broadly classified into three distinct regulatory regions. The region close to the NAMPT transcription start site is the proximal promoter (-1228bp to -628bp). The region adjacent to the proximal promoter is the negative regulatory region (-2428bp to -1228bp) and the distal region (-3028bp to -2428bp). The observation that when the -2428bp to -2128bp region was truncated the mechanical stress inducible region was completely abrogated suggested that the core mechanical stress inducible reside within these residues. Further experiments would be necessary to fine-tune the inducible responses to elucidate the exact mechanical stress response elements in the NAMPT promoter. To understand the conservation of these regulatory regions across species, the region was analyzed In-silico for conservation. The region close to the transcription start site (proximal promoter region) was found to be conserved across the vertebrate species suggesting similar regulatory mechanisms in the basal transcription of this gene. Even though an understanding of the genetic variants (SNP's) and

epigenetic mechanisms (miRNA, DNA demethylation) drive the transcription and post-transcriptional regulation of this pivotal gene, the biochemical factors (sequence-specific DNA-binding factor) are critical for recruitment of basal transcription machinery to the promoter and drive transcription of the gene. The studies conducted in this thesis elucidate the importance of STAT (signal transducer and activator of transcription proteins) family of transcription factors in driving the mechanical stress induced gene transcription. Other studies have previously implicated the STAT family of transcription factors in the regulation of mechanical stress, where b-casein gene expression is regulated by translocation of STAT5 transcription factor to the nucleus(Mammoto and Ingber 2010, Mammoto, Mammoto, and Ingber 2012) and CS also induces the JAK2/STAT5 phosphorylation (Honsho et al. 2009). Support for the implication of STAT transcription factors stems from a recent publication studying the role of STAT3 in the mechanical-stress induced HMGB1 (Wolfson, Mapes, and Garcia 2014). Our In-silico analysis had identified two significant transcription factors binding modules corresponding to STAT in the NAMPT promoter. These were aligned in the mechanical stress inducible region and in the proximal promoter region. Binding assays for the STAT transcription factors assessed that, increased STAT5 bound both binding sites suggesting STAT5 as major transcription factor driving mechanical stress induced NAMPT transcripts. STAT5 mediated transcription regulation is initiated by translocation into the nucleus upon activation (phosphorylation) and binding to DNA sequence specific for the STAT family of transcription factors. The STAT5 transcription factors influence cell differentiation, proliferation and survival (Reich 2013). STAT5 is also known to induce cytokine functions and promote B-cell development (Malin et al. 2010).

STAT5 is also (Kornfeld et al. 2008) known to be influential in activating the PI3-Akt pathway chromatin remodeling during cytokine-induced transcription (Rascle and Lees 2003). Our prior and current studies have demonstrated the intricate involvement of STAT pathway in the excessive mechanical stress and VILI induced NAMPT promoter confirming the activation of NAMPT in VILI (Ye, Simon, et al. 2005, Samal et al. 1994, Ognjanovic et al. 2001, Hong et al. 2008).

ARDS associated SNP's in the NAMPT promoter conferred significant increases in the excessive stress induced NAMPT promoter activity over the wild type. In an independent study the -948G/T variant was found to be a NAMPT SNP associated with low-grade inflammation in diabetes (Zhang et al. 2006). Despite the study, limitations that included small groups of patient's lack of cross validation in the two ARDS cohorts the genetic variants conferring increased NAMPT promoter activity were consistent with increased susceptibility to ARDS. The increases in NAMPT promoter activity remained consistent under basal conditions, however STAT5 silencing was sufficient to decrease mechanical stress induced NAMPT activity, indicating that all three variants were significantly influenced by STAT5 transcription factor binding to the NAMPT promoter. The two SNP's further may differentially bind transcription factors that in turn would synergize with STAT5, affecting transcription. For example, Glucocorticoid receptor acts as coactivator for STAT5 and enhances STAT-dependent transcription (Stöcklin et al. 1996).

8.3 Epigenetic mechanisms regulate NAMPT

8.3.1 DNA Methylation

Genetic and biochemical mechanisms critically influence the upregulation of NAMPT in mechanical stress induced endothelium. Further epigenetic mechanisms have been recently implicated in several pathophysiology's' including cancer and inflammation. Epigenetics defined as inheritable changes in gene expression not at the level of DNA sequence involves three different mechanisms-DNA methylation, histone modifications and microRNA regulation. In-silico analysis of NAMPT promoter revealed significant representation of CpG sites with two distinct CpG islands. To understand the impact of CpG sites on the NAMPT promoter transcription, 5-aza-2' deoxycytidine a potent pharmacological inhibitor was used to demethylate genomic DNA. Pharmacological inhibition of DNA methyltransferases significantly induced dose-dependent increases in NAMPT transcripts suggesting modulation of promoter DNA methylation as a potential mechanism driving NAMPT gene transcription. In addition to increased gene transcription, binding of STAT5 and STAT3 transcription factors as well as acetyl H3K9 were all increased. STAT5 transcription factor bound significantly higher than STAT3 however since STAT5 and STAT3 transcription factors can form heterodimers driving the NAMPT promoter. Supportive of this idea is the fact that STAT5:STAT3 heterodimer formation is induced by M-CSF binding cis-inducible regions to promote STAT5 binding(Novak et al. 1996). The NAMPT promoter is characterized by three separate regulatory regions. To understand DNA methylation occurring in the individual regulatory regions primers specific for bisulfite converted DNA targeted to the specific regulator regions were utilized. Gene expression is inversely correlated with levels of

DNA methylation. Bisulfite sequencing revealed that the proximal promoter region contains several NF-kB binding sites and anti-oxidant response sites and regulates the constitutive transcription of NAMPT. This region was completely demethylated. DNA methylation recruit's chromatin repressive complexes to the region, thus repressing gene transcription and the presence of substantial methylation in this zone is consistent with the regulatory role for negative regulation. The regulatory functions were delineated using promoter luciferase constructs, which lack significant CpG DNA methylation. This suggests that CpG methylation may be an additional factor to repressive factors binding the region or may synergize to contribute to the negative regulatory function. Further bisulfite sequencing of DNA from mechanically stressed EC revealed decreased methylation occurring in the negative regulatory region compared to static cells. Since the STAT transcription factor-binding motif does not contain a CpG site, a CpG site was identified that lay less that 100bp from the STAT5 transcription-binding site that was demethylated. In-silico analysis revealed the binding of Pax5. During maturation of pro-B cells, STAT5 also interacts with PAX5 promoting distal immunoglobulin heavy-chain gene transcription (Bertolino et al. 2005). Similarly, demethylation induced Pax5 binding may synergize with increased STAT5 to drive mechanical stress induced NAMPT promoter activity.

DNA methylation changes occurring in the context of ARDS, VILI and inflammation across clinical samples are lacking. Akin to population based genetic variations contributing to increased transcription and susceptibility, variations in DNA methylation levels can also affect transcription of participating genes leading to ARDS susceptibility. Data from illumina methylation arrays allowed for the characterization of population

specific DNA methylation differences and demonstrated significant differences occurring in the distribution of methylation between European populations and African American populations (Moen et al. 2013, Fraser et al. 2012). This suggests population based methylation variations can contribute to race based ARDS susceptibility. Significant variation occurs in several genes including nmMYLK an important pro-inflammatory molecule, also contributing to cytoskeletal modulation (Szilagyi and Garcia, 2013). The DNA methylation changes occurring in the NAMPT promoter is discussed from the context of inflammatory stimulus and mechanical stimulus in the human lung endothelium.

8.3.2 HDAC Inhibition

In this study, we evaluated the impact of class I and II HDAC's vs the class III HDAC's in the regulation of NAMPT gene transcription. Class I and II are zinc dependent deacetylases that include HDACs1 through 11. Class III HDAC's are NAD dependent deacetylases that include Sirtuins 1 through 7.

Evidence for epigenetic modifiers reducing inflammation *in-vivo* and *in-vitro* by decreasing pro-inflammatory cytokine induction is gaining significance. In this study Trichostatin-A treatment of human lung EC attenuated both basal level NAMPT expression and LPS induced increases in NAMPT gene transcription, consistent with the role of NAMPT as a pro-inflammatory cytokine. These data were confirmed by western blot assays where LPS induced increases in NAMPT protein expression (24hrs) were attenuated by pre-treatment with Trichostatin-A (1000nM, 24hrs). This was mirrored in the case of mechanical stress induced NAMPT expression, where pre-

treatment with TSA (1000nM) attenuated the excessive stress induced NAMPT protein expression. Thus, the impact of HDAC inhibitors seems to decrease NAMPT transcripts and protein. Though the mechanisms that govern the attenuation is not well understood, and LPS mediated STAT5 regulation of the NAMPT promoter is not characterized yet, the decreases may be mediated by the impact of HDAC inhibition influences on STAT5. This is supported by the fact that HDAC activity is essential for the transcription of STAT5 mediated genes and inhibition of deacetylation attenuated STAT5 responsive genes (Rascle, Johnston, and Amati 2003). Considering the fact that trichostatin-A reduces mechanical stress induced NAMPT protein expression; these events may be altered by the influences of HDAC inhibition on STAT5 function. The influences of HDAC activity was not attributed to acetylation status of STAT5 or histones but rather the deacetylase inhibitors induces strong repression of STAT5 mediated transcription via interference of brd2 function (Pinz et al. 2015). NAMPT and VEGF have been suggested to increase the permeability of the human placental amnion (Astern, Collier, and Kendal-Wright 2013). Further, NAMPT has also been found to modulate thrombin induced endothelial barrier dysfunction (Ye, Zhang, et al. 2005). It would be interesting to conduct experiments that would show that decreases in NAMPT mediated by Trichostatin-A may be a potential mechanism through which the decreases in LPS-mediated hyperpermeability occurs.

To compare the roles of HDAC's with that Sirtuins, specific siRNA targeted against Sirt-1 was utilized in human lung endothelial cells and the expression of NAMPT was assayed using qPCR and western blot assays. Surprisingly, silencing of Sirt-1 significantly increased NAMPT gene and protein expression. The increases in protein

expression paralleled increases in the LPS induced NAMPT protein expression. A common target for both LPS induction and Sirt-1 is NF-kB. Upon activation, NF-kB translocates into the nucleus and binds NF-kB elements in their target promoters driving transcription. They are important for pro-inflammatory cytokine transcription (Gilmore 2006). LPS activates NF-kB through the TLR4 pathway where phosphorylation of IkB leads to release of NF-kB and further phosphorylation of NF-kB leading to activation. Sirt-1 alters the stability of p65 by deacetylating p65 and inhibiting NF-kB activity (Yang et al. 2012). In support of interactions between Sirt-1 and p65, LPS mediated decreases in Sirt-1 has been correlated with increased p65 acetylation in macrophage cell lines (Shen et al. 2009) and also led to increased inflammatory gene expression occurring through the activation of JNK and IKK inflammatory pathways (Yoshizaki et al. 2009). These data suggest Sirt-1 may negatively regulate NAMPT transcription in lung endothelial cells by influencing the NF-kB pathway. In relevance for our findings for STAT5 mediated transcription, Sirt-1 can also directly interact with STAT5 and via alterations of the acetylation, status modulates STAT5 responses (Yamamoto et al. 2013).

8.3.3 NAMPT induced hypoacetylation of H3K9

LPS is identified to induce hypoacetylation of histones H3 in brain tissues (Soliman et al. 2012) and in human lung microvascular endothelial cells (Thangjam et al. 2014). Further, in mouse lung fibroblasts, TLR4 mediates decreases in histone acetylation. NAMPT is a pivotal molecule mediating inflammatory processes in VILI by directly binding TLR4. Our data indicate that rhPBEF/extracellular NAMPT induced TLR4-mediated decreases in histone H3K9 acetylation. Human lung EC treated with

extracellular NAMPT decreased H3K9 acetylation, which was rescued by co-treatment with a TLR4 receptor specific inhibitor CLI095. To study the mechanisms of deacetylation, the expression of the upstream ligand for H3K9 deacetylation Sirt-1 was monitored in response to TLR4. Activation of TLR4 via its strong agonist LPS increased the expression of Sirt-1. These data suggest that the expression of Sirt-1 is increased in human lung EC and may cause the decreases in acetylation of its target acetyl H3K9. Thus, NAMPT may potentially modulate the epigenetic landscape in the context of VILI.

8.3.4 NAMPT can be attenuated by microRNA via post-transcriptional regulation

Apart from promoter epigenetic mechanisms, another area of interest is the modulation of the stability of mRNA transcripts mediated by miRNA. The 3'UTR is an important regulatory region moderating the stability, polyadenylation, turnover rates and localization. Several elements target the 3'UTR including microRNA's, RBP's (RNA binding protein), and lncRNA (long non-coding RNA) often modulating immune responses via moderation of the rate of translation of participating immune genes (Schwerk and Savan 2015). microRNAs are 18-30bp long sequence specific binding elements that target the 3' UTR imperfectly, often having multiple evolutionarily conserved binding sites for efficient regulation (Lodish et al. 2008). Since the occurrence of this regulation is primed right ahead of translation, miRNA binding and regulation is optimally placed for quick and efficient degradation or increased turnover of critical genes (Bartel and Chen 2004). Multiple microRNA often overlapped in VILI/ARDS and the ontologies they influence and possibly potentiated the development of ALI through the dysregulation of their target genes (Zhou, Garcia, and Zhang 2011). In-silico analysis of the NAMPT promoter revealed that the 3'-UTR contained several

conserved microRNA-binding sites and is primed for altered stability through miRNA's dysregulated in VILI/ARDS. The search algorithms utilized for potential microRNA binding to the NAMPT promoter revealed several conserved microRNA. In particular, miR-374a and miR-568 were identified and validated as being dysregulated in in-vitro models of stretch and inflammation performed on lung EC. One of the most common observations in miRNA studies is the inverse relationships that exists between mature miRNA levels and their target mRNA molecules. LPS, TNF-alpha and 18% CS each decreased the expression levels of mature miR-374a and miR-568 while increasing the NAMPT mRNA transcripts. Since the miRNAs are downregulated leading to mRNA upregulation, microRNA mimics were transfected into lung EC to test if they alter the stability of LPS or 18% CS augmented NAMPT mRNA transcripts. While the basal level expression remained unaltered, induced increases in NAMPT, mRNA was significantly attenuated. Further utilization of NAMPT 3'UTR luciferase constructs co-transfected with mimics into lung EC paralleled the changes in induced mRNA transcripts and also decreased the basal levels essentially demonstrating that the changes were specifically through the NAMPT 3'UTR.

Utilization of microRNA mimics is often susceptible for false-positives. Thus, we sought to increase the confidence via complementary approaches that would inhibit the targeted miRNA. For these approaches, we again utilized anti-miR's specific for miR-374 and miR-568. By inhibiting the endogenous miRNA, NAMPT mRNA transcripts were upregulated and this was confirmed to be a direct effect on the 3'UTR via NAMPT 3'UTR constructs that reflected similar increases. These data confirmed the regulatory functions of miR-374a or miR-568 in the regulation of NAMPT through their 3' UTR

binding. Experiments conducted with the simultaneous transfection of both miRNAs decreased NAMPT expression higher than the individual miRNA's suggesting that they may co-operate in the downregulation of NAMPT.

8.3.5 Conclusion

In this thesis, the transcriptional regulatory mechanisms governing increased NAMPT transcription have been characterized via biochemical, genetic and epigenetic mechanism relevant to VILI/ARDS. To summarize NAMPT promoter activity is modulated via 5'UTR mechanisms including STAT5 driven increases in mechanical stress inducible NAMPT, confirming its transcriptional upregulation in lung EC. Further STAT5 also influenced genetic variants in the NAMPT promoter conferring increased susceptibility to ARDS. These data suggest the influential role of STAT5 in the regulation of NAMPT promoter. Apart from genetic and biochemical mechanisms, epigenetic mechanisms including DNA methylation has not been previously reported for the NAMPT promoter in lung EC. Our data has linked the mechanical stress and endotoxin challenges to decreases in the NAMPT promoter concomitant with increased NAMPT gene transcription. Further, our data has also shown the importance of increased stability of NAMPT mRNA through dysregulation of miRNA binding the 3'UTR.

Considering that TLR4 pathway has considerable influence on the global histone profiles modulating the transcriptional regulation of target gene promoters and NAMPT binding TLR4 even in the absence of LPS or any other co-factor requirements, the

epigenetic landscape of the endothelium subjected to VILI may be significantly influenced by extracellular NAMPT.

The impact of genetic variants in the NAMPT promoter relevant for epigenetic mechanisms is not discussed in this thesis. However, preliminary data for such studies involving the impact of genetic variants affecting DNA methylation and post-transcriptional regulation of NAMPT suggests the feasibility for conducting such analyses. Thus, the thesis has addressed the transcriptional regulation of NAMPT, a key inflammatory mediator in VILI for generation of novel therapeutic strategies.

The mechanisms observed to regulate NAMPT transcription in response to inflammatory stimuli like LPS or mechanical stress in this thesis can be utilized as therapeutic targets to ameliorate NAMPT mediated influences on VILI. Our experiences with neutralizing antibodies targeting extracellular NAMPT alleviating inflammation mediated ARDS and the feasibility of approaches targeting transcription have been demonstrated via extension to epigenetic modifiers targeting DNA methylation, histone modifications and non-coding RNA.

REFERENCES

Adcock, I. M. 2007. "HDAC inhibitors as anti-inflammatory agents." *Br J Pharmacol* no. 150 (7):829-31. doi: 10.1038/sj.bjp.0707166.

Adyshev, D. M., N. Moldobaeva, B. Mapes, V. Elangovan, and J. G. Garcia. 2013a. "MicroRNA regulation of nonmuscle myosin light chain kinase expression in human lung endothelium." *Am J Respir Cell Mol Biol* no. 49 (1):58-66. doi: 10.1165/rcmb.2012-0397OC.

Adyshev, Djanybek M., Nurgul Moldobaeva, Brandon Mapes, Venkate Elangovan, and Joe G. N. Garcia. 2013b. "MicroRNA Regulation of Nonmuscle Myosin Light Chain Kinase Expression in Human Lung Endothelium." *American Journal of Respiratory Cell and Molecular Biology* no. 49 (1):58-66. doi: 10.1165/rcmb.2012-0397OC.

Albaiceta, G. M., and L. Blanch. 2011. "Beyond volutrauma in ARDS: the critical role of lung tissue deformation." *Crit Care* no. 15 (2):304. doi: 10.1186/cc10052.

Albertine, K. H. 2013. "Progress in understanding the pathogenesis of BPD using the baboon and sheep models." *Semin Perinatol* no. 37 (2):60-8. doi: 10.1053/j.semperi.2013.01.001.

Antequera, F., and A. Bird. 1993. "Number of CpG islands and genes in human and mouse." *Proc Natl Acad Sci U S A* no. 90 (24):11995-9.

Astern, J. M., A. C. Collier, and C. E. Kendal-Wright. 2013. "Pre-B cell colony enhancing factor (PBEF/NAMPT/Visfatin) and vascular endothelial growth factor (VEGF) cooperate to increase the permeability of the human placental amnion." *Placenta* no. 34 (1):42-9. doi: 10.1016/j.placenta.2012.10.008.

Bajwa, E. K., C. L. Yu, M. N. Gong, B. T. Thompson, and D. C. Christiani. 2007. "Pre-B-cell colony-enhancing factor gene polymorphisms and risk of acute respiratory distress syndrome." *Crit Care Med* no. 35 (5):1290-5. doi: 10.1097/01.CCM.0000260243.22758.4F.

Bannister, A. J., and T. Kouzarides. 2011. "Regulation of chromatin by histone modifications." *Cell Res* no. 21 (3):381-95. doi: 10.1038/cr.2011.22.

Barnes, P. J. 2006. "Reduced histone deacetylase in COPD: clinical implications." *Chest* no. 129 (1):151-5. doi: 10.1378/chest.129.1.151.

Barnes, P. J., I. M. Adcock, and K. Ito. 2005. "Histone acetylation and deacetylation: importance in inflammatory lung diseases." *Eur Respir J* no. 25 (3):552-63. doi: 10.1183/09031936.05.00117504.

Bartel, D. P., and C. Z. Chen. 2004. "Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs." *Nat Rev Genet* no. 5 (5):396-400. doi: 10.1038/nrg1328.

Bayarsaihan, D. 2011. "Epigenetic mechanisms in inflammation." *J Dent Res* no. 90 (1):9-17. doi: 10.1177/0022034510378683.

Beiser, D. G., H. Wang, J. Li, X. Wang, V. Yordanova, A. Das, T. Mirzapoiazova, J. G. Garcia, S. A. Stern, and T. L. Vanden Hoek. 2010. "Plasma and myocardial visfatin expression changes are associated with therapeutic hypothermia protection during murine hemorrhagic shock/resuscitation." *Resuscitation* no. 81 (6):742-8. doi: 10.1016/j.resuscitation.2010.02.019.

Benakanakere, M., M. Abdolhosseini, K. Hosur, L. S. Finoti, and D. F. Kinane. 2015. "TLR2 promoter hypermethylation creates innate immune dysbiosis." *J Dent Res* no. 94 (1):183-91. doi: 10.1177/0022034514557545.

Bertolino, E., K. Reddy, K. L. Medina, E. Parganas, J. Ihle, and H. Singh. 2005. "Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5." *Nat Immunol* no. 6 (8):836-43. doi: 10.1038/ni1226.

Birukov, K. G. 2009. "Cyclic stretch, reactive oxygen species, and vascular remodeling." *Antioxid Redox Signal* no. 11 (7):1651-67. doi: 10.1089/ars.2008.2390.

Blank, R., and L. M. Napolitano. 2011. "Epidemiology of ARDS and ALI." *Crit Care Clin* no. 27 (3):439-58. doi: 10.1016/j.ccc.2011.05.005.

Camp, S. M., E. Ceco, C. L. Evenoski, S. M. Danilov, T. Zhou, E. T. Chiang, L. Moreno-Vinasco, B. Mapes, J. Zhao, G. Gursoy, M. E. Brown, D. M. Adyshev, S. S. Siddiqui, H. Quijada, S. Sammani, E. Letsiou, L. Saadat, M. Yousef, T. Wang, J. Liang, and J. G. Garcia. 2015. "Unique Toll-Like Receptor 4 Activation by NAMPT/PBEF Induces NFκB Signaling and Inflammatory Lung Injury." *Sci Rep* no. 5:13135. doi: 10.1038/srep13135.

Chamorro-Jorganes, A., E. Araldi, and Y. Suárez. 2013. "MicroRNAs as pharmacological targets in endothelial cell function and dysfunction." *Pharmacol Res* no. 75:15-27. doi: 10.1016/j.phrs.2013.04.002.

Chang, S., B. D. Young, S. Li, X. Qi, J. A. Richardson, and E. N. Olson. 2006. "Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10." *Cell* no. 126 (2):321-34. doi: 10.1016/j.cell.2006.05.040.

Choi, S. E., T. Fu, S. Seok, D. H. Kim, E. Yu, K. W. Lee, Y. Kang, X. Li, B. Kemper, and J. K. Kemper. 2013. "Elevated microRNA-34a in obesity reduces NAD+ levels

and SIRT1 activity by directly targeting NAMPT." *Aging Cell* no. 12 (6):1062-72. doi: 10.1111/acel.12135.

Dahl, T. B., S. Holm, P. Aukrust, and B. Halvorsen. 2012. "Visfatin/NAMPT: a multifaceted molecule with diverse roles in physiology and pathophysiology." *Annu Rev Nutr* no. 32:229-43. doi: 10.1146/annurev-nutr-071811-150746.

Deaton, A. M., and A. Bird. 2011. "CpG islands and the regulation of transcription." *Genes Dev* no. 25 (10):1010-22. doi: 10.1101/gad.2037511.

Dong, X., and Z. Weng. 2013. "The correlation between histone modifications and gene expression." *Epigenomics* no. 5 (2):113-6. doi: 10.2217/epi.13.13.

Dreyfuss, D., and G. Saumon. 1998. "Ventilator-induced lung injury: lessons from experimental studies." *Am J Respir Crit Care Med* no. 157 (1):294-323. doi: 10.1164/ajrccm.157.1.9604014.

Dudek, S. M., and J. G. Garcia. 2001. "Cytoskeletal regulation of pulmonary vascular permeability." *J Appl Physiol (1985)* no. 91 (4):1487-500.

Dunn, J., H. Qiu, S. Kim, D. Jjingo, R. Hoffman, C. W. Kim, I. Jang, D. J. Son, D. Kim, C. Pan, Y. Fan, I. K. Jordan, and H. Jo. 2014. "Flow-dependent epigenetic DNA methylation regulates endothelial gene expression and atherosclerosis." *J Clin Invest* no. 124 (7):3187-99. doi: 10.1172/JCI74792.

Ehrlich, M. 2002. "DNA methylation in cancer: too much, but also too little." *Oncogene* no. 21 (35):5400-13. doi: 10.1038/sj.onc.1205651.

Eslami-Mossallam, B., H. Schiessel, and J. van Noort. 2016. "Nucleosome dynamics: Sequence matters." *Adv Colloid Interface Sci*. doi: 10.1016/j.cis.2016.01.007.

Fabian, M. R., N. Sonenberg, and W. Filipowicz. 2010. "Regulation of mRNA translation and stability by microRNAs." *Annu Rev Biochem* no. 79:351-79. doi: 10.1146/annurev-biochem-060308-103103.

Fan, E., J. Villar, and A. S. Slutsky. 2013. "Novel approaches to minimize ventilator-induced lung injury." *BMC Med* no. 11:85. doi: 10.1186/1741-7015-11-85.

Fraser, H. B., L. L. Lam, S. M. Neumann, and M. S. Kobor. 2012. "Population-specificity of human DNA methylation." *Genome Biol* no. 13 (2):R8. doi: 10.1186/gb-2012-13-2-r8.

Garcia, J. G. 2005. "Searching for candidate genes in acute lung injury: SNPs, Chips and PBEF." *Trans Am Clin Climatol Assoc* no. 116:205-19; discussion 220.

Gilmore, T. D. 2006. "Introduction to NF-kappaB: players, pathways, perspectives." *Oncogene* no. 25 (51):6680-4. doi: 10.1038/sj.onc.1209954.

González, S., S. Aguilera, U. Urzúa, A. F. Quest, C. Molina, C. Alliende, M. Hermoso, and M. J. González. 2011. "Mechanotransduction and epigenetic control in autoimmune diseases." *Autoimmun Rev* no. 10 (3):175-9. doi: 10.1016/j.autrev.2010.09.022.

Grigoryev, D. N., S. F. Ma, R. A. Irizarry, S. Q. Ye, J. Quackenbush, and J. G. Garcia. 2004. "Orthologous gene-expression profiling in multi-species models: search for candidate genes." *Genome Biol* no. 5 (5):R34. doi: 10.1186/gb-2004-5-5-r34.

Hahn, C., and M. A. Schwartz. 2009. "Mechanotransduction in vascular physiology and atherogenesis." *Nat Rev Mol Cell Biol* no. 10 (1):53-62. doi: 10.1038/nrm2596.

Halbertsma, F. J., M. Vaneker, G. J. Scheffer, and J. G. van der Hoeven. 2005. "Cytokines and biotrauma in ventilator-induced lung injury: a critical review of the literature." *Neth J Med* no. 63 (10):382-92.

Hashimoto, K., R. O. Oreffo, M. B. Gibson, M. B. Goldring, and H. I. Roach. 2009. "DNA demethylation at specific CpG sites in the IL1B promoter in response to inflammatory cytokines in human articular chondrocytes." *Arthritis Rheum* no. 60 (11):3303-13. doi: 10.1002/art.24882.

Hausser, J., and M. Zavolan. 2014. "Identification and consequences of miRNA-target interactions--beyond repression of gene expression." *Nat Rev Genet* no. 15 (9):599-612. doi: 10.1038/nrg3765.

He, X., Z. Jing, and G. Cheng. 2014. "MicroRNAs: new regulators of Toll-like receptor signalling pathways." *Biomed Res Int* no. 2014:945169. doi: 10.1155/2014/945169.

Hickling, K. G., S. J. Henderson, and R. Jackson. 1990. "Low mortality associated with low volume pressure limited ventilation with permissive hypercapnia in severe adult respiratory distress syndrome." *Intensive Care Med* no. 16 (6):372-7.

Hickling, K. G., J. Walsh, S. Henderson, and R. Jackson. 1994. "Low mortality rate in adult respiratory distress syndrome using low-volume, pressure-limited ventilation with permissive hypercapnia: a prospective study." *Crit Care Med* no. 22 (10):1568-78.

Hong, S. B., Y. Huang, L. Moreno-Vinasco, S. Sammani, J. Moitra, J. W. Barnard, S. F. Ma, T. Mirzapoiazova, C. Evenoski, R. R. Reeves, E. T. Chiang, G. D. Lang, A. N. Husain, S. M. Dudek, J. R. Jacobson, S. Q. Ye, Y. A. Lussier, and J. G. Garcia. 2008. "Essential role of pre-B-cell colony enhancing factor in ventilator-

induced lung injury." *Am J Respir Crit Care Med* no. 178 (6):605-17. doi: 10.1164/rccm.200712-1822OC.

Honsho, S., S. Nishikawa, K. Amano, K. Zen, Y. Adachi, E. Kishita, A. Matsui, A. Katsume, S. Yamaguchi, K. Nishikawa, K. Isoda, D. W. Riches, S. Matoba, M. Okigaki, and H. Matsubara. 2009. "Pressure-mediated hypertrophy and mechanical stretch induces IL-1 release and subsequent IGF-1 generation to maintain compensative hypertrophy by affecting Akt and JNK pathways." *Circ Res* no. 105 (11):1149-58. doi: 10.1161/CIRCRESAHA.109.208199.

Illi, B., S. Nanni, A. Scopece, A. Farsetti, P. Biglioli, M. C. Capogrossi, and C. Gaetano. 2003. "Shear stress-mediated chromatin remodeling provides molecular basis for flow-dependent regulation of gene expression." *Circ Res* no. 93 (2):155-61. doi: 10.1161/01.RES.0000080933.82105.29.

Illingworth, R. S., and A. P. Bird. 2009. "CpG islands--'a rough guide'." *FEBS Lett* no. 583 (11):1713-20. doi: 10.1016/j.febslet.2009.04.012.

Imai, Y., T. Kawano, K. Miyasaka, M. Takata, T. Imai, and K. Okuyama. 1994. "Inflammatory chemical mediators during conventional ventilation and during high frequency oscillatory ventilation." *Am J Respir Crit Care Med* no. 150 (6 Pt 1):1550-4. doi: 10.1164/ajrccm.150.6.7952613.

Ishida, K., T. Kobayashi, S. Ito, Y. Komatsu, T. Yokoyama, M. Okada, A. Abe, A. Murasawa, and H. Yoshie. 2012. "Interleukin-6 gene promoter methylation in rheumatoid arthritis and chronic periodontitis." *J Periodontol* no. 83 (7):917-25. doi: 10.1902/jop.2011.110356.

Jamaluddin, M. D., I. Chen, F. Yang, X. Jiang, M. Jan, X. Liu, A. I. Schafer, W. Durante, X. Yang, and H. Wang. 2007. "Homocysteine inhibits endothelial cell growth via DNA hypomethylation of the cyclin A gene." *Blood* no. 110 (10):3648-55. doi: 10.1182/blood-2007-06-096701.

Jeltsch, A. 2002. "Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases." *Chembiochem* no. 3 (4):274-93.

Joss-Moore, L. A., K. H. Albertine, and R. H. Lane. 2011. "Epigenetics and the developmental origins of lung disease." *Mol Genet Metab* no. 104 (1-2):61-6. doi: 10.1016/j.ymgme.2011.07.018.

Kim, H. R., R. X. Han, Y. F. Diao, C. S. Park, and D. I. Jin. 2011. "Epigenetic characterization of the PBEF and TIMP-2 genes in the developing placentae of normal mice." *BMB Rep* no. 44 (8):535-40.

Kim, M. S., H. J. Kwon, Y. M. Lee, J. H. Baek, J. E. Jang, S. W. Lee, E. J. Moon, H. S. Kim, S. K. Lee, H. Y. Chung, C. W. Kim, and K. W. Kim. 2001. "Histone

deacetylases induce angiogenesis by negative regulation of tumor suppressor genes." *Nat Med* no. 7 (4):437-43. doi: 10.1038/86507.

Kishore, A., J. Borucka, J. Petrkova, and M. Petrek. 2014. "Novel insights into miRNA in lung and heart inflammatory diseases." *Mediators Inflamm* no. 2014:259131. doi: 10.1155/2014/259131.

Kornfeld, J. W., F. Grebien, M. A. Kerenyi, K. Friedbichler, B. Kovacic, B. Zankl, A. Hoelbl, H. Nivarti, H. Beug, V. Sexl, M. Muller, L. Kenner, E. W. Mullner, F. Gouilleux, and R. Moriggl. 2008. "The different functions of Stat5 and chromatin alteration through Stat5 proteins." *Front Biosci* no. 13:6237-54.

Kuersten, S., and E. B. Goodwin. 2003. "The power of the 3' UTR: translational control and development." *Nat Rev Genet* no. 4 (8):626-37. doi: 10.1038/nrg1125.

Kumar, S., C. W. Kim, R. D. Simmons, and H. Jo. 2014. "Role of flow-sensitive microRNAs in endothelial dysfunction and atherosclerosis: mechanosensitive athero-miRs." *Arterioscler Thromb Vasc Biol* no. 34 (10):2206-16. doi: 10.1161/ATVBAHA.114.303425.

Lee, R. C., R. L. Feinbaum, and V. Ambros. 1993. "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." *Cell* no. 75 (5):843-54.

Li, Y., and K. V. Kowdley. 2012. "MicroRNAs in common human diseases." *Genomics Proteomics Bioinformatics* no. 10 (5):246-53. doi: 10.1016/j.gpb.2012.07.005.

Lodish, H. F., B. Zhou, G. Liu, and C. Z. Chen. 2008. "Micromanagement of the immune system by microRNAs." *Nat Rev Immunol* no. 8 (2):120-30. doi: 10.1038/nri2252.

Lu, Y. C., W. C. Yeh, and P. S. Ohashi. 2008. "LPS/TLR4 signal transduction pathway." *Cytokine* no. 42 (2):145-51. doi: 10.1016/j.cyto.2008.01.006.

Ma, X., L. E. Becker Buscaglia, J. R. Barker, and Y. Li. 2011. "MicroRNAs in NF-kappaB signaling." *J Mol Cell Biol* no. 3 (3):159-66. doi: 10.1093/jmcb/mjr007.

Malin, S., S. McManus, C. Cobaleda, M. Novatchkova, A. Delogu, P. Bouillet, A. Strasser, and M. Busslinger. 2010. "Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development." *Nat Immunol* no. 11 (2):171-9. doi: 10.1038/ni.1827.

Mammoto, A., T. Mammoto, and D. E. Ingber. 2012. "Mechanosensitive mechanisms in transcriptional regulation." *J Cell Sci* no. 125 (Pt 13):3061-73. doi: 10.1242/jcs.093005.

Mammoto, T., and D. E. Ingber. 2010. "Mechanical control of tissue and organ development." *Development* no. 137 (9):1407-20. doi: 10.1242/dev.024166.

Matouk, C. C., and P. A. Marsden. 2008. "Epigenetic regulation of vascular endothelial gene expression." *Circ Res* no. 102 (8):873-87. doi: 10.1161/CIRCRESAHA.107.171025.

Matthay, M. A., and G. A. Zimmerman. 2005. "Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management." *Am J Respir Cell Mol Biol* no. 33 (4):319-27. doi: 10.1165/rcmb.F305.

McKiernan, P. J., and C. M. Greene. 2015. "MicroRNA Dysregulation in Cystic Fibrosis." *Mediators Inflamm* no. 2015:529642. doi: 10.1155/2015/529642.

Medzhitov, R., and T. Horng. 2009. "Transcriptional control of the inflammatory response." *Nat Rev Immunol* no. 9 (10):692-703. doi: 10.1038/nri2634.

Mendell, J. T., and E. N. Olson. 2012. "MicroRNAs in stress signaling and human disease." *Cell* no. 148 (6):1172-87. doi: 10.1016/j.cell.2012.02.005.

Menssen, A., and H. Hermeking. 2012. "c-MYC and SIRT1 locked in a vicious cycle." *Oncotarget* no. 3 (2):112-3. doi: 10.18632/oncotarget.440.

Mitra, S., M. Khaidakov, J. Lu, S. Ayyadevara, J. Szwedo, X. W. Wang, C. Chen, S. Khaidakov, S. R. Kasula, A. Stone, I. Pogribny, and J. L. Mehta. 2011. "Prior exposure to oxidized low-density lipoprotein limits apoptosis in subsequent generations of endothelial cells by altering promoter methylation." *Am J Physiol Heart Circ Physiol* no. 301 (2):H506-13. doi: 10.1152/ajpheart.00252.2011.

Mitra, S., S. Sammani, T. Wang, D. L. Boone, N. J. Meyer, S. M. Dudek, L. Moreno-Vinasco, J. G. Garcia, and J. R. Jacobson. 2011. "Role of growth arrest and DNA damage-inducible α in Akt phosphorylation and ubiquitination after mechanical stress-induced vascular injury." *Am J Respir Crit Care Med* no. 184 (9):1030-40. doi: 10.1164/rccm.201103-0447OC.

Moen, E. L., X. Zhang, W. Mu, S. M. Delaney, C. Wing, J. McQuade, J. Myers, L. A. Godley, M. E. Dolan, and W. Zhang. 2013. "Genome-wide variation of cytosine modifications between European and African populations and the implications for complex traits." *Genetics* no. 194 (4):987-96. doi: 10.1534/genetics.113.151381.

Mols, G., H. J. Priebe, and J. Guttmann. 2006. "Alveolar recruitment in acute lung injury." *Br J Anaesth* no. 96 (2):156-66. doi: 10.1093/bja/aei299.

Moss, M., and D. M. Mannino. 2002. "Race and gender differences in acute respiratory distress syndrome deaths in the United States: an analysis of multiple-cause mortality data (1979- 1996)." *Crit Care Med* no. 30 (8):1679-85.

Nahid, M. A., M. Satoh, and E. K. Chan. 2011. "MicroRNA in TLR signaling and endotoxin tolerance." *Cell Mol Immunol* no. 8 (5):388-403. doi: 10.1038/cmi.2011.26.

Nemeth, E., L. S. Tashima, Z. Yu, and G. D. Bryant-Greenwood. 2000. "Fetal membrane distention: I. Differentially expressed genes regulated by acute distention in amniotic epithelial (WISH) cells." *Am J Obstet Gynecol* no. 182 (1 Pt 1):50-9.

Nichols, J., E. P. Evans, and A. G. Smith. 1990. "Establishment of germ-line-competent embryonic stem (ES) cells using differentiation inhibiting activity." *Development* no. 110 (4):1341-8.

Novak, U., A. Mui, A. Miyajima, and L. Paradiso. 1996. "Formation of STAT5-containing DNA binding complexes in response to colony-stimulating factor-1 and platelet-derived growth factor." *J Biol Chem* no. 271 (31):18350-4.

O'Connell, R. M., D. S. Rao, and D. Baltimore. 2012. "microRNA regulation of inflammatory responses." *Annu Rev Immunol* no. 30:295-312. doi: 10.1146/annurev-immunol-020711-075013.

O'Connell, R. M., D. S. Rao, A. A. Chaudhuri, and D. Baltimore. 2010. "Physiological and pathological roles for microRNAs in the immune system." *Nat Rev Immunol* no. 10 (2):111-22. doi: 10.1038/nri2708.

Ognjanovic, S., S. Bao, S. Y. Yamamoto, J. Garibay-Tupas, B. Samal, and G. D. Bryant-Greenwood. 2001. "Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes." *J Mol Endocrinol* no. 26 (2):107-17.

Orfanos, S. E., I. Mavrommati, I. Korovesi, and C. Roussos. 2004. "Pulmonary endothelium in acute lung injury: from basic science to the critically ill." *Intensive Care Med* no. 30 (9):1702-14. doi: 10.1007/s00134-004-2370-x.

Pinz, S., S. Unser, D. Buob, P. Fischer, B. Jobst, and A. Rascle. 2015. "Deacetylase inhibitors repress STAT5-mediated transcription by interfering with bromodomain and extra-terminal (BET) protein function." *Nucleic Acids Res* no. 43 (7):3524-45. doi: 10.1093/nar/gkv188.

Pratt, A. J., and I. J. MacRae. 2009. "The RNA-induced silencing complex: a versatile gene-silencing machine." *J Biol Chem* no. 284 (27):17897-901. doi: 10.1074/jbc.R900012200.

Quinn, E. M., J. Wang, and H. P. Redmond. 2012. "The emerging role of microRNA in regulation of endotoxin tolerance." *J Leukoc Biol* no. 91 (5):721-7. doi: 10.1189/jlb.1111571.

Rao, X., J. Zhong, S. Zhang, Y. Zhang, Q. Yu, P. Yang, M. H. Wang, D. J. Fulton, H. Shi, Z. Dong, D. Wang, and C. Y. Wang. 2011. "Loss of methyl-CpG-binding domain protein 2 enhances endothelial angiogenesis and protects mice against hind-limb ischemic injury." *Circulation* no. 123 (25):2964-74. doi: 10.1161/CIRCULATIONAHA.110.966408.

Rascle, A., J. A. Johnston, and B. Amati. 2003. "Deacetylase activity is required for recruitment of the basal transcription machinery and transactivation by STAT5." *Mol Cell Biol* no. 23 (12):4162-73.

Rascle, A., and E. Lees. 2003. "Chromatin acetylation and remodeling at the Cis promoter during STAT5-induced transcription." *Nucleic Acids Res* no. 31 (23):6882-90.

Reich, N. C. 2013. "STATs get their move on." *JAKSTAT* no. 2 (4):e27080. doi: 10.4161/jkst.27080.

Revollo, J. R., A. A. Grimm, and S. Imai. 2004. "The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells." *J Biol Chem* no. 279 (49):50754-63. doi: 10.1074/jbc.M408388200.

Robertson, K. D. 2005. "DNA methylation and human disease." *Nat Rev Genet* no. 6 (8):597-610. doi: 10.1038/nrg1655.

Robertson, K. D., and P. A. Jones. 2000. "DNA methylation: past, present and future directions." *Carcinogenesis* no. 21 (3):461-7.

Samal, B., Y. Sun, G. Stearns, C. Xie, S. Suggs, and I. McNiece. 1994. "Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor." *Mol Cell Biol* no. 14 (2):1431-7.

Saxonov, S., P. Berg, and D. L. Brutlag. 2006. "A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters." *Proc Natl Acad Sci U S A* no. 103 (5):1412-7. doi: 10.1073/pnas.0510310103.

Schmeck, B., W. Beermann, V. van Laak, J. Zahlten, B. Opitz, M. Witzenrath, A. C. Hocke, T. Chakraborty, M. Kracht, S. Rosseau, N. Suttorp, and S. Hippenstiel. 2005. "Intracellular bacteria differentially regulated endothelial cytokine release by MAPK-dependent histone modification." *J Immunol* no. 175 (5):2843-50.

Schones, D. E., K. Cui, S. Cuddapah, T. Y. Roh, A. Barski, Z. Wang, G. Wei, and K. Zhao. 2008. "Dynamic regulation of nucleosome positioning in the human genome." *Cell* no. 132 (5):887-98. doi: 10.1016/j.cell.2008.02.022.

Schwerk, J., and R. Savan. 2015. "Translating the Untranslated Region." *J Immunol* no. 195 (7):2963-71. doi: 10.4049/jimmunol.1500756.

Sessa, R., and A. Hata. 2013. "Role of microRNAs in lung development and pulmonary diseases." *Pulm Circ* no. 3 (2):315-28. doi: 10.4103/2045-8932.114758.

Shen, Z., J. M. Ajmo, C. Q. Rogers, X. Liang, L. Le, M. M. Murr, Y. Peng, and M. You. 2009. "Role of SIRT1 in regulation of LPS- or two ethanol metabolites-induced TNF-alpha production in cultured macrophage cell lines." *Am J Physiol Gastrointest Liver Physiol* no. 296 (5):G1047-53. doi: 10.1152/ajpgi.00016.2009.

Shirodkar, A. V., R. St Bernard, A. Gavryushova, A. Kop, B. J. Knight, M. S. Yan, H. S. Man, M. Sud, R. P. Hebbel, P. Oettgen, W. C. Aird, and P. A. Marsden. 2013. "A mechanistic role for DNA methylation in endothelial cell (EC)-enriched gene expression: relationship with DNA replication timing." *Blood* no. 121 (17):3531-40. doi: 10.1182/blood-2013-01-479170.

Simon, B. A., R. B. Easley, D. N. Grigoryev, S. F. Ma, S. Q. Ye, T. Lavoie, R. M. Tuder, and J. G. Garcia. 2006. "Microarray analysis of regional cellular responses to local mechanical stress in acute lung injury." *Am J Physiol Lung Cell Mol Physiol* no. 291 (5):L851-61. doi: 10.1152/ajplung.00463.2005.

Singer, B. D., J. R. Mock, N. R. Aggarwal, B. T. Garibaldi, V. K. Sidhaye, M. A. Florez, E. Chau, K. W. Gibbs, P. Mandke, A. Tripathi, S. Yegnasubramanian, L. S. King, and F. R. D'Alessio. 2015. "Regulatory T cell DNA methyltransferase inhibition accelerates resolution of lung inflammation." *Am J Respir Cell Mol Biol* no. 52 (5):641-52. doi: 10.1165/rcmb.2014-0327OC.

Slutsky, A. S. 1993. "Mechanical ventilation. American College of Chest Physicians' Consensus Conference." *Chest* no. 104 (6):1833-59.

Soliman, M. L., K. L. Puig, C. K. Combs, and T. A. Rosenberger. 2012. "Acetate reduces microglia inflammatory signaling in vitro." *J Neurochem* no. 123 (4):555-67. doi: 10.1111/j.1471-4159.2012.07955.x.

Song, X. W., Q. Li, L. Lin, X. C. Wang, D. F. Li, G. K. Wang, A. J. Ren, Y. R. Wang, Y. W. Qin, W. J. Yuan, and Q. Jing. 2010. "MicroRNAs are dynamically regulated in hypertrophic hearts, and miR-199a is essential for the maintenance of cell size in cardiomyocytes." *J Cell Physiol* no. 225 (2):437-43. doi: 10.1002/jcp.22217.

Sonneville, F., M. Ruffin, L. Guillot, N. Rousselet, P. Le Rouzic, H. Corvol, and O. Tabary. 2015. "New insights about miRNAs in cystic fibrosis." *Am J Pathol* no. 185 (4):897-908. doi: 10.1016/j.ajpath.2014.12.022.

Stöcklin, E., M. Wissler, F. Gouilleux, and B. Groner. 1996. "Functional interactions between Stat5 and the glucocorticoid receptor." *Nature* no. 383 (6602):726-8. doi: 10.1038/383726a0.

Sullivan, K. E., A. B. Reddy, K. Dietzmann, A. R. Suriano, V. P. Kocieda, M. Stewart, and M. Bhatia. 2007. "Epigenetic regulation of tumor necrosis factor alpha." *Mol Cell Biol* no. 27 (14):5147-60. doi: 10.1128/MCB.02429-06.

Sun, X., B. Icli, A. K. Wara, N. Belkin, S. He, L. Kobzik, G. M. Hunninghake, M. P. Vera, T. S. Blackwell, R. M. Baron, M. W. Feinberg, and MICU Registry. 2012. "MicroRNA-181b regulates NF-κB-mediated vascular inflammation." *J Clin Invest* no. 122 (6):1973-90. doi: 10.1172/JCI61495.

Takahashi, K., Y. Sugi, A. Hosono, and S. Kaminogawa. 2009. "Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis." *J Immunol* no. 183 (10):6522-9. doi: 10.4049/jimmunol.0901271.

Tao, R., D. Wei, H. Gao, Y. Liu, R. A. DePinho, and X. C. Dong. 2011. "Hepatic FoxOs regulate lipid metabolism via modulation of expression of the nicotinamide phosphoribosyltransferase gene." *J Biol Chem* no. 286 (16):14681-90. doi: 10.1074/jbc.M110.201061.

Thangavel, J., A. B. Malik, H. K. Elias, S. Rajasingh, A. D. Simpson, P. K. Sundivakkam, S. M. Vogel, Y. T. Xuan, B. Dawn, and J. Rajasingh. 2014. "Combinatorial therapy with acetylation and methylation modifiers attenuates lung vascular hyperpermeability in endotoxemia-induced mouse inflammatory lung injury." *Am J Pathol* no. 184 (8):2237-49. doi: 10.1016/j.ajpath.2014.05.008.

Thangavel, J., S. Samanta, S. Rajasingh, B. Barani, Y. T. Xuan, B. Dawn, and J. Rajasingh. 2015. "Epigenetic modifiers reduce inflammation and modulate macrophage phenotype during endotoxemia-induced acute lung injury." *J Cell Sci* no. 128 (16):3094-105. doi: 10.1242/jcs.170258.

Thangjam, G. S., C. Dimitropoulou, A. D. Joshi, N. Barabutis, M. C. Shaw, Y. Kovalenkov, C. M. Wallace, D. J. Fulton, V. Patel, and J. D. Catravas. 2014. "Novel mechanism of attenuation of LPS-induced NF-kappaB activation by the heat shock protein 90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin, in human lung microvascular endothelial cells." *Am J Respir Cell Mol Biol* no. 50 (5):942-52. doi: 10.1165/rcmb.2013-0214OC.

Tomankova, T., M. Petrek, and E. Kriegova. 2010. "Involvement of microRNAs in physiological and pathological processes in the lung." *Respir Res* no. 11:159. doi: 10.1186/1465-9921-11-159.

van den Oord, E. J., S. L. Clark, L. Y. Xie, A. A. Shabalin, M. G. Dozmorov, G. Kumar, V. I. Vladimirov, P. K. Magnusson, K. A. Aberg, and Swedish Schizophrenia

Consortium. 2015. "A Whole Methylome CpG-SNP Association Study of Psychosis in Blood and Brain Tissue." *Schizophr Bull*. doi: 10.1093/schbul/sbv182.

van Rooij, E., A. L. Purcell, and A. A. Levin. 2012. "Developing microRNA therapeutics." *Circ Res* no. 110 (3):496-507. doi: 10.1161/CIRCRESAHA.111.247916.

Vaporidi, K., E. Vergadi, E. Kaniaris, M. Hatziapostolou, E. Lagoudaki, D. Georgopoulos, W. M. Zapol, K. D. Bloch, and D. Iliopoulos. 2012. "Pulmonary microRNA profiling in a mouse model of ventilator-induced lung injury." *Am J Physiol Lung Cell Mol Physiol* no. 303 (3):L199-207. doi: 10.1152/ajplung.00370.2011.

Villar, J., J. Blanco, and R. M. Kacmarek. 2016. "Current incidence and outcome of the acute respiratory distress syndrome." *Curr Opin Crit Care* no. 22 (1):1-6. doi: 10.1097/MCC.0000000000000266.

Vlahakis, N. E., and R. D. Hubmayr. 2005. "Cellular stress failure in ventilator-injured lungs." *Am J Respir Crit Care Med* no. 171 (12):1328-42. doi: 10.1164/rccm.200408-1036SO.

Weinhold, B. 2006. "Epigenetics: the science of change." *Environ Health Perspect* no. 114 (3):A160-7.

Wierda, R. J., S. B. Geutskens, J. W. Jukema, P. H. Quax, and P. J. van den Elsen. 2010. "Epigenetics in atherosclerosis and inflammation." *J Cell Mol Med* no. 14 (6A):1225-40. doi: 10.1111/j.1582-4934.2010.01022.x.

Wolfson, R. K., B. Mapes, and J. G. Garcia. 2014. "Excessive mechanical stress increases HMGB1 expression in human lung microvascular endothelial cells via STAT3." *Microvasc Res* no. 92:50-5. doi: 10.1016/j.mvr.2013.12.005.

Wu, F., Z. Yang, and G. Li. 2009. "Role of specific microRNAs for endothelial function and angiogenesis." *Biochem Biophys Res Commun* no. 386 (4):549-53. doi: 10.1016/j.bbrc.2009.06.075.

Xing, S., F. Nie, Q. Xu, Y. Deng, W. Li, Z. Yang, X. Zhao, P. Zhu, X. Wang, Y. Gao, and Z. He. 2015. "HDAC is essential for epigenetic regulation of Thy-1 gene expression during LPS/TLR4-mediated proliferation of lung fibroblasts." *Lab Invest* no. 95 (10):1105-16. doi: 10.1038/labinvest.2015.97.

Yamakuchi, M. 2012. "MicroRNAs in Vascular Biology." *Int J Vasc Med* no. 2012:794898. doi: 10.1155/2012/794898.

Yamamoto, M., G. Iguchi, H. Fukuoka, K. Suda, H. Bando, M. Takahashi, H. Nishizawa, S. Seino, and Y. Takahashi. 2013. "SIRT1 regulates adaptive response of the

growth hormone--insulin-like growth factor-I axis under fasting conditions in liver." *Proc Natl Acad Sci U S A* no. 110 (37):14948-53. doi: 10.1073/pnas.1220606110.

Yang, H., W. Zhang, H. Pan, H. G. Feldser, E. Lainez, C. Miller, S. Leung, Z. Zhong, H. Zhao, S. Sweitzer, T. Considine, T. Riera, V. Suri, B. White, J. L. Ellis, G. P. Vlasuk, and C. Loh. 2012. "SIRT1 activators suppress inflammatory responses through promotion of p65 deacetylation and inhibition of NF-kappaB activity." *PLoS One* no. 7 (9):e46364. doi: 10.1371/journal.pone.0046364.

Ye, H., A. Zhou, Q. Hong, X. Chen, Y. Xin, L. Tang, D. Dai, H. Ji, M. Xu, D. W. Wang, and S. Duan. 2015. "Association of seven thrombotic pathway gene CpG-SNPs with coronary heart disease." *Biomed Pharmacother* no. 72:98-102. doi: 10.1016/j.biopha.2015.04.009.

Ye, S. Q., B. A. Simon, J. P. Maloney, A. Zambelli-Weiner, L. Gao, A. Grant, R. B. Easley, B. J. McVerry, R. M. Tuder, T. Standiford, R. G. Brower, K. C. Barnes, and J. G. Garcia. 2005. "Pre-B-cell colony-enhancing factor as a potential novel biomarker in acute lung injury." *Am J Respir Crit Care Med* no. 171 (4):361-70. doi: 10.1164/rccm.200404-563OC.

Ye, S. Q., L. Q. Zhang, D. Adyshev, P. V. Usatyuk, A. N. Garcia, T. L. Lavoie, A. D. Verin, V. Natarajan, and J. G. Garcia. 2005. "Pre-B-cell-colony-enhancing factor is critically involved in thrombin-induced lung endothelial cell barrier dysregulation." *Microvasc Res* no. 70 (3):142-51. doi: 10.1016/j.mvr.2005.08.003.

Yoshizaki, T., J. C. Milne, T. Imamura, S. Schenk, N. Sonoda, J. L. Babendure, J. C. Lu, J. J. Smith, M. R. Jirousek, and J. M. Olefsky. 2009. "SIRT1 exerts anti-inflammatory effects and improves insulin sensitivity in adipocytes." *Mol Cell Biol* no. 29 (5):1363-74. doi: 10.1128/MCB.00705-08.

Yuan, L., G. C. Chan, D. Beeler, L. Janes, K. C. Spokes, H. Dharaneeswaran, A. Mojiri, W. J. Adams, T. Sciuto, G. Garcia-Cardena, G. Molema, P. M. Kang, N. Jahroudi, P. A. Marsden, A. Dvorak, E. R. Regan, and W. C. Aird. 2016. "A role of stochastic phenotype switching in generating mosaic endothelial cell heterogeneity." *Nat Commun* no. 7:10160. doi: 10.1038/ncomms10160.

Zambon, M., and J. L. Vincent. 2008. "Mortality rates for patients with acute lung injury/ARDS have decreased over time." *Chest* no. 133 (5):1120-7. doi: 10.1378/chest.07-2134.

Zhang, C., J. Tong, and G. Huang. 2013. "Nicotinamide phosphoribosyl transferase (Nampt) is a target of microRNA-26b in colorectal cancer cells." *PLoS One* no. 8 (7):e69963. doi: 10.1371/journal.pone.0069963.

Zhang, X. Q., C. J. Lv, X. Y. Liu, D. Hao, J. Qin, H. H. Tian, Y. Li, and X. Z. Wang. 2013. "Genome‐wide analysis of DNA methylation in rat lungs with

lipopolysaccharide‐induced acute lung injury." *Mol Med Rep* no. 7 (5):1417-24. doi: 10.3892/mmr.2013.1405.

Zhang, Y. Y., L. Gottardo, R. Thompson, C. Powers, D. Nolan, J. Duffy, M. C. Marescotti, A. Avogaro, and A. Doria. 2006. "A visfatin promoter polymorphism is associated with low-grade inflammation and type 2 diabetes." *Obesity (Silver Spring)* no. 14 (12):2119-26. doi: 10.1038/oby.2006.247.

Zhou, T., J. G. Garcia, and W. Zhang. 2011. "Integrating microRNAs into a system biology approach to acute lung injury." *Transl Res* no. 157 (4):180-90. doi: 10.1016/j.trsl.2011.01.010.

Zhou, T., T. Wang, and J. G. Garcia. 2014. "Expression of nicotinamide phosphoribosyltransferase-influenced genes predicts recurrence-free survival in lung and breast cancers." *Sci Rep* no. 4:6107. doi: 10.1038/srep06107.

VITA

Name: Ramamoorthi Elangovan, Venkateswaran
Education and Training

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
School of Engineering and Technology, Bharathidasan University, India	B.Tech	05/2005	Biotechnology
Illinois Institute of Technology, Chicago, IL	M.S.	7/2009	Biotechnology
University of Illinois at Chicago, Chicago, IL	Ph.D.	May 2016	Cellular and Molecular Pharmacology

A. Positions and Honors

Positions and Employment

2008-2009: Research Technologist, The University of Chicago, Chicago, Illinois.

2009-2011: Research Specialist, The University of Illinois at Chicago, Chicago, Illinois.

2011-Current: Research Assistant, The University of Illinois at Chicago, Chicago, Illinois.

2013-Current: Research Specialist, Principal, The University of Arizona, Tucson, Arizona.

Related Peer-Reviewed Publications:

1. Adyshev DM, Moldobaeva NK, **Elangovan VR**, Garcia JG, Dudek SM. Differential involvement of ezrin/radixin/moesin proteins in sphingosine 1-phosphate-induced human pulmonary endothelial cell barrier enhancement. Cell Signal. 2011;23(12):2086-96. doi: 10.1016/j.cellsig.2011.08.003. PubMed PMID: 21864676; PMCID: PMC3651873.

2. Adyshev DM, Moldobaeva N, Mapes B, **Elangovan V**, Garcia JG. MicroRNA regulation of nonmuscle myosin light chain kinase expression in human lung endothelium. Am J Respir Cell Mol Biol. 2013;49(1):58-66. doi: 10.1165/rcmb.2012-0397OC. PubMed PMID: 23492194; PMCID: PMC3727884.

3. Belvitch P, Adyshev D, **Elangovan VR**, Brown ME, Naureckas C, Rizzo AN, Siegler JH, Garcia JG, Dudek SM. Proline-rich region of non-muscle myosin light chain kinase modulates kinase activity and endothelial cytoskeletal dynamics. Microvasc Res. 2014;95:94-102. doi: 10.1016/j.mvr.2014.07.007. PubMed PMID: 25072537; PMCID: PMC4188692.

4. Sun X, **Elangovan VR**, Mapes B, Camp SM, Sammani S, Saadat L, Ceco E, Ma SF, Flores C, MacDougall MS, Quijada H, Liu B, Kempf CL, Wang T, Chiang ET, Garcia JG. The NAMPT promoter is regulated by mechanical stress, signal transducer and activator of transcription 5, and acute respiratory distress syndrome-associated genetic variants. Am J Respir Cell Mol Biol. 2014;51(5):660-7. doi: 10.1165/rcmb.2014-0117OC. ubMed PMID: 24821571; PMCID: PMC4224084.

5. Adyshev DM, **Elangovan VR**, Moldobaeva N, Mapes B, Sun X, Garcia JG. Mechanical stress induces pre-B-cell colony-enhancing factor/NAMPT expression via epigenetic regulation by miR-374a and miR-568 in human lung endothelium. Am J Respir Cell Mol Biol. 2014;50(2):409-18. doi: 10.1165/rcmb.2013-0292OC. PubMed PMID: 24053186; PMCID: PMC3930953.

6. **Elangovan VR**, Wang T, Garcia JG. Characterization of endotoxin and mechanical stress induced DNA methylation changes in the NAMPT/PBEF promoter. (Original manuscript under preparation).

Related Peer-Reviewed Abstracts

1. Djanybek A, Nurgul M, **Elangovan VR**, Joe GG, Steven MD. Ezrin/Radixin/Moesin Proteins Differentially Regulate S1P-Induced Pulmonary Endothelial Cell Barrier Enhancement. C26 REGULATORS OF THE LUNG ENDOTHELIAL BARRIER: American Thoracic Society; 2011. p. A4186-A.

2. Djanybek A, Nurgul M, **Elangovan VR**, Steven D, Joe GNG. Multiple MicroRNAs Regulate Nonmuscle Myosin Light Chain Kinase (nmMLCK) Gene Expression In Pulmonary Endothelium. C71 MAJOR IMPACT OF MICRO-RNA FUNCTION: American Thoracic Society; 2012. p. A4987-A.

3. Jiang T, Mascarenhas JB, **Elangovan VR**, Wang T, Zhang DD, Garcia JG. Nrf2 Signaling Modulates Human Endothelial Cell Barrier Regulation by S1P. Circulation. 2014;130(Suppl 2):A20527-A.

4. Keely LS, Wenbo M, Xu Z, Michael SW, **Elangovan VR**, Ting W, Jeffrey DF, Rick AK, Wei Z, Joe GNG. Characterizing Population-Specific Cytosine Modifications In MYLK For Understanding The Health Disparities In Acute Respiratory Distress Syndrome. C63 LUNG INJURY AND REPAIR: TWO TO TANGO: American Thoracic Society; 2014. p. A5008-A.

5. Mascarenhas JB, Lau SS, Danilov SM, Tchourbanov AY, Granzier HL, **Elangovan VR**, Wang T, Garcia JG. Mechanism of Mechanical Stress-Regulated Non-Muscle Myosin Light Chain Kinase Alternative Splicing, Implication for Acute Lung Inflammation. Circulation. 2014;130(Suppl 2):A20441-A.

6. Probe I, Island C, Mu W, Zhang X, Wade M, **Elangovan VR**, Wang T, Fortman J, Kittles R, Zhang W. Characterizing Population-Specific Cytosine Modifications In Mylk For Understanding The Health Disparities In Acute Respiratory Distress Syndrome. Center for One.2010:34283-21243.

7. Shahid S, Mary B, Patrick AS, **Elangovan VR**, Jaideep M, Saad S, Liliana M-V, Joe GNG. IFITM3 And NmMLCK Are Required In Extracellular PBEF Mediated Activation Of NFkappaB Pathway In Human Lung Microvessel Endothelium. B69 THE ENDOTHELIAL AND EPITHELIAL BARRIERS IN INJURED LUNGS: American Thoracic Society; 2010. p. A3612-A.

8. Ting W, Tao J, Tong Z, Hector Q, Joseph BM, **Elangovan VR**, Louise H, Donna DZ, Joe GNG. The Central Role of Nuclear Factor (Erythroid-Derived 2)-Like 2 in Ventilator-Induced Lung Injury. B43 MECHANISMS OF INJURY IN ARDS AND VILI: American Thoracic Society; 2015. p. A3130-A.

9. **Elangovan VR**, Xiaoguang S, Brandon LM, Steven MD, Joe GNG. LPS- And Cyclic Stretch-Induced Pre B Cell Colony Enhancing Factor Is Epigenetically Regulated. A51 PULMONARY VASCULAR PROLIFERATION AND PERMEABILITY: American Thoracic Society; 2013. p. A1721-A.

10. **Elangovan VR**, Ting W, Joe GNG. Sirtuins Negatively Regulate Nampt/Pre-B Cell Colony-Enhancing Factor Expression in Human Lung Endothelium. C39 REGULATION OF INFLAMMATION AND LUNG INJURY II: American Thoracic Society; 2015. p. A4352-A.

11. Wang T, Jiang T, Quijada H, Mascarenhas JB, **Elangovan VR**, Hecker L, Lussier YA, Yuan JX, Zhang DD, Garcia JG. Nrf2 Signaling Ameliorates Ventilator-Induced Lung Inflammation. Circulation. 2014;130(Suppl 2):A20538-A.

12. Xiaoguang S, Brandon LM, **Elangovan VR**, Michael SW, Jaideep M, Shwu-Fan M, Joe GNG. Transcription Regulation Of Pre-B-Cell Colony-Enhancing Factor And Influences Of Inflammatory Lung Injury Associated Polymorphisms. C78 ACUTE LUNG INJURY: BIOLOGICAL MECHANISMS: American Thoracic Society; 2013. p. A4921-A.

About NEJM Permissions

Submit Your Permission Request Using Rightslink

If you are seeking permission to copy/reproduce/republish content from NEJM and are not the author of that content, you may use the Copyright Clearance Center's Rightslink® service. Simply visit **NEJM.org** and locate the article from which you seek to reuse content.

Once you have located and accessed the article you are looking for on NEJM.org:

- Click on the Permission link in the article toolbox (if you are looking at the full-text), or click on the Permissions link in the middle of the page (if you are viewing the abstract or first 100 words of the article because you don't have a subscription)
- The RightsLink® window will pop up, with information about content you have selected.
- Follow the prompts to obtain a free price quote OR
- Sign-in to your existing RightsLink® account, or create an account if you do not already have one, and follow the prompts to secure permission

Confirmation of your permission and a copy of the permission terms and conditions will be sent to you instantly via e-mail for most requests. Some requests may require Publisher review.

If you have questions about using the Rightslink® service, please contact Rightslink® Customer Support at (877) 622-5543 (toll free) or (978) 777-9929, or e-mail **customercare@copyright.com**.

For other general questions about NEJM Permissions, e-mail **permissions@nejm.org**.

Permission for Authors

If you are the author of the article that was published in The New England Journal of Medicine ("NEJM"), please visit the **NEJM Author Center**.

Reuse of Content within a Thesis or Dissertation

Content (full-text or portions thereof) may be used in print and electronic versions of your dissertation or thesis without formal permission from the Massachusetts Medical Society, Publisher of the New England Journal of Medicine.

> **The following credit line must be printed along with the copyrighted material**:
>
> "Reproduced with permission from (scientific reference citation), Copyright Massachusetts Medical Society.

Third-Party Content

Grants of permissions apply only to copyrighted material that the MMS owns, and not to copyrighted text or illustrations for other sources.

Prohibited Uses

The New England Journal of Medicine (and its logo design) are registered trademarks of the Massachusetts Medical Society. We do not grant permission for our logo, cover or brand identity to be used in materials. Permission will not be granted for photographs depicting identifiable individuals.