

The role of nmMLCK in the regulation of lung vascular barrier integrity during pulmonary inflammation

By

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THESIS

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This dissertation is dedicated to my Grandfather, Kyo Miyake.

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LIST OF ABBREVIATIONS

ABP	Actin binding protein
AJ	Adherens junction
ALI	Acute lung injury
APTI	Airway pressure time index
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
ChIP	Chromatin immunoprecipitation
COPD	Chronic obstructive pulmonary disease
DAG	Diacyl-glycerol
EC	Endothelial cells
ECIS	Electrical cell-substrate impedance sensing system
ECM	Extracellular matrix
eNOS	endothelial nitric oxide synthase
FA	Focal adhesion
FAK	Focal adhesion kinase
HDAC	Histone deacetylase
HMEC	Human microvascular endothelial cells
HPAEC	Human pulmonary artery endothelial cells
IF	Intermediate filament
ITGB4	Integrin beta-4
IPF	Idiopathic pulmonary fibrosis
JAM	Junctional adhesion molecule
KAT	Lysine acetyltransferase
KDR	Kinase domain receptor
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MLCP	Myosin light chain phosphatase
MYPT	Myosin phosphatase targeting protein
nmMLCK	Non-muscle isoform of myosin light chain kinase
OVA	Ovalbumin
PI3	Phosphoinositide-3
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLGF	Platelet growth factor
PTM	Post-translational modification
qPCR	Quantitative polymerase chain reaction
RILI	Radiation-induced lung injury
ROCK	Rho-associated kinase

S1P	Sphingosine 1-phosphate
SNP	Single nucleotide polymorphism
SP1	Specific protein 1
SP3	Specific protein 3
TER	Transendothelial electrical resistance
TJ	Tight junction
VILI	Ventilator-induced lung injury
VEGF	Vascular endothelial growth factor
VPF	Vascular permeability factor
ZO	Zona occluden

SUMMARY

Increased vascular permeability and accumulation of alveolar and airway fluid are cardinal features of lung inflammatory diseases such as ALI/ARDS and asthma. While the two known regulators of vascular barrier regulation, vascular endothelial growth factor (VEGF) and the non-muscle isoform of myosin light chain kinase (nmMLCK) have been shown to be associated with ALI/ARDS and asthma, the direct link between the two molecules in disease pathophysiology have been unclear. In this thesis, I have provided a novel mechanistic linkage between VEGF and nmMLCK. Specifically, I have shown that the nmMLCK gene, *nmMYLK*, is a direct target of the VEGF signaling pathway, with the mRNA and protein expression of nmMLCK being increased by VEGF stimulation in endothelial cells. Using *in vitro* studies I have demonstrated that upon VEGF stimulation the transcription factor, Sp1, binds to the *nmMYLK* promoter and is essential for increasing the expression levels of nmMLCK. In addition, I have provided bioinformatics evidence that SNPs on the *nmMYLK* gene are located within transcription factor binding sites on the promoter and potential candidates for modulating nmMLCK-mediated responses to VEGF stimulation during pulmonary disease. Furthermore, I have shown that nmMLCK activation by VEGF signaling leads to actin-myosin mediated endothelial cell contraction to alter overall vascular barrier function. I have further reinforced the clinical relevance of my *in vitro* findings, by showing that both VEGF and nmMLCK mRNA and protein expression are upregulated in ARDS and asthmatic patients, together with previous data showing variants in the *MYLK* gene are associated with severe asthma in African descent individuals. Taken together, these data provide further mechanistic insights into asthma pathobiology, offer potentially novel therapeutic targets to treat asthma and validate the use of SNPs in the *nmMYLK* gene as guides to future personalized medicine for pulmonary disease.

Chapter I: Introduction

A Overview of pulmonary inflammatory diseases

Increased vascular permeability and accumulation of alveolar and airway fluid are cardinal features of pulmonary inflammatory diseases. The lung pulmonary vasculature extends throughout the tissue with a vast surface area with the inner lining of the pulmonary vasculature comprised of pulmonary endothelium that function as a semipermeable cellular barrier between the vascular compartment and the pulmonary interstitium. The endothelial cell barrier is tightly regulated by physiological signals, which serve to control pulmonary vascular permeability and is dysregulated in the setting of inflammation. Failure to maintain lung vascular integrity contributes to the pathobiology of conditions such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS), radiation-induced lung injury (RILI), and asthma. Increased pulmonary vascular permeability is recognized as a major feature of lung inflammation, and significantly contributes to the profound symptoms observed in these disorders.

Pulmonary inflammatory diseases can be categorized into three phases or stages: acute, sub-acute, and chronic. Acute and sub-acute inflammation such as ALI/ARDS and RILI are more reversible whereas chronic inflammation such as in asthma, is less reversible and can persist throughout life resulting in permanent remodeling of the airway and parenchymal tissues. Given the excessive morbidity and mortality of severe ALI and ARDS as well as asthma, improved understanding of the molecular mechanisms that underlies the pathophysiology of such pulmonary inflammation is urgently needed in order to develop better therapies for these diseases.

i Acute lung inflammation: Acute lung injury and ARDS

ALI and ARDS are inflammatory lung syndromes characterized by diffuse alveolar infiltration, hypoxemia, respiratory failure, and death due to multiorgan failure. Increased pulmonary edema, due to lung vascular hyperpermeability, is a key feature of ALI/ARDS patients, and the flooding of the alveolar air space significantly impairs lung function. Detailed analysis of bronchoalveolar lavage (BAL) samples taken from ALI/ARDS patients has demonstrated that pulmonary edema fluid is protein-rich and contains inflammatory cytokines and inflammatory cells such as neutrophils (Figure 1) (Matthay review, 2005). The ALI/ARDS inducing stimuli that lead to increased pulmonary vascular permeability are varied and include direct contributors such as lung infection and gastric acid aspiration, or indirectly from sepsis, trauma, or blood volume replacement.

Mortality rates in ARDS, the most severe form of ALI, range from 34 to 58% (MacCallum and Evans, 2005) with ~150,000 – 200,000 ALI cases per year in the United States (Ware and Matthay, 2000) and an incidence of 17–34 cases/100,000 people per year in Europe, Australia, and other developed countries. Therefore, ALI and ARDS represent a major healthcare burden due to the intensive and often prolonged intensive care unit (ICU) hospitalizations. Additionally, studies have unveiled race and gender differences in ARDS deaths in the United States over the past several decades demonstrating an increase in incidence and mortality due to sepsis and ALI in African-Americans when compared with Caucasians (Moss and Mannino, 2002).

A variety of experimental models and assays have been developed in an effort to better understand the mechanistic basis for ALI and ARDS. Large animal models such

as porcine models of lung injury enabled measurements of pulmonary and systemic hemodynamic changes after experimental ARDS. Recent studies have utilized small animal models such as genetically-engineered mice where a specific gene is manipulated to examine its involvement and contribution to the development of inflammatory lung injury. Complementing these *in vivo* approaches, novel *in vitro* assay systems, such as measurements of transendothelial electrical resistance (TER), have been developed to enable characterization of the molecular pathways that are involved in lung endothelial permeability as well as for screening of small molecule drugs.

ii Subacute lung inflammation: Radiation-induced lung injury

Microvascular injury and increased vascular leakage are cardinal features of radiation-induced lung injury (RILI) that occurs in 20 to 30% of patients undergoing radiotherapy for thoracic-associated malignancies (Movsas et al. 1997). Radiation-induced lung injury can be divided into two stages: early inflammatory stage (radiation pneumonitis) and later complications of chronic scarring (radiation fibrosis). RILI most often follows radiation therapy administered to cancer patients.

Radiation pneumonitis is characterized by edema, inflammation, and occlusion of airways, air sacs and blood vessels (Movsas et al. 1997). Radiation pneumonitis takes from 1 to 3 months to develop after radiation exposure due to low mitotic index of the pulmonary parenchymal cells and results in a latent period between radiation exposure and radiation-induced pneumonitis (Movsas et al. 1997).

Radiation-induced pulmonary fibrosis is a clinical syndrome that typically evolves over a 6 to 24 month course after the initial radiation exposure, and usually remains

stable after 2 years (Movsas et al. 1997). Pulmonary fibrosis results in scarring of the lung tissues in the area damaged by radiation or in the area that developed pneumonitis caused by radiation. Symptoms of pulmonary fibrosis include shortness of breath, cough, and diminished exercise tolerance. Once lung tissues become fibrotic, it is not reversible and the treatments for severe pulmonary fibrosis such as idiopathic pulmonary fibrosis (IPF) are extremely limited.

Previous studies have confirmed microvascular injury and increased vascular permeability as features of murine RILI where female C57BL/6 mice were exposed to a single dose of whole thoracic radiation (18–25 Gy), and measurements of lung inflammation and vascular permeability assessed at intervals of 4, 6, 8, and 12 weeks. This murine RILI model indicates increased pulmonary leakage in a dose-dependent manner, as shown by the Evans blue dye leakage assay, BAL protein, cells, and lung histology showing edema (Niewald et al. 1986). Additionally, mRNA expression analysis of RILI model has identified deregulation of sphingolipid metabolic pathway genes. Sphingolipids (most prominently sphingosine 1-phosphate, S1P) play critical role in regulation of vascular permeability. Blocking S1P receptor by S1P analog, FTY720 (S)-FTY720-phosphanate attenuates RILI induced lung injury (Mathew et al. 2011).

iii Chronic lung inflammation: Asthma and COPD

Chronic lung inflammation accompanies lung disorders such as asthma and COPD, that are often irreversible and require constant therapeutic interventions. Both asthma and COPD are characterized by airway obstruction as a result of chronic inflammation of the respiratory tract, large and small airway in asthma, and the lower

airway and alveoli in COPD. Asthma, in contrast to COPD, is characterized by reversible airway obstruction and airway hyper-responsiveness, whereas both disorders exhibit varying degrees of remodeling, upper airway in asthma and lower airway in COPD. Chronic inflammation contributes to this remodeling as a consequence of the increased expression of inflammatory cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors.

An estimated 300 million people worldwide suffer from asthma, with 250,000 annual deaths attributed to the disease. The World Health Organization estimates that the number of people with asthma will grow by more than 100 million by 2025. Currently approximately 250,000 people die prematurely each year from asthma (World Health Organization, 2007).

The vasculature in the tracheobronchial region of the lung is thought to be an important contributor toward asthmatic inflammation with increased angiogenesis observed in the airways of asthmatic patients (Feltis et al. 2006). This coincides with increased inflammatory cell infiltration, increased blood flow, as well as increased vascular permeability and edema formation in the airway wall. Consistent with the increase in angiogenesis in asthmatic airways, a study has shown that newly formed vessels in chronically inflamed airways are more permeable and leaky, a process associated with increased leukocyte infiltration and edema formation (McDonald 2001). These changes in the vasculature are correlated with the severity of asthma (Horvath and Wanner 2006). Previous studies have identified several molecules partly responsible for the vascular dysfunction during asthma, which has led to new therapeutic approaches (Horvath and Wanner 2006). Despite this, asthma morbidity

and mortality symptoms are excessive and underscore the need for detailed understanding of the pathobiology of asthma.

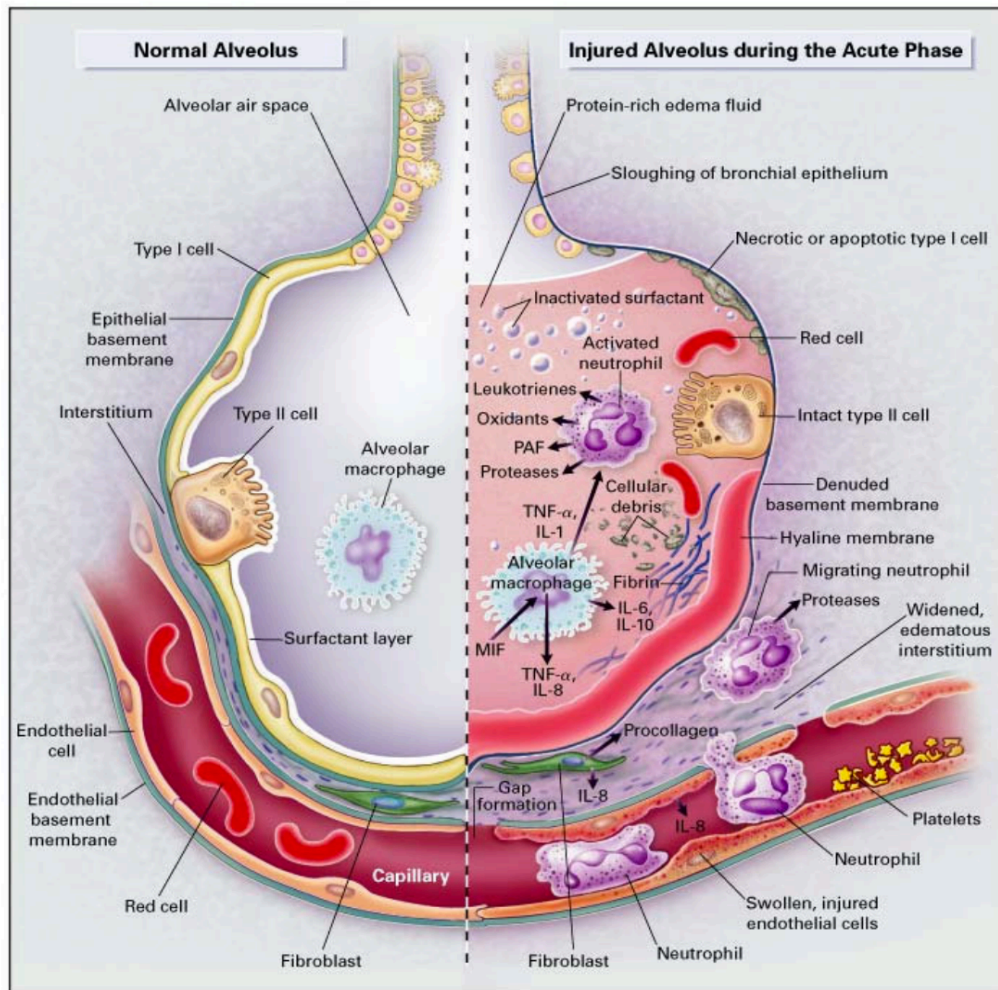


Figure 1: Structure of the normal and injured alveolus.

Schematic demonstration of the normal alveolus (left half) and the injured alveolus in the acute phase of ALI and ARDS (right half) indicating inflammation and edema formation in tissue interstitium and airway space. Reproduced with permission from Matthay MA. *N Engl J Med* 2000; 342:1334-1349, Copyright Massachusetts Medical Society.

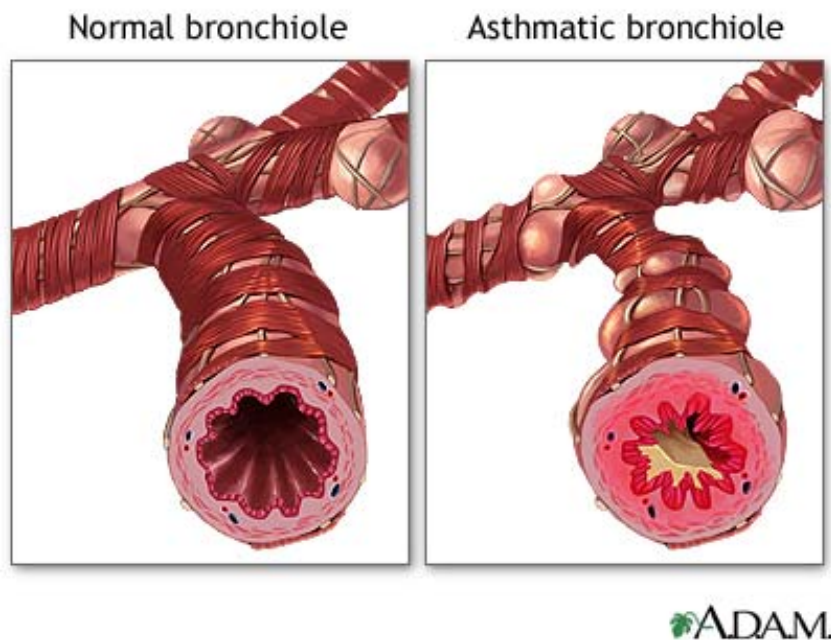


Figure 2: Structure of the normal and asthmatic bronchiole.

Schematic demonstration of the normal bronchiole airway (left half) and the asthmatic bronchiole airway (right half) representing inflammation in airway wall, as well as mucus accumulation and edema formation in the airway space. Taken from Animated Dissection of Anatomy for Medicine (A.D.A.M.).

B Lung vascular barrier regulation

i Overview

Vascular barrier regulation is a key process for systemic homeostasis by allowing the exchange of fluids, solutes, plasma proteins, nutrients, and inflammatory cytokines to tissues throughout the body. Endothelial cells comprise the inner lining of the vessel wall and form a tight cell monolayer, allowing size-selective and a semi-permeable barrier between the blood stream and tissue interstitium. The two modes of transport are 1) transcellular and 2) paracellular transportation. The transcellular pathway allows transport directly through endothelial cells via transcytosis which permits macromolecules such as albumin to selectively transport across the endothelial cell monolayer (Gharib et al. 2012). In contrast, in paracellular transport, gaps are formed between endothelial cells allowing passage of cells and macromolecules that are 3 nm in radius or greater (Pappenheimer et al. 1951). The term vascular permeability describes the process in which the passage of plasma proteins, fluid, and solutes cross the endothelial cell barrier via these two modes of transport.

Majno and Palade observed lung endothelial cell rounding and paracellular gap formation at sites of inflammatory edema within the lung vasculature (Majno and Palade 1961). Their work, led to the ultimate characterization of a barrier-regulatory process based on paracellular gap opening and closing driven by the endothelial cytoskeleton (Figure 3). The dramatic cell shape change that results in paracellular gap formation implicates the direct involvement of endothelial structural components comprised of cytoskeletal proteins such as microfilaments and microtubules. Disruption in the integrity of the EC monolayer is now recognized as a cardinal feature of inflammation, apoptosis,

ischemia-reperfusion injury and angiogenesis. Loss of vascular integrity occurs in response to a variety of inflammatory mediators, mechanical stress factors, and activated neutrophil products that include reactive oxygen species (ROS), proteases and cationic peptides. Thus, the endothelial cell cytoskeleton serves as the major regulator of the endothelial “gate-keeper” function via effects on paracellular gap regulation, leukocyte diapedesis and barrier regulation (Dudek and Garcia 2001, Garcia et al. 1986, Hirata et al. 1995, Majno and Palade 1961).

ii Endothelial cell cytoskeleton

Actin cytoskeleton

A central participant in vascular barrier regulation and maintenance of endothelial cell monolayer integrity is the cytoskeleton. The mammalian cytoskeleton plays an essential role in providing the mechanical support necessary to maintain cell shape, cell motility and movement, and for signaling transducing functions. The key cytoskeletal components are three inter-communicating networking protein filaments: actin microfilaments, microtubules, and intermediate filaments.

It is estimated that ~15-20% of endothelial cell proteins are comprised of cytoskeletal actin and myosin components, portraying the critical importance of actomyosin filament in endothelial cell functions (Gottlieb et al. 1991). Actin filaments bind to plasma membrane proteins to control cell-cell and cell-matrix interactions by stabilizing intercellular junctions, and in doing so, tightly regulate the vascular barrier and therefore control fluid and nutrition exchange between circulating blood and tissues. The major component of the actin filament is the small globular ATP-binding/hydrolyzing

protein, or G-actin. Activation of G-actin monomers by reversible ATP hydrolysis stimulates the assembly of filamentous F-actin polymers. The formation and maintenance of actin filaments is dynamically regulated by actin-associating proteins (**Table 1**) such as the Arp2/3 complex, profilin, and gelsolin (Dudek et al. 2004, Garcia et al. 2001, Shasby et al. 1982). Actin filaments undergo “treadmilling”, a process where the filament length remains approximately constant while actin monomers are added to the plus (+) end and dissociate from the minus (-) end of the polarized filament (DeMali et al. 2002, Gunst 2004). Arp2/3 is a nucleation protein stimulating the formation of new filaments and the branching of existing actin polymers. Arp2/3 complex appears in the leading edge of motile cells along with polymerizing proteins such as profilin that synergistically stimulates the addition of actin monomers at the + end. Capping proteins, such as gelsolin, stabilize the newly formed filaments by inhibiting the addition of G-actin to the polymers, and are well known to be involved in cell movement (Fujita et al. 1997; Spinardi and Witke 2007).

The role of actin filaments in cell motility is well recognized particularly in cell contraction and migration through association with the motor protein, myosin. Migrating cells can be divided into two separate regions during migration: protrusion and retraction, with the actin cytoskeleton playing distinct roles in each segment. Strands of actin filaments align in a parallel manner with + end facing the moving-front of the cells in the protrusions of filopodium or lamellipodium, where actin is polymerized in order to propel the cell forward. In contrast, actin filaments and myosin also form membrane-bound, parallel organized filamentous (F)-actin units termed “stress fibers”, stimulating

myosin sliding along the actin filaments that produces an increase in intracellular tension leading to cell contraction and retraction (Dudek et al. 2004).

Actin and myosin are the main components of stress fibers. Myosins is a member of a family of ATP-dependent motor proteins and play an important role in muscle contraction, although several isoforms of myosin are expressed in non-muscle tissues and have similar functions. Stress fiber formation, like actin cytoskeleton filament formation, undergoes highly dynamic dissociation and association processes mediated by actin binding proteins (ABPs) and focal adhesion (FA)-associated proteins, as well as proteins with kinase activity (ie. PDZ-LIM), or scaffolding protein function (ie. palladin and VASP). Four types of stress fibers exist, with each exhibiting a distinct function that is determined by the composition of each stress fiber type. The most important stress fiber associating protein for cell contraction is myosin. Myosin is the most abundant acting-binding protein in cells and stress fiber contractility is regulated by phosphorylation of the MLC at Thr18 and Ser19. Phosphorylation/dephosphorylation at these sites is mediated by myosin light chain kinase (MLCK) and a myosin phosphatase targeting protein (MYPT). The function of MLCK and MYPT will be focused on later in this chapter.

Microtubules

The microtubule component of the endothelial cytoskeletal network consists of α - and β -tubulin dimers that are compression-resistant hollow rods with diameters of 12-24 nm, and are the largest of the cytoskeletal proteins (Goode et al. 2000; Klymkowsky 1999). The primary role of the microtubule cytoskeleton is to provide mechanical support for the cells to maintain their structure with important roles in cellular processes

such as mitotic spindle formation during cell division, forming the internal structure of cilia and flagella, and providing platforms for intracellular transport including movement of secretory vesicles and organelles. In endothelial cells, together with the actin cytoskeleton, microtubules also contribute to maintaining vascular barrier integrity (Verin et al. 2001).

Intermediate filaments

The remaining cytoskeletal components, intermediate filaments (IF), consist of vimentin and other filament proteins that form structurally conserved subunits in coiled coil filaments that serve to bear tension in nuclear envelope and peripheral cell junctions (Helfand et al. 2003; Helfand et al. 2004). The average diameter of intermediate filaments is 10 nm with IFs able to deform and stretch several times their initial length due to the coiled coil structure of the filaments (Herrmann et al. 2007) attributing to its role in absorbing mechanical stress and integrating cytoskeleton. These cytoskeletal filaments associate with the actomyosin and microtubular elements as well as with neighboring cell and extracellular matrix components through intercellular junctions to aid cells in higher levels of structure and function, however, its contribution to endothelial physiology is not well understood.

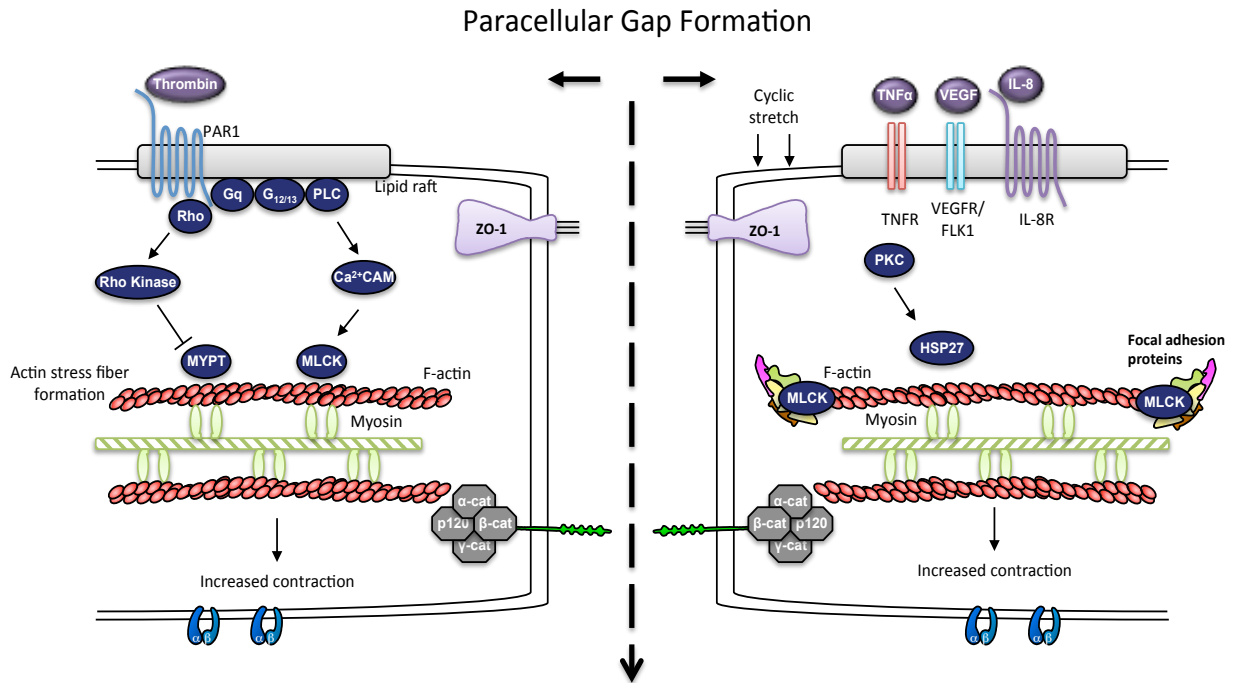


Figure 3: Intracellular signaling pathway activated by contractile agonists

Agonist-mediated formation of actin stress fibers result in increased cell contraction and formation of paracellular gaps. Taken from Shimizu Y and Garcia JG (2013) Chapter 1: The endothelial cell cytoskeleton: multifunctional role of the endothelial actomyosin cytoskeleton. Endothelial cell cytoskeleton. CRC Press, ISBN 9781466590359

iii Junctional protein and cytoskeletal linkages in barrier regulation

Three types of membrane junctional complexes also participate in regulation of endothelial cell monolayer integrity: adherens junctions (AJ), tight junctions (TJ), and focal adhesion (FA) complexes. All three types of complexes share similarity in the composition of the proteins that make up the complex where they consist of a combination of transmembrane proteins and intracellular proteins. In all cases, transmembrane proteins form homodimers via extracellular domains to create cell-cell adhesion, where intracellular proteins have roles in molecular signaling and/or act as an anchor to the cytoskeletal network. Actin cytoskeleton attachment to junctional adhesions is important for transfer of intracellular signals outside the cells and dynamic regulation of junction opening and closure between cells (Dejana 2004). Each complex has a specific role in cell-cell or cell-matrix adhesion, and transmit different cellular downstream signaling.

Adherens Junctions

Adherens junctions are the main junctional complexes in endothelial cells with major functions to initialize and stabilize cell-cell adhesion, to regulate the actin cytoskeleton, and in signal transduction and transcriptional regulation. The core AJ components in endothelial cells are: VE-cadherins and catenin family such as, p120-catenin, β -catenin, and α -catenin. VE-cadherins, a single-pass transmembrane glycoprotein belongs to the classical cadherin superfamily and consist of five extracellular cadherin repeat domains and a conserved transmembrane and cytoplasmic tail. VE-cadherins form cell-cell contacts with neighboring cells in cell-type specific manner by homodimerization of extracellular cadherin repeat domains

(Hartsock and Nelson 2008). The cytoplasmic tails of VE-cadherins interact with catenin proteins, p120-catenin and β -catenin to regulate intracellular signaling and to control actin cytoskeleton. In particular, β -catenin phosphorylation at Y142 upon cell stimulation with agonists such as VEGF, signals the dissociation of β -catenin from the AJ complex and translocation to nucleus for transcriptional activation of target genes such as c-Myc and cyclin D1 (X. L. Chen et al. 2012). Catenin proteins also act to strengthen the cell-cell contact upon interaction with VE-cadherin. α -catenin is the linker protein between adherens junctions and the actin cytoskeleton.

Tight Junctions

In endothelial cells, AJ and TJ localization domains on the cell surface are less defined and are inter-mingled, whereas in polarized epithelial cells their distribution is spatially defined in apical and basal domains. TJ also consist of both transmembrane proteins and intracellular proteins. The main components of TJ are membrane proteins, occludin, claudin family (claudins 1, 5, and 12 in EC), junctional adhesion molecule-A, B and C (JAMs), intracellular proteins zona occludens 1 and 2 (ZO-1, 2). Occludin is a transmembrane protein with four membrane-spanning regions and two extracellular loops (Bazzoni and Dejana 2004). Occludin, like AJ transmembrane proteins, homodimerizes via its extracellular region with occludin on neighboring cells. Occludin interacts directly with ZO-1 via carboxy-terminal domain. Another crucial transmembrane protein in TJ claudins consists of more than 20 members in this family. Like occludin, claudins have four membrane-spanning regions with two extracellular loops and also homodimerizes on its extracellular region with neighboring cells. Both termini are exposed to cytoplasm, where it directly interacts with ZO-1. Intracellular

protein ZO-1 is the most studied protein in TJ. ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) family. ZO-1 binds directly to actin cytoskeleton and acts as an anchor between membrane proteins and the cytoskeletal network. In addition to its role linking TJ and actin cytoskeleton, it also plays important role in the establishment of cell polarity, membrane trafficking, cell signaling, and control of gene expression. When detached from the TJ complex, ZO-1 translocates to the nucleus and modulates transcriptional activity (Dejana 2004). The ezrin, radixin, and moesin (ERM) family of actin-binding proteins also act as both as linkers between the actin cytoskeleton and plasma membrane proteins as well as signal transducers for agonists that induce cytoskeletal remodeling. Despite structural similarities and reported functional redundancy, ERM proteins differentially modulate lung EC permeability (Adyshev et al. 2011).

Focal adhesion complexes

Focal adhesion complexes, unlike AJ and TJ, serve as linkages between the cell and extra-cellular matrix (ECM). In addition, as in cell-cell adhesion, cell-matrix contact complex also play an important role in regulating the integrity of the vascular endothelium and therefore endothelial cell barrier permeability. The focal adhesion complex can be made up of over 100 proteins; nonetheless, the main components of FA mediating cell-ECM adhesion are integrins. Unlike the transmembrane proteins in AJ or TJ, integrins form heterodimers consisting of α and β subunits, and anchors to ECM proteins of the inner vessels such as collagen, fibronectin, and laminin via the extracellular domain. Integrins directly interact with various actin-binding proteins such as α -actinin, talin, filamin, vinculin, paxillin, zyxin, and tensin (Mehta and Malik 2006).

The membrane-spanning integrins are intimately associated with the tyrosine kinases known as focal adhesion kinase (FAK), which are recruited to the complex by tyrosine phosphorylation signaled by permeability-increasing agonists (Burridge et al. 1987; Romer et al. 1992). FAK is critical for the formation of focal adhesions as well as FA turnover. Actin stress fibers anchored to focal adhesion complexes act as fixed points against tension developed by permeability-inducing signals. FAK is known for its critical role in vascular permeability as FAK knockout mice is embryonic lethal due to vascular defects (Furuta et al. 1995; Ilić et al. 1995).

Membrane and junction complex interactions with the endothelial cytoskeleton

In combination with membrane-bound junction proteins, the endothelial cytoskeleton regulates: 1) cell shape and mechanical stability, 2) motility and migration, and 3) cell-cell and cell-matrix interactions; interactions which preserve the integrity of the endothelial monolayer via specialized inter-endothelial contacts (Dudek et al. 2004, Mehta and Malik 2006). The actin microfilament system is focally linked to multiple membrane adhesive proteins such as cadherin, glycocalyx components, functional intercellular proteins of the zona occludens (ZO) and zona adherens, and focal adhesion complex proteins (Dudek et al. 2004). Involved in this actin network are >80 actin-binding proteins that are critical participants in cytoskeletal arrangement and tensile force generation, and serve to provide a high level of fine tuning of cell shape, adhesion, and orchestrated cell migration as well as regulation of endothelial junction stability (Linz-McGillem et al. 2004). Myosin represents the most abundant actin-binding protein and, as noted above, serves as the molecular machinery to generate tension via an actomyosin motor. Focally distributed changes in tension and relaxation can be

accomplished by regulation of the levels of myosin light chain (MLC) phosphorylation and actin stress fiber formation (Dudek et al. 2004). Endothelial specific VE-cadherin is the most prominent protein in the adherens junction complex, forming a complex with catenin proteins (α , β , and γ) linked to an F-actin-based bridge between the cytoskeleton of adjacent cells. The homodimerized extracellular domain of VE-cadherin ensures the regulation of intercellular barriers and enforcing the restricted access of vascular components to the interstitium, a process corrupted during high permeability states associated with paracellular gaps (Corada et al. 1999; Dejana et al. 1999). Permeability-enhancing factors such as VEGF and thrombin transduce signaling to the endothelial cytoskeleton and induce actomyosin cross-bridging that increases contractile elements to retract intercellular junctions, creating gaps between cells (Becker et al. 2003; Birukova et al. 2004; Dudek et al. 2004; Dull and Garcia 2002; Garcia et al. 2001; Petrache et al. 2001).

Actin-Interacting Partners	Function	Molecular Mass (kDa)
α -Actinin	Reorganizes actin cytoskeleton in cell movement. Links actin to plasma membrane and integrins. Part of focal adhesion plaque. Attaches actin to a variety of intracellular structures. Binds vinculin, nebulin, clathrin, and B1 integrins. (Lum and Malik 1994, 1996)	100
Fimbrin	Links actin cytoskeleton to vimentin network at sites of cell adhesion. Present in F-actin adhesion structures as well as microvilli. Calcium regulated. (Dubreuil 1991)	68
Cortactin	Cross-links actin in cell periphery. Involved in actin rearrangement. Substrate for Abl-and Src-modulated tyrosine phosphorylation-associated cytoskeletal rearrangement. (Dudek and Garcia 2003, Dudek et al. 2004, Garcia et al. 1997)	80
Filamin	Participates in vascular permeability. Recognizes peripheral actin. Anchors various transmembrane proteins to actin cytoskeleton. Regulated by cAMP-dependent PKA-mediated phosphorylation and Ca^{2+} /CaM kinase. (Garcia et al. 1995)	280
FAK	Tyrosine kinase. Early recruitment to adhesion sites during cell migration. Activated by autophosphorylation and Src kinases. FAK-Src mediates disassembly of adhesion complex. (Furuta et al. 1995, Ilić et al. 1995)	120
Integrins	Major components of focal adhesion complex. Transmembrane protein that links ECM and actin filaments. (Kornberg et al. 1992, Schaller et al. 1995)	90-160
Paxillin	A focal adhesion protein that interacts with vinculin. Links actin to plasma membrane, recruits other adhesion proteins and signaling regulators. It is tyrosine phosphorylated. (Shikata et al. 2003)	68
nmMLCK	Ca^{2+} /CaM dependent kinase. Regulated by Ser/Thr/Tyr phosphorylation. Key EC barrier regulatory protein involved in both stress fiber and cortical actin formation, angiogenesis and paracellular gap regulation. (Dudek et al. 2004, Garcia et al. 1993, 2000)	210
Catenins (α , β)	Components of cadherin cell adhesion complex, bind to actin and links actin filaments and cadherins. (Taveau et al. 2008)	80, 88
Caldesmon	Binds both actin and myosin. Serves as a nuclear switch for regulation of actomyosin contraction. Phosphorylation by ERK p38 MAPK and PKC facilitates actomyosin contraction. Involved in MLCK independent mechanisms of stress fiber formation. (Bogatcheva and Verin 2008, Mirzapoiazova et al. 2006)	77
Vinculin	Binds actin at adherens junction site and stabilizes cellular adhesions. Involved in thrombin-induced barrier regulations. Substrate for PKC. (Rüdiger 1998, Ziegler et al. 2006, 2008)	130
ERM	Family of proteins that serves as anchor proteins for actin and plasma membrane, involved in regulation of actin dynamic and signal transduction. (Tsukita and Yonemura 1999, Tsukita and Yonemura 1999, Tsukita et al. 1997)	75-82
c-Abl	Non-receptor tyrosinekinase, contains SH2, SH3, DNA-binding and actin-binding domains. Serves as barrier enhancer. Proto-oncogene. (Welch and Wang 1993)	140

Table 1: Actin-binding proteins involved in endothelial cell barrier regulation.

Modified from Shimizu Y and Garcia JG (2013) Chapter 1: The endothelial cell cytoskeleton: multifunctional role of the endothelial actomyosin cytoskeleton. Endothelial cell cytoskeleton. CRC Press, ISBN 9781466590359

C Lung barrier regulatory signals

i Permeability-inducing agonists

A variety of agonists, cytokines, growth factors, and mechanical forces alter the endothelial cytoskeleton to promote the formation of paracellular gaps between endothelial cells and thereby increasing vascular permeability and transmigration of leukocytes. Each permeability-inducing agonist exhibits distinct features of EC permeability induction and therefore influence pulmonary vascular barrier properties in a wide range of pulmonary diseases such as acute lung injury (ALI), acute respiratory distress syndrome (ARDS), ventilator-induced lung injury (VILI), sepsis, asthma, and ischemia reperfusion (I/R)-induced injury (Becker et al. 2003, Birukova et al. 2004, Dudek et al. 2004, Dull and Garcia 2002, Garcia et al. 1999, 2001, Petrache et al. 2001). For example, the vascular leak response to thrombin occurs very rapidly within minutes and persists for up to 2h (Cohen et al. 1999; Moore et al. 2000), whereas the onset of VEGF-mediated lung endothelial cell permeability is ~2hrs and persists for up to 6hrs (Esser et al. 1998; Fischer et al. 1999). Understanding the molecular mechanisms controlling agonist-mediated effects on the EC cytoskeleton is crucial to the development of novel treatments for the vascular dysfunction seen in inflammatory lung injury.

Thrombin

Thrombin is a coagulation factor that increases inflammation, induces intravascular thrombosis, and causes a transient increase in paracellular permeability (Dudek and Garcia 2001; Mehta and Malik 2006; Coughlin 2000; Lum and Malik 1996). Its effects on endothelial permeability are mediated by its activation and proteolytic

cleavage of proteinase-activated receptor 1 (PAR1) at the extracellular NH₂-terminal domain on ECs (Dudek and Garcia 2001; Mehta and Malik 2006; Coughlin 2000; Tiruppathi et al. 2002). PAR1 is a GPCR that signals through multiple G proteins including G_q, G_i, and G_{12/13} (Mehta et al. 2001; Komarova and Malik 2010; Tiruppathi et al. 2002). G_q activation causes downstream activation of PLC β , which catalyzes the conversion of phosphatidylinositol 4,5 bisphosphate (PIP₂) into IP₃ and diacylglycerol (DAG), which ultimately increases intracellular Ca²⁺ flux into the cells that subsequently increases MLC phosphorylation by Rho-mediated MLC phosphatase inhibition and calcium-dependent MLCK activation (Mehta and Malik 2006), causing actomyosin contraction (Dudek and Garcia 2001; Mehta and Malik 2006). Detailed mechanisms of MLCK and MLC regulation will be discussed later in this chapter. The role of nmMLCK in thrombin-induced vascular permeability has been studied to a great extent. Thrombin-induced cytoskeletal remodeling and gap formation has been shown in a variety of cell types including aortic EC (Fu et al. 2012), microvascular EC (Rigor et al. 2013), pulmonary artery EC (Borbiev et al. 2004; Birukova, Smurova, et al. 2004; Dudek et al. 2002), and CV1 fibroblasts (Wadgaonkar et al. 2003). In endothelial cells, thrombin induces tyrosine phosphorylation of multiple proteins including MLCK and calcium-regulatory proteins and inhibition of tyrosine kinase activities by genistein attenuates increases in cytoplasmic Ca²⁺ caused by thrombin challenge. The downstream effector of Ca²⁺ increase is calcium/calmodulin-dependent protein kinase II (CaM Kinase II), which is activated upon Ca²⁺ binding, and activates nmMLCK, leading to phosphorylation of MLC leading to actomyosin contraction, stress fiber formation, and paracellular gap formation (Dudek and Garcia 2001; Borbiev et al. 2004). Current

understanding of thrombin-induced barrier disruption by nmMLCK-mediated stress fiber formation involves p38 MAPK pathway (Borbiev et al. 2004), ERK1/2, and mTORC2 (Fu et al. 2012).

Vascular Endothelial Growth Factor:

Vascular Endothelial Growth Factor (VEGF) is a pro-angiogenic glycoprotein that is essential for variety of cellular processes including angiogenesis, cell proliferation, and cell migration (McDonald 2001b; Ferrara 2004; Weis and Cheresh 2005; Harper and Bates 2008). VEGF increases EC permeability and was originally named “vascular permeability factor” (VPF) for its profound effects on vascular barrier function that was discovered as a tumor-secreted protein that increased small vein hyperpermeability to circulating macromolecules (Dvorak et al. 1995; Dvorak, Orenstein, et al. 1979; Dvorak, Dvorak, et al. 1979). VEGF/VPF has since been shown to be implicated in a wide range of inflammatory diseases including pulmonary inflammation such as ARDS, ALI, VILI, and asthma (Medford and Millar 2006; Barratt et al. 2014; Thickett et al. 2001; Feltis 200; Kanazawa 2002).

VEGFA is the most abundant protein in the family of platelet growth factors (PLGF)/VEGF and is encoded by *VEGFA* gene located on chromosome 6p21.3. The gene consists of 8 exons separated by 7 introns (Vincenti et al. 1996), in which alternative splicing generates several isoforms; VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆ (made of 121, 165, 189, and 206 amino acids respectively) with less frequent variants being VEGF₁₄₅, and VEGF₁₈₃. VEGF₁₆₅ is the predominant isoform and lacks exon 6. VEGF is a heparin-binding homodimeric glycoprotein of ~45 kDa and has a high affinity for cell surface and the ECM. VEGF₁₆₅ has the highest affinity to heparin thereby

exhibiting the greatest stability in physiological conditions in blood stream and tissues, and thus higher availability compared to the other isoforms. For purposes of clarity, we will refer to the VEGF₁₆₅ isoform as VEGF for remainder of this thesis (Ferrara et al. 2003).

While VEGF plays a critical role during organogenesis (Carmeliet et al. 1996; Ferrara et al. 1996), VEGF expression in the lung persists into adulthood, and remains the most predominant source of VEGF in adults (Monacci et al. 1993; Berse et al. 1992). Exposure of endothelial cells to VEGF results in sustained EC permeability via both the induction of actomyosin contractility and the dissociation of AJs (Dudek and Garcia 2001). Both mechanisms are initiated by VEGF binding to its receptor, receptor tyrosine kinase VEGFR2, also known as KDR or Flk-1, with Kd of ~75–125 pM (Ferrara et al. 2003). The critical role of VEGFR2/Flk-1 in angiogenesis and hematopoiesis has been implicated during development; VEGFR2/Flk-1 null mice exhibit embryonic lethality between E8.5 and E9.5 due to lack vasculogenesis and fail to develop blood islands and organized blood vessels. VEGF ligation of VEGFR2 elicits dimerization and ligand-dependent tyrosine phosphorylation at several tyrosine residues (ie. aa 951, 966, 1059, and 1175) (Ferrara 2004), which further initiates downstream signaling cascades such as phosphorylation of VE-cadherin at Y658 and Y731, internalization of VE-cadherin, disruption of AJs and ultimately the formation of paracellular gaps (Dudek and Garcia 2001; Esser et al. 1998; Lampugnani et al. 2006; Tzima et al. 2005). VEGF also affects phosphorylation of β -catenin, γ -catenin/plakoglobin and p120-catenin. In TJs, VEGF affects phosphorylation status of ZO-1 in a Src kinase-dependent manner (Weis and Cheresh 2005). Furthermore, VEGFR2-integrin interactions alter EC permeability in a

manner dependent upon the type of integrin involved with receptor interaction. For example, integrin $\beta 5$ null mice have little or no effect on permeability whereas integrin $\beta 3$ null mice show increased VEGF-induced permeability (Weis and Cheresh 2005). Additionally, VEGF-increased EC permeability involves a mechanism similar to thrombin with PLC γ activation, subsequent IP3 and DAG production, Ca²⁺ release from ER, and CaM kinase-mediated nmMLCK activation (Bates 2010). Although signal transduction from kinases, phosphatases, cell–cell or cell– matrix adhesion molecules, and physical forces are each required for VEGF stimulated vascular leak, it remains poorly understood how these apparently disparate signaling pathways converge to create the hyperpermeability response to VEGF.

The physiological role of VEGF in lung maintenance and homeostasis, and the role of VEGF has been investigated in the pathogenesis of ARDS (Medford and Millar 2006). Several studies have shown that VEGF is present at higher levels in ARDS patient's plasma compared to non-ARDS patients (Thickett et al. 2001) (Mirzapour et al. 2006), as well as phase-dependent increases of VEGF during the initial stage of ARDS (Azamfirei et al. 2010). Additionally, *VEGF* SNP haplotypes are associated with higher mortality ARDS rates (Debruyne et al. 1990).

A recent study by Song and colleague (Song et al 2012) suggested the source of VEGF during asthmatic inflammation as alveolar macrophages (AM). While it has been considered previously that VEGF in the lung is produced by endothelial cells, epithelial cells, CD4⁺ T cells and macrophages, the study by Song et al demonstrated increased expression of VEGF in asthmatic AM isolated from OVA-challenged *in vivo* murine asthma model compared to AM isolated from wild type control. Additionally, they have

demonstrated attenuation of OVA-induced airway hyperresponsiveness and inflammation in lung by pretreating animals with VEGF antibody or depletion of AM (Song et al 2012). Furthermore, VEGF antibody treatment and AM depletion in OVA-challenged mice inhibit production of IL-4, IL-5, and IL-13, known cytokines increased during asthmatic inflammation, strongly suggesting AM as a primary source of VEGF in asthmatic lung.

Histamine

Histamine is a biogenic amine that is released into the blood stream from mast cells and basophils during the inflammatory response (Komarova and Malik 2010). Similar to thrombin, histamine causes a transient increase in vascular permeability; although with significantly less efficacy. Histamine binds three distinct receptors (H1, H2, and H3), however, EC permeability effects are due to activation of the H1 receptor (Dudek and Garcia 2001; Hill 1990), which causes Src-mediated Tyr phosphorylation of VE-cadherin and β -catenin. It is well established that activation of the H1 receptor by histamine leads to a transient increase in intracellular Ca^{2+} concentration via $\text{PLC}\beta$, ultimately leading to activation of nmMLCK, actomyosin contraction, and paracellular gap formation (78-81). In corneal endothelial cells, inhibition of nmMLCK by the chemical inhibitor ML-7 attenuated histamine-induced MLC phosphorylation and subsequent stress fiber formation (Srinivas et al. 2006). However, additional evidence suggests that an alternative mechanism involving MLC phosphatase (MLCP) may also contribute to histamine effects on vascular permeability (Etter et al. 2001; Kolosova et al. 2004). Histamine was found to induce activation of CPI-17 by phosphorylation of Thr38

downstream of PKC α and PKC δ (Kolosova et al. 2004) with histamine-induced endothelial permeability attenuated by depletion of CPI-17. These data suggest that the effects of histamine on endothelial cell permeability are mediated by effects on both MLCK and MLCP.

TNF- α

Cytokines TNF- α and IL-1 β have a prominent effect early in ALI, causing microthrombosis, and eliciting a cascade of inflammatory signals which result in capillary endothelial production of P-selectin, an adhesion molecule which enhances leukocyte-EC migration (Angelini et al. 2006; Mantovani et al. 1997; Nwariaku et al. 2002) and actin reorganization, and paracellular gap formation (Orfanos et al. 2000). The role of TNF- α in endothelial barrier disruption has been well studied, and is a major contributor in LPS-induced lung and kidney injury (Wu et al. 2009). TNF- α rapidly induces RhoA activation and leads to MLC phosphorylation due to RhoA-mediated activation of Rho-associated kinase (ROCK), which inactivates MLCP and leads to stabilization of actin stress fibers (McKenzie and Ridley 2007). Moreover, TNF- α also increases tyrosine phosphorylation of VE-cadherin leading to increased paracellular gaps in human lung endothelium (Angelini et al. 2006). Evidence exists supporting upregulation of nmMLCK protein expression in epithelial cells in immune-mediated colitis with selective knockdown of TNF- α receptor, TNFR2, attenuating nmMLCK upregulation, MLC phosphorylation, and loss of barrier function (Petrache et al. 2003; Su et al. 2013). We recently showed that TNF- α modulates epigenetic regulation of the *nmMYLK* 3'UTR by miRNA with TNF- α markedly increasing human lung EC luciferase activity of a construct

containing *nmMYLK* 3'UTR (Al-Sadi et al. 2013; Wang et al. 2013; Adyshev et al. 2013; Wu et al. 2009) .

Pathologic Cyclic Stress

Pulmonary ECs are exposed to varying levels of cyclic stretch-mediated mechanical stress due to the inflation and deflation of the alveoli that is particularly relevant to critically ill patients receiving mechanical ventilation. Excessive mechanical stress is implicated in ventilator-induced lung injury (VILI), which, similar to ALI, is characterized by vascular leakiness and inflammation. These mechanical forces can be mimicked *in vitro* by exposing ECs grown on a flexible substrate to a vacuum pressure that is either physiologic (5% cyclic stretch, CS) or pathologic (18% CS). Our group and others have established that ECs preconditioned by exposure to pathologic cyclic stretch demonstrate greater paracellular gap formation with increased gap surface area in response to thrombin that correlated with increased levels of MLC phosphorylation (Letsiou et al. 2014; Shikata et al. 2005). Additionally, our findings were associated with significant changes in gene expression of MLCK and pro-inflammatory genes.

ii Barrier-enhancing agonists

Naturally occurring biological molecules that enhance vascular barrier function are abundant to maintain fluid homeostasis and to restrict the passage of plasma proteins and circulating cells into the interstitium. Diminished availability or decreased activity of these molecules promotes vascular barrier dysfunction thereby causing edema or inflammatory cell infiltration into tissues. Understanding the molecular mechanisms underlying endothelial barrier enhancement and restoration is essential in

order to design therapeutic strategies that retard vascular barrier dysfunction. This process of barrier enhancement and restoration involve dynamic processes of actin polymerization, which allows for the rapid reorganization of actin structures with profound functional consequences for barrier regulation that are highly dependent on the exact spatial location of this actin rearrangement. The vascular barrier recovery after inflammation involves development of a cortical actin ring, the most dense form of actin filament, to anchor cellular junctions and a carefully choreographed gap-closing process via formation of Rac GTPase-dependent lamellipodial protrusions into the paracellular space between activated endothelial cells. Lamellipodia consist of actin-binding proteins such as nmMLCK and cortactin as well as focal adhesion complexes, all of which are crucial for signaling barrier restoration processes via modulation of actin cytoskeleton and cell-matrix and cell-cell adhesion (Dudek and Garcia 2001). Several barrier-protective agents such as sphingosine 1-phosphate (S1P), hepatocyte growth factor (HGF), simvastatin, and shear stress share a common and essential feature where polymerized cortical actin is markedly increased in the cell periphery (Birukova, Smurova, et al. 2004; Jacobson et al. 2004; Liu et al. 2002). Detailed below are several examples of EC barrier-enhancing agents that contribute to lung vascular barrier restoration (Birukov et al. 2004; Finigan et al. 2005; Garcia et al. 1986; Liu et al. 2001; Singleton et al. 2006).

Sphingosine 1-phosphate

S1P is an important bioactive sphingolipid that acts as a potent angiogenic factor, and one which the Garcia lab originally described as a potent barrier-enhancing agent (Garcia et al. 2001; Schaphorst et al. 2003; Dudek et al. 2004a; Jacobson and Garcia

2007). S1P is ubiquitously generated with a high content in platelets and released upon platelet activation (Abbasi and Garcia 2013). S1P binds to 5 G protein-coupled receptor (GPCR) S1P receptor subtypes (S1PR1-5) that are differentially expressed based on cell type. The vascular endothelium expresses S1PR1, S1PR2, and S1PR3 (Garcia et al. 2001; Mehta and Malik 2006) and have extensively studied the effects of S1P on the endothelial cytoskeleton and were the first to demonstrate a dose-dependent enhancement of endothelial barrier function by S1P, as measured by *in vitro* transendothelial electrical resistance (TER) (Garcia et al. 2001). S1PR1 is coupled specifically to Gi whereas S1PR2 and S1PR3 couple to Gi, Gq, and G12/13 (Wang et al. 2011). Two major pathways in which S1P regulates endothelial cell barrier enhancement are Rho and Rac-GTPase dependent pathways. The activation of Rac by S1P is essential for translocating nmMLCK to cortical actins that span the cell periphery and where it co-localizes with actin-associating protein cortactin via direct binding to homology domain 3 on cortactin (Dudek et al. 2004). Upon binding to S1PR1, S1P causes Rac activation, cortactin translocation and MLC phosphorylation at the cell periphery, and FA and AJ rearrangements (Dudek et al. 2004a; Jacobson and Garcia 2007). We have shown that these effects are relevant *in vivo* using pre-clinical mouse models of ARDS, which indicate that S1P enhances vascular integrity and attenuates fluid leak into the alveoli and interstitium (Sun et al. 2012; Sammani et al. 2011). Furthermore, the attenuation of vascular permeability *in vivo* using S1P homologues such as SEW2871 and FTY720-P support the role of S1P as an endothelial barrier-enhancing effector and offers an additional target for the development of therapeutics to treat pulmonary edema (Sammani et al. 2010; Natarajan et al. 2013; Camp et al. 2009).

S1P induces cell membrane raft-mediated signaling events and promotes the recruitment of over 20 cell membrane raft proteins exhibiting increased levels of tyrosine phosphorylation, which include known essential barrier-regulatory proteins such as nmMLCK, focal adhesion kinase, cortactin, and c-Abl (Zhou and Murthy 2004).

Hepatocyte growth factor

HGF is a pro-angiogenic protein that plays critical roles in mitogenesis, organogenesis, cell survival, and angiogenesis (Birukova et al. 2007; Liu et al. 2002) and as we have shown, barrier-enhancing effects on pulmonary ECs (Liu et al. 2002). HGF functions via ligation of its receptor, the tyrosine kinase c-Met, and causes rapid activation of phosphatidylinositol 3'-kinase, ERK, p38 mitogen-activated protein kinase, and PKC, with ultimate downstream effects on the small-GTPase Rac1, which underlie its barrier protective effects (Liu et al. 2002). Specifically, HGF promotes recruitment of c-Met to specialized caveolin-rich plasma membrane domain lipid rafts, along with S1PR1 and integrin $\beta 4$ (ITGB4), which promotes the transactivation of S1PR1 and ITGB4 (Ephstein et al. 2013). The complex cell signaling involved in the effects of HGF on endothelial permeability are still incompletely understood; however this remains an area of active investigation.

Simvastatin

Simvastatin is a hydroxyl-3-methylglutaryl(HMG)-CoA reductase inhibitor that is heavily used clinically in the treatment of hypercholesterolemia for its effects on cholesterol synthesis (Grundy 1988). HMG-CoA reductase mediates prenylation pathways including farnesylation and geranylgeranylation that are required for cholesterol synthesis; however, we have shown that these pathways are also critical in

EC permeability via effects on geranylgeranylation of small GTPases (Chen et al. 2008). Our group initially demonstrated that EC treatment with simvastatin causes cytoskeletal rearrangements with attenuated MLC phosphorylation and stress fiber formation, as well as increased Rac GTPase activity and cortical actin (Jacobson et al. 2004). Subsequent studies in our murine model of LPS-induced ALI confirmed the barrier protective effects of statins and verified the translational relevance of statins as a potential therapeutic strategy for pulmonary edema (Jacobson et al. 2005). This high translational potential has led to intense investigation into the mechanisms underlying the effects of simvastatin on endothelial permeability. This work has indicated that EC treatment with simvastatin induces up-regulation of ITGB4, with downstream effects on multiple signaling pathways, including the mitogen-activated protein kinase (MAPK) and SHP-2 pathways (Chen et al. 2010; W. Chen et al. 2012). Additionally, simvastatin treatment was recently shown to increase claudin-5 expression in ECs, which was associated with membrane translocation of VE cadherin (Chen et al. 2014). Thus, statins appear to have pleiotropic effects on the pulmonary vasculature that involve multiple signaling pathways.

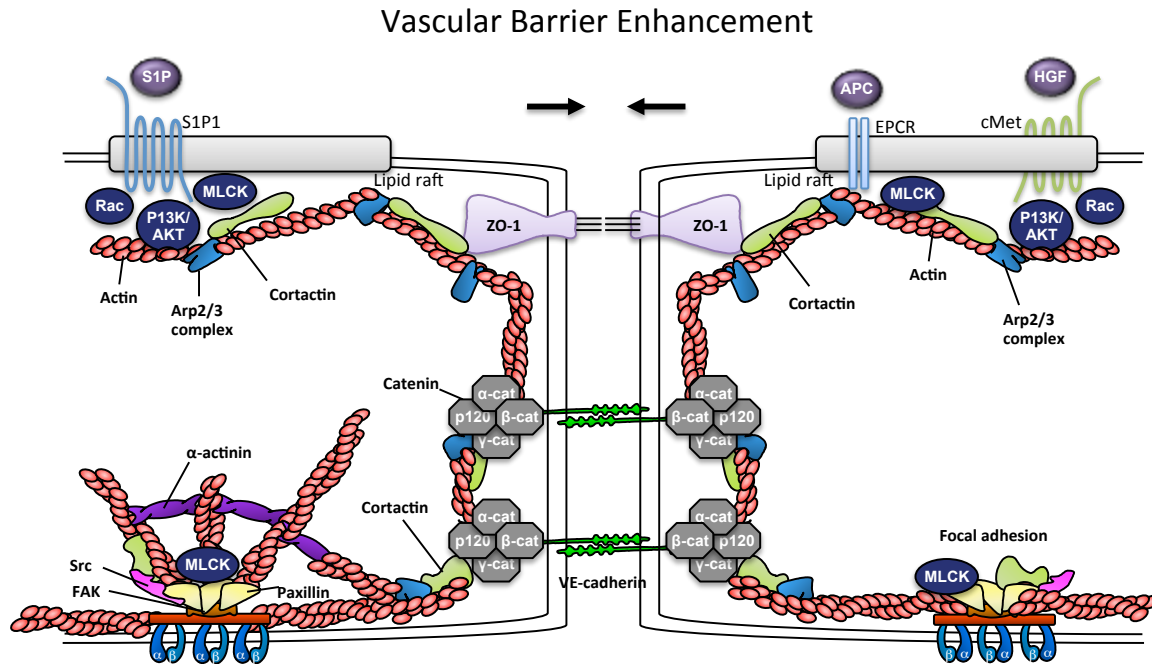


Figure 4: Schematic representation of junction complexes involved in cell-cell and cell-matrix adhesions

Barrier-enhancing agonists activate intracellular signaling pathways that lead to peripheral cortical actin formation, resulting in lung EC barrier enhancement. Taken from Shimizu Y and Garcia JG (2013) Chapter 1: The endothelial cell cytoskeleton: multifunctional role of the endothelial actomyosin cytoskeleton. Endothelial cell cytoskeleton. CRC Press, ISBN 9781466590359

iii Rho family GTPases in actin cytoskeleton rearrangements

Signaling molecules that either increase vascular permeability or enhance vascular barrier integrity do so via small G-protein-activated GTPases. Specifically, when the G α 12/13 subclass of G-proteins is activated by ligand-bound receptor activation, the cytosolic Rho guanine exchange factor (RhoGEF) is allosterically activated, which subsequently activate the Rho GTPases, Rac GTPases, and Cdc42 GTPases, critical regulators of the non-muscle cytoskeleton. These GTPases are intimately involved in cytoskeletal rearrangement and distribution, as well as assembly of intercellular adherens complexes and focal adhesions (FAs) (Wojciak-Stothard and Ridley 2002). For example, S1P-mediated cytoskeletal rearrangement via the GPCR S1PR1, is the key event in S1P-mediated endothelial barrier protection (Garcia et al. 2001). Ligation of cell surface S1P receptors triggers coupling of cytosolic G α 12/13 (Spiegel and Milstien 2003), which then activate Rac GTPase and modulate molecular trafficking to and enzymatic activity at the cell periphery, resulting in peripheral cytoskeletal enhancement and the formation of functional adherens junction complexes.

Previous studies from our group have shown that overexpression of constitutively active Rac enhances peripheral actin polymerization in the cortical ring (Garcia et al. 1986) and induces lamellipodia formation, membrane ruffling, the formation of filaments, and the spreading of ECs (Rodriguez et al. 2003). The role of Rac in barrier enhancement was underscored by inhibition of Rac GTPase, that led to Rho GTPase activation and increased monolayer permeability (Birukov et al. 2004; Dudek et al. 2004; Garcia et al. 1986; Vouret-Craviari et al. 1999). RhoA has been shown to play a different role in cytoskeletal rearrangement upon activation and is responsible for stress

fiber formation (Sah et al. 2000). The primary effector of RhoA is Rho-associated, coiled-coil containing protein kinase (ROCK), an inhibitor of MYPT, leading to stress fiber formation and focal adhesion complex assembly. Consistent with this, overexpression of RhoA leads to increased stress fiber formation (Wójciak-Stothard, Leung et al. 1996) whereas, overexpression of dominant negative mutants of RhoA promotes disassembly of stress fibers in clostridium botulinum (C3) toxin induced actin polymerization (Leung et al. 1996).

D Mechanism of cytoskeletal contractile activation and stress fiber formation

Vascular permeability regulation is a well-orchestrated process in maintaining fluid homeostasis that requires finely tuned responses to various physiological stimuli. Three major and interdependent signaling mechanisms that regulate the increase in paracellular permeability are: c-Src, Ca^{2+} signaling, and Rho GTPase transduction pathways (Komarova and Malik 2010). Activation of c-Src promotes vascular permeability via VE-cadherin-mediated AJ destabilization and actomyosin contraction. Inflammatory agonists such as VEGF, thrombin, and histamine increase cellular Ca^{2+} concentration by store-operated calcium release and extracellular influx and Rho GTPases regulate actin remodeling thereby playing a critical role in vascular barrier enhancement. These processes each affect the function of nmMLCK, a kinase that modulates the properties of actin cytoskeleton. c-Src promotes vascular permeability by direct phosphorylation of nmMLCK, leading to activation of the kinase (discussed further in Chapter IV). Increases in cytosolic calcium via storage operated channels or by IP3 driven mobilization of intracellular Ca^{2+} stores is critical to the development of contractile tension via transcellular actomyosin stress fiber formation, cortical actin ring

disassembly and paracellular gap formation (Kolodney and Wysolmerski 1992; Phillips et al. 1989; Majno and Palade 1961) caused by activation of Ca^{2+} /calmodulin (CaM)-dependent nmMLCK. The Rho family of small GTPases is intimately involved in cytoskeletal rearrangement and the distribution and assembly of intercellular adherens complexes and focal adhesions. Phosphorylation of regulatory myosin light chains (MLC) is primarily catalyzed by MLCK in coordination with the Rho/Rho kinase pathway. Activation of Rho by the guanosine nucleotide exchange factor, p115-RhoGEF, induces phosphorylation of downstream endothelial target MLC phosphatase (MYPT1, Thr686, Thr850), resulting in MYPT1 inactivation and accumulation of dephosphorylated MLC, actin remodeling, and cell contraction (Birukova, Smurova, et al. 2004; Wettschureck and Offermanns 2002).

i Non-muscle isoform of myosin light chain kinase

A key actin-binding protein and central regulator of the EC contractile apparatus is the Ca^{2+} /calmodulin-dependent non-muscle isoform of myosin light chain kinase (nmMLCK) encoded by the human *MLYK* gene located on chromosome 3q21. *MYLK* encodes three MLCK proteins (Figure 3): the nmMLCK isoform, the smooth muscle MLCK isoform (smMLCK, 130-150 kD) and telokin (Garcia et al. 2000; Lazar and Garcia 1999; Potier et al. 1995; Verin et al. 1998). While smMLCK is expressed primarily in smooth muscle tissues, nmMLCK is the predominant form expressed in non-muscle cells such as endothelium (Verin et al. 1998) with a molecular weight of 210kDa (1914 amino acids) compared to smMLCK (130-150kDa) and telokin (17kDa) which retains the C-terminus of both nmMLCK and smMLCK. Endothelial cells respond to Ca^{2+} mobilizing receptor ligands, such as permeability-enhancing thrombin and vascular endothelial growth factor (VEGF), with increased intracellular Ca^{2+} binding to calmodulin (Becker et al. 2003, Garcia et al. 1993). The activated Ca^{2+} /CaM complex interacts with the Ca^{2+} /CaM-binding domain of nmMLCK, resulting in an active conformation and MLC phosphorylation (Thr18, Ser19) which increases actomyosin ATPase activity and shifts the equilibrium between the folded and unfolded myosin forms (Kamisoyama et al. 1994), thus providing the assembling and functioning of the contractile stress fibers.

Smooth muscle smMLCK and nmMLCK are identical in the actin-binding, catalytic, CaM-binding and KRP domains; however, the nmMLCK isoform contains a unique 922 amino acid N-terminal domain, comprising multiple sites for protein-protein interaction (SH2- and SH3-binding domains) as well as potential regulatory phosphorylation sites (Garcia et al. 2000, Lazar and Garcia 1999, Potier et al. 1995,

Verin et al. 1998). For example, we have identified tyrosine residues Y464 and Y471 as p60src phosphorylation sites which upregulate nmMLCK kinase activity (Birukov et al. 2001, Dudek et al. 2010).

The nmMLCK isoform, like the endothelial cytoskeleton in general, is highly multifunctional and serves as an important effector in numerous endothelial processes. In multiple *in vitro* and *in vivo* models of EC permeability, increased MLCK activity produces increased MLC phosphorylation within newly formed stress fibers, paracellular gap formation, cell rounding, intracellular tension, and subsequent EC barrier disruption. Inhibition of nmMLCK attenuates or prevents vascular leak produced by ischemia/reperfusion, neutrophils, TGF β , thrombin, and mechanical stress. (Wu et al. 2009; Borbiev et al. 2004; Adyshev et al. 2013). We have described increased MLC phosphorylation in a cortical distribution during EC barrier enhancement suggesting that spatially-defined MLCK activation can differentially regulate permeability (Garcia et al. 2001). nmMLCK has been implicated in TNF-induced endothelial cell apoptosis, mechanotransduction, and calcium signaling.

ii Alternatively spliced *MYLK* variants.

There are four known *MYLK* splice variants of nmMLCK: nmMLCK1, nmMLCK2, nmMLCK3a, nmMLCK3b, with the existence of a fifth variant, nmMLCK4, strongly implicated. The MLCK1 and MLCK2 variants differ in the display of a single exon 11 in MLCK1 that is deleted in MLCK2 (nucleotides 1428- 1634, 69 amino acids) (Figure 5). In human lung endothelial cells, expression of MLCK2 is comparable to MLCK1 whereas MLCK1 accounts for 97% of MLCK in human GI epithelium. The 69 amino acid

deletion (exon 11) in MLCK2 contains a critical regulatory domain for enzymatic activity via phosphorylation of Tyr464 and Tyr471 residues. Other splice variants lack various domains of the full-length proteins encoded by exon 30 such as MLCK3a (nucleotides 5081-5233), MLCK3b (nucleotides 1428-1634, 5081-5233), and MLCK4 (nucleotides 4534-4737).

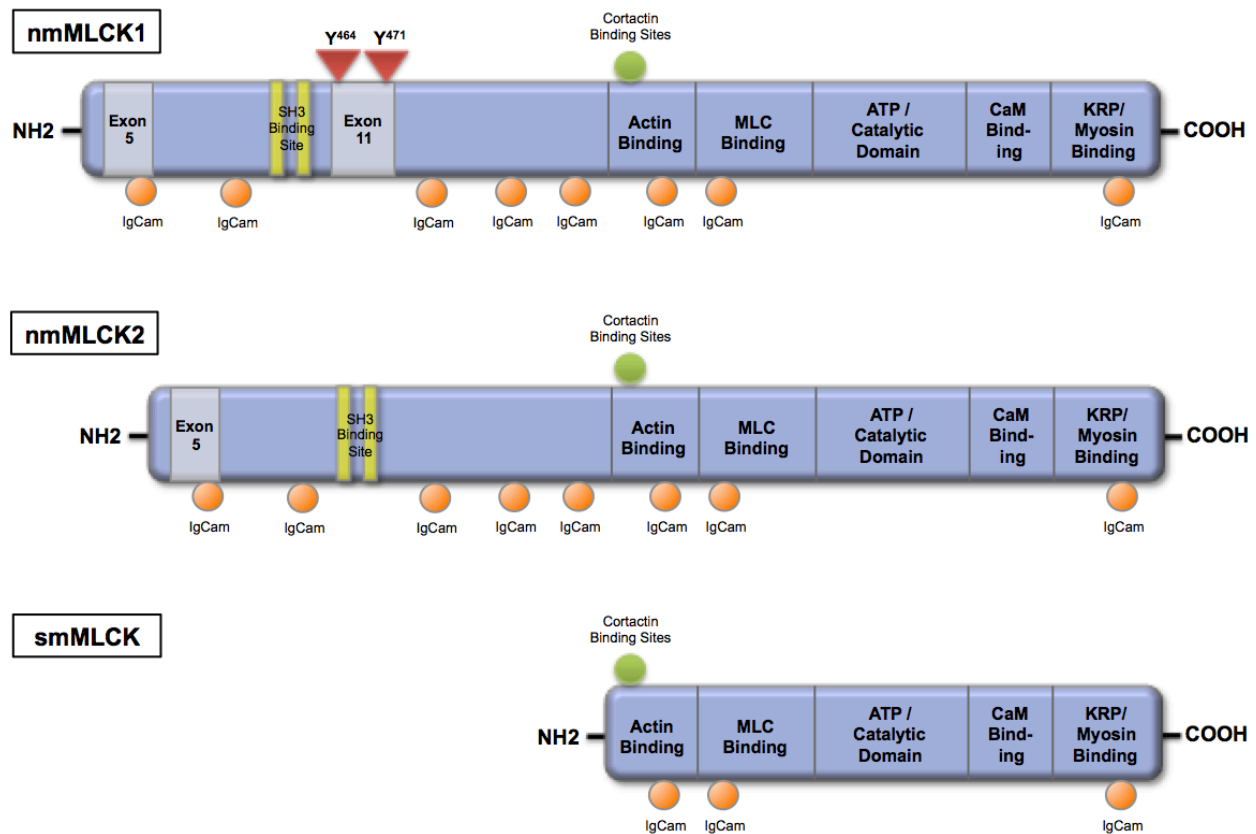


Figure 5: Representation of MLCK isoforms and splice-variants

Protein domain structure of nmMLCK1 (1914aa), nmMLCK2 (1845aa), and the smMLCK isoform (992aa). nmMLCK1 contains SH2- (aa59, aa464) and SH3-binding motifs (aa314-318, aa373-379, yellow bars). Depicted are post-translational modification (PTM) sites (red triangles = novel c-Abl sites, (Zhao et al. 2009), yellow=p60Src sites, (Garcia et al. 1999). Exon 11 (aa 437-506) is lost by alternative splicing to generate nmMLCK2 and thus does not exhibit Y464 and Y471 sites for p60Src & c-Abl-mediated phosphorylation. Also depicted are 8 coding SNPs (orange circles). For amino acid 147, Pro147 is the ancestral allele but Ser147 is the major allele. nmMLCK 2 lack exon 11, and smMLCK consists of exon17-34.

E Involvement of nmMLCK in pulmonary inflammatory diseases

The Garcia lab has previously shown that nmMLCK knockout mice as well as mice treated with an inhibitory peptide to reduce MLCK activity, are protected against ventilator-induced lung injury (Mirzapourzadeh et al. 2006). In addition to *in vivo* transgenic studies, we have previously linked genetic variations (single nucleotide polymorphisms; SNPs) in the *nmMYLK* gene to the severity of asthma. Specifically, our group has demonstrated that SNPs are present in the MYLK gene coding region or 5' UTR, and are associated with asthma susceptibility and severity (Flores et al. 2007). Moreover, the SNP frequency is noticeably higher in African American descent individuals compared to European American individuals. Therefore, the racial disparity in the prevalence and severity exist for asthma pathology. Moreover, studies from our group have also shown that SNPs in *MYLK* confer significant susceptibility to sepsis, and sepsis- and trauma-induced ALI (Gao et al. 2006) in African Americans (Flores et al. 2007).

In effort to examine the potential of targeting nmMLCK in therapeutic scenario, the Garcia lab has also demonstrated the presence of microRNAs (miRNAs) binding to *nmMYLK* 3'UTR, where specific miRNAs can target *nmMYLK* to attenuate TNF α induced nmMLCK expression.

i Genetic variations of *nmMYLK* gene and association with diseases

A variety of our published studies (Tremblay and Slutsky 2006; Shah et al. 2010) have highlighted *MYLK*/nmMLCK as an attractive candidate gene and protein involved in the pathobiology of ARDS. For example, we sequenced *MYLK* in European descent

(ED) and African descent (AD) ARDS subjects and identified population-specific MYLK SNPs that were significantly associated with development of sepsis- and trauma-associated ARDS, thus strongly confirming MYLK as an ARDS candidate gene, particularly in ADs (Lee and Slutsky 2001; Garcia and Sznajder 2013). Our group has confirmed that increased MYLK expression is induced by proinflammatory cytokines and by mechanical stress via transcription activators that bind mechanical stretch response elements (MSRE) (Schnitzer 1992). While the precise regulatory mechanisms of nmMLCK expression are poorly understood, recent preliminary data highlighted the role of epigenetic regulation via DNA methylation within a novel CpG island residing in the non-muscle MYLK promoter (nmMLCK) and our recent publication defined epigenetic regulation of *MYLK* expression via specific microRNAs (Tiruppathi et al. 1997).

F Conclusion

Vascular permeability regulation is a well-orchestrated process in maintaining fluid homeostasis that requires finely tuned responses to various physiological stimuli. Endothelial cells form a tight cell monolayer of the inner lining of the vessel wall representing the key gatekeeper in separating bloodstream components and the tissue interstitium (Komarova and Malik 2010). Accordingly, a failure to maintain such regulation leads to detrimental inflammation in lung and contributes to the untoward outcomes in conditions such as, ALI, ARDS, RILI, and asthma.

Cytoskeletal components are involved in a wide range of biological processes critical for endothelial cell function. Our group and others have demonstrated the

multifunctional role of endothelial actomyosin microfilaments with emphasis on vascular barrier regulation. The association of actin filaments with myosin, a key motor protein, followed by nmMLCK-mediated phosphorylation of the myosin subunit MLC, is essential for myosin conformational changes leading to functional contractile activity and stress fiber formation that in turn lead to paracellular gap formation. Increase in stress fiber assembly is signaled by permeability-increasing factors such as inflammatory mediators and cytokines, which allow cells to make necessary response to outside stimuli. In contrast, vascular barrier enhancement is regulated by nmMLCK-mediated, cortactin-dependent distribution and polymerization of actin at the cell periphery in the cortical ring induced by barrier-enhancing factors such as S1P. A critical mediator in this balance is nmMLCK, a common gateway by multiple signaling, that distinguishes functional output by its spatial specific localization and phosphorylation. This tightly regulated balance between vascular permeability and barrier enhancement becomes impaired in a wide range of pulmonary diseases including ALI/ARDS, VILI, sepsis, asthma, and I/R-induced injury. The precise mechanisms of vascular barrier regulation remain elusive. Deeper understanding of the mechanisms underlying the role of actomyosin in vascular barrier regulation will provide novel therapeutic targets in inflammatory injuries.

Chapter II: Materials and Methods

Mouse models

Male C57BL/6 mice (8–12 weeks of age; Jackson Laboratories, Bar Harbor, ME) were housed in an environmentally controlled animal facility at the University of Illinois at Chicago for the duration of the experiments. All animal procedures were performed under the principles for laboratory animal research outlined by the Animal Welfare Act (1966) and the National Institutes of Health guidelines for the experimental use of animals (Institute of Laboratory Animal Resources 1996), as well as the common guideline of the Institutional Animal Care and Use Committee (University of Illinois, Chicago, IL). All procedures were designed to treat the animals humanely and with regard for the alleviation of suffering.

Two of the genetically engineered mouse lines, nmMLCK^{-/-} and nmMLCK^{ec/ec} were generated as previously described (Rossi et al. 2007; Wainwright et al. 2003; Moitra et al. 2008). Briefly, nmMLCK^{-/-} was generated by inserting neomycin resistance gene (neo) into exon 8 that is only present in non-muscle isoform of MLCK. The neo insertion contains stop codon that terminates translation of nmMLCK protein. Transgenic mice nmMLCK^{ec/ec} that overexpresses nmMLCK2 specifically in endothelial cells was generated by inserting FLAG-tagged human *nmMYLK2* gene fused with mouse VE-cadherin promoter in the genome of C57BL/6.

Mouse asthma model: OVA challenge

Ovalbumin (OVA) administration was utilized to induce experimental asthma in mice as previously described (Ewart et al. 2000) and measured indices of allergic inflammation

and airway responsiveness. Briefly, On Day 0, 6- to 8- month-old C57BL/6 mice (25–30 g) were sensitized intraperitoneally with 20 mg chicken egg OVA (crude grade IV; Sigma, St. Louis, MO) mixed with 1mg alum in 0.2 ml calcium and magnesium-free phosphate-buffered saline (PBS) or an equivalent amount of PBS alone. The same 20 mg sensitization was repeated on Day 7. On Day 14, mice were challenged with 30 mg/kg OVA in 50 μ l PBS or PBS alone. On Day 17, 3 days after OVA challenge, airway hyperresponsiveness (AHR) was determined. Animals were sacrificed for bronchoalveolar lavage (BAL) fluid extraction and tissue harvesting.

Mouse RILI model

Mice were anesthetized with ketamine (100 mg/kg) and acepromazine (1.5 mg/kg) and administered radiation (10-25 Gy) to the thorax as described previously (Mathew et al). Briefly, a 5 mm thick lead block was used to shield the rest of the animal while the thorax, between the clavicles and below sternum, was irradiated with 250 kV x-ray beam at a dose rate of 2 Gy/min using an orthovoltage animal irradiator. Each experimental group consisted of 10 mice irradiated to a single dose of 10 and 20Gy. The variation of the dose delivered within the lung was estimated to be within \pm 5% of the prescribed dose using thermoluminescence dosimeters. Select mice were treated with MLCK inhibitor (ML-7 and PIK) 0.25/gm body wt via intraperitoneal injection 3x/week beginning 1 week prior to irradiation and continuing for a period up to 6 weeks afterwards. Mice were then sacrificed and indices of lung vascular leak and inflammation assessed via BAL fluid protein levels and cell counts at 4-6 weeks as previously described (Wang et al. 2008). Lungs were harvested and stored at -80° C for histologic evaluation.

Airway Responsiveness Measurements (APTI) measurements

Airway reactivity was estimated as the time-integrated change in the peak pressure, referred to as the airway pressure time index (APTI). We assessed airway responsiveness to intravenously administered acetylcholine (ACh) as previously described (Wang et al. 2008). Briefly, Mice were anesthetized by ip ketamine (150 mg/kg) and acetylpromazine (15 mg/kg). Once surgical anesthesia was established, a tracheotomy was performed and a 19-gauge stainless steel cannula was inserted into the trachea. Animals were then paralyzed with pancuronium bromide (4 mg/kg, ip) and placed on a 37°C heating pad, where the cannula was connected to a computer-controlled ventilator. Ventilation was maintained at a rate of 120 breaths/min and a tidal volume of 9 mL/kg, with the heart rate monitored (PowerLab System, AD Instruments, Colorado Springs, CO) to ensure proper anesthetic depth. ACh (1–10 mg/kg) was injected into the inferior vena cava, and changes in airway pressure were recorded for 5 min, followed by calculation of the relative airway pressure time index (APTI)—the percentage increase of APTI within 5 min—to quantify airway responsiveness induced by ACh in various animal groups (Ewart et al. 2000; Grinnan et al. 2006; Wills-Karp et al. 1998).

BAL protein and cell differentiation analysis

At the termination of each experiment, animals were sacrificed in accordance with institutional guidelines for animal care and use. BAL was performed as previously described (Garcia et al. 1989) through the tracheal cannula (after airway hyperreactivity measurements). After the administration anesthesia, bronchoalveolar lavage (BAL) was performed by an intratracheal injection of 1 ml of Hank's balanced salt solution followed

by gentle aspiration. A small aliquot of the BAL fluid from each lobe was taken for cell differential counts and total cell number. Differential cell counts were determined from cytocentrifuge preparations (Cytospin II; Shandon-Southern Instruments, Sewickley, PA), which were stained with a modified Wright-Giemsa stain (Harleco Diff-Quik®; Dade Diagnostics, Aquada, PR). At least 300 cells were counted to enumerate the percentages of macrophages, lymphocytes, neutrophils, and eosinophils present in BAL fluids using morphologic criteria. Total BAL white cell counts were obtained using a hemacytometer, and total numbers of BAL macrophages, neutrophils, and eosinophils were obtained by multiplying the cell differential for each animal. The recovered fluid was also processed for protein concentration (BCA Protein Assay Kit; Pierce Chemical Co, Rockford, IL) and cell count with differential as we described previously (Wang et al. 2008).

Lung histopathology

Murine lungs were excised and the left lung immersed in 10% formalin for 48 hours, washed with 70% ethanol, dehydrated, and embedded in glycol methacrylate. In addition, paraffin-embedded sections were stained for several markers of airway inflammation and remodeling: hematoxylin and eosin for evidence of inflammatory injury in peribronchial and perivascular; periodic acid-Schiff (PAS) for identification of mucus-containing goblet cells; and antisera (EMBP [S-16]: sc-33938; Santa Cruz Biotechnology, Santa Cruz, CA) to major basic protein for determination of lung eosinophil content. Two sections (5–6 mm) from each experimental animal were stained and quantified.

Endothelial cell culture

Human pulmonary artery endothelial cells (HPAECs) were obtained from Lonza (Walkersville, MD). HPAECs were cultured in complete Endothelial Cell Growth Medium Kit (EGM-2 MV, Lonza) supplemented with 10% (vol/vol) FBS and Bullet Kit (Lonza) containing hEGF, hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), VEGF, hFGF-B, R3-IGF-1, and ascorbic acid. Endothelial cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂-95% air and grown to contact-inhibited monolayers with typical cobblestone morphology. Cells from each primary flask were detached with 0.05% trypsin, resuspended in fresh culture medium, cultured in T75 flask to passage 6 to be used for experiments.

Transendothelial Monolayer Electrical Resistance

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 M Ω resistance to approximate a constant current source (~ 1 μ A). 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 (R_0). 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 (32). 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 \pm SE of the mean.

Human recombinant (hr) VEGF reconstitution

Human rVEGF165 was obtained from Sigma-Aldrich, and reconstituted in sterile PBS at 10 μ g/ml concentration. Reconstituted rVEGF165 was stored at -80 °C.

mRNA extraction and cDNA synthesis

mRNA was isolated from cells or mouse lung tissues using Maxwell 16 total RNA isolation kit (Promega Corporation, Madison, Wisconsin). Cultured HPAECs were washed twice with ice-cold PBS and trypsinized with 0.25% trypsin, and collected in Eppendorf tube. Cells were then centrifuged at 800 rpm for 5 min at 4 °C, and pellets were used immediately for RNA isolation or frozen in liquid nitrogen for later use. Harvested lung tissues were frozen and stored in liquid nitrogen until needed for RNA isolation. 50 mg of frozen tissues and 350 μ l of Lysis Buffer were mixed and tissues were homogenized on ice using homogenizer. Lysates were centrifuged at 14,000 rpm for 5 min, and supernatant was used for RNA isolation. Total RNA was isolated using protocol provided by manufacture. The concentration of isolated total RNA was measured using NanoDrop (Thermo Fisher Scientific; Waltham, Massachusetts). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California). 300-1000 ng of RNA was used to synthesize cDNA to be used for qPCR for further quantification analysis.

Quantitative PCR

mRNA expression was measured using quantitative PCR (qPCR) where 1 µl of diluted or undiluted cDNA was mixed with 2x SYBR Select Master Mix (Applied Biosystems, Foster City, California) and 500 nM forward and reverse primers. qPCR reaction was performed using iCycler (Biorad Laboratories, Inc., Hercules, California). qPCR was performed with the following protocol: 50 °C 2 min, 95 °C 2 min, 40 cycles repeat of 95 °C 15 sec and 60 °C 1 min.

Primers for gene expression quantification

Human <i>nmMYLK</i> Forward	CAGCCTTGTGATTCATGCTGTC
Human <i>nmMYLK</i> Reverse	ACCAAGCTGCTTCGCAAAAC
Human Sp1 Forward	CCTCACTGAAGCTGGGTAGC
Human Sp1 Reverse	GCTGCGACCTTTCTTTCATC
Human HNF1 Forward	AATGCGGTGGAACTTCTTG
Human HNF1 Reverse	TCAGCAGAGCAAGAAAAGCA
Human HPRT Forward	AAGCCAGACTTTGTTGGATTT
Human HPRT Reverse	GGCTTTGTATTTTGCTTTTCC
Mouse HPRT Forward	AGGCCAGACTTTGTTGGATTT
Mouse HPRT Reverse	GGCTTTGTATTTGGCTTTTCC

Primers for ChIP quantification

GAPDH Forward	TACTAGCGGTTTTACGGGCG
GAPDH Reverse	TCGAACAGGAGGAGCAGAGAGCGA

<i>nmMYLK</i> Region 1 Forward	CACGCACCAAGACAGTACCA
<i>nmMYLK</i> Region 1 Reverse	CCCTCGCACTTATCCTGCAC
<i>nmMYLK</i> Region 2 Forward	AGGTTTGGCTGCGGATTCTC
<i>nmMYLK</i> Region 2 Reverse	CATTGTCATAGCGCTCGGG

Western Blot

Cultured HPAECs were washed with ice cold PBS, scraped, placed into Eppendorf tube, and centrifuged at 10,000 rpm for 5 min at 4°C. The cell pellets were resuspended in 100 µl of ice-cold RIPA buffer (Thermo Fisher Scientific; Waltham, Massachusetts) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail II and III (Sigma-Aldrich; St. Louis, Missouri). Cells were incubated in RIPA lysis buffer for 15 min on ice with vortexing for 30 sec every 5 min, and the lysates were then centrifuged at 14,000 rpm for 15 min at 4°C. The pellet was discarded. The supernatant was taken to determine the protein concentration by Bradford Protein Assay (BioRad; Hercules, CA) using BSA as a standard. Proteins (10-20 mg) were mixed and boiled in sample buffer (Invitrogen; Carlsbad, California) supplemented with 2-mercaptoethanol (BME, Sigma) reducing agent. Protein lysates were resolved on NuPAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Invitrogen; Carlsbad, California) and transferred onto 0.2 µm pore-size Polyvinylidene Difluoride (PVDF) membranes (BioRad) 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 1 h at 22–24°C in a blocking buffer (0.1% Tween 20 in TBS, (TBST)) containing 5% nonfat dry milk powder or BSA. The membranes were then incubated with primary antibodies diluted in TBST containing 5% milk or BSA, shaking overnight at 4°C. Membranes were

washed three times in TBST for 5 min each, followed by incubation in secondary antibody conjugated to horseradish peroxidase for 2 hrs at room temperature in TBST containing 5% milk or BSA. Membranes were washed 3 times for 5 min each and peroxidase activity was visualized with enhanced chemiluminescence substrate (Thermo Fisher Scientific; Waltham, Massachusetts). Primary antibodies included: anti-Akt (1:1000, Cell Signaling Technology, Inc. Danvers, Massachusetts, 4691S), pAkt S473 (1:1000, Cell Signaling, 4058S), eNOS (1:1000, Cell Signaling, 9586S), p-eNOS S1177 (1:1000, Cell Signaling, 9571S), Src (1:1000, Cell Signaling, 2108S), p-Src Y416 (1:1000, Cell Signaling, 2101S), non-phospho Src Y416 (1:1000, Cell Signaling 2102S), VEGFR2 (1:1000, Cell Signaling, 9698S), p-VEGFR2 Y951 (1:1000, Cell Signaling, 2991S), p-VEGFR2 Y996 (1:1000, Cell Signaling, 2474S), p-VEGFR2 Y1059 (1:1000, Cell Signaling, 3817S), p-VEGFR2 Y1175 (1:1000, Cell Signaling, 3770S), nmMLCK (1:500, Santa Cruz Biotechnology, Inc.; Santa Cruz, California, A20), nmMLCK (1:500, Santa Cruz, A8), Sp1 (1:1000, Santa Cruz, 17824). Secondary antibodies include: β -actin horseradish peroxidase (HRP)-conjugated (A3854, Sigma), GAPDH HRP-conjugated, Donkey anti-goat HRP (1:5000, Santa Cruz, 2020), Goat anti-mouse HRP (1:5000, Santa Cruz, 2005), Goat anti-rabbit HRP (1:5000, Santa Cruz, 2004). Band intensity was quantified with ImageJ, normalized to b-actin or GAPDH control, and expressed as arbitrary units.

Generation of *nmMYLK* promoter plasmids

The promoter region of *nmMYLK* was synthesized by GenScript Co. (Piscataway, NJ), and cloned to pGL3-Basic vector (Promega). Precisely, this region of DNA included 2515 bp of sequence upstream of *nmMYLK* (NM_053025.3) located on

chromosome 3q21 containing 2431 bp of 3' UTR, 97 bp of exon 1, and 7 bp of intron 1, and cloned into pGL3-Basic vector containing *amp^R* gene at multiple cloning site between Mlu 1 and Xho 1. To create promoter deletion constructs, series of primers were designed to amplify the each length (-2441, -2111, -1751, -1471, -1211, -831, -420, -271, -220, -118, TSS) and subcloned into pGL3-Basic vector. Plasmids were then transformed into DH5 α , and plated on LB agar plate containing 100 μ g/ml of ampicillin and grown overnight at 37°C. Individual clones were then amplified in LB media overnight in shaking incubator at 37°C and the plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen), and the inserted DNA was sequenced at UIC DNA Core Facility (Chicago, IL). Once the sequences are verified, plasmid were transfected into DH5 α and grown in 500 ml LB/ampicillin overnight while shaking, and plasmids were purified using EndoFree Plasmid Maxi Kit (Promega). DNA concentration was measured using Nanodrop.

Luciferase promoter assay

All constructs were transfected into HPAECs using Xfect Transfection Reagent (Clontech, Mountain View, California). Cells were cultured to 70-80 % confluency in 6-well plate, and media was replaced with fresh EGM2-MV prior to transfection. 5 μ g *nmMYLK*-Luc or pGL3 control plasmid and 0.05 μ g hRL-TK renilla constructs were transfected according to manufacture's protocol. After 24 hours of transfection, media was removed and replaced with EBM2 containing 0.5 % FBS. After 4 hours of serum starvation, rVEGF165 was added. At the end of the time course, cells were washed with ice-cold PBS for three times and cells were lysed in ice-cold Passive Lysis Buffer (Promega) and collected in eppendorf tubes. Cells were then further lysed in three

cycles of freeze-thaw process. Lysates were centrifuged at 14,000 rpm for 5 min at 4 °C, and supernatant was used to assess the luciferase activity using Dual-Luciferase Reporter Assay System (Promega).

In silico promoter analysis

Genomatix software (www.genomatix.de, Munich, Germany) was used to predict transcription factor binding motif on 2.5kb of *nmMYLK* promoter. The pre-defined transcription factor binding modules were used to predict transcription factor bindings (ModellInspector ref: PMID 9245596, 10222404). These modules in the promoter are determined by the algorithm defined by Frech et al. (Frech et al. 1997) and Klingenhoff et al. (Klingenhoff et al. 1999) that are part of The Promoter Module Library (Genomatix). In regulatory units such as promoters, enhancers or silencers contain functional modules that consist of at least two transcription factor binding sites in conserved order that are separated by a spacer. The module components consist of binding factor, sequential order, matrix similarity, standard orientation, and distance range. The Promoter Module Library is based on a compilation of those elements of multiple transcription factor binding sites, which have been verified experimentally for their function on promoter activity. The list of predicted transcription factors were further sorted out and selected for predicted sites with matrix similarity of > 0.889.

siRNA silencing

All siRNA were purchased from Dharmacon, Inc. (Lafayette, Colorado, USA)

siRNA nmMLCK GGAAAGAGGUGACCAAUGUUU

siRNA Sp1 TARGETplus siRNA, Human SP1 (6667)

siRNA Sp3 TARGETplus siRNA, Human SP3 (6670)

siRNA HNF1 TARGETplus siRNA, Human HNF1

Immunofluorescent staining and confocal microscopy

The CMV-promoter-driven EGFP-tagged nmMLCK1 mammalian expression vector was constructed as previously described (Brown et al. 2010). The construct containing EGFP-nmMLCK1 was transfected into HPAECs using Xfect Transfection Reagent (Clontech, Mountain View, California). Cells were cultured to 70-80 % confluency in 12-well plate containing glass coverslip, and media was replaced with fresh EGM2-MV prior to transfection. 5 µg of plasmid was transfected according to manufacture's protocol. After 24 hours of transfection, media was removed and replaced with EBM2 containing 0.5 % FBS. After 4 hours of serum starvation, rVEGF₁₆₅ was added, and coverslip was removed from the tissue culture plate and washed with ice-cold PBS for three times.

Coverslips were dipped in sterile D-PBS and transferred to wells containing 1 ml of 4% paraformaldehyde/PBS, pH 7.4. Following fixation for 20 min at room temperature, cells were blocked and permeabilized in 0.25% fish skin gelatin/0.01% saponin/0.1% NaN₃/PBS, pH 7.4 (blocking solution), for 30 min at RT. After blocking, actin was stained by incubating the cells in 5 U/ml phalloidin-rhodamine (Life Technologies) /blocking solution for 30 min at RT in the dark. Finally, cells were washed ×3 for 5 min each in blocking solution, then mounted in 10 µL of Prolong Gold with 4, 6-diamidino-2-phenylindole (DAPI) and cured overnight at RT in the dark. Blue fluorescence emitted at 461 nm was used to identify nuclei and red fluorescent emitted at 565 nm was used to display actin filaments. EGFP-nmMLCK1 was visualized with green fluorescent

emitted at 509 nm. Fluorescence was captured by a charge-coupled device camera connected to a fluorescence microscope with a 40x objective lens.

Immunoprecipitation

Cells were grown to confluence in 60 mm dishes as described above. Cells were then treated with either 100 ng/ml VEGF or PBS for 5, 30, or 60 min. Immunoprecipitation was performed as previously described (Belvitch et al. 2014). Briefly, cells were washed three times with ice-cold PBS and lysed with 0.6 ml per dish of IP lysis buffer (20 mM Tris-HCL; 150 mM NaCl; 1% NP-40; 2 mM EDTA; 0.5 mM PMSF; 0.2 mM sodium orthovanadate; 0.5% Protease and 0.5% phosphatase inhibitor cocktail) (Calbiochem). Cells were scraped and allowed to incubate for 15 min on ice. Cell lysates were passed through a 26 G needle 10 times and centrifuged for 10 min at 15 K rpm at 4 °C. Anti-nmMLCK pY464 antibody was added (1:120) and incubated for overnight while rotating at 4 °C. The following day, 50 µl of protein G beads (Life Technologies) was washed three times with Co-IP wash buffer (20 mM Tris-HCL; 150 mM NaCl; 1% NP-40; 2 mM EDTA; 2 mM EGTA; 0.2 mM sodium orthovanadate; 20 mM NaF; 1 mM sodium phosphate). Lysates were then added to beads and rotated at 4 °C for 1.5 h. Beads were washed three times with IP wash buffer and once with 20 mM Tris, pH 7.5. Protein was eluted off the beads with 90 µl 2 × Laemlli sample buffer containing 2-β-mercaptoethanol [1:20] and boiled for 5 min at 100 °C.

Statistical Analysis

For all data reported, values are reported as means. We performed statistical comparisons among treatment groups by randomized-design, two-way ANOVA,

followed by the Newman-Keuls post hoc test for more than two groups, or by an unpaired Student's t test for two groups. In all cases, we defined statistical significance as a P value less than 0.05. The statistical analysis was done using OriginPro 8.1 software (OriginLab Corp., Northampton, MA).

Chapter III: Increased nmMLCK expression via inflammatory signaling

A Overview

As noted earlier, the pulmonary vasculature comprises a vast surface area with the pulmonary endothelium functioning as a semipermeable cellular barrier between the vascular compartment and the interstitium. Consistent with the dynamic process of barrier regulation, increased vascular permeability and accumulation of airway and alveolar fluid are cardinal features of lung inflammatory diseases that include acute lung injury (ALI) and the severe form acute respiratory distress syndrome (ARDS), radiation-induced lung injury (RILI), and asthma. The dynamic cytoskeletal rearrangements are major drivers to the regulation of the lung vascular barrier and to maintenance of lung fluid balance.

The recognition of nmMLCK as a central component in the gate-keeping properties of the endothelial cell barrier (Dudek and Garcia 2001) has led to hypothesis that nmMLCK influences both the induction of lung inflammatory processes as well as participates in resolution from these events (Garcia et al. 1995; Garcia et al. 1996; Dudek and Garcia 2001). nmMLCK is involved in vascular barrier integrity by regulating actin cytoskeleton rearrangement and contraction in Ca^{2+} /Cam-dependent manner. Phosphorylations of MLC at T18 and S19 by nmMLCK modulate assembly of actin monomers into polymers called stress fibers, and facilitates development of the contractile phenotype.. The critical role of nmMLCK during vascular barrier regulation was highlighted *in vivo*, when nmMLCK^{-/-} mice were shown to be protected from acute lung injury induced by LPS (Mirzapoiazova et al. 2011), a representative model for increased lung inflammation and increased pulmonary vascular permeability; in addition,

genetically engineered nmMLCK^{ec/ec} transgenic mice overexpressing the nmMLCK in the vascular endothelium show increased LPS-induced lung injury (Moitra et al. 2008). These *in vivo* studies underscore the critical role of nmMLCK in various inflammatory diseases.

In addition to *in vivo* transgenic studies, our group has previously linked genetic variations (single nucleotide polymorphisms; SNPs) in the *nmMYLK* gene to the severity of asthma. Specifically, we have demonstrated that SNPs are present in the *MYLK* gene both the coding region and 5' UTR and are associated with asthma susceptibility and severity (Flores et al. 2007; Han et al. 2012). Moreover, the SNP frequency is noticeably higher in African descent individuals compared to European descent individuals, a finding with implications for the racial disparity that exists in the prevalence and severity of asthma pathology.

Several studies have shown that VEGF, one of the known agonists that modulate vascular permeability, is present at higher levels in ARDS patient's plasma compared to non-ARDS patients (Thickett et al. 2001), as well as phase-dependent increase of VEGF during the initial stage of ARDS (Azamfirei et al. 2010). Additionally, the *VEGF* SNP haplotype (-460T,+405C,+936T) has been associated with increased ARDS mortality rate (Zhai et al. 2007), whereas, the haplotype (-460C+405G+936T) was associated with lower plasma VEGF levels and reduced mortality, again suggesting that VEGF plays a critical role in ARDS disease severity.

Additional evidence for a role of VEGF in asthma include clinical reports measuring levels of cytokines in blood and sputum samples from asthmatic patients who have demonstrated increased VEGF bioavailability (Feltis et al. 2006; Kanazawa

2002). Moreover, the importance of VEGF in asthmatic outcomes has been validated using a murine model of ectopic human VEGF expression with OVA sensitization that produced profound increases in leukocyte infiltration, airway edema, and vascular remodeling. These results suggest that VEGF exacerbates asthmatic inflammatory responses (Lee et al. 2004).

In spite of the vital role of nmMLCK in vascular barrier regulations, and the implication of both nmMLCK and VEGF in pathogenesis of several pulmonary inflammatory diseases such as ALI/ARDS and asthma, **the exact role of nmMLCK in VEGF-induced vascular permeability remains poorly understood and was the focus of the graduate work presented in this thesis.** I examined nmMLCK mRNA and protein levels in lung tissues taken from ovalbumin (OVA)-challenged asthmatic mice, and a gamma-irradiated murine lung injury model of radiation-induced. Lung injury. Bronchoalveolar lavage (BAL) fluid protein levels and cell count were examined to determine the extent of permeability and inflammation, as well as airway pressure time index (APTI) as a reflection of airway hyper-reactivity. To examine the molecular mechanisms controlling nmMLCK expression, I used *in vitro* VEGF treatment of human lung endothelium to study protein levels, mRNA levels, as well as transcription factor activity at the promoter level.

B nmMLCK expression is increased in asthmatic and radiated lungs

Increased vascular permeability and inflammation are common features of lung diseases such as ALI/ARDS, RILI, and asthma. Common pathological features of the bronchi of asthmatic patients are smooth muscle hypertrophy, tissue remodeling and a hypersensitivity to contraction. Whereas the *MYLK* gene encodes several isoforms, previous asthma studies have largely focused on the role of the smMLCK isoform with only limited information on the role of nmMLCK in asthma. Among these studies was a report from our group which demonstrated the coding SNP Pro147Ser, a SNP almost entirely restricted to African descent individuals, to be significantly associated with severe asthma in African Americans (Flores et al 2007).

i Increased expression of nmMLCK in asthmatic lungs

To further validate the involvement of nmMLCK in asthma pathobiology, I examined nmMLCK mRNA expression levels in lung tissue biopsies obtained from asthmatic patients. Using qPCR and primers designed specifically to detect mRNA levels of the non-muscle MLCK isoform, I observed nearly a 2 fold upregulation of nmMLCK in asthmatic patient's lung compared to healthy controls (Figure 6). In addition, to validate that nmMLCK upregulation is specific to the asthmatic condition, we utilized the commonly accepted OVA-challenged murine asthma model, an asthma model that replicates the human asthmatic condition with increased airway hyperreactivity, increased edema, increased inflammatory cell infiltration, and airway tissue remodeling. Comparatively, nmMLCK mRNA levels in lung tissues taken from OVA-challenged mice were noticeably increased (Figure 6). These findings in human asthmatic patient

samples and a murine model of experimental asthma exhibiting consistent upregulation of nmMLCK mRNA expression, suggests a potentially important role for nmMLCK in asthma pathobiology.

ii Ovalbumin challenge in nmMLCK transgenic mice

Previous studies on *in vivo* models of acute inflammatory lung disease (ALI/VILI) have elucidated the contribution of nmMLCK in exacerbating leukocyte infiltration and edema in the alveolar space, exemplifying the critical role of nmMLCK in pulmonary inflammation processes. While overexpression of nmMLCK has not been reported, both genetic deletion of nmMLCK or silencing of nmMLCK expression (siRNA) *in vivo* in ALI and VILI mouse models have demonstrated a significant attenuation of leukocyte infiltration and pulmonary edema (Mirzapoiazova et al. 2011). In addition, nanocarriers containing peptide inhibitors of nmMLCK kinase activity were protective indicating that enhanced enzymatic function of nmMLCK contributes to the increased permeability response. In the current study, we examined the influence of nmMLCK on the susceptibility and severity of a murine asthmatic phenotype using OVA-challenge asthma model. Two genetically-engineered mouse strains were utilized for these studies: 1) nmMLCK^{-/-} mice lacking non-muscle isoform of MLCK specifically and 2) nmMLCK^{ec/ec} mice selectively overexpressing the non-muscle nmMLCK isoform in endothelial cells (driven by the VE-cadherin promoter). Using a standardized protocol for OVA-challenge in wild type mice, antigen challenge increases airway hyperreactivity measured by airway pressure time index (APTI), airway reactivity to methacholine (Mch), permeability measured by bronchoalveolar lavage (BAL) protein, and leukocyte

infiltration measured by BAL cells (Figure 7). When genetically engineered mice were challenged with OVA, nmMLCK^{-/-} mice showed attenuation in OVA-induced increased APTI while nmMLCK^{ec/ec} exhibited exacerbation of APTI (Figure 7A). Similarly, OVA-challenge mediated airway reactivity to methacholine in nmMLCK^{-/-} mice was significantly attenuated but substantially increased in nmMLCK^{ec/ec} mice in a dose-dependent manner (Figure 7B). OVA-challenged nmMLCK^{ec/ec} mice also showed markedly increased BAL protein concentration (Figure 7C). While we observed strong trend to attenuation of BAL protein concentrations in nmMLCK^{-/-} mice following OVA-challenge, this failed to reach statistical significance. Nonetheless, we believe these results support a role for nmMLCK upregulation in overall asthmatic outcomes by exacerbating airway reactivity and inflammation. Additionally, OVA-challenge increases infiltration of macrophages and eosinophils, inflammatory cell types associated with asthmatic inflammation (Lee et al. 2004), but not neutrophils into airway spaces in wild-type mice. Consistent with BAL protein leakage, OVA-challenge in nmMLCK^{-/-} showed attenuation of neutrophil and eosinophil infiltration while the same challenge in nmMLCK^{ec/ec} exhibited a significant increase in neutrophil and eosinophil infiltration (Figure 7D).

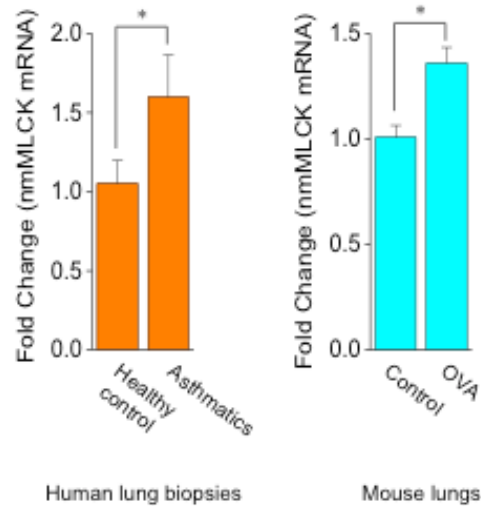


Figure 6: Real-time PCR analysis of nmMLCK mRNAs in human and mouse lung tissues

mRNA of nmMLCK was quantitated using real-time PCR in (Left) human endobronchial biopsy from asthmatic patients and healthy controls and (Right) OVA sensitized mouse model of asthma. mRNA expression levels are normalized to β -actin. * $p < 0.05$ compared with OVA-challenged WT mice; $n \geq 5$ for all groups. Taken from Wang T, Moreno-Vinasco L, Ma SF, Zhou T, **Shimizu Y**, Sammani S, Epshtein Y, Watterson DM, Dudek SM, Garcia JGN. Nonmuscle Myosin Light Chain Kinase Regulates Murine Asthmatic Inflammation, *American Journal of Respiratory Cell and Molecular Biology*, Vol. 50, No. 6 (2014), pp. 1129-1135

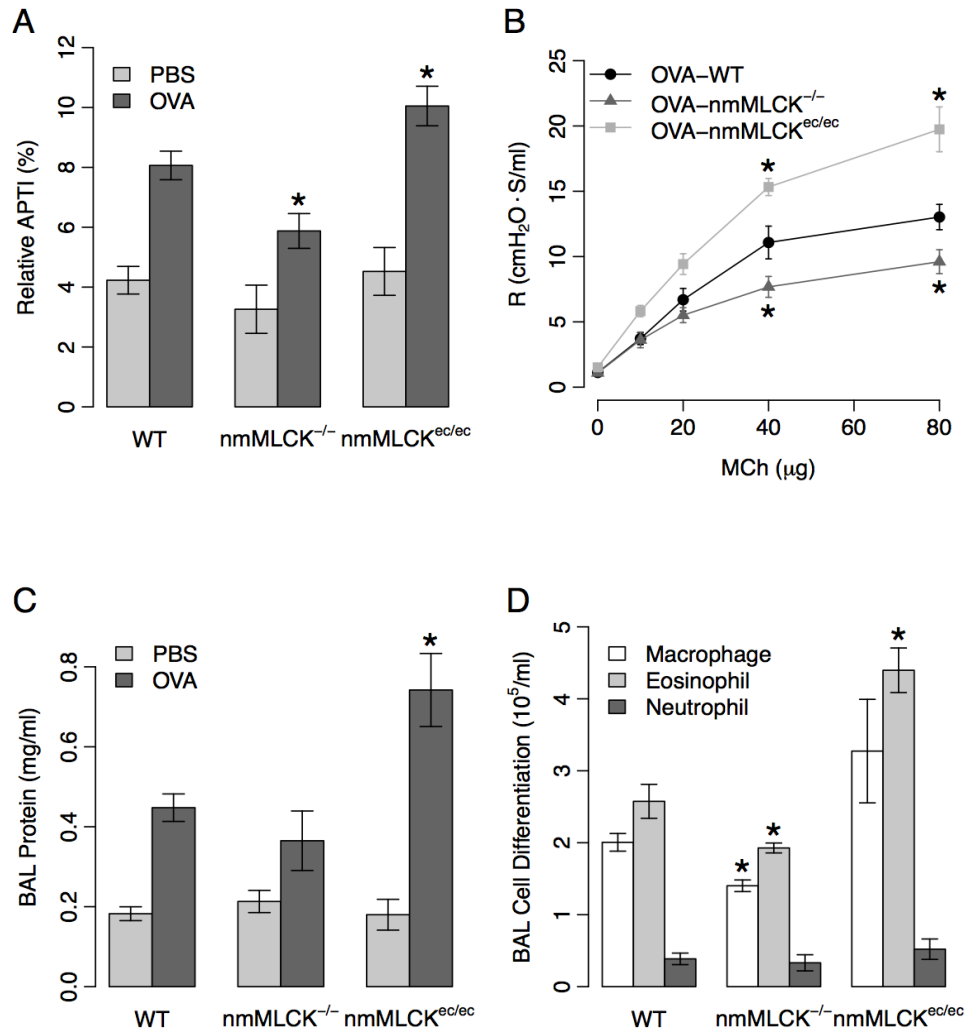


Figure 7: Effect of nmMLCK expression on airway reactivity and inflammation in a murine model of asthmatic inflammation

(A) Airway hyperreactivity was assessed in wild type (WT), nmMLCK^{-/-}, and nmMLCK^{ec/ec} mice using relative airway pressure time index (APTI), a measurement of acetylcholine-induced changes in airway pressures, (B) effects of Mch, (C) effects of nmMLCK expression on BAL protein leakage, and (D) effects of nmMLCK expression on BAL inflammatory leukocyte infiltration in OVA

challenged mice. * $p < 0.05$ compared with OVA-challenged WT mice; $n \geq 5$ for all groups. Taken from Wang T, Moreno-Vinasco L, Ma SF, Zhou T, **Shimizu Y**, Sammani S, Epshtein Y, Watterson DM, Dudek SM, Garcia JGN. Nonmuscle Myosin Light Chain Kinase Regulates Murine Asthmatic Inflammation, *American Journal of Respiratory Cell and Molecular Biology*, Vol. 50, No. 6 (2014), pp. 1129-1135

iii Silencing nmMLCK attenuates asthmatic responses induced by OVA challenge *in vivo*

To further validate the critical role of nmMLCK in asthmatic susceptibility, nmMLCK siRNAs were delivered intravenously to OVA- sensitized asthmatic mice via ACE antibody-tagged liposome delivery to specifically target pulmonary endothelium (5 mg/kg, i.v) (Mirzapoiazova et al. 2011) prior to OVA challenge. Mice receiving nmMLCK siRNAs exhibited significant reductions in OVA-mediated AHR, BAL protein leakage, and inflammatory leukocyte infiltration (Figure 8). As expected, mice challenged with nmMLCK siRNAs failed to alter inflammatory indices in WT mice in the absence of ovalbumin sensitization. Taken together, these findings indicate that availability of the nmMLCK isoform, especially when expressed in lung endothelium, facilitates the migration of inflammatory eosinophils into OVA-challenged murine airways and parenchyma and is a key determinant of airway hyper-responsiveness, mucus production and eosinophilic inflammation. These studies, confirm the involvement of the nmMLCK isoform in asthma pathobiology, and support *MYLK* as a legitimate and novel therapeutic target in severe asthma.

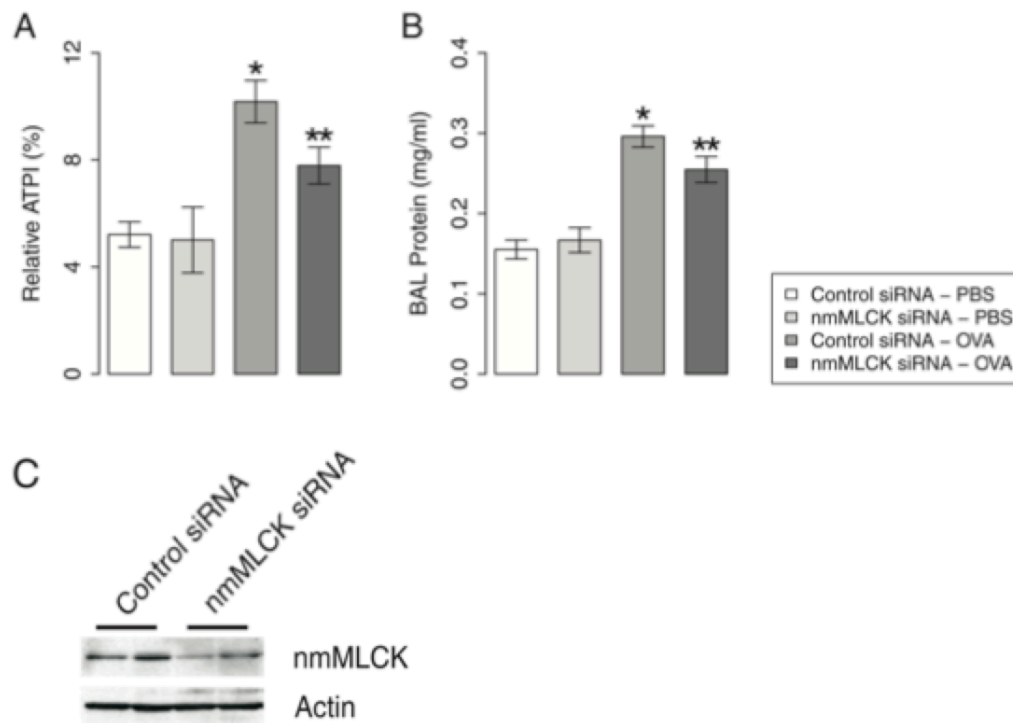


Figure 8: Effect of nmMLCK silencing on asthmatic inflammation in mice

WT mice received nmMLCK small interfering RNA (siRNA) to reduce nmMLCK expression in the lungs, and were challenged by OVA to activate asthmatic inflammation. (A) Effects of nmMLCK siRNA on airway hyperreactivity reflected by acetylcholine-induced APTI. (B) Effects of nmMLCK siRNA on BAL protein leakage. (C) Western blot demonstrating downregulation of nmMLCK by siRNA *in vivo*. * $p < 0.05$ compared with control siRNA–PBS group; ** $p < 0.05$ compared with control siRNA–OVA group; $n = 5$ or 6 . Taken from Wang T, Moreno-Vinasco L, Ma SF, Zhou T, **Shimizu Y**, Sammani S, Epshtein Y, Watterson DM, Dudek SM, Garcia JGN. Nonmuscle Myosin Light Chain Kinase Regulates Murine Asthmatic Inflammation, *American Journal of Respiratory Cell and Molecular Biology*, Vol. 50, No. 6 (2014), pp. 1129-1135

iv nmMLCK expression is increased in the radiation mouse model of lung injury

Thoracic radiation is an effective therapy for treating patients with advanced lung cancers, however, patients who receive radiation therapy often suffer subsequent subacute and chronic radiation-induced lung injury including fibrosis which can develop several years after the initial radiation treatment. Previous studies have confirmed these clinical features in a murine RILI model, where a single dose of whole-thoracic radiation (18-25 Gy) substantially increased lung inflammation and vascular permeability after 4, 6, 8, 12 weeks post radiation (Mathew et al. 2011). In the current study, we examined the potential involvement of nmMLCK during the inflammatory process after radiation exposure.

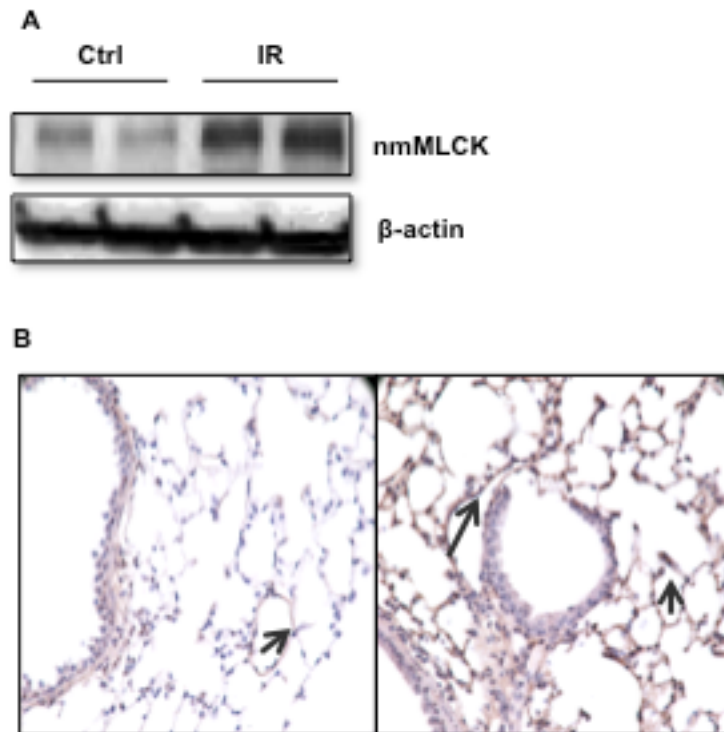


Figure 9: Thoracic radiation increases nmMLCK expression in mice

(A) Western blot demonstrating increased expression of nmMLCK 6 weeks post ionizing radiation exposure (IR) in C57BL6/J (WT) mice with 20Gy single fraction thoracic radiation dose. (B) Immunohistochemistry staining of nmMLCK in lung tissue of radiated mice 6 weeks post radiation. Taken from Matthew, B., **Shimizu, Y.**, Elangovan, V., Rizzo, A., Dudek, S.M., Weichselbaum, R.R., Jacobson, J.R., Garcia, J.G.N. Non-Muscle Myosin Light Chain Kinase modulates radiation-induced lung injury. *Microvascular Research* (submitted), 2014.

C VEGF induces nmMLCK expression *in vitro*

The downstream molecular pathway regulating vascular permeability induced after exposure to pathogenic stimuli is dependent on the type of agonist that endothelial cells are exposed to. In particular, VEGF is thought to play a prominent role because of its profound effect on vascular permeability and its association with multiple pulmonary inflammatory diseases such as ALI, ARDS, and asthma. Furthermore, ectopic expression of VEGF in lung causes leukocyte infiltration, edema, and vascular remodeling (Lee et al. 2004). Importantly, the pathological activation of the VEGF signaling pathway has been shown to alter the transcription of numerous downstream target genes (Ferrara 2002).

i VEGF stimulation upregulates nmMLCK mRNA and protein expression and activates *nmMYLK* promoter activation

Based on previous results demonstrating the upregulation of nmMLCK in both lung biopsies from human asthmatic patients, as well as lungs from OVA-challenged asthmatic mice, I examined whether VEGF contributes to this increased expression of nmMLCK in human lung endothelial cells. ECs were cultured to complete monolayer in low serum condition in EBM-2 (Lonza) containing 0.5% fetal bovine serum (FBS, LifeTechnology) for 4 hours prior to VEGF stimulation. ECs were then stimulated with hrVEGF (100 ng/ ml) for 24 hours, and the change in expression levels of nmMLCK measured using qPCR. VEGF stimulation increased nmMLCK mRNA expression by ~5 fold (Figure 10) compared to control, unstimulated ECs cultured in EGM-2 with 4 hours serum starvation. These results were confirmed by examination of nmMLCK protein

expression (Western blots) which also revealed profound increases in nmMLCK expression in EC after 24 hours of VEGF stimulation (Figure 10). These results suggested that nmMLCK protein expression is increased by VEGF and this change in expression is due to increased production of mRNA in endothelial cells.

I next explored the mechanism for this induction by VEGF and hypothesized that VEGF signaling may induce changes in *nmMYLK* promoter activity. *MYLK* encodes both smMLCK (exon 17-34) and nmMLCK proteins (exon 4-34 with non-coding exon 1-3) via distinct promoters to drive expression of each isoform. In order to examine *nmMYLK* promoter activity, a sequence 2.5 kb upstream of exon 1 of *nmMYLK* gene was cloned into pGL3-basic plasmid (Promega) containing the minimal promoter and the luciferase gene which lacks eukaryotic promoter elements. Dual-Luciferase Reporter Assay System was used to examine the promoter activity. Transfection of pGL3-*nmMYLK*2.5kb plasmid into EC followed by VEGF stimulation showed a significant increase (~3 fold) in *nmMYLK* promoter activity compared to unstimulated endothelial cells (Figure 11). These results, combined with the previous results exhibiting increased mRNA and protein expression, confirm the overall increase of *nmMYLK* gene activation by VEGF in human lung endothelial cells.

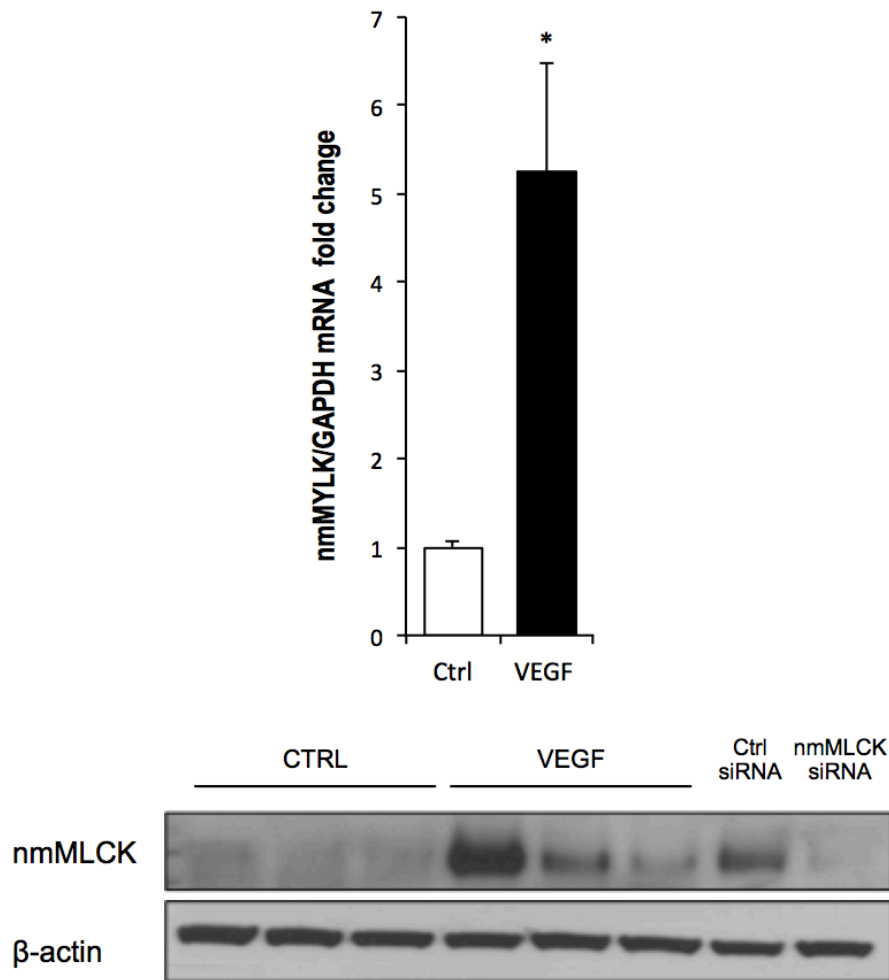


Figure 10: nmMLCK expression is increased by VEGF stimulation

Treatment of human pulmonary artery endothelial cells with VEGF (100 ng/ml, 24 hours) showed a marked increase in *nmMLCK* mRNA levels (Top) and nmMLCK protein levels (bottom) suggesting that VEGF is a potent stimulus for driving increases in nmMLCK expression in the asthmatic lung. * $p < 0.05$

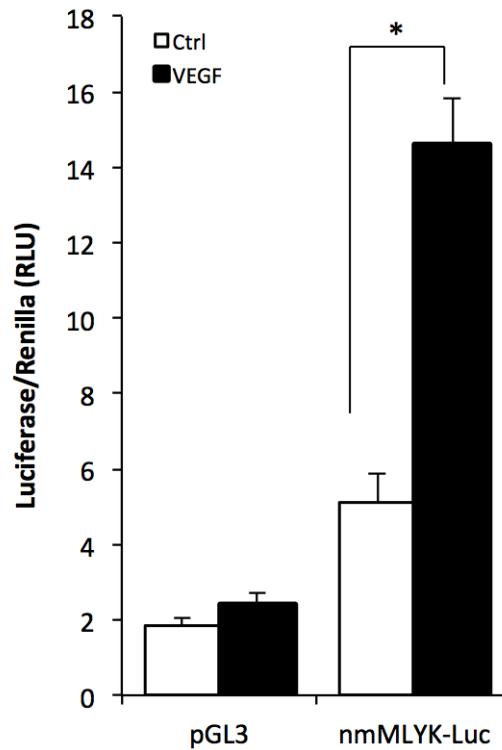


Figure 11: *nmMYLK* promoter activity is increased by VEGF stimulation

Treatment of human pulmonary artery endothelial cells with VEGF (100 ng/ml, 24 hours) dramatically increases *nmMYLK* promoter activity as detected by luciferase reporter activity. * $p < 0.05$ compared to unstimulated nmMYLK-Luc transfected cells

D *nmMYLK* promoter regulation by VEGF signaling

i Determination of critical region of *nmMYLK* promoter *in vitro*

Based on the findings that *nmMYLK* promoter activity is dramatically increased upon VEGF stimulation in human lung EC, I hypothesized that EC stimulation by VEGF involves specific promoter regions and transcription factors and therefore undertook deletion analysis to determine the shortest region of the promoter that is critical for promoter activation in response to VEGF stimulation. Various lengths of *nmMYLK* promoter fragments were cloned upstream of the Luciferase gene in a pGL3-basic vector with promoter activity analyzed by the ability to drive the expression of the luciferase gene after transfection into EC monolayers. A 2.5 kb promoter fragment (-2562 to +104bp) increased relative luciferase activity (luciferase/renilla) by ~3.2-fold in the presence of VEGF compared to untreated cells (Figure 12A). Similarly, 1.3 kb (-1371 to +104bp) and 0.4 kb (-420 to +104bp) fragments significantly increased relative luciferase activity after VEGF stimulation in ECs. In contrast, further deletion of the promoter to a 0.3 kb fragment (-271 to +104bp) demonstrated loss of reduced relative luciferase activity induced by VEGF compared to the larger promoter fragments. Consistently, transfection of plasmids containing smaller fragments of the promoter, 0.2 kb and 0.1 kb, exhibited a similar loss of increased relative luciferase activity induced by VEGF (Figure 12B), compared to the increase in luciferase activity observed with 2.5 kb and 0.4 kb promoter fragments. Based on these results, I concluded that that VEGF increases EC *nmMYLK* promoter activity, and that the critical sequence that responds to VEGF stimulation resides between 0.4 kb and 0.3 kb from nmMLCK exon 1.

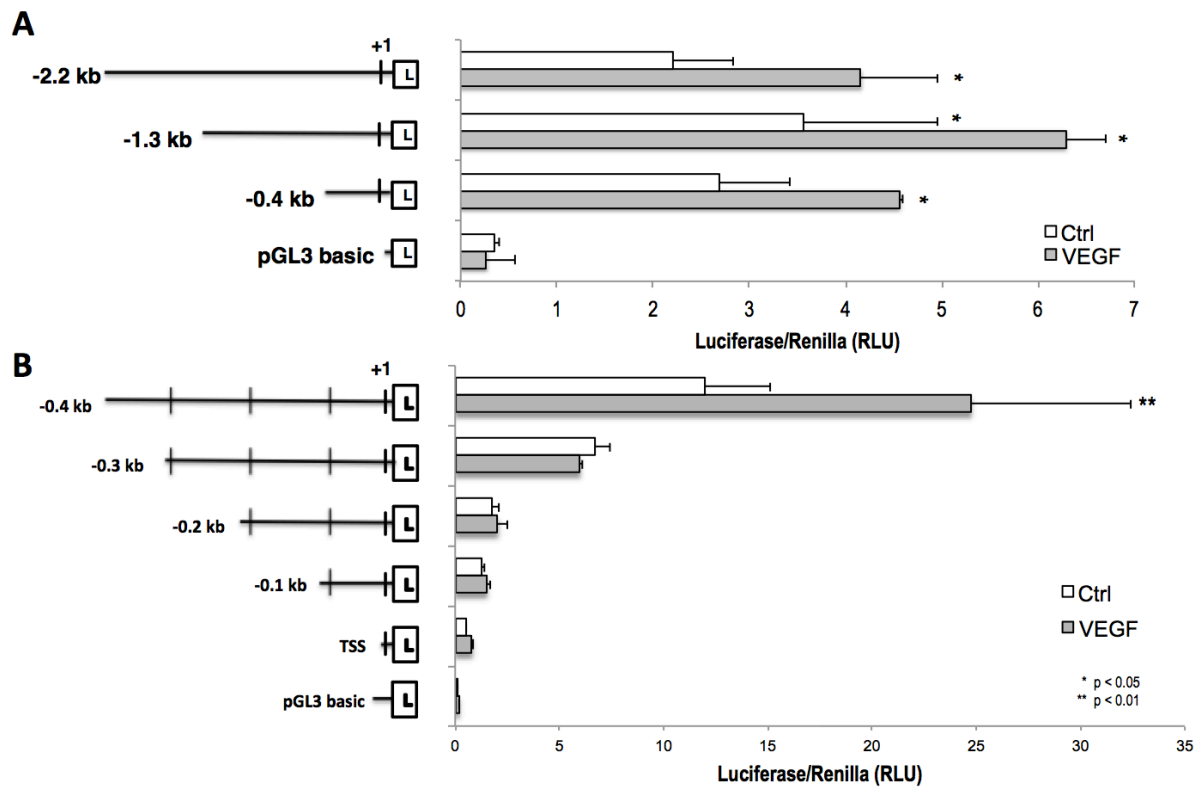


Figure 12: *nmMYLK* promoter truncation decreases VEGF-induced *nmMYLK* promoter activity

(A) Treatment of VEGF (100 ng/mL) in human lung EC shows increased *nmMYLK* promoter luciferase activity in -2.2, -1.3, -0.4 Kb fragments. (B) Deletion of 0.1 kb fragment from 0.4 kb upstream of *nmMYLK* gene show decreased luciferase activity at -0.3 kb of TSS, indicating the area critical for *nmMYLK* transcription by VEGF. *p < 0.05 compared with untreated control; **p < 0.01 compared with untreated control.

CCAGTGTCTCATGCACGCTCACGAAGCTAGCATCAGACCCCGACCTAAGGCCTAGGTCTCCCAGTGTAGG
 -420
 ↓
 ACTAAGACACGCACCAAGACAGTACCAGCCCGCTGACCGCCGCCTGCGAGGAGTTAACT **GGGGCGGTGC**A
 -271
 ↓
 GGATAAGTGCGAGGGGGGCGGGTCTGGCGCTAGGTTTGGCTGCGGATTCTCCGCTCCGGA CTCCGAGG
 -220
 ↓
 CAGAAGGCGGGTGGGCGTGGCGGGAAGGGCAGCAGGTCCACTCCCCGAGCGCTATGCAAATGTCATGCAA
 ATCAGTGCATGGGGGCTGGAGCGGGTGGTTTGAGTCCTGACCCCGTAGCGCCGCGCTCTCACTCGCTCTC
 -118
 ↓
 CTCAGCGCGCTCCGCTCTCCGCTCTCCCACTTGCTCTCTCTCTCGCCGGTTTCGCTCACCCCCGCCCCGC
 CCCGCCCCGCCCCGCCCTGCCAGTCCCGGACGCGCCTCGCGGGCTCCCCGCGCCGCCCGGTGCGCAGCA
GGGCGCTGAGCGAGCTCGGAGCCGCGCTGTGCGCCTGCGGCCGGGGCGCCCCGCCGAGCGCCGGTGCCC
CGGCTCCCGGGCCGCCTTCGCCGCGCGGGTGAGTGAGCCCCGCCCATGCTTTTGTCCAGGCGCGGGCCCT
GGAAGTCAAGG

Figure 13: *nmMYLK* promoter sequence

Sequence of *nmMYLK* promoter 450 bp upstream of TSS. Each arrow indicates the first nucleotide of the clone of the deletion constructs. Boxed sequence indicates Sp1 binding motif. Bold letters indicate Exon 1.

ii ***In silico* prediction of transcription factor binding to the *nmMYLK* promoter**

In parallel with the assessment of luciferase promoter activity, I examined potential transcription factor binding sites on the *nmMYLK* promoter using bioinformatic analyses to further understand the molecular mechanisms that underlie the upregulation of nmMLCK in response to VEGF. For *in silico* analyses, Genomatix software (Munich, Germany) was used to predict transcription factor binding motif on the 2.5kb of *nmMYLK* promoter and predicted a total of 769 potential transcription factor binding sequences within the 2.5kb region of the *nmMYLK* promoter. Of the 769 predicted transcription factor binding sites, 36 sites exhibited sequence similarity to pre-defined transcription factor binding modules determined by the algorithm defined by Frech et al. (Frech et al. 1997) and Klingenhoff et al. (Klingenhoff et al. 1999) that are part of The Promoter Module Library (Genomatix). In regulatory units such as promoters, enhancers or silencers contain functional modules that consist of at least two transcription factor binding sites in conserved order that are separated by a spacer. The module components consist of binding factor, sequential order, matrix similarity, standard orientation, and distance range. The Promoter Module Library is based on a compilation of those elements of multiple transcription factor binding sites that have been verified experimentally for functional effects on promoter activity. All 36 transcription factor binding modules identified in the *nmMYLK* promoter have matrix similarity greater than 0.889 in reference to the perfect match binding modules, with 16 transcription factor binding modules located on the positive strand while the remaining 20 sequences located on the negative strand. Importantly, among the binding module sequences, 20 of the 36 predicted binding sequences were bound by transcription

factors associated with VEGF pathway. This *in silico* analysis of *nmMYLK* promoter, together with the association of the predicted transcription factor binding sites on the promoter, led us to hypothesize that *nmMYLK* promoter activity is evoked by the VEGF signaling pathway leading to increases in the nmMLCK transcripts.

As shown on Figure 14, the only transcription factors predicted to bind to the *nmMYLK* promoter sequence between 0.4 kb to 0.3 kb upstream of TSS, a region that I identified to be critical for VEGF-induced nmMLCK upregulation, were Specific protein 1 (Sp1) transcription factor and hepatocyte nuclear factor 1 (HNF1). This transcription factor-binding module contained Sp1 and GATA, which binds to aactgGGGCggtgcagg sequence on *nmMYLK* promoter (-366/350 from TSS) on the positive strand with high matrix similarity of 0.939.

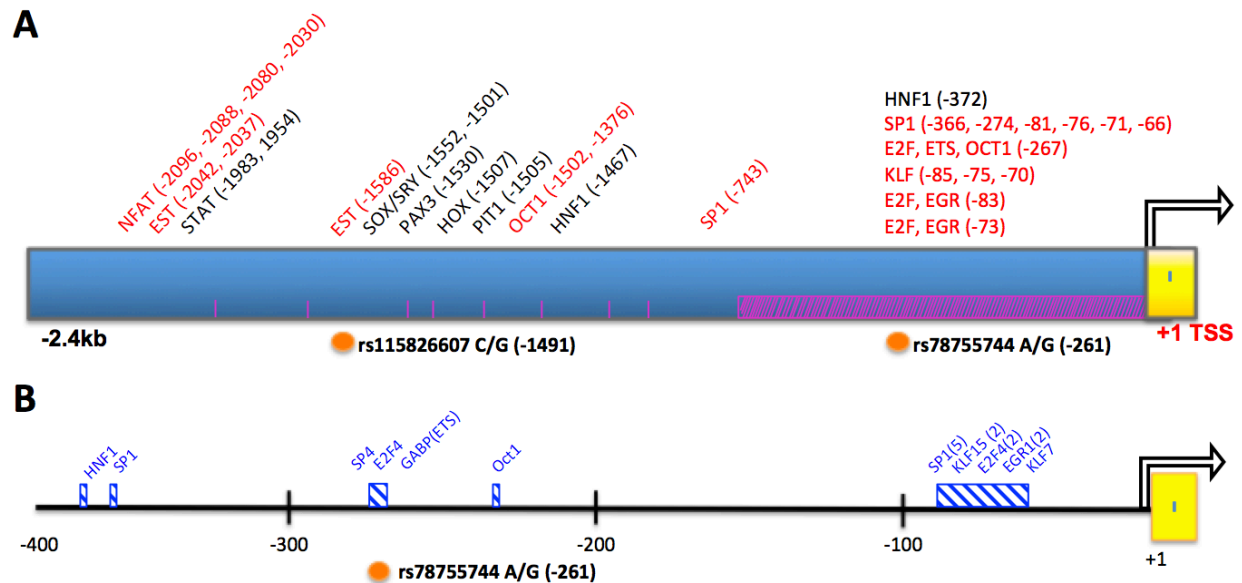


Figure 14: *In silico* analysis of *nmMYLK* promoter

(A) Transcription factor binding sites, CpG island (pink horizontal lines), and SNPs (orange circle) on *nmMYLK* promoter (2.4 kb upstream of TSS and non-coding exon 1). 36 transcription factor-binding modules are shown on top. Red indicates known VEGF effectors. (B) Enlarged view of 400 bp upstream of TSS and locations of potential transcription factor binding site.

iii **Binding of Sp1 transcription factor to the *nmMYLK* promoter**

Sp1 is a C2H2-type zinc finger DNA binding transcription factor involved in biological processes such as cell growth, differentiation, apoptosis, angiogenesis, and immune responses. Sp1 is a member of Sp/KLF family of transcription factors, and recognizes a 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3' sequence (Briggs et al. 1986) with high affinity for the GC-rich sequence. Although it was thought to be expressed ubiquitously in mammalian cells and once considered a housekeeping gene, recent studies have suggested specificity of Sp1 expression to different cell types (Li and Davie 2010) including endothelial cells where Sp1 protein is expressed at 4-24 fold higher levels (Hata et al. 1998). Target genes of Sp1 include cell cycle progression and arrest, pro- and anti-angiogenic factors, and pro- and anti-apoptotic factors, consistent with studies that indicate that Sp1 is primarily involved in cancer invasion and metastasis.

While it has long been thought Sp1 acted solely as transcriptional activator, studies in last decade have elucidated more complex functions, with Sp1 involvement as both transcriptional activator and repressor (Li and Davie 2010). Two isoforms of Sp1 (a and b) consist of 785 amino acids and 778 amino acids respectively, and possess two activity domains that interact with transcriptional activators such as lysine acetyltransferases (KATs) to modify chromatin structure to increase gene access for promoter activation and transcription. In contrast, an inhibitory domain located on N-terminus of Sp1 protein interacts with transrepressor proteins such as histone deacetylases (HDACs) resulting in inhibition of gene activation. Interactions with these proteins ultimately determine Sp1 activity on its target promoter. Additionally, studies

have demonstrated an additional layer of complexity with Sp1 function also dependent on the binding sites within target gene promoter. For example, the distal Sp1/3 sites on adenylate cyclase gene promoter enhance promoter activity whereas the middle sites repress promoter activity (Rui et al. 2008). Furthermore, promoter binding of multiple Sp1 proteins may synergistically enhance gene promoter function by formation of a Sp1 tetramer that binds to multiple Sp1 binding sites on a promoter regions of a gene, increasing recruitment of activation partners to the promoter region for gene activation (Li et al 2004).

Importantly, Sp1's activity is increased upon VEGF stimulation in human microvascular endothelial cells. (Salcedo et al 2003) with an increase in CXCR4 gene expression and promoter activity measured by luciferase promoter assay, whereas, deletion of Sp1 binding sites from the proximal promoter attenuated VEGF-induced increase in CXCR4 promoter activity (Salcedo et al. 2003). Similarly, a study by Bermudez and colleague (Bermudez et al. 2007) demonstrated that Sp1 is required for VEGF-induced telomerase upregulation in ovarian cancer cells. Taken together, the *in silico* analysis of *nmMYLK* promoter revealing Sp1 as a candidate transcription factor in regulating *nmMYLK* gene expression in response to VEGF stimulation, these previous findings support the hypothesis that Sp1 may play a critical role in upregulating nmMLCK expression.

iv ChIP assay to demonstrates direct Sp1 binding to the *nmMYLK* promoter

Based on the transcription factor binding motif prediction, I sought to validate the binding of Sp1 transcription factor on *nmMYLK* promoter between -450 and -331 bp

using chromatin immunoprecipitation (ChIP) techniques. Chromatin immunoprecipitation allows the identification of a specific region on chromatin to which the DNA binding protein such as, a transcription factor, binds. In order to validate Sp1 binding to the *nmMYLK* promoter, EC monolayer were cultured in either 0.5% FBS EBM-2 or VEGF (100 ng/ml) stimulation for 24 hours before harvest. Subsequently, cells were collected, lysed, and sonicated to obtain fragmented chromatin (Komashko et al. 2008). Sonicated lysates were then incubated with magnetic beads conjugated with ChIP grade Sp1 antibody (EMD Millipore). In order to examine the Sp1 binding on the particular region of *nmMYLK* promoter, a pair of primer set was designed to detect the -450 to -331 bp region and the enrichment of the chromatin retrieved by the Sp1 antibody was determined by qPCR.

Quantification of the fragment of *nmMYLK* promoter from the Sp1 antibody ChIP assay reveals an enrichment of a *nmMYLK* promoter fragment containing Sp1 binding sites in low serum control cells compared to negative IgG ChIP controls, suggesting a basal binding of Sp1 to the *nmMYLK* promoter under basal conditions (Figure 15B). Importantly, when ECs are treated with VEGF for 24 hours, the enrichment of *nmMYLK* promoter fragment precipitated with Sp1 antibody increases by ~2 fold compared to low serum controls. These data validate the binding of Sp1 protein directly to the *nmMYLK* promoter at predicted binding sites at -366 to -350 from TSS and confirm our hypothesis that VEGF modulates Sp1 binding in ECs.

The importance of Sp1 in disruption of endothelial cell barrier integrity was validated in permeability assays using TER measurements. Silencing of Sp1 protein by siRNAs significantly attenuated VEGF-induced vascular permeability in ECs (Figure 16).

Taken together, these results confirm Sp1 binding to a critical region of the *nmMYLK* promoter, with Sp1 increasing vascular permeability via increasing expression of nmMLCK.

v Binding of Sp3 transcription factor to the *nmMYLK* promoter

Among the proteins in Sp/KLF transcription factor family, Sp1 and Sp3 share a high homology in protein sequence and structure. Sp1 and Sp3 share 90% homology in the DNA binding zinc finger motif, and bind to the same sequence (Li and Davie 2010). Removing Sp1 or Sp3 in knockout mice have confirmed the redundancy in the function of these two proteins at early developmental stages (Hayashi et al. 2006), however, functional differences were shown later in the developmental stages before embryos die at day 11 post gastrulation (Marin et al. 1997).

Four isoforms of Sp3 are currently known with two long isoforms (a and b) and two short isoforms (c and d) lacking the N-terminus. Both long and short forms of Sp3 are products of the same gene, and have distinct promoters that produce proteins of different sizes. The short isoforms of Sp3 lack activation domain A and might function primarily as repressors. Sp3 is also a ubiquitously expressed transcription factor, yet the expression has been seen 3-7 fold higher in ECs than non-ECs (Li and Davie 2010).

The differences between Sp1 and Sp3 exist in the order of the domain within the protein. While the repressor domain of Sp1 is on N-terminus, the repressor domain on Sp3 is located next to the DNA binding motif. This difference is thought to drive the functional differences between Sp1 and Sp3. Additionally, while Sp1 has been shown to

form a tetramer that leads to synergetic effects on its activity, Sp3 does not form multimers thus lacking the ability to create synergistic activity (Li and Davie 2010).

Neither Sp1 nor Sp3 are specifically activators or repressors since the outcome is dependent on the organization of the promoter binding sequence. Sp1 and Sp3 bind on the same promoter; however, sequential ChIP experiments have suggested that they may not co-occupy (Li and Davie 2008). Moreover, both proteins may occupy distinct promoter regions in a context dependent manner. For example, Sp1 binds specifically to both proximal and distal GC elements of the human topoisomerase II alpha promoter but Sp3 binds only to the distal GC element (Williams et al. 2007). Additional complexities of the interactions between Sp1 and Sp3 are evident where Hata and colleagues reported that overexpression of Sp1 in ECs or non-ECs increased promoter activity of KDR (kinase domain receptor, EC specific gene) by 3-fold. Co-expression of Sp3 attenuated this response (Hata et al. 1998).

Based on the binding sequence that Sp1 and Sp3 shares, I examined whether Sp3 also binds to *nmMYLK* promoter at predicted binding sites at -366/350 from TSS, thereby potentially influencing the regulation of nmMLCK expression. I performed ChIP assay using ChIP grade Sp3 antibody on sonicated chromatin isolated from EC with or without 24 hours of VEGF stimulation as described above. Quantification of fragment of *nmMYLK* promoter indicates ChIP using Sp3 antibody show enrichment of an *nmMYLK* promoter fragment containing Sp3 binding sites in low serum control cells compared to negative IgG ChIP control, suggesting a basal binding of Sp3 on *nmMYLK* promoter during unstimulated conditions (Figure 15). When ECs were treated with VEGF for 24 hours, there was an enrichment of *nmMYLK* promoter fragment precipitated with Sp3

antibody by 2.90 fold compared to low serum control. These data suggest a binding of Sp3 protein on *nmMYLK* at the same region where Sp1 is predicted to bind.

vi Regulation of *nmMYLK* promoter activity by CpG methylation

Another mechanism that can affect promoter activity is epigenetic modification of chromatin. These modifications are crucial in determining whether a particular gene is silenced or is active. Many layers of physiological signaling can contribute to the overall DNA cytosine methylation status, through methylation and acetylation of histones (Jones 2012). These modifications can influence accessibility of other transcriptional co-factors necessary for gene transcription or they can alter chromatin structure to incapacitate transcription initiation.

Approximately 60% of the genes in vertebrates possess CpG-rich regions that regulate gene expression (Jones 2012). Two classes of enzymes regulate cytosine methylation: DNA methyltransferases that methylate cytosine residues and demethylases that remove methyl groups from cytosine bases (Wu and Zhang 2011). CpG islands may be methylated in a tissue specific manner. Methylation in promoter regions has been shown to block the start of the transcription process (Gal-Yam et al. 2006) In most cases, methylation of CpG islands at a specific gene is set during development. However, alterations to DNA methylation have been linked to changes in gene expression during the development of certain cancers (Jones 2012).

Various methods have been developed to study the status of CpG methylation such as, bioinformatics analysis of genomic sequences, *in vitro* bisulfite sequencing and methyl-DNA immunoprecipitation (MeDIP) to verify the methylation at the base level

(Harris et al. 2010). Using these approaches, I have identified potential CpG islands using *in silico* analysis on the *nmMYLK* promoter (Figure 5), and sought to confirm whether the *nmMYLK* promoter activity is altered by methylation of these cytosines.

Using plasmid constructs containing truncated *nmMYLK* promoter regions fused with the *luciferase* gene, I examined the basal level of cytosine methylation in cultured EC. Treatment of EC with DNA methyltransferase inhibitor, 5-aza-2'- deoxycytidine (5-Aza), at 10 mM for 24 hr, demonstrated significant increases in luciferase activity of *nmMYLK* promoter (-2.5, -1.3, -0.4 kb) compared with untreated cells (Figure 17), suggesting the CpG islands on *nmMYLK* promoter is methylated at basal levels and represses *nmMYLK* gene transcription. I further sought to examine whether VEGF signaling alters the CpG methylation status on the *nmMYLK* promoter. Interestingly, pre-treatment of EC with 5-Aza prior to VEGF stimulation further increases the luciferase activity compared to VEGF alone (Figure 17). These results indicate CpG islands on *nmMYLK* promoter are methylated and removal can enhance effects of VEGF stimulation on *nmMYLK* transcription. However, these data raise further questions whether VEGF and CpG demethylation act synergistically, or rather VEGF increases sensitivity to demethylation. This will require further investigation.

E Summary and discussion

Despite the efforts to improve our understanding of the development of various pulmonary inflammatory diseases such as ALI/ARDS and asthma, the molecular mechanisms underlying the pathophysiology of these diseases remain unclear. In an effort to better understand the etiology of pulmonary inflammations and to develop novel pharmacotherapies, our group previously used genomic-intensive approaches to

identify potential ARDS and VILI susceptibility candidate genes (Simon et al. 2006; Grigoryev et al. 2004) and identified *MYLK* gene as a candidate gene for sepsis-associated and trauma-associated ALI. Our previous *in vivo* studies in an LPS-induced ALI model demonstrated that MLCK-dependent MLC phosphorylation resulted in increased actomyosin contraction and reorganization within endothelial cells; this led to disruption of vascular barrier integrity (Jacobson et al. 2006), subsequently causing alveolar flooding and profound vascular leakage (Goldblum et al. 1989; Hirano et al. 2004; Penn and Chisolm 1991). Consistent with the highly multifunctional nature of the *MYLK* gene product in inflammation, our group has previously identified *MYLK* coding SNPs as risk variants in three different asthmatic populations of African descent (i.e., Chicago Collaborative Study on the Genetics of Asthma [CSGA], Baltimore CSGA, and Barbados) (Flores et al. 2007; Gao et al. 2007). Together, these studies underscore the importance of elucidating the exact functional role of nmMLCK in barrier regulation and inflammatory lung injury. However, the molecular mechanism involved in *MYLK* gene regulation as a part of etiology of ARDS and VILI remains poorly understood. VEGF, originally discovered as Vascular Permeability Factor, has profound effects on vascular permeability by junctional protein structure remodeling and cytoskeleton reorganization. VEGF has been associated with pathophysiology of ALI/ARDS as well as asthma (Medford and Millar 2006; Thickett et al. 2001). I sought to investigate the involvement of nmMLCK in pulmonary inflammation in combination with the role of nmMLCK as a novel effector of VEGF-induced increased permeability and inflammation.

I first sought to examine whether the expression of nmMLCK is altered in various pulmonary inflammation. I observed the increased expression of nmMLCK in affected

tissues isolated from patients of asthma, and confirmed this findings using OVA-challenged mouse asthma model. Furthermore, increased expression of nmMLCK protein was observed in *in vivo* RILI model. Additionally, previous studies from our group have demonstrated the use of lipid vehicles to deliver siRNA specifically to endothelial cells has proven beneficial in OVA-challenged mice. This is consistent with previous studies demonstrating nmMLCK silencing attenuated of LPS-induced ALI *in vivo* (Mirzapoiarzova et al. 2011).

Abnormal levels of VEGF have been implicated in a variety of pulmonary inflammatory diseases including ARDS/ALI and asthma. In this study, I demonstrate the activation of VEGF signaling pathway in endothelial cells has a direct influence on nmMLCK expression levels. The product of *nmMYLK* gene are increased at both mRNA and proteins levels. Additionally, a promoter activity assay using pGL3-*nmMYLK*2.5kb plasmid demonstrated profound increases in luciferase signal, implying the activation of *nmMYLK* promoter in endothelial cells upon exposure to VEGF. Examining the promoter activity using a truncated *nmMYLK* promoter to further understand the molecular mechanisms involving VEGF-induced promoter activation, revealed that the -0.4 kb to -0.3 kb region of the promoter is critical for *nmMYLK* promoter activation caused by activation of the VEGF pathway.

Further *in silico* analysis of the *nmMYLK* promoter has revealed the presence of validated transcription factor binding modules of Sp1 within the -0.4/-0.3 kb of the *nmMYLK* promoter. The ubiquitous Sp1 transcription factor has high affinity for GC-rich sequences on the genome, which coincides with the presence of CpG islands on *nmMYLK* promoter. Binding of Sp1 transcription factor on *nmMYLK* promoter was

validated using ChIP assay, where VEGF stimulation on endothelial cells increased the binding of Sp1 transcription factor to the -0.4/-0.3 kb region of the *nmMYLK* promoter. Additionally, I observed binding of another member of Sp family Sp3 to the same region of the *nmMYLK* promoter and its binding was more abundant compared to Sp1 binding on the promoter. As mentioned above, Sp1 and Sp3 bind on the same DNA sequence and have been suggested that they may not co-occupy (Li and Davie 2010). Additional evidences have suggested that the function of both Sp1 and Sp3 are likely to be context-dependent (Williams et al 2007; Hata et al. 1998). I have shown here that silencing Sp1 protein attenuate VEGF-induced increased vascular permeability suggesting the role of Sp1 as a transcriptional activator on *nmMYLK*, however, further examination is needed to validate the role of Sp3 in VEGF-induced vascular permeability.

Unpublished work from our group has identified a number of SNPs on *nmMYLK* promoter, which included SNPs that are associated with ARDS and asthma. One of the SNPs is located within the Sp1/3 binding site. It would be beneficial to examine whether this SNP alter the binding affinity of Sp1 or Sp3 to the *nmMYLK* promoter and if it alter the expression of nmMLCK.

In conclusion, these studies extend a number of previous observations, and further demonstrate that nmMLCK plays a central role in regulating vascular endothelial barrier function. Disruption of this regulation by VEGF causes acute lung injury, vascular leakage, and lung edema via increasing gap formation within the endothelial cell layer. Furthermore, the present study enhances the current mechanistic knowledge of nmMLCK mediated increased vascular permeability with its involvement in VEGF-

induced vascular permeability. Additionally, novel insights regarding the regulation of *nmMYLK* promoter via Sp1 and Sp3 transcription factor extend number of candidate molecules for therapeutic targeting for acute lung injuries.

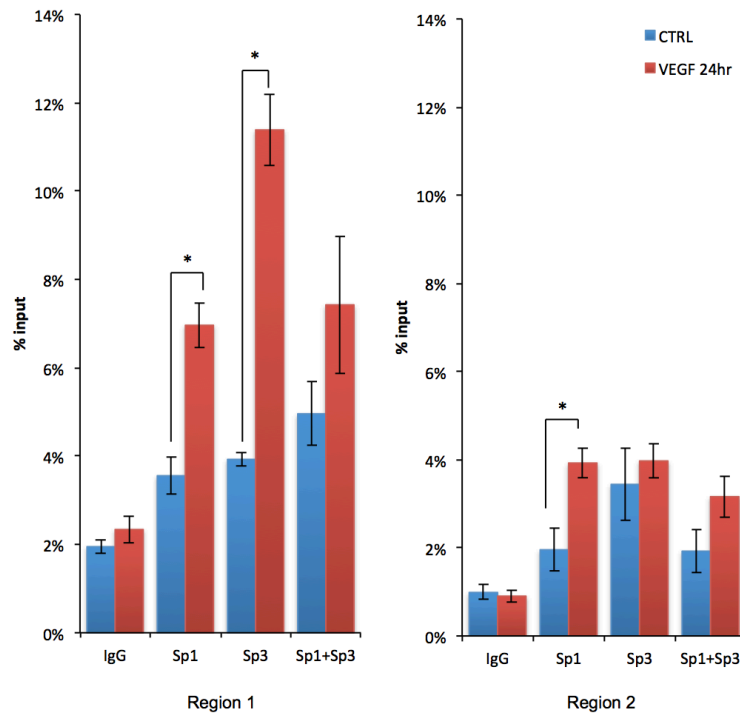
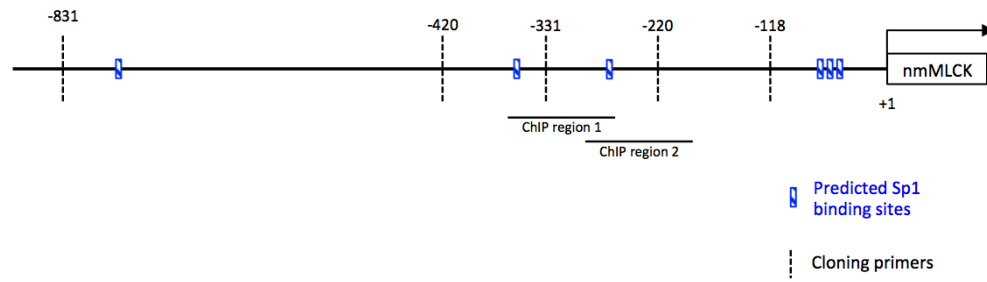


Figure 15: Binding of Sp1 and Sp3 on *nmMYLK* promoter by ChIP assay

(Top) Schematic depiction of the *nmMYLK* promoter with several putative Sp1/Sp3 binding sites (blue rectangles) indicated. Promoter regions analyzed for Sp1/3 binding by ChIP analyses are indicated by the black line in the schematic diagram. Vertical dotted lines indicate the length of the truncated promoter constructs used to determine the critical region of the *nmMYLK* promoter activation by VEGF. (Bottom) ChIP analysis of Sp1 and Sp3 binding to

the *nmMYLK* promoter in human lung EC. qPCR was performed with primers specific to two regions on the *nmMYLK* promoter as indicated in the top diagram. Primers against the -400-base-pair promoter region (Rregion 1) show significant enrichment after normalization to the total input chromatin. *p < 0.05 compared with untreated control group.

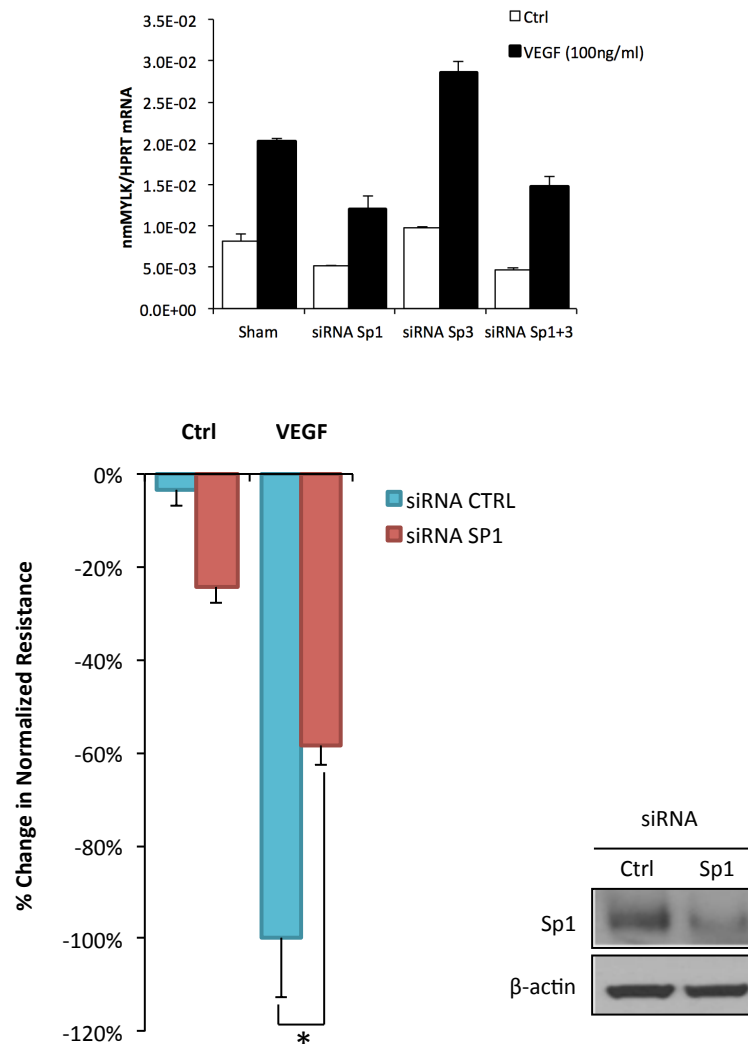


Figure 16: Sp1 silencing attenuates VEGF-induced nmMLCK expression and increased vascular permeability

(Top) Overexpression of nmMLCK mRNA induced by VEGF (100ng/ml, 24 hr) in human lung EC is attenuated by silencing Sp1 by siRNA. Silencing Sp3 increases nmMLCK mRNA levels with VEGF stimulation. Silencing both Sp1 and Sp3 attenuates VEGF-induced increased nmMLCK expression. (Bottom left) Sp1

knockdown by siRNA silencing in human lung EC show attenuation of VEGF-induced Transendothelial Electrical Resistance (TER). (n=4). *p < 0.05 compared with siRNA Sp1 knockdown cells stimulated with VEGF. (Bottom right) Western blot confirming Sp1 protein knockdown.

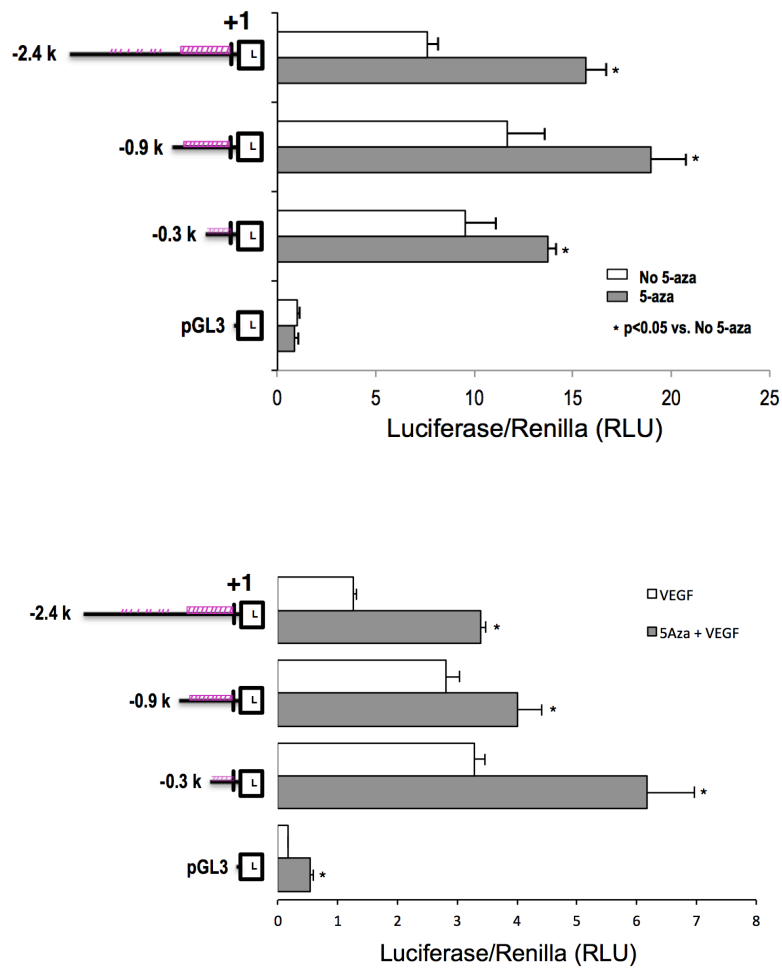


Figure 17: Demethylation increases *nmMYLK* promoter activity

(Top) 5-aza-2'-deoxycytidine (5-Aza) treatment (10 μ M, 24 hours) on human lung EC transfected with various length of *nmMYLK* promoter increased luciferase signals. (Bottom) human lung EC transfected with various length of *nmMYLK* promoter constructs were treated with 5-Aza (10 μ M) and/or VEGF (100 ng/ml, 24 hours). 5-Aza treatment further increases VEGF-induced *nmMYLK* promoter activation. * $p < 0.05$ compared with untreated control group.

Chapter IV: Activation of nmMLCK via inflammatory signals

A Overview

Fluid exchange between blood stream and tissue interstitium across the endothelial cell monolayer requires precise regulation to open and close the endothelial cell gate. Endothelial cells are constantly exposed to a number of naturally occurring biological ligands and molecules, including cytokines and growth factors that regulate vascular permeability. The endothelium has therefore adopted regulatory mechanisms with high specificity to recognize molecules, and selectively activate pathways. Deregulation of this well-orchestrated process promotes vascular barrier dysfunction resulting in increased vascular permeability, thereby causing edema or inflammatory cell infiltration into tissue.

Owing to the intensive efforts to delineate the molecular pathways underlying the mechanisms that regulate vascular barrier functions, we now have a greater understanding of the mechanisms underlying vascular barrier regulation induced by many of the major vascular barrier regulators. Interestingly, each permeability-inducing agonist has different effects on endothelial cell permeability; the effects of histamine and thrombin are transient and reversible, whereas VEGF produces sustained effects on permeability. These molecules are able to influence pulmonary vascular barrier properties in a wide range of pulmonary diseases such as ALI or ARDS, ventilator-induced lung injury, sepsis, asthma, and ischemia reperfusion-induced injury (Becker et al. 2003; Birukova, Liu, et al. 2004; Dudek et al. 2004; Garcia et al. 1999; Petrache et al. 2001; Dull and Garcia 2002). Understanding the molecular mechanisms underlying their effects on increased vascular permeability is crucial to gaining insights into the etiology

of inflammatory induced diseases and to ultimately facilitate the development of novel treatments for the vascular dysfunction seen in inflammatory lung injury.

Despite the existence of a wide variety of molecules that regulate vascular permeability, many share the same downstream signaling mechanisms capable of altering the vascular barrier function. The most common effect of all molecules, is to weaken junctional adhesions and promote the formation of gaps between endothelial cells, which is accomplished by two distinct ways: 1) destabilization of AJs via phosphorylation of AJ complex proteins and 2) reorganization of the actin cytoskeleton to stress fibers and activation of actomyosin contraction. The central component of AJ is VE-cadherin, which is the primary target of the modulation by permeability agonists, and becomes phosphorylated and subsequently internalized upon exposure to different stimuli, resulting in destabilization and dissociation of AJs. Reorganization of the actin cytoskeleton is primarily induced by activation of nmMLCK or inhibition of MYPT, which leads to mechanical contraction and dissociation of cell-cell adhesion between endothelial cells.

Three major, interdependent signaling mechanisms that regulate the increase in paracellular permeability are; c-Src, Ca^{2+} signaling, and RhoGTPase transduction pathways (Komarova and Malik 2010). Activation of c-Src promotes vascular permeability via both VE-cadherin mediated AJ destabilization and actomyosin contraction mediated by direct phosphorylation of nmMLCK. Inflammatory agonists such as VEGF, thrombin, and histamine increase cellular Ca^{2+} concentration by store-operated calcium release and extracellular influx. Increases in intracellular Ca^{2+} induces activation of Ca^{2+} /Cam-dependent nmMLCK to promote actomyosin contraction, as well

as phosphorylation of the AJ component, p120-catenin, to promote VE-cadherin internalization and increases in paracellular permeability. Finally, activation of the RhoGTPase, RhoA, inhibits activity of myosin phosphatase (MYPT) via activation of RhoA/Rho kinase (ROCK) to increase paracellular permeability. VEGF signaling elicits two main pathways known to influence vascular permeability; 1) Src-mediated VE-cadherin internalization, and 2) activation of Ca^{2+} signaling. In this study, I examined the molecular signaling mechanisms induced by VEGF to promote increased vascular permeability. I demonstrate here a novel mechanism whereby VEGF increases vascular permeability by activation of nmMLCK.

B VEGF increases lung endothelial cell (EC) permeability

In 1983, Donald Senger and Harold Dvorak identified a protein that induced vascular leakage while aiming to understand the physiological properties of tumor blood vessels (Senger et al. 1983). Based on the properties of the protein that increased vascular leakage in the Miles assay, the protein was named tumor vascular permeability factor (VPF). In 1989, Napoleone Ferrara and his group had successfully isolated and identified a endothelial cell mitogen from medium that had high levels of mitogenic activity in endothelial cells and *in vivo* (Leung et al. 1989) and was unlike other known cell-bound proteins with similar activity, such as bFGF. Partial sequencing of the newly identified secreted protein confirmed that it was a unique protein that did not match any known proteins in the database and was named vascular endothelial growth factor (VEGF). Because of the technical limitations in protein purification at that time of the study to identify the amino acid sequences, both groups were unable to link the two

proteins as the same protein. Similarly, the same protein was isolated by Jean Plouet and Denis Gospodarowicz and named vasculotropin. However, the cDNA cloning of VEGF and VPF led to the identification of two proteins, and later vasculotropin, as identical proteins (Ferrara 2002). Although VEGF is better known as pro-angiogenic factor, previous studies demonstrated that VEGF has dramatically high vascular permeability effects on endothelial cells compared to its effect on angiogenesis (Dvorak 1995). VEGF is one of the most potent permeability inducers currently known, acting at concentrations that are 50,000 fold higher than the effect of histamine on vascular permeability.

i Dose-dependent effects of VEGF on human lung EC permeability

It has been well established that VEGF-induced increases in vascular permeability occur in a biphasic manner (Bates 2010). The first peak starts as early as a few minutes after ECs are exposed to VEGF stimulation and peaks at 1 hour post exposure, which is considered as acute permeability (Becker et al. 2001). After the transient increase in permeability, the endothelial cell vascular barrier recovers, followed by the second persistent increase in permeability that could last for hours to days depending on the type of endothelial cells and the experimental conditions, and is considered chronic permeability (Bates 2010). Based on this evidence, it is hypothesized that the two phases of increased vascular permeability caused by VEGF are regulated by distinct molecular mechanisms that cause acute or chronic effects.

Additionally, previous studies have demonstrated that VEGF affects vascular permeability in a dose-dependent manner *in vitro* (Mirzapioazova et al. 2006). Here, I aimed to confirm the dose-dependent effects of human rVEGF165 in human lung EC

using our experimental conditions. In order to examine the degree of vascular permeability under these experimental conditions, I utilized a transendothelial electrical resistance (TER) system. In this assay, EC monolayers TER measurements across endothelial cell monolayer in polycarbonate wells containing gold microelectrodes were performed using an electrical cell-substrate impedance sensing system (ECIS), which measures the electrical resistance across the endothelial cell monolayer. The recorded resistance throughout the measurement time period was then normalized to the resistance at the time of the start of the experiment (Time 0). VEGF stimulation at 5 ng/ml concentration on human lung EC shows increased resistance in TER measurement (Figure 18) indicating decreased vascular permeability and improvement in vascular barrier integrity. This is consistent with a previous study demonstrating the protective effect of low VEGF (10 ng/ml) concentration (Mirzapoiazova et al. 2006). Stimulation of human lung ECs with higher concentrations of VEGF at 50, 100, and 200 ng/ml demonstrated dose-dependent decrease in TER measurement, indicating EC complete monolayer is disrupted and permeability is increased by VEGF in a dose-dependent manner (Figure 18). I observed sufficient increases in vascular permeability in our experimental system at 100 ng/ml VEGF concentration, therefore 100 ng/ml recombinant VEGF concentration was used for the remaining of the *in vitro* experiments performed in this study.

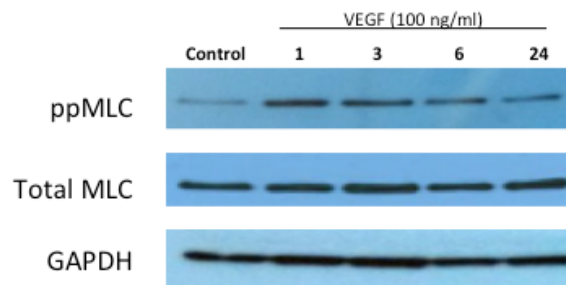
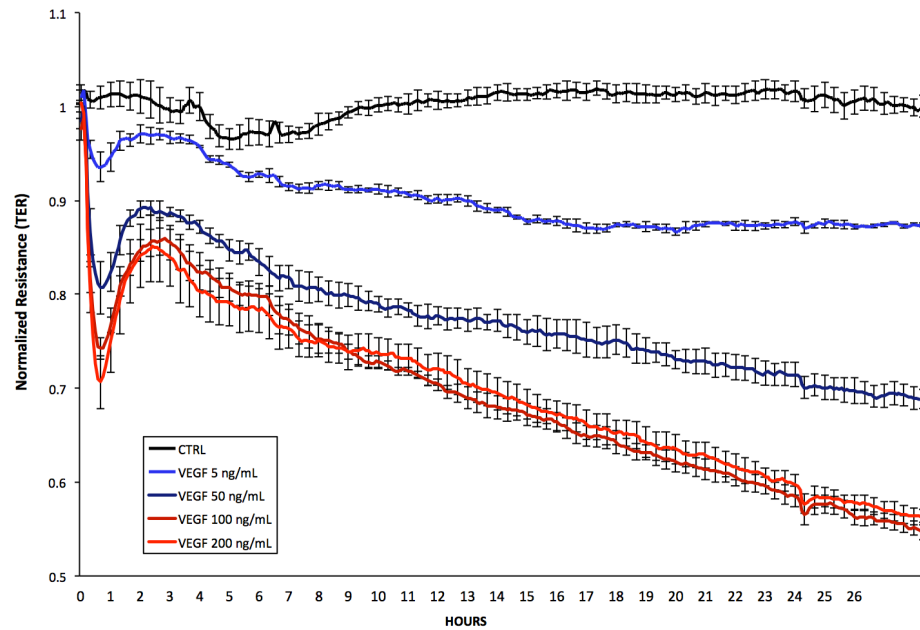


Figure 18: Dose- and time-dependent effects of VEGF on human pulmonary artery endothelial cells

(Top) TER of HPAEC after administration of recombinant human VEGF (5, 50, 100, 200 ng/ml). Data are expressed as in-phase voltage (proportional to resistance) normalized to initial voltage. VEGF increases vascular permeability in dose-dependent manner. (Bottom) Western blot showing a time course of MLC phosphorylation at Ser18 and Thr19 after VEGF treatment. Phosphorylation of MLC is increased with VEGF stimulation and peaks at 5 min after the stimulation.

C Activation of VEGF in lung endothelium

Multiple mechanisms are known to induce vascular permeability in ECs that are driven by VEGF. Exposure of cells to VEGF activates downstream signaling by binding to membrane surface receptors that are selectively expressed on the cell membrane of the various cell types, such as endothelial cells and mast cells. VEGF-A, including VEGF165, primarily binds to VEGFR1 (flt-1) and VEGFR2 (flk-1 in mice and KDR in human).

VEGF receptors (VEGFR) belong to the family of tyrosine receptor kinases. VEGFR 1 and 2 form homodimers or heterodimers, although functional significance of heterodimerization compared to homodimerized VEGFR1 and 2 remains to be determined. Previous studies have demonstrated that VEGFR2 is the predominant receptor in endothelial cells, and is the driving force for subsequent downstream signaling cascades to regulate vascular permeability. The structure of VEGFR1 and 2 are conserved and possess extracellular domains consisting of approximately 750 amino acids that are organized into seven immunoglobulin (Ig)-like folds that function as VEGF ligand-binding sites. Each of the Ig domains has specific functions, where Ig domain-2 functions mainly as ligand-binding sites whereas Ig domain-3 plays an important role in determining ligand-binding specificity (Olsson et al. 2006). Both VEGFRs possess single transmembrane domains followed by intracellular domains that contain a series of tyrosine residues important in receptor activation, recruitment of binding proteins, and signal transduction. This recruitment of interacting proteins is promoted by initial dimerization of the VEGFRs, which leads to activation of receptor-kinase activity via autophosphorylation of the receptors.

i Time-dependent phosphorylation of VEGFR2 in human lung EC

Studies have elucidated a number of tyrosine residues on the cytoplasmic tail of VEGFR2 that plays a specific role in a variety of cellular responses upon binding of VEGF. VEGFR2 is a member of a conserved receptor tyrosine kinase (RTK) family and possesses a unique kinase insert domain (KID) on its intracellular domain unique from other members of the RTK family. Within the KID, two phosphorylation sites at Tyr951 and Tyr996 have functional roles in response to VEGF stimulation. Phosphorylation at Tyr951 and Tyr996 recruits T cell specific adaptor protein (TSAd) via SH2 of the TSAd protein, which is subsequently phosphorylated to recruit and activate Src kinase (Locascio and Donoghue 2013). Previous studies have demonstrated that the activation of Src by VEGF in endothelial cells causes actin reorganization and induces vascular permeability (Sinha et al. 2009).

Two additional autophosphorylation sites known to be phosphorylated after binding of VEGF are, Tyr1054 and Tyr1059, that are located within the kinase catalytic domain on the intracellular tail. Biochemical studies have shown that phosphorylation at Tyr1054 and Tyr1059 is required for maximal kinase activity of the VEGFR2 (Olsson et al. 2006; Dougher and Terman 1999), where expression of proteins with mutations at Tyr1054 and Tyr1059 shows overall reduction in autophosphorylation of the receptor and a dramatic reduction in kinase activity (Dougher and Terman 1999). An additional piece of evidence highlighting the importance of these tyrosine residues on VEGFR2, arises from a study that showed kinase activity is inhibited when Tyr1054 and Tyr1059 are unphosphorylated, an inhibition relieved with autophosphorylation of these tyrosines.

Phosphorylation at Tyr1175 (Tyr1173 in mouse) recruits and promotes the binding of PLC γ mediating the activation of mitogen-activated protein kinase (MAPK) and extracellular-signal-related kinase-1/2 (ERK1/2). This interaction leads to activation of protein kinase C (PKC) that subsequently activates diacyl-glycerol (DAG) to induce increases in intracellular calcium concentrations. This signaling pathway further activates endothelial nitric oxide (NO) synthase (eNOS), which as a result, increases vascular permeability. Furthermore, a mouse expressing the mutated Tyr1173Phe residue is embryonic lethal at E8.5 – E9.5 due to vascular defects on blood-island formation and vasculogenesis that are similar to mice lacking VEGFR2 on both alleles, further demonstrating its importance. The adapter protein SH2 domain-containing adapter protein B (Shb) also binds to VEGFR2 at Tyr1175, which leads to activation of PI3K and Akt, resulting in promotion of endothelial cell survival. Akt activates eNOS to increase NO, leading to increased vascular permeability.

I sought to determine the phosphorylation status of VEGFR2 at various time points in both acute and chronic stages of stimulation in HPAECs. Treatment of confluent HPAECs *in vitro* with 100 ng/ml of recombinant VEGF, demonstrated increased phosphorylation at Tyr951 and Tyr996 after 1 hr, and this phosphorylation persisted up to 24 hr post stimulation (Figure 18). However, examining the phosphorylation status of Tyr951 and Tyr996 at shorter time points failed to show alterations in phosphorylation status. In contrast, Tyr1059 elicited a dramatic increase in phosphorylation at acute stages of VEGF stimulation within 1 min. This phosphorylation at Tyr1059 was sustained for 30 min post stimulation and eventually returned to basal levels of phosphorylation (Figure 19). Examining the phosphorylation status of Tyr1095

at longer time-points exhibited undetectable levels of phosphorylation after 1 hr of stimulation. Similarly, phosphorylation at Tyr1175 was profoundly increased in the acute phase of stimulation within 1 min after the treatment peaking at 5 to 10 min post exposure to VEGF. This strong phosphorylation response persisted until 30 min after the treatment, however, at Tyr1175 as opposed to phosphorylation at Tyr1059, weak phosphorylation persisted up to 24 hrs post stimulation (Figure 19). The total expression levels of VEGFR2 were unchanged up to 24 hrs after VEGF stimulation in human lung ECs. As previous studies have established the role of Tyr1175 in the regulation of vascular permeability upon VEGF stimulation, these results suggest the weak but persistent phosphorylation at Tyr1175 may be critical for the sustained VEGF-induced increase in vascular permeability that was observed in Ch3.2 (Figure 18).

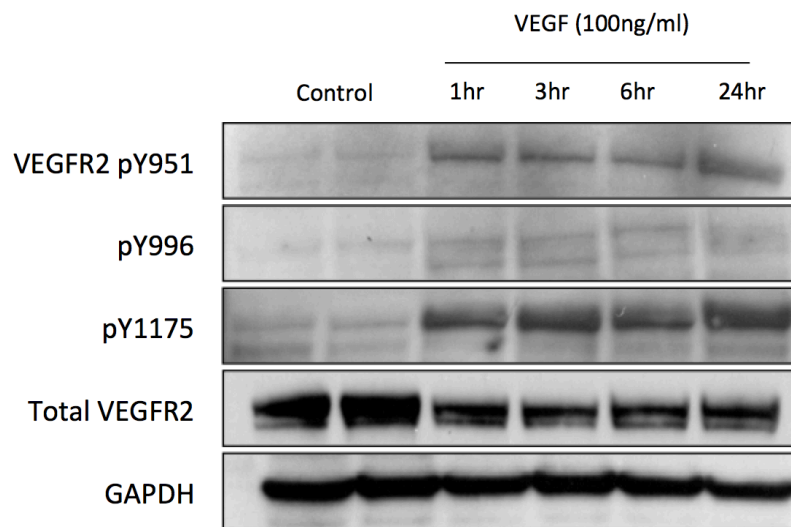
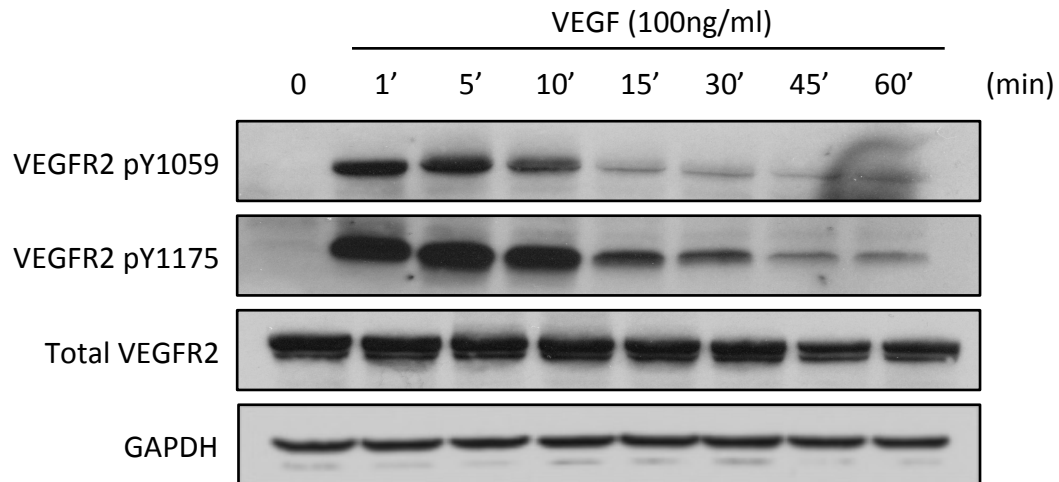


Figure 19: Differential tyrosine phosphorylation of VEGFR2 with VEGF stimulation in human lung endothelium

Human lung ECs were treated with 100 ng/ml VEGF for indicated time and lysates were analyzed for phosphorylation of tyrosine residues of VEGFR2 at Y951, Y996, Y1059, Y1175 and normalized to total VEGFR2 and GAPDH.

D Silencing nmMLCK attenuates VEGF-induced lung endothelial cell permeability

nmMLCK is an essential component of the endothelial barrier regulatory machinery that controls leukocyte infiltrations and edema into the interstitium and alveolar airspace, and is implicated in the exacerbation of vascular permeability during inflammatory injury (Tremblay and Slutsky 2006; Shah et al. 2010; Schnitzer 1992). nmMLCK is a well established molecule involved in gate-keeper function of the endothelial cell barrier. For example, overexpression of nmMLCK exacerbates thrombin-induced increases in vascular permeability (Figure 20A), whereas siRNA silencing of nmMLCK attenuates the permeability effect caused by thrombin. Similarly, increased permeability caused by both *in vitro* and *in vivo* treatment with LPS is attenuated by silencing nmMLCK (Mirzapoiazova et al. 2011). Additional evidence using *in vivo* engineered mouse models further prove the importance of nmMLCK in LPS induced acute lung injury. Our studies have previously shown that mice overexpressing nmMLCK in endothelial cells, nmMLCK^{ec/ec} mice, had exacerbated leakage in lung after LPS treatment compared to wild type mice, as demonstrated by the Evans blue dye permeability assay (Figure S1). In contrast, LPS treatment in nmMLCK^{-/-} mice showed significantly less Evans blue dye leakage in the lungs of these mice. These data strongly demonstrate the importance of nmMLCK during the process of vascular permeability, specifically in lung.

Based on the correlation between nmMLCK expression and the extent of increased vascular permeability *in vitro*, together with the state of lung injuries *in vivo* and the increased expression of nmMLCK in endothelial cells stimulated with VEGF, I

hypothesized that nmMLCK plays a critical role in regulating vascular barrier function and permeability. To test this hypothesis, I utilized an *in vitro* TER permeability assay. Human lung ECs were transfected with siRNA specifically targeting non-muscle isoform of MLCK and were grown to a complete monolayer in ECIS plates coated with gold microelectrodes; the electric resistance across the cultured endothelial cell monolayers was measured across groups. TER measurement of nmMLCK silenced endothelial cells show attenuation of the decreased resistance induced by VEGF stimulation compared to those endothelial cells transfected with scrambled siRNA (Figure 20). These results suggest that nmMLCK plays a major role in regulating endothelial barrier integrity and exacerbating increased vascular permeability caused by VEGF in human lung ECs monolayers.

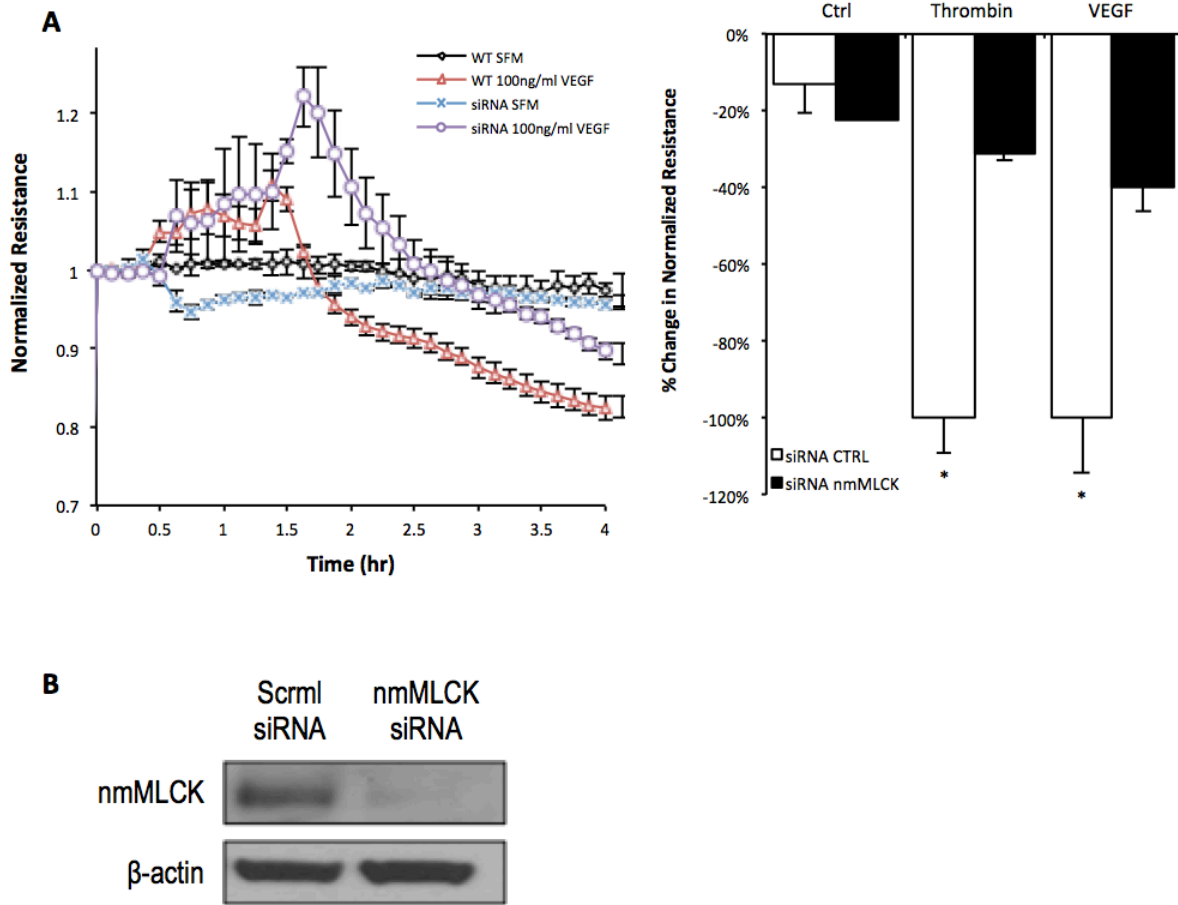


Figure 20: VEGF-induced increased vascular permeability is nmMLCK-dependent

(A) nmMLCK knockdown by siRNA silencing in human lung EC show attenuation of VEGF-induced Transendothelial Electrical Resistance (TER) (n=4). (B) Western blot demonstrating nmMLCK protein silencing. *p < 0.05 compared with control siRNA group.

E Activation of nmMLCK by VEGF signaling in EC

i VEGF induces translocation of nmMLCK in EC

In addition to the kinase activity of nmMLCK on formation of actin stress fibers, nmMLCK also plays key roles in the maintenance of endothelial cell barrier integrity. This dual function of nmMLCK is regulated by post-translational modifications (PTM) ie tyrosine phosphorylation, catalyzed by p60src kinase, c-Abl kinase, or by inhibition of tyrosine phosphatases (vanadate). These PTMs selectively increase nmMLCK activity (Carbajal and Schaeffer 1998; Dudek et al. 2004; Garcia et al. 1997). The vascular barrier enhancement function of the nmMLCK is mediated by the cortical actin-binding protein, cortactin, a 80/85 kDa protein involved in barrier regulation (Dudek and Garcia 2001, Dudek et al. 2004) that localizes to numerous cortical structures within cells (Garcia et al. 1997). Cortactin contains an N-terminal acidic region that stimulates actin polymerization by binding the Arp2/3 complex, which enables the direct binding of F-actin, a proline- and tyrosine-rich site for p60src phosphorylation. In addition, the Cortactin also contains a C-terminal SH3 domain (Weed and Parsons 2001) that binds junctional proteins such as ZO-1 as well as key cytoskeletal effectors such as nmMLCK (Dudek and Garcia 2001; Garcia et al. 1993). This interaction between cortactin and nmMLCK enhances cortical actin formation and tensile strength. The central region of cortactin binds and cross-links actin filaments, with its C-terminus being the site for p60src kinase-mediated phosphorylation to reduce cross-linking activity. Tyrosine phosphorylation of cortactin by p60src potentiates and stabilizes actin polymerization, as well as strengthens cortactin-nmMLCK interactions (Dudek et al. 2002) and is a key

step in a sequence of events that produce cytoskeletal changes, reassembly of adherens junctions, and barrier restoration during lung inflammation.

ii VEGF stimulation signals translocation of nmMLCK to stress fibers

Because of the dual functions of the nmMLCK, the localization of the protein within the cell is a critical determinant of the function of the protein. For example, nmMLCK colocalizes with cortactin at cell periphery to function as a barrier enhancer when endothelial cells are stimulated with barrier enhancing agonists such as S1P. On the other hand, when endothelial cells experience stimulation by agonists that promote vascular permeability such as thrombin and histamine, nmMLCK translocates onto cytosolic actin stress fibers to induce cell contraction. Therefore, it is critical to determine the precise localization of nmMLCK within the cells to determine the specific barrier response to the various stimuli. Here, I examined the localization of nmMLCK in HPAEC after exposure to 100 ng/ml of VEGF using overexpression of EGFP-tagged nmMLCK2 isoform and visualization with confocal microscopy.

Consistent with previous findings, immunofluorescent staining of actin fibers using phalloidin show formation of stress fibers in pulmonary endothelial cells upon VEGF stimulation as short as 5 min post stimulation and that this persists for up to 60 min (Figure 21). In order to examine whether VEGF induces nmMLCK translocation to stress fibers, GFP-tagged nmMLCK2 was overexpressed and followed the movement of nmMLCK in cells. Without VEGF, GFP-tagged nmMLCK is spread out in the cytosol and also present at cell peripheries along with cortical actin (at time 0, Figure 21). When cells are treated with VEGF, nmMLCK translocates to form a fibrous network beginning at 15 min post stimulation, and co-localizes with actin stress fibers, indicated by yellow

on Figure 19. This co-localization of nmMLCK and stress fibers persists for up to 60 min for the duration of the experiment. Furthermore, paracellular gap formation is visible at 60 min post VEGF stimulation between the endothelial cells (Figure 21 ARROW).

Additionally, I confirmed the catalytic activity of nmMLCK in human lung EC after VEGF stimulation using western blot for di-phospho MLC (ppMLC). Phosphorylation of MLC at Ser18 and Thr19 were increased upon VEGF stimulation, indicating increased kinase activity of nmMLCK as well as the formation of actin stress fibers (Figure 21). These results demonstrate that nmMLCK stimulates actin stress fiber formation via phosphorylation of MLC at Ser18 and Thr19 to increase the endothelial cell contractility, which ultimately results in gap formation between endothelial cells.

iii VEGF stimulation increases nmMLCK phosphorylation is increased by VEGF stimulation

As mentioned in the previous section, PTM of nmMLCK is an important modulator of the protein's activity. We previously used mass spectroscopy to show that agonist-stimulated Tyr464 and Tyr471 phosphorylation (unique nmMLCK sites) catalyzed by p60Src and c-Abl, (Figure 5) (Dudek et al. 2010; Yuan 2010; Gilbert-McClain et al. 1998) results in a 3-fold increased nmMLCK1 kinase activity (Yuan 2010; Dudek et al. 2010). Tyr464 and Tyr471 phosphorylation during endothelial cell barrier recovery and enhancement (S1P, HGF) also drives rapid nmMLCK1 translocation (5 min) to lamellipodia (Dudek et al. 2010; Zhao et al. 2009). These studies suggest Tyr464 within an SH2-binding domain regulates specific nmMLCK interactions with cytoskeletal regulatory proteins.

In order to determine whether VEGF alters PTM of nmMLCK, I examined the phosphorylation of nmMLCK at Tyr464 at various time points after VEGF stimulation. Immunoprecipitation using an anti-phosphotyrosine primary antibody followed by western blotting of samples probing for phospho-nmMLCK Tyr464 revealed a robust phosphorylation of nmMLCK at Tyr464 after 5 min of VEGF stimulation (Figure 22) with phosphorylation returning to baseline by 30 min.

Our long-term goal is to better understand the role of nmMLCK in molecular mechanisms underlying increased vascular permeability and inflammation in pathological conditions such as, asthma. Therefore, I sought to determine the phosphorylation status of nmMLCK at Tyr464 in lung tissue samples taken from asthmatic patients. I have observed a marked increase in Tyr464 phosphorylation in asthmatic tissue containing both airways and lung vasculature, whereas staining was minimal in normal human airways (Figure 23). These data are consistent with the involvement and activation of nmMLCK during development of asthmatic inflammation.

F Summary and discussion

VEGF/VPF is a potent permeability-inducing molecule with a pivotal role during physiological and pathological inflammation. Expression of VEGF in adults remains high in lung tissues compared to other tissues, which has been shown to be further increased during pulmonary inflammation such as ALI/ARDS and asthma (Thickett et al. 2001; Feltis et al. 2006; Kanazawa 2002). In the previous chapter, I have demonstrated that the expression of nmMLCK, a key kinase regulating vascular permeability, and have shown increased levels upon VEGF stimulation. In this present study, I sought to

examine the role of VEGF in altering the activity of nmMLCK in pulmonary endothelial cells.

As I have previously shown using an *in vitro* TER permeability assay system, that VEGF is able to induce vascular permeability in a biphasic and dose-dependent manner. Endothelial cells respond to VEGF in an acute phase within seconds after the exposure and causes transient increases in vascular permeability, which becomes resolved within minutes. However, vascular permeability increases again after 1 hr of the initial exposure to VEGF; this chronic phase is sustained for 24 hours and beyond after stimulation and likely to be regulated by different mechanisms compared to the acute phase. Increased phosphorylation of MLC at Ser18 and Thr19 was observed after 24 hrs of exposure to VEGF, indicating actin cytoskeleton remain arranged into contractile stress fibers for prolonged periods of time despite the short half life (90 min) of VEGF in physiological conditions (Kleinheinz et al. 2010). Interestingly, VEGF decreases vascular permeability at low concentrations at 5-10 ng/ml on human lung EC. However, the mechanisms that underlie this observation remain unclear.

Multiple phosphorylation sites on VEGFR2 have been identified, and each phosphorylation site serves to signal distinct downstream effector proteins and signaling pathways. Our findings suggest that phosphorylation at each tyrosine residue occurs with different timing. Two tyrosine residues at aa951 and aa996 that reside within KID are autophosphorylated upon VEGF binding to VEGFR2, and this phosphorylation is sustained for 24 hrs after the stimulation. Phosphorylation at these residues induces Src-mediated increase in vascular permeability, and the sustained phosphorylation suggests the chronic phase of VEGF-induced vascular hyperpermeability is Src

dependent. Similar observations have been made for the phosphorylation status of Tyr1175, where transient increases in phosphorylation status occur within minutes after stimulation. Phosphorylation at this residue has also been shown to be associated with increased vascular permeability, however, by a mechanism involving PLC γ mediated eNOS activation. Contrary to Tyr 951 and 996, phosphorylation at Tyr1175 persisted for longer time, however at much lower levels than the initial acute stage, suggesting modification at this residue may play a role in the chronic stage of VEGF-induced vascular permeability. Phosphorylation at Tyr1175 induce eNOS mediated vascular permeability via Shb and Akt. Further studies using inhibitory chemicals or siRNAs is necessary in order to delineate the exact mechanisms associated with VEGF-induced vascular hyperpermeability mediated by nmMLCK. Additionally, two autophosphorylated tyrosine residues are Tyr1054 and 1059 located in the catalytic domain. Phosphorylation at these sites is required for maximum kinase activity of VEGFR2, and our findings show that the transient phosphorylation at these residues from VEGF stimulation is greatest within 30 min of exposure, suggesting its involvement in the acute phase. However, the association of such modifications on tyrosine residues in the kinase catalytic domain with increased vascular permeability remains to be determined.

The VEGF/VEGFR2 complex formation can also induce increases in cytosolic Ca²⁺ concentrations. This is achieved by two ways: 1) release of ER stored Ca²⁺ mediated by PLC γ that hydrolyzes PIP2 into IP3 and DAG (Nilius and Droogmans 2001; Tiruppathi et al. 2006), and 2) Ca²⁺ influx from the extracellular space that is mediated by membrane cation channels such as, the TRIPC family (Chakrabarti and Chakrabarti 2006; Nilius et al. 2003). Increases in cytosolic calcium via storage operated channels

or by IP3 driven mobilization of intracellular Ca^{2+} is critical to the development of contractile tension via transcellular actomyosin stress fiber formation, cortical actin ring disassembly and paracellular gap formation (Kolodney and Wysolmerski 1992, Phillips et al. 1989, Majno and Palade 1961). Phosphorylation of regulatory MLC is primarily catalyzed by Ca^{2+} /calmodulin (CaM)-dependent MLCK in closely coordinated activity with the Rho/Rho kinase pathway. Therefore, in addition to direct phosphorylation of nmMLCK by Src or cAbl activated by VEGF, stimulation promotes actomyosin contraction via activation of nmMLCK by Ca^{2+} binding.

I have provided strong evidence to support a critical role of nmMLCK in VEGF-induced vascular permeability: 1) the decrease in TER caused by VEGF was attenuated in endothelial cells that had been silenced for nmMLCK using siRNA; 2) VEGF stimulated translocation of nmMLCK onto stress fibers within endothelial cells; 3) increased phosphorylation levels on VEGFR2 at Tyr464 was observed, suggesting increased kinase activity of nmMLCK; and 4) I observed increased phosphorylation of nmMLCK at Tyr464 within the arteries of lung biopsies taken from asthmatic patients compared to those of healthy individuals, indicating the clinical importance of activation of nmMLCK in asthma pathophysiology.

Together this study suggests that nmMLCK plays an important role in inducing vascular permeability when endothelial cells are exposed to VEGF. Several inhibitors have been used to block nmMLCK's activity *in vitro* (ML7, ML9, and inhibitor peptides) and to attenuate vascular hyperpermeability induced by various agonists both *in vitro* and *in vivo*. Specifically, the use of lipid vehicles to deliver siRNA specifically to endothelial cells has proven beneficial in a mouse model of ALI and asthma. However,

the efficacy of using such inhibitors and gene silencing method for therapeutic purposes needs to be further examined.

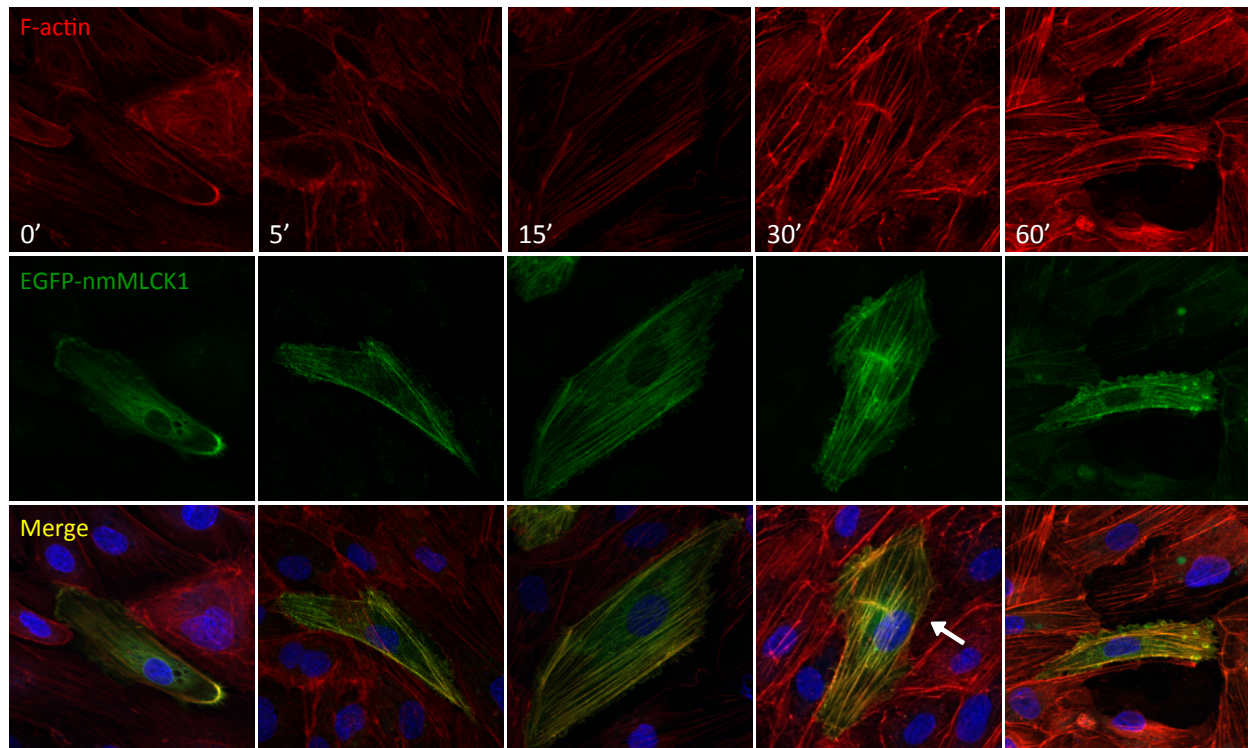


Figure 21: VEGF stimulates translocation of nmMLCK protein

EGFP conjugated nmMLCK were ectopically expressed in human lung EC, treated with VEGF (100ng/ml) for 0, 5, 15, 30, and 60 min. nmMLCK is initially translocated to cell periphery (5'), followed by translocation and co-localization with actin stress fiber (< 15').

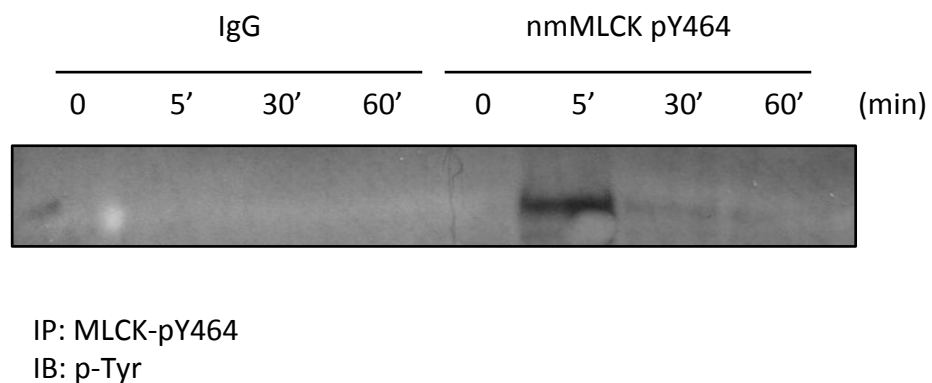


Figure 22: Phosphorylation of nmMLCK evoked by VEGF

Representative western blot of lysates of human lung EC treated with VEGF after nmMLCK immunoprecipitation using anti-phospho Y464 nmMLCK antibody and blotted for phospho-tyrosine.

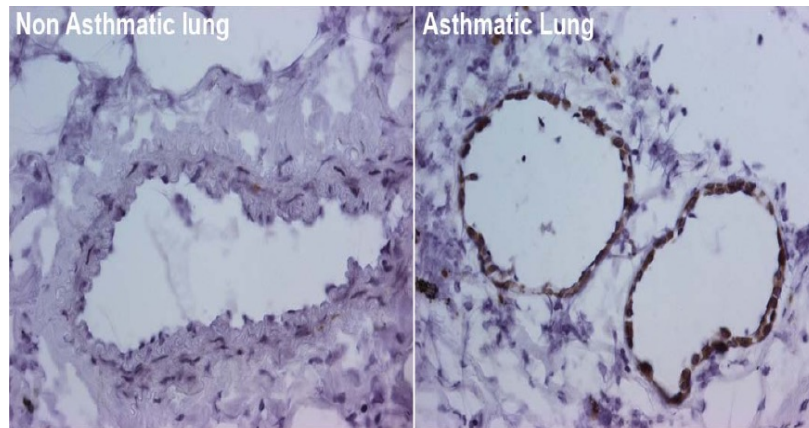


Figure 23 Detection of nmMLCK Y464 phosphorylation in endothelium in human lung endothelium of asthmatic lung tissues

(Left) Representative lung tissue from 5 non-asthmatic human subjects showing absence of immunoreactivity (0/5). (Right) Representative lung tissue from 5 asthmatic subjects demonstrating strong phospho-Y464 immunoreactivity in lung vascular endothelium (4/5).

Chapter V: Conclusions

Pulmonary inflammatory diseases are significant causes of illness and death, and continue to be a significant healthcare problem affecting approximately 1 billion individuals annually worldwide. In the US alone, hospital visits for ALI measure 200,000 cases per year with a high mortality rate at up to 58% once progressed to ARDS, and an estimation of 74,500 deaths every year (Sweeney et al. 2013). In addition, 300 million people worldwide suffer from asthma, with 250,000 annual deaths attributed to the disease (Pawankar 2014).

Given the high incidence of hospital admissions and death due to ALI and ARDS, there have been great efforts spent to develop therapies to treat and reduce the mortality rate of ALI/ARDS. For example, various therapies have been investigated such as, anti-inflammatory agents, diuretics, β_2 agonists, pharmaco-nutrients, anti-oxidants, anti-proteases, and cell-based therapies, however, none of these are able to decrease mortality. Of these, β_2 agonists are the most studied pharmacological agents for pulmonary disease and are currently used to treat asthma symptoms, by inducing smooth muscle relaxation and vasodilatation. *In vivo* and early clinical studies have shown the positive effects of β_2 agonists in ALI/ARDS; which have been attributed to improved clearance of alveolar fluid (Shale 1987). However, a recent larger study failed to demonstrate significant benefit of β_2 agonists on clinical outcomes (ie. ventilator-free days and death rate) in patients with ALI/ARDS (Matthay et al. 2011). The lack of positive outcomes using pharmacological agents in these previous clinical trials, suggests that these agents fail to specifically target the disease and underscores the importance of further understanding the ALI/ARDS disease etiology. This will potentially

enable the development of more disease specific agents that are more likely to alleviate symptoms and reduce mortality.

Increased vascular permeability and accumulation of alveolar and airway fluid are cardinal features of pulmonary inflammatory diseases such as ALI/ARDS, RILI, and asthma. The pulmonary edema fluid that is accumulated during pathological conditions is protein-rich and contains inflammatory cytokines, endotoxins, and cells such as granulocytes and other leukocytes (Matthay and Zimmerman 2005); and such fluid itself can serve to further contribute to the inflammatory process. The major mediator of edema during lung inflammation is increased pulmonary vascular permeability, and has been linked to disease severity and is thought to be responsible for the profound symptoms observed in these disorders.

Under normal circumstances, vascular barrier regulation is a key process for systemic homeostasis by allowing the exchange of fluids, solutes, plasma proteins, inflammatory cytokines, and nutrients to tissues throughout the body. This dynamic regulation is controlled by the endothelial cells that comprise the inner lining of the vessel wall and form a tight cell monolayer. The permeability of this vascular barrier is tightly regulated by physiological signals, and allows size-selective and semi-permeable fluid exchange between the blood stream and tissue interstitium (Komarova and Malik 2010). Numerous studies have established that a failure to maintain such regulation can lead to detrimental lung inflammation and progression to conditions such as, ALI/ARDS, RILI, and asthma. In addition to pulmonary inflammation, disruption in the integrity of the EC monolayer is now recognized as a major player in local and systemic inflammation, ischemia-reperfusion injury, apoptosis, and angiogenesis. The mechanisms underlying

the disruption in EC integrity include: mechanical stress factors, inflammatory mediators, activated neutrophil products such as reactive oxygen species, proteases and cationic peptides. In this study, I focused on the mechanisms that regulate paracellular vascular permeability leading to inflammation in the lung.

A central player in vascular barrier regulation and maintenance of the integrity of endothelial cell monolayers is the actin cytoskeleton. Actin and myosin are the most abundant proteins in the endothelial cells, and form actin filaments that control a variety of biological processes including maintenance of the cell shape, movement, and contraction. Actin filaments bind to plasma membrane proteins to control cell-cell and cell-matrix interactions by stabilizing intercellular junctions, and in doing so, tightly regulate the vascular barrier, which enables a controlled fluid and nutrition exchange between circulating blood and tissues. Formation of contractile stress fibers as a result of exposure to pathological extracellular stimuli is the critical step in promoting formation of paracellular gaps between endothelial cells to increase vascular permeability.

Factors affecting barrier permeability are upstream mediators such as thrombin and VEGF. In particular, VEGF has been linked to both ALI/ARDS and asthma and has been associated with disease severity. Indeed, several studies have shown that VEGF is present at higher levels in the plasma of ARDS patient's compared to non-ARDS patients (Thickett et al. 2001), as well as phase-dependent increases of VEGF during the initial stage of ARDS (Azamfirei et al. 2010). Additionally, the SNP haplotype (-460T+405C+936T) on the VEGF gene has been associated with a high mortality rate for ARDS (Zhai et al. 2007), whereas, the haplotype (-460C+405G+936T) was less associated - consistent with lower plasma VEGF levels. Similarly, clinical reports

measuring levels of cytokines BAL in blood (226 vs 177 pg/ml, $p < 0.05$) and sputum (7051 vs 1345 pg/ml, $p < 0.001$) samples taken from asthmatic patients have demonstrated that VEGF bioavailability is increased (Feltis et al. 2006; Kanazawa 2002).

Major efforts have been aimed at elucidating downstream pathways activated by these molecules. Our lab previously discovered the *nmMYLK* gene and has provided substantial evidence for a role for nmMLCK in controlling vascular permeability. Previous studies from our group have demonstrated that nmMLCK serves as the major gate-keeper of the endothelial cell barrier in a number of *in vitro* and *in vivo* models. Specifically, nmMLCK plays a critical role in controlling vascular barrier regulation upon activation by extracellular agonists by leading stress fibers formation. nmMLCK protein has been shown to bind to numerous actin-associating proteins such cortactin, paxillin, FAK, Arp2/3 (Dudek and Garcia 2001). Our studies have identified nmMLCK as one of two main proteins that control the phosphorylation of MLC, therefore driving the formation of stress fibers and actomyosin contraction. Importantly, inhibition of nmMLCK activity by the chemical inhibitors ML7 or ML9 attenuates thrombin- or TNF- α -induced vascular permeability and phosphorylation of MLC *in vivo* (Petrache et al. 2001; Kawkitinarong et al. 2004).

Our previous studies using an *in vivo* ALI mouse model challenged with LPS demonstrated that nmMLCK^{-/-} mice are protected from Evans blue dye extravasation caused LPS whereas nmMLCK^{ec/ec} mice experienced significantly worse vascular leak (Figure S1). These data clearly show that nmMLCK mediates vascular leakiness in the lungs during edema formation and inflammation. Consistent with this, we have further demonstrated that nmMLCK^{-/-} mice could be protected from acute lung injury induced by

LPS (Mirzapoiazova et al. 2011); in contrast, edema formation and inflammation are exacerbated in LPS-treated nmMLCK^{ec/ec} mice overexpressing nmMLCK.

However, the additional molecules that cooperate with nmMLCK to control the vascular barrier response are yet to be determined. The elucidation of novel molecules orchestrating this vascular barrier response through nmMLCK would increase the number of potential therapeutic targets and may lead to the development of more disease specific therapies with greater efficacy. It was therefore the aim of this thesis to determine the genetic mechanisms underlying the modulation of nmMLCK expression and activity during conditions of vascular barrier dysfunction.

Our initial examination of the expression levels of nmMLCK showed nmMLCK to be increased in tissue samples from asthma patients. Additional *in vivo* studies using experimental models of asthma and RILI were consistent with this by demonstrating that nmMLCK levels are increased at both the mRNA and proteins levels. The common feature of asthma and RILI is inflammation; suggesting inflammation leads to increased expression levels of nmMLCK. In addition to this, various pulmonary inflammatory diseases such as ALI/ARDS and asthma share features that is associated with alterations in vascular barrier integrity. For example, both in ALI and asthma, increased VEGF levels are detected in plasma and sputum, strongly suggesting VEGF plays a critical role in modifying the mechanisms of vascular permeability under inflammatory conditions.

I herein demonstrate that VEGF can in fact influence on nmMLCK expression directly in endothelial cells by modulating the promoter activity of the *nmMYLK* gene. Detailed analysis of the *nmMYLK* promoter using *in vitro* luciferase promoter activity

assay in combination with *in silico* transcription factor binding predictions, have identified Sp1 and Sp3 as a critical transcription factors that promote nmMLCK expression upon VEGF stimulation. Furthermore, binding by these transcription factors on *nmMYLK* promoter were confirmed using ChIP assay. Previous studies have demonstrated that gene regulation of Sp1 and Sp3 target genes is achieved by binding of co-factors. Numerous binding partners have been identified for Sp1 and Sp3 including histone acetyltransferases, HDACs, co-activator proteins p300/CBP, as well as DNA binding proteins (ER α , E2F) and chromatin remodeling proteins such as SWI/SNF. In this study, I have identified the critical binding region on *nmMYLK* promoter that Sp1 and Sp3 binds to and confirmed that binding is increased with VEGF stimulation of endothelial cells. Further studies are necessary to determine the exact mechanism underlying *nmMYLK* gene activation by Sp1 and Sp3. Possible mechanisms may include, Sp1/3 interactions with their binding partners that could lead to either promotion or repression of transcription by various mechanisms such as: 1) enhancing binding/recruitment of other activator proteins, 2) opening chromatin to make gene promoter regions accessible, 3) blocking DNA binding when bound to other proteins, and 4) binding to both HDAC and p300 and establish dynamic chromatin modification. Future experiments designed to test these mechanistic hypotheses will provide further detailed understanding of the control of the *nmMYLK* gene by Sp1 and Sp3 transcription factors.

Sp1/3 bind to their common consensus binding sequences and have high affinity for GC rich sequences. Interestingly, binding to their consensus binding sequence is not reduced with CpG methylation (Zhu et al. 2003). Our *in silico* data indicates that the

nmMYLK promoter is methylated with presence of CpG islands within 600 bp from the TSS. Preliminary *in vitro* experiments using 5-Aza treatment on luciferase promoter constructs containing *nmMYLK* promoter suggest overall CpG island methylation at basal levels. Additional preliminary results from *in vitro* experiments using bisulfite conversion of genomic DNA from pulmonary EC suggests that the cytosine at the critical Sp1 binding site at -366 shows hypermethylation when treated with VEGF compared with the unstimulated EC. In spite of the increased methylation of the promoter activity of *nmMYLK*, the expression of the *nmMYLK* gene is increased with VEGF stimulation, which is consistent with the notion that methylation of CpG specifically at the Sp1/3 consensus binding site do not influence Sp1 binding. These data may suggest alternative roles for methylation at this site. Interestingly, methylation on sites surrounding the Sp1/3 consensus binding site have been shown to influence Sp1/3 binding (Zhu et al. 2003; Harrington et al. 1988; Douet et al. 2007). Specifically, there are two CpG sites immediately upstream of the -366 Sp1 binding sequence showing decreased methylation with VEGF stimulation compared to unstimulated cells (33% to 0%) These data suggest CpG sites are more likely to play an important role in determining the binding of Sp1 and/or Sp3 to influence the transcriptional activity of *nmMYLK*. This hypothesis can be examined in future experiments by creating site-specific methylation mutant constructs using *in vitro* methylation methods on mutated luciferase promoter constructs (Mamrut et al. 2013).

Another possible mechanism is that Sp1 increases its activity to enable increased expression. For example, post-translational modifications, such as phosphorylation, glycosylation, sumoylation, acetylation (Tan and Khachigian 2009)

could potentially alter transcriptional activity. Phosphorylation sites for Sp1 have been identified, yet whether VEGF signaling alters PTM of Sp1 has not been studied. Therefore, it will be worthwhile examining the phosphorylation status of Sp1 upon VEGF stimulation in part to determine whether these PTM of nmMLCK underlay the activation of *nmMYLK* promoter by Sp1.

I have shown that, silencing Sp1 attenuates the increased expression of nmMLCK and increased permeability induced by VEGF, which verifies the critical role of Sp1 in VEGF induced vascular hyperpermeability mediated by nmMLCK. Interestingly, silencing Sp1 does not completely reverse the induced vascular permeability *in vitro*, suggesting additional factors are involved in the process of enhanced vascular permeability by VEGF. Although reducing expression of Sp1 protects the endothelial cell monolayer from increasing vascular permeability, Sp1 is a ubiquitously expressed transcription factor that is essential for variety of biological processes. Therefore targeting Sp1 for therapeutic purposes might not be suitable in systemic manner. Alternatively, targeting nmMLCK by pharmacological inhibitors such as PIK or ML-7 locally in pulmonary tissue may be a better alternative to reduce inflammation in lung diseases.

Future perspectives

Novel therapies are desperately needed to target the pulmonary inflammation in order to reduce side effects and increase efficacy. In this study, I have demonstrated the association of increased nmMLCK expression and activity in asthmatic lung tissues and in disease models of pulmonary inflammatory diseases including ALI/ARDS, RILI,

and asthma. Therefore, targeting nmMLCK to inhibit its activity or to reduce expression could be considered as a novel therapy for lung inflammatory diseases. We have previously considered nmMLCK as a druggable target and have conducted complementary *in vivo* studies using targeted MLCK inhibitors (PIK) as well as nmMLCK siRNA delivered selectively to the pulmonary endothelium using a novel ACE antibody-conjugated liposomal delivery system. Both methods conferred similar improvements in barrier function with nmMLCK inhibition (Mirzapoiazova et al. 2011).

I further demonstrated that methylation status perhaps adversely affects ARDS and asthma, and is likely to enhance nmMLCK expression. The use of such drugs to target the epigenetic machinery has attracted pharmaceutical interest, particularly for cancers. Indeed, targeting specific epigenetic modifications in certain cancer types has shown promise, with some drugs entering Phase I clinical trials (Helin and Dhanak 2013). A similar approach could be used to treat pulmonary inflammatory diseases.

Within the epigenetic machinery are different classes of molecules (i.e. HDACs) and specific inhibitors for each class or family of proteins are being developed (Gräff and Tsai 2013). Since our data strongly point to the endothelial cell monolayer being the critical mediator of disease in ALI/ARDS, the lack of cell specificity of these drugs may be an obstacle to achieving true efficacy without having significant adverse effects. Perhaps cell-specific-marker antigens could be used for delivery to specific cell types such as endothelial cells, which has been tried elsewhere (Mirzapoiazova et al. 2011). Finally, a more thorough understanding of the epigenetic regulatory mechanisms in ALI/ARDS and asthma may provide further candidate molecules or aid the ARDS/ALI drug development process. Importantly, drugs are being developed elsewhere that

reverse the methylation status on genes other than *nmMLCK* and have shown therapeutic potential (Carey et al. 2011). Given our findings in this study, it would be useful to begin testing these compounds under experimental conditions similar to the ones described here. These experiments may lead to direct translation of these compounds for therapeutic use in the setting of ALI/ARDS.

This study has provided a more detailed picture of how *nmMYLK* can be activated to promote disease progression in ALI/ARDS thereby setting a more solid foundation for using pharmacogenomic approaches using *nmMYLK* SNP haplotypes to tailor specific therapies to individuals from different racial backgrounds (European descents, African descents, Hispanic, Native Americans, etc). A pharmacogenomics approach has already proven beneficial for the treatment of asthma with β_2 -adrenergic receptor agonists (Ortega, VE). Further candidate genes profiles such as those of *nmMYLK* could be combined with those of other genes to improve personalized treatment options for ARDS/ALI and asthma patients. It will be important to confirm the mechanism by which SNPs are able to alter *nmMYLK* behavior and ultimately disease severity. A simple first step would be to repeat the luciferase experiments with *nmMYLK* promoter containing SNPs to compare the transcriptional efficiencies across SNP profiles.

The regulation of vascular permeability is a well-orchestrated process in maintaining fluid homeostasis that requires finely tuned responses to various physiological stimuli. Endothelial cells form a tight cell monolayer lining the inner vessel, and wall is the key gatekeeper separating the blood stream and tissue interstitium (Komarova, Malik). A failure to maintain such regulation leads to detrimental

inflammation in the lung and development of conditions such as, ALI, ARDS, RILI, and asthma.

Cytoskeletal components are involved in a wide range of biological processes critical for endothelial cell function. Our group and others have demonstrated the multifunctional role of endothelial actomyosin microfilaments with a particular emphasis on vascular barrier regulation. We have demonstrated here that nmMLCK, is a common gateway that regulates vascular permeability, and is involved in pathogenesis of ALI/ARDS and asthma by exhibiting increased endothelial cell expression and activity. We have unveiled key regulatory mechanisms by which nmMLCK expression is altered, and have provided potential novel targets for pulmonary inflammatory diseases. Taken together, development of pharmacological agents targeting nmMLCK or factors that alter nmMLCK expression and activity, may enable the development of improved therapies for ALI/ARDS and asthma to reduce the significant morbidity and mortality associated with these diseases.

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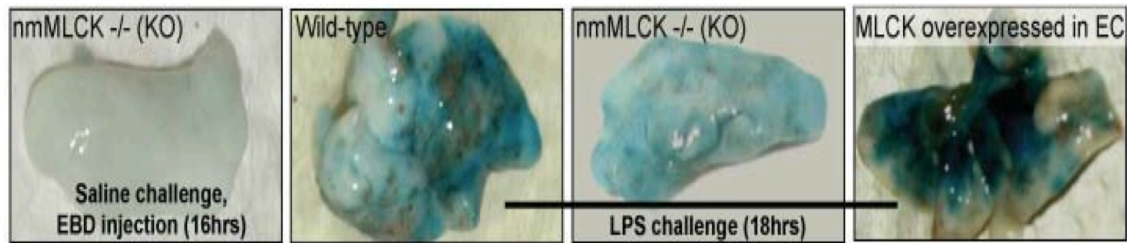
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APPENDIX A



Supplemental Figure 1

Evans Blue dye extravasation in wild type mice- saline (Panel A). Wild type mice (Panel B), nmMLCK-/- (KO) mice (Panel C) or nmMLCK2 overexpressing mice confined to ECs (Panel D) receiving LPS. Representative lung Images.

APPENDIX B



Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

March 20, 2013

Joe G.N. Garcia
Medicine/Pulmonary, Critical Care & Sleep Medicine
M/C 719

Dear Dr. Garcia:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **2/19/2013**. *The protocol was not initiated until final clarifications were reviewed and approved on 3/18/2013. The protocol is approved for a period of 3 years with annual continuation.*

Title of Application: Toxicogenomic Study of Particulate Matter Air Pollution
ACC Number: 13-019
Initial Approval Period: 3/18/2013 to 2/19/2014

Current Funding: *Portions of this protocol are supported by the funding sources indicated in the table below.*

Number of funding sources: 1

Funding Agency	Funding Title	Portion of Proposal Matched		
Parker B. Francis	Mechanisms of Particulate Matter Induced Cardiac Arrhythmias	All matched		
Funding Number	Current Status	UIC PAF NO.	Performance Site	Funding PI
N/A	Funded	2012-01074	UIC	Ting Wang

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the*

Use of Animals" (<http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf>) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,



Bradley Merrill, PhD
Chair, Animal Care Committee

BM/mbb

cc: BRL, ACC File, Ting Wang, Liliana Moreno, Carrie Kempf, PAF 2012-01074



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2. Matthew, B., **Shimizu, Y.**, Elangovan, V., Rizzo, A., Dudek, S.M., Weichselbaum, R.R., Jacobson, J.R., Garcia, J.G.N. Non-Muscle Myosin Light Chain Kinase modulates radiation-induced lung injury. *Microvascular Research* (submitted), 2014.
3. **Shimizu, Y.**, Garcia, J.G.N., (2013) *Chapter 1. Multifunctional Role of the Endothelial Actomyosin Cytoskeleton*, Endothelial Cytoskeleton (Science Publishers)
4. **Shimizu, Y.**, Mhakta, M.S., Segal D.J. (2009) Restricted spacer tolerance of a zinc finger nuclease with a six amino acid liner. *Bioorg Med Chem Lett*, 19, 3970-3392
5. **Shimizu, Y.**, Solli, C., Meckler, J.F., Adriaenssens, A., Zykovich, A., Cathomen, T., Segal, D.J. (2011) Adding fingers to an engineered zinc fingers can reduce activity. *Biochemistry*, 5(22), 5033-5041

IN PREPARATION

1. **Shimizu, Y.**, Sun, X., Zhou, T., Wang, T., Garcia J.G.N. nmMLCK mediates VEGF induced vascular permeability in pulmonary artery endothelial cells (In preparation)
2. Rizzo, A.N.*, **Shimizu, Y.***, Garcia, J.G.N. Regulation of Pulmonary Vascular Permeability by the Non-Muscle Isoform of Myosin Light Chain Kinase, Review. (In preparation)
3. Wang T*, **Shimizu Y***, Wang L, Garcia, J.G.N. Particulate Matter Disrupts Endothelial Barrier via Rho-dependent Pathways (In preparation)

PRESENTATIONS

- **Shimizu, Y.**, Sun, X.G., Zhou, T., Wang, T., Garcia, J.G.N. Increased Expression Of Non-Muscle Myosin Light Chain Kinase In VEGF-Induced Vascular Hyperpermeability During Asthmatic Inflammation. American Thoracic Society Annual Meeting 2014 (San Diego, California) (Oral abstract presentation)
- **Shimizu, Y.**, Sun, X.G., Zhou, T., Wang, T., Garcia, J.G.N. Increased Expression Of Non-Muscle Myosin Light Chain Kinase In VEGF-Induced Vascular Hyperpermeability During Asthmatic Inflammation. International Vascular Biology Meeting 2014 (Kyoto, Japan) (Poster presentation)

- **Shimizu, Y.**, Sun, X.G., Zhou, T., Wang, T., Garcia, J.G.N. *Non-Muscle Myosin Light Chain Kinase Mediates Vascular Endothelial Growth Factor-Induced Vascular Hyperpermeability via Specificity Protein 1 During Asthmatic Inflammation*. American Heart Association Annual Meeting 2013 (Dallas, Texas) (Oral abstract presentation)
- **Shimizu, Y.**, Sun, X.G., Zhou, T., Wang, T., Garcia, J.G.N. *The role of nmMLCK in VEGF-induced hyperpermeability of Asthma*. American Thoracic Society Annual Meeting 2013 (Philadelphia, Pennsylvania) (Poster presentation)
- **Shimizu, Y.**, Sun, X.G., Wang, T., Garcia, J.G.N. *The role of nmMLCK in VEGF-induced hyperpermeability of Asthma*. Health Disparities in Lung Disease Symposium 2012 (Chicago, Illinois) (Poster presentation)
- **Shimizu, Y.**, Sollu, C., Cathomen, T., Segal, D.J. *Is more the better? –Influence of Zinc Finger Numbers on the Activity of Engineered Zinc Finger Nuclease*. American Society of Gene Therapy 2008 (Seattle, Washington) (Poster presentation)
- Bhakta M.S., Brayer K.J., Lockwood S.H., Ooi A.T., **Shimizu Y.**, Zykovich A., Segal D.J. *Engineering zinc finger DNA-binding proteins for diagnostics and gene therapy*. Annual Biochemistry and Molecular Biology Graduate Group Colloquium 2007 (Davis, California) (Poster presentation)
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