MicroRNA Profiling in Experimental Sepsis-Induced Acute Lung Injury

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

MicroRNA Profiling in Experimental Sepsis-Induced Acute Lung Injury

Master of Science

Year of Convocation – 2014

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Institute of Medical Science

University of Toronto

Introduction: Currently, there are no specific pharmacological treatments for sepsis-induced acute respiratory distress syndrome (ARDS). And mesenchymal stem cells (MSCs) have shown reparative potential in both sepsis and ARDS.

Objectives: To determine the role of MSC administration in the modulation of pulmonary host-responses to sepsis via differential regulation of regulatory microRNAs (miRNAs/miRs).

Methods: MicroRNA and mRNA profiling was performed to identify differential expression. Quantitative real time polymerase chain reaction (qRT-PCR), trans-endothelial electrical resistance (TEER) measurements, and luciferase activity assay were used.

Results: MicroRNA expression was examined in Human Pulmonary Microvascular Endothelial Cells (HPMECs). One miRNA – miR-193b-5p, targets occludin, a tight junction protein associated with endothelial leakage. A specific regulatory relationship between miR-193b-5p and occludin...
was identified. The loss in endothelial integrity was rescued when miR-193b-5p inhibitor was transfected.

**Conclusion**: miR-193b-5p is a suppressor of occludin. Studying transcriptional changes allows identification of therapeutically relevant mediators for ARDS/ALI treatment.
Acknowledgements

It would not have been possible to write this Master’s thesis without the help and support of the people around me, to only some of whom it is possible to give particular mention here.

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Dun Zhou

Aug 6, 2013
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<tbody>
<tr>
<td>3’UTR</td>
<td>3’ untranslated region</td>
</tr>
<tr>
<td>5’UTR</td>
<td>5’ untranslated region</td>
</tr>
<tr>
<td>Ago2</td>
<td>argonaute 2</td>
</tr>
<tr>
<td>ALI</td>
<td>acute lung injury</td>
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<tr>
<td>Ang-1</td>
<td>angiopoietin 1</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>APACHE II</td>
<td>acute physiology and chronic health evaluation II</td>
</tr>
<tr>
<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAL</td>
<td>broncoalveolar lavage</td>
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<tr>
<td>CIP</td>
<td>calf-intestinal alkaline phosphatase</td>
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<td>CLP</td>
<td>cecal ligation and perforation</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3C chemokine receptor 1</td>
</tr>
<tr>
<td>DAD</td>
<td>diffuse alveolar damage</td>
</tr>
<tr>
<td>DGCR8</td>
<td>microprocessor complex subunit DGCR8</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>EBM-2</td>
<td>Endothelial Cell Basal Medium MV 2</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>eIF4G</td>
<td>eukaryotic translation initiation factor 4G</td>
</tr>
<tr>
<td>eIF6</td>
<td>eukaryotic initiation factor 6</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<tr>
<td>FDR</td>
<td>false discovery rate</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-----------------------------------------------------------------------------</td>
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<tr>
<td>MiRISC</td>
<td>miRNA-incorporated RNA-induced silencing complex</td>
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<tr>
<td>MSCs</td>
<td>mesenchymal stem cells</td>
</tr>
<tr>
<td>MSC-CdM</td>
<td>mesenchymal stem cell conditioned medium</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OCC2</td>
<td>occludin second extracellular domain peptide</td>
</tr>
<tr>
<td>OCLN</td>
<td>occludin</td>
</tr>
<tr>
<td>PACT</td>
<td>protein kinase R activating protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>principal components analysis</td>
</tr>
<tr>
<td>pCO₂</td>
<td>partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>pO₂</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNase III</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SMAD1</td>
<td>SMAD family member 1</td>
</tr>
<tr>
<td>SOFA</td>
<td>sequential organ failure assessment</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TC</td>
<td>tissue culture</td>
</tr>
<tr>
<td>TER</td>
<td>transepithelial resistance</td>
</tr>
<tr>
<td>TEER</td>
<td>transendothelial electrical resistance</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TJ</td>
<td>tight junction</td>
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<tr>
<td>TLR2</td>
<td>toll-like receptor 2</td>
</tr>
<tr>
<td>TLR3</td>
<td>toll-like receptor 3</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNFa</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFRSF1B</td>
<td>tumour necrosis factor receptor super family 1 B</td>
</tr>
<tr>
<td>TNFRSF11B</td>
<td>tumour necrosis factor receptor super family 11 B</td>
</tr>
<tr>
<td>TRBP</td>
<td>TAR RNA binding protein</td>
</tr>
<tr>
<td>VAP-33</td>
<td>vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa</td>
</tr>
<tr>
<td>VA/Q ratio</td>
<td>ventilation/perfusion ratio</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VILI</td>
<td>ventilator induced lung injury</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>ZIP8</td>
<td>zinc transporter ZIP8</td>
</tr>
<tr>
<td>ZO-1</td>
<td>tight junction protein 1</td>
</tr>
<tr>
<td>ZO-2</td>
<td>tight junction protein 2</td>
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<td>ZO-3</td>
<td>tight junction protein 3</td>
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1.1 Acute Respiratory Distress Syndrome (ARDS)

1.1.1 Definition and Background of ARDS

Acute respiratory distress syndrome, is characterized by increased permeability of the alveolar-capillary barrier, and accumulation of a protein-rich pulmonary edema, and infiltration of neutrophils, macrophages, and cytokines. The term acute lung injury encompasses a spectrum of bilateral pulmonary damage (endothelial and epithelial), which can be initiated by numerous conditions. Clinically, acute lung injury manifests as acute respiratory distress syndrome (ARDS): (1) acute onset of dyspnea, (2) decreased arterial oxygen pressure (hypoxemia), and (3) development of bilateral pulmonary infiltrates on the chest radiograph, in the absence of clinical evidence of primary left-sided heart failure [1, 2]. Histologically, diffuse alveolar damage is the major feature of ARDS and this is in turn characterized by damage to the alveolar capillary membrane, inflammatory cell infiltration, exudative pulmonary edema and hyaline membrane deposition.

The incidence ARDS is variable among studies, it ranges from 5 to 86 cases per 100,000 person-years [3-5]. Overall, in the United States, ALI has a high incidence of approximately 200,000 per year. Moreover, mortality remains high ARDS. The 2011 Berlin definition revised the 1994 criteria, aiming to address issues of reality and validity that had emerged over the years [2, 6]. Acute lung injury (ALI) was eliminated as a distinct category and ARDS was re-classified on the basis of severity of hypoxemia: mild \((200 \text{ mm Hg} < \frac{\text{PaO}_2}{\text{FiO}_2} \leq 300 \text{ mm Hg})\), moderate \((100 \text{ mm Hg} < \frac{\text{PaO}_2}{\text{FiO}_2} \leq 200 \text{ mm Hg})\), and severe \((\frac{\text{PaO}_2}{\text{FiO}_2} \leq 100 \text{ mm Hg})\).
Patient level meta-analysis of 4188 ARDS patients from four multicentre clinical data sets was used to evaluate the Berlin definition [7]. Under the Berlin definition, mild, moderate, and severe stages of ARDS were associated with increased mortality (24%-30%; 29%-34%; and 42%-48%, respectively; P < .001), as well as lengthened median duration of mechanical ventilation in survivors (5 days; interquartile [IQR], 2-11; 7 days; IQR, 4-14; and 9 days; IQR, 5-17, respectively; P < .001).

Clinically, ARDS involves a series of diverse processes that produce widespread alveolar damage. In an injured alveolar capillary barrier, there will be exudation of fluids, cells and proteinaceous contents across the membrane, leading to the subsequent alveolar edema to cause refractory hypoxemia the cardinal physiological manifestation of this syndrome. The American/ European Consensus Conference in 1994 [8] defined ARDS as: acute onset of hypoxemia, associated with bilateral infiltrates on chest X-ray (CXR) in the absence of left ventricular failure.

1.1.2 Endothelial and Epithelial Injury in ARDS

ARDS is characterized histologically by diffuse alveolar damage (DAD). This is a final common pathology caused by a large variety of insults including respiratory infections, sepsis, shock, aspiration of gastric contents, inhalation of toxic gases, near-drowning, radiation pneumonitis and many drugs and other chemicals. These conditions are linked by the fact that they can all injure alveolar epithelial and endothelial cells, thereby producing DAD.
About 90 percent of the alveolar surface area is made up of flat type I epithelial cells that are easily injured, and cuboidal type II cells make up less than 10% of the alveolar surface area. The main functions of type II cells include surfactant production, ion transport, proliferation and differentiation to type I cells after injury. In ARDS, epithelial integrity is severely compromised. Normally, the endothelial barrier is much more permeable than the epithelial barrier [9, 10]. As a result, epithelial injury can directly contribute to alveolar flooding. Also, the loss of epithelial integrity and injury to type II cells critically disrupt normal epithelial fluid transport, impairing the removal of edema fluid from the alveolar space [11, 12]. Moreover, ARDS also leads to reduced production and turnover of surfactant as type II cells are injured [13].

Under normal conditions, both epithelial and endothelial barriers are intact, whereas in disease conditions, gaps are formed in the endothelium and or epithelium. Pulmonary edema is produced as the barrier functions are compromised in the epithelium and endothelium. There is leakage of water, protein, inflammatory cells, and red blood cells into the interstitium and alveolar lumen [13].

1.1.3 Mechanisms of Increased Permeability

At the site of gas exchange, the capillary-alveolar barrier acts to prevent air bubble formation in the blood, and to ensure that blood does not enter the alveolar space. The capillary-alveolar barrier is permeable to various gases, including oxygen and carbon dioxide. In ARDS development, destruction of endothelial cells and damaged pulmonary capillary endothelium are two characteristics [14, 15].
In terms of capillary fluid flux in the lung, endothelial activation affects the process in several ways. In a more general pathway, activated endothelium releases inflammatory mediators that amplify endothelial injury directly or indirectly by recruiting inflammatory cells into the vascular, interstitial, and alveolar spaces. [16]. Some mediators such as, angiotensins, bradykinin, and endothelin, have crucial effects on vascular regulation, and damage in the endothelium may substantially impair their metabolism. As a result, there may be interstitial fluid flux through physiologic mechanisms that are normally closely regulated. In addition to these vaso-regulatory mediators, reactive nitrogen and oxygen species may further deteriorate microcirculatory dysfunction in ARDS. If these reactive species enhance local blood flow or increase postcapillary resistance, they may increase capillary pressure and worsen pulmonary edema [17].

Increased endothelial permeability and reduction of alveolar liquid clearance capacity are two leading pathogenic mechanisms of pulmonary edema, which is a major complication of acute lung injury. Understanding of fundamental mechanisms involved in regulation of endothelial permeability is essential for development of barrier protective therapeutic strategies.

The endothelial barrier function is regulated via two pathways: the transcellular and the paracellular pathways. The transcellular pathway, also known as transcytosis, is a constitutive process which transports macromolecules larger than 3nM through the cells via vesicles [18, 19]. Paracellular pathway passively allows transport of solutes ions smaller than 3nM in
diameter [19, 20]. The paracellular pathway is regulated by interendothelial junctions (IEJs) that are responsible for maintaining cell–cell contacts and will be the focus of this thesis.

1.1.4 Signaling Mechanisms Increasing Paracellular Permeability

1. Intracellular Calcium

Intracellular calcium is known to play a role in the regulation of membrane permeability. Transient receptor potential family (TRP) members are involved in calcium-mediated channels for permeability [21]. TRPC1 and TRPC4 are the two ion channels with greatest effects on increasing lung endothelial cellular permeability as activation of calcium entry through its stores. TRPC1 and TRPC4 channels exert most of their influence on the lung extraalveolar endothelial barrier, and TRPV4 channels are more involved in lung capillary endothelial barrier function [22, 23]. When the alveolar septal barrier is compromised, it can lead to alveolar flooding and impaired gas exchange [21, 23].

2. Role of Rho GTPases

One established pathway known to be involved in endothelial contractility is the Rho/ Rho kinase (ROCK) pathway. The small GTPase RhoA and ROCK are known to regulate cellular adherence, migration, and proliferation through control of the actin–cytoskeletal assembly and cell contraction [24, 25] Rho kinases have been identified to have a variety of functions, including regulatory role in T cell transendothelial migration, prevention of thrombin-induced intercellular adhesion molecule 1 (ICAM-1) expression and inhibition of nuclear factor NF-kB activity [26]. Specifically for the endothelium, background Rho kinase activity is fundamental for
the maintenance of endothelial barrier integrity [27] When RhoA/ROCK is over-activated, it can lead to cytoskeletal rearrangement, consequently leading to barrier dysfunction in vascular endothelium [27, 28] When endothelial integrity is no longer maintained, there is an imbalance between endothelium-derived relaxing factors (EDRF) and endothelium-derived constricting factors (EDCF). When endothelial function is dramatically compromised, EDCFs mediate endothelium-dependent contraction by activating thromboxane–prostanoid (TP) receptors on vascular SMCs [29, 30], and one such signaling molecule activated by TP receptor in smooth muscle is Rho kinase [31]. Also, ROCK has been shown to contribute to endothelium-dependent contraction to Ach and enhanced vasocontractile activity [32]. Therefore, studies that focus on inhibition of the RhoA/ROCK pathway may have significant clinical implications.

Experiments addressing the mechanisms of endothelial permeability increases in acute lung injury and ARDS have focused on endothelial responses to agonists such as thrombin or histamine. Binding of such mediators to their cell-surface receptors elicits a series of signaling events that culminate in cell rounding and interendothelial gap formation, which represents disruption of interendothelial junctions [33, 34].

3. Role of Myosin Light Chain Kinases

Normally, the tension between actin-myosin interaction plays a critical role in controlling the semi-permeable nature of the microvasculature. Myosin light chain kinase (MLCK) phosphorylates myosin light chain (MLC), which elicits the actin-myosin interaction and reduces endothelial cell-cell interaction. In ARDS conditions, inflammatory or angiogenic mediators can
cause vascular hyperpermeability [35]. MLCK inhibitors have been shown to reverse the initial increase in endothelial permeability when MLCK is activated. MLCK210 knockout mice have also been used to associate MLCK with endothelial hyperpermeability. Mediators, including thrombin, TNF-alpha, and LPS, activate MLCK, and GTPase RhoA (introduced in the previous section). Ca2+ activation of MLCK and RhoA disrupts junctions [36].

1.1.5 Signaling Mechanisms Restoring Normal Paracellular Permeability

1. Sphingosine Kinases

Sphingolipids can act as intracellular second messengers and extracellular mediators that have critical roles in cellular processes. Sphingosine-1-phosphate (S1P) has received the greatest attention, as it is a potent angiogenic factor that elevates lung endothelial cell integrity, and it has been shown to prevent vascular permeability and alveolar flooding in preclinical animal models of ALI [37]. S1P is produced from sphingosine by two kinases, SphK1 and SphK2, which have been identified as possible drug targets for the treatment of ARDS [38]. Another important protein in S1P response is focal adhesion kinase (FAK) [39]. Endothelial FAK is stimulated by S1P – tyrosine phosphorylation results in FAK activation and dynamic interactions with other effector molecules, all of which act to improve endothelial barrier function.
2. Role of S1P Lyase

As an endogenous lipid regulator of endothelial permeability, S1P enhances barrier function through actin and junctional protein rearrangement, leading to cytoskeletal changes [37, 40]. S1P lyase is the enzyme that cleaves S1P to permanently degrade the molecule. Zhao et al. have demonstrated that inhibition of S1PL could ameliorate ALI in murine models [41]. The Zhao group also illustrated that intratracheal instillation of LPS to mice exacerbated S1PL expression, which led to a decreased S1P level in lung tissue, and the resultant enhanced level lung inflammation and injury.

1.1.6 Approach to ARDS Treatment

Despite the fact that improvement in the supportive care of patients with ARDS has been seen, its mortality rate of ARDS remains high. Treatment approaches have extended from modulating inflammation to a careful search for the underlying causes, with particular attention paid to sepsis or pneumonia. Several novel treatment strategies have been evaluated based on the field’s increased level of understanding of ARDS pathogenesis.

There has been a large number of pharmacological treatments, including glucocorticoids, surfactants, inhaled nitric oxide, antioxidants, protease inhibitors, and a variety of other anti-inflammatory treatments, all of which have been assessed in various Phase II and III clinical trials for ARDS. However, none of these aforementioned pharmacological therapies has proven to be effective over the majority of patients [42, 43]. Given the lack of a specific pharmacologic
treatment, lung-protective ventilation has continued to be the most widely used supportive care therapy, and has reduced the mortality of ALI from 40% in 2000 to 25% in 2006 [44].

Statin therapy for ARDS patients has been proven to be effective to a certain extent, particularly in patients with sepsis-induced ARDS. Also, given the fact that sepsis is the most harmful cause of ARDS, recent studies have looked at how directly combatting with sepsis may have significance in treating ARDS, notably by testing a Toll-4 receptor inhibitor in patients with sepsis [45].

Cell-based approaches are the most promising new therapies, and are receiving the most attention. In preclinical studies, research in mouse models of ARDS has shown that mesenchymal stem cells may be of value in treating ALI as well as sepsis. MSCs have been shown to have immune-modulatory effects in the lung, and a recent study reported that MSCs decreased pro-inflammatory cytokines and increased in anti-inflammatory cytokines, particularly IL-10 [46]. Also administration of MSCs was shown to alleviate epithelial permeability to protein, to reduce pulmonary edema, and to increase alveolar fluid clearance [47]. Moreover, MSCs have been found to release paracrine factors which confer protective effect [48]. More preclinical studies are conducted to evaluate the protective mechanisms of MSCs in ALI treatment.
1.2 Transcriptomic Analysis

1.2.1 Background
The most fundamental advantage associated with microarray technology is the ability to simultaneously monitor thousands of mRNA transcripts at the same time, what is also known as transcriptomics. RNA is reverse transcribed into cDNA, and a labeling molecule is incorporated for detection. Once labelled, the cDNA targets are applied to a chip/plate that already contains nucleotide sequences that correspond to specific probes. Then, hybridization occurs between the probes and targets, and the amount of hybridization will be representative of the abundance of a specific mRNA. Raw mRNA abundance data are retrieved once the chip/plate has been scanned.

Data analysis for microarray is a constantly evolving subject. Commonly, it involves data normalization, statistical comparison across groups by analysis of variance, and correction for multiple comparisons [49]. The initial data normalization allows comparisons across samples by reducing technical errors, so that the true biological variations can be acknowledged [50, 51]. Then, by analysis of variance, statistical comparisons across multiple groups of interest can be performed. This step is critical but can be troublesome in terms of generating consistent lists of differentially regulated gene. This is largely due to the number of available statistical tests that could be used, and from the same data set, depending on the test used, a different list of differentially expressed genes could be produced [52, 53] This corrects for the multiple comparisons performed in each individual test.
Some of the most popular deciphering techniques include hierarchical clustering, principal component analysis, expression mosaic map, etc. Hierarchical clustering will generate heat maps which group genes and samples together based on their relatedness of expression. Principal component analysis groups samples together and attempt to account for their variances within the group and across different treatment groups. Both of these methods help interpret and visualize a picture of global gene expression patterns, and can help identify outliers and disease state expression based on differential analysis [54]. The gene mosaic approach analyzes the microarray data based on pattern recognition, and this method has been clinically applied to categorize patients into relevant subclasses [55, 56].

By statistical tests, genes with differential expression can be identified from the microarray experiment. However, understanding the meaning of such genes may be a challenging process. To understand about the meaning of genes, data mining is an approach that can reveal abundant potential interactions and relevance of gene sets under specific conditions. To designate biological function to genes, both public and proprietary databases have been set up. Once submitted, the microarray data will be examined to determine whether certain gene lists are functionally or structurally related based on literature. These databases could be used to help identify possible biological functions of genes in a given context, and generate P values based on the evaluation of the likelihood of how a gene is enriched for a particular biological function. Biological meaning of a single gene also depends on gene networks and interactions [57].
1.2.2 Microarray and Sepsis

In the past two decades, microarray technology has become increasingly popular in studying trends of gene expression. Researchers have utilized the available microarray technology in combination with various bioinformatics techniques to a number of complex diseases, including cancer and inflammation.

Specifically for clinical sepsis, genome-wide analysis allows for the ability to simultaneously and efficiently measure mRNA abundance of thousands of transcripts from a given tissue source across different treatments or conditions, and it has inspired an unmatched opportunity to gain a broader, genome level understanding of complex and heterogeneous clinical syndromes such as sepsis [57, 58]. Microarray-based transcriptome-level analysis has led to a genome level understanding of the complexity of disease, identified novel candidate targets and pathways for potential therapeutic intervention, pinpointed candidate diagnostic biomarkers, enabled stratification of patients based on their expression of sepsis-related genes [59]. Also, the genome-level screening approach has been effective on reducing researcher’s bias. Since the entire genomic library is being closely examined rather than a predetermined set of genes that could be arbitrarily handpicked by the research, the discovery of potentially meaningful molecular targets and tools is much broader and more reliable.

Specifically for sepsis, as a disease that displays tremendous complexity in its gene expression, microarray screening could be an extremely rewarding approach. One group was able to uncover that a large number of transcripts related to inflammation and innate immunity were
substantially up-regulated in response to endotoxin when mononuclear cell-specific RNA was used in their microarray study [60]. In accordance with the acute phase of ARDS development, the researchers found the peak transcriptomic response in less than 24h following a single endotoxin administration. On top of that, the investigators also identified genes that exhibited differential expression in response to endotoxin exposure that were not previously thought to have any role in acute inflammation [57, 60]. Using network analysis, a large number of gene pathways and networks that are related to mitochondrial function, namely, energy production, showed substantial down-regulation in their expression trend – some of the genes involved in the electron transport chain, NADH dehydrogenase 1, ATP synthase were significantly knocked down. As an end result, protein synthesis related genes also had reduced gene expression, such as ribosomal protein L3, ribosomal protein S8, eukaryotic translation initiation factor. These findings in the repression of mitochondrial genes have been confirmed in human sepsis patients [61, 62].

Adaptive immunity has been implicated to have a key role in the development of acute inflammation as well as ARDS. Not surprisingly, exposure to endotoxin was shown to lead to suppression of a list of genes directly involved in the proper functioning of adaptive immunity.

Studies that involve human endotoxemia can provide results that aid in the advancement of knowledge in sepsis at a transcriptomic level. Nonetheless, this model of endotoxemia does not fully replicate the complex and heterogeneous syndrome seen at the bedside following infection with live microbes [63].
In response to better characterize the global gene expression changes in sepsis, various investigators have attempted microarray-based studies in critically ill patients with sepsis and septic shock. Despite the fact that inherent heterogeneity exists in clinical sepsis, several studies have provided novel insight into the overall genome-level response to sepsis [64-66].

A widespread pattern that has been found in many microarray-based sepsis studies is the considerable overexpression of genes associated with inflammation and innate immunity. Microarray data generated from these patients are largely in accordance with what is already known with sepsis, such as the initial hyperactive inflammatory response, making the microarray data highly plausible in further explaining clinical sepsis.

Microarray based studies have also been able proven effective in documenting gene expression changes associated with adaptive immune system and the inability to clear infection [67]. Previously, in a mouse experimental model of sepsis, the administration of an anti-apoptotic cytokine, IL-7, led to lymphocyte survival and expansion which ultimately led to improved survival in the animals [68].

In patients with sepsis, early repression of adaptive immunity genes has been reported [66, 69]. And even in children with sepsis, a similar trend of early repression of adaptive immunity-related genes has been documented several times, such genes include T cell receptors, and genes that respond to the receptor [64]. Collectively, microarray screening on the genome level
has been able to document and support the existing understanding of adaptive immune
dysfunction as an early and prominent feature of clinical sepsis.

1.2.3 Novel Genes, Pathways, and Networks

The ability to closely examine the entire human genome constitutes a novel approach to
discover individual molecules, as well as gene pathways and networks that may play role in
sepsis. Recently, several studies have demonstrated the potential of genome-wide screening of
gene profiles in uncovering novel targets for sepsis treatment. For instance, interleukin-6 was
identified as a critical contributor to myocardial depression in patients with meningococcal
sepsis, using microarray-guided in vitro approaches. The relatively homogenous patient
samples further ensured the result of the study.

As one of pioneer studies that used microarray to identify genes with differential expression
between survivors and non-survivors, the chemokine receptor CX3CR1 was the most highly
expressed in survivors [70, 71]. The researchers conducted in vitro experiments and found that
dys-regulation of CX3CR1 in monocytes contributed to immune paralysis in human sepsis [72,
73]. Therefore, there are considerable potential that lies in the use of the microarray approach
to discover novel pathways through gene expression profiling.

As a beneficial strategy proven in experimental sepsis zinc supplementation has not reached
clinical trials due to safety concerns, despite the fact that decreased plasma zinc concentrations
have been found in patients with sepsis, and that low plasma zinc concentrations correlate with
higher illness severity [74]. Since normal zinc homeostasis is essential to immune function, zinc supplementation has been raised as a potential treatment for sepsis. Interestingly, microarray-based studies in children with septic shock have reported high levels of ZIP8, a zinc transporter, expression in non-survivors, relative to survivors [75, 76]. More experiments will be necessary to fully establish the networks of genes that interact with zinc.

Also, metalloproteinase (MMP)-8 has shown marked increases in its expression in patients with septic shock, compared to control patients [64, 77]. Moreover, among patients with sepsis, the ones with more severe sepsis expressed a greater amount of MMP-8; similarly, non-survivors also showed more MMP-8 expression than survivors. It is known that chemokines and cytokines contribute to MMP-8 production. Recently, studies have demonstrated that genetically truncated MMP-8 or direct inhibition of MMP-8 activity may provide significant survival advantage in an animal experimental model of sepsis [78].

1.2.4 Microarray Application and ARDS

Microarray-based genome screening has remained a new technology to the field of ARDS. Based on microarray data of gene expression from animal models of lung injury, Hu et al., performed a meta-analysis to identify potential injury-specific gene expression signatures that may help predict the development of lung injury in humans [79]. From the study, two injury-specific lists of differentially expressed genes were discovered from animal lung injury models. External data sets and prospective data from animal models of VILI validated the results. By examining the potential pathways of gene sets, it was identified that a large number of
differentially regulated genes are involved VILI-related pathways. Gene expression signatures identified in animal models of lung injury were used to predict development of primary graft failure in lung transplant recipients, and achieved greater than 80% accuracy. The data suggests that microarray analysis of gene expression data allows for the detection of “injury” gene predictors that can classify lung injury samples and identify patients at risk for clinically relevant lung injury complications.

Also, another recent study from our own group identified three commons effects of MSC administration in an experimental animal model of sepsis-induced inflammation and organ injury. By using a network knowledge-based approach, common effects of MSC in five different organs were discovered, including, i) attenuation of sepsis-induced mitochondrial-related functional derangement, ii down-regulation of endotoxin/Toll-like receptor innate immune proinflammatory transcriptional responses, and iii) coordinated expression of transcriptional programs implicated in the preservation of endothelial/vascular integrity. Conclusively, transcriptomic analysis has shown that the beneficial effects of MSC therapy in sepsis are not limited to a single mediator or pathway, but rather involves a substantial number of biological networks playing critical roles metabolism and inflammatory response of the cell [80].

In a recent study published by our lab, genome level screening identified transcriptional responses of target organs to MSC therapy [80]. Rather than individual genes that exhibited altered levels of expression, a large number of gene sets and gene pathways showed significant changes in their expression following sepsis and MSC administration. Thus, it is highly plausible
that the beneficial effect of MSC therapy is not attributed to single mediators or pathways, instead, a series of networks and activities could be involved in the biological control of the host system in response to systemic inflammation. miRNAs are the ideal candidates for studies that involve large numbers of targets/intermediates, as each miRNA can potentially have hundreds of target mRNAs, and each mRNA can be regulated by a number of miRNAs under different conditions [81]. In contrast to a proteomics study, the combination of miRNA and mRNA microarrays can yield much information on the interaction between miRNA and mRNA prior to translation after which proteins are produced. The expression signature obtained from such studies will be much more dynamic than proteomics alone. Thus, the focus of miRNA and miRNA pairing/interaction will be much more reflective of the changes in expression, as well as downstream phenotypes, making the approach more representative of the nature of sepsis and sepsis-induced ARDS.

In summary, these aforementioned molecules came to the sight of sepsis researchers through the use of microarray-based genome screening. Even though many of these studies demand further validation experiments to confirm the proposed relationship, the novel approach of genome-based analysis has yielded a new approach to identify targets that may have potential therapeutic value in the clinical setting.
1.3 Mesenchymal stem cells

1.3.1 Background

Mesenchymal stem cells (MSCs) are multipotent, self-renewing progenitor cells. MSCs can be isolated from the bone marrow, umbilical blood, placenta, fat, skeletal muscles and tendons [82-84]. MSCs have a very slow rate of differentiation. And there are three major criteria to characterize MSCs, (a) adherence to plastic, (b) expression of CD105, CD73, and CD90; lack of expression of CD45, CD34, CD14, CD11b, CD79α, CD19, HLA II, and (c) ability to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [85, 86].

MSCs have the ability to differentiate into any other cell type in the body, and previous studies have examined how such potential could aid in the repair of various tissues. Recently, however, MSCs have received much attention due to their potent immunomodulatory effects. A wide variety of molecules are produced by MSCs, such as hematopoietic factors, chemokines, and angiogenic factors [87, 88]. It has been found that MSCs are able to modulate the immune response [89]. One critical characteristic of MSCs is their lack of MHC Class II antigens, allowing them to evade immune destruction and enabling them to be used in gene therapies [90].

In preclinical trials, MSCs have been used widely and found to have beneficial protective effects; and the feasibility of pursuing cell based therapies is currently being explored for a variety of lung diseases.
1.3.2 MSC and ALI/ARDS

In the past decade, mesenchymal stem cells have been used in the treatment for experimental ARDS. MSCs have been shown to possess both anti-inflammatory as well as reparative potential. Since the repair process and attenuation of inflammatory responses are important for ARDS treatment, the ability of MSCs to release growth factors, such as keratinocyte growth factor (KGF), hepatocyte growth factor, to induce repair in the lung parenchyma makes the approach very promising [79]. In fact, MSC has been shown to repair damaged lung tissue, its effects include re-epithelialization, replacement of injured vascular endothelium, promotion of endothelial cell growth, induce generation of fibroblasts and macrophages, and even angiogenesis [80].

Under disease conditions, following endothelial and epithelial injury, the alveolus contains a large amount of edema and infiltration of inflammatory cells. MSCs release a number of paracrine factors to help mediate the effects of the disease, including Ang-1, PGE 2, IL-1ra, TGF-b, and KGF [78].

In animal models, intrapulmonary MSC administration has been shown to improve survival and reduce pulmonary edema formation. Moreover, there was a significant increase in lung function in the animals, accompanied with elevated repair mechanism in the epithelium and endothelium [47, 91]. Also, when MSC or MSC conditioned medium was administered to cells, the systemic inflammatory response seen in LPS-induced models of ARDS was mitigated, and a balance between the release of pro- and anti-inflammatory mediators was established [92].
A number of studies have pointed out that pulmonary engraftment of MSC in the lungs is very limited, the beneficial effects seen in MSC administration rests with paracrine effects, the ability to secrete soluble factors to modulate immune responses of different diseases [93]. The effects of paracrine secretion were demonstrated through the use of MSC conditioned medium. Even though the exact paracrine constituents responsible for the therapeutic effects identified in cells or animals following MSC conditioned medium treatment remain to be elucidated, there has been evidence that suggests using the conditioned medium, rich in IL-1, growth factors, such as VEGF-A, and TGF-b promoted attenuation of inflammatory response and stimulated endothelial cell migration, contributing to repair of vascular tissue injured in an ischemic muscle injury model [94].

1.3.3 MSC Confers Protective Benefits in Animal Models of ALI

Some of the most widely used approaches to induce ALI in animal models include, oleic acid, endotoxin, acid aspiration, hyperoxia, CLP, and bleomycin. MSC administration has been found beneficial in a number of such models. LPS is the most widely used endotoxin to elicit acute lung injury in animal models.

In a recent work by Gupta et al., intratracheal MSC administration to mice following intratracheal LPS administration showed significantly improved survival [47]. Histologically, there was much less pulmonary edema in tissues; as well as a lowered wet-to-dry ratio. In another study, when LPS was administered intratracheally, intravenous MSC infusion produced
a significant drop in total BAL and neutrophil counts, 72h after the initial insult [95]. Moreover, the Mei group also found that there was a significant decrease in inflammatory infiltrates, interalveolar septal thickening, and interstitial edema, by examining the histology of lung slices. The same group found that in a CLP model, MSC infusion resulted in decreased BAL fluid cell count and decreased total protein, and large proteins, ie. albumin. There was also an attenuated level of lung infiltrates and interstitial edema. Most interesting was that in animals that received antibiotic therapy, MSC treatment led to a 50% reduction in animal mortality [96]. Also, there was reduced kidney and liver injury, improved glycogen storage and increased bacterial clearance in the spleen.
1.4 MicroRNA

1.4.1 Background

The discovery of miRNAs was made twenty years ago as a major breakthrough. In 1993 the first miRNA was identified in C.elegans [97], and eight years later, the highly conserved let-7 was found to be crucial for developmental timing in C.elegans [98].

MicroRNAs are small, endogenous RNAs, approximately 20-22 nucleotides long. miRNAs regulate gene expression at a post-transcriptional level and have direct effects on gene expression by inducing mRNA degradation or translation inhibition. Recently, miRNAs have also been documented to exert global effects indirectly through alteration of methylation and transcription factor activity [99]. Over the past decade, interest in miRNAs has increased exponentially, unfolding more of their importance in the regulation of biological processes – development, proliferation, apoptosis etc. In comparison to the known number of miRNAs in animals, relatively few miRNAs have been studied in detail, and their biological significance still remains to be fully understood. In humans, close to half of the genome may be regulated by miRNAs. Expression levels of miRNAs vary greatly among tissues and it is believed that dysregulation of miRNAs can contribute to disease pathology [100, 101].

1.4.2 miRNA Processing and Function

Genes for miRNAs can be found in introns or exons of protein-coding genes, or in non-coding regions of the genome. miRNA genes are much longer than the biologically active form, mature miRNAs are only produced after a multi-step process [102] Once activated, the transcription is
carried out by RNA polymerase II or III, giving rise to a primary miRNA (pri-miR) structure that contains a hairpin loop. Inside the nucleus, both ends of the pri-miR are cut by the Drosha/DGCR8 complex, leading to the precursor of mature miR (pre-miR), with an approximate size of 70–100 nucleotides [103, 104]

Transport of the pre-miR to the cytoplasm is via Exportin 5 where pre-miRNAs are subsequently cleaved by RNase III Dicer into ~22-nt miRNA duplexes [102]. One strand of the miRNA duplex is degraded, otherwise known as the "passenger" strand, indicated as miR*; whereas the other "guide" strand, or simply miR, is incorporated into the RNA-induced silencing complex (RISC) and serves as a functional, mature miRNA [105]. Selection of the "guide" strand is based on the base pairing stability of both dsRNA ends [106]. The argonaute (Ago) 2 protein, also known as “slicer,” has the capacity to cleave target mRNAs [107].

Fundamentally, miRNAs are repressors of genic expression at the post-transcriptional level, by degrading messenger RNA (mRNA) or inhibiting protein translation. Once miR is incorporated into the RISC (miRISC), through the interaction between the complementary regions of the miR and mRNA, the complex is able to recognize the target mRNAs. To date, most of the binding sites between miR and mRNA are at the 3’ end of the non-coding region of the mRNA (3’UTR). In animals, partial sequence complementarity between the miRNA and its target will guide the complex to bind to miRNAs target sites, mainly within the 3’-UTR of genes. A “seed region” of 2-8 nucleotides long exists in all miRNAs, and it is critical for miRNA target selection [102]. The
seed region is utilized by mature miRNAs to bind with specificity to miRNA recognition elements within the 3'UTR of mRNAs.

miRNAs also function to inhibit translation initiation, which requires both the cap and poly(A) tail structures in eukaryotes [108]. Translation is facilitated by both of these structures. It has been demonstrated that miRNAs inhibit translation by hindering the assembly of the 80S ribosomal complex in a cap (m7GpppG)-dependent manner [109]. Partially, this effect could also be attributed to the recruitment of the anti-association factor elf6 by RISC, which acts to impede the assembly of the 80S complex [110]. Moreover, the Ago2 protein within the RISC complex contains a cap-binding domain that is in direct competition with elf4E and inhibits binding of the initiation complex to the m7GpppG cap. Ago2 also has the capacity to compete with elf4E for its interaction with elf4G, which further disrupts assembly of the initiation complex [111].

Certain mRNA can be a shared target of many miRNAs, and each miR can suppress tens or hundreds of genes. In addition, there is evidence that suggests that miRNAs can be transferred from one cell to another, generating an interesting mechanism of intercellular regulation and communication [112].

Given the fact that miRNAs have an enormous capability to influence the expression of a large part of the genome, their deregulation also has significant impacts on the homeostasis of the
organism. An ever growing number of miRNAs have been shown to be involved in different lung
diseases, thus, it is critical that we gain a better understanding on their pathogenesis.

1.4.3 miRNA Regulation

miRNA regulation and expression is thought to have multiple levels of control. O'Connell et al proposed three levels of regulation, namely at the stages of "(i) transcription, (ii) processing and (iii) subcellular localization" [113]. At the level of transcription, induction of miRNA expression can be modulated by transcription factors in response to inflammatory stimuli and cellular stresses. During processing of miRNA, dicer inhibition or post-transcriptional modifications may lead to a dramatic slow-down [114]. When miRNAs localize to stress granules and p-bodies to alter local transcriptional levels, the exact mechanism for regulation on miRNA still remains unknown. Epigenetic mechanisms for miRNA expression also exist.

Selective post-transcriptional modification on pre-miR can change a miRNA's targeting capacity. Adenosine can be converted to inosine in this through RNA-editing [115]. The first such miRNA was pre-miR-22 that was edited at various sites. Approximately, about 6% of human miRNAs are subject to post-transcriptional editing [116, 117]. Interestingly, some of the edited sites have been found within the “seed” region, which would most likely lead to an altered set of targets for the miRNA.
1.4.4 Targets of miRNAs

As mentioned previously, single miRNAs can regulate the expression of many mRNAs, and each mRNA is also likely to be regulated by several miRNAs simultaneously [118]. Hence, successful identification of miRNA target genes is challenging and of great importance. A number of computational algorithms are available to predict miRNA targets [119, 120]. Algorithms take thermodynamic stability and conservation analysis into account to maximize specificity when predicting targets. Recently, many web-based applications have combined prediction programs with functional databases to better forecast the role and effect of miRNAs [121].

The effect of a particular miRNA during health or disease rests in the function of its target mRNAs. Several computational target prediction tools have been developed based on distinct but overlapping algorithms, which are continuously modified and updated as more empirical validation comes about. Generally, these algorithms rely on base pairing between the “seed sequence” of the miRNA and the 3′-UTR of its target, on top of evolutionary conservation of the targeted sequence. Although most studies have focused on functional mRNA-miRNA pairing that resides in the 3′-UTRs, miRNA target sites can also reside in the coding region of a gene, the close proximity of the arrangements of miRNAs and their targets may offer regulatory and organizational advantage [122].

It may be beneficial to search multiple databases for potential targets of the same miRNA. Nevertheless, not all putative targets are biologically genuine due to spatial or temporal restrictions. Moreover, binding of the miRNA to the targeting site might be modulated by 3′-
UTR cis-acting sequences. Again, a single 3′-UTR may be targeted by multiple miRNA. Taken together, the level of a mRNA or its translation product is governed collectively by these factors that may act on its targeting miRNA.

Prediction algorithms identify putative miRNA:target pairing based on structural complementarity, experimental validation is still necessary. Brennecke et al. [123] empirically confirmed that nucleotides 2–8 from the 5′-end of a miRNA are the most essential in base pairing with its target mRNA. The groups also found that the position of the base pairing was a greater determinant in the functional efficiency of the miRNA than the pairing energy.

Due to high similarities in miRNA sequences, usually, a large number of putative miRNA binding sites, as well as mRNA targets are produced by computational algorithms [124]. Thus, experimental validation in biological system remains fundamental and essential to complete the understanding of target prediction [121, 125]; antagomir studies or immunoprecipitation of Ago-bound mRNAs have been specifically developed for miRNA-mRNA studies. Thus, using highly specific monoclonal antibodies against members of the Ago protein family, Ago-bound mRNAs can be co-immunoprecipitated [126]

### 1.4.5 Role of miRNAs in the Lung

In the lung, there is a very specific miRNA profile that shows highly conservation across mammalian species [127]. Nonetheless, the role of miRNAs in physiological and pathological conditions in the lung remains to be elucidated.
In normal lung development, a number of miRNAs have been shown to play a role. The \textit{mir-17-92 cluster} has been proposed to regulate lung development. Its expression is high in the embryo, and steadily diminishes through development into adulthood [128]. It has been demonstrated in mice deficient in the \textit{mir-17-92 cluster}, such animals were not viable and showed lung hypoplasia/ventricular septal defects. Moreover, B cells were much more inhibited in the absence of the miRNA cluster [128, 129]. Interestingly, when the miRNA cluster was overexpressed in murine models, terminal air sacs were absent in the lung, instead, highly proliferative, undifferentiated pulmonary epithelium developed. As for miR-26a, its expression has been associated with bronchial and alveolar epithelial cells in murine lung [130]. Transcription factor SMAD1 is one of the targets of miR-26a, making the miRNA a critical regulator in lung development and pulmonary vascular remodelling [131]. Also, \textit{mir-155} is crucially involved in the differentiation of naive T-cells into Th1 and Th2 cells [132, 133]. Animals that lacked \textit{mir-155} were immune-deficient and exhibited increased lung remodelling, higher bronchoalveolar leukocytes and impaired T- and B-cell responses to inflammatory stimuli [132]. As a key factor for normal granulocyte development in the lung, \textit{mutant mice for miR-223} spontaneously developed neutrophilic lung inflammation with tissue destruction after exposure to endotoxin.

\subsection*{1.4.6 miRNAs as Potential Molecular Therapies and Biomarkers in ARDS}

Increasing amounts of evidence have shown that changes in miRNA expression are associated with immune response, inflammation pathways, and the pathogenesis of inflammatory lung
disease including ARDS, miRNAs have great prospects to become a group of potential novel therapeutic agents. The key function of miRNA regulation is through altering the abundance of copy numbers of mRNAs [134]. Even with a moderate change in expression of a target gene, the downstream effects could be potentially amplified, which may influence various physiological pathways. For example, miRNAs that are responsible for down-regulating genes in the inflammation pathway could be used to avoid over-expression of cytokines to repress acute inflammatory response in patients. Moreover, miRNAs involed in ARDS pathogenesis could be knocked down based on sequence complementarity [135].

microRNA expression is a dynamic process that changes in response to the ever-changing cellular environment. Therefore, miRNAs could act as biomarkers to pinpoint pathophysiological processes in specific disease states or development stages. With the expected advances in understanding the relationships between miRNAs and ALI/ARDS through genome-wide miRNA profiling, more miRNA biomarkers associated with immune response, inflammation pathways, disease development, and disease severity could be evaluated as a novel tool in the diagnosis and monitor for inflammatory lung disease.
1.5 Endothelial Permeability and Occludin

1.5.1 Background

In the endothelial and epithelial cell lining, the tight junction (TJ) forms a continuous, circumferential belt separating apical and basolateral plasma membrane domains, constitutes the principal barrier to passive movement of fluid, electrolytes, macromolecules and cells through the paracellular pathway, and works as a barrier within the intercellular space and as a fence within the plasma membrane. The paracellular pathway is defined as the space between the cells and comprises both the TJ and the lateral intercellular space [136, 137].

Recently, the physical composition of TJ transmembrane molecules has received a lot of attention, which has yielded much knowledge to the structure and function of TJs. Freeze-fracture electron microscopy has been utilized to analyze the morphology of TJs — a set of continuous, trans-membranous particle strands on the inner leaflet of the plasma membrane with complementary vacant grooves on the outer leaflet [138]. Depending on the cell type, the number and complexity of ramification of the TJ network varies, leading to differences in permeability barrier function between different tissues. The permeability barrier formed by polarized epithelial and endothelial cells is of fundamental importance in cell biology. [138, 139].

1.5.2 Regulation of Tight Junction Permeability

As a physical barrier, TJ control physiological fluxes. A wide range of growth factors, cytokines, drugs, and hormones regulate the TJ and its barrier function. Some of the most well documented regulators that enhance the barrier function include, glucocorticoid
hydrocortisone, prolactin, and unsaturated fatty acids, all of which increase the expression of occludin in endothelial and epithelial cells [139]. It has also been found that astrocyte-conditioned media is capable of elevating barrier properties and the expression of ZO-1 in retinal and brain-derived endothelial cells through a mechanism that remains unknown [140]. On the other hand, cytokines and several growth factors have been demonstrated to increase permeability and mediate discontinuous cell border staining of ZO-1 and/or occludin in endothelial and epithelial cells, such as tumor necrosis factor-α, interferon-γ, interleukin-1β, transforming growth factor-α, and platelet-derived growth factor [141, 142]. Additionally, hepatocyte growth factor, VEGF, and histamine increase permeability as well through a similar mechanism that leads to reduced expression of occludin or ZO-1 in vascular endothelial cells [143]. Clearly, a diverse group of extracellular factors can stimulate changes in intracellular signalling cascades which affect tight junction permeability. To date, the exact mechanism that links signaling pathways to enhanced permeability in TJ are yet to be elucidated.

1.5.3 Occludin as a Membrane Component of the Tight Junction

Occludin was the first identified transmembrane component localized in the TJ. Its discovery was made in chicken and has a molecular weight of 65 kDa [144]. Later on, occludin was found in mammals as well [145]. In the protein, its transmembrane domains cross the membrane four times, and the protein has a short cytoplasmic N-terminus and a much longer C-terminal cytoplasmic domain. The protein consists of two extracellular sections – an N-terminal cytosolic domain, and one at the C-terminus [144]. Occludin also has three cytoplasmic domains: one intracellular short turn, a small amino terminal domain and a long carboxyl terminal region.
Occludin protein has shown slightly variable molecular weights in different animals. However, in all species, occludin resolves as multiple bands on SDS–PAGE gel with the lowest being the most abundant. The higher bands on the gel correspond to phosphorylation states of occludin – bands of higher molecular weight are in the more phosphorylated state. In the extracellular loops, a large number of tyrosine residues can be located; more significantly, more than half of the residues are tyrosine and glycine in the first extracellular loop. Previously, studies have shown that occludin enters a hyperphosphorylated state as the tight junction assembles itself. Also, the main form of occludin that is found in the tight junction is hyperphosphorylated, and the hyperphosphorylation leads to a substantial detergent insolubility [146, 147].

1.5.4 Occludin’s Interaction in the Tight Junction

In the TJ, occludin is associated with a number of structural proteins and functional proteins. It has been reported previously, that occludin’s cytoplasmic domain, the C-terminus was able bind to the GuK region of ZO-1 in vitro, strongly suggesting a possible physical link between occludin to the actin cytoskeleton [147, 148]. Also, in parallel in vitro studies, ZO-2 and ZO-3 have been identified to bind to the C-terminus of occludin [149] Moreover, occludin has been demonstrated to interact with a gap junction protein, connexin-32; and the co-localization between the two proteins have been proven to exist at tight junctions. Kojima et al also showed that exogenous expression of connexin-32 in mouse hepatocytes raised the expression of occludin and increased the number of tight junction strands [150]. Aside from interactions with
other junctional proteins, occludin molecules also homodimerize via a C-terminal coiled-coil domain [151].

Besides interactions between occludin and structural proteins, there has been established interactions between occludin and a number of regulatory proteins at tight junctions. With the use of yeast two-hybrid screening, c-Yes, connexin-26, protein kinase C, and p85 were shown to bind to the C-terminus of occludin. These protein-protein interaction relationships were identified using only a 27 amino acid sequence of the C-terminus of occludin that gives rise to a putative coiled-coiled domain. However, the functional significance of these interactions has yet to be elucidated [151]. For p85, as the regulatory subunit of phosphatidylinositol 3-kinase, its interaction with occludin may lead to amplified downstream effects. Also, it has been proposed that as a nonreceptor tyrosine kinase, the association between c-Yes and occludin in an in vivo setting may potentially be responsible for tyrosine phosphorylation of occludin [152]. Moreover, occludin’s C-terminus has been found to bind to VAP-33. VAP-33 co-localized with occludin both at the tight junction and in an intracellular vesicle pool. And when overexpressed, VAP-33 led to a changed pattern of endogenous occludin localization. Lapierre and colleagues proposed that VAP-33 either regulates vesicle targeting to tight junctions or the localization of occludin at tight junctions [153]. At the other end of the protein, occludin may interact with other molecules via its N-terminal cytoplasmic region. The E3 ubiquitin-protein ligase, Itch, was found to associate with the N-terminus of occludin in vitro and in vivo, a possible ubiquitination mechanism in occludin regulation. Taken together, occludin interacts with a wide array of cellular proteins and may be regulated by diverse cellular signaling pathways.
1.5.5 Functional Role of Occludin in the Tight Junction

Functionally, occludin has been suggested to play a significant role in the regulation of tight junction barrier function, and there is a large amount of evidence in support of it. In MDCK cells, expression of chicken occludin substantially increased TER and increased the number of tight junction strands compared to untreated cells [154] [155]. In contrast, when the second extracellular loop of occludin (OCC2) was targeted by synthetic peptides, significantly decreased TER and increased flux of several paracellular tracers in confluent monolayers of an *xenopus* kidney epithelial cell line were documented [156]. Similarly, with the aid of TER measurement, synthetic peptides homologous to regions of the first extracellular loop of occludin were able to impede junction re-sealing following calcium depletion and re-addition [157]. These aforementioned experiments indicate that the extracellular loops of occludin exhibit adhesive properties and are critical in the formation of paracellular barriers.

In fact, on top of playing a critical role in regulating paracellular barriers, occludin may have a vital role in the formation of paracellular channels. Overexpression of occludin led to increased TER [154]. Overexpression of an occludin construct containing a C-terminal deletion also led to increased TER, associated with an even higher amount of paracellular flux than overexpression of the wild-type occludin. Interestingly, cells expressing occludin mutants with short deletions within the extracellular loops were able to form electrically tight monolayers, without increase paracellular permeability [155]. These lines of evidence have highlighted that intact
extracellular loops are essential for occludin to form paracellular channels, and the C-terminus of occludin may act as a vital regulatory spot for flux through paracellular channels.

The functional role of the C-terminal cytoplasmic portion of occludin has received intense research and been partly elucidated. In Madin-Darby bovine kidney cells, expression of C-terminal deletion occludin mutants identified that the distal half of the C-terminus, or amino acid residues 358–504 were critical and necessary for proper localization of occludin to tight junctions [158]. The same amino acids were necessary for the interaction between occludin and ZO-1. These studies demonstrated that ZO-1 binding was required for occludin localization at tight junctions; however, binding to ZO-1 was not sufficient for the localization of occludin to tight junctions, indicating that other factors may also be involved [158]. Moreover, a different group also reported that the proximal region of the C-terminal tail to the plasma membrane lacking the ZO-1 binding region was not sufficient to target occludin to tight junctions [159]. It has been demonstrated that the binding of the C-terminus of occludin to ZO-1, is required for proper localization of occludin at tight junctions, and for targeting occludin to the basolateral membrane [160].

Cellular adhesion has been shown to partly depend on occludin expression. Through cross-species transfection studies, Furuse et al have demonstrated that chicken occludin was able to promote cellular adhesion in insect cells [161]. Due to a lack of ZO-1 expression in insect cells, although occludin did not localize to cell borders, occludin was detected in multilamellar cytoplasmic structures that resembled tight junctions. In a parallel study, occludin was
introduced to several kinds of occludin-deficient fibroblasts, to establish its role in cell–cell adhesion. In fibroblast cell lines that expressed adherens-like junctions and ZO-1 at sites of cell–cell contact, exogenous occludin was able to localize to cell–cell contact surfaces and led to cell–cell adhesion [162]. In contrary, as identified by the same group of researchers, in a fibroblast cell line that lacked adherens-like cell junctions or localization of ZO-1 at cell–cell contacts, exogenous occludin was not able to localize to cell–cell contacts or confer cell–cell adhesion. This evidence suggests that ZO-1 was required to recruit occludin to cell junctions. It was also found that the Cell–cell adhesion in fibroblasts could be eliminated when cells were treated with synthetic peptides corresponding to the first extracellular loop of occludin, stressing the key role played by this region of occludin in cell adhesion [162]. Given its ability to promote cell adhesion, occludin may likely play critical roles in the formation of paracellular barriers.

1.5.6 Disturbance of the Barrier Function

Because tight junctions are located at the apical most areas of basolateral spaces between epithelial, or endothelial cells, they are involved in a wide variety of pathological conditions where the normal physiological regulation of passage of ions, molecules, and inflammatory cells may be compromised. It is particularly clear that tight junctions are targets of pathogens such as bacteria, viruses, allergens, etc.

Cytokine production can be identified in nearly all kinds of pathological conditions. For instance, the proinflammatory cytokines TNF-α and interferon-γ downregulate the expression of occludin,
causing dysfunction of tight junctions [163]. Also, both factors can cause redistribution of JAM in endothelial cells, which is also another key player in maintaining tight junction barrier function [164]. In certain inflammatory diseases, such as the inflammatory bowel disease, there are increases in intestinal permeability, likely due to reduction in occludin expression [165, 166]. Generally, inflammation is always accompanied by an increase in vascular permeability, which can be partly attributed to the increased expression of vascular endothelial growth factor (VEGF), which primarily affects the barrier function of tight junctions by both phosphorylation and down-regulation of occludin [167, 168]
Objectives and Hypotheses

In sepsis treatment, we do not have a complete understanding of the molecular responses to injury, the development of immunosuppressive complications, the limitations of delivery systems to specific organs, and the role of timing and combination therapy \(^{34-37}\). And due to our lack of knowledge in the molecular mechanisms of sepsis, a considerable number of clinical trials have failed \(^{16,31-33}\). Recently, MSC administration has been shown to have immune-regulatory and beneficial effects in a number of animal models of lung diseases, however, the exact mechanism by which the beneficial effects are conferred by MSCs remains unknown.

General Objective of the Project

Despite the fact that there are no specific pharmacological treatments for ARDS, post-translational gene regulatory processes offer the hope for new interventions. In this project, we used an informed target discovery approach on genome-wide analysis of functional transcriptional regulation in response to MSC treatment of sepsis, aiming to identify molecules/pathways that play a role in MSC-conferred protection from sepsis-induced mortality and ARDS, and to better understand the protective mechanisms of benefit by MSC administration.

With the use of microarray-based genome-wide analysis, we aim to determine the role of MSC administration in the modulation of pulmonary host-responses to sepsis via differential transcription of regulatory microRNAs.
By analyzing the transcriptional response to polymicrobial sepsis, the molecular phenotype associated with the disease can be defined. In our experimental model of sepsis, the cecal ligation puncture model, mice were fluid resuscitated and antibiotic treated. MSC administration in sepsis-induced mice was shown to reduce mortality and increase bacterial clearance. In order to better understand the exact mechanism by which MSCs confer their beneficial effects, we used microarray to examine the global gene expression, and identify expression signatures that may potentially be therapeutically relevant for ARDS.

In order to address the overall hypothesis, we established the following aims to examine individually.

**Aim 1: Identification of miRNAs that are differentially expressed in mouse septic lungs after treatment with MSCs**

We hypothesized that by combining miRNA and mRNA profiles from murine model of CLP-induced ARDS treated with either vehicle or MSCs will provide novel insight into the regulation of gene expression during sepsis.

First, to validate our in-silico hypothesis described above, we screened for differentially expressed miRNAs in the lung. miRNA profiling was performed using the Exiqon miRCURY LNA™ microRNA Arrays. To gain insight into miRNAs involved in the regulation of the transcriptional response to polymicrobial sepsis, specifically in the case of sepsis-induced ARDS, we performed organ-specific analyses to examine expression profiles of miRNAs. Two main comparisons were
considered: 1) Sham vs. CLP+placebo (CLP); and 2) CLP+placebo (CLP) vs. CLP+MSC (MSC). Results were normalized using Lowess normalization. Determination of differential miRNA expression was performed in R/bioconductor using the LIMMA package. miRNAs with a false discovery rate (FDR) of ≤ 0.05 were considered differentially expressed.

**Aim 2: Selection of candidate miRNAs for “functional validation” based on mRNA:miRNA profiling**

Recently, our group successfully used genome-wide analyses to identify 3 prominent effects of MSC administration on sepsis-induced MODS in target organs. These effects are 1) reconstituted transcription of mitochondrial related genes; 2) down-regulated innate immune pro-inflammatory transcriptional responses; and 3) coordinated expression of transcriptional programs implicated in the preservation of endothelial/vascular integrity.

Microarray analysis was used to identify expression profiles associated with common pathways of MSC-mediated protection and repair from sepsis-induced ARDS in the lung. Similar to the miRNA microarray, two main comparisons were considered: 1) Sham vs. CLP+placebo (CLP); and 2) CLP+placebo (CLP) vs. CLP+MSC (MSC). Results were normalized using Lowess normalization. Determination of differential miRNA expression was performed in R/bioconductor using the LIMMA package. Differentially expressed genes were defined as those having a False Discovery Rate (FDR)-corrected p-value <0.05 (adjusted p-value, corrected for multiple comparisons).
To generate a list of putative target genes, we used a prediction algorithm, miRDB, for miRNAs that displayed the most substantial expression changes. This list was then merged against the list of differentially expressed genes as determined using the two comparisons: 1) Sham vs. CLP+placebo (CLP); and 2) CLP+placebo (CLP) vs. CLP+MSC (MSC).

Based on the merged list, miRNAs that have differentially regulated target genes that are relevant to the ARDS would be selected for functional validation. Putative target genes were screened to determine whether changes in their expression would elicit a corresponding phenotype associated with ARDS.

**Aim 3: Determination of molecular regulation of candidate miRNAs and their putative targets in vitro and in vivo**

Based on our bioinformatics data, we were able to determine that miR-193b-5p was differentially up-regulated in mice subjected to CLP surgeries, and was also responsive to MSC administration following the CLP insult. With mirdb.org, an online prediction algorithm, we were able to identify occludin, a tight junction protein found in the endothelium, as a putative target gene of miR-193b-5p.

We hypothesized that through manipulation of miR-193b-5p expression, endothelial/vascular integrity can be maintained in response to TNFa in the HPMEC cell model.
Thus, the potential association between miR-193b-5p and occludin made it possible for miR-193b-5p to be a critical regulator of the endothelial integrity. Moreover, the mechanism of our miRNA of interest may be able to explain, in part, the beneficial effects that MSCs exert on the lung in sepsis-induced acute lung injury.

To validate our in silico findings, we selected HPMEC cells as our in vitro model. HPMEC cells were treated with TNFa to simulate an inflammatory state. Through overexpression and knockdown of miR-193b-5p, with or without TNFa stimulation, permeability measurements, and luciferase reporter assay, we aimed to establish a regulatory relationship between miR-193b-5p and occludin.
**Materials and Methods**

**RNA Isolation**

Total RNA was isolated from lung tissues of mice treated with Sham, CLP, and CLP + MSC, for microarray, as well as for qRT-PCR studies. For tissue samples, 1mL of Trizol (Life Technologies, Rockville, MD) was added for every 1000mg of tissue. For in vitro cell experiments, once the treatment was completed, medium was removed, and 0.5 ml of TRIzol reagent was added to each well in a 6-well plate. Cells were placed at 4°C, and total RNA extraction was performed. Chloroform was added to the samples at a 1:5 ratio with the volume of Trizol. Samples were centrifuged at 12,000g for 25min to separate RNA from DNA and proteins. Supernatant of each sample was transferred into a new eppendorf tube, and 100% isopropanol was used to first wash the RNA at 12,000g for 25min. The supernatant was removed and 95% ethanol was added to the pellet to complete the second wash at 7,500g for 5min. The final pellet product was air-dried then re-suspended in 20ul of DEPC RNase free water. RNA quality was ensured by spectrophotometric analysis (OD260/280).

**mRNA Microarray**

Total RNA extracted from mouse lung tissues was isolated using Trizol and purified using Qiagen was hybridized to the Illumina Mouse WG 6v1.1 expression bead array as per manufacturer’s specifications (n=5 animals/group). Probe based analysis was performed in the R-project for Bioconductor. Variance-stabilization transformation was used to refine normalization.
Principal component analysis was performed in Partek (St. Louis, Missouri). For clustering, Log2-transformed normalized expression data were gene-centered (mean). Hierarchical clustering was performed in JMP and in Partek using a correlation matrix and Ward linkage.

**microRNA Microarray**

MicroRNA profiling was performed using the Exiqon miRCURY LNA™ microRNA Arrays (http://www.exiqon.com/microrna-microarray-analysis-microrna-array). The RNA samples isolated from mouse lung tissues were labeled using the miRCURY LNA™ microRNA Hi-Power Labeling Kit. First, 5’-phosphates were removed from the microRNA termini using Calf Intestinal Alkaline Phosphatase (CIP). Second, a fluorescent label was attached enzymatically to the 3’-end of the microRNAs in the total RNA sample. This was followed by an enzyme inactivation step after which the sample was ready for hybridization to the microRNA Array (6th gen) can measure the abundance of 1081 possible features/miRNAs. Results were normalized and determination of differential miRNA expression was performed in R/bioconductor using the LIMMA package. miRNAs with a FDR of ≤ 0.05 were considered differentially expressed. Target genes were predicted for differentially expressed miRNAs according to the algorithm of mirdb and then compared to changes in genome-wide expression levels.

Before the preprocessing, we kept only the mice-specific probes on the arrays, which includes 4324 probes. Each miRNA has printed 4 times on the arrays so we have total 1081 miRNAs. We took the median of the within-array normalized intensities of the 4 probes for a given miRNA.
To gain insight into miRNAs involved in the regulation of the transcriptional response to polymicrobial sepsis, we analyzed the 3’UTR region of MSC-responsive genes in the lung to identify enrichment for putative miRNAs.

**Microarray Data Analysis and Normalization**

Differential gene expression in the lung was determined by computing empirical Bayes moderated t-statistics with the LIMMA package. Two main comparisons were considered: 1) Sham vs. CLP+placebo (CLP); and 2) CLP+placebo (CLP) vs. CLP+MSC (MSC). Differentially expressed genes were defined as those having a False Discovery Rate (FDR)-corrected p-value <0.05 (adjusted p-value, corrected for multiple comparisons).

The LIMMA R package was used for preprocessing and differential analysis. For preprocessing, we performed background correction using “normexp” option and set the offset to be 50. This method adjusts the foreground adaptively for the background intensities and results in strictly positive adjusted intensities. The use of an offset damps the variation of the log-ratios for very low intensities spots towards zero [169]. Within-array normalization was performed using Loess normalization approach which assumes that the bulk of the probes on the array are not differentially expressed. It doesn’t assume that that there are equal numbers of up and down regulated genes or that differential expression is symmetric about zero [170]. Between-array normalization was carried out using the Quantile normalization approach. The differential analysis is based on an Empirical Bayes approach [170]. The false discovery rate was controlled using Benjamini-Hochberg approach [171].
**CLP Model of Sepsis**

C57 mice were anesthetized with 200 mg/kg ketamine (Ketalean, 100 mg/ml; Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) and 10 mg/kg xylazine hydrochloride (Rompun, 20 mg/ml; Bayer Inc., Toronto, ON, Canada) via intraperitoneal injection. The mice were shaven and cleaned with 70% ethanol. The cecum was brought outside of the abdomen and was ligated 1 cm from the apex with 3–0 silk suture and double punctured with an 18-gauge needle. The abdomen was closed with a 4–0 nylon suture. For sham surgery, where the cecum was exteriorized but not ligated or punctured, was performed in control animals. Animals were fluid resuscitated with 50 ml/kg saline injected subcutaneously. Twenty-four hours after initial procedures, mice were killed to collect tissues for analysis.

**MSC Administration**

Mice that had undergone CLP procedures were given either saline or MSCs 6 hours after initial surgeries. Animals were anesthetized by xylazine and ketamine via intraperitoneal injection. By blunt dissection, the jugular vein was exposed and a small canula (PE10) was placed into the vein. Saline or $2.5 \times 10^5$ male MSCs were slowly injected 6 hours after sham operation or CLP. When the injection was completed, the vein was ligated, and the incision was closed using nylon suture.

**Quantification of miRNA and mRNA by qRT-PCR, Primer Design**

All the quantitative realtime polymerase chain reaction (PCR) was performed with ViiA PCR system (Applied Biosystems, Foster City, CA, USA) in triplicate. Total RNA was extracted from
HPMEC cells or mouse lung tissues with Trizol. For quantification of miRNAs, 500 ng of total RNA was reverse transcribed to cDNA following a miRNA-specific approach [172] using Superscript II RT Kit (Invitrogen) with miR-191 as loading control. miR-191 was previously reported to be the most suitable gene for endogenous control due to the lowest stability values, and the least amount of intergroup variation in its expression level [173].

For quantification of mRNAs, 500 ng of total RNA was reverse-transcribed using Superscript II RT Kit (Invitrogen) and PCR was performed with SYBR Master Mix (Life Technologies, Rockville, MD) with GAPDH as loading control. GAPDH was examined in our mouse lung tissue samples and cell samples, and showed consistent expression levels across various treatment groups in our model. All the real-time PCR analyses were performed in triplicate.

For mature miRNAs, miRNA-specific primers are listed in Table 1 below. Primers used in the reverse transcription step were designed so that the last 8bp would be reverse complementary to the last 8bp of the mature miRNA sequence, offering specificity in the binding; and the preceding sequence of the RT primer would form a stem loop which helps maintain the stability of structure. For the PCR primers, the forward primer was designed to be complementary to the mature miRNA sequence that lied before the 8bp reverse complementary to the RT primer. And the reverse primers were designed to contain a sequence that was entirely the same as a segment of the RT primer.
For immature precursor forms of miRNAs, the RT primer and the reverse PCR primer were the same. Also, the forward and reverse PCR primers were designed to largely overlap with the mature miRNA sequences in the precursor step loop structure. The forward primer shared a substantial overlap with the 5p mature sequence, the reverse with the 3p mature sequence.

<table>
<thead>
<tr>
<th>miR</th>
<th>RT</th>
<th>PCR Forward</th>
<th>PCR Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>193b-3p</td>
<td>CTC AACT GTTGCTGGGAGTGGCAATTCAAGAGCTGG</td>
<td>ACGCTCAGCTGGGAACCTGGCCCTCAAA</td>
<td>TGG TGT CGT GGA GTC G</td>
</tr>
<tr>
<td>193b-5p</td>
<td>CTC AAC TGG TGT CGT GGA GTC GGC</td>
<td>ACA CTC CAG CTG GGC</td>
<td>TGG TGT CGT GGA GTC G</td>
</tr>
<tr>
<td>323a-5p</td>
<td>CTCAACTGTGGGTGGGAGTGGCAATTCAAGAGCTGG</td>
<td>ACGCTCAGCTGGGAACCTGGCCCTCAAA</td>
<td>TGG TGT CGT GGA GTC G</td>
</tr>
<tr>
<td>423-5p</td>
<td>CTC AACT GTTGCTGGGAGTGGCAATTCAAGAGCTGG</td>
<td>ACA CTC CAG CTG GGT GAG GGG CAG AGA GC</td>
<td>TGG TGT CGT GGA GTC G</td>
</tr>
<tr>
<td>762</td>
<td>CTCAACTGTGGGTGGGAGTGGCAATTCAAGAGCTGG</td>
<td>ACGCTCAGCTGGGAACCTGGCCCTCAAA</td>
<td>TGG TGT CGT GGA GTC G</td>
</tr>
<tr>
<td>883a-5p</td>
<td>CTC AACT GTTGCTGGGAGTGGCAATTCAAGAGCTGG</td>
<td>ACA CTC CAG CTG GGT GAG GGG CAG AGA GC</td>
<td>TGG TGT CGT GGA GTC G</td>
</tr>
<tr>
<td>191-5p</td>
<td>CTCAACTGTGGGTGGGAGTGGCAATTCAAGAGCTGG</td>
<td>ACGCTCAGCTGGGAACCTGGCCCTCAAA</td>
<td>TGG TGT CGT GGA GTC G</td>
</tr>
<tr>
<td>pre-193b</td>
<td>ACCCACAAGCGGGACCTTTTGAGGGCCAGG</td>
<td>TCTCCAGATCGGTTTGTAGG</td>
<td>ACCCACAAGCGGGACCTTTTGAGGGCCAGG</td>
</tr>
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Table 1. miRNA-specific primers for cDNA synthesis and PCR.
**General Cell Culture**

HPMEC cells were selected as our in vitro model to validate the in silico findings. Lung endothelial injury is one of the key characteristics of ARDS. And, recent studies have shown that MSCs secrete paracrine factors that modulate immune responses and alter the responses of the endothelium to injury. These two pieces of knowledge make the choice of HPMEC as our in vitro model highly suitable to imitate the insult and rescue what would otherwise be observed when sepsis was inflicted in mice. HPMEC cells were obtained from Promocell (Heidelberg, Germany). Cells were cultured in Endothelial Cell Basal Medium MV 2 (EBM-2, C-22221; PromoCell) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (Lonza), hEGF, hydrocortisone, gentamicin, amphotericin-B, VEGF, hFGF-B, R3-IGF-1, and ascorbic acid. Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO2. All experiments were conducted with cells younger than passage six.

**Transfection – Gain and Loss of Function**

Our miRNA of interest, miR-193b-5p has complete sequence homology between mouse and human. In the transfection experiments, the reagents used, including anti-hsa-miR-193b-5p miScript miRNA Inhibitor (Cat# MIN0004767), hsa-miRNA inhibitor negative control (Cat# 1027271), Syn-hsa-miR-193b-5p miScript, hsa-miRNA Mimic (Cat# MSY0004767) and AllStars siRNA Negative Control (Cat # 1027280) were purchased from Qiagen. Transfection experiments were carried out in triplicate as per manufacturer’s instructions using HiPerFect Transfection Reagent (HPF, QIAGEN Cat# 301705). 10^6 Cells were seeded in 6-well plates and allowed to grow to >80% confluency prior to any transfection. A final concentration of 50nM for
miR-193b-5p inhibitor was used, and 5nM for miR-193b-5p mimic transfection. In a 6-well plate (Falcon, BD Biosciences), 18uL of miR-193b-5p inhibitor was diluted into 100uL of EBM-2 along with 18uL of HiPerFect transfection reagent. The transfection mixture was incubated for 10 min at room temperature (RT) before being added to 500uL of serum free culture medium in each well. Five hours into the transfection process, 500uL of additional serum-containing culture medium was added to each well. Twenty four hours later, the cells were treated with recombinant TNFα (10ng/ml) (Life Technologies, Rockville, MD) for an additional 24 hours before RNA or protein was collected.

Transfection – Co-transfection of Luciferase Reporter Construct and miRNA Mimic/Inhibitor

For co-transfection of luciferase reporter constructs with miRNA mimic, inhibitor, or non-targeting control, cells were in a 96-well white TC plate in 100μl total volume of antibiotic free media. The transfection experiments were conducted as per manufacturer’s manual (Switchgeargenomics, Menlo Park, CA). Two mixtures were added to each well. In mixture 1, 100ng of luciferase reporter construct, and 50nM (final concentration) of miR-193b-5p inhibitor or 5nM (final concentration) of miR-193b-5p mimic were used, and serum free media topped up the volume to 10uL. In mixture 2, 0.5uL of HiPerFect transfection reagent was added to 9.5uL of serum free media. The mixtures were incubated at room temperature for 10 minutes before added to each well.
**Luciferase Reporter Assays**

Whether miR-193b-5p would directly inhibit the activity of a luciferase reporter construct, pLightSwitch_3UTR (SwitchGearGenomics), constructs that contained the human OCLN-3’UTR and constructs that contained the control 3’UTR from a non-target gene were compared against each other. HPMEC cells (2*10^4 per well) were seeded onto 96-well plates. The following morning, cells were transiently transfected using HiPerFect reagent (Qiagen, Venlo, Netherlands) with luciferase reporter that contained either (a) the complementary seed sequence for miR-193b-5p in the 3’UTR of occludin, or (b) a mutated version of the 3’UTR sequence of occludin where the complementary seed sequence had been removed. Prior to reading the plate, 100uL of LightSwitch Assay Reagents were added to each well and incubated in a dark room at room temperature for 30min. For co-transfection experiments with miR-193b-5p mimic, the plate was read with a spectrophotometer (Thermoscientific, Waltham, MA) 24 hours post-transfection; for co-transfection experiments with miR-193b-5p inhibitor, the reading took place 48 hours post-transfection.

The size of the reporter prior to 3’UTR insertion is approximately 3910bp long. After the addition of the OCLN 3’UTR, the total length of the reporter construct will reach a length less than 5200bp. RenSP expression is responsible for the chemo-luminescence, and the 3’UTR will be found upstream to it.
**Western Blot**

Tissue and cell lysates were analyzed by SDS-PAGE using 10% polyacrylamide gels (Biorad, Hercules, CA). Proteins were transferred to nitrocellulose membranes, blocked for 1 hour, and probed overnight with the primary antibody (1:1000 for occludin) at 4°C. After washing in TBS with Tween 20, blots were incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour, washed, and then visualized. For protein quantification, films were scanned and analyzed using Image J (National Institutes of Health). The image of the gel was converted to grey scale, and a rectangular box was drawn around the first lane and applied to the other lanes in the image. The density of each of the bands was then converted to a peak, and the “straight line” tool was employed to close the peak to generate an enclosed area representative of the band’s density. The “wand” tool was used to determine the area of the peak. The integrated intensity was normalized to the amount of protein loaded.

**Cell Viability Assay**

The effect of hsa-193b-5p mimic or inhibitor on cellular viability in the presence or absence of TNFα was determined using MTT assay (Sigma, St. Louis, MO). Cells were seeded at a density of 10^4 cells/100 µl in a 96-well plate. After treatments of transfection and TNFα insult, cells were incubated overnight. After washing twice with PBS, cells were given MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) solution (100 ul/well of 5 mg/ml solution in PBS) for 3 h. This assay was used to assess cell viability by examining the activity of cellular enzymes that reduced the yellow tetrazolium dye, MTT, to the insoluble formazan, giving rise to the observed purple color in living cells. Next, 100 µl/well DMSO (Sigma, St. Louis,
MO) was added to dissolve the converted purple formazan, and the absorbance of formazan was measured at 570 nm using an ELISA reader (BioTek, Winooski, VT, USA). Absorbance values were normalized to media control.

**Transwell Assays of HPMECs**

HPMECs, seeded on 0.4 µm-pore polyester transwells (Costar, Tewksbury, MA) that were coated with Attachment Factor (Invitrogen), were grown until confluency 2–3 days later. Confluency of the monolayer was determined using transendothelial electrical resistance (TEER) and microscopic observations. FITC-dextran (70kDa) and TEER were the two independent approaches employed to measure the changes in endothelial monolayer integrity.

Following TNFα and/or transfection of the miRNA inhibitor/mimic, FITC-dextran was added to the top chamber of the transwell. Then, transwells were incubated at room temperature for 3 hours, then the media in the top and bottom portions of the transwells were collected and transferred into a 96-well plate for reading, using spectrophotometer (Thermoscientific, Waltham, MA). Dextran permeability readings were then measured and compared to the control baseline.

TEER was measured using the Endohm-12 (WPI, Florida). When the monolayer of HPMECs was determined to be near confluency, cells were transfected with miR-193b-5p inhibitor/mimic, followed by treatment of TNFα (10ng/mL) for 24 hours. TEER was measured at different time
points following the treatment, 0h, 3h, 5h, and 24h. TEER readings were then measured and compared to baseline.

**Statistics**

All experiments were performed at least 3 times unless otherwise stated. Data are expressed as mean. Statistical values were defined using two-way ANOVAs as appropriate. A p value <0.05 was considered significant.
Results

In order to identify the regulatory relationship between septic stimuli and tissue function in the lung, we undertook two microarray profiling studies, (one for mRNA, the other for miRNA), using total RNA extracted from murine lungs that had been subjected to Sham, CLP surgeries for 24h and treated with either placebo or MSCs.

Effect of MSC Treatment on Global Gene Expression Profiles of Lungs in Experimental Sepsis

A total of 45282 genes from 5 different animals per group were profiled using Illumina Mouse WG 6v1.1 expression bead arrays. Three electronic experiments were conducted based on the microarray data. First, all of the probes in the lung were processed with zero filtering, and we obtained a global expression signature of the 45282 genes. Second, probes that showed differential expression from the LIMMA analysis (adj p <0.05), in the CLP vs. Sham comparison were plotted. Lastly, probes that showed differential expression from the LIMMA analysis (adj p <0.05), in the CLP vs. MSC comparison.

Principal component analysis (PCA) is a mathematical procedure where orthogonal transformation is employed convert a set of observations of possibly correlated variables into linearly uncorrelated variables. PCA is a principal axis rotation of the original variables that preserves the variation in the data. In PCA, the data points are rotated around their mean in order to align with the principal components. Therefore, the total variance of the original variables is equal to the total variance of the principal components. The eigenvectors and eigenvalues define the rotation and variation. Eigenvalues are the variances of the principal
components. The eigenvectors are the direction cosines of the new axes, PCs, relative to the old, original variables, thus they define the rotations of the original axes.

PCA was used to account for the variances among samples, and normalized unfiltered genes revealed considerable differences in apparent gene expression in the lung tissues profiled. We decided to first determine the genes that were responsive to the CLP insult. Between sham and CLP, the first three components explain 74.7% of the variability in the data, and a total of 11451 probes were significantly different between the two treatment groups (Figure 1). As for between CLP and MSC, a total of 4752 probes were different, and the first three components were able to explain 79.0% of the variability in the data (Figure 3). PCA was used to determine the molecular responses to CLP treatment based on gene expression profiles. As shown in hierarchical clustering plots, MSC treatment restored transcriptional responses to an expression profile intermediate to both the sham and CLP profiles in the lungs (Figure 2).
Figure 1. Principal component analysis (PCA) of the mRNA data that characterizes the trends of global gene expression between Sham and cecal ligation puncture (CLP). As exhibited by the gene profiles of Sham (green), cecal ligation perforation (CLP, red), and cecal ligation perforation followed by mesenchymal stem cell treatment (MSC, blue), each mouse lung sample is represented by one spot, or the eigenvalue vector, and each color represents a different type of sample. PCA finds the 3 most important components from all the genes and uses them to create the 3d view. The percentage of the variance in genetic distance explained by each PC is shown on the axis, and a total of 74.7% could be explained by the first 3 axes.
Figure 2. Hierarchical clustering using two-channel microarray data of genes that displayed differential expression between Sham and CLP (adj P < 0.05). The rows represent individual genes, and the mouse samples are shown as columns (1 column per sample). The color represents the expression level of the gene. By convention, relative to the global change in gene expression, red represents up-regulation, while green down-regulation. The dendrograms constitute a quantitative means of assessing the distance between individual genes and mouse lung samples. The figure was generated by Partek V6.6.
Figure 3. Principal component analysis (PCA) of the mRNA data that characterizes the trends of global gene expression between cecal ligation puncture (CLP) and cecal ligation perforation followed by mesenchymal stem cell treatment (MSC). As exhibited by the gene profiles of Sham (green), CLP (red), and MSC (blue), each dot represents a mouse lung sample and each color represents a different type of sample. The percentage of the variance in genetic distance explained by each PC is shown on the axis, and a total of 79% could be explained by the first 3 axes. CLP shows the greatest ellipse, while sham shows the smallest cluster. The ellipses enclose data points of the same class. It is drawn at a distance beyond which the probability of a point belonging to the class is low, and can be thought of as a class boundary. Along the first principal component, CLP shows the greatest variance, and the MSC ellipse lies in between the Sham and CLP.
**Effect of CLP and MSC Treatment on miRNA Expression Profiles of Lungs in Experimental Sepsis**

A total of 1081 miRNAs from 5 animals per group were profiled using Exiqon bead arrays. Volcano plots were generated based on fold change and adjusted p value from LIMMA analysis for individual miRNAs. Between sham and CLP, as shown in Figure 4, up- or down-regulation was observed depending on the miRNA, and the orange line represents a cut-off that selects only the top 100 miRNAs. miR-193b-5p was observed to be up-regulated. Similarly, between CLP and MSC, up- or down-regulation was observed depending on the miRNA, and the orange line represents a cut-off that selects only the top 100 miRNAs (Figure 5), and miR-193b-5p showed down-regulation. In order to study the role of miRNAs in ALI, we intended to look for the ones that were differentially expressed following CLP surgeries, but also showed significant responses to MSC treatment. Therefore, the expression of the miRNA expression following MSC treatment would be on a similar level compared to that of the Sham group (Figure 6). As shown in Figures 4 and 5, miR-193b-5p shows differential expression following CLP insult, as well as in response to MSC treatment. Moreover, miR-193b-5p exhibits a complete homology between its human and mouse sequences, making it highly relevant for future clinical studies.
Figure 4. Volcano plot of statistical significance against fold change between Sham, and CLP.

Each dot corresponds to one miRNA. The log fold change is plotted on the x-axis and the negative log10 p-value is plotted on the y-axis. Note that not all genes with a fold change of 1.5 or more have significant adjusted p values (the adjusted P values are shown on the vertical axis of the volcano plot), conversely, not all the genes with significant P values satisfy the fold change cut-off. False discovery rate (FDR) was used as a cut-off, the corresponding adjusted P value was 0.05
Figure 5. Volcano plot of statistical significance against fold change between CLP and MSC.

Each dot corresponds to one miRNA. The log fold change is plotted on the x-axis and the negative log10 p-value is plotted on the y-axis. Note that not all genes with a fold change of 1.5 or more have significant adjusted p values (the adjusted P values are shown on the vertical axis of the volcano plot), conversely, not all the genes with significant P values satisfy the fold change cut-off. False discovery rate (FDR) was used as a cut-off, the corresponding adjusted P value was 0.05.
Figure 6. **Volcano plot of statistical significance against fold change between Sham vs. MSC.**

Each dot corresponds to one miRNA. The log fold change is plotted on the x-axis and the negative log10 p-value is plotted on the y-axis. As shown in the plot, miR-193b-5p did not exhibit an expression that was statistically significant or past the fold change off, displaying a similar expression pattern between Sham and MSC. Note that not all genes with a fold change of 1.5 or more have significant adjusted p values (the adjusted P values are shown on the vertical axis of the volcano plot), conversely, not all the genes with significant P values satisfy the fold change cut-off. False discovery rate (FDR) was used as a cut-off, the corresponding adjusted P value was 0.05.
Functional Prediction of a Differentially Expressed miRNA to MSC Treatment of Experimental Sepsis

To develop a functional role of the identified hsa-miR-193b-5p, we used prediction algorithms to determine putative candidate target genes that our miRNA of interest could potentially regulate. After entering the name of the miRNA into the mirdb.org database, the putative targets were prompted. The prediction algorithm used the seed region of the miRNA, 2nd to the 8th base, as a criterion of complementarity between the miRNA and mRNA. Out of the 268 putative targets of hsa-miR-193b-5p predicted by mirdb.org, 23 of them were in fact also documented in our list of mRNAs in the lung that showed differential expression, one of such targets was occludin, with a target score of 58.

To further validate the prediction, we joined our list of differentially expressed mRNAs in response to MSC treatment of experimental sepsis against the list putative targets of hsa-miR-193b-5p generated by mirdb.org. Occludin was also confirmed as one of the top predicted target genes of hsa-miR-193b-5p, with a fold change of 1.50, and adjusted p value of 0.034 (Table 2). With the use of hierarchical clustering, it was obvious that MSC treatment returned occludin’s expression profile to one that lies intermediate to both sham and CLP (Figure 7).
Figure 7. Heat map of occludin mRNA expression in Sham, CLP, and MSC mouse lung tissues.

Hierarchical clustering depicts the relatedness of the 15 samples, and the mouse samples are shown as columns (1 column per sample). The color represents the expression level of occludin mRNA. Red represents high expression, while green low expression. The dendrogram constitutes a quantitative means of assessing the similarity between individual samples. As indicated by the dendrogram, the lung tissues that were given MSC treatment display closer relatedness to the Sham lung samples, in terms of occludin expression. The figure was generated by Partek V6.6.
Table 2. Targets of miR-193b-5p with differential expression in the mRNA microarray.

Occludin exhibits a fold change of 1.4990, adj p < 0.05. This shows occludin is potentially strongly regulated by miR-193b-5p. Some of the top regulated mRNAs are also listed here.
miR-193b-5p Expression is Responsive to both CLP and MSC and Correlates with Occludin

Expression

By screening the miRNAs, we focused on the top 100 miRNAs that showed differential expression following CLP and were also responsive to MSC treatment. Out of the top 100 miRNAs, we examined the ones that showed 100% sequence homology between mouse and human. And miR-193b-5p was one such miRNA. From the in silico data analyses, expression of miR-193b-5p and its putative target gene occludin are shown (Figures 8, 9), when miR-193b-5p expression level was stimulated by CLP, occludin mRNA level was significantly down-regulated. On the other hand, when miR-193b-5p expression was rescued towards one similar to that of sham’s, occludin showed a significant attenuation in its expression.
Figure 8. Gene expression of miR-193b-5p by normalized raw intensity values across Sham, CLP and MSC. As exhibited by the gene profiles of Sham (green), CLP (red), and MSC (blue), miR-193b-5p expression was elevated by CLP, and suppressed in CLP + MSC samples. Results were normalized using Lowess normalization. Determination of differential miRNA expression was performed in R/bioconductor using mainly the LIMMA package. The values plotted are means ± SEMs of N=4-5 independent experiments for each treatment group.
Figure 9. Gene expression of occludin by normalized raw intensity values across Sham, CLP and MSC. Results were normalized using Lowess normalization. Determination of differential miRNA expression was performed in R/bioconductor using mainly the LIMMA package. As exhibited by the gene profiles of Sham (green), CLP (red), and MSC (blue), occludin expression was significantly down-regulated by CLP (p<0.05), and alleviated in CLP + MSC samples (p<0.05). The values plotted are means ± SEMs of N=5 independent experiments for each treatment group.
To confirm this finding that we extracted from microarray analyses, it was necessary to quantify the genes by qRT-PCR. In the internal validation experiment, using the same RNA samples in the microarray experiment, it was clear that miR-193b-5p was significantly up-regulated by more than 3 fold, and down-regulated to a level similar to sham following MSC treatment (Figure 10).

In line with the expression level from the microarray data, occludin mRNA expression in mouse lung tissue was regulated in a fashion complementary to miR-193b-5p expression trend (Figure 11).

In an external separate set of experimental septic mice, we noted the same correlation between miR-193b-5p and occludin 24h after the initial CLP surgeries (Figures 12, 13). However, by 48h, the surge in miR-193b-5p, and the decrease in occludin had subsided to pre-surgery levels (Figures 12, 13). To monitor protein level change, western blotting was used and we detected a significant reduction in occludin in the CLP mice (Figure 14).
Figure 10. Quantitative RT-PCR analysis of miR-193b-5p. RNA samples from the same lung tissues for the microarray were used. The expression of miR-193b-5p was corrected for expression of the control gene, miR-191. It was noted that CLP led to significantly up-regulated expression of miR-193b-5p (P<0.05), and MSC reversed the expression (P<0.05). The values plotted are means ± SEMs of N=4-6 independent experiments for each treatment group.
Figure 11. Quantitative RT-PCR analysis of occludin. RNA samples from the same lung tissues for the microarray were used. The expression of occludin was corrected for expression of the control gene, GAPDH. It was noted that CLP led to significantly down-regulated expression of occludin (P<0.05), and MSC rescued the expression (P<0.05). The values plotted are means ± SEMs of N=4-6 independent experiments for each treatment group.
**Figure 12. Quantitative RT-PCR analysis of miR-193b-5p.** We aimed to confirm our in silico findings using a different set of mice. RNA samples were extracted from lung tissues of this external group of mice that underwent CLP surgeries. The expression of miR-193b-5p was corrected for expression of the control gene, miR-191. It was noted that CLP (24h) led to significantly up-regulated expression of miR-193b-5p (P<0.05), but CLP (48h) showed a subsided expression (P<0.05). The values plotted are means ± SEMs of N=4-6 independent experiments for each treatment group.
Figure 13. Quantitative RT-PCR analysis of occludin. RNA samples from an entirely different set of mouse lung tissues were used. The expression of occludin was corrected for expression of the control gene, GAPDH. It was noted that CLP (24h) led to significantly down-regulated expression of occludin (P<0.05), but CLP (48h) showed a recovered expression (P<0.05). The values plotted are means ± SEMs of N=4-6 independent experiments for each treatment group.
Figure 14. Down-regulation of occludin protein in mouse lung tissue. Lysates from tissue samples were analyzed by SDS-PAGE using 10% polyacrylamide gels. The Western blot is a representative image for 3 independent experiments. For densitometry, films were scanned and analyzed using Image J, and the integrated intensity was normalized to the control protein – GAPDH. Occludin expression was significantly reduced 24h after the initial CLP surgery.
miR-193b-5p Expression is Up-Regulated by TNFa and Correlated to Loss of Occludin in HPMECs

HPMEC cells were selected to be the in vitro model to further validate our findings from the in silico analyses for the following reasons. First, lung endothelial injury is one of the key characteristics of ARDS. Also, recent studies have shown that MSCs secrete paracrine factors that modulate immune responses and alter the responses of the endothelium to injury. These two pieces of knowledge make the choice of HPMEC as our in vitro model highly suitable to imitate the insult and rescue that would be similarly observed when CLP and MSCs were applied in mice. When the cells were treated with TNFa for 24h, there was a significant and pronounced rise in miR-193b-5p expression (Figure 15). As predicted by previous experiments, occludin mRNA expression was significantly down-regulated (Figure 16). This was also confirmed using western blotting, and we noted a 63% reduction in occludin protein (Figure 17).
**Figure 15. Quantitative RT-PCR analysis of miR-193b-5p.** Sub-confluent proliferating HPMEC cells were treated with TNFa at 10 ng/ml for T=24 hr, in serum-free culture medium, and then harvested for quantitative RT-PCR assays (see Methods section). The expression of miR-193b-5p was corrected for expression of the control gene, miR-191. TNFa treatment for 24h led to a significantly overexpression of miR-193b-5p (P<0.05). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 16. Quantitative RT-PCR analysis of occludin. Sub-confluent proliferating HPMEC cells were treated with TNFa at 10 ng/ml for T=24 hr, in serum-free culture medium, and then harvested for quantitative RT-PCR assays (see Methods section). The expression of occludin was corrected for expression of the control gene, GAPDH. TNFa treatment for 24h led to a significantly suppressed expression of occludin (P<0.05). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 17. A) Western blot analysis of HPMEC cells treated with TNFa. Cells were treated with TNFa (10ng/mL, for 24h). Lysates were analyzed by SDS-PAGE using 10% polyacrylamide gels. B) For densitometry, films were scanned and analyzed using Image J, and the integrated intensity was normalized to the control protein – GAPDH. Occludin expression was significantly reduced 24h after the initial TNFa administration.
**Overexpression and Knockdown of miR-193b-5p Correlates with Occludin Expression**

Microarray analysis identified a putative regulatory relationship between miR-193b-5p and occludin in the lung. Through confirmation by qRT-PCR in a greater number of animals, we suspected that the regulatory relationship may in fact exist. To test this, gain- and loss-of-function experiments were carried out. When the mimic of miR-193b-5p was transfected into HPMEC cells, we noted a significant change in miR-193b-5p expression compared to control, greater than 2000 fold. Cells treated with TNFa 24h following the initial transfection did not have any significant statistical difference compared to cells transfected with the mimic alone (Figure 18). Expectedly, for occludin, cells that were treated with TNFa, mimic only, or TNFa + mimic, all showed significant decrease in occludin mRNA levels (p<0.001). However, the resulting occludin expression levels were not statistically significant between TNFa, mimic only, or TNFa + mimic, while TNFa + mimic treated cells had the lowest level of occludin (Figure 19).

Transfection of the inhibitor alone led to no statistically significant change in miR-193b-5p expression level (Figure 21), and a slight increase in occludin mRNA expression (Figure 21). When the inhibitor of miR-193b-5p was transfected into HPMEC cells, the effect of TNFa stimulation on miR-193b-5p was much attenuated (p<0.01) (Figure 20). And this transfection reduced the loss of occludin due to TNFa significantly (p<0.01) (Figure 21).

Using western blotting, we found that after transfection of miR-193b-5p inhibitor into HPMEC cells, in the presence of TNFa, occludin protein expression was also rescued significantly compared to a level that was almost entirely knocked out (Figure 22).
Figure 18. Quantitative RT-PCR analysis of miR-193b-5p. Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p mimic, in serum-free culture medium, one group was also treated with TNFa at 10 ng/ml for 24 hr. Cells were then harvested for quantitative RT-PCR assays. The expression of miRNA was corrected to expression of the control gene, miR-191. The mimic of miR-193b-5p was able to be detected following transfection, showing a significant difference compared to the control group (P<0.001), and mimic transfection in combination with TNFa stimulation led to significant overexpression of miR-193b-5p compared to the group that only received TNFa(P<0.001). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 19. Quantitative RT-PCR analysis of occludin. Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p mimic in serum-free culture medium, one group was also treated with TNFa at 10 ng/ml for 24 hr. Cells were then harvested for quantitative RT-PCR assays. The expression of miRNA was corrected to expression of the control gene, GAPDH. Cells that were overexpressed with miR-193b-5p displayed significant down-regulation in occludin expression compared to the control group (P<0.001), and mimic transfection in combination with TNFa stimulation was also associated with significant reduction in occludin (P<0.001). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 20. Quantitative RT-PCR analysis of miR-193b-5p. Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p inhibitor in serum-free culture medium, one group was also treated with TNFa at 10 ng/ml for 24 hr. Cells were then harvested for quantitative RT-PCR assays. The expression of miRNA was corrected for expression of the control gene, miR-191. Cells transfected with the miRNA inhibitor did not show significant differences in miR-193b-5p expression compared to the control group, however, the inhibitor was able to lower miR-193b-5p expression in response to TNFa stimulation (P<0.01) that would otherwise be observed when TNFa was given to the cells alone. The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 21. Quantitative RT-PCR analysis of occludin. Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p inhibitor in serum-free culture medium, one group was also treated with TNFa at 10 ng/ml for 24 hr. Cells were then harvested for quantitative RT-PCR assays. The expression of miRNA was corrected for expression of the control gene, GAPDH. Cells transfected with the miRNA inhibitor did not show significant differences in occludin expression compared to the control group, however, to a large degree, the inhibitor prevented the decreased level of occludin expression in the response to TNFa stimulation (P<0.01). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 22. A) Western blot analysis of occludin expression HPMECs after miR-193b-5p knockdown. Cells were transfected with either miR-193b-5p inhibitor or scrambled control, allowed to grow for 24h, then treated with TNFa (10ng/mL, for 24h). Lysates were analyzed by SDS-PAGE using 10% polyacrylamide gels. B) For densitometry, films were scanned and analyzed using Image J, and the integrated intensity was normalized to the control protein – GAPDH. Occludin expression was significantly reduced 24h after the initial TNFa administration, but rescued in cells transfected with miR-193b-5p.
Effect of miR-193b-5p Inhibitor on HPMEC Cell Permeability

Gain-and loss-of-function experiments described previously have suggested a strong correlation in expression between miR-193b-5p and its putative target occludin. To determine whether the manipulation of miR-193b-5p levels led to any functional effects, we performed permeability measurements across the monolayer of HPMEC cells.

The first experiment that we did was to examine whether there would be altered cellular viability among various treatment conditions. Apoptosis would be a confounding factor in evaluating permeability across a cell monolayer as increasing gaps would exist due to increased cell loss. With the use of a spectrophotometer, we found no statistically significant differences among cells that underwent different treatment conditions (Figure 23).

FITC-dextran was used first to measure monolayer permeability. As shown in Figure 24, at 24h after the initial transfection of the miR-193b-5p inhibitor, there were not significant differences between the groups. However, when TNFa was administered to the cells accordingly, non-transfected cells displayed marked increases in cellular leakage, as indicated by the amount of FITC-dextran measured. miR-193b-5p inhibitor transfection was able to reduce the leakage to less than half compared to cells that did not receive a knock down in miR-193b-5p (p<0.05).

We also used transendothelial electrical resistance as a marker for paracellular and transcellular leakage. HPMEC cells that were given TNFa only and cells given TNFa plus the scambled control showed the greatest loss in their monolayer electrical resistance at 24h, there was no statistical
significant difference between the two groups. When cells were transfected with the miR-193b-5p inhibitor alone, there was a slight increase in electrical resistance at the 24h mark compared to the control group. Most importantly, transfection of miR-193b-5p inhibitor followed by TNFa stimulation displayed a significant difference compared to the scrambled control plus TNFa group (p<0.05). Also, there was no statistically significant difference between the inhibitor plus TNFa and control cells (Figure 25).
Figure 23. Effect of transfection on HPMEC cell viability. HPMEC cells were transfected with respective mimic, inhibitor, or scrambled control, followed by TNFa treatment (10ng/mL for 24h) if indicated. The viability of the cells was assessed by MTT assay (see Methods). Results are expressed as enrichment factor relative to DMSO-treated control. Each experiment was done at least twice in triplicate and representative data from a single experiment are shown. Columns, mean (n=3); there was no statistical significance different (P<0.05) between the indicated groups by paired t-test.
Figure 24. **Effect of miR-193b-5p inhibitor on permeability.** HPMEC cells were seeded in transwells and allowed to grow to near confluency prior to transfection (2-3 days). Following transfection, cells were given TNFa at 10ng/mL for 24h. FITC-dextran was given to all cells, and measured in both control cells and transfected cells. The inhibitor of the miRNA was able to maintain a similar level of FITC-dextran that leaked across the cell monolayer compared to the control at 24h, and showed significant improvement compared to the negative control treated with TNFa (P<0.05). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 25. **Effect of miR-193b-5p inhibitor on permeability.** HPMEC cells were seeded in transwells and allowed to grow to near confluency prior to transfection (2-3 days). Following transfection, cells were given TNFa at 10ng/mL for 24h. TEER was measured at 4 different time points as indicated in the figure. The inhibitor of the miRNA was able to maintain a similar level of TEER compared to the control at 24h, and showed significant improvement compared to the scrambled control (P<0.05). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group. Also, to assess whether a tight monolayer had formed, we repeated measured the TEER prior to any transfection or treatment, a confluent monolayer displayed a resistance of 320-350 ohms cm$^2$. 

![Graph showing effects of miR-193b-5p inhibitor on HPMEC permeability](image-url)
miR-193b-5p Knockdown Inhibits Loss in Occludin

The ability to limit the extent of pulmonary edema is key in treating ARDS. So far, we have hypothesized down-regulated miR-193b-5p activity as a potential player in conferring beneficial effects in the lung to reduce cellular leakage. In the 3’UTR region of the occludin gene, a binding sequence for the 7-mer seed sequence in miR-193b-5p is identified there. When occludin-3’UTR-luciferase reporter and miR-193b-5p mimic were co-transfected into HPMEC cells, increased miR-193b-5p led to decreased level of chemoluminescence, indicating a decreased level of luciferase expression due to the occludin-3′UTR contained in the reporter (Figure 26). On the other hand, when transient co-transfection of an occludin-3’UTR-luciferase reporter and miR-193b-5p inhibitor was performed on HPMEC cells, there was no significant loss in chemoluminescence of luciferase, even in the presence of TNFa. More importantly, mutating the binding site for the seed sequence in the 3’UTR of occludin in the plasmid prevented miR-193b-5p from suppressing luciferase expression in HPMEC cells (Figure 27).
Figure 26. miR-193b-5p activity in HPMEC cells expressing occludin: HPMEC cells were transiently transfected with luciferase construct plasmids that contained a wildtype 3’UTR or a mutated 3’UTR of occludin, and the mimic of miR-193b-5p for 24hr. Cells were harvested and luciferase activity was determined using the Luciferase Assay (Switchgear genomics) per the manufacturer’s protocol. The two controls used in the experiment were, ACTB that stands for beta actin was used as the housekeeping gene, and R01 was used as a random sequence control, it contains non-conserved, non-genic, and non-repetitive human genomic fragments. The treatment of miR-193b-5p mimic (24h) led to a significant reduction in luciferase activity (P<0.05). This effect was not observed in cells expressing mutated plasmids.
**Figure 27. miR-193b-5p activity in HPMEC cells expressing occludin:** HPMEC cells were transiently transfected with luciferase construct plasmids that contained a wildtype 3’UTR or a mutated 3’UTR of occludin, and the inhibitor of miR-193b-5p for 48hr, followed by a 24h TNFa treatment. Cells were harvested and luciferase activity was determined using the Luciferase Assay (Switchgear genomics) per the manufacturer’s protocol. The two controls used in the experiment were, ACTB that stands for beta actin was used as the housekeeping gene, and R01 was used as a random sequence control, it contains non-conserved, non-genic, and non-repetitive human genomic fragments. The treatment of TNFa (24h) led to a significant reduction in luciferase activity (P<0.05), and the inhibitor of miR-193b-5p was able to rescue it (P<0.05). This effect was not observed in cells expressing mutated plasmids.
Precursor and Complementary Mature Forms of miR-193b-5p Are Affected by Transfection of miR-193b-5p Mimic and Inhibitor

When the mimic of miR-193b-5p was transfected into HPMEC cells, both pre- miR-193b and miR-193b-3p showed significantly increased levels of expression, while the transfection of miR-193b-5p inhibitor did not lead to statistically significant changes in either one of the two (Figures 28, 30).

Similar to the changes in miR-193b-5p expression level in response miR-193b-5p inhibitor transfection, we noted substantially diminished expression in both pre- miR-193b and miR-193b-3p. Moreover, miR-193b-5p inhibitor transfection in the presence of TNFa maintained a level of miR-193b-3p expression nearly unchanged compared to the control cells in response to TNFa stimuli (Figures 29, 31).
Figure 28. Quantitative RT-PCR analysis of pre-miR-193b. Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p mimic or inhibitor in serum-free culture medium, allowed to grow for 24h, and then harvested for quantitative RT-PCR assays (see Methods section). The expression of miRNA was corrected for expression of the control gene, pre-miR-191. Cells transfected with the miRNA inhibitor did not show significant differences in pre-miR-193b expression compared to the control group, however, a significant up-regulation in pre-miR-193b was observed in cells treated with TNFa and cells transfection with miR-193b-5p mimic (P<0.05). The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
**Figure 29. Quantitative RT-PCR analysis of pre-miR-193b.** Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p mimic or inhibitor in serum-free culture medium, allowed to grow for 24h, given TNFa (10ng/mL) for another 24h, and then harvested for quantitative RT-PCR assays (see Methods section). The expression of miRNA was corrected for expression of the control gene, pre-miR-191. Unlike the mature form of miR-193b-5p, cells transfected with the miRNA inhibitor did not return pre-miR-193b to a level similar to the control, and showed no significant differences in pre-miR-193b expression compared to the TNFa treated group. The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 30. **Quantitative RT-PCR analysis of miR-193b-3p.** Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p mimic or inhibitor in serum-free culture medium, allowed to grow for 24h, and then harvested for quantitative RT-PCR assays (see Methods section). The expression of miRNA was corrected for expression of the control gene, miR-191. Like the mature form of miR-193b-5p, cells transfected with the miRNA mimic displayed an overexpression of miR-193b-3p (P<0.05), while the inhibitor of miR-193b-5p did not have a significant decrease on the 3p expression. The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 31. Quantitative RT-PCR analysis of miR-193b-3p. Sub-confluent proliferating HPMEC cells were transfected with miR-193b-5p scrambled control or inhibitor in serum-free culture medium, allowed to grow for 24h, given TNFα (10ng/mL) for another 24h, and then harvested for quantitative RT-PCR assays (see Methods section). The expression of miRNA was corrected for expression of the control gene, miR-191. Like the mature form of miR-193b-5p, cells transfected with the mir-193b-5p inhibitor showed a markedly reduced level of miR-193b-3p under TNFα stimulation (P<0.05), while the scrambled control of miR-193b-5p did not have a significant decrease on the 3p expression. The values plotted are means ± SEVs of N=3-4 independent experiments for each treatment group.
**Wildtype MSC Conditioned Medium (MSC-CdM) Altered miR-193b-5p Expression**

With the established regulatory relationship between miR-193b-5p and occludin, we then aimed to determine whether the use of wildtype MSC conditioned medium would be able to elicit the change brought about by the inhibitor. As shown in Figure 32, MSC-CdM did not induce a change in miR-193b-5p expression compared to the control medium when there was no external stimuli. However, in the presence of TNFa, the MSC-CdM group maintained a similar level of miR-193b-5p expression, whereas the control group exhibited a significantly increased amount of miR-193b-5p. On the other hand, occludin expression was significantly downregulated in the control group following TNFa stimulation, but the loss in occludin was significantly reduced when the cells were immerse in MSC-CdM (Figure 33). In animals that received control medium after CLP, miR-193b-5p expression was significantly increased; while MSC-CdM was able to attenuate much of the hike in miR-193b-5p expression following CLP (Figure 34). Similar to the HPMECs, animals injected with MSC-CdM showed a much more mild reduction in occludin following CLP insult (Figure 35).
Figure 32. Quantitative RT-PCR analysis of miR-193b-5p in conditioned medium treated HPMECs. HPMECs were treated with control medium or wildtype MSC-CdM, with or without TNFa (10ng/mL) stimulus for 24h, and then harvested for quantitative RT-PCR assays (see Methods section). Cells stimulated with TNFa in the presence of the control medium showed a marked increase in miR-193b-5p expression, while cells exposed to MSC-CdM showed a level of miR-193b-5p similar to that of control’s. Expression of miRNA was corrected for expression of the control gene, miR-191. The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 33. Quantitative RT-PCR analysis of occludin in conditioned medium treated HPMECs. HPMECs were treated with control medium or wildtype MSC-CdM, with or without TNFa (10ng/mL) stimulus for 24h, and then harvested for quantitative RT-PCR assays (see Methods section). Cells stimulated with TNFa in the presence of the control medium showed a marked decrease in occludin expression, while cells exposed to MSC-CdM exhibited an attenuated level of loss of occludin mRNA expression. Expression of miRNA was corrected for expression of the control gene, GAPDH. The values plotted are means ± SEMs of N=3-4 independent experiments for each treatment group.
Figure 34. Quantitative RT-PCR analysis of miR-193b-5p in conditioned medium treated mice.

Wildtype C57 6J mice were administered control medium or wildtype MSC-CdM, following sham or CLP surgery, for 24h, and then lung tissues were harvested for quantitative RT-PCR assays (see Methods section). CLP-operated mice showed a marked increase in miR-193b-5p expression when control medium was given. Sham-operated mice displayed no significant difference in miR-193b-5p expression when injected with either control or conditioned medium. CLP-operated mice that received MSC-CdM showed a noticeable decrease in miR-193b-5p expression. Expression of miRNA was corrected for expression of the control gene, miR-191. The values plotted are means ± SEMs of N=2 independent experiments for each treatment group.
Figure 35. Quantitative RT-PCR analysis of occludin in conditioned medium treated mice.

Wildtype C57 6J mice were administered control medium or wildtype MSC-CdM, following sham or CLP surgery, for 24h, and then lung tissues were harvested for quantitative RT-PCR assays (see Methods section). CLP-operated mice showed a marked decrease in occludin expression when control medium was given. Sham-operated mice displayed no significant difference in occludin expression when injected with either control or conditioned medium. CLP-operated mice that received MSC-CdM showed an evidently increased level of occludin compared to mice that were given control medium. Expression of miRNA was corrected for expression of the control gene, GAPDH. The values plotted are means ± SEMs of N=2 independent experiments for each treatment group.
Discussion

In the past decade, miRNAs have been demonstrated to play key regulatory roles in inflammatory and immune responses, including development and differentiation of B and T cells, proliferation of monocytes and neutrophils, antibody production, and the release of inflammatory mediators [174-176]. Also, miRNAs have been implicated in the pathogenesis of various inflammatory lung diseases, making them potential candidates to contribute to the pathogenesis of ARDS as well [81, 177, 178].

The main molecular effects that miRNAs exert are represented by gene expression variation. These alterations in the amount of gene copies produced by miRNAs are usually moderate, but the consequences potentially could affect numerous target genes [178, 179], which in turn may influence various physiological pathways in normal and disease states. miRNA can be used to inhibit mRNA production and function of disease-related genes. For instance, miRNAs that keep the expression of inflammation pathway genes in control could be used to avoid overexpression of cytokines, thereby to limit or suppress acute inflammatory response in patients. In addition, miRNAs that directly participate in the pathogenesis of ALI/ARDS could be down-regulated by antisense oligonucleotides, taking advantage of the sequence structure of these small RNA molecules [180].

**miRNAs and Sepsis**

Also, miRNAs can be detected in a variety of sources, including tissue, blood, and body fluids. Despite their short lengths, they are reasonably stable which increases their potential as clinical
biomarkers [178]. Recently, miRNAs were identified in serum and plasma as biomarkers for diagnosing and monitoring several diseases including cancer, cardiovascular diseases, and rheumatic diseases [181]. In septic patients, circulating miR-146a and miR-223 were shown to be significantly reduced compared with healthy controls [182]. miR-146a and miR-223 have been demonstrated by Wang et al to serve as potential biomarkers with high specificity and sensitivity for sepsis. In contrast, Vasilescu and colleagues reported that the expression of miR-150 correlated with the aggressiveness of sepsis; therefore, they suggested that miR-150 could be a plasma prognostic marker in patients with sepsis [183]. Similarly, the expression of miR-147 has been proven critical for endotoxin-induced tolerance [184]. miR-147 has been shown to become induced in LPS-treated murine peritoneal macrophages, and in LPS-exposed murine lungs via the stimulation of multiple TLRs. Such TLRs include TLR2, TLR3, and TLR4, all of which enable inflammatory cells to recognize invading microbial pathogens. Also, activated TLR3, TLR4, and TLR9 induced the expression of miR-148 and miR-152, which inhibited the production of pro-inflammatory mediators, including IL-12, IL-6, and TNFa [185]. Moreover, as a predicted suppressor for NF-kB, miR-9 was induced in human polymorphonuclear neutrophil and monocytes following TLR4 activation [186]. Therefore, a negative feedback loop exists in which TLR stimulation induces miRNAs such as miR-147, miR-9, miR-148, and miR-152 to prevent excessive inflammatory responses, and such a mechanism may contribute to immune homeostasis and immune regulation.
Microarray, miRNAs, and ARDS

With the use of high-throughput miRNA profiling, and quantitative real time polymerase chain reaction, more miRNAs associated with immune response, inflammation pathways, disease development, and disease severity could be evaluated as a novel tool in diagnosis monitoring, or as therapy in inflammatory lung diseases. More specifically, paired miRNA and mRNA microarrays to could be used identify the ones that exhibit differential expression in contributing to immune responses in the context of experimental sepsis-induced ARDS. Recently, in a microarray-based study, miR-181b has been demonstrated to regulate NFkB-mediated endothelial cell activation and vascular inflammation in response to pro-inflammatory stimuli and that rescue of miR-181b expression could provide a new target for anti-inflammatory therapy and critical illness [187]. In addition, the entire list of putative target genes of miR-181b contained 6 biological signalling pathways associated with NF-kB activation. In another microarray-based study, through the screening, miR-127 was identified to be involved in lung inflammation in ARDS. During lung injury, miR-127 was predicted to be down-regulated. By targeting IgG FcγRI, miR-127 successfully reduces cytokine release by macrophages [188]. The researchers were able to translate their in silico findings to their in vitro and in vivo models, and validated the anti-inflammatory effects of miR-127 in their IgG immune complex model.

miR-193b on Endothelial Integrity

Previously, functional studies on miR-193b have largely focused on its role as a potential tumor suppressor [189-191]. miR-193b was demonstrated to be a negative regulator of uPA in breast
cancerous tissues [190]. In metastatic breast cancer tissues, miR-193b was downregulated, which in turn upregulated uPA expression and contributed to the development of breast cancer. Also, miR-193b has been proven to down-regulate myeloid cell leukemia sequence 1 (Mcl-1) in melanoma cells [189]. From miR microarray profiling, miR-193b expression was significantly lower in malignant melanoma than in normal tissues. In a survey of melanoma samples, the level of Mcl-1 is inversely correlated with the level of miR-193b and that down-regulation of miR-193b in vivo could be an early event in melanoma progression. Moreover, miR-193b has previously been suggested to be epigenetically regulated in lymph node metastatic cell lines originating from colon, skin and head and neck cancers [192].

miR-193b has not been investigated in depth in the field of sepsis or ARDS. Only very recently, in a clinical publication, miR-193b was reported to be a biomarker for septic patients [193]. Wang and colleagues reported a significant overexpression in miR-193b in human serum in the non-survivors, and generated a composite score based on the expression of six miRNAs that had differential expression in patients with septic shock. The group discovered that the predictive value of miR-193b for sepsis mortality was better than SOFA scores and APACHE II scores, both of which are established composites that integrate numerous sepsis indicators. They concluded that a combination of miR-15a, miR-16, miR-193b, miR-483-5p, SOFA scores, APACHE II scores, would have a much better predictive value for sepsis mortality. In fact, all four of these miRNAs suggested by the Wang group were identified in our miRNA microarray analysis.
Endothelial cells perform multiple critical functions in vascular homeostasis, including controlling leukocyte trafficking, regulating vessel wall permeability, and maintaining blood fluidity. Pulmonary edema is a hallmark of ARDS. Herein, we provide evidence that miR-193b-5p is dynamically regulated in response to sepsis and TNFa, and functions to suppress the expression of a critical protein found in the tight junction, occludin. We have clearly demonstrated the ability of alterations that inflammatory stimuli have on the function of tight junctions. Here, we significantly expand this theme by demonstrating that manipulation of the expression of miR-193b-5p in an acute inflammatory response could alleviate the loss of permeability barrier function by rescuing occludin protein expression. In support, using complementary gain-and loss-of-function approaches, we demonstrated that knockdown of miR-193b-5p inhibits the degradation of occludin in HPMEC cells. Furthermore, overexpression of miR-193b-5p through mimic transfection was able to significantly suppress the activity of luciferase reporter plasmids that contained 3’UTR of occludin. Taken together, these studies identify miR-193b-5p as a novel regulator of vascular permeability.

However, it is important to note that individual miRs regulate multiple mRNAs, and likewise, each mRNA is often targeted by multiple miRs. Thus, it is likely that the collective actions of a number of miRs acting on a host of target mRNAs ultimately contribute to the pro-inflammatory effects of TNFa. Therefore, future studies need to map the combined actions of miR networks regulated by CLP/TNFa stimulation and to determine how these events may have an additive effect on endothelial integrity.
Interestingly, as predicted by the miRDB algorithm, we noted that the inhibition of some of the other putative targets of miR-193-5p may in fact also have a role in anti-inflammatory mechanisms and endothelial integrity. TNFRSF11B, and TNFRSF1B, both of which belong to the super family of tumor necrosis factor signalling, which has key roles in the development and regulation of the immune system [mirdb.org]. Members of the TNF receptor superfamily contain cysteine-rich domains in their extracellular portion and bind to ligands of the TNFSF. The TNFRSF has key functions in the development and regulation of the immune system and knockout mice of different family members usually have some form of immunopathology [194, 195]. Moreover, TNFSF ligands frequently activate NF-κB and loss of this NF-κB response is likely to contribute to immune dysfunction. Furthermore, loss of NF-κB signalling, for example in inhibitor of nuclear factor κ-B kinase (IKK) knockouts, mimics some of the effects seen in TNFSF knockouts [196, 197]. Thus, the up-regulation of miR-193b-5p brought about by MSC administration in our mouse model could potentially act to diminish the pro-inflammatory effects caused by TNFa signalling.

**Limitations on Microarray Studies**

Some of the limitations of microarray studies based on genome-level analyses include the fact that such methods only generate data extracted from a particular point in time, at a steady state condition [49, 198]. This may in fact overlook the dynamic changes that take place continuously in cells or tissues. In our validation experiment by qRT-PCR, as shown in Figure 12, we identified an initial surge in the expression of miR-193b-5p at 24h. However, this overexpression of the miRNA waned by 48h when we examined again. This subsiding trend
observed in miR-193b-5p expression could likely be attributed to a survivor bias, as miR-193b-5p has been shown to play a role in detrimental to endothelial integrity. On the other hand, if the true expression of miR-193b-5p did see a decrease from 24h to 48h, this would have been an overlooked phenomenon.

One other limitation to microarray studies is that there is no direct information about protein abundance, or post-translational modifications, such as phosphorylation or glycation. To address this weakness, a proteomic screening process could be used in parallel to the microarray screen [191]. When the transcriptome level data are examined in conjunction with the proteome level data, first, the end result of the miRNA down regulation can be directly determined, second, a combinatorial effect that takes into account knock-down of multiple genes can better address the broader impact of miR-193b-5p inhibitor on other gene pathways and networks that may assist in its function of preserving endothelial integrity.

Also, it is common to use RNA samples that are relatively homogenous and closely represent the disease condition, and RNA samples that come from a single cell type are widely used in microarray studies [69, 198]. Even though this approach provides a much more homogenous source of RNA, important and biologically relevant gene expression profiles could potentially be overlooked from this experimental setup. In our study, total RNA was extracted from whole lung tissues, making the expression signatures were representative to treat the organ as one entity.
Other Mechanisms by Which MSCs May Act on Occludin to Confer Protection

Another possible mechanism by which MSCs confer protective benefits to improve endothelial integrity in sepsis or sepsis-induced ARDS is through the interaction angiopoietin-1 and occludin. Increased permeability of the endothelium and epithelium, and decreased alveolar fluid clearance, may both contribute to the formation of edema in the alveoli. At the site of gas exchange, the alveolar epithelium normally forms a tighter barrier than the endothelium, and its loss of integrity in ALI is of great significance [199, 200]. Commonly, to measure the degree of lung endothelial and epithelial permeability, BAL albumin and protein are used as markers [95]. Several groups of researchers have documented beneficial effects of MSC administration on improving endothelial integrity.

One possible mechanism involves angiopoietin-1 (Ang-1). Mei and colleagues found that intratracheal administration of LPS led to increased BAL albumin, total protein, and IgM, and this was attenuated by intravenous MSC following the injury [95]. Previously, Ang-1 has been shown to reduce permeability and promote endothelial cell survival [201, 202] Two groups were able to determine that MSCs overexpressing Ang-1 led to significant reduction in BAL protein, and albumin. It was proposed that Ang-1 was able to diminish the influx of inflammatory cells and plasma protein leakage by acting on the vascular endothelium. Two groups were able to determine that MSCs overexpressing Ang-1 led to significant reduction in BAL protein, and albumin. It was proposed that Ang-1 was able to diminish the influx of inflammatory cells and plasma protein leakage by acting on the vascular endothelium [203,
These findings suggest that MSCs, acting in part through angiopoietin, improve the critical barrier function of the alveolar epithelium in ALI.

A previous study using rat brain capillary cells discovered that pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression. The up-regulation observed in occludin expression was significantly inhibited by angiopoietin-1-neutralizing antibody [205]. In addition, immunoprecipitation and western blot analyses confirmed that multimeric angiopoietin-1 induced occludin mRNA in endothelial cells. Also, Yu and colleagues reported that angiopoietin-1 ameliorates the expressions of ZO-1, occludin, VE-cadherin, and PKCα signaling after focal cerebral ischemia/reperfusion in rats [206]. Ang-1 injection significantly decreased blood brain barrier permeability, and increased the protein expressions of ZO-1, occludin, and VE-cadherin. Similar findings on Ang-1 and occludin interaction have also emerged from retinal microvascular endothelial cells [207]. Taken together, these studies have demonstrated that MSCs may act on Ang-1 - occludin interaction to help decrease the permeability of endothelial cells in response to TNFa or polymicrobial insult.

**DicER and miRNA Processing**

While we were able to establish a direct regulatory relationship by miR-193b-5p on occludin mRNA expression, which also led to a change in its protein levels in the tight junction, it was quite interesting for us to find that miR-193b-3p and the precursor were both responsive to the overexpression and knock down of the 5p mature form. As shown in Figure 28, when miR-193b-5p was overexpressed through transfection, we noted a significant increase in the expression
level of the precursor. And when the inhibitor of miR193b-5p was used along with TNFa stimulation, the increase in precursor copy number was dramatically reduced (Figure 29). Likewise, similar trends were observed in miR-193b-3p expression, as miR-193b-5p was manipulated through transfection (Figures 30, 31). The data suggest that there could in fact be a negative feedback pathway in the miRNA processing machinery that regulates both the precursor and mature forms of the miRNA, and the Dicer protein could have significant roles in such process.

Given our findings in this current study, it would be interesting to test the hypothesis that other cellular stresses or stimuli also lead to changes in the miRNA processing machinery. Indeed, it has been reported that Dicer expression is down-regulated by multiple stresses, such as hypoxia [208], reactive oxygen species, and many others [114]. Importantly, the biological significance of Dicer’s down regulation has not been understood in these cases. Thus, the functional changes associated with changes in dicer expression under stress need to be addressed in future studies. As produced in our current study, measurements of precursor as well as mature miRNA species may be a potentially useful indicator.

As established in the literature, biogenesis of miRNAs is a multistep process, and this process critically depends on Dicer for proper functioning. A specific set of proteins governs the formation and maturation of miRNAs from long, primary transcripts, to medium-length precursor forms, to the final stage of ~22 basepair mature miRNAs. Sequentially, the long initial miRNA transcript that contains a 7-methylguanosine cap and a poly-(A) tail, and more than one
stable step loop structures, encounters the RNAse III enzyme Drosha, in complex with DeGiorgio Critical Region 8 together with several other cofactors binds and gets cleaved. The step loop regions of the pri-miRNA then give rise to the precursor miRNA [209, 210]. At a length of less than 100 basepairs, the precursor is home to the 5′ or 3′ arm of the mature forms of miRNAs. The two arms are highly conserved, and this sequence complementarity found in the opposing arms is key for the recognition by exportin 5 for miRNAs to be transported to the cytoplasm for their final functions [211]. The pre-miRNA stem-loop is processed into a dsRNA duplex containing only the 5p and 3p miRNA sequences by another RNAse III enzyme, Dicer [212]. Usually, the more stable strand of the two processed mature miRNAs will be loaded into an argonaute (Ago) protein for target binding/degradation. Argonaute proteins mediate the effector function of the miRNAs through inhibiting translation of the target mRNA or, in the case of Ago2, by directly degrading the mRNA transcript [213].

Based on the essential role that Dicer plays in the miRNA processing, we investigated its potential function in our experimental sepsis model. In fact, we have also found that mesenchymal stem cell conditioned medium (MSC CdM) has been able to change Dicer and miR-193b-5p expression in a complementary pattern for miRNA function. From our unpublished data, we noted that MSC CdM administered to HPMEC cells that also received TNFa stimulation had significant impacts on the tight junction protein occludin and the miRNA processing machinery. For one thing, MSC CdM was able to alleviate the significant drop in occludin expression following CLP. Also, a number of proteins involved in the processing machinery, dicer, ago2, and exportin 5 all exhibited MSC CdM dependent expression -- MSC
CdM was able to suppress Dicer, Ago2, and exportin 5 expressions in response to TNFa, at both the mRNA level, as well as the protein level. We hypothesized that Dicer could act as a critical point in the entire miRNA regulatory mechanism.

It has been demonstrated that, at the catalytic core of the pre-to-mature miRNA processing complex, Dicer can also serve as an important site for regulation of miRNA biogenesis, which is in full support of our experimental findings. In fact, sequence association factors that bind to the pre-miRNA step loop have been proven involved in the promotion of miRNA maturation [214, 215]. Moreover, transactivating response RNA binding protein (TRBP) has been identified as an important component of the Dicer complex [216]. From HEK293 cells, with the use of gain- and loss-of-fucntion experiments, and microarray analyses, another group has demonstrated that phosphorylatable TRBP is critical for the expression of a variety of miRNAs, including miR-17, miR-20a and miR-92a, that were induced upon phosphorylation. TRBP proteins are phosphorylated by extracellular signal-regulated kinase, ERK1/2. The ERK pathway is a fundamental modulating component of various cellular pathways, thus, TRBP recruitment to the Dicer complex may act as a regulatory switch activated by ERK signaling to promote expression of miRNAs [217, 218].

As we know, ERK has been well established to have important roles in the process of inflammation. It has been reported that ERK is involved in controlling endothelial NF-kB activation and inflammatory responses. In human endothelial cells, vascular endothelial growth factor (VEGF) induces NF-kB-dependent transcription of cell adhesion molecules and monocyte
adhesion. Consistently, inhibition of ERK significantly increased nuclear translocation of NF-kB induced by VEGF, whereas overexpression of ERK resulted in the loss of these responses to VEGF. Using two protein kinase C inhibitors has demonstrated that VEGF downregulates its negative regulatory signal ERK through protein kinase C that lies downstream. Strikingly, elevation of ERK in endothelial cells markedly inhibited NF-kB activation as well as monocyte adhesion induced by IL-1β and TNFα. The data collectively suggest that ERK serves as an anti-inflammatory signal that suppresses expression of NF-kB-dependent inflammatory genes in endothelial cells.

Therefore, under an overall inflammatory response, miRNA levels could potentially be altered by Dicer expression, which in turn may be regulated by an ERK-NF-kB interaction/feedback [218]. The use of MSC-CdM may exert its effect on this interaction through its secreted paracrine factors, altering ERK’s levels, producing a much less inflammatory state in the endothelial cells, and a reduced amount of Dicer available for miRNA processing. Thus, measuring the existence of ERK activity in vascular endothelial cells may be useful for predicting the feasibility and potency of inflammatory reactions in the vasculature, and could act as an indicator for miR-193b-5p expression/activity.

Moreover, another component of the Dicer complex, the protein kinase R activating protein (PACT) could also have a critical role in the activation of pre-miRNA processing. Despite being a non-essential component in the process of miRNA maturation, inhibition of PACT has been demonstrated to decrease expression levels of a number of mature miRNAs [219]. Interestingly,
knocking down any components of the Dicer complex inhibits the levels of both pre-miRNAs and mature miRNAs [220]. Again, this evidence reinforces our hypothesis of an existing feedback loop by which mature miRNAs could act to modulate the levels of their pre-miRNAs. In C. elegans, the feedback mechanism was found to affect for let-7 expression where the argonaute protein can bind a conserved region in the 3’ end of pri-let-7 to promote its processing [220].

However, similar to the Drosha microprocessor complex, the Dicer complex may still be bypassed in miRNA biogenesis in many cases. From mice that were deficient in Dicer, sequencing analyses identified significant enrichment of one specific mature miRNA: miR-451. Pre-miR-451 has a unique, highly conserved structure that allows it to associate with Ago2, which contains RNase activity. Loading of pre-miR-451 into Ago2 results in slicing of pre-miR-451 and generates a 30 nt intermediate miR-451, which is then further trimmed to become a mature miRNA [221].

**Other Potential Microarray Approaches**

As discussed in the Methods section, our microarrays were performed using bead-based technologies. Despite being the most widely used approach in the field, in the near future, new or adapted platforms that address newly recognized challenges in miRNA detection are expected to be developed.
One such challenge in miRNA studies is the detection of miRNA from sources that yield only low amounts of miRNA, for instance, plasma or serum; or where miRNA may be difficult to extract, including formalin-fixed tissue blocks. Even though in our experiment, none of the two sources of miRNAs was in fact a difficulty for us, the lung still represents a tremendously heterogeneous tissue with many different cell types that are capable of producing a large variety of miRNAs, a great number of which are only present at extremely low copy numbers. To address the difficulty in RNA extraction, methods that eliminate the need of RNA extraction have received increasing amount of attention. HTG Molecular Diagnostics has recently released the quantitative nuclease protection technology system (http://www.htgmolecular.com/proof-for-ffpe-samples-drug-discovery-news/). In this technique, RNA and DNA oligonucleotides complementary to the miRNAs hybridize to their targets. S1 nuclease is added to the sample to remove non-protected RNA and excess oligonucleotides. Once the RNA is removed, a specific DNA oligonucleotide library is generated. The oligonucleotides are then bound to specific oligonucleotide linkers on the array and are detected with a horseradish peroxidase probe that produces measurable luminescence in the presence of substrate. Studies involving miRNAs extracted from formalin-fixed tissues have successfully used this technique to detect miRNA expression levels. Using this approach may potentially provide us with an even more precisely documented global miRNA expression profile in the lung, in ARDS.

**Future Outlook**

To further validate our experimental findings, both in silico and in vitro, we aim to translate these results into an in vivo model to establish biological significance of the modulation of miR-
193b-5p. The same kind of wildtype C576J mice would be used as the ones in the microarray. LPS will be administered intratracheally, and the inhibitor of miR-193b-5p will be injected through the tail vein 6h after LPS has been given. We will assess membrane permeability in the lung by the extravasation of Evans blue dye. Histologically, the permeability and occludin expression will also be examined. Moreover, we will use wet-to-dry ratio as a factor to help determine the overall impact of miR-193b-5p inhibitor on the entire organ.

In addition, miR-193b-5p expression will be investigated in mice that are subjected to CLP surgeries and given MSC conditioned medium as treatment. We will further analyze the impact that MSC-CdM administration can have on the miRNA processing machinery, miR-193b-5p, 3p, precursor, and tight junction proteins. This in vivo experiment may help corroborate our established findings in the in silico and in vitro experiments, and elucidate the changes that MSC-CdM may have on the global gene expression profile much higher upstream due to its potential impact and regulation on the miRNA processing machinery since thousands of genes’ expression could be resultantly changed.

Currently, we have human lung tissues from patients that died from ARDS. We are very interested in finding out the genetic profiles exhibited in these septic patients compared to normal ICU controls. As demonstrated in a previous study in ICU septic patients, miR-193b has been found to exhibit changes in the serum, the findings obtained here will serve as the first critical piece of evidence to complement the protective beneficial effects that MSCs confer in experimental models of sepsis [193].
Summary

Collectively, understanding how miRNAs regulate lung inflammation may represent an attractive way to modulate ARDS-associated symptoms. In summary, we have identified miR-193b-5p as a MSC-responsive miRNA that regulates the expression of a key tight junction protein, occludin, involved in the endothelial integrity in response to inflammation in vitro and in vivo. These findings support the possibility that miR-193b-5p may serve as an important miRNA involved in inflammation by controlling essential aspects of endothelial cell homeostasis in the lung under physiologic and/or pathologic conditions. These studies also revealed that strategies that keep miR-193b-5p expression in check, eg.the inhibitor of miR-193b-5p, as potential therapies for the treatment against ARDS. Our current study provides the initial framework to identify and characterize the role of other potential miRNAs as candidate therapeutic tools. Taken together, the combination of high-throughput microarrays and bioinformatics analyses constitutes a valid approach for molecular target identification to limit acute inflammatory disease states.
References


