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Role of Micrornas in Intestinal Inflammation and Barrier Homeostasis after Alcohol and Burn Injury

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ROLE OF MICRORNAS IN INTESTINAL INFLAMMATION AND BARRIER HOMEOSTASIS AFTER ALCOHOL AND BURN INJURY

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LIST OF ABBREVIATIONS

AMP	Anti-Microbial Peptide
ANOVA	Analysis of Variance
APC	Antigen Presenting Cells
BAC	Blood Alcohol Concentration
CXCL	Chemokine Ligand
DAMP	Damage Associated Molecular Pattern
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbant Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GI	Gastrointestinal
HBSS	Hank's Balance Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia Inducible Factor
HRP	Horseradish Peroxidase
IEC	Intestinal Epithelial Cell
IL	Interleukin
i.p.	Intraperitoneal
KC	Keratinocytes-Derived Chemokine (Also known as CXCL1)

LNA	Locked Nucleic Acid
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
miRISC	miRNA-Induced Silencing Complex
MOF	Multiple Organ Failure
MyD88	Myeloid Differentiation primary response 88
NLRP	NLR Family Pyrin Domain Containing
PAMPS	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PVDF	Polyvinylidene Difluoride
RT-qPCR	Quantitative Real-time Polymerase Chain Reaction
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
STAT	Signal Transducer and Activator of Transcription
TBSA	Total Body Surface Area
TGF	Transforming Growth Factor
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF Receptor Associated Factor
UTR	Untranslated Region
WHO	World Health Organization
ZO	Zonula Occludin

CHAPTER 1

INTRODUCTION

Burn injury is one of the leading causes of accidental injury and death in the United States[1]. The pathophysiology of severe burn injury is systemic and complex, with consequences on almost every organ system throughout the body. In particular, severe burn patients are at risk of multiple organ failure (MOF) and sepsis, which significantly contribute to mortality[2,3]. Nearly half of reported burn injuries occur under the influence of alcohol[4–6]. Compared to patients with similar burn size and depth, individuals intoxicated at the time of burn injury require longer hospital stays and surgical procedures. Additionally, they have an increased risk of infection, MOF, sepsis, and ultimately higher mortality rates[6–10]. In order to study how alcohol exacerbates burn pathophysiology, our laboratory utilizes a well-established model of acute alcohol intoxication and burn injury. In short, mice receive a single gavage of 0.4 mL 25% ethanol, resulting in a blood alcohol content of 90-100 mg/dL four hours later. At this time mice receive an approximately 12.5% total body surface area scald burn injury on their shaved dorsum. This dosage is clinically relevant as traumatic injuries are more often associated with acute, episodic drinking as opposed to chronic alcohol consumption[11]. As alcohol is a key mediator in exacerbating post-burn pathogenesis, severe pathological changes are only seen when burn is combined with alcohol exposure. Using this model, our laboratory and others have shown alcohol and burn injury's impact on several systems, including the gastrointestinal (GI) tract, lungs, liver, and metabolism[12–15]. Gut dysfunction is common in trauma injuries and has emerged as a critical instigator of post-burn co-morbidities, including sepsis and multiple organ

failure[7,8,16,17]. Acute intestinal inflammation, which has been shown to include excessive intestinal inflammatory cytokines, including IL-6, along with increased neutrophil accumulation can negatively impact gut barrier integrity after alcohol and burn injury[18–20]. Translocation of bacteria and pro-inflammatory pathogen associated molecular patterns (PAMPs) across the compromised intestinal barrier results in system inflammation and distant organ damage[16,18,21].

MicroRNAs (miRNAs) are small noncoding RNAs which post-transcriptionally regulate gene expression. MiRNAs recognize target mRNAs via complementary binding to the 3' untranslated region (UTR) and result in translational repression or mRNA degradation[22,23]. Over half of the genome is estimated to be regulated by miRNAs and a single miRNA can regulate multiple targets[22–24]. Therefore, miRNAs are an important regulatory mechanism that coordinate several key signaling networks within cells. In particular, miRNAs are critical regulators of normal gut homeostasis and barrier integrity[25–29]. Aberrant miRNA expression is associated with intestinal inflammation and gut barrier disruption, contributing to pathophysiology associated with inflammatory disorders of the intestine such as colorectal cancer and inflammatory bowel disease (IBD)[30–33]. While traumatic injury and alcohol exposure are known to alter miRNA expression, their role in gut barrier integrity following alcohol and burn injury is poorly understood[34–37].

This led to our central hypothesis that alteration of miRNA expression in intestinal epithelial cells drive inflammation and gut barrier dysfunction following alcohol and burn injury. To address this, we developed three specific aims. Aim 1 profiles anti-inflammatory miRNA expression in small intestinal epithelial cells after alcohol and burn injury and investigates the impact of reduced miR-146a expression on intestinal epithelial cell inflammation. Aim 2 evaluates the effects of miR-146a mimic administration on intestinal inflammation and barrier

integrity following alcohol intoxication and burn injury. Finally, aim 3 investigates global changes in the miRNA and gene expression profile of intestinal epithelial cells after alcohol and burn to elucidate miRNA–mRNA interactions that regulate critical pathways for gut barrier function.

To begin, small intestinal epithelial cell expression levels for a panel of anti-inflammatory miRNAs were assessed by RT-qPCR and significant downregulation of miR-146a was identified one day following alcohol intoxication and burn injury. *In vitro* studies demonstrate that miR-146a expression regulates intestinal epithelial cell LPS-induced inflammatory cytokine expression by regulation of p38 MAPK signaling and targeting of TRAF6. We then sought to assess the therapeutic potential of targeting miR-146a downregulation to reduce intestinal inflammation and promote barrier integrity following alcohol and burn injury. Small intestinal epithelial cell expression of miR-146a was elevated following alcohol and burn injury via intraperitoneal injection of miR-146a mimic the day prior to injury. Our results show that overexpression of miR-146a in small intestinal epithelial cells significantly reduced intestinal inflammation one day after alcohol and burn injury. We also found that *in vivo* miR-146a overexpression promoted intestinal epithelial cell proliferation and tight junction protein expression but was unable to significantly reduce intestinal permeability. To broaden our understanding of the contributions of aberrant miRNA expression on intestinal barrier disruption following alcohol intoxication and burn injury, we then performed an integrated analysis of miRNA and gene expression in small intestinal epithelial cells isolated one day following combined injury. Our results identify several miRNAs which could promote gut barrier disruption after alcohol and burn injury, including upregulation of miR-98-3p and miR-381-3p, which is associated with reduced proliferation, upregulation of miR-29a-3p, miR-429-3p and

miR3535, which can reduce cellular adhesion, and downregulation of Let-7d-5p and miR-130b-5p, which is linked to apoptosis. Together, these studies demonstrate the critical role that miR-146a downregulation could play in promoting excessive intestinal inflammation following alcohol intoxication and burn injury, thereby contributing to severe consequences including sepsis and multiple organ failure. Our findings also provide new insights into the contributions of aberrant miRNA to intestinal dysfunction in general and presents several potential miRNA targets of interest which could provide a new avenue of therapy for patients after alcohol intoxication and burn injury.

CHAPTER 2

REVIEW OF RELATED LITERATURE: MICRORNA REGULATION OF INTESTINAL INFLAMMATION AND BARRIER HOMEOSTASIS AFTER ALCOHOL AND BURN INJURY

Introduction to MicroRNAs

MicroRNAs (miRNAs) were first discovered as important regulatory molecules in the widely studied model organism *Caenorhabditis elegans*[38]. Since then, miRNAs have been shown to be highly conserved in their regulation of target mRNAs across animal species, highlighting their importance as essential components of normal physiology. Due to the nature of miRNA target recognition, a single miRNA can regulate the expression of several mRNA targets[39,40]. Likewise, a single mRNA can be targeted by several miRNAs. Studies have estimated that almost half of the human genome is regulated by miRNAs[24,39–41]. As such, even small changes in miRNA levels can have a dramatic impact on cellular processes and aberrant miRNA expression has been associated with a variety of diseases including cancer, neurodegenerative disorders, diabetes, and heart disease[42].

Biogenesis of miRNAs is a highly regulated process that was previously thought to be a single pathway universal to all miRNAs. Although further investigations have revealed a variety of alternative processes that impact miRNA biogenesis, most functional miRNAs share the same general biogenesis pathway[22,43,44]. First transcribed similarly to mRNAs, the primary transcript, or pri-miRNA, includes a hairpin structure which contains the final mature miRNA sequence within. The initial step of miRNAs processing is performed by a complex containing Drosha,

which is the main enzyme responsible for excising the hairpin to generate a precursor molecule, or pre-miRNA[45]. Once exported out of the nucleus via Exportin 5, the hairpin precursor is further processed by Dicer to remove the looped end of the hairpin[46–48]. One strand of the resulting duplex is then selectively loaded into Argonaute to form the final miRNA-Induced Silencing Complex (miRISC)[49,50]. The final mature miRNA can originate from either the 5' or 3' strand of the duplex, which is delineated in current miRNA nomenclature as -5p or -3p respectively. Although both strands can generate functional miRNAs, studies indicate that one strand is often preferentially loaded into Argonaute. Therefore, previous nomenclature has referred to the dominant strand as the guide miRNA (designated miR-) while the strand more often degraded is the passenger strand (designated miR*-)[50].

The final miRISC complex recognizes its target mRNA by imperfect base pairing of the miRNA and the 3' untranslated region (UTR) of the target mRNA, resulting in mRNA expression downregulation. Although a mature miRNA is approximately 22 nucleotides, the specificity of a mature miRNA for its target mRNA is determined by its seed sequence (nucleotides 2-7)[40,41]. miRISC mediated downregulation of gene expression has been shown to require GW182 proteins within the RISC complex[51]. Ultimately, mRNA downregulation via miRISC can occur by either preventing mRNA translation or triggering mRNA degradation[49,52]. Inhibition of mRNA translation primarily occurs via disassociation of the 5' methylated cap from the 3' poly-A tail, which de-circularizes the target mRNA and suppresses initiation of its translation. Although this process is enough the downregulate gene expression, some miRISC complexes promote the degradation of their target mRNA. This occurs through the recruit of de-adenylase proteins, which shorten the poly-A tail. Target mRNAs with shortened tails then

recruit decapping enzymes and are ultimately targeted for degradation by cytoplasmic 5' to 3' exoribonuclease[53,54].

Functional mature miRNAs are regulated at nearly every step of their biogenesis to tightly control their expression and safeguard miRNA homeostasis. This is crucially important because slight changes in miRNA expression have been shown to have widespread impacts on cellular function, contributing to human disease. Similar to mRNAs, transcription factors are heavily involved in the regulation of miRNA transcription. This allows for increased or decreased expression of miRNAs in response to cellular stimuli and generates feedback loops in which miRNAs target transcription factors that regulate them[55,56]. Following transcription, miRNAs are processed first by Drosha in the nuclear and then Dicer in the cytoplasm. Changes in the protein levels of these key enzymes can modulate miRNA processing. In addition, recognition of miRNAs by these processing enzymes can be regulated by RNA binding proteins which bind miRNA transcripts and/or accessory proteins which associate with the processing enzyme complex. Export of the pre-miRNA molecule from the nucleus to the cytoplasm can be regulated by modulation of Exportin 5 activity, which requires hydrolysis of its cofactor Ran-GTP to release its RNA cargo[57]. Binding to Exportin 5 also stabilizes the pre-miRNA and protect it from nuclease degradation[52,58]. Although Argonaute does not play a primary role in processing miRNAs, strand selection and the loading of mature miRNAs into Argonaute is a complex process that is not fully understood[50]. Furthermore, the stability of miRNAs and their degradation by nucleases appears to be primarily regulated by their association with Argonaute and target mRNAs[59]. Finally, more recent studies suggest that miRNAs can also regulate themselves by binding to their pri-miRNA sequence and either positively or negatively regulating their processing into pre-miRNA[52,60].

MicroRNAs and Intestinal Homeostasis

Investigation of the intestinal miRNA transcriptome has demonstrated the crucial role that miRNAs play in normal gut homeostasis and intestinal epithelial cell function. Under normal conditions, intestinal epithelial cells support gut homeostasis via maintenance of the intestinal barrier. In order to sustain a healthy barrier, intestinal epithelial cells are constantly being sloughed off at the top of villi structures and replaced by new epithelial cells originating from stem cells present in crypts. This process requires strict control of intestinal epithelial cell proliferation, differentiation, migration, and apoptosis[61]. In addition, intestinal epithelial cells form intercellular junctions to regulate paracellular permeability. Among these intercellular junctions, tight junctions are the main determinant of intestinal permeability and are composed of transmembrane proteins Claudin and Occludin, in addition to intracellular accessory proteins such as ZO-1[62].

Early studies of miRNA function within the intestinal epithelium focused on specific miRNAs identified by their contributions to colorectal cancer. For example, Let-7 miRNAs were found to be downregulated in colon cancer tumors and further investigation confirmed their tumor suppressor capacity[63]. Eventually, studies were performed to demonstrate the crucial role that miRNAs play in regulating and maintaining normal gut homeostasis. Due to the major role that the Dicer enzyme plays in miRNA processing, studies utilized Dicer knockout within the intestinal epithelium to investigate the general impact of disrupting miRNAs on intestinal function. McKenna et al demonstrated that Dicer knockout in Villin-Cre mice resulted in dramatic effects on intestinal homeostasis, including impaired intestinal epithelial cell differentiation leading to expanded crypt zones, lower goblet cell counts, and increased epithelial cell apoptosis[27]. Interestingly, microarray analysis on intestinal epithelial cells revealed that

immune associated genes were the largest category impacted by Dicer knockout. This was associated with elevated intestinal inflammation, increased neutrophil infiltration, and ultimately disruption of intestinal epithelial cell tight junctions, indicating the important role miRNAs play in gut barrier integrity[27]. Other studies have gone on to elucidate the contributions of several individual miRNAs to intestinal homeostasis barrier integrity, including miRNAs that directly target tight junction proteins [25,26,28,29,64].

MicroRNA Contributions to Intestinal Inflammation and Barrier Disruption

Intestinal epithelial cells have been increasingly recognized for their immunological functions and capacity to mediate inflammatory signaling. In particular, their expression of innate immune receptors results in inflammatory signaling that play a crucial role in intestinal barrier function via regulating intestinal epithelial cell proliferation, apoptosis, and tight junction integrity[65–68]. Due to the close proximity of intestinal epithelial cells to gut bacteria, toll like receptor (TLR) signaling especially plays in important role in regulating intestinal inflammation and subsequent barrier function[66,69]. For example, studies using knockout of the adaptor protein myeloid differentiation primary response 88 (MyD88), resulting in loss of TLR signaling, demonstrate reduced intestinal barrier integrity due to loss of ZO-1 protein and impaired epithelial cell differentiation[66,70,71]. On the other hand, too much TLR signaling can perpetuate inflammation and also has detrimental effects on the gut barrier[18,69,72]. Dysregulated TLR signaling that leads to intestinal inflammation has been shown to promote chronic disease, including inflammatory bowel disease (IBD) and colorectal cancer[65,66,69]. Intestinal epithelial cell inflammatory signaling also contributes to overall intestinal immunity via crosstalk with intestinal immune cells present within the epithelium and in the underlying lamina propria. Pro-inflammatory cytokines, including IL-6, IL-18, and TNFα, or anti-inflammatory cytokine, such

as IL-10 or TGF-β, are released by intestinal epithelial cells and can impact a variety of immune cell populations. While anti-inflammatory cytokines are important for immune tolerance and resolution of inflammation, elevated pro-inflammatory cytokines can promote innate immune cell recruitment and modulate T cell function, thereby exacerbating intestinal inflammation. For example, IL-6 is overexpressed in the inflamed intestinal epithelium and may contribute to the pathogenesis of colitis associated cancers by promoting inflammatory macrophage polarization[73]. In addition, the accumulation of activated innate immune cells, such as neutrophils, in the intestine has been shown to promote inflammation and result in tissue damage and barrier disruption[19,74]. Overall, studies have shown that numerous inflammatory intestinal disorders exhibit disruption of the intestinal barrier, thus supporting the critical association between inflammatory signaling and loss of intestinal barrier integrity[30,31,33].

Regulation of key inflammatory signaling pathways by miRNAs has been primarily studied in the context of immune cell signaling. Although less is known about miRNA regulation of inflammation within the intestinal epithelium, several studies indicate that miRNAs are fundamentally involved in regulating the balance between intestinal inflammation and gut barrier function. As previously described, Dicer knockout within intestinal epithelial cells is highly associated with disruption of genes related to immunity. Studies using these genetic mouse background show increased intestinal inflammation, characterized by elevated inflammatory cytokine production and increased immune cell infiltration[27]. In addition, several studies have reported aberrant miRNA expression in inflamed intestinal tissues. For example, microRNA expression profiles can be used to track active flares in patients with inflammatory bowel disease and differentiate between disease states[72,75]. Due to the increased risk that IBD patients have to develop colorectal cancer, miRNAs have become attractive biomarker and prognostic tools.

Further investigations into miRNAs of interest have begun to elucidate the molecular pathways responsible for miRNA mediated regulation of intestinal inflammation, providing both mechanistic insight and potential therapeutic targets[32,75].

Targeting miRNAs in Diagnostics and Therapeutics

Since the discovery of miRNAs and their crucial role in maintain normal cellular homeostasis, miRNAs have been studied for their association with disease. Numerous miRNAs have been identified for their involvement in disease onset and their expression can change depending on disease progression. Early diagnosis is critical for the prognosis of patients with diseases such as cancer. Therefore, the development of non-invasive and sensitive biomarker panels using miRNAs have been of particular interest for the early identification of disease onset. In addition, miRNAs have been explored for their use as biomarkers of patient prognosis and responsiveness to therapy. Circulating miRNAs have been the focus of several biomarker studies due to the ease of obtaining blood and other body fluid samples from patients. Research has shown that miRNAs can be secreted from cells by packaging them into exosomes or they can be secreted bound to RNA binding proteins like Argonaute (Ago2). Although the mechanism of miRNA packing and secretion is not fully understood, these circulating miRNAs serve an important purpose for intercellular communication. As more miRNAs are identified for their association with disease and our ability to detect circulating miRNAs advances, several miRNA-based diagnostic tools have made their way into the clinic. These tools range from tests that assess tumor biopsy miRNA expression to characterize malignancy, to those that readily assess circulating miRNAs as early detectors of disease. ThyraMir is a 10-miRNA panel used in conjunction with a standard oncogene expression panel on thyroid biopsy aspirates to differentiate between benign and cancerous thyroid nodules, which has been shown to

significantly decrease unnecessary surgical interventions. Other cancer related tools include the miRNA library miRviewTM Mets, which was developed to test metastatic tumor biopsies and ascertain unknown tissues of origin. The use of miRNA biomarkers has also led to the development of tools outside of cancer diagnosis, including the OsteomiR panel of 19 miRNAs detected in the blood to ascertain fracture risk in patients suffering from osteoporosis or type-2 diabetes. In addition, ThrombomiR detects 11 miRNAs in patient serum to assess platelet reactivity and has been used to analyze the risk for certain cardiovascular events or for the early diagnosis of type-2 diabetes.

Identification of miRNAs as markers of disease has naturally developed into studies characterizing the functional impacts of altered miRNA expression on disease pathology. In addition, several studies have shown that the expression of circulating miRNAs can correlate with miRNA expression in tissue. This allows researchers to utilize circulating miRNAs as an easy means of identifying miRNAs changes in disease, correlate them with expression changes in the tissue of interest, and then study the mechanistic role these miRNAs may play in disease pathogenesis. For example, miR-21-5p was identified as a plasma diagnostic marker for colorectal cancer which was also significantly increased in colorectal cancer tissue compared to normal colonic tissue[76]. Further studies have shown that elevated miR-21-5p in colonic tissue promotes colorectal cancer and that circulating miR-21-5p is a promising, easily detectable biomarker[77].

As our understanding of miRNA associated pathophysiology expands, the therapeutic targeting of aberrant miRNA expression has expanded as an area of interest. The two main strategies for miRNA therapy include miRNA restoration and miRNA inhibition-based therapies[42,78,79]. Restoration based therapies, in most cases, utilize miRNA mimics to restore the

expression of beneficial miRNAs which are downregulated in disease. These miRNA mimics are double-stranded RNA molecules that are essentially a copy of the mature miRNA duplex. Upon entering a cell, the guide strand from the duplex is incorporated into the miRISC complex while the passenger strand is degraded. Mimic molecules can be modified to promote incorporation of the targeted guide strand and to reduce the risk of off target effects from improper incorporation of the passenger strand into the miRISC complex. To this purpose, passenger strands can be cleaved into two parts, both of which are too small to generate a functional miRISC complex. Inhibitor-based miRNA therapies are used to silence target miRNAs which are overexpressed and promote disease. Antagomirs are single stranded RNA oligonucleotides that have a complementary sequence to the miRNA of interest, blocking the interaction between the miRNA and its target mRNAs[80]. Studies investigating and utilizing of miRNA therapeutics have led to several advancements that overcome the intrinsic difficulties of administering RNA oligonucleotides. Two common problems associated with miRNA therapeutics include the abundance of RNases in the body which can readily degrade oligonucleotides and side effects associated with innate immune system activation via Toll-like receptor recognition of RNA molecules. The most common modification to prevent these issues for both miRNA mimics and antagomirs is the alteration of the 2’ -OH position in the ribose sugar backbone via addition of a methyl group. A well-studied variation includes the locked nucleic acid (LNA), which is an oligonucleotide containing one or more backbone monomers with a 2’-C,4’-C-oxy-methylene link[81]. Modifications like these reduce RNase recognition of administered miRNA therapies, which increases their stability therefore also reduces innate immune activation.

Although there are currently no approved miRNA-based therapies, the field of miRNA therapeutics is promising. Several miRNA-based therapeutics have reached clinical trials,

including as treatments for cancer, viral infection, and inflammatory bowel disease. Miravirsen is one of the farthest developed miRNA-based therapy, currently in phase II clinical trials for the treatment of hepatitis C[42,78,79]. This LNA antagomir targets miR-122, which is highly expressed in the liver and promotes hepatitis C virus stability and propagation[82]. Clinical trials have demonstrated the efficacy and safety of miravirsen, including its ability as a monotherapy to produce long-last inhibition of viral activity[83,84]. There are also alternative studies that utilize drugs to alter miRNA expression instead of administering miRNA mimics or antagomirs. ABX464 is a quinoline drug that induces miR-124 expression in immune cells and is currently in clinical trials for inflammatory bowel disease[85]. Although originally developed for its ability to inhibit HIV replication, ABX464 was shown to have anti-inflammatory properties that were dependent on its induction of miR-124[86]. Studies have shown that miR-124 is a crucial regulator of inflammation that is downregulated in the colons of patients suffering from active ulcerative colitis[87]. Early clinical trials have demonstrated that ABX464 treatment is well tolerated and effective, with one study showing 70% of patients achieving clinical remission following 8 weeks compared to 33.3% in the placebo group[85,88]. Studies such as these highlight the incredible potential present in the field of miRNA targeted therapeutics.

Alcohol Intoxication and Burn Injury

Burn injuries are a subset of traumatic injuries to the skin that can be caused by a number of different insults. This includes thermal, chemical, radiation, electrical, friction, and cold burns (frostbite). The majority of burns are caused by thermal insults, including fire, hot objects, or hot liquids/scalding. As of 2016, an estimated 486,000 people in the United States received medical care related to a burn injury, with 40,000 people hospitalized due to their injury[1]. Furthermore, incidents of burn injury are significantly higher in lower income countries with the WHO

attributing up to 90% of the 11 million burn injuries world-wide in low-income countries and are a major cause of morbidity in the world[89].

Burn injury severity is one the main indicators of prognosis and is categorized by the depth and size of the wound. First degree burns have the injury limited to the epidermis, or outermost and superficial layer of the skin. Second degree or partial thickness burns are defined by the injury extending past the outermost layer into the dermis and often form blisters that are extremely painful. Depending on the depth of the partial thickness burn, surgery may be required. Third- and fourth-degree burns penetrate the full thickness of the dermis, with fourth degree burns causing deeper damage to underlying muscle or bone. Third- and fourth-degree burns are not painful due to destroyed nerve endings and require surgery and careful management of the burn area to prevent infection. Along with the depth of the injury, the total surface area is an important component of determining burn injury severity. If the burn covers less than 10% of the total body surface area (TBSA), it is categorized as a minor burn. The classification for major burns is less well-defined, however, the following guidelines are often used to indicate a major burn: greater than 20% TBSA in adults, greater than 30% TBSA in children, and greater than 10% TBSA in elderly patients[16].

The initial burn injury causes numerous detrimental effects not only to the immediate burn area but leads to a cascade of responses in the entire body that can lead to severe consequences such as shock and multiple organ failure. These systemic responses can cause serious detriment to the patient and their recovery. Burn injury usually leads to distributive shock, in which capillaries become leaky and fluid is lost to the extravascular space. This loss of fluid from the circulatory system results in edema, fluid accumulation in tissues, reduced cardiac output, and compromised delivery of oxygen to numerous bodily organs, including the

gastrointestinal tract[90,91]. Immediately following injury there is also systemic release of catecholamines, cortisone, and several inflammatory cytokines into circulation. This systemic inflammatory phase is characterized by release of inflammatory cytokines such as IL-1, IL-6, IL-18, and tumor necrosis factor (TNF), leading to further detrimental effects in numerous organ systems[92]. Uncontrolled inflammatory responses and the release of cytokines can lead to systemic inflammatory response syndrome (SIRS), characterized by overactivation of the immune system[93]. Uncontrolled activation of the inflammatory responses leads to organ tissue damage, which exacerbates the injury itself. Under these circumstances, the inflammatory mediators produced by the host are causing more damage to organ systems rather than eliminating subsequent infections.

Most instances of burn injury do not occur in isolation and are complicated by factors such as the patient alcohol and/or drug usage. In nearly half of all burn injuries, patients are intoxicated at the time of admission following burn injury[94]. Alcohol leads to lowered inhibition, loss of dexterity and balance, and leads to increased accidental injuries, including burn injury[95]. It is well-established that alcohol intoxication at the time of burn injury leads to worse outcomes, including increased hospital stay, increased infection rates, and a higher rate of surgical procedures[7]. Alcohol alone is a risk factor and one of the leading causes of morbidity and mortality worldwide[96]. Furthermore, alcohol usage can disrupt the microbiome, leading to dysbiosis, as well as alter permeability of the intestinal barrier. Studies have shown increased levels of endotoxin in alcoholic patients compared to controls, as well as increased small intestinal bacterial overgrowth[97–99]. Ethanol exposure also alters immune responses, such as decreasing NLRP3 activation and cytokine production[100–102]. Taken together, alcohol exposure at

the time of burn injury potentiates end organ damage, including the lungs, liver, and gastrointestinal tract[103–107].

Gastrointestinal Consequences Following Alcohol and Burn Injury

Some of the most serious consequences of alcohol and burn injury are the result of remote organ injury, such as inflammation in the lungs, kidney, gastrointestinal system, and bone marrow[108–113]. The gut-origin hypothesis of sepsis, SIRS, and multiple organ dysfunction after burn injury states that breakdown of the gut barrier and hyperpermeability leads to the transport of toxic agents derived from gut bacteria into the portal circulation and mesenteric lymph[114]. Toxic agents can include bacterial products like peptidoglycan or endotoxin, which can travel to distant organs to cause inflammation and organ failure. In support of this hypothesis, animal models of trauma have found that ligation of the mesenteric lymph duct can prevent acute lung and renal injury following intraperitoneal injection of LPS and models of hemorrhagic shock in rodents [115–117]. Similarly, mice given prophylactic antibiotics prior to burn injury reduced hepatosteatosis and liver injury markers[118]. Numerous abnormalities in the GI system have been documented in burn patients, including increased gut permeability, decreased gut motility/transit, and increased gut bacteria translocation[119–125]. Several studies have implicated the gastrointestinal tract as a source of bacterial endotoxin products and infection due to its large reservoir of bacteria[126–129]. Bacterial products and bacteria themselves have been detected in the mesenteric lymph node, liver, and lungs following injury[126,129,130]. This infiltration of bacteria can exacerbate the tissue damage by recruiting more neutrophils who continue to release ROS and free radicals[19,131,132].

Following a alcohol and burn injury, the gastrointestinal system is adversely impacted by the initial hypoxia, the subsequent free radical injury and inflammation, leading to deficits in

gastrointestinal (GI) barrier integrity and immune function[109,123,124,133]. The gastrointestinal tract is the largest mucosal surface in the body, responsible for regulating nutrient absorption while maintaining a barrier against environmental toxins and pathogens. The proximity of resident microbiota ensures that the barrier formed by intestinal epithelial cells is crucial for maintaining a homeostatic relationship between the host immune response and resident microbes. This intestinal epithelial barrier includes tight junction proteins that maintain close association of epithelial cells with each other, in addition to antimicrobial peptides (AMPs) and mucus which prevent bacterial overgrowth and invasion[68,134]. These components make intestinal epithelial cells the primary physical barrier that prevents enteric infections. Consequently, disruption of the intestinal barrier can dramatically impact health and is associated with a wide variety of gastrointestinal disorders[135]. As shown in Figure 1, nearly every aspect of the intestinal epithelial barrier is affected by alcohol and burn. Studies show that alcohol and burn injury reduces epithelial cell tight junction protein expression, disrupts tight junction localization, reduces mucus production, and inhibits the expression of AMPs[136,137]. Overall, these changes result in increased intestinal permeability[136,138]. Bacterial translocation resulting from intestinal barrier disruption is thought to contribute to severe consequences after burn injury, including sepsis and multiple organ failure[139]. Burn injury has been reported not only to harm the gastrointestinal organs but alters the microbial populations themselves. Animal models of burn injury and reports from patients have shown that bacterial diversity is diminished and beneficial species, such as Bifidobacterium, are decreased compared to healthy controls[127,140,141]. GI functional deficiencies can be complicated by treatments used for the burn injury, including opioid analgesics, which may inhibit gut transit and reduce motility[142]. This slowed transit of digestion products can lead

to increased bacterial overgrowth in the intestines and overgrowth of pathogenic bacteria, in particular Enterobacteriaceae[130,143114].

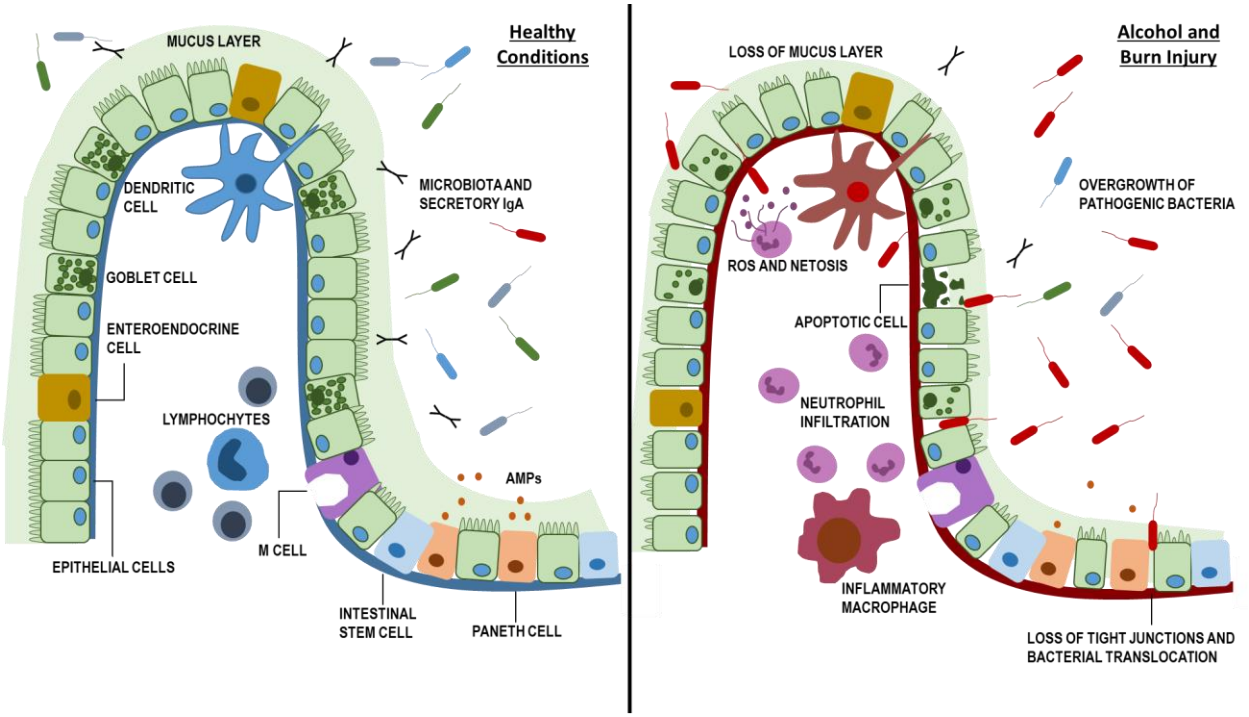

Figure 1. Representation of the Intestinal Barrier Under Healthy and Alcohol Burn Conditions. Under healthy conditions, the intestinal barrier is intact and composed of intestinal epithelial cells (IEC) that are tightly bound together by tight junction proteins. Goblet cells that produce mucus (light green) and enteroendocrine cells (yellow) are interspersed between IECs. In the intestinal crypt, there are intestinal stem cells (blue) that continuously replicate and regenerate IECs that are shed in the lumen. Paneth cells (orange) producing anti-microbial peptides (AMPs). M-cells (purple) are continuously testing luminal contents for uptake by any resident antigen presenting cells. Under the IEC barrier is the lamina propria, which contains a variety of immune cells, including dendritic cells, monocytes, and T/B cells (blue). Secretory IgA is continuously passed into the lumen to maintain homeostatic conditions with the microbiome. Under healthy conditions, there is a large diversity of intestinal microbiota with few pathogenic species. Following alcohol and burn injury (right panel), there is a loss of tight junction proteins and the mucus barrier, along with increased IEC apoptosis, leading to a leaky gut. Increased inflammatory cell infiltration by neutrophils and macrophages leads to production of reactive oxygen species (ROS) and further damage of the barrier, allowing for intestinal bacterial and bacterial products to translocate into systemic circulation. Furthermore, there is overgrowth of pathogenic bacterial species and loss of bacterial diversity in the lumen. Adapted from Luck, Herrnreiter et al[144].

Current Treatments for Alcohol Intoxication and Burn Injury

Current standards in burn treatment require immediate fluid resuscitation to account for this hypovolemic state. Fluid requirements are calculated based on several different formulas; however, initial rates are dependent on the size of the burn area, the patient's body weight, and eventual fluid output by the patient (urine volume)[145–147]. The purpose of fluid resuscitation is to adequately perfuse and oxygenate organs to avoid complications such as renal failure[148,149]. A delicate balance must be maintained while resuscitating burn patients, as over-resuscitation combined with endothelial leakage observed can lead to "fluid creep" and severe consequences such as abdominal compartment syndrome, pulmonary edema, and decreased perfusion of the burn wound itself[146,150].

The type of fluid used in resuscitation must also be considered: isotonic crystalloids, hypertonic solutions, colloids, and increasingly plasma is being used. Crystalloids, including Ringer's lactate (RL) and normal saline, are readily available products and commonly used. However, over-resuscitation with RL has been associated with increased neutrophil activation after hemorrhage, and high-volume administration of saline can lead to hyperchloremic acidosis[151,152]. Due to problems associated with over-resuscitation and edema, hypertonic solutions have also been used but require close monitoring due to risks of hypernatremia and subsequent renal failure[153]. Recently, there has been an increased shift to using blood products, including plasma, as it is a physiologic fluid that may help prevent excess vascular leakage after burn injury[154]. A rat model of burn injury demonstrated that addition of fresh frozen plasma to resuscitation fluid helped diminish endothelial leakage[155]. However, there are limited studies on the effect of the type of resuscitation fluids on the burn microbiome. One study using a swine burn model demonstrated that resuscitation volumes could influence the gut microbiota, such as

a dose-dependent increase in Bacteroidetes and ameliorating growth of harmful Proteobacteria populations[156]. A similar study used different resuscitation fluid paradigms in a swine model and found that while all groups experienced intestinal microbial dysbiosis following burn injury, limited-volume crystalloid (LV-Cr) resuscitation led to the most drastic dysbiosis and hepatocellular damage[157]. However, further research needs to be done to fully understand the impact of burn resuscitation protocols on the microbiome.

While fluid resuscitation is paramount to burn treatment, it does not fully restore organ function. Burn patients still have numerous systemic abnormalities that must be closely monitored and treated. Inflammatory cytokine levels are consistently elevated in mouse models of burn injury for several days and a similar trajectory was observed in pediatric burn patients[104,131,158,159]. Although several studies have demonstrated that inhibiting excessive inflammation can reduce intestinal dysfunction and remote organ damage following alcohol and burn injury, there are currently no therapies in use that target this period of acute inflammation. Investigation of potential therapeutics that support gut barrier function and reduce intestinal inflammation could provide more treatment options to burn patients and reduce their risk for serious complications such as sepsis and multiple organ failure[160]. More recently studies have begun to investigate the use of miRNAs as predictors of poor outcomes in burn patients. Several miRNAs have been associated with insulin resistance and cardiac dysfunction following burn injury. Additionally, circulating miRNAs have been studied for their association with sepsis incidence and severity. Further study of these important miRNAs could provide new therapeutic targets to prevent or reduce the severity of complications after burn injury. Even after sufficient fluid resuscitation, patients still have experienced significant organ ischemia, and secondly, aggressive infusion of fluids leads to rapid re-introduction of oxygen to ischemic tissues,

production of reactive oxygen species (ROS), and formation of free radicals. A major source of free radicals are neutrophils activated by damage associated molecular patterns (DAMPs) derived from injured tissue, leading to oxidative stress and injury of organs[161–164]. Free radical-mediated injury after burn injury has been documented in numerous organs, including the lungs, liver, and the gastrointestinal tract[131,132,165,166]. Numerous studies have investigated antioxidants such as Vitamin C as a supplemental therapy to combat the severe oxidative stress encountered after a burn injury[167,168].

Conclusions

Previous studies have shown that alcohol intoxication and burn injury results in acute intestinal inflammation, which promotes gut barrier disruption and leads to serious complications including sepsis and multiple organ failure. Therefore, the prevention of acute intestinal inflammation following alcohol and burn injury is a promising therapeutic strategy to improve patient outcomes. MicroRNAs play a crucial role in gut homeostasis, however their impact on intestinal dysfunction after alcohol and burn injury is poorly understood. Therefore, it is important to investigate the changes in intestinal epithelial cell miRNA expression and elucidate their contributions to intestinal inflammation and gut barrier integrity following alcohol and burn injury. These studies could broaden our understanding of the mechanisms by which miRNAs regulate intestinal homeostasis in disease, as well as provide prospective therapeutic targets for promoting gut function across a variety of diseases involving intestinal inflammation.

CHAPTER 3

INVESTIGATING THE IMPACT OF REDUCED INTESTINAL EPITHELIAL CELL MIR-146A EXPRESSION AFTER ALCOHOL AND BURN INJURY

Abstract

Previous findings from our laboratory have shown that alcohol intoxication at the time of burn injury promotes intestinal inflammation and gut barrier disruption. MicroRNAs (miRNAs) are small noncoding RNA molecules that negatively regulate gene expression and play a central role in intestinal epithelial cell homeostasis. In this study, we examined the impact of dysregulated miRNA expression on intestinal epithelial cell inflammatory responses to further understand their contributions to gut barrier disruption after alcohol and burn injury. Utilizing a mouse model of acute alcohol intoxication and burn injury, we found that small intestinal epithelial cell expression of anti-inflammatory miR-146a and miR-150 were significantly reduced in alcohol and burn mice compared to vehicle sham ($p < 0.05$). Although miR-146a is well characterized in innate immune cells as an important brake to control inflammation, there is less known about its role in regulating inflammatory signaling in small intestinal epithelial cells. Therefore, we sought to analyze the mechanism by which downregulation of miR-146a could contribute to intestinal inflammation and gut barrier disruption after alcohol and burn injury. Inhibition of miR-146a in MODE-K cells significantly elevated, while miR-146a overexpression significantly reduced, IL-6 and KC expression and secretion in response to LPS. Results from further *in vitro* experimentation suggest that miR-146a expression modulates small intestinal epithelial cell inflammatory responses via regulation of p38 MAPK signaling and targeting of

TRAF6. Overall, these findings indicate that abnormal downregulation of anti-inflammatory miRNAs, particularly miR-146a, after alcohol and burn injury could exacerbate intestinal inflammation and thereby contribute to gut barrier disruption.

Introduction

Every year in the United States nearly 500,000 people receive medical treatment for burn injuries and 40,000 of these cases require some form of hospitalization[1]. In addition, the prevalence of alcohol use at the time of burn injury can be as high as 50%[4]. Alcohol intoxication is associated with poorer prognosis in burn patients, resulting in more adverse outcomes, longer hospitalization, and increased mortality[4,8,17]. Disruption of the small intestinal barrier is a significant issue that arises following alcohol intoxication and burn injury, and is known to contribute to post-burn co-morbidities, including sepsis and multiple organ failure[7,8,16,17]. Intestinal epithelial cells form a crucial component of the gut's physical barrier, maintaining tight junctions that prevent translocation of bacteria and their pro-inflammatory pathogen associated molecular patterns (PAMPs) to the underlying immune compartment and systemic circulation[21]. Previous studies have shown that following severe burn injury, toll-like receptor 4 (TLR4) activation by bacteria products can promote excessive small intestinal inflammation[18]. This intestinal inflammatory response is characterized by elevated levels of pro-inflammatory cytokines, such as IL-6, and neutrophil infiltration can occur between hours to one day after injury[19,169]. Acute intestinal inflammation contributes to intestinal barrier disruption, which results in translocation of bacteria and PAMPs across the intestinal barrier, system inflammation, and distant organ injury[16,18,21].

MicroRNAs (miRNAs) are small noncoding RNA first transcribed from the genome as pri-miRNA molecules. Following cleavage by Drosha into a pre-miRNA molecule and export

from the nucleus, the miRNA is further cleaved into a miRNA duplex by Dicer. A functional mature miRNA is then generated by selection and loading of one strand into the RISC complex. The mature miRNA can then associate with the 3' UTR of target mRNAs via complementary sequence binding, resulting in repression of target mRNA expression[22]. Within the intestinal epithelium, miRNAs have been shown to play a key regulatory role in gut homeostasis[27–29]. Changes in miRNA expression have been implicated in the pathogenesis of several disorders involving intestinal inflammation and barrier disruption, including intestinal ischemia, Crohn's disease, ulcerative colitis, sepsis, and colorectal cancer[29,64]. However, the role of altered miRNA expression within the intestinal epithelium following trauma, including alcohol intoxication and burn injury, remains largely unexplored.

In this study, we hypothesized that downregulation of key anti-inflammatory miRNAs would promote excessive intestinal inflammation and GI dysfunction following alcohol and burn injury. To begin, we utilized our lab's well characterized mouse model of acute ethanol intoxication and burn injury to profile the expression of several anti-inflammatory miRNAs. Among those assessed, we identified significant reductions in small intestinal epithelial cell expression of miR-146a and miR-150 one day after ethanol and burn injury.

Several studies have shown that miR-146a plays a critical role in controlling inflammatory responses of immune cells, particularly macrophages[170–172]. Furthermore, some studies indicate that miR-146a expression can reduce epithelial cell inflammation following cytokine or LPS stimulation[173,174]. Therefore, we sought to investigate the mechanism by which downregulation of miR-146a could contribute to intestinal inflammation and gut barrier disruption after alcohol and burn injury. Our results demonstrate that inhibition of miR-146a in small intestinal epithelial cells enhances the production of pro-inflammatory cytokine IL-6 and neutrophil chemokine KC

(also known as CXCL1) in response to LPS stimulation. Elevated LPS induced pro-inflammatory cytokine expression due to miR-146a repression was significantly dampened by pharmacological inhibition of p38 MAPK activity. Additionally, we found that miR-146a overexpression reduces intestinal epithelial cell levels of TRAF6, which is a well characterized and validated target of miR-146a[171,173,174]. Enhanced pro-inflammatory cytokine expression via miR-146a inhibition is prevented by TRAF6 knockdown via siRNA. Overall, these studies indicate that downregulation of miR-146a following alcohol and burn injury promotes intestinal epithelial cell inflammatory cytokine expression by promoting p38 MAPK signaling via increased levels of its target TRAF6. The results of this study highlight the importance of miRNA mediated regulation of intestinal homeostasis and further our understanding of the contributions of altered miRNA expression on post-burn pathophysiology.

Materials and Methods

Animals

10–12-week-old C57BL/6 male mice (23–25 g body weight) were obtained from Charles River Laboratories and maintained in animal housing facilities at Loyola University Chicago Health Sciences Division, Maywood, Illinois, USA.

Mouse Model of Acute Ethanol Intoxication and Burn Injury

Male C57/BL6 mice (10-12 weeks old, 22–26 g) were randomly assigned into one of four experimental groups: sham injury + vehicle treatment (sham vehicle, SV), sham injury + ethanol treatment (sham ethanol, SE), burn injury + vehicle treatment (burn vehicle, BV), or burn injury + ethanol treatment (ethanol burn, EB). On the day of injury, ethanol mice were gavaged with 400 µL of 25% ethanol in water (2.9 g/kg), while vehicle animals were gavaged with 400 µL water. Three hours following the gavage, mice were given 1 mg/kg buprenorphine

subcutaneously for pain management. Four hours following the gavage, mice were anesthetized with a ketamine hydrochloride/xylazine cocktail (~ 80 mg/kg and ~ 1.2 mg/kg respectively) via intraperitoneal injection. The dorsal surface of each mouse was shaved before placing the mice in a prefabricated template exposing ~ 12.5% total body surface area, calculated using Meeh's formula[175]. Burn group animals were immersed in ~85 °C water bath for ~7 seconds to induce a full-thickness scald burn injury. Sham animals were placed in a 37 °C, lukewarm water bath for an equal length of time. Following burn or sham injury, animals were dried gently and given 1.0 mL normal saline resuscitation by intraperitoneal injection. Animals were returned to their cages, which were placed on heating pads to help maintain their body temperature and observed to ensure recovery from anesthesia. Mice were then returned to their normal housing and allowed food and water ad libitum. All animal experiments were conducted in accordance with the guidelines set forth by the Animal Welfare Act and approved by the Institutional Animal Care and Use Committee (IACUC) at Loyola University Health Sciences Division.

Small Intestinal Epithelial Cell Isolation

One day after injury, mice were euthanized, and the abdominal cavity was exposed via midline incision. Approximately ~8 cm of the distal small intestine was harvested and opened longitudinally and washed twice in ice cold PBS + 100 U/mL penicillin + 100 μg/mL streptomycin. Small intestines were then incubated in HBSS buffer without phenol red supplemented with 10 mMol/L HEPES, 50 μg/mL gentamicin, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM EDTA and 1 mM DTT (pre-digestion solution) for 20 min at 37 °C with agitation at 250 rpm. Samples were vortexed to disrupt epithelial cells from the lamina propria and epithelial cells were collected through a 100 μm strainer. This process of pre-digestion solution incubation and epithelial cell collection was repeated a second time, pooling isolated

epithelial cells[106,176]. Epithelial cells were then washed with PBS twice to remove pre-digestion solution and then used in downstream applications.

Total RNA Isolation

Total RNA for miRNA or mRNA analysis was extracted from small intestinal epithelial cells using the mirVana miRNA Isolation Kit (Invitrogen) according to manufacturer's instructions. If only mRNA analysis was being performed, then total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer (ThermoScientific).

qRT-PCR Analysis of microRNA Expression

Using total RNA isolated via the mirVana miRNA Isolation Kit, reverse transcription and cDNA amplification was performed using the miRCURY LNA RT Kit (Qiagen). Relative expression of target miRNAs was then assessed via quantitative real time PCR (qPCR) using miRCURY LNA SYBR Green PCR Kit and miRCURY LNA PCR Assay primers (Qiagen) specific to each target miRNA. Each sample's target miRNA Ct cycle values were normalized to SNORD68 housekeeping control and relative expression was calculated using the ΔΔCT method.

Murine Duodenal Epithelial Cell (MODE-K) Culture

To investigate miRNA regulation of small intestinal epithelial inflammation in vitro, MODE-K cells were obtained from the laboratory of Dr. Jin Mo Park (Harvard Medical School, Mass General Research Institute, Charlestown, MA). MODE-K cells are small intestinal epithelial cells isolated from mouse duodenum tissue and immortalized with SV40 large T gene. There are widely used in literature and exhibit normal enterocyte characteristics, including the formation of intercellular tight junction and inflammatory cytokine responses to TLR ligands such as LPS[67,177,178]. MODE-K cells were cultured in Dulbecco's Modified Eagle Media

(DMEM) containing 4.5 g/L glucose, 1 mM L-glutamine, 1 mM sodium pyruvate (Gibco, ThermoFisher Scientific) and supplemented with 1% penicillin-streptomyocin cocktail (ThermoFisher Scientific), 1 mM HEPES, and 5% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2.

Overexpression or Inhibition of miR-146a and LPS Stimulation

MODE-K cells were seeded into 6-well plates at 200,000 cells/well to obtain ~30-40% confluency the next day. One day after seeding, culture media was replaced, and cells were transfected with 10 pmol scramble or miR-146a specific miRCURY LNA mimics or 50 pmol scramble or miR-146a specific miRCURY LNA inhibitors (Qiagen) using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific) according to manufacturer instructions. After 24 hours, culture media was replaced, and cells were treated with 1 ug/mL LPS. For pro-inflammatory cytokine expression, media supernatant and cells were collected 24 hours later for ELISA analysis or RNA isolation and RT-qPCR analysis. For western blot analysis of pro-inflammatory signaling, cells were collected for protein isolation after 30 minutes, 1 hour, 2 hours, 3 hours, or 4 hours of LPS stimulation.

qRT-PCR Analysis of mRNA Expression

Using total RNA isolated via either the mirVana miRNA Isolation Kit or RNeasy Mini Kit, reverse transcription and cDNA amplification was performed using the High Capacity Reverse Transcription Kit (Applied Biosystems). Relative expression of target genes was then assessed via quantitative real time PCR (qPCR) using TaqMan Fast Advanced Master Mix and FAM TaqMan primer probes (Life Technologies) specific to each target gene. Each well's target gene Ct cycle values were normalized to either Beta Actin or GAPDH housekeeping control Ct

values using VIC Taqman primer probes in the same reaction. Relative expression was calculated using the ΔΔCT method.

ELISA

Media was collected from MODE-K cells 24 hours after LPS stimulation and centrifuged at 10,000 x g for 10 min. Supernatants were then analyzed for IL-6 (R&D Systems) and KC (R&D Systems) using their respective ELISA kits according to manufacturer instructions. Cytokine levels were expressed per milliliter of supernatant.

Protein Isolation and Western Blot

Cell pellets from MODE-K cell culture or small intestinal epithelial cell isolation were lysed in Cell Lysis Buffer (Cell Signaling Technology) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) added. Lysates were homogenized and centrifuged at 10,000 x g for 10 min. Protein concentration was measured by DC Protein Assay (BioRad). Equal amounts of protein were then loaded and run on an SDS-PAGE gel and transferred to a PVDF membrane for blotting. Membranes were blocked for 1 hour at room temperature with 5% blocking grade milk (BioRad) in TBS-T (0.1% Tween 20 in TBS). After washing membranes twice for 5 min in TBS-T to remove excess milk, membranes were incubate with desired primary antibody overnight at 4°C. Primary antibodies used include anti-phospho-p38 MAPK (Cell Signaling), anti-p38 MAPK (Cell Signaling), anti-phospho-STAT3 (Cell Signaling), anti-STAT3 (Cell Signaling), and anti-Beta-Actin (Cell Signaling). Membranes were then washed three times for 10 min in TBS-T and incubated in the appropriate secondary antibody conjugate to horseradish peroxidase for one hour at room temperature. Triplicate washes were then repeated, and membranes were developed using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and exposed on a ChemiDoc (BioRad) for imaging. Densitometric analysis was

performed using Image Lab software (BioRad). Bands were normalized to Beta Actin and expressed as densitometric units. Levels of phosphorylated protein were expressed relative to its total protein after normalization to Beta Actin.

Pharmacological Inhibition of Pro-inflammatory Signaling

Inhibitors specific for p38 MAPK (SB 203580), NF-κB (CAPE), and STAT3 (Stattic) were obtained from R&D Systems. Pharmacological inhibitors were reconstituted in DMSO and stored at recommended stock concentrations at -20°C. 24 hours after transfection with scramble or miR-146a specific inhibitors, MODE-K cells were treated with either 10 µM SB 203580, 20 µg/mL CAPE, 10 µM Stattic, or DMSO control in culture media. Following one hour of pre-treatment, 1 ug/mL LPS was added, and cells were incubated for 24 hours. Media supernatant was collected for ELISA analysis and cells were lysed for RNA isolation and RT-qPCR analysis.

TRAF6 Knockdown

Predesigned scramble and TRAF6 specific siRNAs (Ambion Silencer Select) were obtained from ThermoFisher Scientific. MODE-K cells were co-transfected with 10 pmol siRNA and 50 pmol miRNA inhibitors using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific) according to manufacturer instructions. The following day, culture media was replaced, and cells were treated with 1 ug/mL LPS overnight. Media supernatant was collected for ELISA analysis and cells were lysed for either protein and western blot analysis or RNA and RT-qCPR analysis. Knockdown was confirmed via western blot analysis of TRAF6 protein level 24 hours after siRNA transfection.

Statistics

Data is presented as means ± standard error of the mean (SEM). *In vitro* experiments were performed in at least duplicate and repeated 3 independent times. Statistical analysis was

performed using GraphPad Prism 7 as defined in figures legends. Briefly, experiments containing 2 groups were analyzed via student's t test. Experiments containing more than two groups were analyzed via 2-way ANOVA with the p values from t tests between two groups being adjusted for false discovery rate via two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. P-values or adjusted p-values of less than 0.05 were considered significant.

Results

Acute intestinal inflammation following alcohol intoxication and burn injury contributes to intestinal barrier disruption and promotes serious post-burn consequences, including sepsis and multiple organ failure[16,18,21]. To identify miRNAs that could contribute to elevated intestinal inflammation, we sought to identify anti-inflammatory miRNAs with downregulated expression following ethanol and burn injury. Following literature review, a panel of anti-inflammatory miRNAs were selected for RT-qPCR analysis, including miR-146a, miR-150, miR-194, miR-199a, miR-381, miR-495, miR-574 and miR-671. As shown in Figure 2, preliminary analysis of miRNA expression in intestinal epithelial cells one day following injury reveals significant downregulation of miR-146a, miR-150, and miR-194 expression in ethanol burn compared to sham vehicle. To determine the impact of ethanol or burn alone versus combined ethanol and burn injury on the expression of these miRNAs, small intestinal epithelial cells were isolated one day after injury from mice belonging to four experimental groups: sham vehicle, sham ethanol, burn vehicle, and ethanol burn. Figure 3 shows that both miR-146a and miR-150 were confirmed to be reduced in intestinal epithelial cells after combined ethanol and burn injury, while miR-194 expression was not significantly changed.

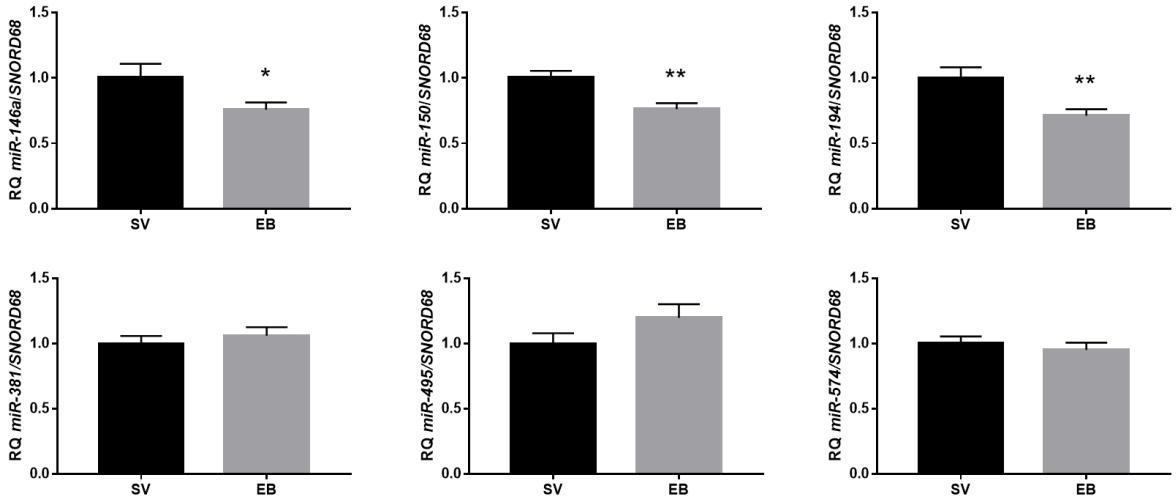

Figure 2. Profiling Expression of Anti-Inflammatory miRNAs in Small Intestinal Epithelial Cells One Day After Ethanol and Burn Injury. Small intestinal epithelial cells were isolated one day after ethanol and burn injury and total RNA extracted for RT-qPCR analysis of miRNA expression using primers specific for miR-146a-5p, miR-150-5p, miR-194-5p, miR-381-3p, miR-495-3p, miR-574-3p. Expression is depicted relative to sham vehicle control, Snord68 was used as housekeeping. Values across three individual mouse experiments, n=18-24 animals per group. Statistical analysis via student's t test, * $p<0.05$, ** $p<0.01$.

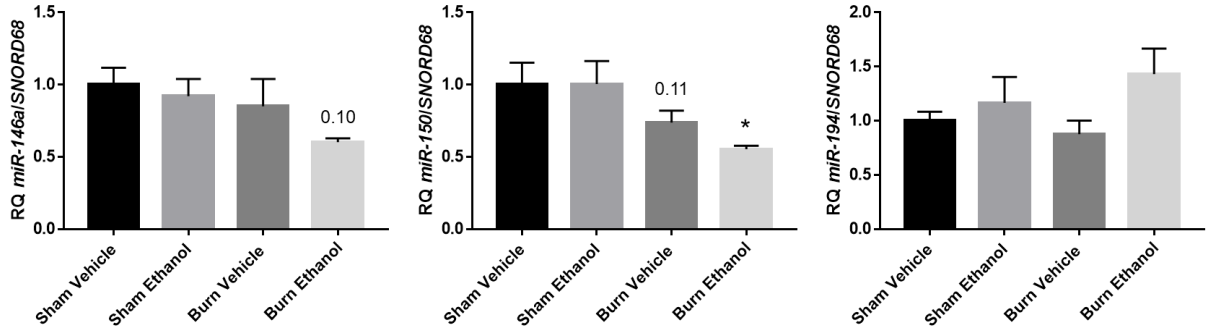

Figure 3. Reduced Expression of miR-146a and miR-150 in Small Intestinal Epithelial Cells One Day After Ethanol and Burn Injury. Small intestinal epithelial cells were isolated one day after ethanol and burn injury and total RNA extracted for RT-qPCR analysis of miRNA expression using primers specific for (A) miR-146a-5p, (B) miR-150-5p, (C) miR-194-5p, Expression is depicted relative to sham vehicle control, Snord68 was used as housekeeping. n=5-8 animals per group. Statistical analysis via two-way ANOVA with on graph significance depicting individual t tests compared to sham vehicle with p values adjusted for false discovery rate, * $p<0.05$.

Although the reduction in miR-146a with ethanol and burn injury did not reach statistical significance in this experiment (padj = 0.10), there is a clear decrease in ethanol burn compared to all other treatment groups and miR-146a expression does not appear to be reduced with burn

alone. The expression of miR-150 is significantly reduced in small intestinal epithelial cells following ethanol and burn injury ($p < 0.05$), but also appears to be affected by burn injury alone, with a trending (padj = 0.11) decrease in expression compared to sham vehicle.

Several studies have shown that miR-146a is an important brake to control inflammation and innate immunity, while others suggest that miR-146a also plays a regulatory role in epithelial cell inflammatory responses[170–174]. Therefore, we sought to determine if miR-146a expression regulates pro-inflammatory signaling in small intestinal epithelial cells. MODE-K cells are a mouse duodenal epithelial cell line commonly used in literature which exhibit normal enterocyte characteristics, including intercellular junction proteins and cytokine responses to TLR ligands such as LPS[177]. To investigate the impact of miR146a expression on small intestinal inflammation after ethanol and burn injury, MODE-K cells were stimulated with LPS for 24 hours and then IL-6 and KC were measured to assess inflammation. Cells were transfected with miR-146a antagomir inhibitor molecules to imitate reduced miR-146a expression seen in our *in vivo* mouse model, or mimic molecules to study the impact of miR-146a overexpression on small intestinal epithelial cell inflammation. Figure 4 demonstrates that LPS stimulation of MODE-K cells significantly upregulates IL-6 and KC gene expression, measured via RT-qPCR, and protein secretion, measured via ELISA ($p < 0.05$). Inhibition of miR-146a results in a significant enhancement in gene expression ($p < 0.01$) and secretion ($p < 0.01$) of IL-6 compared to LPS alone. Gene expression, but not overall secretion, of KC is also significantly enhanced ($p < 0.01$) after LPS stimulation by miR-146a inhibition. As shown in Figure 5, overexpression of miR-146a via miR-146a mimic transfection significantly reduces baseline expression of IL-6 ($p < 0.01$). Overexpression of miR-146a also significantly reduces IL-6 and KC gene expression and secretion in response to LPS ($p < 0.01$).

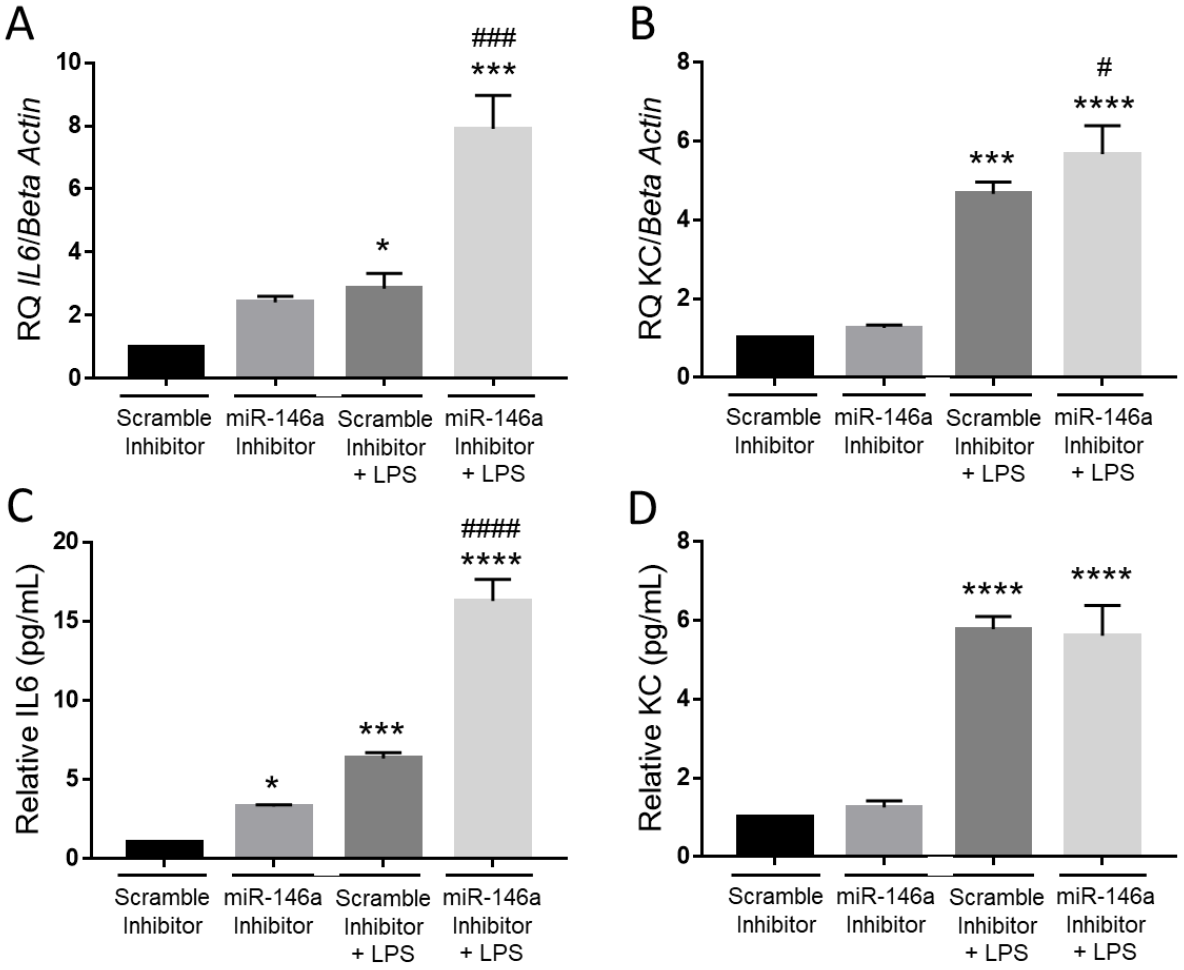

Figure 4. Inhibition of miR-146a Promotes LPS Induced Small Intestinal Epithelial Cell Inflammation. MODE-K cells were transfected with scramble or miR-146a specific antagomir inhibitor and stimulated the next day with 1 μg/mL LPS for 24 hours. RNA was extracted for RT-qPCR analysis of gene expression using primers specific for (A) IL-6 or (B) KC. Expression is depicted relative to sham vehicle control, Beta Actin was used as housekeeping. Graphs are means ± SEM, n=3. Media supernatant collected and ELISA performed for quantification of (C) IL-6 or (D) KC concentration. Concentrations in pg/mL depicted relative to scramble inhibitor control. Graphs are means ± SEM, n=4. Statistical analysis via two-way ANOVA with on graph significance depicting individual t tests compared to sham vehicle with p values adjusted for false discovery rate, * padj < 0.05, ** padj < 0.01, *** padj < 0.001, **** padj < 0.0001 as compared to Scramble Inh or # padj < 0.05, ## padj < 0.01, ### padj < 0.001, #### padj < 0.0001 as compared to Scramble Inh + LPS.

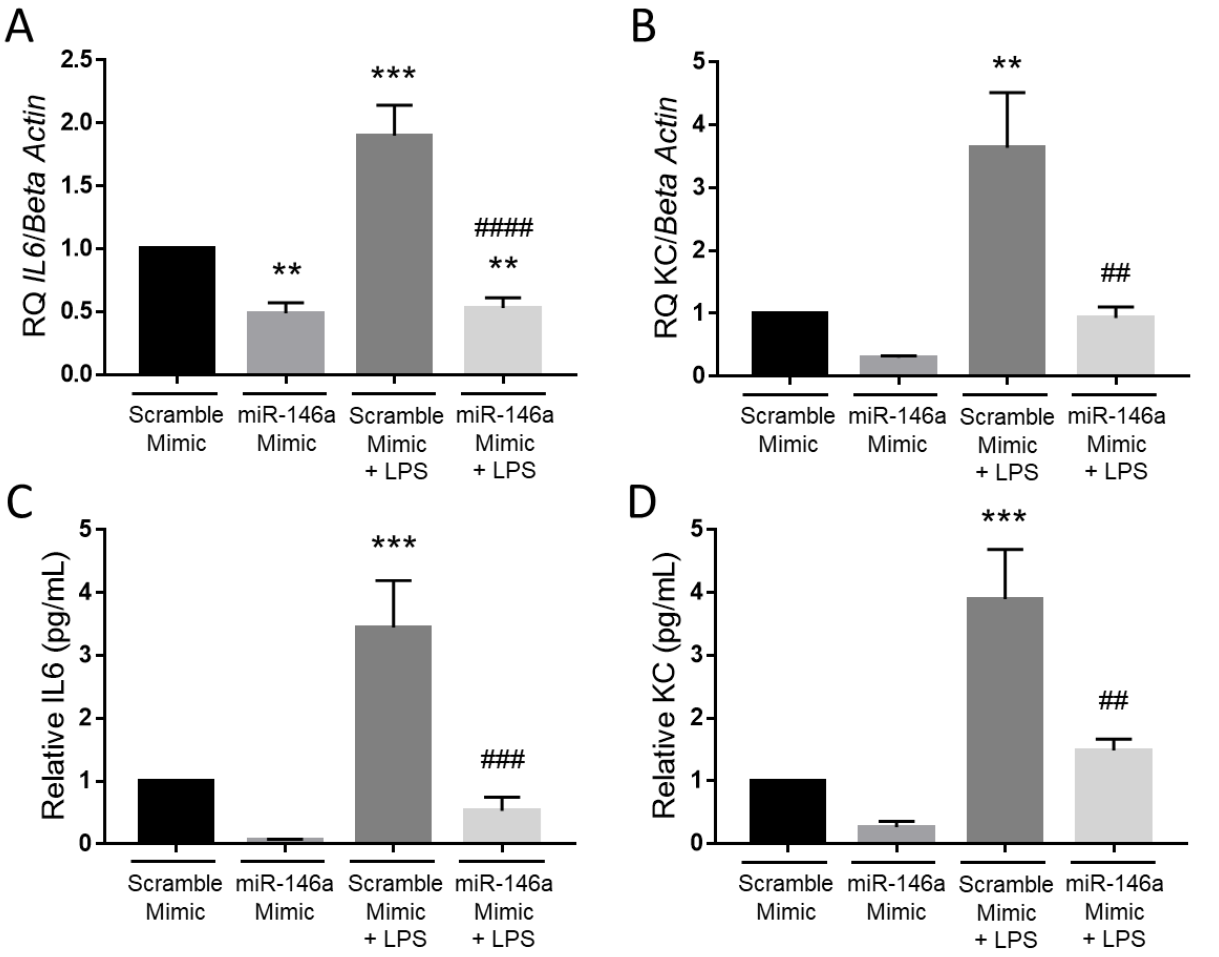

Figure 5. Overexpression of miR-146a Inhibits LPS Induced Small Intestinal Epithelial Cell Inflammation. MODE-K cells were transfected with scramble or miR-146a specific mimic and the next day stimulated with 1 μg/mL LPS for 24 hours. RNA was extracted for RT-qPCR analysis of gene expression using primers specific for (A) IL-6 or (B) KC. Expression is depicted relative to sham vehicle control, Beta Actin was used as housekeeping. Graphs are means ± SEM, n=3. Media supernatant collected and ELISA performed for quantification of (C) IL-6 or (D) KC concentration. Concentrations in pg/mL depicted relative to scramble inhibitor control. Graphs are means ± SEM, n=4. Statistical analysis via two-way ANOVA with on graph significance depicting individual t tests compared to sham vehicle with p values adjusted for false discovery rate, * padj < 0.05, ** padj < 0.01, *** padj < 0.001, **** padj < 0.0001 as compared to Scramble Mimic or # padj < 0.05, ## padj < 0.01, ### padj < 0.001, #### padj < 0.0001 as compared to Scramble Mimic + LPS.

Our results clearly indicate miR-146a expression in small intestinal epithelial cells can regulate inflammatory responses, therefore we next sought to determine which signaling pathways are involved in miR-146a mediated regulation of IL-6 and KC production. There are several signaling pathways activated following LPS stimulation that could be regulated by miR-

146a expression. Activation of p38 MAPK and NF-κB can occur directly downstream of LPS stimulation of TLR4 signaling. In addition, LPS stimulation can result in STAT3 activation via indirect mechanisms, including IL-6 autocrine signaling. Activation of these pathways can cooperatively or independently promote pro-inflammatory cytokine expression in small intestinal epithelial cells. To examine which pro-inflammatory signaling pathways are required for miR-146a mediated elevation of LPS induced cytokine expression, MODE-K cells were transfected with miR-146a inhibitor antagomirs and pretreated with 10 µM SB 203580 (p38 MAPK inhibitor), 20 µg/mL CAPE (NF-κB inhibitor), or 10 µM Stattic (STAT3 inhibitor) for one hour prior to LPS stimulation. IL-6 and KC gene expression after 24 hours of LPS are shown in Figure 6.

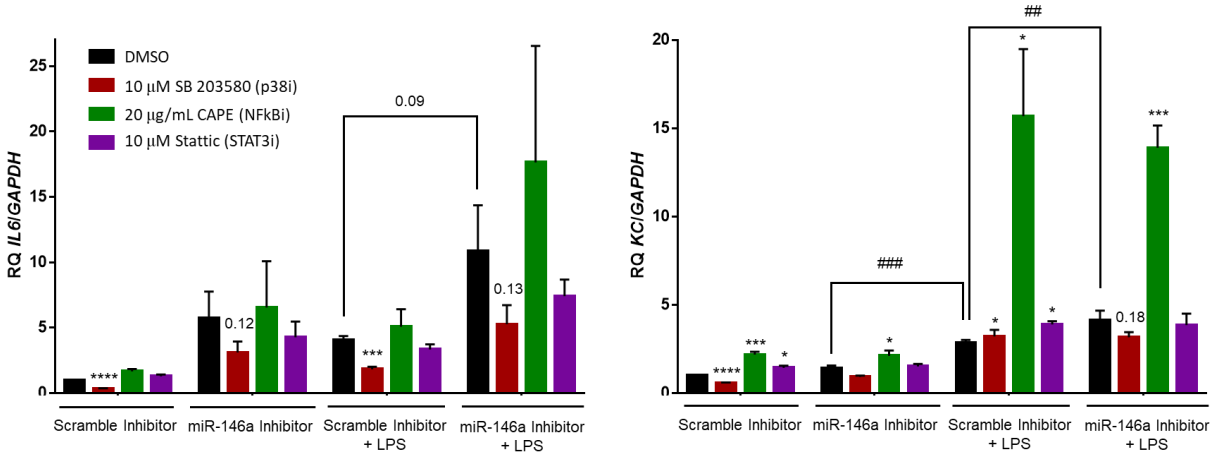

Figure 6. Pharmacological Inhibition of p38 MAPK Suppresses Small Intestinal Epithelial Cell Inflammation Induced by miR-146a Inhibition. MODE-K cells were transfected with scramble or miR-146a specific antagomir inhibitor and the next day pretreated with DMSO control (black), 10 µM SB 203580, 20 µg/mL CAPE, or 10 µM Stattic for 1 hour before stimulation with 1 µg/mL LPS for 24 hours. RNA was extracted for RT-qPCR analysis of gene expression using primers specific for IL-6 or KC. Expression is depicted relative to scramble inhibitor DMSO control, GAPDH was used as housekeeping. Graphs are means ± SEM, n=5. Statistical analysis via two-way ANOVA with on graph significance depicting individual t tests compared to sham vehicle with p values adjusted for false discovery rate, * padj < 0.05, ** padj < 0.01, *** padj < 0.001, **** padj < 0.0001 as compared to DMSO control within the same group or # padj < 0.05, ## padj < 0.01, ### padj < 0.001, #### padj < 0.0001 for comparisons as indicated.

As previously demonstrated, miR-146a inhibition enhances IL-6 and KC expression in response to LPS. Only pretreatment with p38 MAPK inhibitor SB 203580 was able to significantly reduce LPS induced IL-6 expression ($padj<0.001$) and also slightly reduced IL-6 expression after the addition of miR-146a inhibitor ($padj=0.13$). Inhibition of STAT3 appears to slightly reduce IL-6 expression but not as significantly as p38 MAPK inhibition. Although both p38 MAPK and STAT3 inhibition slightly, but significantly, elevated KC expression induced by LPS ($padj<0.05$), inhibition of p38 MAPK alone was able to reduce KC expression after miR-146a inhibition to any degree ($padj=0.18$). Inhibition of NF-κB showed the opposite effect, resulting in slightly higher IL-6 expression when combined with miR-146a inhibition and significantly elevating KC expression after LPS stimulation with ($padj<0.05$) or without ($padj<0.001$) miR-146a inhibition.

To further explore the role of p38 MAPK and STAT3 in miR-146a mediated regulation of pro-inflammatory cytokine expression, MODE-K cells were transfected with either miR-146a mimic or inhibitors following LPS stimulation for a time series ranging from 15 min to 4 hours. Western blot analysis of total protein was performed to assess activation of these pathways via p38 MAPK and STAT3 phosphorylation. As shown in Figure 7, phosphorylation of p38 MAPK increases within 30 minutes of LPS stimulation while phosphorylation of STAT3 increases after approximately 4 hours. Overexpression of miR-146a inhibits LPS induced phosphorylation of both p38 MAPK and STAT3. On the other hand, Figure 8 demonstrates that inhibition of miR-146a primarily increases LPS induced phosphorylation of p38 MAPK but has less effect on STAT3 phosphorylation.

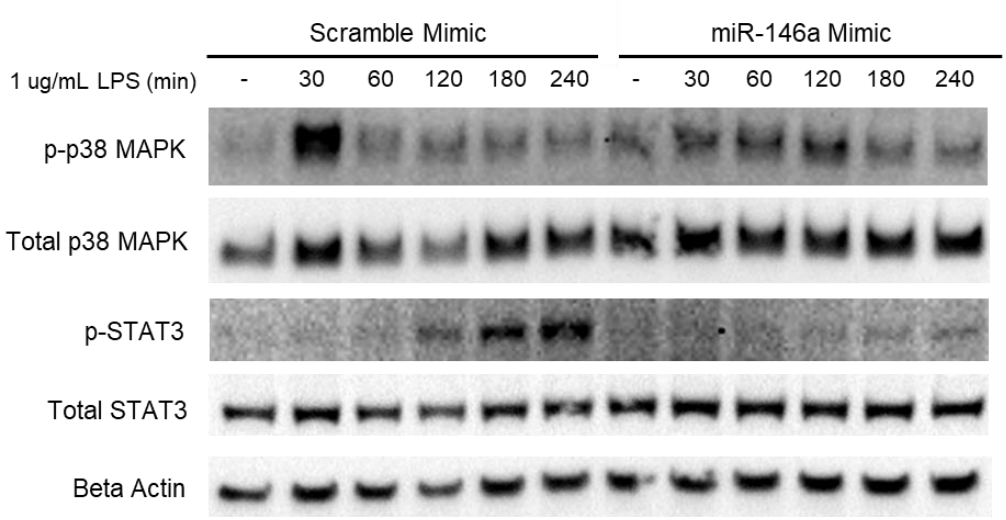

Figure 7. Overexpression of miR-146a Reduces p38 MAPK and STAT3 Phosphorylation in Small Intestinal Epithelial Cells Stimulated with LPS. MODE-K cells were transfected with scramble or miR-146a specific mimic and the next day stimulated with 1 μg/mL LPS for 24 hours. Total protein was isolated and western blot analysis performed to compare relative levels of p38 MAPK and STAT3 phosphorylation following LPS stimulation.

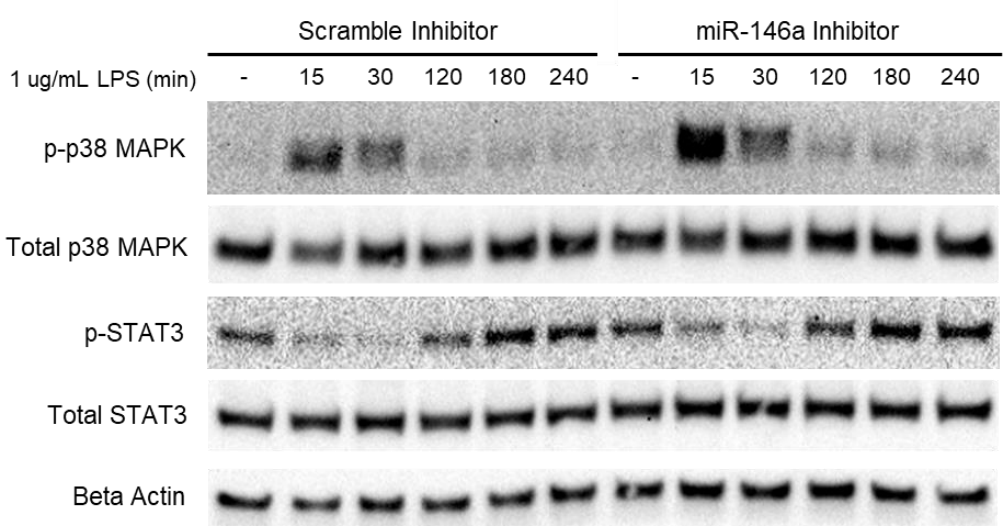

Figure 8. Inhibition of miR-146a Increases p38 MAPK Phosphorylation in Small Intestinal Epithelial Cells Stimulated with LPS. MODE-K cells were transfected with scramble or miR-146a specific inhibitor and the next day stimulated with 1 μg/mL LPS for 24 hours. Total protein was isolated and western blot analysis performed to compare relative levels of p38 MAPK and STAT3 phosphorylation following LPS stimulation.

As our results indicate that miR-146a likely controls small intestinal epithelial cell inflammation by regulating a target upstream of p38 MAPK signaling, we next assessed protein

levels of upstream signaling proteins to identify a potential target of miR-146a. Several studies have validated miR-146a targeting of both IRAK1 and TRAF6, which are downstream of LPS induced TLR4 stimulation and can lead to p38 MAPK and NF-κB activation [171,173,174]. As shown in Figure 9A, miR-146a overexpression in MODE-K cells significantly decreases TRAF6 protein levels, supporting the targeting of TRAF6 by miR-146a in small intestinal epithelial cells. Other studies have validated that TRAF6 is a direct target of miR-146a mediated repression of inflammatory signaling[170–174]. The binding site of miR-146a within the 3' UTR of TRAF6 is shown in Figure 9B. Although IRAK1 is expressed at lower levels than TRAF6, we also see a slight decrease in IRAK1 protein following miR-146a overexpression.

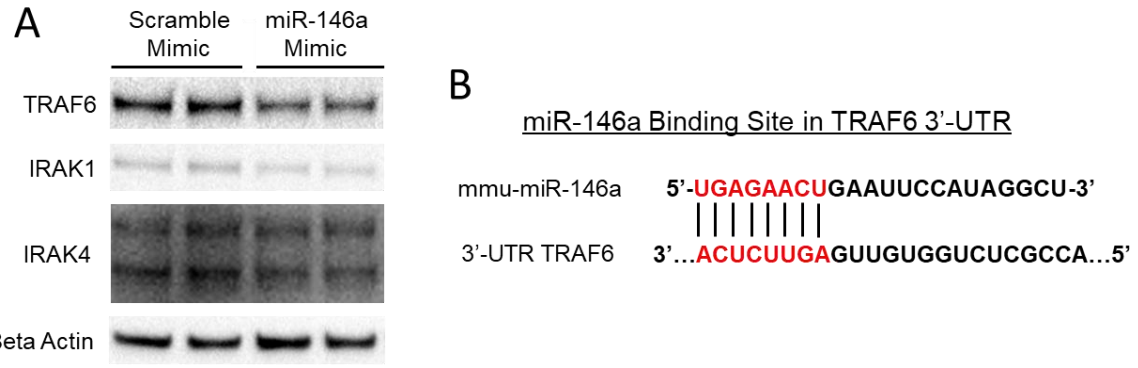

Figure 9. miR-146a Overexpression Reduces Small Intestinal Epithelial Cell TRAF6 Protein. (A) MODE-K cells were transfected with scramble or miR-146a specific mimic and total protein was isolated 24 hours for western blot analysis of proteins upstream of p38 MAPK activation. (B) The miR-146a binding site found within the TRAF6 3' UTR found via complementary binding prediction and validated in immune cells via previous studies[170–174].

To determine if miR-146a downregulation promotes small intestinal epithelial cell inflammation via targeting of TRAF6, MODE-K cells were co-transfected with miR-146a inhibitor and TRAF6 siRNA and stimulated with LPS. Figure 10 demonstrates successful knockdown of TRAF6 in MODE-K cells transfected with as little as 10 pmol siRNA compared to scramble control transfected cells. As shown in Figure 11, knockdown of TRAF6 recapitulates the effect of miR-146a overexpression by inhibiting the expression of pro-inflammatory

cytokines IL-6 and CXCL1 in response to LPS. Furthermore, enhanced pro-inflammatory cytokine expression resulting from miR-146a inhibition in prevented by TRAF6 knockdown.

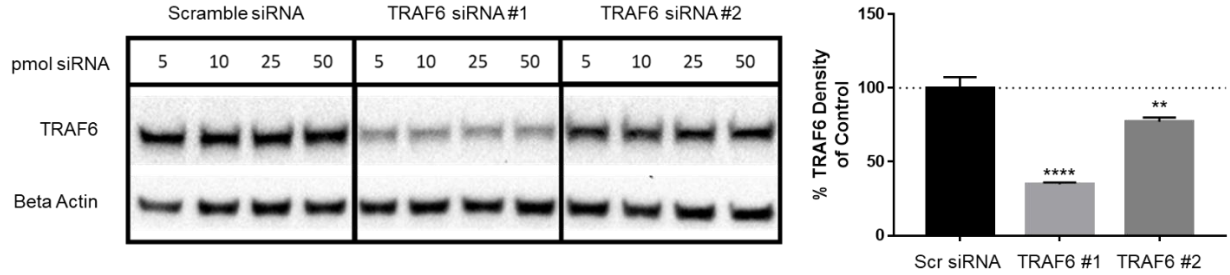

Figure 10. Validation of TRAF6 siRNA Knockdown. MODE-K cells were transfected with scramble or TRAF6 specific siRNA constructs. Total protein was isolated after 24 hours for western blot analysis of TRAF6 knockdown. Graph displays densitometry quantification of TRAF6 normalized to beta actin and then presented as a percentage relative to scramble siRNA control with mean ± SEM, n=3. Statistical analysis via student's t test compared to Scramble siRNA, ** p<0.01, **** p<0.0001.

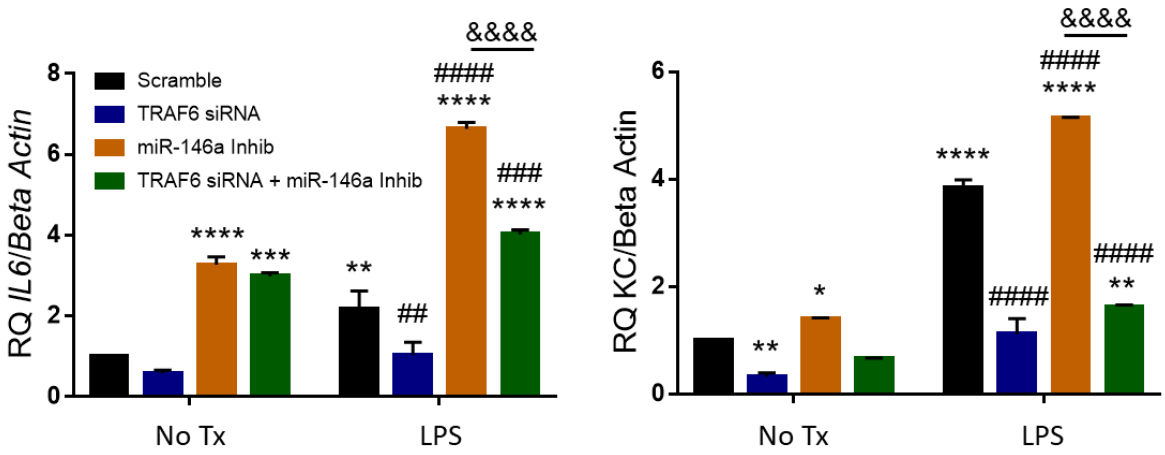

Figure 11. TRAF6 Knockdown Inhibits Small Intestinal Epithelial Cell Inflammation Following miR-146a Inhibition and LPS Stimulation. MODE-K cells were transfected with scramble siRNA or TRAF6 siRNA #1 in combination with scramble or miR-146a specific inhibitor and then stimulated with 1 µg/mL LPS for 24 hours. RNA was extracted for RT-qPCR analysis of gene expression using primers specific for (A) IL-6 or (B) KC. Expression depicted relative to scramble siRNA scramble inhibitor, Beta Actin used as housekeeping. Graphs are means ± SEM, n=2-4. Statistical analysis via two-way ANOVA with on graph significance depicting individual t tests compared to sham vehicle with p values adjusted for false discovery rate * padj < 0.05, ** padj < 0.01, *** padj < 0.001, **** padj < 0.0001 as compared to Scramble No Tx control, # padj < 0.05, ## padj < 0.01, ### padj < 0.001, #### padj < 0.0001 as compared to Scramble + LPS control, or &&&& p<0.0001 for comparison shown.

Summary

In this study, we characterized small intestinal epithelial cell expression of anti-inflammatory miRNAs and investigated their potential role in elevating intestinal inflammation following alcohol and burn injury. Our results show, in mouse model of acute ethanol intoxication and burn injury, that small intestinal epithelial expression of miR-146a and miR-150 are significantly downregulated one day following combined insult. To understand the potential impacts of reduced miR-146a expression after alcohol and burn injury, mechanistic studies were performed *in vitro* using MODE-K cells. We found that inhibition of miR-146a significantly elevated LPS induced small intestinal epithelial cell inflammation, characterized by increased expression of IL-6 and KC. Furthermore, overexpression of miR-146a significantly inhibited LPS induced inflammation in small intestinal epithelial cells. Additionally, our results suggest that miR-146a mediated control of small intestinal epithelial cell inflammation is primarily through targeting of TRAF6 and regulation of p38 MAPK signaling. Altogether, our findings reveal miR-146a downregulation after alcohol and burn injury may substantially contribute to intestinal inflammation and therefore promote gut barrier disruption. Moreover, this study supports the beneficial impact of targeting miRNA regulation of inflammation as a therapeutic target following burn injury.

CHAPTER 4

EFFECTS OF *IN VIVO* MIR-146A MIMIC ADMINISTRATION ON SMALL INTESTINAL INFLAMMATION AND BARRIER INTEGRITY AFTER ALCOHOL AND BURN INJURY

Abstract

MicroRNAs are small noncoding RNA molecules that negatively regulate gene expression and are important regulators critical for maintaining intestinal homeostasis. Studies have shown that alcohol intoxication and burn injury increases intestinal inflammation and promotes gut barrier disruption, but the contribution of miRNA expression changes to these processes require further investigation. In this study, we sought to determine if *in vivo* restoration of intestinal miR-146a expression after alcohol and burn injury would improve gut barrier integrity by reducing intestinal inflammation. Male C57BL/6 mice were given an intraperitoneal injection of 50 ug scramble or miR-146a mimic in in vivo-jetPEI/5% glucose (Polyplus Transfection) one day prior to alcohol and burn injury. Small intestinal tissue and small intestinal epithelial cells were isolated one day after injury and processed to obtain both total RNA and protein lysate. Our results demonstrate that restoration of miR-146a expression significantly suppressed small intestinal inflammation following ethanol and burn injury, characterized by reduced intestinal epithelial cell expression of IL-6 and phosphorylation of pro-inflammatory signaling molecules p38 MAPK and NF-κB. In addition, expression of CyclinD1 and Occludin were reduced in intestinal epithelial cells of alcohol burn mice receiving scramble mimic but significantly enhanced in alcohol burn mice receiving miR-146a mimic, indicating enhanced cell

cycling and increased tight junction proteins. Although these results suggest that miR-146a expression suppresses intestinal epithelial cell inflammation and promotes barrier integrity, miR-146a mimic administration did not significantly reduce intestinal permeability following ethanol and burn injury. Nevertheless, these finding suggest a critical role for miR-146a in intestinal inflammation and barrier integrity after alcohol and burn injury and thus highlights the therapeutic potential of targeting miRNAs for maintaining intestinal homeostasis after alcohol and burn injury.

Introduction

MicroRNAs (miRNAs) are small noncoding RNA molecules that negatively regulate gene expression by complementary binding of their sequence with the 3' UTR of target mRNAs, resulting is repression of translation or mRNA degradation[22]. Individual miRNAs are capable of regulating numerous mRNA targets and studies indicate that 30-60% of the human genome is regulated by miRNAs[24,39–41]. Due to the expansive nature of miRNA regulation, small changes in miRNA expression can have dramatic consequences for normal cellular process. Unsurprisingly, altered miRNA expression has been associated with a wide variety of diseases including cancer, neurodegenerative disorders, diabetes, and heart disease[42]. As our understanding of miRNA associated pathophysiology expands, increased interest in targeting abnormal miRNA expression as a therapeutic intervention has developed. The most common therapeutic tactics involve delivery of exogenous miRNA mimics to overcome downregulation of important miRNAs or antagomir molecules which inhibit mature miRNA function to block the impacts of elevated miRNA expression[42,78]. Several studies have shown the potential efficacy of different miRNA therapeutics and the therapeutic potential of miRNAs has become widely recognized. However,

the field is still relatively new with only a few miRNA therapeutics having reached pre-clinical development or clinical trials.

Disruption of the intestinal barrier is associated with a wide variety of gastrointestinal disorders and is a major contributing factor to mortality and morbidity following severe burn injury[135,139]. Alcohol intoxication at the time of burn injury promotes intestinal inflammation, which exacerbates disruption of the gut barrier and contributes to severe consequences of burn injury, including sepsis and multiple organ failure[20,139,179,180]. Studies suggest that reducing inflammatory cytokines, including IL-6 and IL-18, can promote intestinal barrier function following alcohol and burn injury[181,182]. It has also been shown that intestinal inflammation after alcohol and burn injury can mediate tissue damage and barrier disruption via the recruitment of neutrophils to intestinal tissue[19,131,132]. Within the intestinal epithelium specifically, miRNAs play a critical role in gut homeostasis and aberrant miRNA expression has been implicated in various disorders associated with intestinal inflammation and barrier disruption, including ulcerative colitis, sepsis, and colorectal cancer[27–29,64]. Although miRNAs heavily regulate inflammatory signaling and gut barrier homeostasis, the role of altered miRNA expression within the intestinal epithelium following alcohol intoxication and burn injury is poorly understood.

Several studies have shown that miR-146a plays a critical role in controlling inflammatory responses of immune cells, particularly macrophages[170–172]. Furthermore, some studies indicate that miR-146a expression can reduce epithelial cell inflammation following cytokine or LPS stimulation[173,174]. Using a mouse model of acute ethanol intoxication and burn injury, our studies show that miR-146a expression is significantly downregulated within small intestinal epithelial cells one day following combined injury, potentially contributing to excessive inflammation and intestinal barrier disruption. Therefore, we sought to investigate the

therapeutic potential of miR-146a restoration via intraperitoneal injection of *in vivo* ready miRNA mimic in our mouse model of combined ethanol and burn injury. Our results demonstrate that *in vivo* miR-146a overexpression significantly inhibits intestinal inflammation and supports intestinal barrier homeostasis one day following combined injury, although it was unable to fully prevent intestinal permeability. Overall, this study highlights the important impact that miRNA expression can have on intestinal homeostasis and the valuable potential of harnessing aberrant miRNA expression as a therapeutic target to control intestinal inflammation.

Materials and Methods

Animals

10–12-week-old C57BL/6 male mice (23–25 g body weight) were obtained from Charles River Laboratories and maintained in animal housing facilities at Loyola University Chicago Health Sciences Division, Maywood, Illinois, USA.

Mouse Model of Acute Ethanol Intoxication and Burn Injury

Male C57/BL6 mice (10-12 weeks old, 22–26 g) were randomly assigned into four experimental groups: Sham Vehicle + Scramble Mimic, Sham Vehicle + miR-146a Mimic, Burn Ethanol + Scramble Mimic, or Burn Ethanol + miR-146a Mimic. One day prior to injury, mice were given an intraperitoneal injection containing 50 ug of either scramble or miR-146a mirVana mimic (ThermoFisher Scientific, HPLC purified, in vivo ready) with 6 uL in vivo-jetPEI reagent in 250 µL 5% glucose (Polyplus Transfection). On the day of injury, ethanol burn mice (EB) were gavaged with 400 µL of 25% ethanol in water (2.9 g/kg), while sham vehicle (SV) animals were gavaged with 400 µL water. Three hours following the gavage, mice were given 1 mg/kg buprenorphine subcutaneously for pain management. Four hours following the gavage, mice were anesthetized with a ketamine hydrochloride/xylazine cocktail (~ 80 mg/kg and ~ 1.2 mg/kg

respectively) via intraperitoneal injection. The dorsal surface of each mouse was shaved before placing the mice in a prefabricated template exposing ~ 12.5% total body surface area, calculated using Meeh's formula[175]. Burn group animals were immersed in ~85 °C water bath for ~7 seconds to induce a full-thickness scald burn injury. Sham animals were placed in a 37 °C, lukewarm water bath for an equal length of time. Following burn or sham injury, animals were dried gently and given 1.0 mL normal saline resuscitation by intraperitoneal injection. Animals were returned to their cages, which were placed on heating pads to help maintain their body temperature and observed to ensure recovery from anesthesia. Mice were then returned to their normal housing and allowed food and water ad libitum. All animal experiments were conducted in accordance with the guidelines set forth by the Animal Welfare Act and approved by the Institutional Animal Care and Use Committee (IACUC) at Loyola University Health Sciences Division.

Small Intestinal Epithelial Cell Isolation

One day after injury, mice were euthanized, and the abdominal cavity was exposed via midline incision. Approximately ~8 cm of the distal small intestine was harvested and opened longitudinally and washed twice in ice cold PBS + 100 U/mL penicillin + 100 μg/mL streptomycin. Small intestines were then incubated in HBSS buffer without phenol red supplemented with 10 mMol/L HEPES, 50 μg/mL gentamicin, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM EDTA and 1 mM DTT (pre-digestion solution) for 20 min at 37 °C with agitation at 250 rpm. Samples were vortexed to disrupt epithelial cells from the lamina propria and epithelial cells were collected through a 100 μm strainer. This process of pre-digestion solution incubation and epithelial cell collection was repeated a second time, pooling isolated

epithelial cells[106,176]. Epithelial cells were then washed with PBS twice to remove pre-digestion solution and then used in downstream applications.

Total RNA Isolation and RT-qPCR Analysis

Total RNA was extracted from small intestinal tissue or isolated small intestinal epithelial cells using the mirVana miRNA Isolation Kit (Invitrogen) according to manufacturer's instructions. RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer (ThermoScientific). For analysis of miR-146a expression, reverse transcription and cDNA amplification was performed using the miRCURY LNA RT Kit (Qiagen). Relative expression of miR-146a was determine via quantitative real time PCR (qPCR) using miRCURY LNA SYBR Green PCR Kit and miRCURY LNA PCR Assay primers (Qiagen). miR-146a Ct cycle values were normalized to SNORD68 housekeeping control and relative expression was calculated using the ΔΔCT method. For mRNA expression analysis, reverse transcription and cDNA amplification was performed using the High Capacity Reverse Transcription Kit (Applied Biosystems). Relative expression of target genes was then assessed via quantitative real time PCR (qPCR) using TaqMan Fast Advanced Master Mix and FAM TaqMan primer probes (Life Technologies) specific to each target gene. Each well's target gene Ct cycle values were normalized to Beta Actin housekeeping control Ct values using VIC Taqman primer probes in the same reaction. Relative expression was calculated using the ΔΔCT method.

Protein Isolation and Western Blot

Cell pellets from small intestinal epithelial cell isolation were lysed in Cell Lysis Buffer (Cell Signaling Technology) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) added. Lysates were homogenized and centrifuged at 10,000 x g for 10 min. Protein

concentration was measured by DC Protein Assay (BioRad). Equal amounts of protein were then loaded and run on an SDS-PAGE gel and transferred to a PVDF membrane for blotting. Membranes were blocked for 1 hour at room temperature with 5% blocking grade milk (BioRad) in TBS-T (0.1% Tween 20 in TBS). After washing membranes twice for 5 min in TBS-T to remove excess milk, membranes were incubate with desired primary antibody overnight at 4°C. Primary antibodies used include anti-phospho-p38 MAPK (Cell Signaling), anti-p38 MAPK (Cell Signaling), anti-phospho-NFκB (Cell Signaling), anti-NFκB (Cell Signaling), anti-Occludin (Invitrogen), anti-Claudin-4 (Invitrogen), anti-Claudin-2 (Invitrogen), and anti-Beta-Actin (Cell Signaling). Membranes were then washed three times for 10 min in TBS-T and incubated in the appropriate secondary antibody conjugate to horseradish peroxidase (anti-rabbit or anti-mouse) for one hour at room temperature. Triplicate washes were then repeated, and membranes were developed using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and exposed on a ChemiDoc (BioRad) for imaging. Densitometric analysis was performed using Image Lab software (BioRad). Bands were normalized to Beta Actin and expressed as densitometric units. Levels of phosphorylated protein were expressed relative to its total protein after normalization to Beta Actin.

Measurement of Intestinal Barrier Permeability

One day following alcohol gavage and burn injury, mice were given a gavage of 0.4 ml Fluorescein Isothiocyanate (FITC)-dextran at 22 mg/ml in PBS. 3 hours later, mice were euthanized, and blood was collected by cardiac puncture into a heparin coated syringe. Blood was centrifuged at 8,000 rpm for 10 min at 4°C to collect plasma. Samples were assessed via fluorescent spectrophotometry (480 nm excitation and 520 nm emission wavelengths) against a standard curve to quantify plasma FITC-dextran levels as µg FITC-dextran per µl plasma.

Statistics

Data is presented as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 7 as defined in figures legends. Briefly, experiments containing 2 groups were analyzed via student's t test. Experiments containing more than two groups were analyzed via 2-way ANOVA with the p-values from t tests between two groups being adjusted for false discovery rate via two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. Adjusted p-values or p-values of less than 0.05 were considered significant and represented as *p < 0.05, ** p < 0.01, * p < 0.001, and **** p < 0.0001.

Results

To validate miR-146a overexpression following *in vivo* mimic administration, miR-146a expression was analyzed in small intestinal tissue and isolated epithelial cells one day after ethanol and burn injury. As shown in Figure 12, mice receiving miR-146a mimic had significantly higher miR-146a expression compared to mice receiving scramble mimic. This overexpression successfully elevated miR-146a expression following ethanol and burn injury.

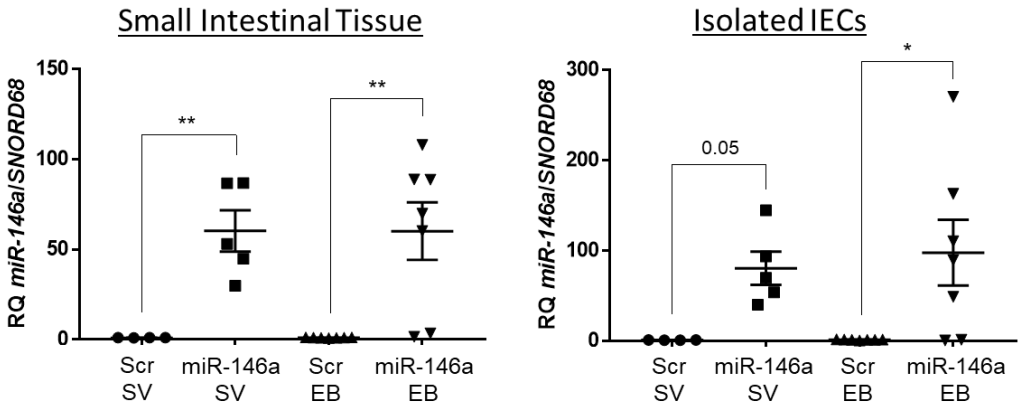

Figure 12. *In Vivo* Administration of miR-146a Mimic Significantly Increases miR-146a Expression in Small Intestine. (A) Small intestinal tissue or (B) small intestinal epithelial cells were isolated one day after ethanol and burn injury and total RNA extracted for RT-qPCR analysis of miRNA expression using primers specific for miR-146a-5p. Expression is depicted relative to scramble sham vehicle control, Snord68 was used as housekeeping. n=4-7 animals per group. Statistical analysis via two-way ANOVA with significance depicting individual t test p values adjusted for false discovery rate, * padj<0.05, ** padj<0.01.

Previous studies in our laboratory have demonstrated that elevated intestinal inflammation following ethanol and burn injury is characterized by increased levels of pro-inflammatory mediators, including pro-inflammatory cytokine IL-6[179]. To assess the overall impact of miR-146a overexpression on intestinal inflammation following ethanol and burn injury, gene expression of IL-6 was measured in small intestinal tissue. Figure 13 demonstrates significantly elevated IL-6 gene expression in small intestinal tissue one day following ethanol and burn injury. Overexpression of miR-146a almost entirely inhibited IL-6 expression, firmly supporting the anti-inflammatory capacity of miR-146a. We also found that IL-6 expression was significantly reduced by miR-146a overexpression in isolated small intestinal epithelial cells, indicating that epithelial cell expression of miR-146a effectively strongly contributes to the observed reduction in intestinal inflammation.

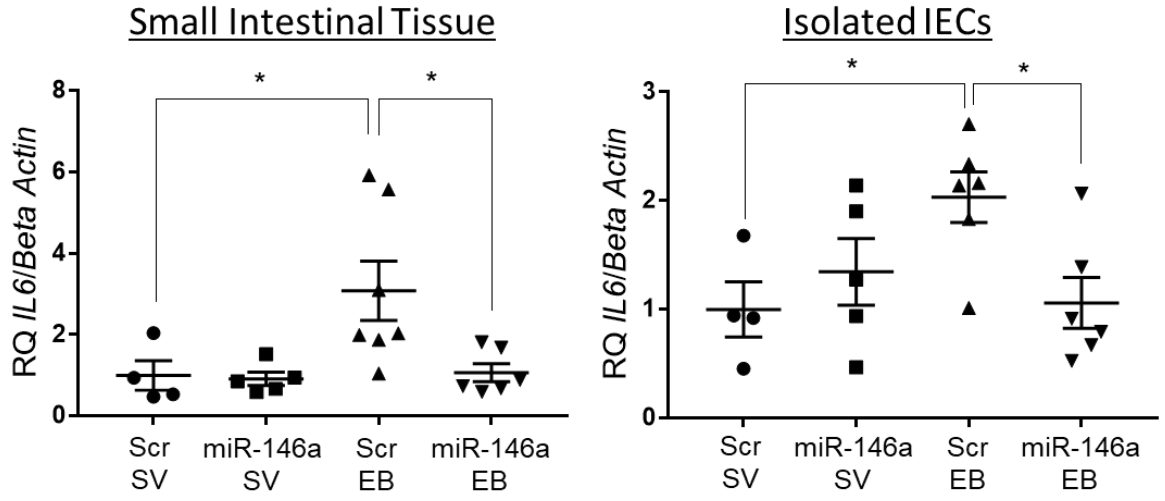

Figure 13. *In Vivo* Overexpression of miR-146a Inhibits Intestinal Inflammation Following Ethanol and Burn Injury. Small intestinal tissue or small intestinal epithelial cells were isolated one day after ethanol and burn injury and total RNA extracted for RT-qPCR analysis of miRNA expression using primers specific for IL-6. Expression is depicted relative to scramble sham vehicle control with Beta Actin was used as housekeeping. n=4-7 animals per group. Statistical analysis via two-way ANOVA with significance depicting individual t test p values adjusted for false discovery rate, * padj<0.05.

Expression of miR-146a has been previously studied for its regulation of inflammatory signaling, where its expression reduced inflammatory cytokine expression via inhibition of NF-κB signaling[170,174,183]. In addition, our *in vitro* studies suggest that p38 MAPK signaling is required for miR-146a mediated regulation of pro-inflammatory cytokine expression in small intestinal epithelial cells. To further characterize the impact of *in vivo* miR-146a overexpression on intestinal inflammation following ethanol and burn injury, activation of pro-inflammatory signaling within small intestinal epithelial cells was assessed by western blot analysis of p38 MAPK and NF-κB p65 phosphorylation. As shown in Figure 14, elevated phosphorylation of p38 MAPK and NF-κB p65 in small intestinal epithelial cells one day after ethanol and burn injury appears to be reduced by miR-146a overexpression.

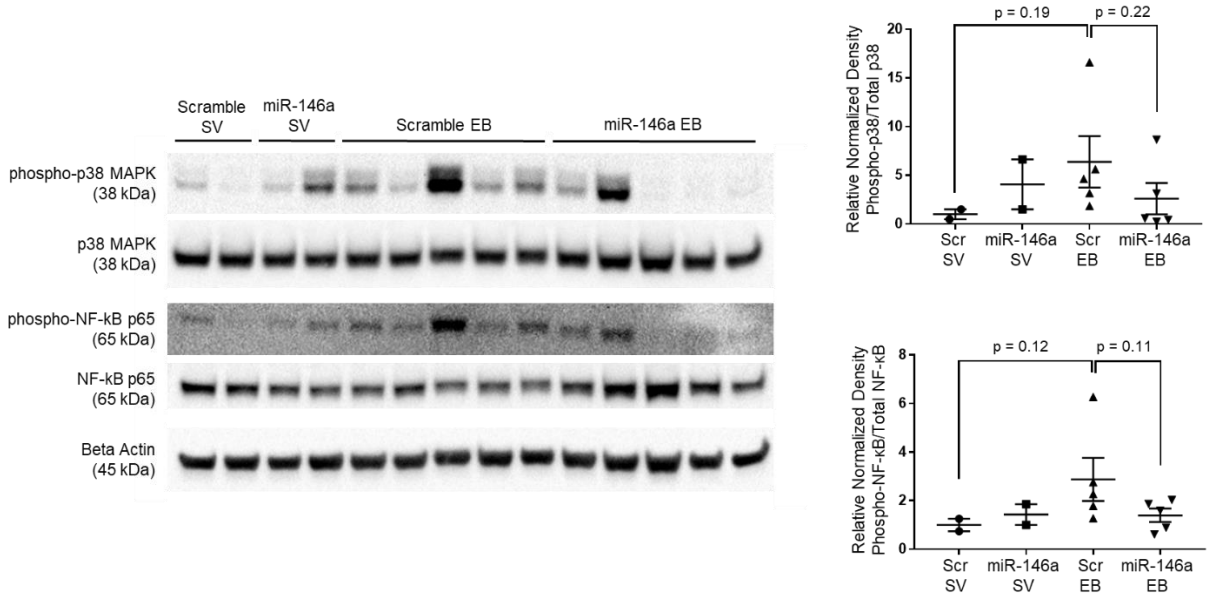

Figure 14. *In Vivo* Overexpression of miR-146a Inhibits Small Intestinal Epithelial Cell Inflammatory Signaling Following Ethanol and Burn Injury. Small intestinal epithelial cells were isolated one day after ethanol and burn injury. Western blot analysis was performed to analyze activation of inflammatory signaling via quantification of phosphorylated p38 MAPK and NF-κB. Graphs depict density of phospho- relative to total protein bands normalized to beta actin and presented relative to average Scramble SV, with mean ± SEM (n = 2-5 mice per group). Significance on graphs depict student's t tests between comparisons shown.

Studies have shown that neutrophil accumulation contributes to intestinal tissue damage and inflammation after ethanol and burn injury[19,131]. Elevated levels of the neutrophil chemokine KC (also known as CXCL1) can promote neutrophil infiltration. Figure 15 demonstrates that, although observed changes fail to reach significance, KC gene expression in small intestinal tissue one day following ethanol and burn injury is considerably inhibited by miR-146a overexpression. To further evaluate neutrophil infiltration of intestinal tissue, gene expression of neutrophil marker Ly6G was assess via RT-qPCR. In addition, we assessed gene expression of the neutrophil effector enzyme Lcn2, which is stored in specific granules and is induced by inflammatory cytokines and bacterial products. Small intestinal expression of Ly6g and Lcn2, shown in Figure 15, are significantly elevated following ethanol and burn injury. Overexpression of miR-146a dramatically decreases Ly6g and Lcn2 expression after ethanol and burn injury, indicating reduced neutrophil infiltration, which could contribute to reduced intestinal inflammation and tissue damage.

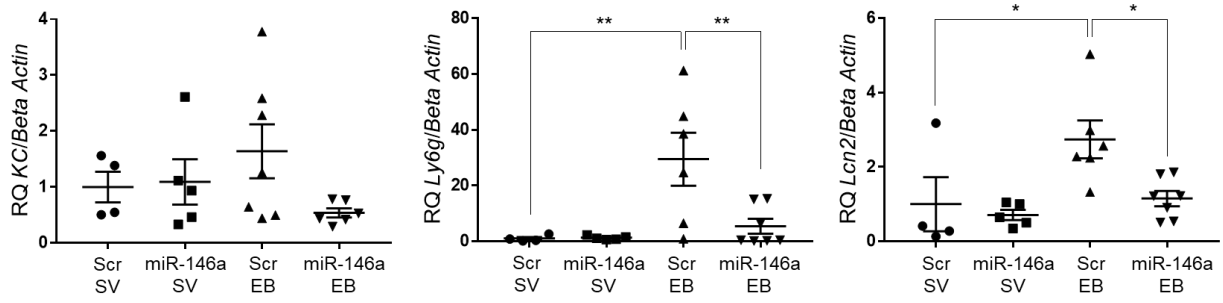

Figure 15. In Vivo Overexpression of miR-146a Reduces Small Intestinal Neutrophil Infiltration Following Ethanol and Burn Injury. Small intestinal tissue was isolated one day after ethanol and burn injury and total RNA extracted for RT-qPCR analysis of miRNA expression using primers specific for neutrophil markers KC, Ly6g or Lcn2. Expression is depicted relative to scramble sham vehicle control, beta actin was used as housekeeping. n=4-7 animals per group. Statistical analysis via two-way ANOVA with significance depicting individual t test p values adjusted for false discovery rate, * padj<0.05, ** padj<0.01.

Intestinal inflammation and neutrophil infiltration can result in gut barrier disruption and promote consequences including systemic inflammation and remote organ injury. The intestinal

barrier is maintained by a variety of mechanisms, including the formation of tight junctions holding intestinal epithelial cells closely together and the constant regeneration of the intestinal epithelial layer. To analyze the proliferative state of intestinal epithelial cells following ethanol and burn injury, RT-qPCR and western blot analysis of cell cycling protein CyclinD1 was performed. Figure 16A demonstrates that reduced intestinal epithelial cell proliferation, marked by CyclinD1 gene expression one day after ethanol and burn injury, is dramatically and significantly elevated by miR-146a overexpression.

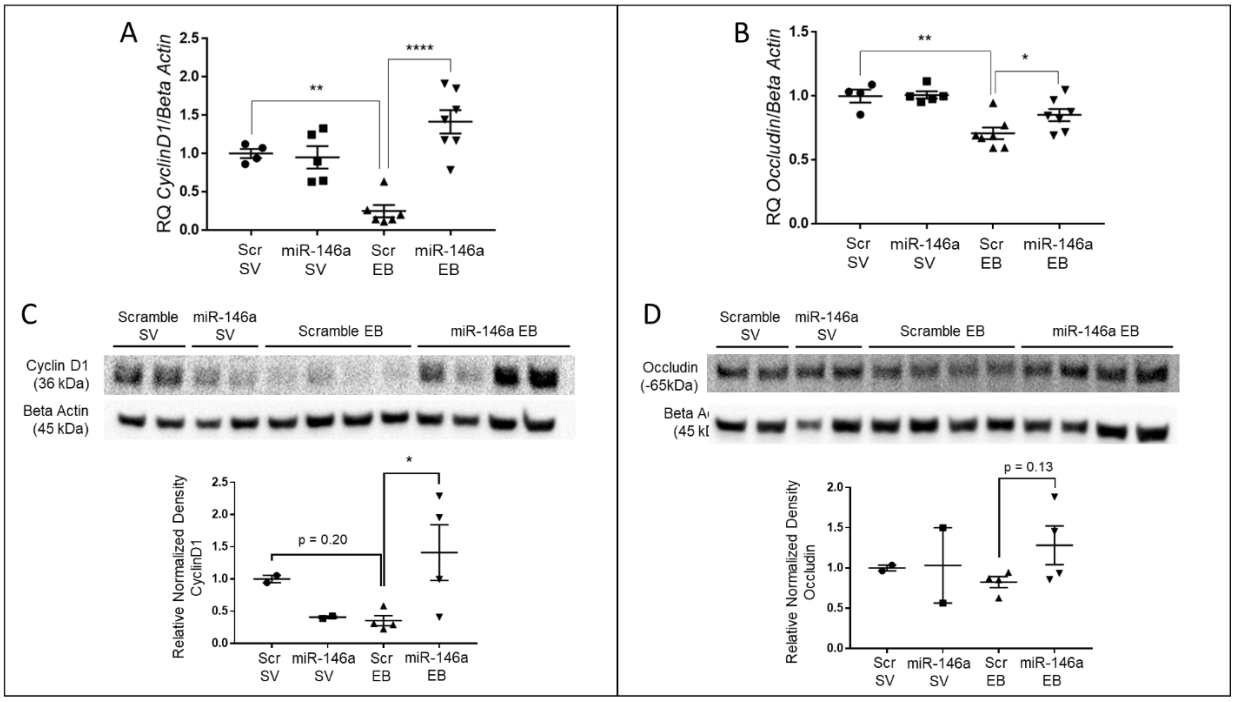

Figure 16. In Vivo Overexpression of miR-146a Promotes Small Intestinal Epithelial Cell Proliferation and Tight Junctions Following Ethanol and Burn Injury. Small intestinal epithelial cells were isolated one day after ethanol and burn injury. Total RNA extracted for RT-qPCR analysis of miRNA expression using primers specific for (A) CyclinD1 or (B) Occludin. Expression is depicted relative to scramble sham vehicle control. Beta Actin was used as housekeeping. Graphs show mean ± SEM (n = 4-7 mice per group). Statistical analysis via two-way ANOVA with significance depicting individual t test p values adjusted for false discovery rate, * padj<0.05, ** padj<0.01, **** padj<0.0001. Western blot analysis was performed to assess (C) CyclinD1 and (D) Occludin protein levels. Graphs depict density normalized to beta actin and presented relative to average Scramble SV, with mean ± SEM (n = 2-4 mice per group). Significance on graphs depict student's t tests between comparisons shown.

Western blot analysis of CyclinD1 protein levels, shown in Figure 16C, are also significantly increased one day after combined injury when miR-146a is overexpressed. Additionally, our results indicate that miR-146a overexpression enhances intestinal epithelial cell tight junction protein expression. RT-qPCR, shown in Figure 16B, and western blot analysis, shown in Figure 16D, demonstrate reduced expression of Occludin, a crucial component of tight junctions, one day after ethanol and burn injury. Occludin expression after ethanol and burn injury is enhanced by miR-146a overexpression. These results demonstrate that miR-146a overexpression may promote the intestinal barrier by increasing epithelial cell proliferation and tight junction protein expression.

Following ethanol and burn injury, intestinal inflammation and disruption of the epithelial barrier culminates in heightened intestinal permeability and gut leakiness. This allows for the translocation of bacteria and inflammatory products such as endotoxin, which has been shown to contribute to serious pathologies following ethanol and burn injury including sepsis and multiple organ failure. To measure intestinal permeability, mice were gavaged to introduce FITC-dextran to the gastrointestinal tract. Blood was collected three hours later to quantify the amount of FITC-dextran that was able to leak from the intestinal tract into systemic circulation. As shown in Figure 17, intestinal permeability is significantly increased one day following ethanol and burn injury. Overexpression of miR-146a may slightly impact intestinal permeability, however no significant reduction in serum FITC-dextran levels were found. This suggests that although miR-146a overexpression may significantly impact individual components of the intestinal barrier, such as inflammation or small intestinal epithelial cell proliferation, administration of miR-146a mimic alone is unable to fully restore gut barrier integrity following ethanol and burn injury.

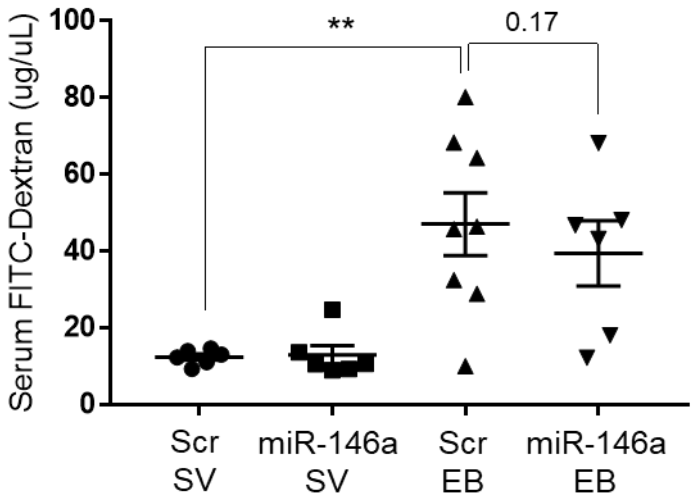

Figure 17. In Vivo Overexpression of miR-146a Fails to Fully Restore Intestinal Barrier Integrity. To assess intestinal permeability, mice were gavaged with FITC-Dextran one day after ethanol and burn injury. Three hours later, mice were euthanized, and blood collected for spectrophotometric analysis of serum FITC-dextran levels. Concentration of FITC-dextran shown as μg FITC-dextran per μL serum, with mean ± SEM (n = 4-7 mice per group). Statistical analysis via two-way ANOVA with significance depicting individual t tests p values adjusted for false discovery rate, ** padj<0.01.

Summary

The goal of this study was to elucidate that impact of aberrant miR-146a expression on intestinal homeostasis and to assess the therapeutic potential of targeting miR-146a expression to reduce intestinal inflammation and promote barrier integrity after ethanol and burn injury. Our results demonstrate that successful overexpression of miR-146a in small intestinal tissue and epithelial cells via intraperitoneal injection of miR-146a mimic significantly reduced intestinal inflammation one day following ethanol and burn injury. In particular, we observed downregulation of intestinal IL-6 gene expression and reduced phosphorylation of important pro-inflammatory signaling molecules p38 MAPK and NF-κB p65 in intestinal epithelial cells from mice receiving miR-146a mimic compared to scramble. Furthermore, we results suggest that *in vivo* miR-146a overexpression inhibited neutrophil recruitment to intestinal tissue following ethanol and burn injury. Elevated miR-146a expression also promoted intestinal epithelial cell

proliferation, marked by increased CyclinD1 expression, and supported tight junctions via improved expression of the tight junction protein Occludin. Although our findings suggest that restoration of miR-146a expression in the intestinal epithelium successfully reduces intestinal inflammation and supports barrier homeostasis, increased miR-146a expression was unable to significantly lower intestinal permeability one day after ethanol and burn injury. Taken together, this study illustrates that downregulation of miR-146a is a potential therapeutic target to prevent excessive intestinal inflammation following alcohol intoxication and burn injury but may not enough on its own to fully restore gut barrier integrity.

CHAPTER 5

INTEGRATED ANALYSIS OF DYSREGULATED MICRORNA AND MRNA EXPRESSION IN SMALL INTESTINAL EPITHELIAL CELLS AFTER ETHANOL AND BURN INJURY

Abstract

Gut barrier dysfunction is often implicated in pathology following alcohol intoxication and burn injury. MicroRNAs (miRNAs) are negative regulators of gene expression that play a central role in gut homeostasis, although their role after alcohol and burn injury is poorly understood. We performed an integrated analysis of miRNA and RNA sequencing data to identify a network of interactions within small intestinal epithelial cells (IECs) which could promote gut barrier disruption. Mice were gavaged with ~ 2.9 g/kg ethanol and four hours later given a ~ 12.5% TBSA full thickness scald injury. One day later, IECs were harvested, and total RNA extracted for RNA-seq and miRNA-seq. RNA sequencing showed 712 differentially expressed genes (DEGs) (padj < 0.05) in IECs following alcohol and burn injury. Furthermore, miRNA sequencing revealed 17 differentially expressed miRNAs (DEMs) (padj < 0.1). Utilizing the miRNet, miRDB and TargetScan databases, we identified both validated and predicted miRNA gene targets. Integration of small RNA sequencing data with mRNA sequencing results identified correlated changes in miRNA and target expression. Upregulated miRNAs were associated with decreased proliferation (miR-98-3p and miR-381-3p) and cellular adhesion (miR-29a-3p, miR-429-3p and miR3535), while downregulated miRNAs were connected to upregulation of apoptosis (Let-7d-5p and miR-130b-5p) and metabolism (miR-674-3p and miR-185-5p). Overall, these findings suggest that alcohol and burn injury significantly alters the

mRNA and miRNA expression profile of IECs and reveals numerous miRNA–mRNA interactions that regulate critical pathways for gut barrier function after alcohol and burn injury.

Introduction

Burn injury is one of the leading causes of accidental injury in the United States, contributing to approximately half a million cases and 40,000 hospitalizations each year[1]. Sepsis and multiple organ failure, resulting from gut barrier disruption and bacterial translocation, are the most prominent cause of death among patients with severe burn injury[68,136,138,139]. Alcohol use is a common confounding factor in many traumas, as it increases the risk of accidental injury[95]. Similar to other traumas, nearly half of reported burn injuries occur under the influence of alcohol[94]. Compared to patients with similar burn size and depth, individuals intoxicated at the time of burn injury require longer hospital stays and more surgical procedures. Additionally, they have an increased risk of infection, sepsis, multiple organ failure, and ultimately higher mortality rates[7,94,95,104,107]. Therefore, alcohol intoxication at the time of burn injury is not only prevalent, but also significantly contributes to worsened patient outcomes. Consequently, the mechanisms underlying this worsened pathology are an important facet of trauma research that requires further study. To investigate how alcohol exacerbates burn pathophysiology, our laboratory utilizes a well-established mouse model of acute alcohol intoxication and burn injury. Using this model, our laboratory and others have shown that alcohol worsens intestinal inflammation, gut barrier disruption and bacterial dysbiosis after burn injury, which can contribute to the increased risk of sepsis seen in patients[7,104,107].

MicroRNAs (miRNAs) are small noncoding RNAs which post-transcriptionally regulate gene expression via complementary binding to the 3′ untranslated region (UTR) of their target mRNAs. This interaction negatively impacts gene expression by either translational repression or

mRNA degradation[184,185]. Over half of the genome is estimated to be regulated by miRNAs which regulate key signaling networks in a wide variety of cell types[24,186]. Recent studies demonstrate that miRNAs play a crucial role in regulating intestinal homeostasis and inflammation[27,28]. In addition, dysregulation of numerous miRNAs has been linked to worsened disease in models of chronic intestinal inflammation, including Inflammatory Bowel Disease (IBD) and colorectal cancer[30,31,33]. Far less is known about the roles of miRNA in models of acute injury and inflammation, such as trauma and burn injury. Moreover, the contributions of miRNA in regulating intestinal barrier dysfunction following alcohol and burn injury are poorly understood. Previous studies in our laboratory show decreased expression of miR-150 in intestinal epithelial cells (IECs) after alcohol and burn injury[187]. Additionally, we see decreased expression of Drosha and Argonaute 2 (Ago2) in intestinal epithelial cells after alcohol and burn injury[187]. Both Drosha and Ago2 are components of the miRNA biogenesis pathway and changes in their expression could have a significant impact on the levels of numerous miRNAs. Furthermore, studies looking at intestinal knockout of Dicer-1, an essential miRNA processing enzyme, show disruption of tight junction protein expression and localization, increased apoptosis, and increased intestinal inflammation[27]. This indicates that global changes in miRNA expression can adversely affect intestinal inflammation and barrier integrity and highlights the need for further research into the impact of miRNA on post-burn intestinal dysfunction.

To assess the global profile of miRNA and begin to evaluate miRNA as a molecular mechanism behind intestinal barrier disruption after alcohol and burn injury, we performed an integrated analysis of miRNA and mRNA expression in intestinal epithelial cells using our well-established mouse model. Small RNA sequencing analysis, also known as miRNA-seq, was used to generate a miRNA expression profile of intestinal epithelial cells one day following alcohol

intoxication and burn injury. In addition, we performed mRNA sequencing (RNA-seq) in parallel to assess gene expression. To understand how changes in miRNA expression would impact diverse networks of gene expression, we then integrated miRNA-seq data with the RNA sequencing data to assess correlated changes in miRNA expression and their gene targets. Our findings suggest that IEC miRNA expression is considerably and globally impacted by alcohol and burn injury and is therefore likely to significantly contribute to post-burn pathogenesis. Furthermore, integrated analysis performed using correlated changes in gene and miRNA expression alongside databases of both validated and predicted miRNA gene targets, allowed us to identify mRNA–miRNA interactions relevant to intestinal barrier function, which could play a critical role in perpetuating gut barrier disruption following alcohol exposure and burn injury.

Materials and Methods

Animals

10–12-week-old C57BL/6 male mice (23–25 g body weight) were obtained from Charles River Laboratories and maintained in animal housing facilities at Loyola University Chicago Health Sciences Division, Maywood, Illinois, USA.

Mouse Model of Acute Ethanol Intoxication and Burn Injury

Male C57/BL6 mice (10-12 weeks old, 22–26 g) were randomly assigned into two experimental groups: Sham injury + vehicle treatment or burn injury + ethanol treatment. On the day of injury, ethanol burn mice (EB) were gavaged with 400 µL of 25% ethanol in water (2.9 g/kg), while sham vehicle (SV) animals were gavaged with 400 µL water. Three hours following the gavage, mice were given 1 mg/kg buprenorphine subcutaneously for pain management. Four hours following the gavage, mice were anesthetized with a ketamine hydrochloride/xylazine cocktail (~ 80 mg/kg and ~ 1.2 mg/kg respectively) via intraperitoneal injection. The dorsal

surface of each mouse was shaved before placing the mice in a prefabricated template exposing ~ 12.5% total body surface area, calculated using Meeh's formula[175]. Burn group animals were immersed in ~85 °C water bath for ~7 seconds to induce a full-thickness scald burn injury. Sham animals were placed in a 37 °C, lukewarm water bath for an equal length of time. Following burn or sham injury, animals were dried gently and given 1.0 mL normal saline resuscitation by intraperitoneal injection. Animals were returned to their cages, which were placed on heating pads to help maintain their body temperature and observed to ensure recovery from anesthesia. Mice were then returned to their normal housing and allowed food and water ad libitum. All animal experiments were conducted in accordance with the guidelines set forth by the Animal Welfare Act and approved by the Institutional Animal Care and Use Committee (IACUC) at Loyola University Health Sciences Division.

Small Intestinal Epithelial Cell Isolation

One day after injury, mice were euthanized, and the abdominal cavity was exposed via midline incision. Approximately ~8 cm of the distal small intestine was harvested and opened longitudinally and washed twice in ice cold PBS + 100 U/mL penicillin + 100 μg/mL streptomycin. Small intestines were then incubated in HBSS buffer without phenol red supplemented with 10 mMol/L HEPES, 50 μg/mL gentamicin, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM EDTA and 1 mM DTT (pre-digestion solution) for 20 min at 37 °C with agitation at 250 rpm. Samples were vortexed to disrupt epithelial cells from the lamina propria and epithelial cells were collected through a 100 μm strainer. This process of pre-digestion solution incubation and epithelial cell collection was repeated a second time, pooling isolated epithelial cells[106,176]. Epithelial cells were then washed with PBS twice to remove pre-digestion solution and then used in downstream applications.

Total RNA Isolation

Total RNA was extracted from small intestinal epithelial cells using the mirVana miRNA Isolation Kit (Invitrogen) according to manufacturer's instructions. Total RNA concentration and purity were assessed using a Nanodrop 2000 spectrophotometer (ThermoScientific).

MicroRNA Sequencing Analysis

Isolated total RNA from small intestinal epithelial cells (low quality RNA samples excluded and n = 5 per group chosen randomly) was submitted to Northwestern University Chicago's NUSeq Core Facility where quality control via an Agilent 2100 BioAnalyzer, library preparation and miRNA sequencing was performed. Analysis, including read clean up, genome mapping, and differential expression analysis, were performed at Loyola University by Dr. Michael Zilliox. Briefly, small RNA libraries were prepared using TruSeq Small RNA Library Preparation Kit according to manufacturer's instructions. Resulting libraries were then sequenced at 100 million raw reads per sample using 75 base pair, single read sequencing via the NextSeq Illumina Platform. Raw sequencing reads were then cleaned using Cutadapt software to remove adaptor sequences and low-quality reads[188]. The resulting clean reads were then mapped to the mouse genome (mm10). An annotation file from miRBase (release 22.1) describing miRNA coordinates, and the sequencing alignment mappings were used as input for the Python package HTSeq to generate raw counts of miRNAs observed in the alignments[189–191]. Differential expression analysis was performed using the DESeq2 R package[192]. Gene set enrichment analysis was performed using validated miRNA gene targets from the miRNet database and their built-in software for Gene Ontology (GO) analysis[193–195]. KEGG pathway enrichment analysis was performed using g:Profiler software[195–197].

RNA Sequencing Analysis

Isolated total RNA from small intestinal epithelial cells was submitted to Novogene for library preparation and RNA sequencing analysis (same samples as those sent for miRNA sequencing). Briefly, samples were enriched for mRNAs using oligo(dT) beads targeting the 3′ polyA tail. Enriched mRNAs were then fragmented randomly, and non-stranded, non-directional library preparation performed using NEBNext kit according to manufacturer's instructions. Resulting libraries were sequenced at 20 million raw reads per sample using 150 base pair, pair-ended sequencing via the Illumina Platform. Raw reads were obtained by CASAVA base recognition (Base Calling) and then filtered for adaptor contamination and low-quality score. The resulting clean reads were then mapped to the mouse genome using STAR software60. FPKM (expected number of Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) was used to quantify mRNA expression and differential expression analysis was performed using the DESeq2 R package[192].

Statistics

Differentially expressed miRNAs (DEMs) and genes (DEGs) were identified via DESeq2 using Wald test p-values both before (p) and after adjustment (padj) using Benjamini and Hochberg's approach to control for the false discovery rate. DEMs with a p value <0.1 or padj value <0.1 and DEGs with a padj value <0.05 were considered significant for the purpose of different analyses. Functional gene set enrichment analysis of DEM gene targets, prior to DEG integration, was performed using miRNet's built in hypergeometric testing for GO annotated pathways of biological process (BP) terms [193–195]. Pathways with a p-value <0.1 were considered significantly enriched. Once DEGs were integrated into the DEM gene target lists, KEGG and GO-BP pathway enrichment analysis was performed using g:Profiler software with g:SCS

multiple testing correction[195–197]. Following this multiple testing correction, pathways with a padj value <0.05 were considered significantly enriched.

Results

To examine the overall impact of ethanol intoxication and burn injury on miRNA expression in the gut, we performed miRNA-seq analysis of small intestinal epithelial cells isolated 24 h after either vehicle treatment and sham injury (SV, n = 5) or ethanol treatment and burn injury (EB, n = 5). Preliminary differential expression analysis ($p < 0.1$) yielded 65 microRNAs with altered expression in ethanol burn mice compared to sham vehicle. Figure 18 shows a volcano plot depicting the significance and magnitude fold change for each of these 65 microRNAs.

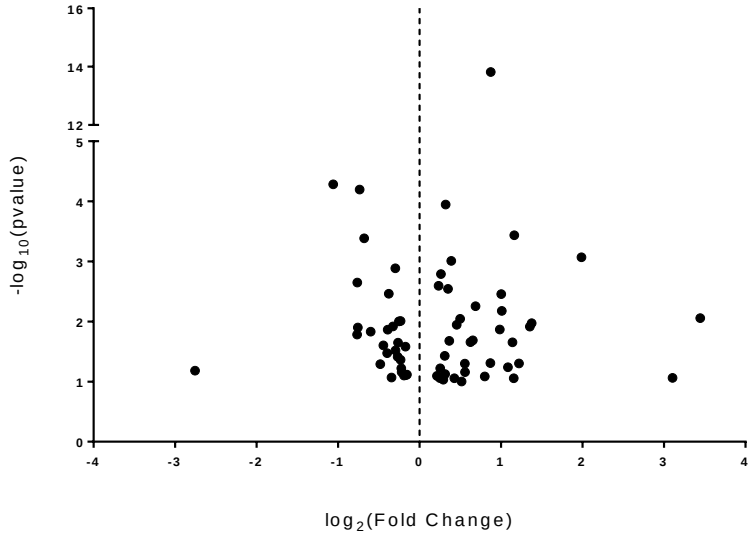

Figure 18. Volcano Plot of Initial 65 DEMs in Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Small RNA sequencing analysis identification of 65 differentially expressed miRNAs in small intestinal epithelial cells 1 day following alcohol and burn injury. Volcano plot demonstrates differentially expressed miRNAs ($p<0.1$) where each point on the graph represents an individual miRNA. Log2 fold change between average ethanol burn and average sham vehicle treated mice is plotted on the x-axis and the –log10 of the p value is plotted on the y-axis.

To further narrow our results, we adjusted p-values using Benjamin–Hochburg to control for false discovery rate. Using this method, we identified 17 differentially expressed microRNA

(DEMs), 11 of which were significantly upregulated and 6 of which were significantly downregulated (padj < 0.1). A heat map displaying fold change in expression of these 17 DEMs for each EB sample relative to the average SV control is shown in Figure 19. Overall, the majority of differentially expressed miRNAs were upregulated following ethanol and burn injury, with miR-429-5p displaying the greatest upregulation.

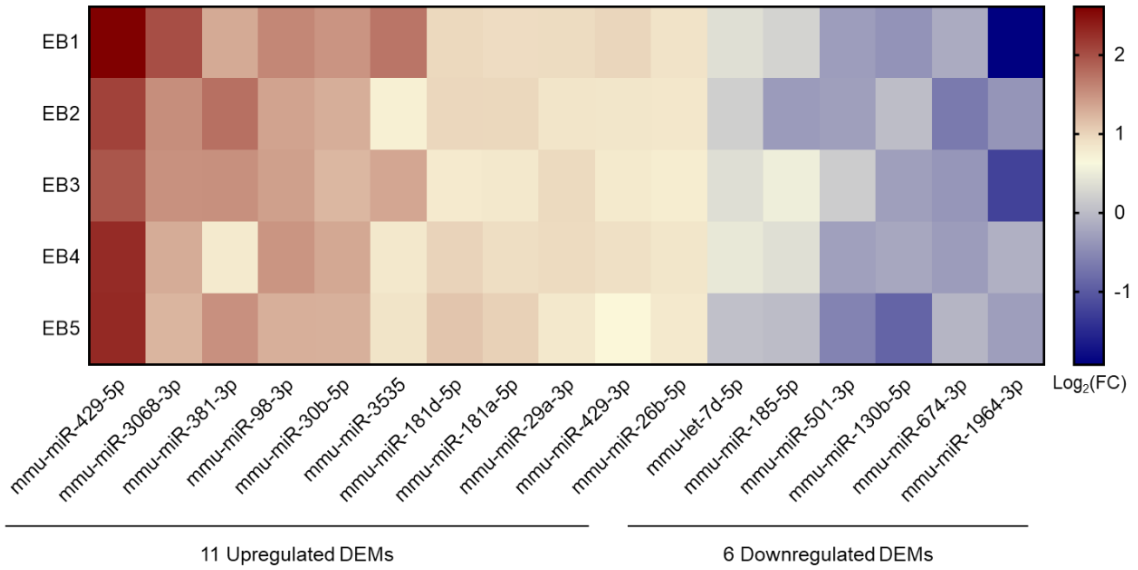

Figure 19. Heat Map of 17 Major DEMs Identified in Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Heat map of fold change expression (log scale) of 17 differentially expressed miRNAs (DEMs) in each ethanol burn sample (EB1-5) relative to sham vehicle controls. Each row shows the log2 fold change of individual DEMs which were significant (padj < 0.1) following Benjamin–Hochburg adjustment to control for false discovery rate. Color grading shows changes ranging from downregulated (blue) to upregulated (red).

We then utilized the miRNet database of experimentally validated miRNA gene targets to understand the potential role of these DEMs in intestinal homeostasis after ethanol and burn injury[193,194]. Among our 17 DEMs, 12 miRNAs (miR-1964-3p, miR-501-3p, Let-7d-5p, miR-185-5p, miR-30b-5p, miR-29a-3p, miR-429-5p, miR181d-5p, miR-429-3p, miR-26b-5p, miR-181a-5p, and miR-381-3p) were annotated in the miRNet database. In total, the 5,068 validated gene targets were identified for these 12 DEMs. To distinguish between pathways that are

potentially upregulated or downregulated after alcohol and burn injury, we separated gene targets of upregulated DEMs from gene targets of DEMs that were downregulated and performed pathway enrichment analysis. Figure 20 shows that gene targets of our upregulated DEMs were highly associated with proliferation and cell differentiation, as well as the cytoskeleton and actin organization. Due to the negative regulation of target gene expression that is mediated by miRNAs, this suggests that upregulated miRNAs after alcohol and burn injury may negatively impact the intestinal barrier by negatively regulating epithelial cell regeneration and adhesion. As shown in Figure 21, pathway analysis of the gene targets of significantly downregulated miRNAs are associated with potential upregulation of metabolism, cell cycle arrest, and cell death processes.

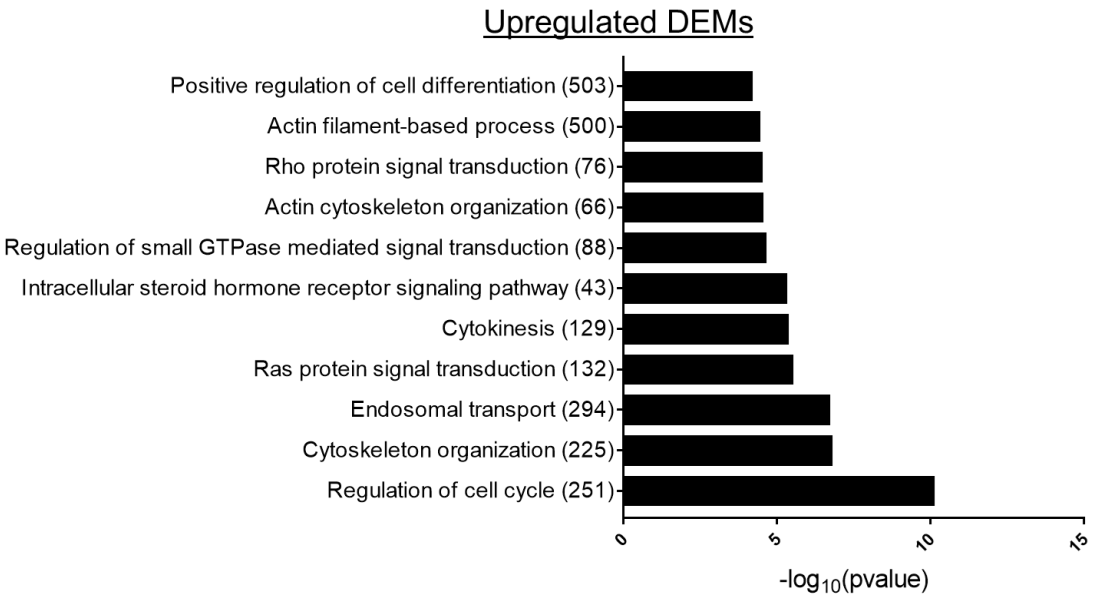

Figure 20. Functional Gene Ontology Enrichment Analysis of Validated miRNA Gene Targets for Upregulated DEMs. Validated miRNA gene targets for differentially expressed miRNAs were separated into those associated with upregulated DEMs versus downregulated DEMs. GO-BP gene set enrichment analysis was then performed using miRnet built in hypergeometric testing. Significantly enriched (p-value < 0.1) pathways of interest are depicted in the bar graph above for gene targets of significantly upregulated microRNAs (DEMs). Numbers in parentheses for each pathway represent to number of identified gene targets associated with the GO-BP pathway term. The –log10 of the p value is plotted along the axis.

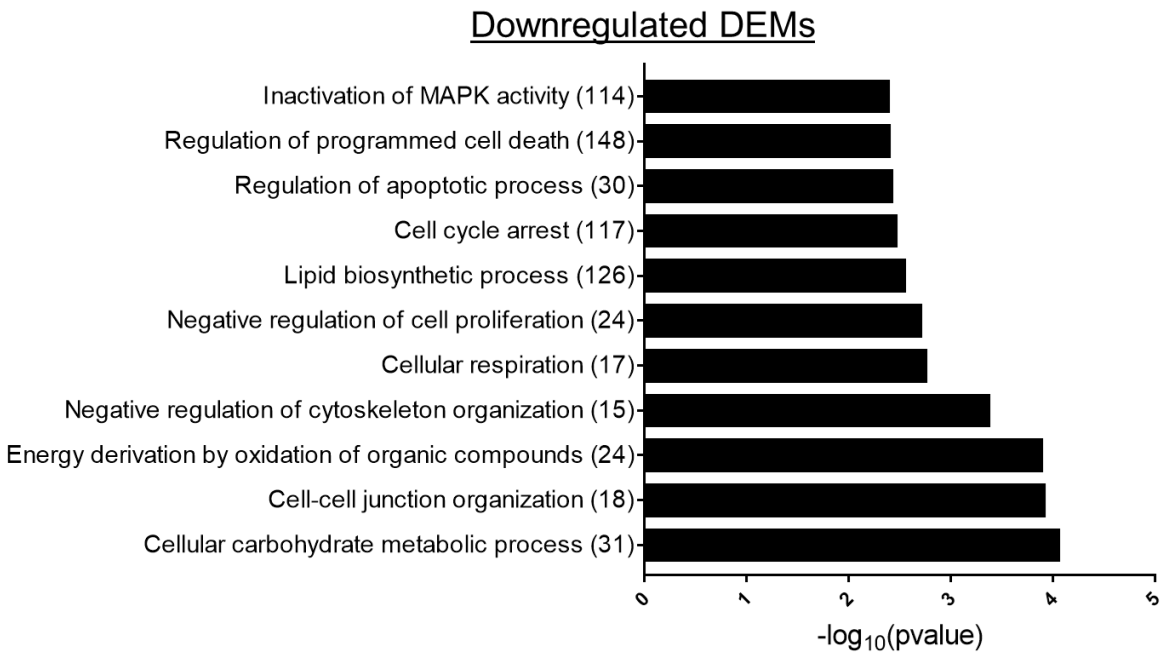

Figure 21. Functional Gene Ontology Enrichment Analysis of Validated miRNA Gene Targets for Downregulated DEMs. Validated miRNA gene targets for differentially expressed miRNAs were separated into those associated with upregulated DEMs versus downregulated DEMs. GO-BP gene set enrichment analysis was then performed using miRnet built in hypergeometric testing. Significantly enriched (p-value < 0.1) pathways of interest are depicted in the bar graph above for gene targets of significantly downregulated microRNAs (DEMs). Numbers in parentheses for each pathway represent to number of identified gene targets associated with the GO-BP pathway term. The –log10 of the p value is plotted along the axis.

To further delineate the impact of alcohol and burn injury on gene expression, we performed RNA-seq alongside the miRNA sequencing, using the same samples to again compare small intestinal epithelial cells from sham vehicle mice (n = 5) and ethanol burn mice (n = 5). Sequencing achieved an average of 45.7 million cleaned reads per sample and an average mapping rate of 94.9%. In total, we identified 11,809 expressed genes (FPKM > 1), of which 11,078 were expressed by both treatment groups (93.8%). Overall, differential gene expression analysis identified 712 differentially expressed genes (DEGs) in ethanol burn mice compared to sham vehicle (padj < 0.05). The volcano plot in Figure 23 shows the significance and magnitude fold change of each DEG, including 349 genes which are significantly upregulated and 363 genes which are significantly downregulated after ethanol and burn injury.

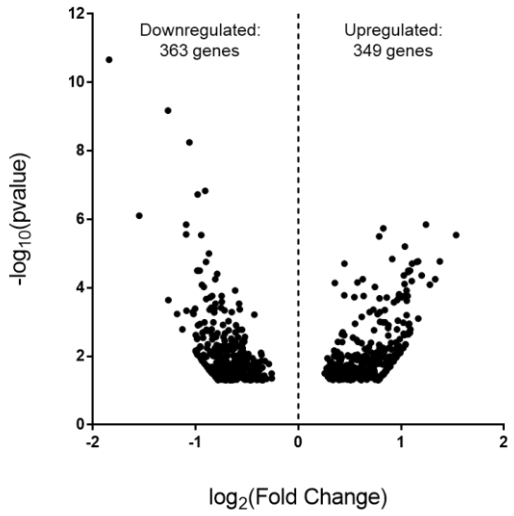

Figure 22. Volcano Plot of Differentially Expressed Genes in Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Volcano plot demonstrates differentially expressed mRNAs (padj<0.05) where each point on the graph represents an individual gene. Log2 fold change between average ethanol burn and average sham vehicle treated mice is plotted on the x-axis and the –log10 of the p value is plotted on the y-axis. Overall, sequencing analysis identified 712 differentially expressed genes (including 349 upregulated genes and 363 downregulated genes).

Complex networks of miRNA and their gene targets regulate a wide range of signaling pathways. Consequently, networks of miRNAs cooperate to significantly impact gene expression and subsequent cellular signaling. To visualize the global range of miRNA and gene target interactions most impacted by alcohol and burn injury, we constructed an interaction network that integrates both our miRNA and mRNA sequencing data. To begin, we assessed the 5068 validated gene targets identified previously for our DEMs utilizing the miRNet database for overlap with genes identified as differentially expressed by mRNA sequencing. Of the 712 DEGs identified via sequencing, 188 (26.4%) genes were validated targets of 9 of our DEMs. The miRNA–target interaction network generated from this overlap via miRNet, in Figure 23, can be used to visualize the interactions between dysregulated genes and miRNAs after alcohol and burn injury.

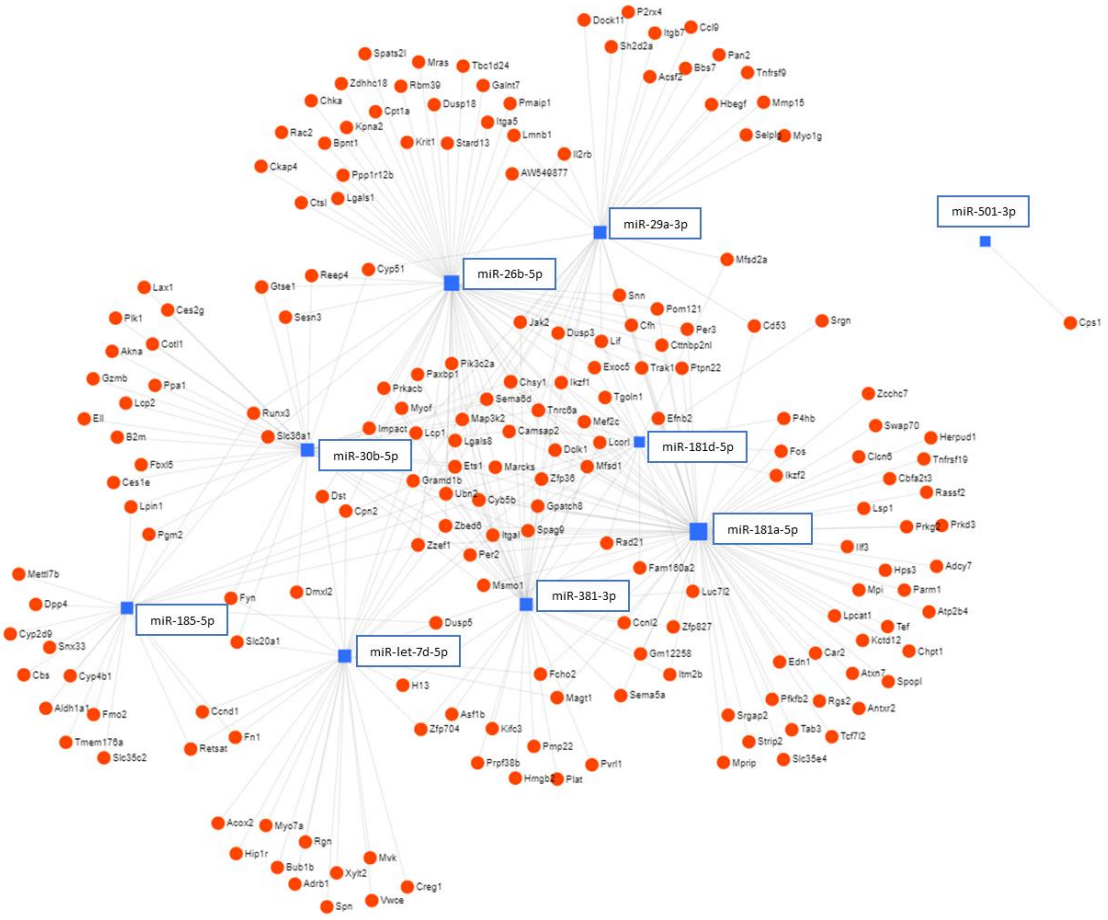

Figure 23. Integrated Network of DEMs and Validated Differentially Expressed Gene Targets in Small Intestinal Epithelial Cells One Day after Ethanol and Burn Injury. miRNA-mRNA interaction network constructed using miRNet containing DEMs (blue squares) and their validated miRNA gene targets which are differentially expressed genes (DEGs) via mRNA sequencing (red circles). Validated gene targets of our 17 major DEMs were identified using the miRNet database and overlapped with genes identified as differentially expressed via sequencing (DEGs). Subsequently, an interaction network was then constructed using miRNet software containing 9 DEMs and 188 differentially expressed validated gene targets.

The miRNet database provides several measurements of network connectivity to describe the interactions of miRNAs and gene targets within the network. The number of total nodes directly connected to any particular node is given by the node degree. Betweenness centrality is calculated using the number of shortest paths through a node and gives sense of the density of surrounding node clusters. To assess the functional impact of the network's most connected, core DEMs and their gene targets, we performed GO-BP enrichment analysis on central network

genes (degree > 3, betweenness > 100) using g:Profiler with g:SCS multiple testing correction method[197]. Significantly enriched pathways (padj < 0.05) and their associated miRNA gene targets are described in Table 1. These include cell adhesion, proliferation, metabolism, and signaling pathways associated with stress and inflammatory responses.

Table 1. Integrated miRNA-mRNA Network Analysis. GO-BP database analysis of central network genes (Degree > 3, Betweenness centrality > 100) was performed using g:Profiler with g:SCS multiple testing correction method. Each term identified in the table are among those significantly enriched (padj > 0.05) with important impacts on intestinal barrier integrity. All significantly enriched pathways are not displayed.

GO:BP Term	GO:BP ID	padj	Intersections
Cell Differentiation	GO:0030154	9.16E-07	Ets1, Chsy1, Zbed6, Spag9, Ikzf1, Lif, Lgals8, Dclk1, Zfp36, Efnb2, Mef2c, Impact, Jak2, Sema6d, Camsap2
Cellular Metabolic Process	GO:0044237	4.44E-05	Tnrc6a, Cyb5b, Ets1, Chsy1, Zbed6, Spag9, Ikzf1, Map3k2, Lif, Lgals8, Dclk1, Zfp36, Efnb2, Mef2c, Lcorl, Impact, Jak2, Dusp5, Per2
Cell Migration	GO:0016477	5.64E-05	Ets1, Spag9, Lgals8, Dclk1, Efnb2, Mef2c, Jak2, Sema6d, Itgal
Cell Development	GO:0048468	0.00022	Chsy1, Spag9, Lif, Dclk1, Efnb2, Mef2c, Impact, Jak2, Sema6d, Camsap2
Regulation of Cell Adhesion	GO:0030155	0.00218	Ets1, Lif, Lgals8, Efnb2, Jak2, Itgal
MAPK Cascade	GO:0000165	0.00359	Spag9, Map3k2, Lif, Zfp36, Mef2c, Dusp5
Cell Activation	GO:0001775	0.02699	Ikzf1, Lgals8, Efnb2, Mef2c, Jak2, Itgal
Regulation of Cell Population Proliferation	GO:0042127	0.03526	Ets1, Lif, Zfp36, Efnb2, Mef2c, Jak2, Itgal
Regulation of Cell Death	GO:0010941	0.03538	Ets1, Zbed6, Zfp36, Efnb2, Mef2c, Impact, Jak2
Cellular Response to Stress	GO:0033554	0.04257	Tnrc6a, Ets1, Spag9, Zfp36, Mef2c, Impact, Jak2

As an alternative approach to elucidate potentially important gene targets for individual DEMs after alcohol and burn injury, we performed a separate integrated analysis for each individual DEM with our RNA sequencing data. As some of our DEMs did not have experimentally validated gene targets in the miRNet database, we expanded our analysis to include predicted gene targets for each DEM from the TargetScan and miRDB databases[198,199]. We then extracted targets of downregulated DEMs which were upregulated DEGs or targets of upregulated DEMs which were downregulated DEGs. In order to understand the role that individual DEMs might play in gut barrier dysfunction after alcohol and burn injury, we performed KEGG pathway enrichment analysis on the extracted gene targets for each DEM individually. Table 2 demonstrates that downregulated DEMs miR-674-3p and miR-185-5p exhibited the highest number of significantly associated KEGG pathways.

Table 2. Individual Integrated Analysis of Downregulated DEMs and Upregulated Gene Targets. KEGG pathway enrichment analysis upregulated gene target lists for each individual downregulated DEM using G:profiler with g:SCS multiple testing correction. All significantly enriched (padj < 0.1) pathways are outlined in the table below.

Downregulated DEMs	KEGG Pathways Associated with Upregulated DEG Targets	KEGG ID	padj
miR-1964-3p	none	none	none
miR-130b-5p	none	none	none
miR-501-3p	Amphetamine addiction	KEGG:05031	0.09649
Let-7d-5p	Peroxisome	KEGG:04146	0.07558
miR-674-3p	Proteoglycans in cancer	KEGG:05205	0.02157
	Carbohydrate digestion and absorption	KEGG:04973	0.02874
	Regulation of actin cytoskeleton	KEGG:04810	0.02900
	Pathways in cancer	KEGG:05200	0.03249
	Gastric acid secretion	KEGG:04971	0.07090
	Insulin secretion	KEGG:04911	0.08894
miR-185-5p	Bacterial invasion of epithelial cells	KEGG:05100	0.02753
	Retinol metabolism	KEGG:00830	0.04314
	AGE-RAGE signaling pathway in diabetic complications	KEGG:04933	0.0478
	Protein digestion and absorption	KEGG:04974	0.05577
	TNF signaling pathway	KEGG:04668	0.05786

In particular, upregulated DEGs of miR-185-5p were significantly associated with pathways that could disrupt intestinal barrier integrity, including bacterial invasion of epithelial cells and TNF signaling. Other downregulated DEMs exhibited either no significant pathway enrichment among upregulated gene targets, or just one significantly enriched pathway. Table 3 demonstrates the plethora of KEGG pathways significantly associated with the downregulated gene targets of our upregulated DEMs. The most common KEGG pathways associated with upregulated DEMs included cell adhesion and tight junctions (miR-29a-3p, miR-429-3p, and miR-3535), Ras signaling (miR-429-3p and miR-26b-5p) and the cell cycle (miR-98-3p and miR-381-3p). In addition, we saw significant enrichment in hormone signaling pathways associated with GI motility and mucosal function, including oxytocin (miR-181a-5p, miR-98-3p and miR-381-3p), aldosterone (miR-429-5p, miR-181a-5p and miR-98-3p), and parathyroid hormone (miR-98-3p and miR-381-3p).

Table 3. Individual Integrated Analysis of Upregulated DEMs and Downregulated Gene Targets. KEGG pathway enrichment analysis downregulated gene target lists for each individual upregulated DEM using G:profiler with g:SCS multiple testing correction. All significantly enriched (padj < 0.1) pathways are outlined in the table below.

Upregulated DEMs	KEGG Pathways Associated with Downregulated DEG Targets	KEGG ID	padj
miR-30b-5p	T cell receptor signaling pathway	KEGG:04660	0.00094
	Natural killer cell mediated cytotoxicity	KEGG:04650	0.00140
	Fc epsilon RI signaling pathway	KEGG:04664	0.02673
	p53 signaling pathway	KEGG:04115	0.03277
	Antigen processing and presentation	KEGG:04612	0.04143
	Platelet activation	KEGG:04611	0.08874
	Osteoclast differentiation	KEGG:04380	0.09022
miR-29a-3p	Viral protein interaction with cytokine and cytokine receptor	KEGG:04061	0.00188
	Cytokine-cytokine receptor interaction	KEGG:04060	0.00246
	Cell adhesion molecules	KEGG:04514	0.01007
	Intestinal immune network for IgA production	KEGG:04672	0.01962
	Calcium signaling pathway	KEGG:04020	0.03096
	Arrhythmogenic right ventricular cardiomyopathy	KEGG:05412	0.06069

miR-3068-3p	none	none	none
miR-429-5p	Aldosterone synthesis and secretion	KEGG:04925	0.00198
	Primary immunodeficiency	KEGG:05340	0.01279
	Intestinal immune network for IgA production	KEGG:04672	0.01744
	cAMP signaling pathway	KEGG:04024	0.01773
	Calcium signaling pathway	KEGG:04020	0.02554
	Endocrine and other factor-regulated calcium reabsorption	KEGG:04961	0.03559
	Cytokine-cytokine receptor interaction	KEGG:04060	0.04390
	Salivary secretion	KEGG:04970	0.07104
	Longevity regulating pathway	KEGG:04211	0.07608
miR-181d-5p	none	none	none
miR-181a-5p	cGMP-PKG signaling pathway	KEGG:04022	0.00039
	Aldosterone synthesis and secretion	KEGG:04925	0.00296
	Pancreatic secretion	KEGG:04972	0.00426
	Oxytocin signaling pathway	KEGG:04921	0.00928
	Rap1 signaling pathway	KEGG:04015	0.02673
	Human T-cell leukemia virus 1 infection	KEGG:05166	0.03698
	Gastric acid secretion	KEGG:04971	0.07090
	Salivary secretion	KEGG:04970	0.09322
	Longevity regulating pathway	KEGG:04211	0.09982
miR-429-3p	Cholinergic synapse	KEGG:04725	0.01293
	Platelet activation	KEGG:04611	0.01513
	Tight junction	KEGG:04530	0.02931
	Proteoglycans in cancer	KEGG:05205	0.04073
	Ras signaling pathway	KEGG:04014	0.05538
	Human T-cell leukemia virus 1 infection	KEGG:05166	0.05877
	Calcium signaling pathway	KEGG:04020	0.05976
	Prion disease	KEGG:05020	0.07166
miR-26b-5p	Regulation of actin cytoskeleton	KEGG:04810	0.00058
	MAPK signaling pathway	KEGG:04010	0.00170
	Apelin signaling pathway	KEGG:04371	0.00459
	Chemokine signaling pathway	KEGG:04062	0.01201
	Proteoglycans in cancer	KEGG:05205	0.01379
	Rap1 signaling pathway	KEGG:04015	0.01711
	Pathways in cancer	KEGG:05200	0.01809
	Ras signaling pathway	KEGG:04014	0.02172
	Human T-cell leukemia virus 1 infection	KEGG:05166	0.02371
	p53 signaling pathway	KEGG:04115	0.04998
	Viral myocarditis	KEGG:05416	0.06159
	Th1 and Th2 cell differentiation	KEGG:04658	0.07271

	Longevity regulating pathway	KEGG:04211	0.07437
	Fc gamma R-mediated phagocytosis	KEGG:04666	0.07605
	Dilated cardiomyopathy	KEGG:05414	0.07947
	Viral protein interaction with cytokine and cytokine receptor	KEGG:04061	0.08120
miR-98-3p	Progesterone-mediated oocyte maturation	KEGG:04914	0.02906
	Aldosterone synthesis and secretion	KEGG:04925	0.03922
	Parathyroid hormone synthesis, secretion, and action	KEGG:04928	0.04320
	Oocyte meiosis	KEGG:04114	0.05085
	Cell cycle	KEGG:04110	0.05722
	Apelin signaling pathway	KEGG:04371	0.07104
	Oxytocin signaling pathway	KEGG:04921	0.08403
miR-3535	Cell adhesion molecules	KEGG:04514	0.01235
	NOD-like receptor signaling pathway	KEGG:04621	0.02254
	Rap1 signaling pathway	KEGG:04015	0.02673
	Regulation of actin cytoskeleton	KEGG:04810	0.02900
	Pertussis	KEGG:05133	0.07474
miR-381-3p	MAPK signaling pathway	KEGG:04010	0.01083
	Progesterone-mediated oocyte maturation	KEGG:04914	0.02906
	Parathyroid hormone synthesis, secretion, and action	KEGG:04928	0.04320
	Oocyte meiosis	KEGG:04114	0.05085
	Cell cycle	KEGG:04110	0.05722
	Apelin signaling pathway	KEGG:04371	0.07104
	Oxytocin signaling pathway	KEGG:04921	0.08403

To gain a broader understanding of the regulatory network, we also combined all identified targets for downregulated versus upregulated DEMs and assessed them separately. Figure 24 shows the KEGG pathways significantly associated with the gene targets of either upregulated or downregulated DEMs. For upregulated DEMs, we found a total of 167 predicted gene targets that were downregulated by RNA sequencing, which were associated with Rap1 signaling, cellular adhesion, calcium signaling, and hormone signaling, including aldosterone, parathyroid hormone and apelin. On the other hand, we identified 121 upregulated predicted gene targets for downregulated miRNAs, which were linked to metabolic pathways, TNF signaling, IL-17 signaling, and genes associated with colorectal cancer.

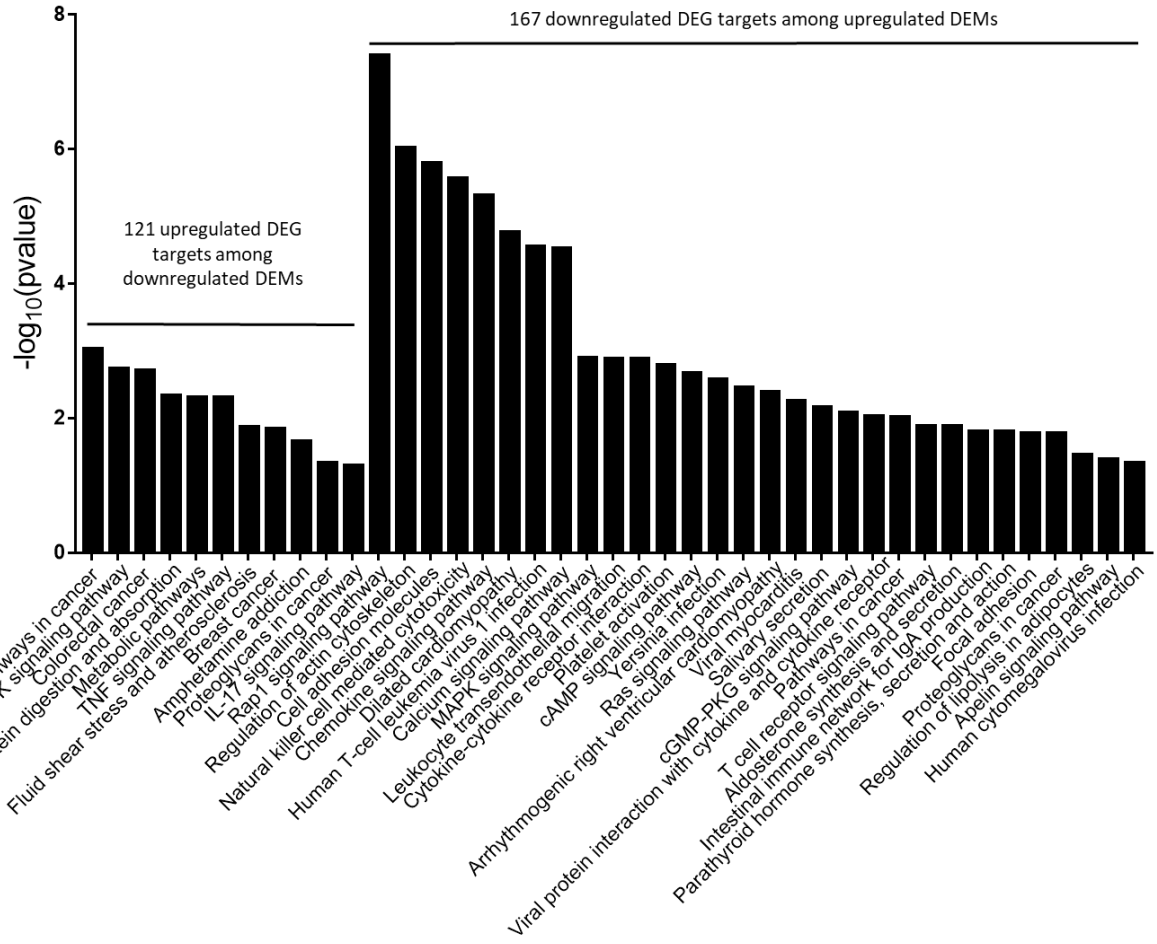

Figure 24. Pathway Enrichment Analysis of DEMs and Gene Targets with Correlated Expression Changes. Validated and predicted gene targets for each identified DEM were collected from miRNet, TargetScan, and miRDB databases. Gene target lists were then integrated with gene expression data from mRNA sequencing. Gene targets with significantly upregulated expression were extracted for each downregulated DEM, while significantly downregulated gene targets were extracted for each upregulated DEM. The resulting upregulated and downregulated gene targets were compiled separately, and KEGG pathway enrichment analysis was performed using G:profiler with g:SCS multiple testing correction. All significantly enriched (padj < 0.05) pathways are shown in the bar graph above.

Summary

The goal of this report was to assess the global impact of alcohol and burn injury on intestinal epithelial cell miRNA expression and gain deeper insight into the potential mechanisms by which miRNA could contribute to gut barrier disruption following combined injury. The results from this study demonstrate widespread changes in miRNAs and their target

gene expression within intestinal epithelial cells after alcohol and burn injury. Moreover, integration of miRNA and mRNA sequencing data revealed several interactions which are highly associated with intestinal barrier integrity and provide evidence that changes in miRNA expression following alcohol intoxication and burn injury are an important component of post-burn pathogenesis. In particular, we revealed several miRNAs of interest which likely play an important role in decreasing gut barrier integrity by reducing intestinal proliferation via upregulating miR-98-3p and miR-381-3p, disrupting epithelial cell tight junctions via upregulating miR-29a-3p, miR-429-3p and miR-3535, and increasing intestinal apoptosis via downregulating Let-7d-5p and miR-130b-5p. In addition, we found that downregulation of miRNAs including miR-674-3p and miR-185-5p could promote a hypermetabolic phenotype in intestinal epithelial cells. Overall, these findings open the door to future mechanistic studies that could increase our understanding of how miRNAs regulate and control gut barrier function and intestinal homeostasis.

CHAPTER 6

DISCUSSION

New Contributions to the Field of Alcohol and Burn Injury

Intestinal dysfunction is commonly seen following traumatic injury, including severe burns, and is established to be a significant contributing factor to systemic consequences that can lead to mortality, including sepsis and multiple organ failure[7,8,16,17]. Alcohol intoxication is commonly associated with burn injury, occurring in as many as half of all reported burn injuries[4–6]. Studies have shown that alcohol intoxication of the time of burn injury worsens intestinal dysfunction and increases the risk of poor outcomes[6–10]. Although miRNAs are essential regulators of intestinal homeostasis, their role in intestinal inflammation and barrier integrity following alcohol intoxication and burn injury remains unexplored. The overall purpose of dissertation project was to identify aberrantly expressed miRNAs in small intestinal epithelial cells following alcohol and burn injury which would provide future therapeutic targets to reduce intestinal dysfunction following burn injury. Furthermore, this project sought to gain further insight into the mechanism by which reduced anti-inflammatory miRNAs, such as miR-146a, contribute to excessive intestinal inflammation and gut barrier disruption following alcohol intoxication and burn injury.

Our results demonstrate significant downregulation of small intestinal epithelial cell expression of both miR-146a and miR-150 one day after alcohol and burn injury. Previous reports have validated miR-146a as an important regulator of inflammatory signaling in immune cells and have also indicated miR-146a may regulate similar responses in intestinal epithelial

cells[170–174]. In our *in vitro* studies, we show that miR-146a expression reduces small intestinal epithelial induction of pro-inflammatory cytokines following LPS stimulation via regulation of the TRAF6/p38MAPK signaling pathway. Furthermore, *in vivo* overexpression of miR-146a significantly inhibited intestinal inflammation, enhanced small intestinal cell proliferation, and promoted epithelial cell tight junction protein expression. Although miR-146a overexpression alone did not significantly reduce overall intestinal permeability, we were able to demonstrate that targeting miR-146a or other miRNAs of interest holds therapeutic promise in promoting intestinal function following alcohol and burn injury. Finally, our integrated analysis of small intestinal epithelial cell miRNA and gene expression identified several miRNA targets for future studies into their mechanism and therapeutic potential for regulating intestinal dysfunction after alcohol intoxication and burn injury.

Aberrant miR-146a Expression and Intestinal Inflammation after Alcohol and Burn Injury

Although miRNAs have been shown to impact wound healing, insulin resistance, and cardiac dysfunction after severe burn, the contributions of miRNA expression to intestinal inflammation and barrier disruption following alcohol and burn injury have remained largely unexplored[200–203]. Within the intestinal epithelium, miRNAs play a critical role in maintaining gut homeostasis[27–29]. Changes in miRNA expression have been implicated in the pathogenesis of several disorders involving intestinal inflammation and barrier disruption[29,64]. While less is known regarding the impact of miRNAs on gut dysfunction following alcohol and burn injury, several miRNAs have been studied as candidate biomarkers for sepsis, which can result from intestinal barrier disruption after burn injury. Reduced levels of circulating miR-150, miR-381, miR-495, and miR-574 are associated with inflammation and sepsis severity[204–211]. Additionally, previous studies have shown that toll-like receptor activation by bacteria products, such as LPS,

can promote excessive intestinal inflammation following burn injury[18]. Several anti-inflammatory miRNAs regulate toll-like receptor signaling to control inflammatory responses, particularly in immune cells. Studies have shown that expression of miR-146a, miR-150, miR-194, miR-495, and miR-574 can significantly reduce LPS induced inflammation[170,173,187,210,212–217]. Due to these associations, we sought to determine if reduced expression of these anti-inflammatory miRNAs promotes excessive intestinal inflammation and gut dysfunction following alcohol and burn injury. Using a well characterized mouse model of acute ethanol intoxication and burn injury, we found significantly reduced expression of miR-146a and miR-150 in small intestinal epithelial cells one day following ethanol and burn injury. These results validate a previous study demonstrating reduced miR-150 intestinal epithelial cell expression after ethanol and burn injury, which also showed that miR-150 expression can regulate intestinal epithelial cell inflammatory cytokine production in response to LPS[187]. First identified as a lipopolysaccharide (LPS) responsive gene in monocytes, miR-146a was shown to be an important brake for macrophage inflammation with important roles in autoimmunity and endotoxin-induced tolerace[170–172,218]. In monocytes, miR-146a expression is induced by inflammatory stimuli and then miR-146a targets MyD88 adaptor molecules, including TNF receptor associated factor 6 (TRAF6), to limit over-activation of inflammatory pathways[171]. More recent studies indicate that miR-146a may be similarly important in intestinal epithelial cells. Using rat and human colonic epithelial cell lines, Anzola et al demonstrated that miR-146a expression inhibits LPS or IL-1β induced production of MCP-1 and IL-8[173]. In addition, He et al revealed the protective effect of miR-146a expression on intestinal injury following ischemia-reperfusion and linked reduced injury to lower TRAF6 levels and reduced NF-κB activation[174]. If miR-146a does indeed play an important role in regulating intestinal epithelial cell inflammatory

signaling, then changes in miR-146a could have significantly contribute to gut dysfunction in disease. Therefore, we sought to further elucidate the impact the miR-146a downregulation could have on intestinal inflammation following alcohol intoxication and burn injury.

Activation of toll-like receptor 4 (TLR4) signaling has been shown to mediate intestinal barrier disruption following burn injury[18,66]. Therefore, we recapitulated activation of this crucial inflammatory pathway *in vitro* by stimulating MODE-K small intestinal epithelial cells with LPS. Stimulation of MODE-K cells for 24 hours significantly induced the expression of the pro-inflammatory cytokine IL-6 and the neutrophil chemokine KC (also known as CXCL1). These pro-inflammatory mediators promote intestinal inflammation, neutrophil recruitment, and barrier disruption following ethanol and burn injury[19,179,180,182]. Our results demonstrate that miR-146a inhibition in MODE-K cells significantly elevates LPS induced IL-6 and KC expression, while miR-146a overexpression significantly dampens this inflammatory response. Previous studies have established that miR-146a regulates inflammation by inhibiting NF-κB signaling via targeting of TRAF6 or other targets including IRAK1/4 or TAB1[171,174,183]. However, we found that pharmacological inhibition of NF-κB in MODE-K cells had no significant impact on IL-6 expression while significantly enhancing KC expression induced by miR-146a inhibition and LPS stimulation. Although this may appear counterintuitive, NF-κB signaling has been shown to play different roles in epithelial and immune cells. While generally considered pro-inflammatory in immune cells, NF-κB signaling is critical for homeostasis of the intestinal epithelium and therefore loss of NF-κB within intestinal epithelial cells has been associated with increased inflammation[65,219]. Instead, we revealed that miR-146a inhibitor mediated increases in LPS induced IL-6 and KC expression was blocked most significantly by p38 MAPK inhibition, while STAT3 inhibition appeared to have no significant effect. Furthermore, western blot analysis of

p38 MAPK and STAT3 phosphorylation following LPS stimulation of MODE-K cells demonstrates that while miR-146a overexpression inhibits phosphorylation of both p38 MAPK and STAT3, miR-146a inhibition only significantly promoted LPS induced phosphorylation of p38 MAPK. The difference observed between miR-146a overexpression and miR-146a inhibition is likely due to the overwhelming increase in miR-146a expression that occurs following miR-146a mimic treatment. Inhibition of miR-146a, instead, reduces endogenous miR-146a and is more physiologically relevant to the downregulation of miR-146a seen after alcohol and burn injury.

The most well characterized and validated target of miR-146a is TRAF6, which has generally been demonstrated as the primary target of miR-146a responsible for regulation of NF-κB signaling[170–174]. Although our results indicate that miR-146a primarily modulates inflammation of MODE-K cells via the p38 MAPK pathway, TRAF6 is also upstream of p38 MAPK activation and therefore is still the most likely direct target of miR-146a in our model. We found that overexpression of miR-146a does indeed reduce TRAF6 protein levels. Furthermore, the results from our TRAF6 knockdown experiments support targeting of TRAF6 by miR-146a significantly contributes to miR-146a mediated regulation of LPS induced cytokine production in MODE-K cells. Overall, our results suggest that reduced expression of miR-146a in intestinal epithelial cells one day after alcohol and burn injury potentiates intestinal inflammation via the TRAF6/p38 MAPK pathway. Studies have shown that inhibition of p38 MAPK can protect intestinal barrier integrity and reduce intestinal permeability after burn injury[220,221]. In addition, activation of p38 MAPK in Kupffer cells has been linked to pulmonary and hepatic inflammation following alcohol and burn injury[103]. Therefore, our studies also support the therapeutic potential of targeting this miR-146a/TRAF6/p38 MAPK pathway to

promote intestinal barrier integrity after alcohol and burn injury, potentially reducing poor patient outcomes associated with sepsis and multiple organ failure.

Therapeutic Potential of miR-146a Mimic Administration

As insight into miRNA associated pathophysiology expands, targeting of miRNA expression has emerged as a promising therapeutic strategy. Although miRNA-based therapies have yet to reach federal approval, several have demonstrated the efficacy of targeting miRNA expression in clinical trials. Studies have shown that miRNA-based therapies can be used to control excessive activation of inflammatory signaling, including in diseases associated with intestinal inflammation[32,42,79]. Indeed, clinical trials are currently ongoing for the drug ABX464 which treats ulcerative colitis by inducing miR-124 expression[85–88]. Therapeutic targeting of intestinal inflammation has the potential to reduce gut barrier disruption and prevent serious consequences following alcohol and burn injury, including sepsis and multiple organ failure[160]. Although activation of toll-like receptor 4 (TLR4) signaling and subsequent pro-inflammatory cytokine production have been connected to intestinal barrier disruption following burn injury, therapeutic targeting of these pathways may have unintended consequences within the intestinal epithelium[18,66]. Studies have shown that loss of Myd88, a signaling molecule immediately downstream of TLR4 activation, or downstream NF-κB signaling are both associated with increased inflammation and intestinal barrier disruption[65,219]. Due to the more fine tuned regulation that miRNAs provide, targeting of this pathway via modulation of key miRNAs that regulate its activity may provide a more promising therapeutic strategy to control intestinal inflammation and support barrier integrity following alcohol intoxication and burn injury. In a mouse model of acute ethanol intoxication and burn injury, we found significantly reduced expression of miR-146a in small intestinal epithelial cells isolated one day after insult. This anti-

inflammatory miRNA is an established brake mechanism that controls activation of innate immune signaling by negatively regulating target mRNAs, including its most well characterized target TRAF6[170,172–174,183]. Our *in vitro* studies suggest that downregulation of miR-146a within small intestinal epithelial cells promotes LPS induced inflammation via the TLR4/TRAF6/p38 MAPK signaling axis, which indicates that restoration of miR-146a expression could reduce intestinal inflammation and promote barrier integrity following ethanol and burn injury. Our results demonstrate that *in vivo* administration of a miR-146a mimic significantly elevates miR-146a expression in both small intestinal tissue and epithelial cells one day after ethanol and burn injury. This *in vivo* overexpression significantly reduced intestinal inflammatory cytokine expression and lowered intestinal neutrophil marker levels, indicating that miR-146a administration successfully prevents intestinal inflammation following alcohol and burn injury. Additionally, we saw increased intestinal epithelial cell expression of CyclinD1 and Occludin after alcohol and burn injury in mice receiving miR-146a mimic compared the scramble control. Although these improvements were unable to fully prevent intestinal permeability following ethanol and burn injury, our findings suggest that miR-146a expression may impact intestinal epithelial cell barrier function either as a secondary result from reducing inflammatory signaling or via direct regulation of intestinal epithelial cell proliferation or tight junction protein expression. Further characterization, both *in vitro* and *in vivo*, is required to understand these mechanisms and the impact they may have on intestinal epithelial barrier integrity after alcohol intoxication and burn injury.

Although our mouse model does not use a large burn area (only ~12.5% TBSA) and does not exhibit intestinal pathology unless combined with alcohol intoxication, some cytokines, including IL-6, have been observed to be elevated by burn injury alone[179,180]. However, the

addition of alcohol does significantly elevate inflammatory cytokine levels compared to burn alone, which leads to the recruitment of inflammatory immune cells to the intestine and results in increased intestinal permeability. Other studies have shown that inhibiting inflammation via anti-IL-6 antibody treatment prevents neutrophil accumulation, promotes tight junction organization, reduces intestinal damage, and prevents bacterial translocation after alcohol and burn injury[182]. While our studies demonstrate that miR-146a expression significantly reduced intestinal inflammation, including reduced IL-6 and neutrophil marker expression, administration of miR-146a mimic alone was unable to fully restore the gut barrier and prevent significant intestinal permeability. Inflammation is just one of the many factors that impact intestinal barrier integrity following alcohol and burn injury. Other studies have shown that the loss of intestinal IL-22 after alcohol and burn injury contributes to reduced intestinal proliferation and barrier disruption[105,106]. Like miR-146a mimic treatment, however, recombinant IL-22 treatment alone was unable to significantly reduce intestinal permeability following alcohol and burn injury[105]. Administration of miR-146a mimic reducing intestinal inflammation and recombinant IL-22 treatment promoting intestinal barrier integrity could work together to successfully prevent intestinal permeability after alcohol and burn injury and therefore is a promising option for a combination therapy.

It is important to consider a major limitation to the conclusions that can be drawn from this study regarding the role of intestinal epithelial cell miR-146a expression on intestinal inflammation and barrier integrity after alcohol and burn injury. Administration of miRNA mimics, including via intraperitoneal injection, can result in miRNA expression in a variety of organs and cell types. In the current study, we are unable to clarify the contribution that increased miR-146a expression in immune cells, present both in circulation and within intestinal

tissue, versus epithelial cells of the intestinal epithelium might have on mitigating intestinal inflammation. Both inflammatory immune cell infiltration and inflammatory cytokine production from epithelial cells have been implicated in barrier disruption following alcohol and burn injury[19,132,181,182]. While our results support the conclusion that restoration of intestinal epithelial cell miR-146a expression contributes to reduced intestinal inflammation and prevents recruitment of neutrophils to the intestine, it is likely that miR-146a expression in immune cells also plays a role in the observed outcomes. Further studies evaluating restoration of miR-146a specifically in intestinal epithelial cells or the immune cell compartment are required to fully understand the contributions of miR-146a expression in different cell type. While our study demonstrates the impact of miR-146a downregulation on intestinal inflammation following alcohol and burn injury, there are limitations to the conclusions that can be drawn regarding miR-146a mimic administration as a viable treatment option. In our study, mice were injected with miR-146a mimic one day prior to burn injury. Although miR-146a expression is upregulated 24 hours after injury and significantly controls intestinal inflammation, we are unable to conclude that miR-146a treatment immediately following alcohol intoxication and burn injury would provide similar results. A more thorough study exploring miR-146a administration at several time points within the first 24 hours following burn injury would provide more concrete support of the therapeutic potential of targeting miR-146a expression and deeper understanding of its limitations.

Altered miRNAs as a Mechanism of Intestinal Dysfunction after Alcohol and Burn Injury

Intestinal epithelial cells form a crucial physical barrier, which maintains homeostasis between pathogens present in the microbiome and the host defense response[68,134]. Disruption of this barrier promotes severe complications after burn injury, including sepsis and multiple organ

failure[136,139]. Following alcohol and burn injury, intestinal barrier disruption is primarily instigated by heightened inflammation, loss of tight junction proteins, reduced IEC proliferation and increased IEC apoptosis[18,19,136,179,181,222]. In line with the literature, our RNA sequencing data shows significant association between downregulated genes and cellular adhesion pathways. Under normal conditions, intestinal homeostasis is maintained, in part, through complex interactions between miRNAs and their gene targets, which coordinate signaling pathways critical for the regulation of IEC apoptotic and inflammatory signaling, in addition to the preservation of tight junction stability[27]. Our findings show that widespread changes in miRNA expression after alcohol intoxication and burn injury likely contribute to disruption of normal gut homeostasis. Utilizing miRNet, a database of experimentally validated miRNA gene targets, we found that several of our differentially expressed miRNAs regulate gene targets associated with intestinal barrier integrity. Specifically, pathways associated with cellular adhesion, including organization of the actin cytoskeleton and Rho-GTPase signaling, were enriched among predicted gene targets of our upregulated DEMs. Additionally, predicted gene targets associated with negative regulation of cytoskeleton organization are enriched among downregulated DEMs. While cell adhesion is critical for maintaining an intact intestinal barrier, intestinal epithelial cells are constantly being sloughed off and replaced. This process makes the balance between cellular proliferation and cell death very important. We found that downregulated DEMs were significantly associated with cell cycle arrest and programmed cell death, including apoptosis, while cellular division and the cell cycle were among the enriched pathways for the gene targets of upregulated DEMs. As miRNAs inhibit the translation of their mRNA targets, we anticipated that these identified changes to the miRNA expression profile of IECs after alcohol and burn injury would consequently promote cell death while reducing proliferation and cellular adhesion.

To further elucidate which of these DEMs are most likely contributing to intestinal dysfunction after alcohol and burn injury, we utilized two separate methods to assess correlated changes in the expression of DEMs and their gene targets. From this, we identified numerous interactions related to regulation of gut barrier integrity, including interactions which would reduce cellular proliferation and adhesion while increasing inflammation. Ultimately, our findings indicate that altered miRNA expression after alcohol and burn injury likely contributes to intestinal barrier disruption by hindering IEC adhesion processes, reducing proliferation, and promoting cell cycle arrest and apoptosis.

Following the acute phase of injury, burn patients must content with systemic and chronic hypermetabolic responses for up to years after injury. This hypermetabolic state is characterized by increased energy expenditure and metabolic rates, full body catabolism, muscle protein degradation, elevated adipose lipolysis, and insulin resistance[223–225]. Studies suggest this process is mediated by increases in both plasma catecholamine and corticosteroid levels and elevated system pro-inflammatory mediators[12,223,226]. It is also known that increased inflammation following chronic alcohol consumption negatively impacts insulin signaling, thereby also contributing to increases in metabolic processes[33,34]. Although both burn injury and chronic alcohol consumption promote hypermetabolism, there are few studies which explore the impact of combined alcohol intoxication and burn injury on metabolic outcomes. One recent study revealed significant alterations in metabolism after combined insult, including hyperglycemia and changes in serum insulin, glucagon and gastrointestinal hormones in alcohol and burn mice compared to sham or burn alone[227]. In our study, almost all KEGG pathways significantly associated with upregulated genes in IECs after alcohol and burn injury were metabolic in nature. Taken together, these findings suggest that intestinal hypermetabolism after alcohol and

burn injury is a major component of the systemic and chronic hypermetabolic state observed in burn patients. The gastrointestinal tract is the initial sight of nutrient absorption and metabolism in the body. Proper metabolic function of intestinal epithelial cells is critical for both gut homeostasis and barrier function. For example, glucagon-like peptide-2 (GLP-2) is a hormone produced in the gastrointestinal epithelium that is important for nutrient absorption, lipid metabolism and energy homeostasis. However, studies also show GLP-2 promotes intestinal barrier integrity by stimulating IEC proliferation, inhibiting apoptosis, and increasing tight junction protein expression[228,229]. In addition to the levels of a variety of nutrients, lipid and bile acid metabolism have also been shown to impact gut barrier function and inflammation[230–233]. In the last decade, miRNAs have emerged as crucial regulators of metabolism in the liver, pancreas, muscle, and adipose tissue. Dysregulation of miRNA expression has been linked to several metabolic disorders, including obesity and diabetes[234,235]. More recent studies demonstrate a clear relationship between intestinal epithelial cell metabolism and miRNAs. Small intestinal miRNA expression is significantly altered by high fat diet and excessive lipid exposure[235,236]. Furthermore, a study utilizing a mouse model of intestinal Dicer-1 knockout showed that disruption of intestinal miRNA expression results in changes to lipid absorption and accumulation[237]. Other studies have confirmed extensive regulation of lipid metabolism by intestinal miRNAs[238]. Although research exploring miRNA mediated regulation of intestinal metabolism in the context of alcohol and burn injury is lacking, our findings highlight intestinal epithelial cell metabolism as an important network impacted by miRNA changes after alcohol and burn injury. In accordance with the repressive nature of miRNAs, we found metabolic pathways (lipid biosynthesis, cellular respiration, energy derivation by oxidation of organic compounds, carbohydrate and protein metabolism) enriched among the miRNet derived

validated gene targets of our downregulated DEMs. When we integrate gene expression data from RNA sequencing and extract the validated gene targets which also exhibit differential expression, the resulting network also exhibited significant association with regulation of metabolic processes. This association with metabolism was further demonstrated in a separate integrated analysis which integrated both validated and predicted gene targets of DEMs with RNA sequencing data. Individual assessment of each DEM identified miR-674-3p and miR-185-5p as critical downregulated miRNAs with upregulated gene targets associated with lipid and protein metabolism. Future studies investigating the mechanism by which these miRNAs influence IEC metabolism after alcohol and burn injury could provide new insights into their impact on gut barrier integrity.

Final Conclusions

Although we've only begun to lay the foundations, these studies address an important gap in our current understanding of the mechanisms underlying intestinal dysfunction in patients exhibiting alcohol intoxication at the time of burn injury. Our studies identify numerous miRNAs which are crucial regulators of intestinal homeostasis and highlight their potential contributions to intestinal dysfunction after alcohol intoxication and burn injury. In particular, we demonstrate significantly reduced expression of miR-146a in intestinal epithelial cells one day following the combined insult and elucidate the mechanism by which decreased intestinal epithelial cell miR-146a expression perpetuates intestinal inflammation and potentially impacts gut barrier integrity. Future studies will continue to illuminate the role of altered miRNA expression in gut dysfunction and the therapeutic potential of harnessing these important miRNAs to reduce intestinal inflammation and improve outcomes after alcohol intoxication and burn injury.

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