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Role of Micrornas in Impaired Gut Permeability Following Ethanol and Burn Injury

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ROLE OF MICRORNAS IN IMPAIRED GUT PERMEABILITY FOLLOWING ETHANOL AND BURN INJURY

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ago	Argonaute
AMPs	Antimicrobial Peptides
ANOVA	Analysis of Variance
BSA	Bovine Serum Albumin
CFU	Colony Forming Units
DAPI	(4’,6-Diamidino-2-Phenylindole, Dihydrochloride)
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DIM	Diindolylmethase
DSS	Dextran Sodium Sulfate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GI	Gastrointestinal
HDAC	Histone Deacetylase
HBSS	Hank’s Balanced Salt Solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIF	Hypoxia Inducible Factor
HRP	Horseradish Peroxidase

IEC	Intestine Epithelial Cells
IL	Interleukin
I.P.	Intraperitoneal
I/R	Ischemia/Reperfusion
LPS	Lipopolysaccharide
miR	microRNA
miRISC	miRNA-Induced Silencing Complex
MODs	Multiple Organ Dysfunction
MOF	Multiple Organ Failure
ODDD	Oxygen Dependent Degradation Domain
PACT	Protein Activator of PKR
PBS	Phosphate Buffered Saline
pri-miR	Primary microRNA
pre-miR	Precursor microRNA
pVHL	von Hippel Lindau
PVDF	Polyvinylidene Difluoride
qPCR	Quantitative Real Time Polymerase Chain Reaction
RhoB	Ras Homolog Gene Family, Member B
RPMI	Roswell Park Memorial Institute Medium
RNA	Ribonucleic Acid
RNAi	RNA Interference
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEM	Standard Error of the Mean
TBSA	Total Body Surface Area
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline-Tween 20
TRBP	Trans-Activation Response RNA-Binding Protein
XRN1	Exoribonuclease 1
XRN2	Exoribonuclease 2
UTR	Untranslated Region
YAMC	Young Adult Mouse Colonocytes
ZO	Zonula Occludens

CHAPTER ONE

INTRODUCTION

Every year there are nearly 500,000 reported burn injuries in the United States, half of which occur under the influence of alcohol[1-3]. Clinical studies have shown that burn patients who are intoxicated at the time of injury have a worse prognosis[4,5]. Patients intoxicated at the time of injury have increased susceptibility to infection, exhibit delayed wound healing, and have longer hospital stays[4,5]. Furthermore, patients who are intoxicated at the time of burn injury have elevated risk of developing multiple organ failure (MOF) and sepsis. Intoxicated patients also have significantly higher mortality rates[2,3,6,7]. The etiology of these pathological consequences of ethanol and burn injury remains to be elucidated. The Gut-lymph hypothesis of multiple organ dysfunction (MODs) theorizes that trauma (e.g. ethanol and burn injury) results in a redistribution of blood flow to protect more vital organs which leads to ischemia/hypoxia (diminished oxygen delivery) in the intestines[8]. This contributes to the impaired intestinal barrier function which could lead to sepsis and MODs.

An intact intestinal barrier is particularly important as the intestines are the largest reservoir of bacteria within the human body. Any breakdown of this barrier could potentially lead to translocation of bacteria or bacterial endotoxins resulting in systemic infection and inflammation. Studies from our laboratory provide support that there is a breakdown in the intestinal barrier following ethanol and burn injury[9,10]. Furthermore, we observed that ethanol and burn injury leads to an increase in intestinal inflammation (IL-6, IL-18 and KC), hypoxia,

microbial dysbiosis (increased total bacteria and *Enterobacteriaceae*) and modifications of some other cellular processes (e.g. apoptosis, proliferation, and mucins)[11-14]. These changes could negatively impact the intestinal barrier, leading to the pathology following the combined insult of ethanol and burn injury. Ultimately, the major goal of our studies is to determine the underlying mechanism by which ethanol and burn injury impairs the intestinal barrier. Major cellular regulators-microRNAs (miRs) likely have a role in adverse effects following ethanol and burn injury. Therefore, the mechanism underlying the altered intestinal cellular responses and the role that miRs play in these processes remains the major focus of our current studies.

microRNAs are small noncoding RNAs sequences that control gene expression at the post-transcriptional level[15,16]. They mediate their gene silencing ability by complementary binding to the 3' Untranslated Region (UTR) of their target. Binding of the miR to the 3'UTR results in either mRNA degradation or translational repression of their target[15-17]. miRs are estimated to regulate nearly 60% of the genome, therefore, alterations in their expression could potentially affect thousands of genes[18,19]. To function as mature microRNAs, the small RNAs undergo two processing events: nuclear cleavage by drosha and cytoplasmic cleavage by dicer. Argonaute is a main component of the miRNA-Induced Silencing Complex (miRISC) which mediate microRNA gene silencing[15-17]. Alterations in expression of drosha and dicer, microRNA transport protein (Exportin-5) and the miRISC component (argonaute) may lead to diminished microRNA processing resulting in elevated intestinal inflammation and diminished intestinal barrier function. microRNA levels and biogenesis are affected by a number of factors including trauma/injury, ethanol, and disease[20-26]. The role of microRNAs following ethanol and burn injury, however, remains largely unknown.

This dissertation focuses on examining whether hypoxia following ethanol and burn injury modulates expression of microRNA biogenesis components and microRNAs resulting in increased inflammation and intestinal barrier disruption. The central hypothesis is that, hypoxic insult following acute ethanol intoxication and burn injury disrupts microRNA biogenesis resulting in decreased miR-150 expression in isolated small intestinal epithelial cells and increased intestinal permeability *in vivo*. To test this hypothesis, we developed three specific aims. **Specific Aim 1:** Profile microRNA biogenesis components following ethanol and burn injury in murine cells isolated from the terminal small intestine. **Specific Aim 2:** Determine whether hypoxia modulates microRNA biogenesis and if inhibition of hypoxia inducible factor (HIF)-1α restores microRNA biogenesis components, miR-150 expression in epithelial cells isolated from the terminal small intestine resulting in increased intestinal barrier integrity. **Specific Aim 3:** Evaluate whether miR-150 alters inflammation in the young adult mouse colonocytes *in vitro* which results in impaired paracellular permeability.

In order to determine the role of hypoxia on microRNA biogenesis and intestinal barrier function we administered PX-478 a HIF-1α inhibitor via intraperitoneal (I.P.) injection at the time of resuscitation. HIF-1α expression was measured in small intestinal tissue sections by immunofluorescence. Enzyme-Linked Immunosorbent Assays (ELISA) were used to quantify the presence of inflammatory mediators including IL-6, KC, IL-18 in small intestinal tissue. Intestinal epithelial cells (IECs) were isolated and used to evaluate drosha, dicer, and argonaute-2 by quantitative real-time polymerase chain reaction (qPCR) and western blot. Furthermore, IECs were used to measure expression of tight junctions, mucins, and microRNAs by qPCR. Additionally, microbiome alterations were assessed by 16S rRNA by qPCR for total bacteria and *Enterobacteriaceae*. Together these studies will elucidate the role of HIF-1α in intestine barrier

disruption following the combined insult which may lead to a potential therapeutic option for patients intoxicated at the time of burn injury.

CHAPTER TWO

REVIEW OF RELATED LITERATURE: ROLE OF MICRORNAS ON THE INTESTINAL BARRIER AND MICROBIOME

Ethanol and Burn Injury

Alcohol (ethanol) abuse remains a major economic and health problem. In 2010, excessive alcohol misuse cost the United States nearly 250 billion dollars[27]. Furthermore, each year three million people die worldwide from alcohol abuse or complications associated with alcohol exposure. Alcohol is widely considered to be the fourth ranking preventable cause of death[28]. Alcohol abuse also increases risky behaviors which enhances the likelihood of incurring traumatic injuries (e.g. burn injuries).

Nearly 500,000 burn injuries are reported each year in the United States, resulting in approximately 40,000 hospitalizations and 4000 deaths[1]. Approximately 50% of these injuries occur under the influence of ethanol[1-3]. Alcohol intoxication at the time of burn injury delays wound healing, results in longer hospitalization, and increases susceptibility to infection compared to patients who did not consume alcohol prior to burn injury[4,5]. Patients who are intoxicated at the time of burn injury also have an increased risk of developing MOF and sepsis. Additionally, intoxicated patients have higher mortality rates and die from smaller burn injuries[2,3,6,7].

Studies carried out in animal models suggest that the adverse effects seen in patient studies may arise from gut barrier disruption following ethanol and burn injury[10,29]. Furthermore, ethanol and burn injury increases intestinal inflammation which can contribute to

barrier disruption[9-14,30,31]. However, the mechanism behind these observed effects following ethanol and burn injury have not been elucidated. Therefore, the central goal of our laboratory is to decipher the mechanism by which ethanol exacerbates post-burn pathogenesis and determine a therapeutic option for implementing treatment following ethanol and burn injury.

The Gastrointestinal (GI) tract performs multiple functions including digestion, and nutrient absorption, as well as maintaining an interface to prevent the translocation of bacteria living in the GI tract[32,33]. Constant exposure to antigens from the diet and commensal bacteria (up to 10^{12} microorganisms per gram of tissue), requires the gut to employ physical and immunological defense barriers which limit luminal bacteria translocation[33-38]. As shown in Figure 1, the intestine forms a semi-permeable barrier composed of a single layer of columnar epithelial cells that are sealed by tight junction proteins. While the majority of columnar epithelial cells are absorptive enterocytes, there are other cell types (e.g. goblet cells, M cells, and Paneth cells) that participate in intestinal defense[33,34,39]. Additionally, the intestine contains a mucus barrier composed of mucins secreted by goblet cells which prevents the luminal bacteria from adhering to the epithelial cells[34]. Paneth cells contribute to gut homeostasis by producing a large number of antimicrobial peptides (AMPs). The immune component of the intestine includes a layer of loose connective tissue (lamina propria) beneath the epithelial cells and intestinal lymphoid tissue called Peyer's patches[33,40]. Together, these components maintain intestinal homeostasis by providing a physical and immunological barrier. Disruption in any of these barrier functions due to disease, misuse of alcohol or trauma (e.g. burn injury) may compromise the barrier integrity resulting in increased intestinal permeability[9,41]. Major cellular regulators such as microRNAs may have a role in intestinal barrier integrity.

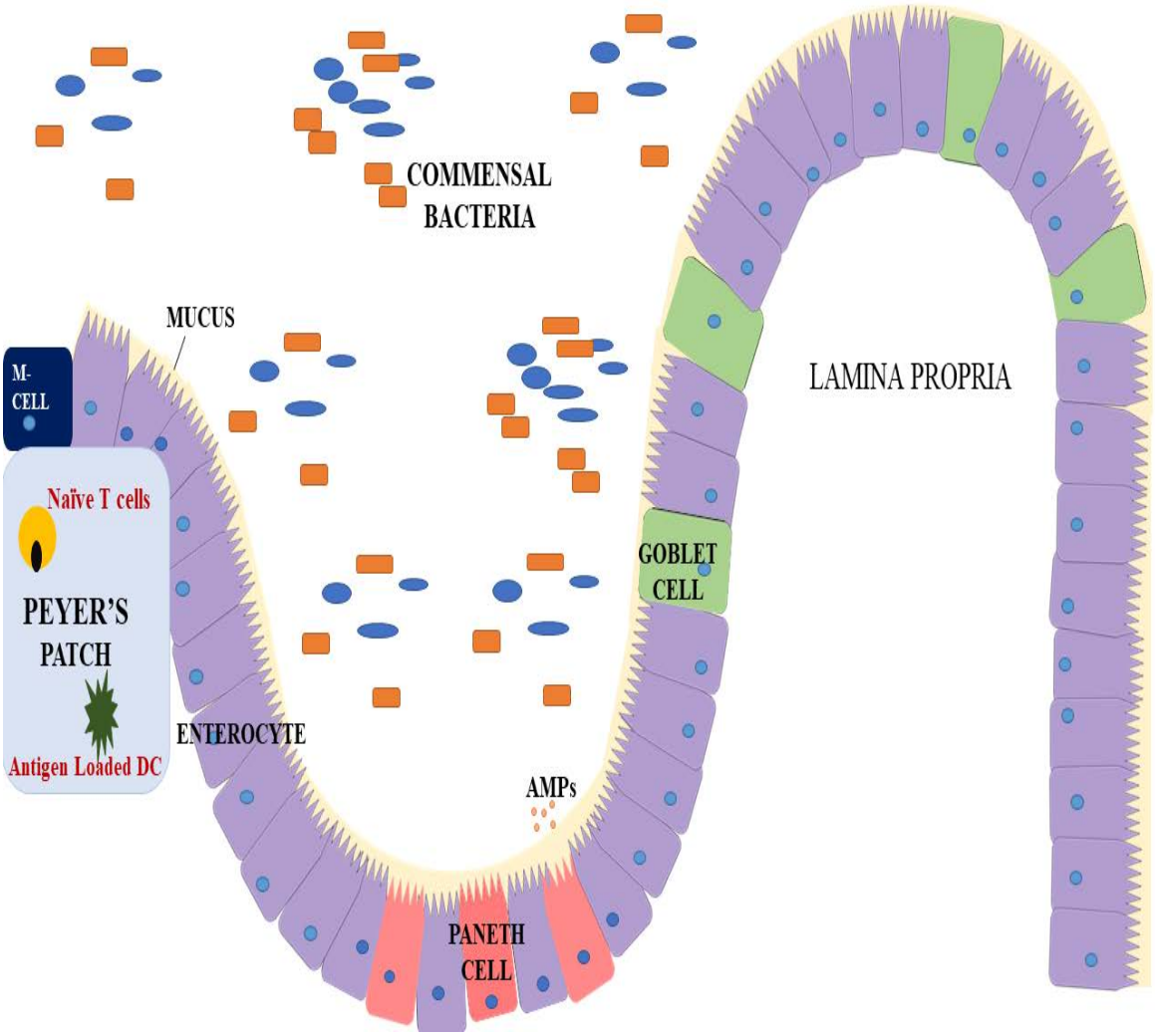

Figure 1. Overview of Intestinal Barrier Components and Microbiome. The intestinal barrier consists of a single layer of columnar epithelial cells (enterocyte) that are sealed together by tight junction proteins which aids in limiting luminal bacteria (orange and blue) translocation between adjacent cells. The columnar epithelial cells are encased in a mucus layer (yellow) that are made by mucin secreting goblet cells (green). The immunological component of the intestinal barrier comprises of Paneth cells (pink) that are located at the base of the intestinal crypts which secrete AMPs. The lamina propria is an area of connective tissue directly below the layer of enterocytes, which is rich in immune cells which allows for a rapid immune response to invading pathogens. M cells (Dark blue) permit passage into the Peyer's patches (light blue).

Introduction to microRNAs

microRNAs are excellent candidates for providing a mechanism behind many of the outcomes resulting in impaired gut barrier integrity following ethanol and burn injury as they control many cellular processes (e.g. apoptosis/proliferation, tight junction proteins and

inflammation). miRs are small noncoding RNAs which control gene expression at the post-transcriptional level[15,16,42]. They bind to the 3' UTR region of their target mRNA resulting in gene silencing[15,16,42]. microRNAs must undergo a maturation process in order to function as gene silencers. microRNAs are transcribed by RNA polymerase II forming a primary microRNA (pri-miR). The pri-miR undergoes a nuclear cleavage by a microprocessor composed of drosha and its co-factor DiGeorge syndrome critical region gene 8 (DGCR8). The nuclear cleavage by the microprocessor forms a 60–70 nucleotide precursor microRNA (pre-miR) which is exported into the cytoplasm by the ran-GTP dependent exportin-5. In the cytoplasm the pre-miR is cleaved by dicer and its cofactors trans-activation response RNA-binding protein (TRBP) and protein activator of PKR (PACT). The second cleavage forms a 21-24 nucleotide duplex microRNA consisting of a guide and passenger strand. The passenger strand is usually degraded whereas, the guide strand and an argonaute protein form miRISC. TRBP aids in the loading of the microRNA into the miRISC, as it functions as a sensor for the thermodynamically asymmetrical strand, the less thermodynamically stable paired 5' end is loaded onto the argonaute protein. The formation of the miRISC permits imperfect base pairing binding of the seed region of the microRNA to its target resulting in mRNA degradation or translational repression (Figure 2)[15-17].

microRNAs are estimated to target over 60% of all genes and each miR can have multiple target genes[18,19]. Aberrant microRNA expression can impair normal organ function including the intestinal barrier function. Villin-specific dicer knockout mice exhibited altered intestinal morphology, decreased differentiation, increased intestinal inflammation and apoptosis. Furthermore, ablation of dicer-1 led to diminished expression and mislocalization of tight junction proteins[43,44]. These observations coincided with diminished barrier integrity

providing evidence of the indispensable role of microRNAs in the intestine and demonstrate the need to explore these molecules following ethanol and burn injury.

microRNA levels and biogenesis are affected by a number of factors including trauma/injury, ethanol, and disease[20-26]. Furthermore, as microRNAs are ubiquitously expressed in both circulation and tissues their differential expression can be used as diagnostic tools and for possible therapeutic interventions[24,25,45]. This review will summarize the current knowledge on the role of microRNAs in the maintenance of intestinal barrier integrity and discuss the gap in knowledge in the area of microRNAs and their role in pathology associated with ethanol and burn injury.

Drosha.

The endonucleolytic enzyme drosha is an essential component of canonical RNA interference (RNAi). Drosha recognizes and cleaves secondary stem-loop structures, which allows it to have microRNA independent functions and the ability to influence expression of other types of RNAs (e.g. mRNAs, pre-rRNAs, and DNA damage induced RNAs). Cleavage of its co-factor DGCR8 was identified as drosha's first non-microRNA dependent function[46]. Drosha has been shown to cleave DGCR8 mRNA resulting in its destabilization. This relationship is likely a mechanism of autoregulation of the microprocessor. Furthermore, drosha's ability to process other RNAs has been demonstrated to control cell cycle. Knockdown of drosha led to an inability to progress the cell cycle which resulted in stalling the cell in the G1 phase due to the accumulation of pre-rRNAs. Interestingly, however, knockout of drosha did have any effect of rRNA levels[46].

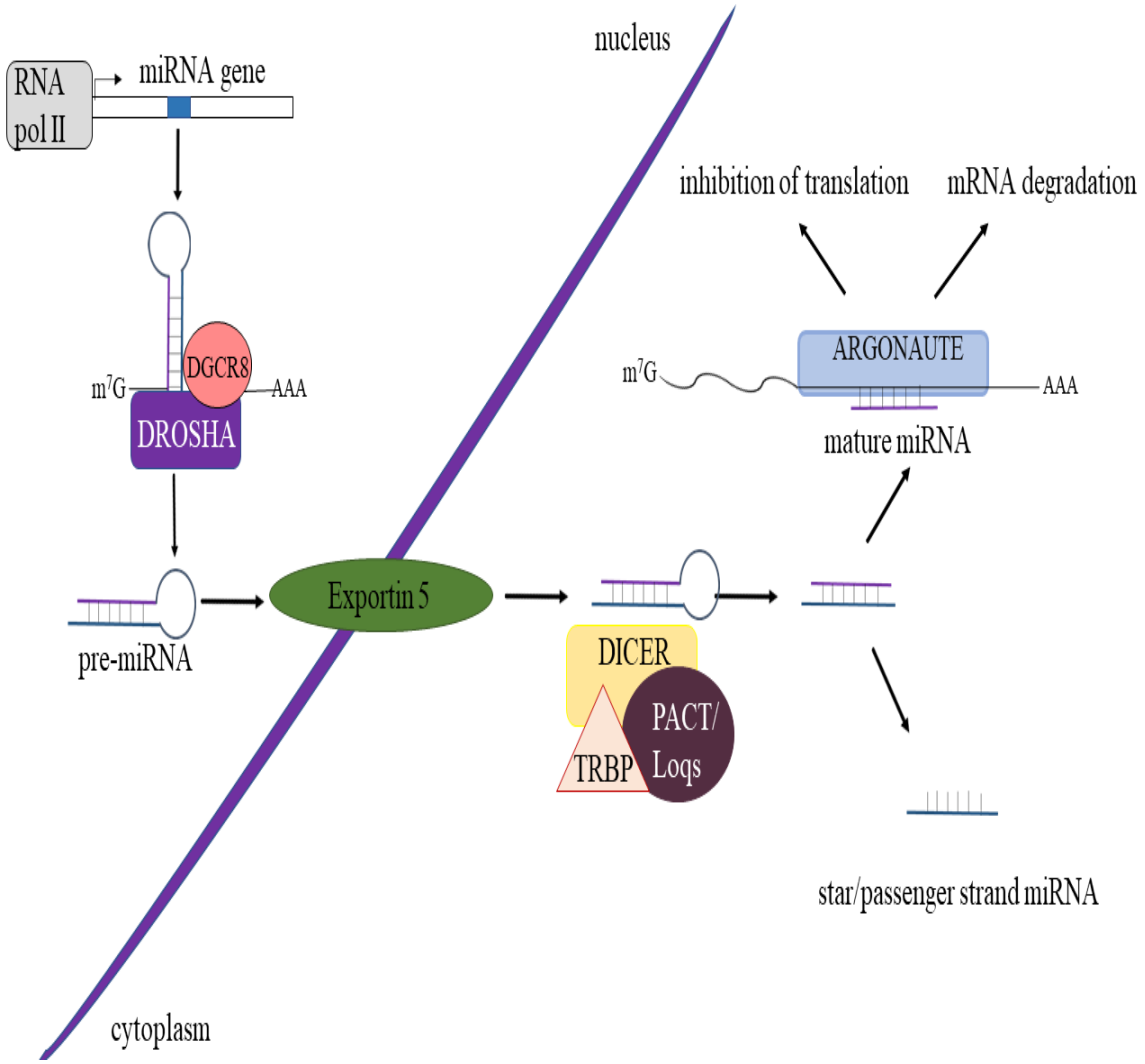

Figure 2. microRNA Biogenesis. microRNAs are transcribed by RNA polymerase II forming a pri-miRNA. The pri-miR undergoes its first cleavage in the nucleus by the microprocessor consisting of the protein drosha and its co-factor DGCR8. Nuclear cleavage by the microprocessor forms a pre-miRNA. The pre-miRNA is translocated from the nucleus into the cytoplasm by exportin-5. Once in the cytoplasm the pre-miRNA undergoes cleavage by dicer and its co-factors TRBP and PACT/loqs forming a duplex structure. The duplex formed from dicer cleavage contains a passenger strand which is usually degraded and a mature miRNA strand. The mature miRNA strand is loaded onto argonaute forming the miRISC where the mature microRNA can mediate its gene silencing ability (inhibition of translation or mRNA degradation).

Dicer.

Knockout of dicer is embryonically lethal in mice[47]. Furthermore, tissue-specific knockout of dicer has been shown to lead to developmental defects and organ dysfunction[47,48].

Villin-specific dicer knockout resulted in elevated inflammation, cell death, and decreased differentiation. Furthermore, villin-specific knockout impaired intestinal morphology, and resulted in reduced expression and mislocalization of tight junction proteins[43,44]. These studies suggest an indispensable role of dicer and resulting expression of microRNAs in normal functioning of the cell, tissue and organ. Dicer is an obligatory molecule in the siRNA and miRNA pathway in vertebrates. However, in addition to its role in RNA as a riboendouclease, dicer has been shown to process a number of other RNAs including dsRNAs, siRNA precursors, DNA-damage induced RNAs, SINE-derived RNAs, and triplet repeat RNAs[46]. Furthermore, in *C. elegans*, caspase CED-3 mediated cleavage of dicer showed that it has a DNase capacity where it binds and cleave one strand of dsDNA. This cleavage resulted in DNA degradation and apoptosis, however, it is not known if this occurs in other organisms[46].

Argonaute.

In humans, the argonaute proteins are a family of proteins consisting of argonautes 1-4. Ago-1, ago-3, and ago-4 are non-catalytic members of the argonaute family. Ago-2 is the only catalytic member of the argonaute family in mammals. They are highly specialized proteins that bind to small RNAs. Argonaute proteins play an essential role in microRNA mediated silencing as they are the core proteins of the miRISC complex. Various post-transcriptional modifications affect the function of the argonaute proteins. Additionally, ago-2 has been shown to protect single stranded microRNAs from degradation[49,50]. There is some evidence that the functions of argonautes may extend more than their role in microRNA mediated silencing. Interestingly, argonaute proteins have been found both in the nucleus and cytoplasm. In addition, to their role in RNAi, argonaute proteins have been shown to influence chromatin modification, alternative splicing and double strand break repair[51].

microRNAs and the Epithelial Layer

microRNAs are regulators of normal cellular homeostasis and numerous studies have demonstrated that they play a major role in intestinal barrier maintenance. microRNAs have been shown to affect apoptosis[52-57], proliferation[58-64], tight junction protein expression[23,65-71], ischemia/hypoxia[20,57,72], inflammation[20,73-82] and the microbiome[83] (Figure 3) all of which can impact the intestinal barrier function.

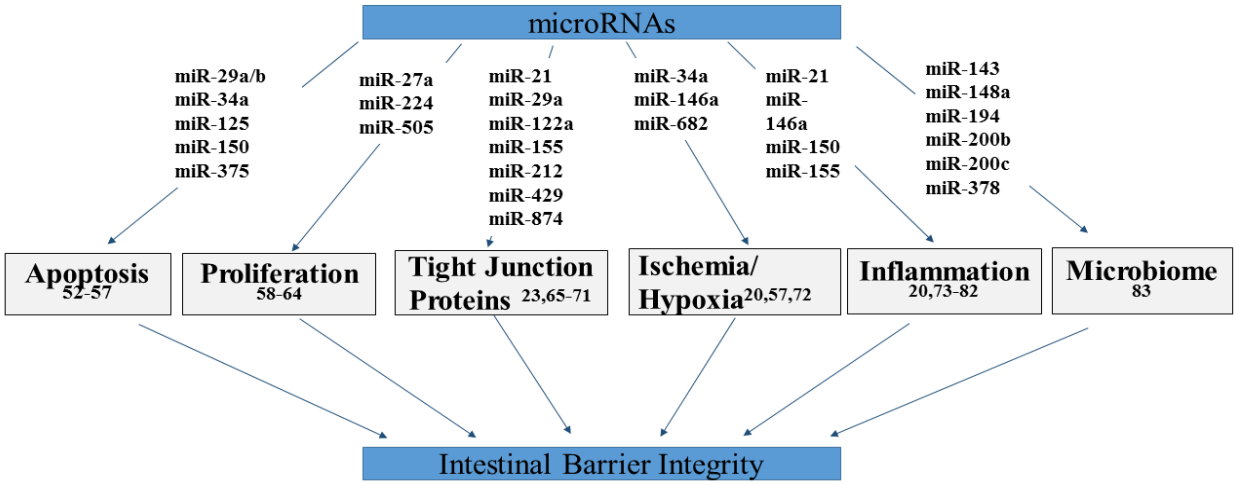

Figure 3. microRNAs in Intestinal Barrier Integrity. microRNAs can directly and indirectly affect intestinal barrier integrity by impacting apoptosis, proliferation, tight junction protein expression, ischemia/hypoxia, inflammation and microbiota composition in the intestine[20,23,52-83].

microRNAs Role in Apoptosis.

Apoptosis is a natural physiological process resulting in death and removal of unwanted cells[84-86]. Apoptosis is essential for normal maintenance of the GI tract and is required to counterbalance the constant cell turnover of proliferating cells[87]. Apoptosis therefore, is crucial to enable normal structure and function of the GI tract [86]. Increased levels of apoptosis beyond this normal cellular maintenance however, results in an impaired intestinal barrier[86]. It is widely accepted that increased intestinal epithelial cell apoptosis disrupts the intestinal barrier resulting in increased intestinal permeability.

In particular, our laboratory observed that ethanol and burn injury resulted in increased intestinal apoptosis[10]. The mechanism of this increased apoptosis following ethanol and burn injury is unknown but it is likely linked to microRNAs as many microRNAs have a role in intestinal apoptosis[52-54,56,57,72]. Our laboratory has demonstrated that ethanol and burn injury delays neutrophil apoptosis compared to sham injured animals[88,89] and increased intestinal epithelial cell death (Hammer *et al*. (2016) unpublished data), all of which could lead to tissue damage and impaired intestinal barrier function. Apoptosis can arise due to the intrinsic apoptotic pathway which results in the release of cytochrome c into the cytoplasm from the mitochondria. The release occurs after activation of pro-apoptotic proteins (e.g. Bax). Following the release cytochrome c, the apoptosome forms with procaspase-9 and apoptotic protease-activating factor 1. This process can be averted by anti-apoptotic proteins Bcl-2 which prevents cytochrome c release[86]. Although, it is established that ethanol and burn injury influences apoptosis, the mechanism remains elusive.

microRNAs can influence apoptosis by gene silencing members of the anti-apoptotic Bcl-2 family. miR-150 was significantly elevated in the dextran sodium sulfate (DSS) model of colitis in mice and in active ulcerative colitis in human colonic tissue which resulted in apoptosis by downregulation of c-Myb. Reduction of c-Myb led to decreased levels of the anti-apoptotic protein Bcl-2 which resulted in increased apoptosis[52]. In contrast, Christensen *et al*. observed that miR-150 did not alter cellular viability in certain colorectal cancer cell lines suggesting that the apoptotic ability of miR-150 may be cell type dependent[53]. Furthermore, miR-375 expression was decreased in patient samples and in multiple colorectal cell lines. miR-375 targeted Yes-associated protein 1 whose downstream targets are BIRC5 and BCL2L1 (also known as Bcl-xl) resulting in apoptosis[53].

microRNAs can also impact apoptosis by regulation of other anti-apoptotic proteins such as Mcl-1. miR-29a was increased e in a mouse model of DSS-induced ulcerative colitis which resulted in decreased levels of Mcl-1. *In vitro* analysis in colonic epithelial cells HT29 showed that miR-29a caused apoptosis by downregulation of its target molecule Mcl-1 which activated caspase-3[54]. As mRNAs can have numerous binding sites allowing for binding of different miRs it is not surprising that other miRs (miR-125 and miR-29b) have been shown to reduce Mcl-1 levels[55,56]. These studies illustrate that aberrant microRNA expression in the intestines can directly and indirectly target the apoptotic pathway. Furthermore, these studies suggest that microRNAs could play a role in increased small intestinal apoptosis following ethanol and burn injury.

microRNAs Role in Proliferation.

The intestinal epithelium is one of the most rapidly renewing tissues within the body. The intestine is renewed every 2-3 days in mice and 3-5 days in humans[90]. Intestinal epithelial stem cells develop into transit amplifying progenitor cells and differentiate into various subtypes of intestinal cells. Proliferation occurs in the crypts, allowing for differentiation and migration up the intestinal villi. Proliferation and apoptosis is tightly regulated in order to maintain the intestinal barrier function.

Burn injury alone or in combination with ethanol decreases intestinal proliferation. Furthermore, ki67 staining was significantly decreased following ethanol and burn injury compared to sham animals suggesting that the combined insult diminished intestinal epithelial cell proliferation[29]. microRNAs can provide insight into diminished proliferation following ethanol and burn injury as numerous studies suggest they have a role in cell cycle regulation[91,92]. As alterations of cell proliferation is a hallmark of cancer, the majority of work on microRNAs

and proliferation is performed in cancer models. Overexpression of miR-27a, and miR-505 influenced cell proliferation and invasion of colonic cancer cells[63,64]. Numerous studies have shown that miR-224 is increased in colorectal cancer patients[58-62]. *In vitro* studies using HCT-116 and SW-480 colon cancer cell lines demonstrated that upregulation of miR-224 increased proliferation, and cell migration while promoting cell cycle progression[58,59,61,62]. Furthermore, microRNAs can be used for prognosis and predictions of colorectal cancer relapse. microRNAs can increase proliferation and contribute to the development of colonic cancer[58,59,61,62]. As we observed a reduction in intestinal epithelial cells proliferation (Hammer *et al*. (2016). unpublished data), perhaps these microRNAs may have a role in decreased proliferation in the intestine following ethanol and burn injury. Thus, investigation into these microRNAs following the combined insult could potentially provide some insight into the mechanism underlying the reduction in proliferation.

microRNAs and Tight Junction Proteins.

Tight junction proteins play an indispensable role in maintenance of intestinal barrier integrity[34]. Tight junction proteins are major components of apical junctional complex which seal adjacent cells limiting paracellular flux[93-95]. Tight junction complexes forms a semi-permeable protein complex comprised of transmembrane, scaffold and adaptor proteins[34,39,94,96]. The Claudin family and occludin proteins make up the essential tetra-span transmembrane proteins[95]. Occludin is a 65 kDa protein widely believed to function in regulation of paracellular flux between intestinal epithelial cells[97]. Claudins and occludin proteins attach to adaptor proteins Zonula occludens (ZO) which anchor occludin and claudin proteins to actin cytoskeleton and adherens junction[39,93,95,96]. Formation of tight junction complexes is necessary to maintain the integrity of the intestinal barrier[34]. Numerous studies show that altered

expression of microRNAs (miR-21,miR-122a, miR-155, miR-429 and miR-874) can directly and indirectly modulate tight junction proteins and apical junctional complexes resulting in increased intestinal permeability[65-71].

Tight junction proteins (occludin, claudins-4, and 8) are significantly reduced following the combined insult of ethanol and burn injury compared to shams. Additionally, the leaky claudin, claudin-2 (that allows transport of water and ions) was significantly increased following ethanol and burn injury compared to sham injured animals[10,13] . Furthermore, phosphorylation of tight junctions (occludin and claudin-1) and expression of the adapter molecule ZO-1 was significantly decreased in the intestine following ethanol and burn injury compared to shams[10,30,31]. The role that microRNAs play in modulating these proteins following ethanol and burn injury requires more investigation as they have been shown to influence expression of many tight junction proteins.

miR-122a has been shown to directly target occludin resulting in degradation of its mRNA which negatively impacted intestinal barrier function[66,71]. Administration of 10^9 colony forming unit (CFU)/day of probiotics *L. rhamnosus Gorbach-Goldin* per day for four weeks ablated chronic ethanol exposure effects on intestinal permeability. The probiotic treatment reduced miR-122a expression resulting in increased occludin protein levels[71]. A similar study investigating the role of ethanol exposure and microRNAs showed that exposure of Caco-2 cells to 0.1 to 1% ethanol for three hours resulted in increased expression of miR-212 in a time and dose dependent manner which corresponded with decreased ZO-1 expression[23]. Reduced ZO-1 levels is associated with impaired intestinal barrier function[23,98]. Studies such as these are particularly important and demonstrate the need to investigative the relationship between microRNAs and tight junction protein expression in the context of ethanol and burn injury. The

relationship requires further examination as ethanol and burn injury diminishes both occludin and ZO-1 expression in the intestine[10,30,31]. Furthermore, as ethanol exposure resulted in similar deleterious effects on occludin and ZO-1 levels as observed following ethanol and burn injury in the gut perhaps these ethanol mediated effects are both being mediated by miRs.

microRNAs can target molecules which indirectly result in reduced tight junction expression. miR-21 was upregulated in both the colon and serum of patients with ulcerative colitis, which is associated with decreased expression of occludin, ZO-1, and Ras homolog gene family, member B (RhoB)[67]. Overexpression of miR-21 in Caco-2 cells resulted in a loss of both occludin, ZO-1, and RhoB protein levels while increasing epithelial permeability. The group showed that miR-21 influenced RhoB expression and that siRNA-mediated ablation of RhoB resulted in decreased occludin and increased permeability[67]. Similarly, occludin can be indirectly targeted by miR-874. *In vitro* overexpression of miR-874 resulted in increased paracellular permeability and bacterial translocation and diminished occludin and claudin-1 levels[69]. Although, it is not known whether RhoB is affected by ethanol and burn injury, the combined insult does diminish occludin and claudin-1 expression. These studies establish that microRNAs could directly or indirectly regulate tight junction protein expression following ethanol and burn injury.

microRNAs and the Pathophysiological Consequences of Ethanol and Burn Injury

microRNAs Role in the Microbiome.

The gut microbiota is a relatively constant microbe population comprising over 100 trillion organisms consisting of 1000 bacterial species[99,100]. These bacteria play an important role ranging from metabolism to developing the intestinal immune system. Alterations in the gut microbiome can lead to pathological conditions (inflammatory bowel disease, obesity and

diabetes)[100-103]. In particular, trauma (e.g. 20% burn injury or ethanol intoxication prior to burn injury) has the ability to alter the intestinal microbiome and increase gut bacterial load[13,104]. Numerous studies profiling microRNA expression utilizing either germ free or antibiotic treated mice show that the microbiota composition influences expression of microRNAs[83,105-108]. Germ free mice infected with the food born pathogen *Listeria monocytogenes* had reduced ability to clear the bacterial counts in multiple tissues compared to conventional (C57BL6/J) mice suggesting that the microbiota is protective against infection. Furthermore, microRNAs (miR-143, miR-148a, miR-194, miR-200b, miR-200c, and miR-378) were significantly decreased in conventional mice infected with *Listeria* which coincided with increased expression in several of their predicted targets. These changes were not observed in germ free mice infected with *Listeria*, with the exception of miR-378 which was significantly elevated 72 hours post infection[83]. As the microbiota/host relationship is so interconnected it is not surprising that host microRNAs can influence the microbiota composition[43].

The host can influence microbiota composition and growth through intestinal epithelial cell and intestinal epithelial +4 niche stem cells expressing cell derived microRNAs. These microRNAs are released via extracellular vesicles into the feces where they can enter bacteria and control bacterial growth and gene expression. It is unclear as to how the miRs enter bacteria and how the microRNAs are processed by the bacteria however, the fecal miRs could be binding to complementary binding site on bacterial genes. Furthermore, dysregulation of host derived microRNAs using mice with an intestinal epithelial cell deficiency in dicer expression resulted in gut microbiota dysbiosis exacerbating DSS induced colitis[43]. These studies display the essential symbiotic relationship between the host and the microbiota which in part is shaped by microRNAs. As ethanol and burn injury results in microbiota dysbiosis[13] it is possible that these

changes are being mediated by the microRNAs, alternatively the observed microbiota changes could be influencing host microRNA expression. These studies reveal that the interplay between the host and microbiota is mediated through microRNAs shaping the intestinal microbiota and the microbiota in turn modifies microRNA expression. This suggests, that the microbiota/microRNA crosstalk play an important role in cultivating our gut microbiota composition. Future studies are necessary to examine whether microRNA levels and microbiota dysbiosis and bacterial growth are interconnected following ethanol and burn injury.

microRNAs Role in Ischemia/Hypoxia.

Ischemia is a major consequence in the intestine following trauma and burn injury, where blood flow is redistributed to more vital organs resulting in hypoxia (diminished oxygen delivery) in the gut[8,109-112]. Elevated levels of hypoxia inducible factor (HIF-1α), a marker of hypoxia, in the gut has been associated with diminished intestinal barrier function (altered tight junction expression), cell death and inflammation. Our laboratory has demonstrated that ethanol exposure at the time of burn injury results in an ischemic condition in the gut due to a reduction in intestinal oxygen delivery[14].

HIF-1 is a transcription factor that induces expression of genes that enables cell survival during shifts of oxygen levels. There are three alpha isoforms (-1α, -2α and -3α) which share the same binding partner (HIF-1β). HIF-1α is ubiquitously expressed and provides a quick response to oxygen deficiency (2-24 hours) during periods of severe hypoxia (<0.1% O_2). HIF-2α has 48% amino acid homology with HIF-1α however, its expression is more prevalent in the carotid body, lung and endothelium and is active under milder hypoxic insult (<5%O_2) and remains active after 48-72 hours of hypoxia[113,114]. Finally, HIF-3α is believed to act as a negative feedback regulator for HIF-1.

Under normoxic conditions HIF-1α has a relatively short half-life (~5minutes) where it undergoes hydroxylation of two prolines and acetylation of a lysine on the oxygen dependent degradation domain (ODDD) which triggers the signaling pathway. These post-translational modifications triggers binding with von Hippel Lindau (pVHL) E3 ligase complex tagging HIF-1α, resulting in its degradation by the ubiquitin-proteasome pathway. However, under hypoxic conditions HIF-1α is stabilized and translocates to the nucleus and binds to HIF-1β where the complex binds to the HRE region of its targets where it regulates gene expression[113].

Hypoxia/ischemia influences both expression of microRNAs and microRNA biogenesis components[57,69,72,115-117]. Intestinal ischemia/reperfusion (I/R) injury leads to intestinal injury through increased inflammation, overproduction of reactive oxygen species and apoptosis. miR-34a is significantly upregulated following I/R. Knockdown of miR-34a was shown to increase Sirtuin-1 which reduced I/R related oxidative relative damage and apoptosis[57]. In a similar study, miR-682 mediated intestinal I/R injury by targeting phosphatase and tensin homolog. *In vivo* overexpression of miR-682 prior to I/R injury significantly reduced apoptosis and other effects of I/R injury[72]. Chassin *et al*., utilized a mouse model of I/R to show the connection between miR-146a and IRAK1 expression. The group demonstrated that increased IRAK1 due to I/R injury resulted in increased expression of the pro-inflammatory chemokine CXCL2, apoptosis and intestinal permeability[20]. *In vivo* induction of miR-146a via diindolylmethase (DIM) a miR-146a inducing agent or administration of miR-146a reduced I/R mediated inflammation and IRAK1 elevation. Furthermore, exposure of human intestinal tissues *ex vivo* to hypoxic conditions elevated IRAK1 which was attenuated with DIM treatment and reduced CXCL8 mRNA expression following lipopolysaccharide (LPS) stimulation[20]. These studies demonstrate the relationship between hypoxia and inflammation. As we observed significant

increases in both hypoxia and inflammation following ethanol and burn injury it would interesting to ascertain if the link between hypoxia and inflammation is due to microRNAs.

microRNAs Role in Inflammation.

Intestinal inflammation is a hallmark of intestinal pathology. Numerous disease conditions (e.g. ulcerative colitis, Crohn's disease, ethanol and burn injury) are associated with excess inflammation and exhibit modulation of microRNA biogenesis and/or microRNA expression[24,25,77,80,118,119]. Inflammation in the gut contributes to increased apoptosis, tissue damage and dismantling tight junction complexes. In particular, we observed an increase in IL-6, IL-18 and KC within 24 hours after ethanol and burn injury[12]. Increased IL-6 and IL-18 are linked to increased intestinal permeability by negatively impacting tight junction protein levels and functions[10,120]. Similarly, other models of ethanol and burn injury have illustrated that mice treated with IL-6 antibodies after the combined insult diminished intestinal morphological changes, bacterial translocation, and reestablished tight junction protein localization. While complete knockout of IL-6 using IL-6 knockout mice improved intestinal damage it did not improve bacterial translocation and tight junction protein localization suggesting complete loss of IL-6 is not advantageous following the combined insult[30]. KC is a neutrophil chemokine, which likely contributes to increased neutrophil infiltration observed following ethanol and burn injury. Similarly, IL-18 has been shown to delay neutrophil apoptosis, increase their O^{2-} production and neutrophil recruitment to the intestine following ethanol and burn injury compared to sham injured animals[88,109,121]. Elevated IL-18 altered permeability by decreasing the levels of tight junction proteins (occludin) and activation (phosphorylation) of claudin-1 and occludin following the combined insult compared to shams[10]. These findings suggest that intestinal inflammation following ethanol and burn injury is a major contributor of increased

intestinal permeability. Attenuating intestinal inflammation following ethanol and burn injury therefore could be instrumental in providing therapeutic treatments following the combined insult.

microRNAs can disrupt inflammatory pathways contributing to increased inflammation following disease and trauma. Our laboratory observed that ethanol and burn injury, significantly reduced drosha and argonaute-2 expression one day following the combined insult in small intestinal epithelial cells compared to sham animals. Diminished expression of microRNA biogenesis components correlated with reduced expression of miR-150. Overexpression of miR-150 in young adult mouse colonocytes (YAMCs) reduced LPS mediated inflammation (IL-6 and KC) compared to empty vector controls[81]. Other models have also demonstrated a similar relationship between miR-150 and inflammatory mediators[45,122-125]. The changes in microRNA biogenesis and miR-150 are particularly important as miR-150 levels was reduced in plasma from sepsis patients a major adverse effect following ethanol and burn injury[45].

Immunomodulatory miRs (miR-21, miR-146a and miR-155) have been shown to be overexpressed in patients with inflammatory bowel disease, *vibrio cholera* infection, and acute intestinal obstruction[73,75,77,80,82]. Knockout of immunomodulatory miR-21 resulted in a reduction in intestinal inflammation (TNF-α and MIP-2) in a model of DSS induced colitis while improving survival[75]. Several studies have implicated miR-155 and miR-146a as negative feedback regulators of the inflammatory response modulating signal molecules in the NFκB pathway[20,73,74].

Our laboratory investigated whether miR-155 influenced splenic T cell release of IFN-γ in a murine model following acute ethanol exposure prior to burn injury. We observed a

significant reduction in miR-155 in T cells following ethanol and burn injury compared to sham injured animals. Furthermore, we observed that there was no difference in *ex vivo* T cell release of IFN-γ between miR-155 knockout mice and wild type mice one day following ethanol and burn injury[76]. These studies suggest that the immunomodulatory role of miR-155 in the gut may be cell type specific. Interestingly, miR-146a and miR-155 are upregulated in inflammatory bowel disease and *vibrio cholera* infection[77,78,80], suggesting that these microRNAs are not involved in the hyperinflammatory response associated with the diseases, but may have a role in the resolution of the disease state. Chronic ethanol exposure (Lieber-DeCarli diet for five weeks) significantly elevated miR-155 while significantly reducing the AMP, Reg3β. Knockout of miR-155 in the presence of chronic ethanol intoxication, reduced ethanol mediated increases in serum endotoxins levels, NFκB activation and inflammation (TNF-α) in the small intestine[79]. In contrast, acute ethanol exposure (5 g/kg ethanol in water for three days) resulted in elevated AMPs but did not alter miR-155 expression or TNF-α protein levels however it significantly increased NFκB activation[79].

These studies demonstrate that microRNAs are particularly important in regulating the inflammatory response in the gut. Interestingly, atypical microRNA expression may be both protective to the gut or contribute to the pathology of the disease. As inflammation is paramount for intestinal barrier dysfunction following ethanol and burn injury more investigation is required to elucidate the relationship between microRNAs in ethanol and burn injury mediated inflammation.

Conclusions

Collectively, the findings in this review exemplify the role of microRNAs in the regulation of intestinal barrier function. These studies demonstrate that microRNAs can directly

and indirectly affect intestinal apoptosis, proliferation, tight junction protein expression, ischemia/hypoxia, inflammation and microbiota composition (Figure 3). There are studies that have investigated the role of ethanol or trauma including burn injury on microRNA expression in other organ systems. There is however a big gap in research examining how microRNAs can be influenced or influence ethanol's effects on trauma particularly in the context of burn injury in the gut. Furthermore, aberrant microRNAs can be further exploited as microRNAs could be used as biomarkers or for therapeutic design.

CHAPTER THREE

REGIONAL EFFECTS OF ETHANOL AND BURN INJURY ON THE EXPRESSION OF PRO-INFLAMMATORY MEDIATORS[12]

Abstract

The intestinal tract is the second largest immune organ that is segmented into separate and functionally distinct parts. The intestines consist of the duodenum, jejunum, ileum and colon. These compartments have different functions and bacterial content. The current study evaluated whether the inflammatory response varied throughout the intestinal tract following ethanol and burn injury. Male (C57BL/6) mice were gavaged with ~2.9 g/kg of ethanol four hours prior to receiving a ~12.5% total body surface area (TBSA) full thickness burn. Mice were euthanized days one, three and seven after the insult. Following euthanasia, intestinal tissue segments (duodenum, jejunum, ileum, and colon) were collected. The total tissue was homogenized, and inflammatory mediators were analyzed using their respective ELISAs. The pro-inflammatory mediators (IL-6, IL-18 and KC) exhibited differential expression levels throughout the intestinal tract. We observed significant increases in KC levels in the jejunum, ileum and colon following the combined insult of ethanol and burn injury compared to sham injured animals. Furthermore, the KC levels were ~30-fold higher in the colon as compared to ethanol and burn injury in the duodenum. Additionally, both IL-6 and IL-18 levels were significantly elevated following ethanol and burn injury in the ileum and colon compared to shams. There was a ~7-fold increase in IL-6 levels in the distal intestinal tract (colon) compared

to the duodenum following ethanol and burn injury. Similarly, IL-18 levels were ~1.5-fold higher in the colon compared to the ileum following the combined insult of ethanol and burn injury. Collectively, these data suggest that ethanol and burn injury differentially affects the distinct compartments of the intestinal tract.

Introduction

The intestine is the second largest immunological organ consisting of functionally discrete compartments – duodenum, jejunum, ileum, and colon. Additionally, the intestine is the largest bacterial reservoir within the body. Collectively, the intestine is responsible for nutrition absorption and maintaining an interface to prevent gut bacterial translocation[33]. Regions of the intestine (duodenum, jejunum, ileum, and colon) are functionally distinct and contain regional variations in antigen-presenting cells and bacterial content[33,126,127]. It is well established that the intestinal bacterial content increases progressively from the duodenum to the colon, where the colon contains the largest bacterial population[128,129]. Both ethanol exposure and burn injury alone perturb intestinal structural and functional integrity [8,130,131]. Similarly, ethanol combined with burn injury has been demonstrated to cause increased intestinal permeability and bacterial translocation[9,10,30,132]. Previously, our lab showed that acute ethanol exposure prior to burn injury results in significantly increased levels in pro-inflammatory mediators in the terminal ileum[11,133]. The present study examined whether ethanol combined with burn injury differentially influences the expression of pro-inflammatory mediators in various parts of the intestine (duodenum, jejunum, ileum and colon).

Materials and Methods

Animals.

8–10-week-old male C57BL/6 mice (22–25 g) were obtained from Harlan Laboratories

(Indianapolis, IN). Mice were housed and acclimated for two weeks prior to experimentation. All animal procedures were conducted in accordance with the Animal Care and Use Committee at Loyola University Chicago Health Sciences Division.

Mouse Model of Ethanol Intoxication and Burn Injury.

Mice were randomly divided into four groups: sham vehicle (n =10–12), sham ethanol (n= 10–12), burn vehicle (n =5–8), and burn ethanol (n=6–8). As described previously, ethanol- or water-treated mice were gavaged with 0.4 mL of 25% ethanol in water (~2.9 g/kg) or water, respectively[11]. Four hours after the gavage, mice were anesthetized by I.P. injection with a cocktail of ketamine and xylazine (80 mg/kg and 1.25 mg/kg, respectively). The dorsal surface was shaved, and mice were transferred to a template, which is fabricated to expose ~12.5% of the TBSA. Mice in the burn group were immersed in 85–90°C water for 7–8 seconds. The mice were then dried and resuscitated with an I.P. injection of 1.0 mL physiological saline[11] before being returned to their cages and given water and food *ad libitum*.

Tissue Harvesting.

Groups of mice were euthanized one, three and seven days after injury. The duodenum, jejunum, ileum, and colon were removed and immediately transferred into liquid nitrogen.

Preparation of Tissue Homogenates.

For the measurement of inflammatory mediators, tissue from the various groups was sonicated on ice in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). Homogenates were cleared by centrifuging at 9,000 RPM at 4 °C for 30 min and stored at −80 °C.

Measurement of Cytokines in Tissue Homogenates.

IL-6, IL-18, and KC levels in tissue homogenates were determined by ELISA kits

according to the manufacturer's instructions. Mouse IL-6 and KC ELISA kits were purchased from R&D Systems (Minneapolis, MN), and mouse IL-18 ELISA kit was purchased from eBioscience (Santa Clara, CA).

Statistical Analysis.

Data is presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using wherever applicable Analysis of Variance (ANOVA) with Tukey-Kramer's post-hoc test or student's t-test. (GraphPad Prism 7 Software, La Jolla, CA). A $p < 0.05$ was considered statistically significant.

Results

We observed no significant changes in IL-6 in the duodenum and jejunum following the combined insult of ethanol and burn injury compared to animals in the sham vehicle group (Figures 4A-B). Consistent with our previous findings[11], IL-6 levels were significantly elevated in the ileum day one following burn injury alone compared to the shams groups (Figure 4C). Similarly, IL-6 was significantly increased day one after the combined insult of ethanol and burn injury in the ileum compared to the sham groups. We did not however, observed any significant changes between the burn vehicle and burn ethanol groups in the ileum (Figures 4C). IL-6 levels were significantly elevated in the colon day one after the combined insult of ethanol and burn injury compared to all groups (Figure 4D). We did not detect any changes in IL-6 levels days three and seven following the combined insult of ethanol and burn injury in the duodenum, jejunum, ileum or colon compared to the sham vehicle group (Figure 4).

Next, we evaluated IL-18 levels days one, three and seven following the combined insult of ethanol and burn injury. IL-18 was of particular interest as it is a pro-inflammatory cytokine associated with intestinal tissue damage[89,134]. We observed no changes in IL-18 levels in the

duodenum or jejunum following ethanol and burn injury compared to animals in the sham vehicle group (Figures 5A-B). IL-18 levels were significantly increased in the ileum and colon after ethanol and burn injury compared to all groups (Figures 5C-D). We did not find any changes in IL-18 levels days three and seven following ethanol and burn injury in the duodenum, jejunum, ileum or colon compared to the sham vehicle group (Figure 5).

To examine whether chemokine levels were also differentially affected in the GI tract following the combined insult of ethanol and burn injury, we analyzed KC levels in the intestine tissues days one, three and seven after ethanol and burn injury. Similar to cytokine levels (IL-6 and IL-18), we determined that KC exhibited differential expression throughout the GI tract. KC levels were not changed in the duodenum following ethanol and burn injury compared to the sham vehicle group (Figure 6A). Burn injury alone significantly elevated KC levels in the ileum and colon compared to sham vehicle animals (Figures 6C-D). Furthermore, KC levels were further elevated following ethanol and burn injury in the jejunum and ileum day one following the combined insult of ethanol and burn injury compared to the sham groups (Figures 6B-C). KC levels were significantly higher in the colon compared to all groups tested (Figure 6D). We did not detect any changes in KC levels days three and seven following ethanol and burn injury in the duodenum, jejunum, ileum or colon compared to the sham vehicle group (Figure 6).

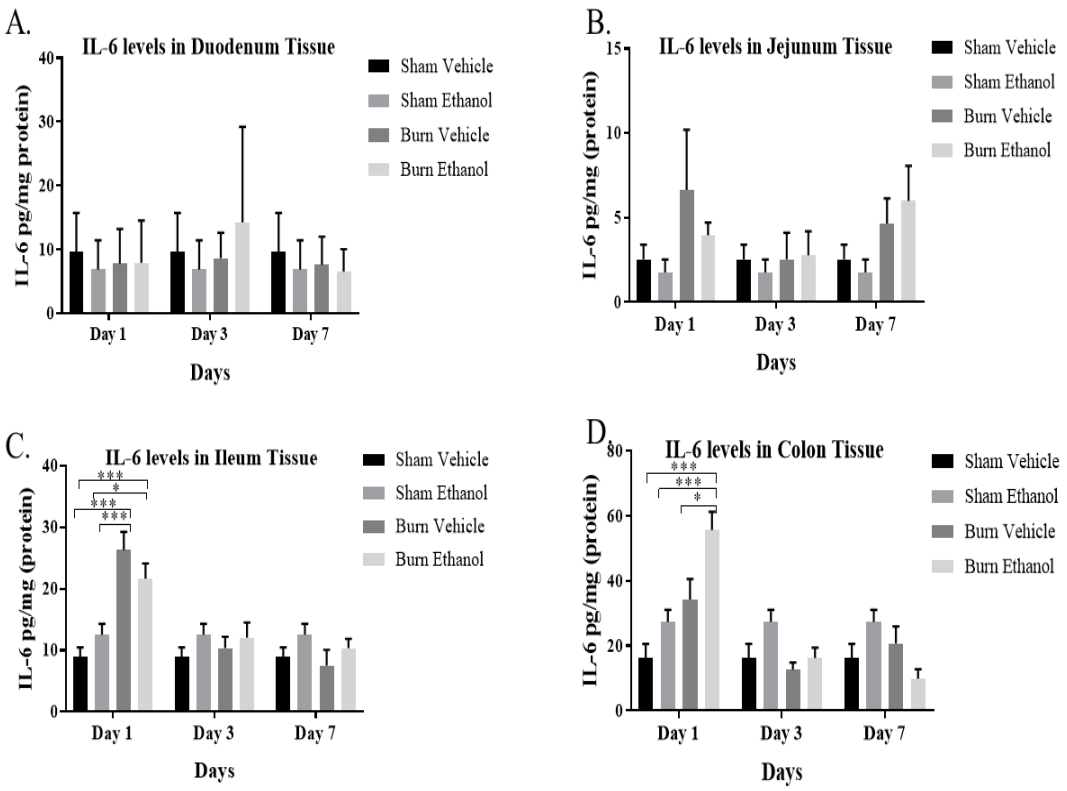

Figure 4. Effects of Ethanol and Burn Injury on IL-6 Levels in Intestinal Tissues Days One, Three and Seven Following Injury. Intestinal tissues (A. duodenum, B. jejunum, C. ileum, and D. colon) were collected days one, three and seven following ethanol and burn injury and used to measure IL-6 levels. *$p<0.05$, ***$p < 0.001$, by Two-Way ANOVA. The data shown are mean ± SEM of n = 5–12 animals per group. All sham vehicle and sham ethanol animals from all the days tested were pooled together.

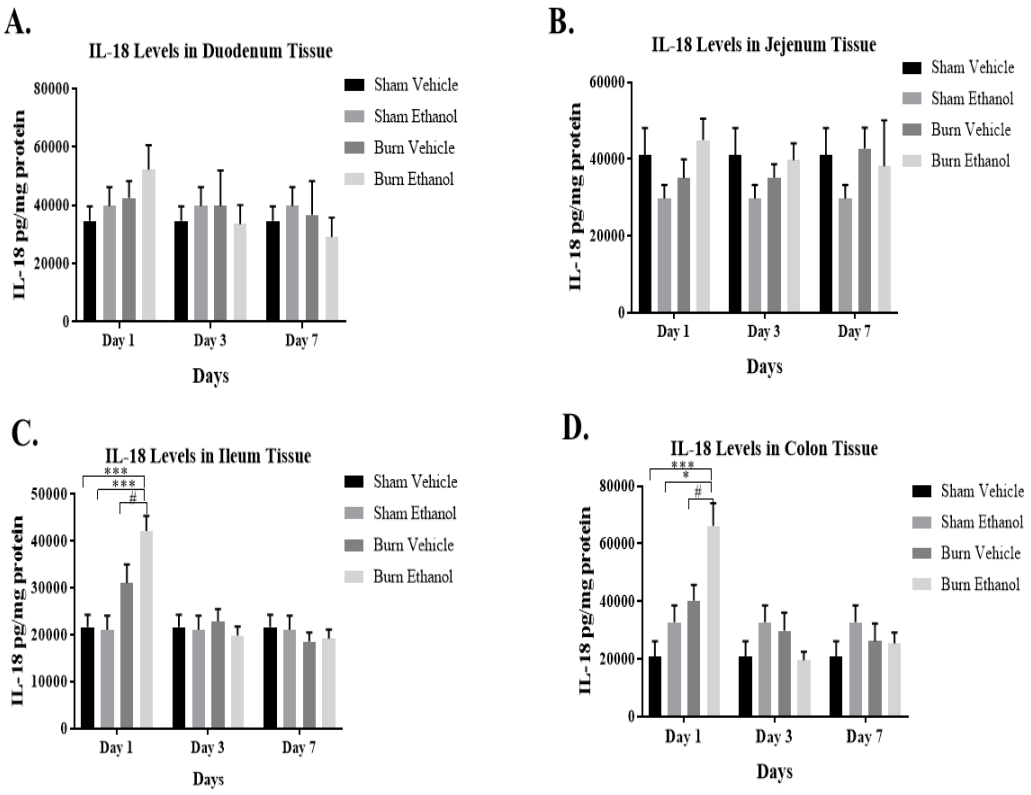

Figure 5. Effects of Ethanol and Burn Injury on IL-18 Levels in Intestinal Tissues Days One, Three and Seven Following Injury. Intestinal tissues (A. duodenum, B. jejunum, C. ileum, and D. colon) were collected days one, three and seven following ethanol and burn injury and used to measure IL-18 levels. *$p<0.05$, ***$p<0.001$ by Two-Way ANOVA, # by student's t-test compared to burn vehicle. The data shown are mean ± SEM of n = 5–12 animals per group. All sham vehicle and sham ethanol animals from all the days tested were pooled together.

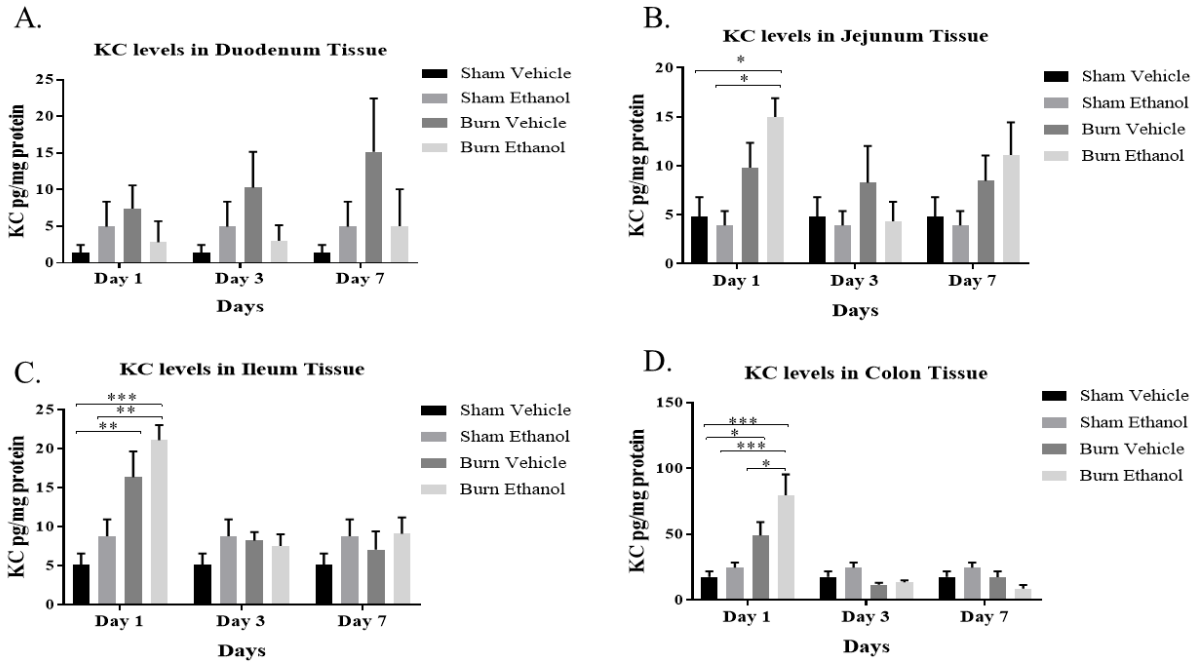

Figure 6. Effects of Ethanol and Burn Injury on KC Levels in Intestinal Tissues Days One, Three, and Seven Following Injury. Intestinal tissues (A. duodenum, B. jejunum, C. ileum, and D. colon) were collected days one, three and seven following ethanol and burn injury and used to measure KC levels. *p<0.05, **p<0.01, ***p<0.001 by Two-Way ANOVA. The data shown are mean ± SEM of n = 5–12 animals per group. All sham vehicle and sham ethanol animals from all the days tested were pooled together.

We reevaluated the data collected (Figures 4-6) to examine whether levels of pro-inflammatory mediators were differentially affected by region (duodenum, jejunum, ileum or colon). Interestingly, we observed that the levels of pro-inflammatory mediators (IL-6, IL-18 and KC) increased distally, with levels being higher in the colon. This coincided with the increasing bacterial content in the GI tract which also increases distally, with the highest levels being in the colon. The duodenum and jejunum contain 10^2–10^4 CFU/g, whereas the ileum's bacterial content is 10^{10} CFU/g. The colon's bacterial content is 10^{10}–10^{12} CFU/g[128,129]. While IL-6 levels increased similar in the ileum and colon (~2.5-fold) following ethanol and burn injury compared to the sham vehicle injured animals. The net IL-6 levels were significantly higher in the colon compared to the small intestine (e.g. duodenum, jejunum and ileum) (Figure

7A). Further analysis of the data demonstrated a ~7-fold increase in IL-6 levels in the colon following the combined insult of ethanol and burn injury compared to the duodenum and a ~3-fold increase when compared to the ileum. Similarly, IL-18 levels increased more distally, with higher levels in the colon however in contrast to IL-6, the net elevation was only ~1.5-fold higher in the colon compared to the ileum after ethanol and burn injury and this was not found to be significantly different (Figure 7B). In agreement with our other findings, the pro-inflammatory mediator KC also increased distally, with higher levels in the colon. KC levels were significantly elevated in the colon compared to the duodenum ~30-fold and the ileum ~4-fold following the combined insult of ethanol and burn injury (Figure 7C).

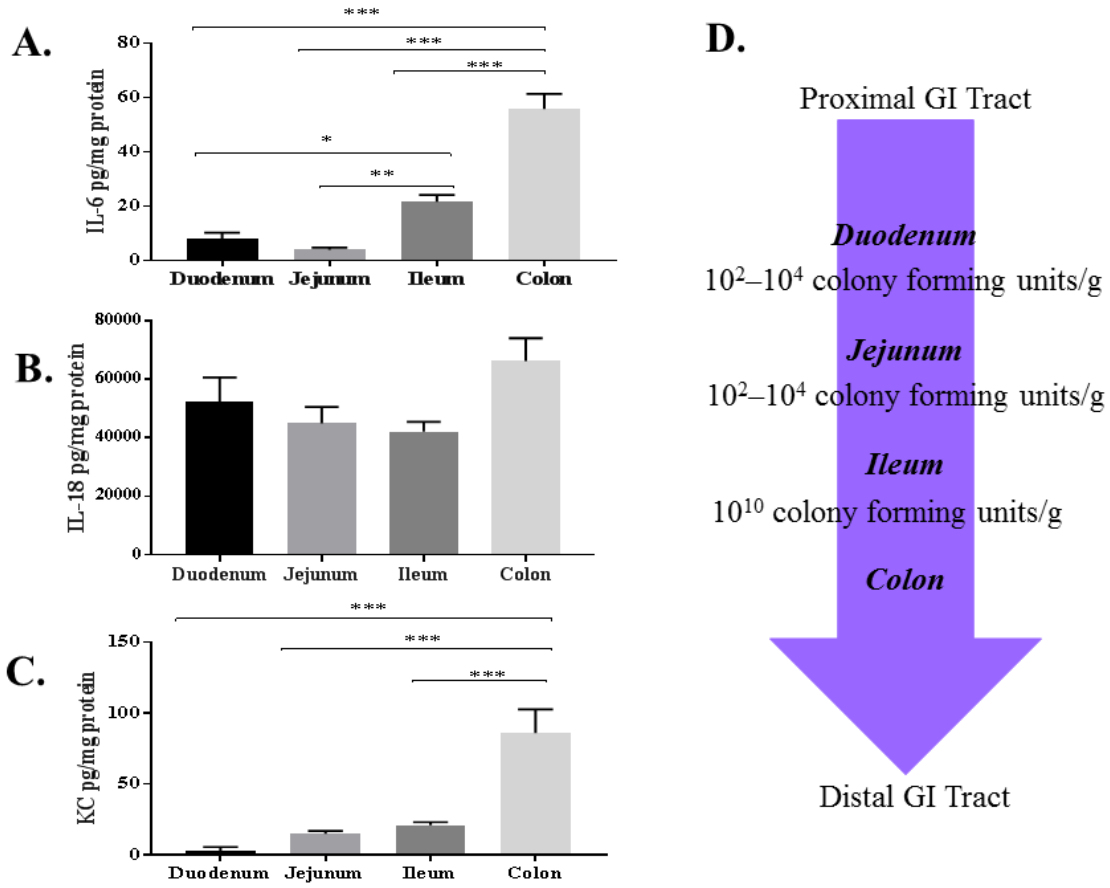

Figure 7. Expression of Pro-Inflammatory Mediators One Day Following Ethanol and Burn Injury Intestinal Tissues. Reevaluated data collected from Figures 4-6 to investigate whether levels of pro-inflammatory mediators (A. IL-6, B. IL-18, and C. KC), were differentially affected by region (duodenum, jejunum, ileum, and colon) day one following ethanol and burn injury. Panel D was generated from O'Hara & Shanahan. *EMBO Rep*. 2006;7(7):688-693 and Hakansson & Molin. *Nutrients*. 2011;3(6):637-682[128,129] *p<0.05, **p<0.01, ***0.001 by One-Way ANOVA compared to the respective sham vehicle group. The data shown are mean ± SEM of n = 7-8 animals per group.

Summary

In the present study, we examined pro-inflammatory mediators in various parts of the intestine days one, three, and seven following ethanol and burn injury. These data suggest that ethanol and burn injury significantly increased pro-inflammatory mediators (IL-6, IL-18 and KC) in intestinal tissue following the combined insult of ethanol and burn injury. Interestingly, we discovered that the levels of the pro-inflammatory mediators progressively increase down the GI tract with larger net levels in the colon. As the levels of bacterial content also follows this trend,

this suggests that the microbiota may influence the levels of the pro-inflammatory mediators following the combined insult. Although further investigation is needed to validate this relationship, collectively, these data suggest that ethanol and burn injury differentially effects levels of pro-inflammatory mediators in different parts of the GI tract following the combined insult of ethanol and burn injury.

Although we observed higher levels of pro-inflammatory mediators in the colon, we observed similar responses in both small and large intestines in other parameters (e.g. expression of tight junction proteins). This provides further evidence that the levels of the inflammatory responses are strongly linked to the bacterial content following ethanol and burn injury. As our laboratory historically evaluate changes following ethanol and burn injury in the ileum, we only used the ileum or isolated epithelial cells from the ileum for downstream experiments.

CHAPTER FOUR

EFFECTS ON ETHANOL AND BURN INJURY ON EXPRESSION OF MICRORNA BIOGENESIS COMPONENTS[81]

Abstract

Ethanol exposure at the time of burn injury is a major contributor to post-burn pathogenesis. Many of the adverse effects associated with ethanol and burn injury are linked to an impaired intestinal barrier. The combined insult causes intestinal inflammation, resulting in tissue damage, altered tight junction protein expression, and increased intestinal permeability. microRNAs are key regulators of cellular homeostasis and play a critical role in the maintenance of intestinal barrier function. Specifically, miR-150 has been shown to impact levels of inflammatory mediators which can contribute to gut barrier disruption. The present study examined whether ethanol and burn injury alters expression of microRNA processing enzymes (drosha, dicer, and argonaute-2) and microRNAs in the small intestinal epithelial cells (IECs). Male mice were gavaged with ethanol (~2.9 g/kg) 4 hours prior to receiving a ~12.5% total body surface area full thickness burn. One day after injury, mice were euthanized and IECs were isolated and analyzed for expression of miR biogenesis components (drosha, dicer and argonaute-2) and miRs (-7a, -22, -150, -210, and -375). Dicer mRNA and protein levels were not changed following the combined insult compared to sham injured animals. Drosha and argonaute-2 mRNA and protein levels were significantly reduced in IECs one day after injury. There was a trend for decrease expression of all tested miRs following ethanol and burn injury compared to shams however, only miR-7a and miR-150 were significantly reduced. To

substantiate the role of miR-150 in intestinal inflammation, young adult mouse colonocytes were transfected with a miR-150 plasmid and stimulated with LPS (100ng/mL). miR-150 overexpression significantly reduced IL-6 and KC protein levels compared to vector control cells challenged with LPS. These results suggest that altered microRNA biogenesis and associated decrease in miR-150 likely contributes to increased intestinal inflammation following ethanol and burn injury.

Introduction

Recent findings have demonstrated that the gut barrier disruption following ethanol and burn injury is widely associated with excess inflammation[10,11,30,135]. These studies suggest that increases in intestinal inflammatory mediators such as IL-6, IL-18 or other chemokines (e.g. KC) can directly or via recruitment of neutrophils cause intestinal tissue damage and alter tight junction protein expression[10,30,31,88,134,135]. microRNAs (miRs) are small noncoding RNA sequences, which control gene expression at the post-transcriptional level[15,16,18,42], and dysregulation of microRNA expression has resulted in numerous pathological conditions. Many studies have shown a relationship between microRNAs and tissue inflammation following ethanol exposure or tissue injury. However, whether microRNAs play a role following ethanol and burn injury remains unknown. Therefore, the present study assessed the effects of ethanol combined with burn injury on the biogenesis of microRNAs in intestinal epithelial cells (IECs).

Biogenesis of microRNAs occurs in several steps, starting with transcription by RNA polymerase II forming a primary microRNA (pri-miRNA), which is then cleaved by drosha (a RNase III enzyme) resulting in a precursor miRNA (pre-miRNA). The pre-miRNA is exported from the nucleus by exportin-5, where it is cleaved by dicer[15,16]. Cleavage by dicer results in a duplex miRNA complex containing both the guide and passenger strand. The guide strand is

loaded onto an argonaute (ago) protein forming miRISC, while the passenger strand is usually degraded. The guide miRNA uses partial base pairing to guide miRISC to its target mRNA. Binding of miRISC to the target mRNA allows for miRNA mediated gene regulation [15-17].

Numerous studies have illustrated the importance of microRNAs in maintenance of the intestinal barrier[23,66,136]. Thus, altered expression of microRNAs could negatively affect the intestinal barrier. Changes in microRNAs expression as a result of ethanol and burn injury could potentially alter the levels of pro-inflammatory cytokines, which have been associated with tissue damage and altered tight junction protein expression[10,30,31,88,134]. Specifically, miR-150 has been shown to regulate levels of inflammatory mediators and is downregulated in sepsis patients and following exposure of cells to bacterial LPS *in vitro*[45,124,125].

Therefore, we examined whether ethanol and burn injury modulates expression of microRNAs and biogenesis components in small intestinal epithelial cells. We hypothesized that expression of microRNAs and biogenesis components would be diminished following ethanol and burn injury. Our data suggest that ethanol and burn injury diminished drosha and ago-2 expression. We determined that ethanol and burn injury reduced expression of miRs (-7a and-150). Furthermore, *in vitro* overexpression of miR-150 illustrated that the microRNA decreased levels of pro-inflammatory mediators. Taken together, our results demonstrate the ethanol and burn injury negatively affects expression of microRNA biogenesis components and miRs (-7a and -150) compared to sham injured animals, which may provide a mechanism behind the elevated inflammatory response following ethanol and burn injury.

Materials and Methods

Mouse Model of Acute Ethanol Intoxication and Burn Injury.

Adult male C57BL/6 mice (22-25g) were purchased from Charles River Laboratories (Wilmington, MA). The mice were randomly divided into four groups: sham vehicle (n = 5-8), sham ethanol (n = 3-9), burn vehicle (n = 4-11), and burn ethanol (n=7-12). Mice were gavaged with 0.4ml of 25% ethanol in water (~2.9 g/Kg) or water (vehicle). Four hours following the gavage, mice were anesthetized by I.P. with ketamine and xylazine (80 mg/Kg and 1.25 mg/Kg, respectively). The mice were transferred to a template fabricated to expose ~12.5% of the total body surface area. For burn injury, mice were immersed in 85-90°C water bath for 7-8 sec. Mice were dried and resuscitated with an I.P. of 1.0 ml physiological saline and returned to their cages and given water and food *ad libitum*[11]. All animal procedures were carried out in accordance with the Animal Care and Use Committee at Loyola University Chicago Health Sciences Division.

Small Intestinal Epithelial Cell Isolation and RNA Isolation.

Day one after injury, mice were euthanized, and small intestine tissue was harvested and washed in ice cold 1x PBS containing a cocktail of Gentamycin (50mg/ml) and 1x penicillin-streptomycin. The small intestine tissue was further processed to isolate small intestinal epithelial cells as described by Weigmann *et al*.[137]. The small intestines were then incubated in a pre-digestion solution (1x Hank's Balanced Salt Solution (HBSS), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5mM Ethylenediaminetetraacetic acid (EDTA) and 1mM Dithiothreitol (DTT)) and incubated at 37 °C for 20 minutes. Following the incubation, the supernatant was passed through a 100 µm strainer to collect epithelial cells. The incubation in the pre-digestion solution was performed twice to maximize cell count. The collected cells were centrifuged for 10 minutes at 4°C at 1500 RPM and washed in 1x PBS. IECs were lysed and large RNA and enriched small RNA were isolated using mirVana miRNA Isolation Kit (Life

Technologies, Carlsbad, CA) according to the manufacturer's instructions. A nanodrop spectrophotometer (Thermo Scientific) was used to determine RNA concentration.

Determination of microRNA Biogenesis Components (Drosha, Dicer and Argonaute-2) and Degradation Components (Exoribonuclease 1 and Exoribonuclease 2).

Large RNA was used to make cDNA using the High-Capacity cDNA Reverse Transcription Kit from Life Technologies (Carlsbad, CA). Expression of drosha, dicer and ago-2, exoribonuclease 1 (XRN1) and exoribonuclease 2 (XRN2) was analyzed by qPCR using their respective primers obtained from Life Technologies (Carlsbad, CA). GAPDH was used as the endogenous control for qPCR experiments. The target genes Ct cycle values were normalized to GAPDH Ct values. Data were calculated using the ΔΔCT method and expressed relative to the average of sham vehicle group[13].

Determination of microRNA Biogenesis Components (Drosha, Dicer and Argonaute-2) Protein Expression.

To determine protein levels of microRNA biogenesis components in epithelial cells day one following ethanol and burn injury, lysates were analyzed by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and were transferred to either Polyvinylidene Difluoride (PVDF) or nitrocellulose membranes. The membranes were blocked for one hour at room temperature with 5% Bovine Serum Albumin (BSA) in TBS-T (0.05% Tween 20 in Tris Buffer Solution). Following blocking the membranes were incubated with antibodies specific to dicer (Santa Cruz Biotechnology, Santa Cruz, CA), drosha or argonaute-2 (Cell Signaling Technology, Danvers, MA) overnight at 4°C. Membranes were washed five times for five minutes with TBS-T. Following the last wash, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (HRP) for one hour. After the incubation in the

secondary antibody, the membrane was washed five times for five minutes in TBS-T and one time for 10 minutes in TBS. Following the final wash, the membranes were probed using Western Lightning™ Chemiluminescence Reagent Plus from PerkinElmer (Norwalk, CT). The membrane was visualized using a ChemiDoc System from BioRad (Hercules, CA).

Determination of Expression of microRNAs.

Enriched small RNAs were used for cDNA synthesis using miScript II RT Kit from Qiagen (Valencia, Ca). Expression of microRNAs (-7a, -22, -150, -210 and -375) was examined by qPCR using Qiagen miScript Primer Assays. Small nucleolar RNA (Snord68) was used as an endogenous control for microRNA qPCR experiments. miR Ct cycle values were normalized to snord68 Ct values. Data were calculated using the ΔΔCT method and expressed relative to the average of sham vehicle group. As we observed no changes in mRNA or protein levels of microRNA biogenesis components, miR expression was examined in two groups: sham vehicle (n=3-4), and ethanol burn (n=3-4).

miR-150 Overexpression and Assessment of IL-6 and KC.

To establish the role of miR-150 on intestinal inflammation, 6 x 10^5 YAMCs were seeded in Roswell Park Memorial Institute medium (RPMI) 1640 containing 50μg/ml gentamicin, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% ITS+ Premix, 5% fetal bovine serum (FBS) and 1x glutamine. One day after plating, the cells were transfected with 250ng of miR-150 expression plasmid (miR-150 plasmid) or PCMVMIR empty vector control (vector) from Origene (Rockville, MD) and Lipofectamine 2000 from Invitrogen (Carlsbad, CA). The cells were incubated for 48 hours in the Lipofectamine 2000/DNA mixture. Following the incubation, the cells were washed with PBS and then treated with LPS (100ng/ml) for 6 hours. After the 6-hour LPS treatment both the supernatant and cells were collected. The cells were lysed and used

for RNA isolation, total and enriched RNA were used for cDNA synthesis and subsequent qPCR (miR-150, IL-6 and KC). Snord68 was used as an endogenous control for miR-150 qPCR, while β-Actin was used as an endogenous control for IL-6 and KC qPCR. IL-6 and KC levels from the supernatant were determined by ELISA kits according to the manufacturer's instructions. Mouse IL-6 and KC ELISA kits were purchased from BD Biosciences (Bedford, MA) and R&D Systems (Minneapolis, MN) respectively. Transfection experiments were performed three times in duplicates. Due to variability between IL-6 and KC levels on different days, IL-6 and KC values were normalized to vector LPS.

Statistical Analysis.

All statistical analysis is presented as mean ± SEM. Analysis was performed with Two-Way ANOVA with Tukey-Kramer Multiple Comparisons Test or student's t-test (GraphPad Prism 7 Software, La Jolla, CA). A $p<0.05$ was considered statistically significant.

Results

To determine whether ethanol and burn injury modulates expression of microRNA biogenesis components, we examined expression of drosha. The first step in microRNA maturation is cleavage of pri-miR by drosha in the nucleus[15]. Drosha mRNA expression was significantly diminished day one following ethanol and burn injury in isolated small intestinal epithelial cells compared to all groups (Figure 8A). Consistent with mRNA expression, we found a significant decrease in drosha protein levels in small intestinal epithelial cells day one following the combined insult compared to the sham vehicle and burn vehicle groups (Figure 8B).

Next, we examined expression of dicer to further assess whether ethanol and burn injury disrupts expression of microRNA biogenesis components in small intestinal epithelial cells.

Cytoplasmic cleavage of the pre-miR by dicer is the next maturation step in microRNA biogenesis[15]. There was no change in IEC dicer mRNA expression day one following the combined insult of ethanol and burn injury compared to all groups (Figure 9A). Additionally, dicer protein levels were not changed day one following ethanol and burn injury in small intestinal epithelial cells compared to all groups (Figure 9B).

Next, we examined whether the combined insult affects ago-2 expression. Argonaute proteins are the core component of the miRISC complex, and ago-2 is the only member of the argonaute family with catalytic ability[15]. Furthermore, ago-2 has been shown to protect the single stranded mature microRNA from cleavage by ribonucleases[49,50]. Therefore, altered expression of ago-2 could influence microRNA levels. There was a significant decrease in ago-2 mRNA expression day one following the combined insult in small intestinal epithelial cells compared to all groups (Figure 10A). Furthermore, ago-2 protein levels were significantly reduced day one following the combined insult compared to the sham vehicle and burn vehicle groups (Figure 10B).

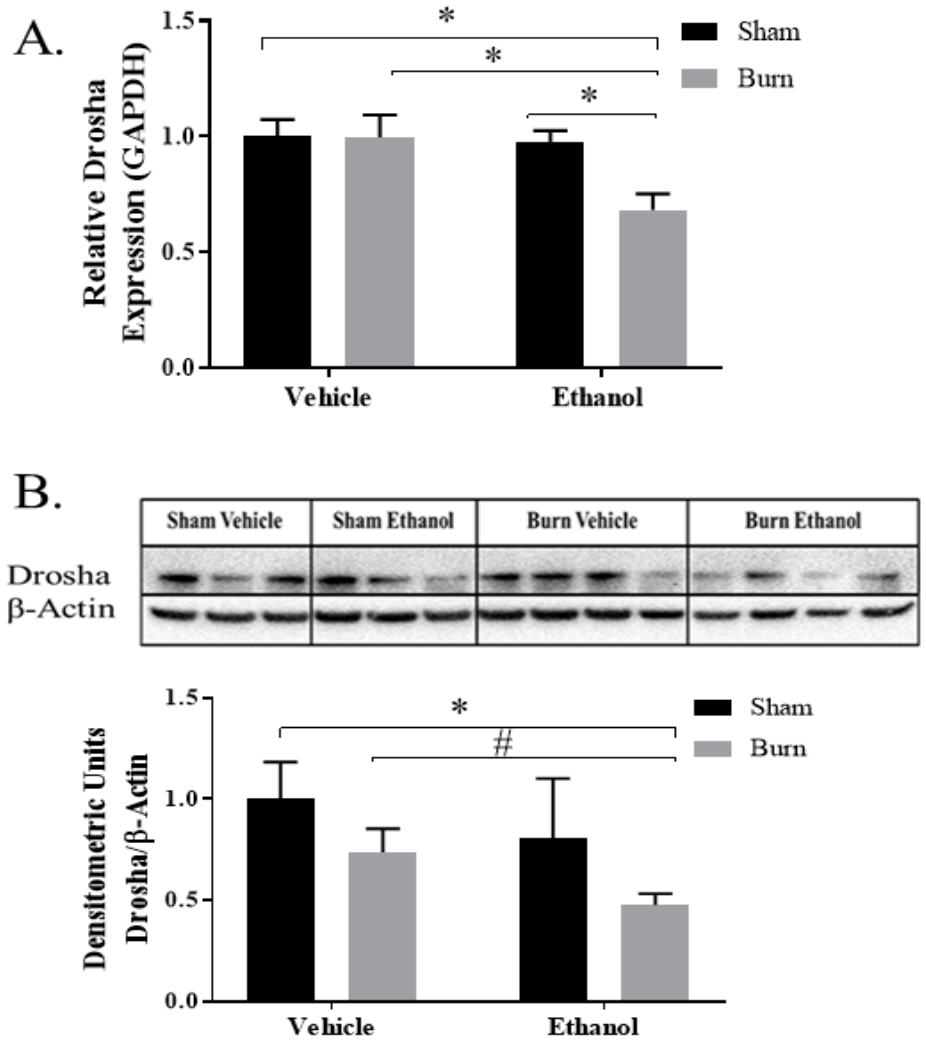

Figure 8. Drosha mRNA and Protein Levels in Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine drosha A.) mRNA expression, and B.) protein levels day one following ethanol and burn injury. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. GAPDH was used as an endogenous control. Densitometry measurements for each protein are given as a ratio of the protein density to β-Actin. The data shown are mean ± SEM of duplicate experiments. *p < 0.05 by Two-Way ANOVA or #p< by student's t-test compared to burn vehicle. n = 3–12 animals per group.

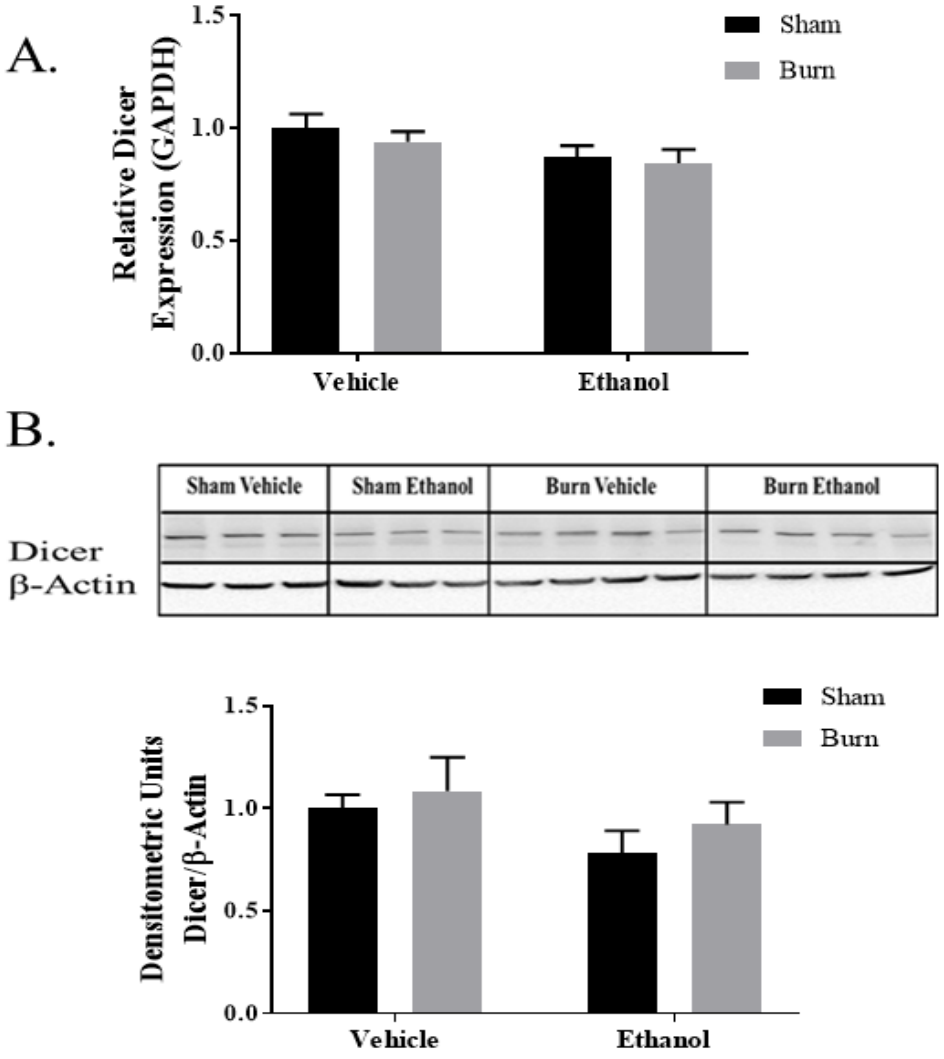

Figure 9. Dicer mRNA and Protein Levels in Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine dicer A.) mRNA expression, and B.) protein levels day one following ethanol and burn injury. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. GAPDH was used as an endogenous control. Densitometry measurements for each protein are given as a ratio of the protein density to β-Actin. The data shown are mean ± SEM of duplicate experiments. Statistical analysis was performed by Two-Way ANOVA. n = 3–12 animals per group.

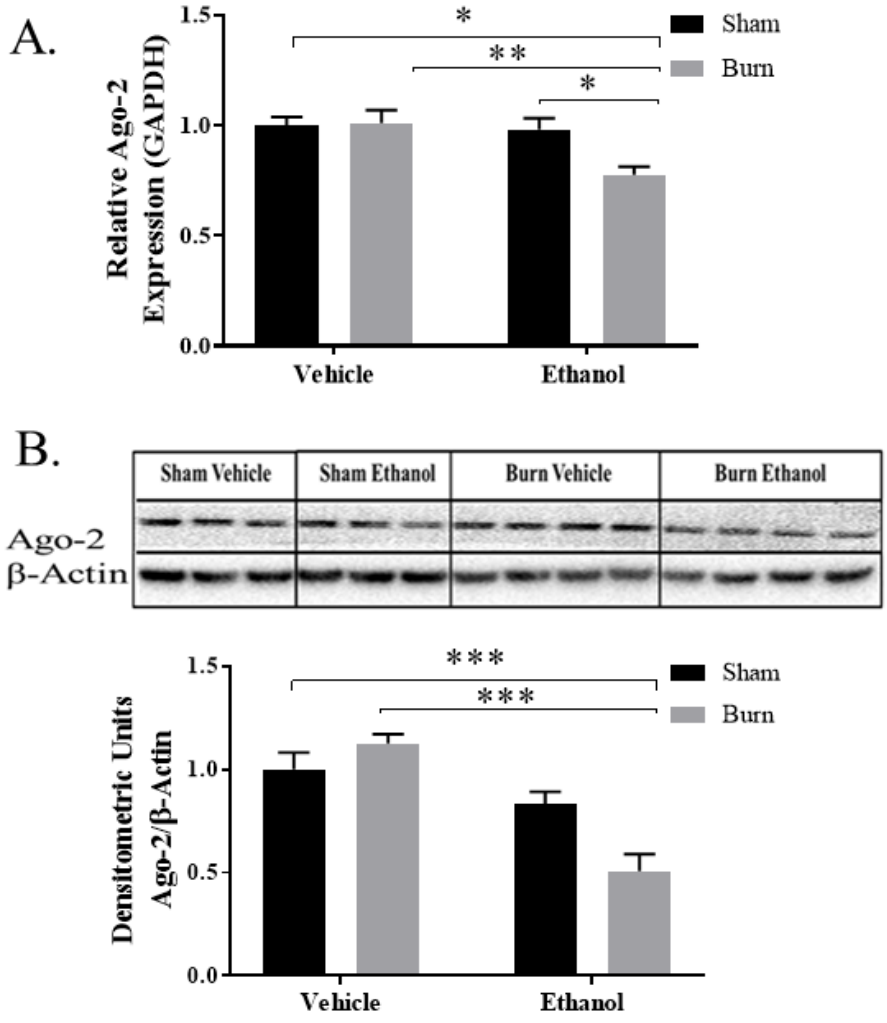

Figure 10. Ago-2 mRNA and Protein Levels in the Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine ago-2 A.) mRNA expression, and B.) protein levels day one following ethanol and burn injury. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. GAPDH was used as an endogenous control. Densitometry measurements for each protein are given as a ratio of the protein density to β-Actin. The data shown are mean ± SEM of duplicate experiments of duplicate experiments. *$p < 0.05$, **$p<0.01$, ***$p<0.001$ by Two-Way ANOVA. n = 3–12 animals per group.

To establish whether ethanol and burn injury modulates microRNA expression we evaluated expression of miR-7a, miR-22, miR-150, miR-210 and miR-375. We chose to examine expression of five microRNAs (miR-7a, miR-22, miR-150, miR-375, and miR-210) based on their predicted targets. miR-7a was selected because this microRNA play an important role in

proliferation, apoptosis and inflammation[138-140]. Similarly, microRNAs (miR-22, miR-150 and miR-210) were selected for evaluation due to their involvement in inflammation[45,122,124,141], which is a major adverse effect following ethanol and burn injury[11,12,30]. Furthermore, miR-375 was selected due to its role in goblet cell differentiation[136]. Although, all microRNAs examined exhibited a trend towards reduced expression following the combined insult, miR-7a and miR-150 were the only miRs whose expression was significantly reduced compared to sham vehicle animals (Figure 11). miR-7a expression was reduced by 60%, while miR-150 expression was reduced by 65% compared to the sham vehicle group (Figure 11A and 11C).

5′ to 3′ exoribonucleases (XRN1 and XRN2) are believed to be involved in microRNA degradation [15,21]. We evaluated whether ethanol and burn injury modulates expression of these exoribonucleases. We observed no changes in XRN1 or XRN2 mRNA expression following ethanol and burn injury compared to all groups (Figures 12A-B), suggesting that degradation is not contributing to the observed decrease expression of select microRNAs.

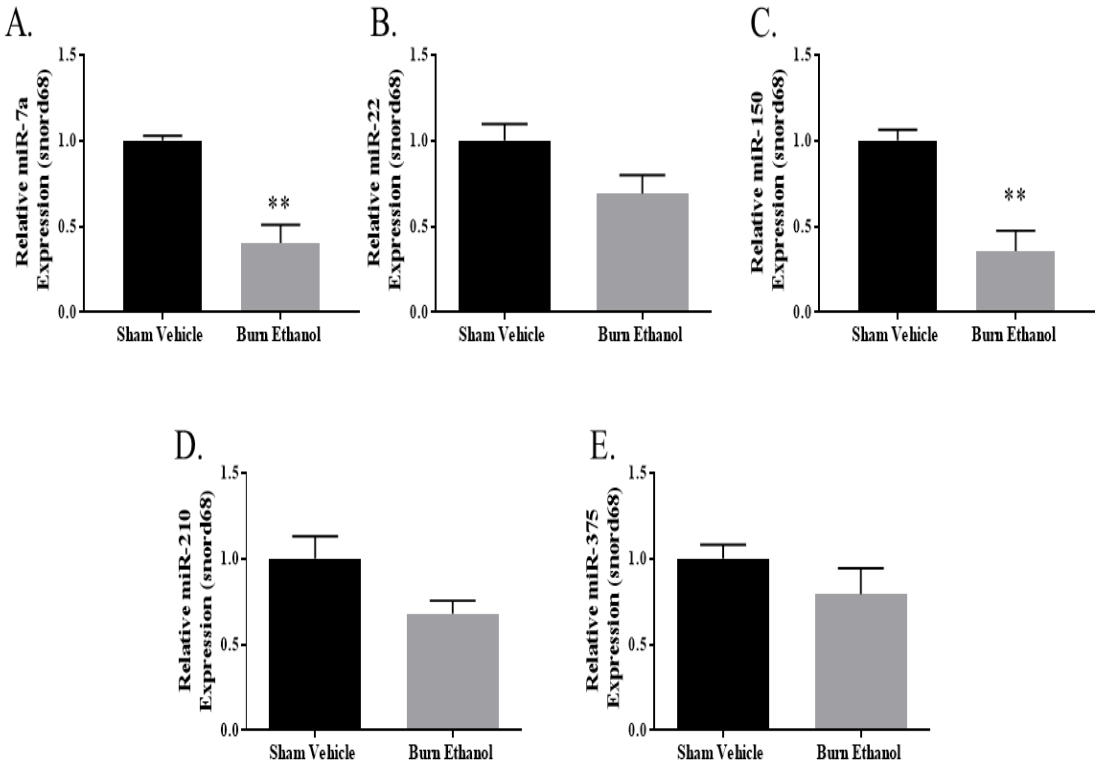

Figure 11. Expression of miRNAs in Isolated Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine A.) miR-7a, B.) miR-22, C.) miR-150, D.) miR-210 and E.) miR-375 day one following ethanol and burn injury. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. Snord68 was used as an endogenous control. **$p<0.01$ by student's t-test compared to sham vehicle. The data shown are mean ± SEM of n = 3–4 animals per group.

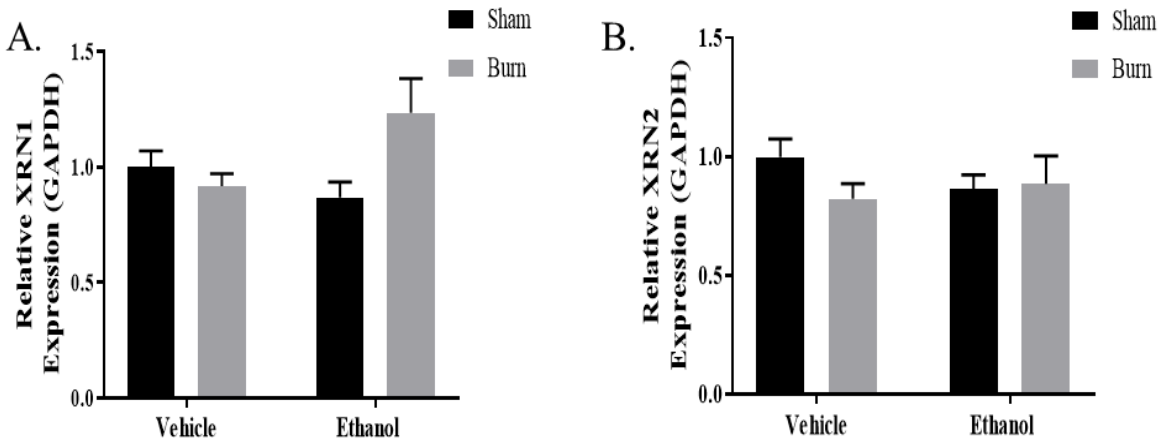

Figure 12. XRN1 and XRN2 Expression in the Small Intestinal Epithelial Cells Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine A.) XRN1 and B.) XRN2 day one following ethanol and burn injury. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. GAPDH was used as an endogenous control. Statistical analysis was performed by Two-Way ANOVA. The data shown are mean ± SEM of n = 5-7 animals per group.

Although, we observed significant reduction in both miRs (-7a and -150), we chose to further investigate the role of miR-150. miR-150 was of particular interest due to its involvement of inflammation and sepsis which are major adverse effects following ethanol and burn injury. We used an *in vitro* approach in which young adult mouse colonocytes were transfected with a miR-150 plasmid for 48 hours prior to a 6-hour LPS (100ng/ml) treatment (Figure 13A). The 48-hour transfection significantly increased miR-150 expression in cells transfected with the miR-150 plasmid compared to cells transfected with the empty vector (Figure 13B). Treatment of cells containing the empty vector with LPS resulted in a significant increase in IL-6 (12-fold) and KC (48-fold) expression. Overexpression of miR-150 did not affect IL-6 or KC expression (Figures 13C and 13E). IL-6 protein levels were significantly reduced following transfection with miR-150 compared to vector LPS treated cells (Figure 13D). Furthermore, KC was significantly reduced in cells transfected with the miR-150 plasmid and challenged with LPS compared to vector LPS cells (Figure 13F).

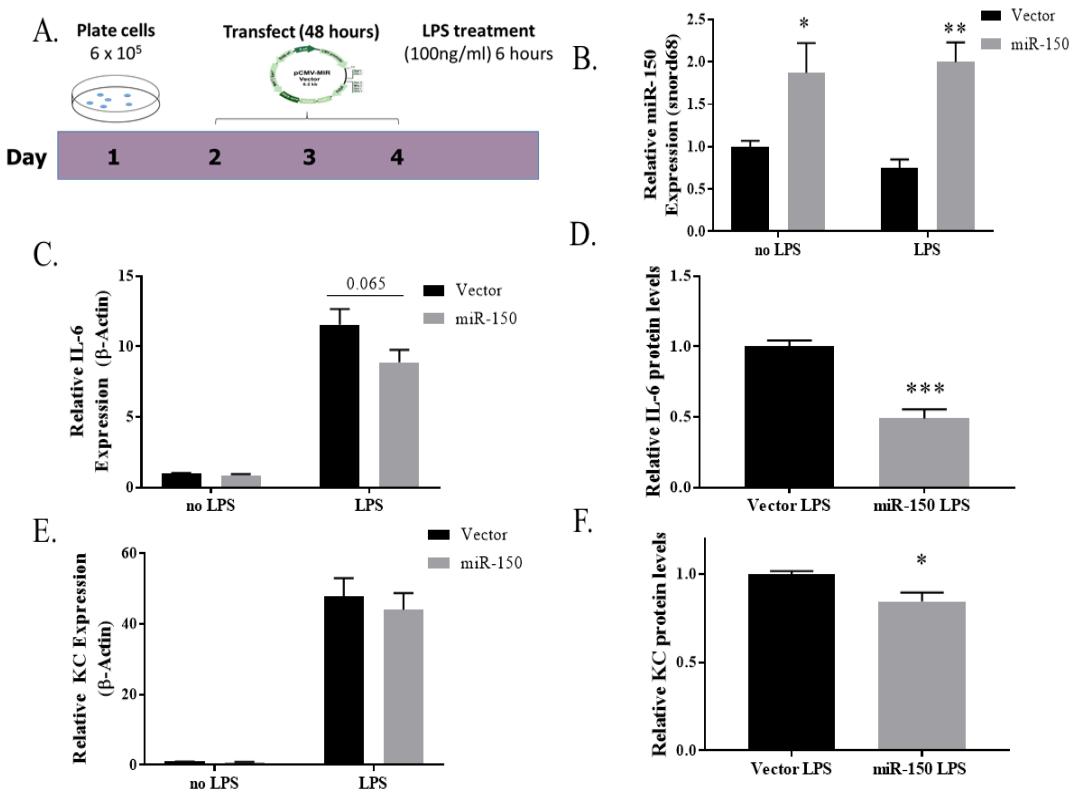

Figure 13. Effects of miR-150 Overexpression on Pro-Inflammatory Mediators. A.) Schematic of time course of YAMCs transfection experiment: YAMCs transiently transfected with a miR-150 plasmid, 48 hours later cells were treated for 6-hour LPS (100ng/ml). **B.)** Expression of miR-150 following transfection and LPS challenge. Values were calculated using a ΔΔCT method and normalized to cells containing the empty vector. Snord68 was used as an endogenous control for miRNA. RNA isolated from YAMCs was used to examine IL-6 (C) and KC (E) mRNA expression. Values were calculated using a ΔΔCT method and normalized to cells containing the empty vector. β-Actin was used as an endogenous control for mRNA. Secreted IL-6 (D) and KC (F) levels were examined by ELISA, results were normalized to vector LPS (n = 3 individual experiments performed in duplicate). *p < 0.05, **p < 0.01, ***p<0.001 by Two-Way ANOVA or student's t-test compared to vector or vector LPS.

Summary

In this study, we demonstrate that ethanol exposure at the time of burn injury negatively impacts expression of microRNA biogenesis components (drosha and ago-2) in small intestinal epithelial cells compared to shams. Furthermore, the reduction in expression microRNA components correlates with reduced miR-7a and miR-150 expression. Our findings further show that miR-150 influences levels of inflammatory mediators. *In vitro* overexpression of miR-150 and subsequent LPS stimulation reduced pro-inflammatory mediators (KC and IL-6) following

ethanol and burn injury. Together these findings suggest that diminished expression of microRNA biogenesis and subsequent reduction in miRs (e.g. miR-150) could contribute to the observed elevated inflammation following ethanol and burn injury.

CHAPTER FIVE

ROLE OF HIF-1α IN THE GUT FOLLOWING ETHANOL AND BURN INJURY

Abstract

Ethanol remains a major confounder in the pathology associated with burn injury. Experimental studies have widely linked these adverse effects to an impaired intestinal barrier. Our laboratory has previously demonstrated that there is reduced intestinal oxygen delivery (hypoxia) to the gut following ethanol and burn injury. Furthermore, we observed altered microRNA expression in small intestinal epithelial cells after the combined insult of ethanol and burn injury. Others have shown that hypoxia can influence expression of both microRNAs and microRNA biogenesis components. In this study, we sought to examine whether hypoxia has any role in altered expression of microRNA biogenesis components (drosha, dicer and argonaute-2) and miRs (-7a and -150) following ethanol and burn injury. Additionally, we evaluated whether improvements in expression of miRs could impact other parameters that are disrupted following ethanol and burn injury (tight junction expression, bacterial outgrowth, and intestinal permeability). Male mice were gavaged with ethanol (~2.9 g/kg) four hours before receiving a ~12.5% total body surface full thickness burn. Immediately after burn, mice were resuscitated with 1 mL of normal saline with or without 5 mg/Kg PX-478 a HIF-1α inhibitor. One day following injury mice were euthanized, luminal contents were collected from the distal ileum and small intestinal tissue was harvested and processed for isolation of IECs. Ethanol and burn injury significantly reduced expression of drosha and argonaute-2 in IECs. This correlated

with reduced expression of miRs (-7a and -150), occludin and claudin-4 compared to sham injured animals. This was accompanied with an increase in intestinal permeability. Furthermore, we observed an increase in total bacteria and *Enterobacteriaceae* populations following the combined injury compared to sham vehicle animals. Treatment of mice with PX-478 improved expression of drosha, argonaute-2 and miRs (-7a and -150). PX-478 treatment increased occludin, claudin-4, ZO-1 expression, while reducing bacterial dysbiosis, and intestinal permeability. Taken together, these data suggest that PX-478 improves microRNA biogenesis which improves expression of miRs, barrier integrity while reducing bacteria dysbiosis following ethanol and burn injury.

Introduction

Experimental findings have widely linked an impaired gut barrier to ethanol and burn injury pathology[9,29]. One factor that is modulated following ethanol and burn injury and likely contributes to this pathology is the ischemic condition and resulting hypoxia that occurs in the gut[14]. Our laboratory has demonstrated that the combined insult of ethanol and burn injury reduces intestinal oxygen delivery to the gut (hypoxia)[14]. Hypoxia can result in increased inflammation, altered tight junction expression and tissue damage[110-112]. Furthermore, hypoxia after burn injury has been associated with tissue damage while reducing tight junction protein expression and increased intestinal permeability[110-112,142,143]. Additionally, hypoxia has been shown in other models to reduce expression of microRNAs and their biogenesis components[115,116,144-147]. As microRNAs are major regulators of cellular homeostasis aberrant expression due to hypoxia can lead to detrimental effects.

Hypoxia has been shown to result in decreased drosha expression in a HIF-1α dependent manner. Interestingly, hypoxia decreased dicer expression in a HIF-1α independent manner[115].

The effects on argonaute expression as a consequence of hypoxic insult are conflicting, one study suggest that hypoxia potentiates argonaute expression while another study demonstrated that hypoxia diminishes microRNA loading onto argonaute[144,145]. Together, these studies illustrate that hypoxia can impact expression of microRNA biogenesis components and subsequently expression of microRNAs. Alterations of these key regulators due to the hypoxic condition in the gut could contribute to the observed negative effects following ethanol and burn injury.

As microRNAs are central to many cellular functions disruption of these molecules due to hypoxic insult could influence tissue damage, tight junction protein expression and inflammation. Furthermore, as ethanol and burn injury reduces expression of microRNA biogenesis components (drosha and argonaute-2)[81], we examined whether the hypoxic insult in the gut following injury mediate these effects. We hypothesized that inhibiting the hypoxic signaling pathway using PX-478 (HIF-1α inhibitor) will restore expression of microRNA biogenesis components and parameters negatively impacted following ethanol and burn injury.

Materials and Methods

Animals.

Adult (8-10-week-old) C57BL/6 male mice (~22-25g body weight) were purchased from Charles River Laboratories (Wilmington, MA). Mice were housed and acclimated for one week before experimentation. All animal procedures were conducted in accordance to the Animal Care and Use Committee at Loyola University Chicago Health Science Division, Maywood, IL.

Mouse Model of Acute Ethanol Intoxication and Burn Injury and PX-478 Treatment.

Mice were randomly divided into four groups: sham vehicle (saline) (n=5-6), sham

vehicle (PX-478) (n=4-6), burn ethanol (saline) (n=6-10) and burn ethanol (PX-478) (n=5-10). Mice were gavaged either with water (vehicle group) or with (~2.9 g/Kg) 0.4ml of 25% ethanol in water (ethanol group). Four hours after the gavage, the mice were anesthetized by I.P. with a cocktail of ketamine and xylazine (80 mg/Kg and 1.25 mg/Kg, respectively). The mice were then placed into a template which is fabricated to expose ~12.5% of the TBSA. Mice in the burn group were immersed in a water bath (85-90°C) for 7-8 seconds. Immediately after the injury mice were dried and resuscitated with an I.P. injection of 1.0 ml physiological saline (saline group) or 5mg/kg of PX-478[148] in saline (PX-478 group). Mice also received the analgesic Buprenorphine (1mg/kg) subcutaneously. Mice were returned to their cages and received water and food *ad libitum*[11]. One day following the gavage, mice were euthanized, luminal contents and small intestine tissue were collected.

Small Intestinal Epithelial Cell Isolation & RNA Isolation.

Harvested small intestinal tissue was washed in ice cold 1x PBS containing Gentamycin (50mg/ml) and 1x penicillin-streptomycin. Small intestinal tissue was incubated at 37 °C for 20 minutes in pre-digestion solution (1x HBSS, 1% HEPES, 5mM EDTA and 1mM DTT). The epithelial cells were collected by passing the supernatant through a 100 µm strainer. Incubation of the small intestinal tissue in the pre-digestion solution was preformed twice to maximize cell count. Cells were centrifuged for ten minutes at 4°C at 1500 RPM before being washed in 1x PBS. Enriched IECs were lysed and used for downstream experimentation.

Determination of Expression in Isolated Small Intestinal Epithelial Cells.

A mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA) was used according to the manufacturer's instructions to isolated large RNA and enriched small RNA. A nanodrop spectrophotometer (Thermo Scientific, Waltham, MA) was used to determine RNA

concentration. A high-Capacity cDNA Reverse Transcription Kit from Life Technologies (Carlsbad, CA) was used to make cDNA from large RNA. Expression of drosha, dicer, argonaute-2 (ago-2), occludin, claudin-4 and ZO-1 was analyzed by qPCR using their respective primers from Life Technologies (Carlsbad, CA). Data were calculated using the ΔΔCT method, GAPDH was used as an endogenous control. The Ct cycle values for the target genes were normalized to Ct values for GAPDH. The data are expressed relative to the average of the sham vehicle group[13].

Determination of Drosha, Dicer, and Argonaute-2 Protein Expression.

Lysates from isolated IECs were analyzed by SDS-PAGE and were transferred to PVDF membranes to determine protein levels of drosha, dicer and argonaute-2. The membranes were blocked for one hour at room temperature in 5% BSA in TBS-T (0.05% Tween 20 in TBS) before incubation overnight at 4°C with the desired antibody dicer (Santa Cruz Biotechnology, Santa Cruz, CA), drosha and argonaute-2 (Cell Signaling Technology, Danvers, MA). The membranes were washed five times for five minutes in TBS-T following the overnight incubation before the membrane was incubated for an hour in the secondary antibody conjugated with HRP. The membranes were then washed five times for five minutes in TBS-T and once in TBS for ten minutes. The membranes were then probed using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer, Norwalk, CT) and visualized using a ChemiDoc System.

Determination of Expression of microRNAs.

A miScript II RT Kit (Qiagen, Valencia, CA) was used to make cDNA from enriched small RNAs. miRs (-7a and -150) expression was examined by qPCR using their respective Qiagen miScript Primer Assays. Data were calculated using the ΔΔCT method, snord68 was

used as an endogenous control. The Ct cycle values for the target genes were normalized to Ct values for snord68. The data are expressed relative to the average of sham vehicle group.

Immunofluorescent Tissue Staining.

HIF-1α staining was performed on frozen tissues from mice in four groups: sham vehicle (n = 3), sham ethanol (n = 4), burn vehicle (n =5), and burn ethanol (n=8). Frozen sections were stored in optimal cutting temperature (O.C.T.) medium. Sections were cut to 5μm using a Cryostar NX50 Cryosectioner (Thermo Fisher Scientific, Waltham, MA) and mounted on glass slides. Mouse anti-HIF-1α antibody (SantaCruz, Santa Cruz, CA) were used with an Alexa-455 conjugated secondary antibody (Invitrogen, Carlsbad, CA). ProLong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad, CA) was used to visualize the cell nuclei. Images were captured using a Zeiss Axiovert 200m (200X total magnification). Tissue sections were analyzed in a blinded fashion and a minimum of three were randomly obtained. Brightness and contrast of images were adjusted using Photoshop CC. Quantification of the fluorescent intensity was determined using Image J (NIH, Bethesda, MD). Fluorescent intensity=Integrated Density– (Selected Area x Mean fluorescent reading of background).

Isolation of Luminal Contents Genomic DNA.

Luminal contents were aseptically collected from the distal 5 cm of the ileum. Luminal genomic DNA QIAmp DNA Stool Isolation Kit (Qiagen, Valencia, CA) was used according to the manufacturer's instructions, contents were incubated at 95°C to improve bacterial cell lysis. DNA concentration was determined by NanoDrop 2000 spectrophotometer.

16S rRNA Bacterial Quantitative Real-Time PCR.

As described previously, specific primer sets which target small subunit (SSU) 16S rRNA of total bacteria and *Enterobacteriaceae* were used for qPCR[104]. The primers were

purchased from Thermo Fisher Scientific. The sequences used for total bacteria primers: 340F (ACTCCTACGGGAGGCAGCAGT) and 514R (ATTACCGCGGCTGCTGGC) for total bacteria. While the primers used for *Enterobacteriaceae* and 515F (GTGCCAGCAGCCGCGGTAA) 826R (GCCTCAAGGGCACAACCTCCAAG). Serial dilutions were made from purified genomic DNA from reference bacteria: total bacteria (*Blautia producta* strain VPI 4299 [ATCC 27340D-5] and *Enterobacteriaceae* (*Escherichia coli* strain K-12 [ATCC 1098D-5] purchased from ATCC (Manassas, VA) to generate standard curves for PCR quantification. qPCR reactions were performed using 1X iTaq Universal SYBR Green Supermix (Bio-Rad) 300nM of forward and reverse primers. Equal amount of fecal DNA was loaded, and data are presented as changes in bacterial copies.

Determination of Intestinal Permeability.

To determine intestinal permeability, one day after injury mice received a gavage of 0.4 ml of 22mg/ml Fluorescein Isothiocyanate (FITC)-dextran (4D) in PBS. 90 minutes following the gavage, the mice were euthanized, and blood was collected to measure levels of FITC-dextran in circulation. Data presented are the average of two independent experiments.

Statistics.

The data are presented as means ± SEM and were analyzed using Two-Way Analysis of variance (ANOVA) with Tukey's post-hoc test (GraphPad Prism 7 Software, La Jolla, CA). A p-value of <0.05 was considered statistically significant.

Results

Previous findings from our laboratory have shown a significant reduction in oxygen delivery and blood flow to the intestine following ethanol and burn injury[14]. Such a decrease in oxygen delivery can cause hypoxia. Here, we measured expression of the hypoxic marker, HIF-

1α in small intestinal tissue using a mouse anti-HIF-1α antibody. The results shown in Figure 14 clearly demonstrate a significant increase in HIF-1α following ethanol and burn injury. To provide additional evidence of the hypoxic insult in the gut following ethanol and burn injury, we measured HO-1 and VEGF-α, downstream targets of HIF-1α. We observed a trend of an increase in VEGF-α one day following ethanol and burn injury, however, this was not found to be significantly different (Appendix-A Figure 26B). HO-1 expression was significantly elevated one day following ethanol and burn injury compared to all groups (Appendix A-Figure 26A). This together with our previous observations suggest that there is a hypoxic insult to the gut following the combined insult of ethanol and burn injury.

In a recent study, we observed a decrease in microRNA biogenesis components (drosha and argonaute-2) following ethanol and burn injury[81]. Additionally, studies have shown that hypoxia and its signaling molecule HIF-1α can influence microRNA biogenesis[115]. Here, we sought to determine whether inhibition of HIF-1α via PX-478 would increase expression of drosha, dicer and argonaute-2 in small intestinal epithelial cells one day following ethanol and burn injury. To provide evidence of the ability of PX-478 to reduce levels of HIF-1α, we examined HIF-1α immunostaining. We found reduced immunostaining of HIF-1α in small intestinal tissue following PX-478 treatment at the time of injury (Appendix A-Figure 27). Furthermore, we observed a significant reduction in expression of the HIF-1α downstream target HO-1 in IECs following treatment of mice at the time of burn injury with PX-478 compared to all groups (Appendix A-Figure 28). Next, we examined the relationship between HIF-1α and expression of microRNA biogenesis components. Similar to our previous studies, drosha mRNA expression (~38%) and protein levels (~44%) were significantly reduced following the combined insult of ethanol and burn injury in IECs compared to animals in the sham vehicle group (Figure 15). Dicer mRNA

expression and protein levels remained unchanged in small intestinal epithelial cells following ethanol and burn injury compared to sham vehicle (Figure 16). Argonaute-2 mRNA expression (~23%) and protein (~48%) levels were significantly decreased in small intestinal epithelial cells following the combined insult compared to sham vehicle animals (Figure 15A). Expression of drosha was normalized to sham values after treatment with PX-478 as we observed an increase in expression (~35%) and protein levels (~30%) in IECs (Figure 15) compared to ethanol and burn injured animals. Dicer mRNA expression and protein levels remained unchanged in small intestinal epithelial cells following treatment of mice with PX-478 at the time of burn injury (Figure 16). We did observe however, that there was a significant increase in dicer mRNA in the sham treated group compared to ethanol and burn injured animals (Figure 16A). PX-478 treatment at the time of burn injury enhanced argonaute-2 expression (~23%) and protein (~22%) levels in small intestinal epithelial cells (Figure 17) compared to animals in the ethanol and burn group. Together, these data illustrate that HIF-1α has a role in regulating expression of drosha and argonaute-2 following the combined insult.

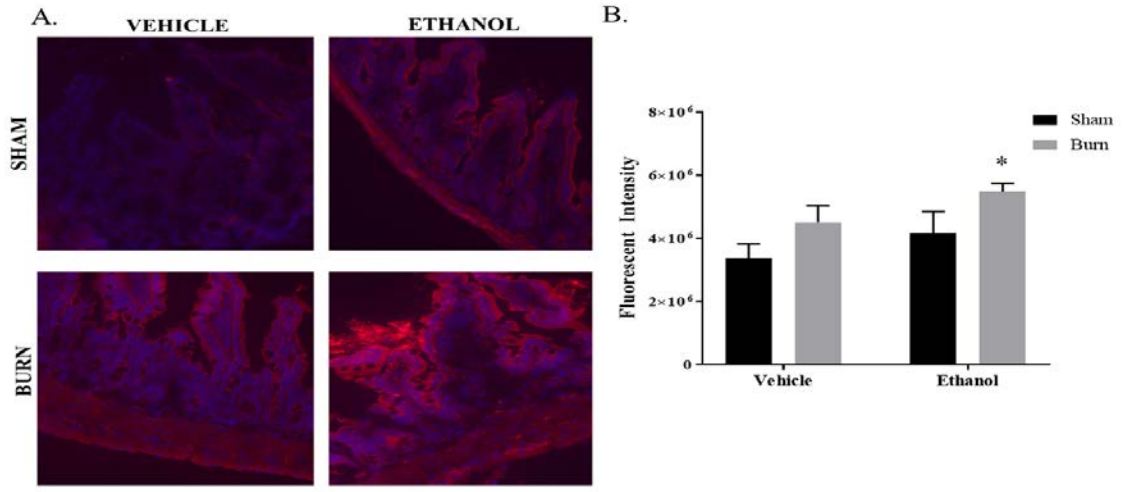

Figure 14. HIF-1α Levels in Small Intestinal Tissue Day One Following Ethanol and Burn Injury. Immunofluorescent staining was performed on 5μm frozen sections using anti-HIF-1α to assess HIF-1α levels in small intestinal tissue one day following the ethanol and burn injury $*p < 0.05$, by Two Way ANOVA. (Red-HIF-1α and Blue-Dapi). The data shown are mean ± SEM of n = 3-8 animals per group.

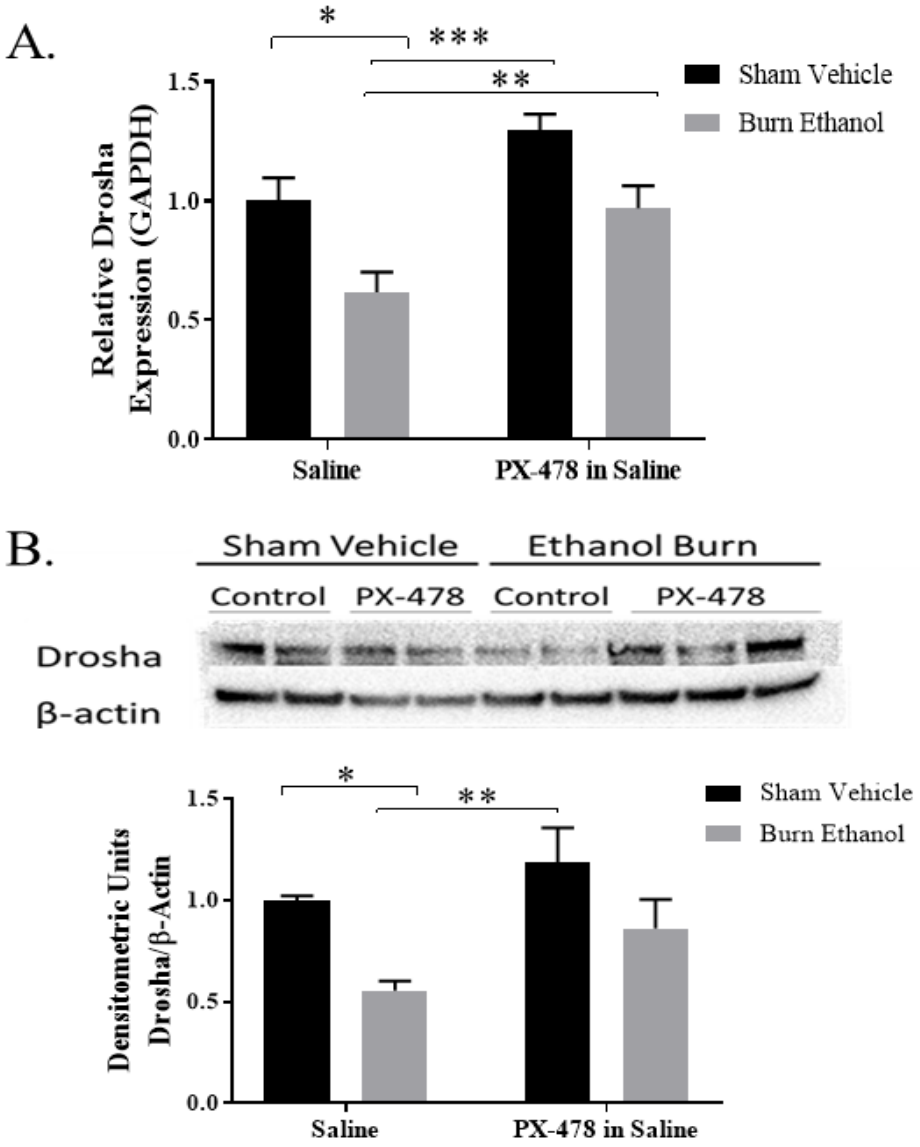

Figure 15. Effects of PX-478 Treatment on Drosha Expression in Small Intestinal Epithelial Cells One Day Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine drosha A.) mRNA expression and B.) protein levels day one following ethanol and burn injury with or without PX-478. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. GAPDH was used as an endogenous control. Densitometry measurements for each protein are given as a ratio of the protein density to β-Actin. *$p < 0.05$, **$p<0.01$, ***$p<0.001$ by Two Way ANOVA. The data shown are mean ± SEM of n = 4-8 animals per group.

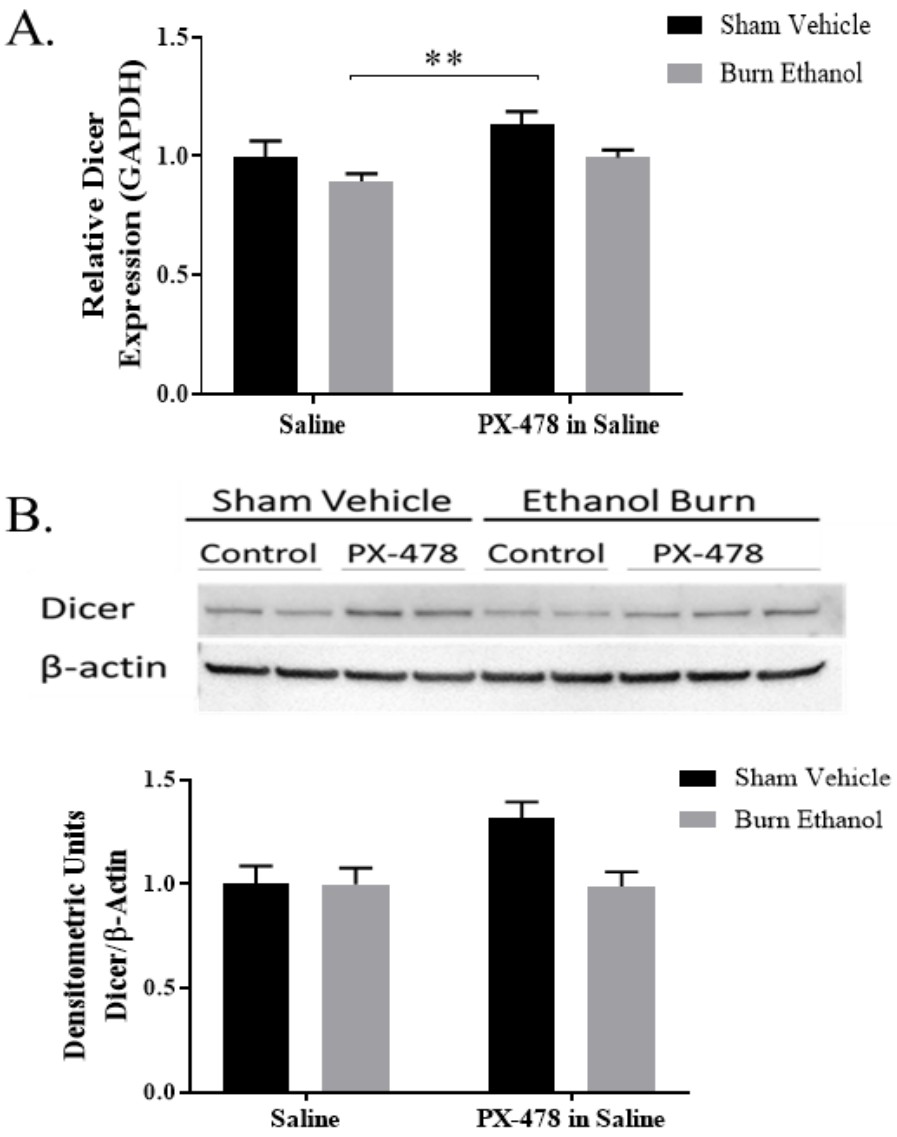

Figure 16. Effects of PX-478 Treatment on Dicer Expression in Small Intestinal Epithelial Cells One Day Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine dicer A.) mRNA expression and B.) protein levels day one following ethanol and burn injury with or without PX-478. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. GAPDH was used as an endogenous control. Densitometry measurements for each protein are given as a ratio of the protein density to β-Actin. **p<0.01 by Two Way ANOVA. The data shown are mean ± SEM of *n* = 4-8 animals per group.

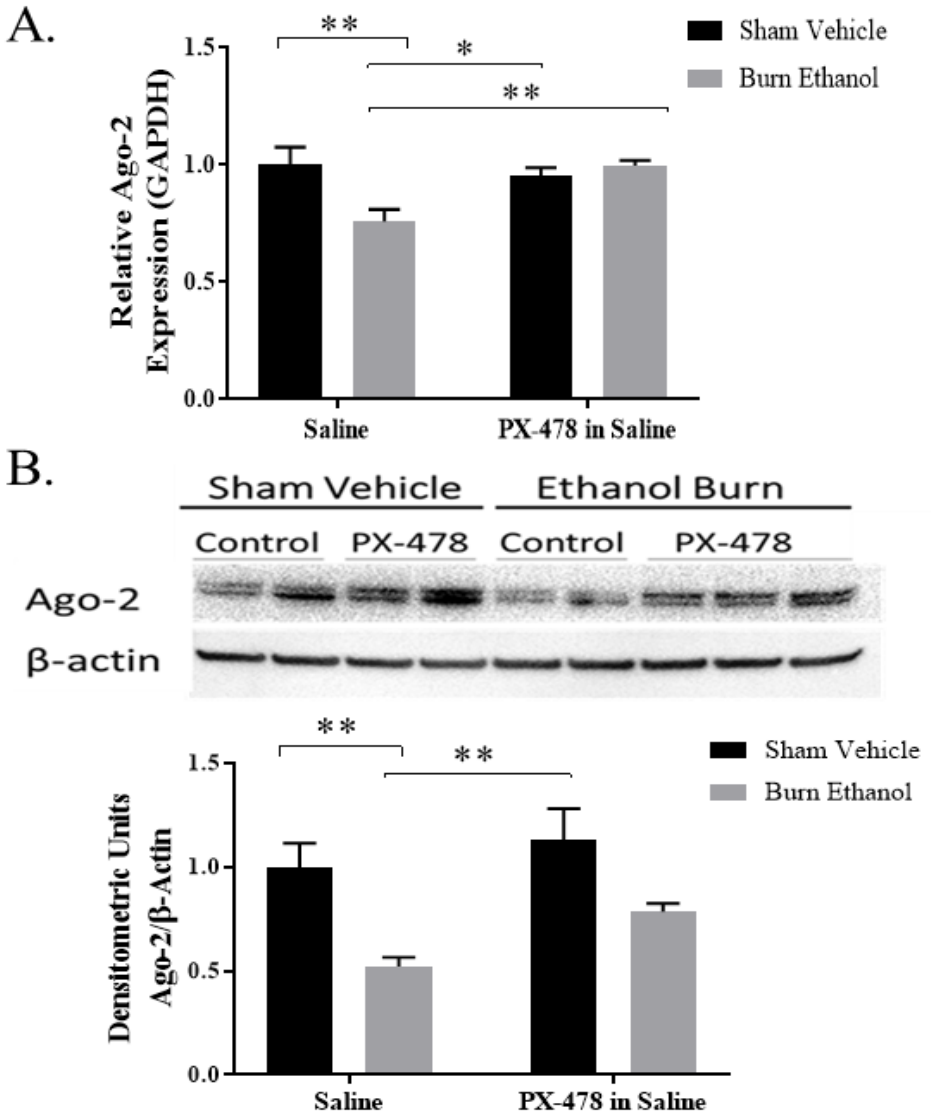

Figure 17. Effects of PX-478 Treatment on Ago-2 Expression in Small Intestinal Epithelial Cells One Day Following Ethanol and Burn Injury. Isolated intestinal epithelial cells were used to examine ago-2 A.) mRNA expression and B.) protein levels day one following ethanol and burn injury with or without PX-478. Values were calculated using a ΔΔCT method and normalized to sham vehicle animals. GAPDH was used as an endogenous control. Densitometry measurements for each protein are given as a ratio of the protein density to β-Actin. *$p < 0.05$, **$p<0.01$ by Two Way ANOVA. The data shown are mean ± SEM of n = 4-8 animals per group.

As previously demonstrated, ethanol and burn injury reduced expression of selected microRNAs relative to animals in the sham vehicle group. Here, we sought to determine whether improved expression of drosha and argonaute-2 following PX-478 extended to

improving expression of miRs (-7a and -150). Similar to our previous studies, we observed significant reductions in expression of miRs (-7a and -150) compared to sham injured animals. Treatment of mice at the time of burn injury with PX-478 increased expression of miRs (-7a and -150) (Figures 18A-B). This suggests that increased expression of microRNA biogenesis components (drosha and argonaute-2) following treatment with PX-478 at the time of burn injury resulted in increased expression of miRs (-7a and -150).

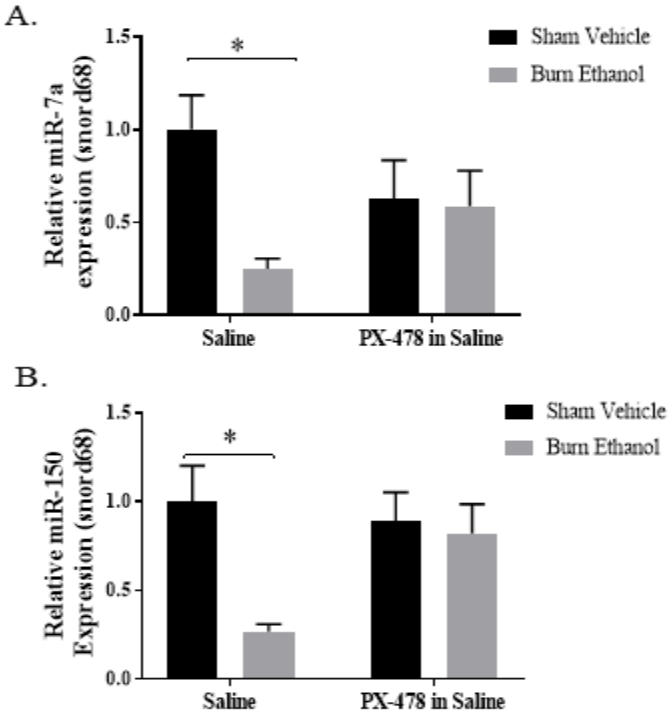

Figure 18. Effects of PX-478 Treatment on microRNA Expression in Small Intestinal Epithelial Cells One Day Following Ethanol and Burn Injury. A.) miR-7a and B.) miR-150 expression was examined in small intestinal epithelial cells day one following injury *$p < 0.05$, by Two Way ANOVA. The data shown are mean ± SEM of $n = 6$ animals per group.

microRNAs are known to regulate cellular homeostasis and specifically they have been shown to have indispensible roles in intestinal barrier maintenance. Therefore, we sought to determine whether restoration of microRNA biogeneis components and select miRs aided in

improving intestinal barrier components that are disrupted following the combined insult of ethanol and burn injury.

It has been established by our laboratory and others that ethanol and burn injury reduces expression of tight junction proteins[13,30,31]. Consistent with these findings, we observed significant reductions in (~47%) occludin, (~48%) claudin-4 and (~32%) ZO-1 expression in small intestinal epithelial cells following ethanol and burn injury compared to sham animals (Figures 19A-C). Treatment of mice immediately after the burn injury with the HIF-1α inhibitor PX-478 improved expression of these tight junction proteins in small intestinal epithelial cells. Occludin expression increased by (~29%) and claudin-4 expresssion was increased by (~23%) in mice treated with PX-478 at the time of burn injury compared to ethanol and burn injured animals who were not treated with PX-478. Similarly, ZO-1 expression increased by (~26%) after PX-478 treatment at the time of burn injury compared to untreated ethanol and burn injured animals. Additionally, other parameters of intestinal barrier such as mucins (-2 and -4), and proliferation (PCNA) were reduced following ethanol and burn injury (Appendix A-Figures 29 and 30). Treatment of mice at the time of injury increased expression of mucin-2, but did not increase expression of mucin-4 (Appendix A-Figure 29). Furthermore, PX-478 treatment increased PCNA levels suggesting that PX-478 treatment increased proliferation following the combined insult of ethanol and burn injury.

Previously our laboratory has demonstrated that ethanol and burn injury results in intestinal microbial dysbiosis and increased growth of total bacteria and *Enterobacteriaceae* relative to all groups[13,39]. Consistent with our previous finding, we saw a significant increase in both total bacteria (Figure 20A) and *Enterobacteriaceae* (Figure 20B) in small intestinal luminal contents following the combined insult of ethanol and burn injury. Our findings further show that PX-478 treatment of mice at the time of burn injury attenuates the increase in both total bacteria and

Enterobacteriaceae (Figure 20). These data illustrate that inhibition of HIF-1α prevents bacterial dysbiosis following the combined insult of ethanol and burn injury.

As PX-478 treatment at the time of burn injury improved components that can influence the intestinal barrier (miRs, and tight junction proteins), we sought to determine whether PX-478 treatment would reduce intestinal permeability. Mice were gavaged with FITC-dextran one day following the injury. 90 minutes following the FITC-dextran gavage blood was collected and levels of FITC-dextran in plasma was evaluated to determine intestinal permeability. The data show that there were significantly higher FITC-dextran levels in circulation following ethanol and burn injury (Figure 21). Consistent with our other findings PX-478 given at the time of burn injury normalized intestinal permeability compared to shams animals. Together, these data demonstrate a role of HIF-1α in intestinal permeability following ethanol and burn injury. These observed changes are likely to be due to increased levels of drosha and argonaute-2 following treatment of PX-478 at the time of burn injury which parallels with elevated miR expression, improved expression of these cellular regulators could be contributing to the reduction in intestinal permeability.

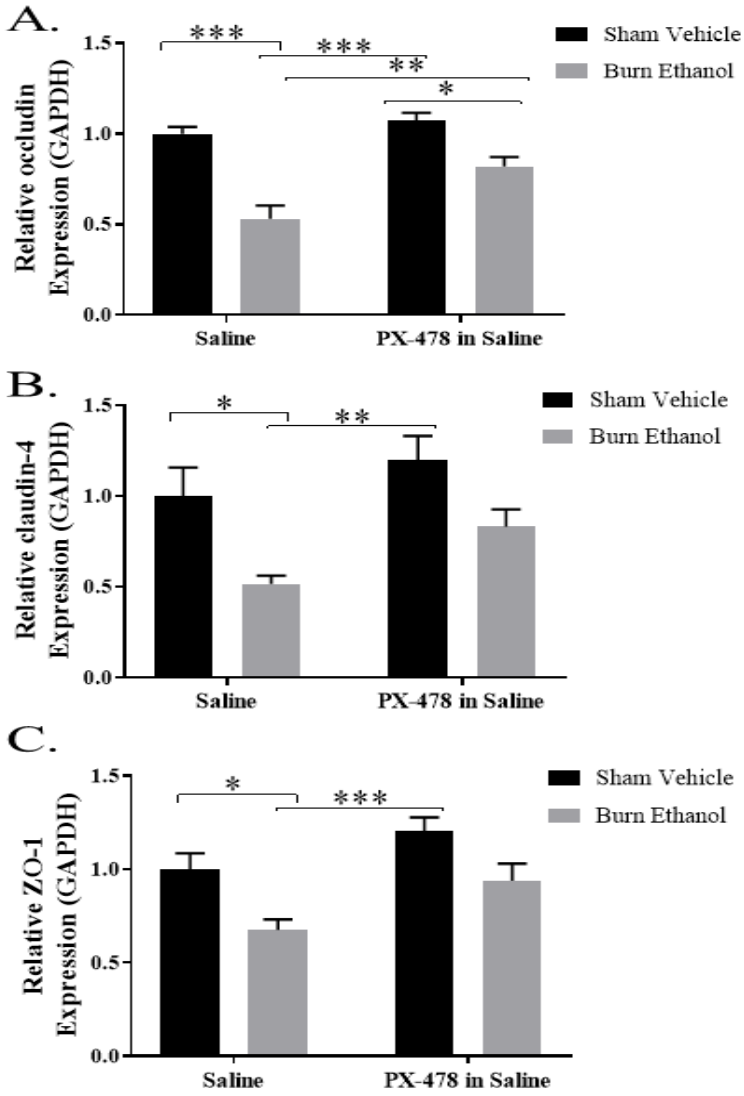

Figure 19. Effects of PX-478 Treatment on Tight Junction Protein Expression in Small Intestinal Epithelial Cells One Day Following Ethanol and Burn Injury. A.) occludin, B.) claudin-4, and C.) ZO-1 expression were examined in small intestinal epithelial cells day one following injury. *$p < 0.05$, **p<0.01, ***0.001 by Two-Way. The data shown are mean ± SEM of n = 5-8 animals per group.

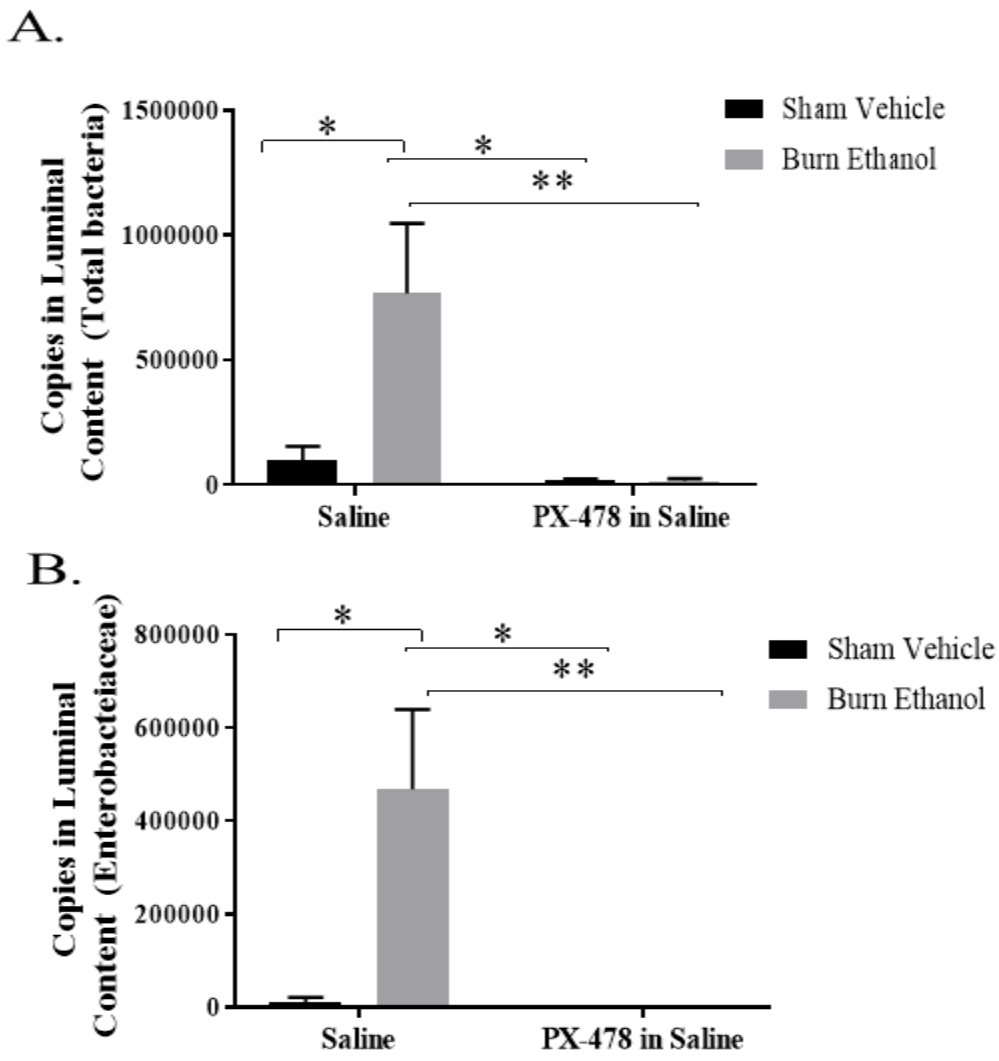

Figure 20. Effects of PX-478 Treatment on Total Bacteria and *Enterobacteriaceae* Levels One Day Following Ethanol and Burn Injury. Changes in A.) total bacteria and B.) *Enterobacteriaceae* populations within the small intestinal luminal content were analyzed one day following injury using specific primers by qPCR. *$p < 0.05$, **$p<0.01$ by Two Way ANOVA. Data are expressed as copies of 16S rRNA in luminal content relative to the sham vehicle group. The data shown are mean ± SEM of n = 4-10 animals per group.

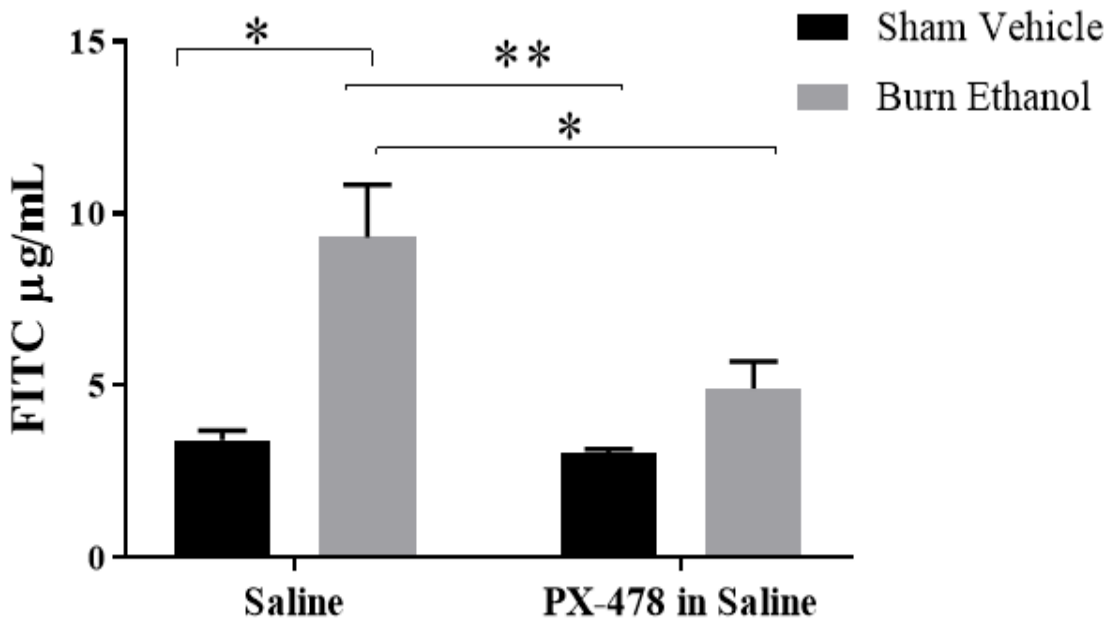

Figure 21. Effects of PX-478 Treatment on Intestinal Permeability One Day Following Ethanol and Burn Injury. Mice were gavaged with FITC-dextran one day following the injury, 90 minutes later blood was collected, and FITC-dextran levels were determined in plasma by absorbance. *$p < 0.05$, **$p<0.01$ by Two-Way ANOVA. The data shown is a combination of two independent experiments and data are mean ± SEM of n = 9-19 animals per group.

Summary

These data suggest that there is a hypoxic insult in the gut following ethanol and burn injury as there is a significant increase in HIF-1α, a marker of hypoxia. We showed that treatment of mice at the time of burn injury with PX-478, an inhibitor of HIF-1α, increased expression of microRNA biogenesis components: drosha and argonaute-2. These changes corresponded with increased expression of miRs (-7a and -150). As microRNAs regulate normal cellular homeostasis we expanded our studies to examine whether restoration of microRNA biogenesis and expression of miRs improved parameters of intestinal barrier function following the combined insult of ethanol and burn injury. Treatment of mice with a HIF-1α inhibitor at the time of burn injury improved expression of tight junction proteins. Furthermore, improvements

in these parameters due to PX-478 treatment prevented microbial dysbiosis and decreased intestinal permeability one day following injury. Together these data suggest that HIF-1α has a distinct role in expression of microRNA biogenesis components: drosha and argonaute-2 which could impact many other functions of the intestinal barrier.

CHAPTER SIX

DISCUSSION

New Contributions to the Ethanol and Burn Injury Field

The overall aim of this dissertation project was to examine the role of microRNAs in intestinal barrier disruption following ethanol and burn injury, and to identify a therapeutic approach for lessening the negative effects of ethanol and burn injury. The adverse effects following the combined insult of ethanol and burn injury are widely known, however, there is a gap in the burn field examining the contributions of microRNAs and how they can influence ethanol and burn injury pathology. The current project provided evidence that there is an increased inflammatory response day one following injury, the levels of the pro-inflammatory mediators increases distally with higher levels in the colon. Furthermore, ethanol and burn injury diminished expression of drosha and argonaute-2 proteins which correlated with reduced expression of miRs (-7a and -150). Ethanol and burn injury also significantly increased HIF-1α expression which has been shown to effect expression of microRNA biogenesis components. Administration of PX-478, an inhibitor of HIF-1α, at the time of burn injury reestablished expression of drosha, argonaute-2 and miRs (-7a and -150). PX-478 treatment also increased tight junction expression and these changes accompanied restoration of the intestinal microbiome and intestinal permeability following the combined insult of ethanol and burn injury.

Intestinal Inflammation

The major role of the intestine is the facilitation of nutrient and water absorption. In

addition to this function the intestine also has an importation role in maintenance of an immunological and physical barrier which prevents translocation of gut bacteria or their products to extra-intestinal sites. The intestine is compartmentalized into four anatomically and functionally distinct segments (duodenum, jejunum, ileum and colon)[33]. These intestinal parts have different bacterial content, with the bacterial number increasing progressively from the duodenum to the colon. The bacterial content in the duodenum and jejunum is 10^2–10^4 CFU/g content, whereas, the ileum's is 10^{10}CFU/g. The colon's bacterial content is 10^{10}–10^{12} CFU/g content [128,129]. These commensal bacteria normally reside in the gut and are protective and aid in digestion. However, dysbiosis or increased permeability in the intestine can allow for translocation into otherwise extra-intestinal sites which could lead to MOF or sepsis formation. It has been demonstrated that ethanol and burn injury negatively impacts the intestinal barrier resulting in increased bacterial translocation[149]. Due to the regional variations in bacterial content and increased bacterial translocation due to ethanol and burn injury it is important to determine if there are any differences in the inflammatory response in different parts of the intestine.

Pro-inflammatory cytokine levels were increased in total tissue homogenates collected from the jejunum, ileum and colon. Interestingly, levels were several folds higher in the colon compared to jejunum or ileum as a consequence of ethanol and burn injury. While the definitive cause for differential expression of these cytokines/chemokine in various parts of the intestine remains to be established, the progressive increase in bacterial density from duodenum to the colon is likely to contribute to an excess inflammatory response in the distal part (e.g. colon) compared to jejunum or ileum.

The neutrophil chemokine, KC increased in the distal gastrointestinal tract with higher KC levels seen in the colon following ethanol and burn injury. Circulating neutrophil sequestration to the site of injury following the combined insult is believed to be a major contributing factor to MOF. Neutrophils are the immediate responders following injury. Earlier studies from our laboratory have shown an increase in neutrophil infiltration into the tissue following a combined insult of ethanol and burn injury[89]. We observed ethanol and burn insult significantly elevated KC levels in the jejunum, ileum and colon compared to shams within 24 hours after injury. Such an increase in KC is likely to cause neutrophil recruitment to the intestine as observed in our previous studies. Similarly, the increase in IL-6 levels following ethanol and burn injury has also been associated with increased intestinal permeability[120]. Additionally, findings from both patients and animal models indicate a relationship between the elevated levels of IL-6 and the development of septic complications[150,151].

IL-18 has also been demonstrated to have a role in increased gut leakiness following combined ethanol and burn injury. Results from that study showed a role of IL-18 in decreased claudin-1 phosphorylation and occludin expression and phosphorylation compared to sham animals[10]. Similarly, occludin dephosphorylation has been demonstrated to increase intestinal permeability[135]. Additionally, *in vitro* treatment of YAMCs with recombinant IL-18 increased paracellular permeability[10]. We showed that IL-18 levels were elevated in the ileum and colon following ethanol and burn injury. We have previously demonstrated a role of IL-18 in increased neutrophil O_2^- production and their recruitment to the intestine tissue. Furthermore, IL-18 delays neutrophil apoptosis[134]. Such an increase in IL-18-dependent neutrophil infiltration into the intestine may contribute to intestinal tissue damage following ethanol and burn injury.

Ethanol and Burn Injury Effects on Expression of microRNA Biogenesis Components

microRNAs are non-coding RNAs which control gene expression at the post-transcriptional levels. The gene silencing ability of miRs allow microRNAs to regulate normal cellular homeostasis and function[15,16,18,42]. Aberrant expression of microRNAs contributes to development of pathological conditions. Numerous studies have illustrated that microRNAs can influence inflammation following exposure to ethanol and tissue injury[75,79]. Interestingly, the role of microRNAs following the two-hit model of ethanol and burn injury remains unknown.

microRNAs have been shown to regulate intestinal barrier maintenance[23,66,136], as such, aberrant miR expression could impact the intestinal barrier function. Furthermore, modulation of miR expression following ethanol and burn injury could alter levels of pro-inflammatory mediators, which are linked to tissue damage and diminished expression of tight junction proteins [10,30,31,88,134,135]. Specifically, miR-150 is reduced in sepsis patients and after *in vitro* exposure of cells to bacterial LPS[45,125]. Additionally, miR-150 is involved in regulation levels of pro-inflammatory mediators[124].

Therefore, we examined whether ethanol and burn injury modulates expression of microRNA biogenesis components and microRNAs. We observed decreased expression in mRNA and protein levels of microRNA biogenesis components (drosha and ago-2) day one following ethanol and burn injury in IECs. This was accompanied with a trend of a reduction in all miRs (miR-7a, miR-22, miR-150, miR-210 and miR-375) tested however, only miRs (-7a and-150) expression was significantly reduced in IECs day one following ethanol and burn injury. Using an *in vitro* overexpression approach, our findings further suggest that the decrease in miR-150 could potentially contribute to increased intestinal inflammation. These data suggest that altered microRNA expression and biogenesis components following ethanol and burn injury could result in increased intestinal inflammation.

microRNAs are promiscuous, each microRNA has the ability to regulate hundreds of different genes based on the complementary binding of the microRNA to its target[18]. Therefore, it is quite possible and likely true that miR-150 has targets in addition to the targets listed here. Furthermore, we cannot discount that disruption in microRNA biogenesis likely reduces expression of many microRNAs, therefore, more microRNAs maybe in play in the pathology associated with ethanol and burn injury.

Ethanol and burn injury increased levels of pro-inflammatory mediators (IL-6, IL-18 and KC) day one following injury, which are normalized to sham values day three following injury[11,12]. These elevated pro-inflammatory mediators within the intestine can contribute to intestinal tissue damage and barrier disruption following ethanol and burn injury[10,30]. These studies correlate well with the present data as we observed a decrease in miR-150 which accompanied altered microRNA biogenesis day one following the combined insult of ethanol and burn injury. Interestingly, miR-150 which had reduced expression day one following injury, has been shown to have an inverse relationships between their expression and levels of inflammatory mediators[45,122-125,138,140]. These findings agree with our *in vitro* study. We observed that overexpression of miR-150 significantly reduces levels of IL-6 (50%) and KC (20%) following stimulation with LPS. Therefore, reduced miR-150 levels observed following ethanol and burn injury likely contributes to the increased levels of pro-inflammatory mediators.

One limitation of the current study is that the *in vitro* transfection of miR-150 was measured in colonocytes and the *in vivo* studies were carried out in small intestinal epithelial cells. YAMCs were generated using 18-day old transgenic mice which have a temperature sensitive simian virus 40 large tumor antigen mutation. This mutation allowed for the creation of conditionally immortalized epithelial cell cultures. Crypts were isolated from the colonic

mucosa and they stain positive for keratin and synthesize brush border disaccharidase and peptidases[152]. While there is a possibility that small and large intestinal epithelial cells respond differently to a stimulus such as LPS, our laboratory has shown a similar trend in inflammation in both the small and large intestines (colon). The inflammatory response however, after injury was relatively of a higher magnitude in the large intestine[12]. Based on this observation, we believe that colonocytes could be used for *in vitro* studies to address the role of miR-150. Together these observations suggest that overexpression of miR-150 can cause a decrease in colonocytes release of IL-6 and KC. While, these findings remain to be confirmed in epithelial cells from the small intestine, it is likely that small intestinal epithelial cells will follow a similar trend.

microRNAs regulate key components of the intestinal barrier function including the expression of tight junction proteins, inflammatory mediators, apoptosis and proliferation[23,26,56,66,70,122,124,141]. Environmental factors (e.g. injury, ethanol, and disease) can impair microRNA expression and biogenesis[20-26], which may adversely affect the components of the intestinal barrier. McKenna *et al*. utilized dicer1$^{loxP/loxP,}$ villin –Cre mutant mice which lack the obligatory microRNA processing enzyme in the small and large intestinal epithelium. The ablation of dicer-1 altered intestinal morphology and number of goblet cells. Similarly, dicer-1 deficiency increased number of apoptotic cells and intestinal inflammation, while decreasing differentiation. Ablation of dicer-1 led to tight junction mislocalization (Claudin-7) and disruption of protein levels (Claudin-4) which coincided with diminished barrier integrity[44] . These data clearly illustrate a vital role that microRNAs play in intestinal barrier maintenance. Gaulke *et al*. have shown that environmental factors (SIV infection) also can impact microRNA biogenesis components (dicer-1 and argonaute-2) contributing to SIV mediated enteropathy [21].

Furthermore, impairment of microRNA biogenesis and increased levels of microRNA degradation machinery resulted in decreased microRNA expression. Although the present study did not observe any changes in dicer-1 expression, these studies illustrate the importance of microRNAs and their processing enzymes for normal intestine function. Therefore, the reduction of drosha and argonaute-2 following the combined insult is likely to influence normal intestinal homeostasis

Taken together, these data suggest that ethanol and burn injury decreases the expression of microRNA biogenesis components and miRs (-7a and -150) in IECs. Such a decrease in miR-150 can lead to an increase in intestinal inflammation including increased IL-6 and KC which in turn can contribute to intestinal barrier disruption observed after ethanol and burn injury.

Role of HIF-1α Following the Combined Insult of Ethanol and Burn Injury

Hypoxia causes cellular adaptation in which processes that are energy and oxygen dependent are downregulated, while increasing genes that lead to angiogenesis and stress survival. HIF-1α is a major protein elevated following hypoxia and as such widely considered to be a marker of hypoxia. Studies have determined that ethanol and burn injury alone elevates intestinal expression of HIF-1α[110,112,148]. Our laboratory has previously shown using Strotinum85 labelled microspheres that there is a ~20% decrease in intestinal blood flow day one following ethanol and burn injury. Similarly, we observed a ~25% reduction in oxygen delivery to the small intestine day one following ethanol and burn injury[14]. In the current study, we confirmed the hypoxic insult in the gut as HIF-1α is significantly elevated (Figure 14) following the combination of ethanol and burn injury.

HIF-1 is a heterodimer of HIF-1α and HIF-1β which acts as a transcription factor that regulates genes necessary to allow for cell survival and adaptation during hypoxic conditions.

HIF-1α is constitutively expressed however, under instances of normoxia (~21% oxygen), the protein is post-translationally modified. Under normoxic conditions HIF-1α has a half-life of ~5 minutes where the protein is hydroxylated on two proline residues and acetylated on a lysine reside within the ODDD on HIF-1α. This triggers association of HIF-1α to the pVHL ubiquitin E3 ligase complex which results in its ubiquitin-proteasome pathway dependent degradation. Under hypoxic conditions (~1% oxygen), HIF-1α is stabilized where it binds to HIF-1β forming HIF-1 which acts as a transcription factor[113,153].

In addition to HIF-1α, there are also HIF-2α and HIF-3α that can interact with HIF-1β. HIF-1α is the most active isoform and is active in fast (2–24 h) and severe hypoxic conditions. HIF-2α is more slower acting (48-72h) and drives the response during chronic hypoxia. HIF-2α has 48% amino acid homology with HIF-1α however, unlike its counterpart HIF-2α is not ubiquitous and is mainly expressed in the endothelium, carotid body and the lung[113,114]. While HIF-3α also binds to HIF-1β, its splice variant is believed to have an inhibitory effect, preventing the binding of HIF-1α[113,153].

In the current study, we examined the relationship of HIF-1α on expression of microRNA biogenesis components (drosha and argonaute-2) and whether this influences intestinal barrier integrity. Mice were treated with PX-478 at the time of burn injury to assess the role of HIF-1α in reduced expression of drosha and arognaute-2 following the combined insult. Although, the mechanisms of action for this small molecule has not been elucidated, PX-478 (*S*-2-amino-3-[4′-*N*,*N*,-bis(chloroethyl)amino]phenyl propionic acid *N*-oxide dihydrochloride) has been shown to decrease expression of HIF-1α mRNA, protein levels, deubiquitination and activity[153,154]. While we did not measure HIF-1β protein levels, PX-478 is not believed to affect its expression[153]. Koh *et al*. exposed MCF-7 cells to hypoxia, which had maximum expression of HIF-1α after 8

hours of exposure. Interestingly, inhibition of HIF-1α was not observed until 8 hours of hypoxic exposure and complete inhibition was not observed until 16 hours after hypoxia but was maintained for up to 20 hours[153]. These data illustrated that PX-478 has a delayed effect in inhibiting HIF-1α however it is long lasting.

Elevated levels of HIF-1α has been associated with increased histone deacetylase (HDAC) activity[155]. Furthermore, use of the HDAC inhibitor valproic acid has been shown to reduce expression of HIF-1α and improve deleterious effects (e.g. diminished expression of tight junction proteins) of elevated HIF-1α[110]. The relationship between HDAC and HIF-1α is one that requires more exploration as it affects many processes including expression of microRNA biogenesis components[115,116]. Rupaimoole *et al*. demonstrated that increased hypoxia downregulates expression of drosha in a HIF-1α dependent mechanism via ETS1 and ELK1 recruitment of HDAC1 and ARID4B onto its promoter region. Treatment with an HDAC inhibitor (valproic acid) normalized drosha expression[115]. Together these studies suggest that valproic acid reduces HIF-1α expression, which improves drosha and subsequently expression of miRs which reduces harmful effects of elevated HIF-1α such as diminished tight junction protein expression. This study supports our data, as we saw diminished reduction in drosha which coincided with elevated HIF-1α. Furthermore, the group found that hypoxia did affect dicer expression but in a HIF-1α independent fashion. We observed no changes in dicer following ethanol and burn injury and no improvement with inhibition of HIF-1α via PX-478. There are conflicting reports that hypoxia may help or hinder argonaute expression and miR loading onto the argonaute protein[144-146]. Our studies show that following ethanol and burn injury there is diminished expression of argonaute-2 which is normalized to sham values when mice are treated with PX-478. These findings suggest that HIF-1α negatively impacts

expression of drosha and argonaute-2 following ethanol and burn injury, which is normalized with treatment of PX-478.

It is not surprising that as expression of microRNA biogenesis components is negatively impacted by hypoxia so is expression of microRNAs. Rupaimoole *et al*. showed that hypoxia led to >40% reduction in precursor miRs, and >60% reduction in mature miRNAs compared to normoxic cells[115]. As we did not observed changes in both drosha and dicer we were not surprised that we did not observe global downregulation of miRs. We determined however, that miR-150 is significantly diminished following ethanol and burn injury. Yu *et al*. demonstrated that miR-150 has an inverse relationship with HIF-1α and is significantly reduced during hypoxia, as it is predicted to target VEGF-α which is a downstream target of HIF-1α[156]. Consistent with these findings use of the HIF-1α inhibitor at the time of burn injury increased expression of miR-150.

HIF-1α has conflicting roles within the body. Some studies attest to the beneficial effects of HIF-1α. While other have shown that it is detrimental[110,112,148]. Yun *et al*. determined that inhibition of HIF-1α via PX-478 following binge ethanol exposure decreased levels of 3-nitrotyrosine and apoptosis in hepatocytes[148]. Studies have demostrated that large burn injury significantly elevates HIF-1α in the gut while reducing expression of tight junction proteins[110,112]. Furthermore, Luo *et al*. demonstrated that treatment of rats with valproic acid a histone deaceytalase inhibitor (HDACIs) represses HIF-1α expression and improved expression of tight junction proteins and intestinal barrier[110]. Consistent with these findings, we observed that HIF-1α is elevated following ethanol and burn injury (Figure 14), while tight junction proteins exhibit reduced expression[13,30,31]. Furthermore, treatment at the time of burn injury with PX-478 improved expression of tight junctions and improved intestinal barrier (Figure 19

and Figure 21), this likely occurs through improved microRNA expression. microRNAs have been shown to regulate numerous cellular processes including tight junction protein expression[23,66,71].

It is widely accepted that hypoxia increases inflammation and NFκB activation however, inflammation can also stabilize HIF-1α resulting in "inflammatory hypoxia"[157-159]. Furthermore, our laboratory and others have shown an inverse relationship of miR-150 and inflammatory mediators[45,81,124]. Together these results suggest that elevated HIF-1α levels following ethanol and burn injury reduces expression of microRNA biogenesis components (drosha and ago-2) resulting in diminished miR-150 expression which leads to elevated intesinal inflammation following the combined insult.

Given the strict oxygen requirements of the gut microbiome, it not suprising that shifts in oxygen levels could result in microbiome dysbiosis. We observed significant elevation in bacterial copies of both total bacteria and *Enterobacteriaceae* following ethanol and burn injury using specific primers that target the 16S rRNA region of total bacteria and the family of Gram-negative bacteria *Enterobacteriaceae*. Studies have suggested that anaerobic intestinal bacteria are more abundant than the gram-negative enteric bacteria which prevents the gram-negative enteric bacteria from attaching to the intestinal epithelium[109,160]. We observed a significant increase in *Enterobacteriaceae*, a family of gram-negative bacteria following ethanol and burn injury which could be shifting this relationship (Figure 20). Treatment of mice with PX-478 attenuated the observed increase in total bacteria and *Enterobacteriaceae*. Currently, we are unsure, why this phenomenon is occurring as treatment with PX-478 does not reduce hypoxia in the gut following ethanol and burn injury but prevents the hypoxic signaling pathway through inhibition of HIF-1α. These changes could be occurring due to the restoration of the intestinal

barrier. Furthermore, these changes could be a result of normalization of microRNAs expression, as microRNAs have been shown to regulate intestinal bacteria[43].

As depicted in Figure 22, ethanol and burn injury results in diminished expression of microRNA biogeneis components (drosha and ago-2) and miRs (-7a and -150) compared to shams in small intestinal epithelial cells. This phenomenon, likely occures due to the hypoxic insult in small intestinal epithelial cells (shown as a red cell to portray hypoxic insult on small intestinal epithelial cells) following the combined insult. Use of the HIF-1α inhibitor PX-478 at the time of burn injury, restores expression of microRNA biogenesis components:drosha and argonaute-2 in small intestinal epitheilal cells (shown as a white cell to represent inhibition of HIF-1α signaling) (Figure 23). This restoration coincides with elevation of miR-150 which has been shown to negatively impact levels of inflammatory mediators. Changes in microRNA expression likely influences downstream effects of ethanol and burn injury including improved expression of tight junction proteins, microbial dysbiosis and intsesinal permeability.

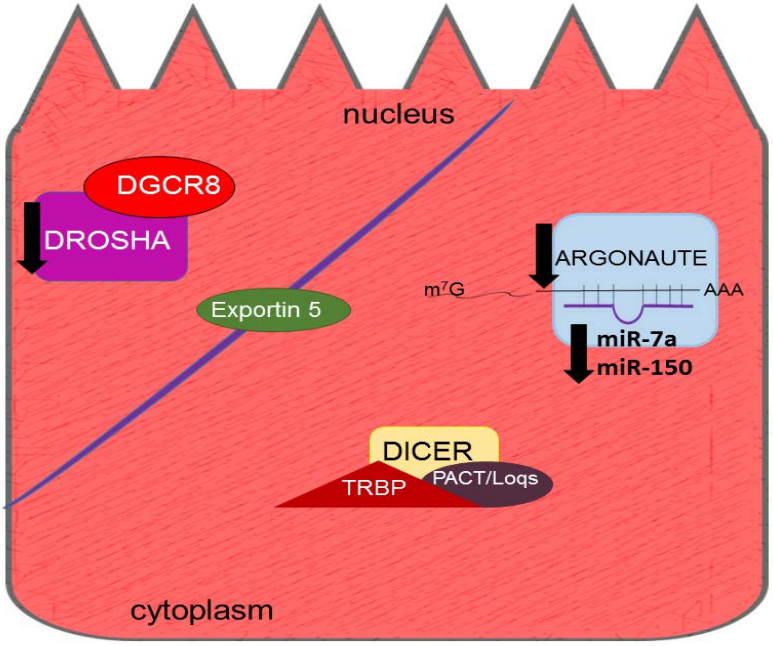

Figure 22. Schematic Depicting Relationship Between HIF-1α and microRNA Biogenesis. Ethanol and burn injury results in elevated levels of HIF-1α in small intestinal epithelial cells (red cell denotes hypoxia insult) which leads to diminished expression of drosha and argonaute-2 which coincides with reduced expression of miRs (-7a and 150).

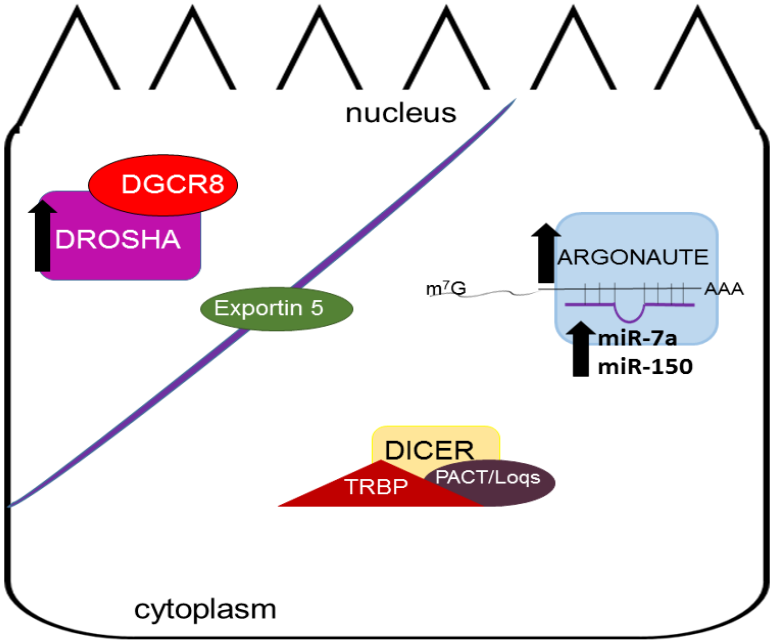

Figure 23. Schematic Depicting Treatment of Mice with PX-478 at the Time of Burn Injury. Treatment of mice at the time of burn injury with PX-478 to inhibit HIF-1α in small intestinal epithelial cells (white cell denotes inhibition of hypoxic signaling) resulted in increased expression of drosha and argonaute-2 which coincided with elevated expression of miRs (-7a and 150).

Limitations

Unfortunately, there are numerous limitations to the studies associated with this dissertation project. As the ultimate aim of research is to be translational, we feel that our work would have greatly benefited from the use of human samples after ethanol and burn injury. Unfortunately, due to limited resources as patients do not commonly need to undergo intestinal biopsies or non-invasive procedures we were not able to include these in our studies. Another limitation on our studies is that our experiments were only performed using male mice. We currently do not include female mice in our studies due to the necessity of aligning female mice estrous cycles as estrogen levels could influence our results. Our laboratory will be including both female and male mice in future studies as the National Institutes of Health require female and male mice to be used.

One major limitation in our study to definitively link diminished miR expression to the adverse effects observed following ethanol and burn injury would be that miR mimics were not used. miR mimics are chemically synthesized double-stranded RNA oligonucleotides which emulate the function of endogenous mature miRNAs. Our studies would have greatly profited if we had pretreated mice by oral gavage with a miR-150 mimic prior to ethanol and burn injury. This could have showed a direct relationship between miR-150 and intestinal damage following the combined insult of ethanol and burn injury.

Final Conclusions

Our current work has introduced a molecule of interest in the pathology of ethanol and burn injury. We showed that there is an inflammatory response throughout the GI tract following the combined insult of ethanol and burn injury. The magnitude of the inflammatory response increases progressively in the distal end of the gastrointestinal tract (colon) which

interestingly follows the pattern of the bacterial concentration in the GI tract. Furthermore, we observed that there is a significant microbial dysbiosis in the small intestine following the combined insult compared to sham injured animals. As the intestinal microbiome is now linked to many disease states this relationship needs to be further explored following ethanol and burn injury.

Interestingly, we also confirmed that drosha, argonaute-2 and miRs (-7a and-150) are significantly reduced following ethanol and burn injury compared to the sham vehicle group (Figure 24). Additionally, *in vitro* overexpression of miR-150 in YAMCs illustrates that the miR has a role in regulation of the inflammatory response. Previously, studies have demonstrated an inverse relationship between hypoxia and expression of microRNA biogenesis components. Treatment of mice at the time of injury with a HIF-1α specific inhibitor PX-478 improved expression of drosha, argonaute-2 and miRs (-7a and -150) (Figure 25). Furthermore, we saw improvements of many parameters disrupted following the combined insult of ethanol and burn injury (tight junction protein expression, microbial dysbiosis, and intestinal permeability) compared to shams (Figure 25). More work is needed to definitively show that improvements in these components are directly related to miR expression following ethanol and burn injury.

Together, this work has illuminated the relationship of hypoxia and microRNAs and their role in regulation of the intestinal barrier. Furthermore, in providing a potential mechanism behind the adverse effects following ethanol and burn injury, we have shown two potential therapeutic strategies (miR-150 and PX-478) for patients who experience ethanol and burn injury. Future research will continue to focus on exploring this relationship and add to the wealth of knowledge into the role of ethanol in pathology of burn injury.

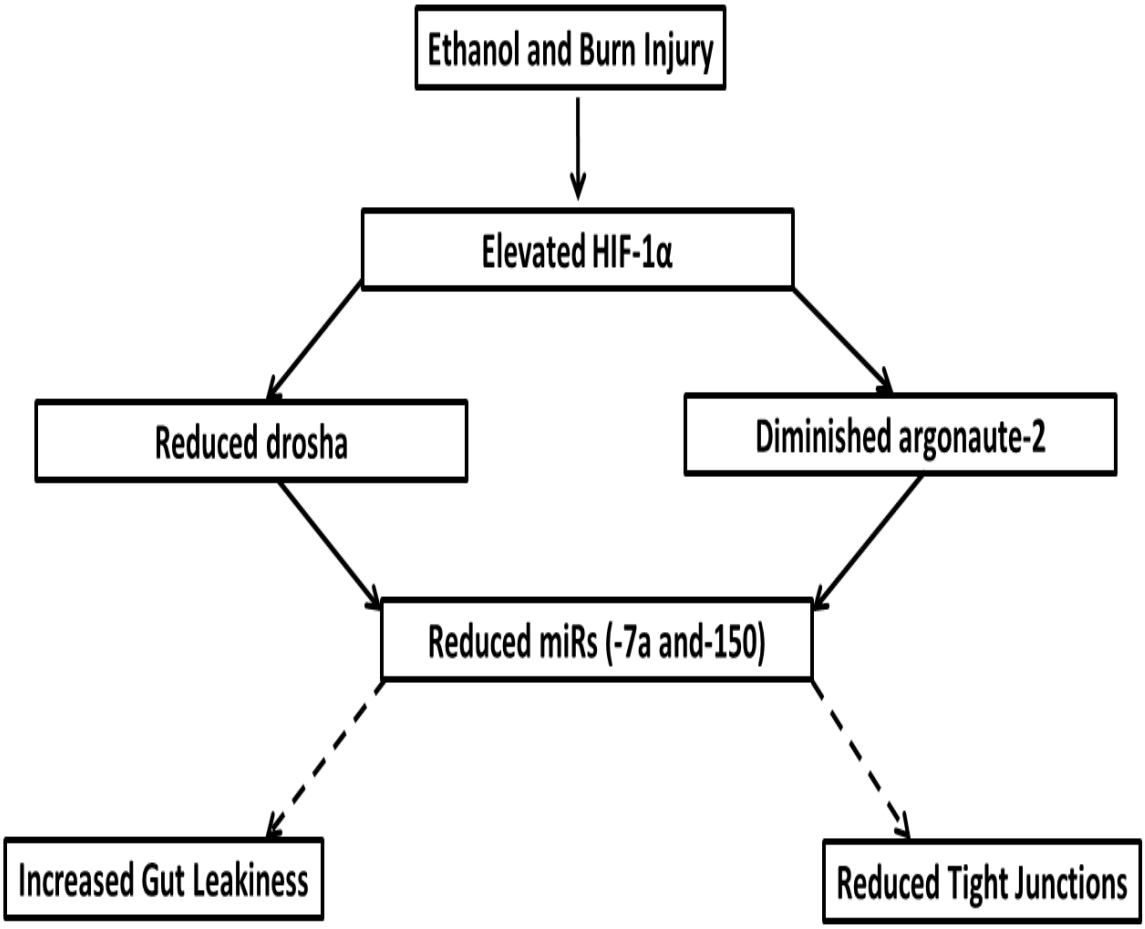

Figure 24. Schematic Depicting Major Findings Following the Combined Insult of Ethanol and Burn Injury. Ethanol and burn injury results in diminished expression of drosha and argonaute-2 which coincides with reduced expression of miRs (-7a and -150) which parallels with increased gut leakiness and reduced tight junction protein expression following ethanol and burn injury.

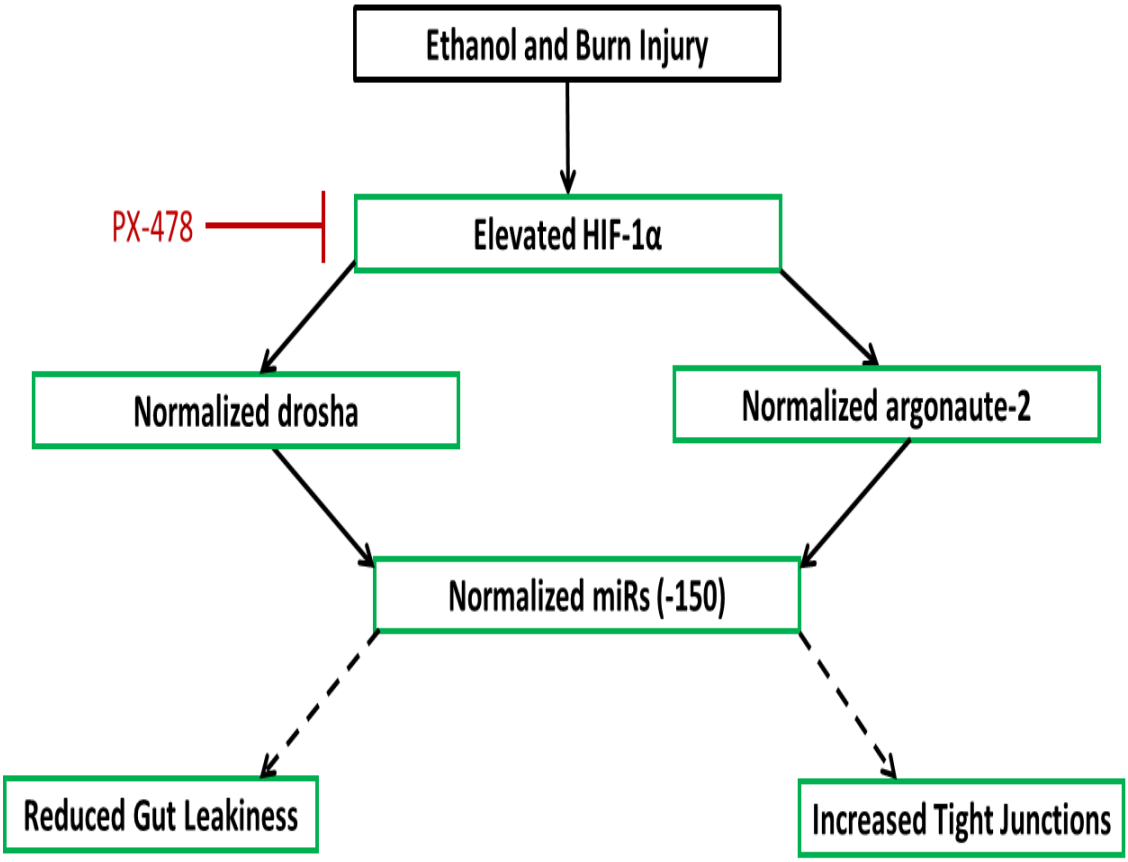

Figure 25. Schematic Depicting Major Findings Following Ethanol and Burn injury with PX-478 Treatment. Treatment of mice at the time of burn injury with PX-478 led to improved expression of drosha, argonaute-2 and miR-150. Furthermore, PX-478 treatment increased tight junction protein expression and reduced gut leakiness following the combined insult of ethanol and burn injury.

APPENDIX A

SUPPLEMENTAL FIGURES

To confirm that HIF-1α is significantly elevated following ethanol and burn injury, we measured expression of VEGF-α and HO-1 downstream targets of HIF-1α. We observed that HO-1 mRNA expression was significantly elevated in small intestinal epithelial cells following ethanol and burn injury compared to all groups (Figure 26A). Furthermore, there was a trend of increased expression of VEGF-α in small intestinal epithelial cells day one following the combined insult of ethanol and burn injury however, this was not found to be significantly different from the sham vehicle group (Figure 26B). These data provide further evidence that there is a hypoxic insult occurring in the gut following ethanol and burn injury.

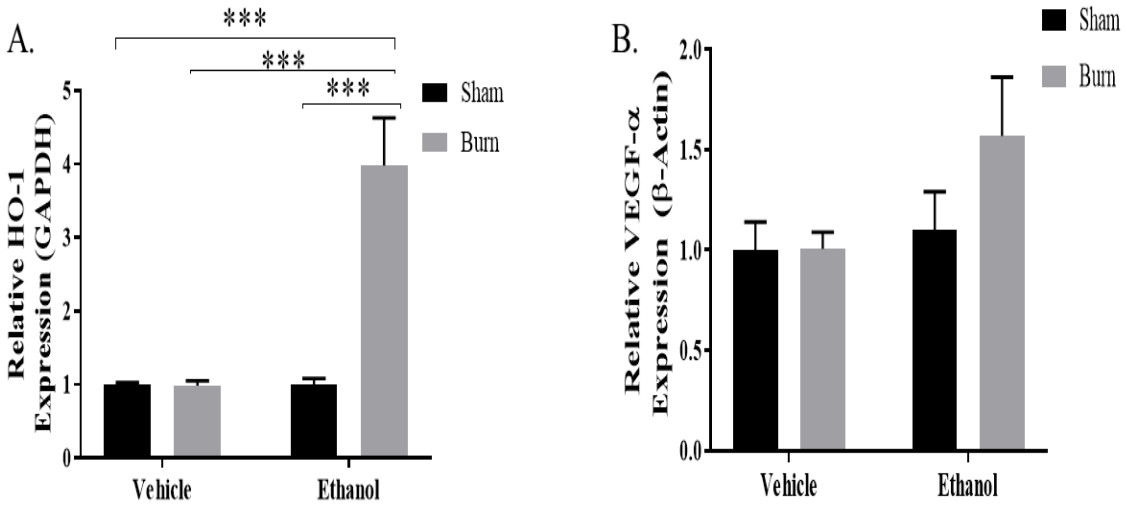

Figure 26. Effects of Ethanol and Burn Injury on HIF-1α Downstream Targets in Small Intestinal Epithelial Cells. A.) HO-1 and B.) VEGF-α levels were measured in small intestinal epithelial cells day one following injury. ***$p<0.001$ by Two Way ANOVA. The data shown are mean ± SEM of n = 5-7 animals per group.

Mice were treated with PX-478 immediately after burn injury to determine the role of HIF-1α in modulating expression of microRNAs biogenesis components and microRNAs. We evaluated HIF-1α expression following treatment by immunofluorescent staining. We observed reduced HIF-1α staining following PX-478 treatment administered at the time of burn injury (Figure 27). Furthermore, we saw a significant reduction in the HIF-1α downstream target HO-1

in small intestinal epithelial cells day one following ethanol and burn injury with PX-478 treatment (Figure 28).

As microRNAs are major regulators of normal cellular homeostasis we investigated whether the improved expression of drosha and argonaute mediated by PX-478 extended to improved other parameters disrupted following ethanol and burn injury. We measured expression of mucin-2 and mucin-4 in IECs day one following the combined insult of ethanol and burn injury. Consistent with previous findings the mucins (-2 and -4) were significantly reduced day one following the combined insult compared to sham vehicle mice. We observed however, that only mucin-2 expression was improved with treatment of mice at the time of burn injury with PX-478 (Figure 29). Furthermore, we evaluated levels of proliferating cell nuclear antigen (PCNA), a marker of proliferation in IECs day one following ethanol and burn injury. PCNA expression was reduced following the combined insult of ethanol and burn injury which was elevated with treatment of mice at the time of injury with PX-478 (Figure 30). Together, these data provide additional evidence that inhibition of HIF-1α via PX-478 improves numerous components necessary for maintenance of the intestinal barrier and these changes are likely due to the restoration of drosha, argonaute-2 and microRNAs.

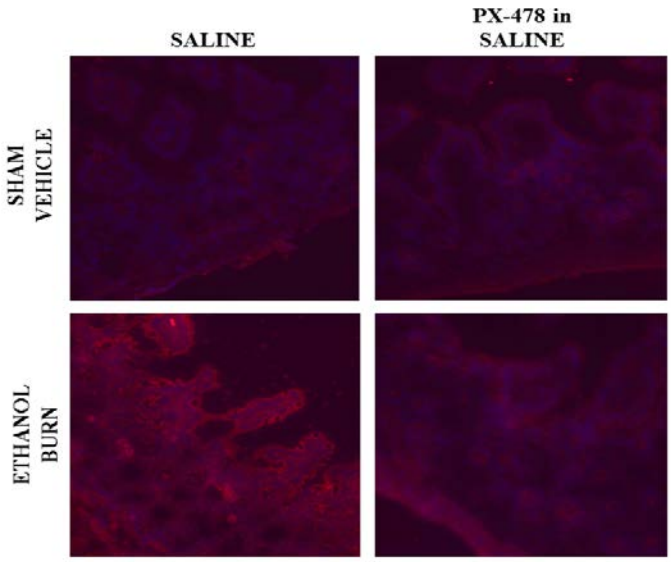

Figure 27. Effects of PX-478 Treatment on HIF-1α Immunostaining One Day Following Ethanol and Burn Injury. Immunofluorescent staining was performed on 5μm frozen sections using anti-HIF-1α to assess HIF-1α levels in small intestinal tissue one day following the ethanol and burn injury.

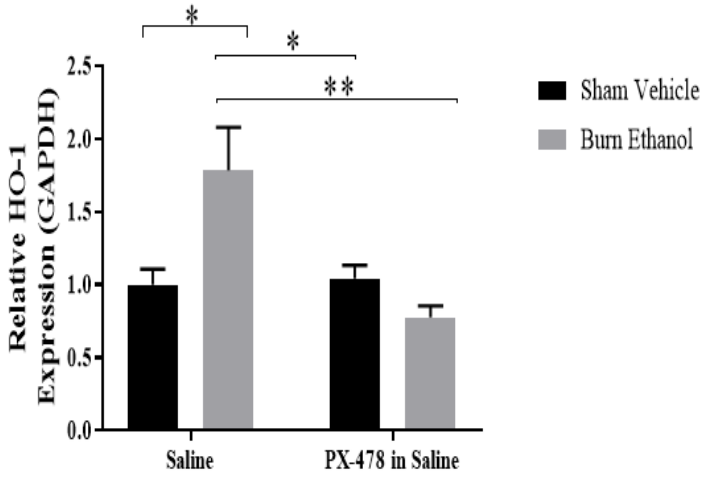

Figure 28. Effects of PX-478 Treatment on HO-1 Expression One Day Following Ethanol and Burn Injury. Expression of HO-1, a HIF-1α downstream target was examined in small intestinal epithelial cells day one after ethanol and burn injury. *$p < 0.05$, **p<0.01 by Two Way ANOVA. The data shown are mean ± SEM of n = 4-8 animals per group.

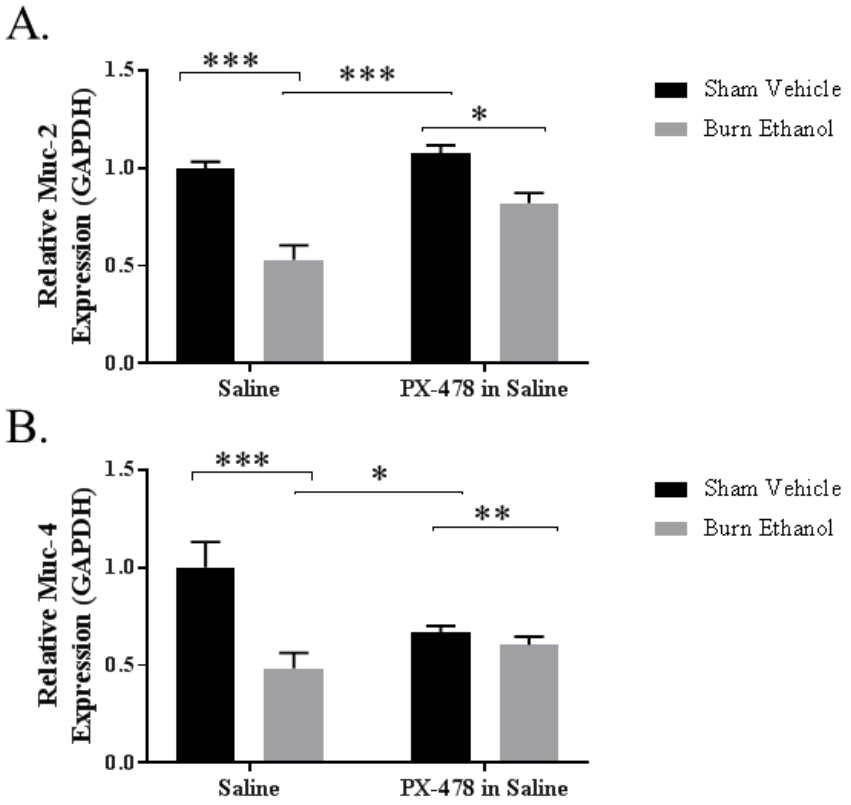

Figure 29. Effects of PX-478 Treatment on Expression of Mucins One Day Following Ethanol and Burn Injury. A.) mucin-2 and B.) mucin-4 expression was evaluated in small intestinal epithelial cells day one following injury. A*p<0.05, **p<0.01, ***p<0.001 by Two Way ANOVA. The data shown are mean ± SEM of *n* = 6-8 animals per group.

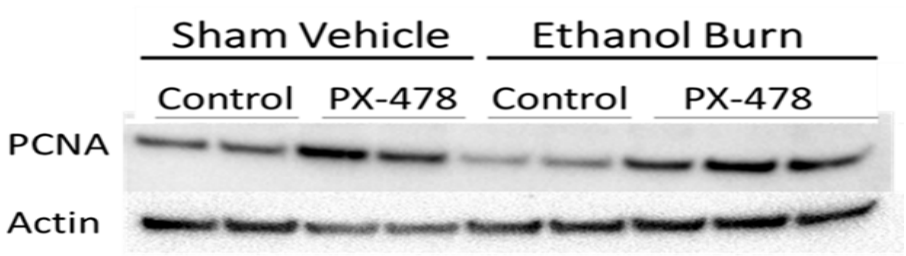

Figure 30. Effects of PX-478 Treatment on PCNA Expression in Small Intestinal Epithelial Cells One Day Following Ethanol and Burn Injury. Intestinal epithelial cells isolated from small intestine tissue were probed for PCNA protein expression by Western blot.

APPENDIX B

DETAILED MATERIALS AND METHODS

Mouse Model of Acute Ethanol Intoxication and Burn Injury

Materials and Reagents.

- Two Water baths
- Timer
- Thermometer
- Absorbent pads
- Template fabricated to expose ~12.5% surface area for 25g mouse
- 1 mL syringes with 20-oral gavage needle
- 1 mL syringes with 27-gauge precision glide needles
- 3 mL syringes with 27-gauge precision glide needles
- Ethanol 200 Proof
- Sterile Water
- Heating pads
- Hair clippers
- Scale
- Bacteriostatic 0.9% sodium chloride (sterile)
- Ketamine
- Xylazine

Protocol.

1. Fill and heat one water bath (for burn group) to 85°C, the other water bath (for sham group) fill with lukewarm water.

2. Prepare a 25% ethanol solution in sterile water (for ethanol group) and water (for vehicle group).

3. Tail mark mice and gavage mice in ethanol group with 0.4ml of the 25% ethanol solution. Gavage mice in the vehicle group with 0.4ml of sterile water. Return mice to their cages in the animal facility.

4. Four hours later, weigh the mice. Return mice to their cages and place cages on warm heating pads.

5. Give mice a cocktail of anesthesia of 80 mg/kg ketamine hydrochloride and 1.2 mg/kg xylazine by intraperitoneal injection.

6. After the mice are sleep, shave the dorsal surface of the mice.

7. Place mice onto a template fabricated to expose ~12.5% of their dorsal surface.

8. Mice undergo a burn injury by placing mice contained in the burn template into 85°C water bath allowing only the dorsal surface of the mouse to make contact with the water for 7 seconds.

9. After 7 seconds, remove mice from the water and the burn template and dry on absorbent pad.

10. Give mice 1mL of normal saline resuscitation by intraperitoneal injection and return them to their cages where they can be monitored for 3-4 hours. After this period the cages can be returned to the mouse room.

11. Perform steps 7-10 with sham animals but using the lukewarm water bath.

Intestinal Epithelial Cell Isolation

Materials and Reagents.

- Water bath
- Vortex
- 50 mL conical tubes
- 100um filters
- 37°C shaker incubator
- Petri dishes
- Forceps
- centrifuge set to 4°C
- chilled sterile 1X PBS
- Hemocytometer
- Microscope
- Trypan blue
- <u>PBS antibiotics solution:</u>

  1X PBS (sterile), 500ml

  1% penicillin-streptomycin cocktail

  Gentamycin (50mg/ml)

- <u>Pre-digestion solution:</u>

  1X HBSS (without $Ca2^{+}$ and Mg^{2+})

  5% FBS (Heat inactivated)

  Gentamicin (50mg/ml)

  1% HEPES (1M)

1% penicillin-streptomycin cocktail

5mM EDTA

1 mM DTT

Protocol.

1. Prepare pre-digestion solution in sterile bottle and warm to 37°C in water bath.

2. Chill centrifuge to 4°C

3. Harvest intestine tissue without fat and open longitudinally. Place collected tissue in a 50ml conical tube containing 20ml of ice cold PBS antibiotics solution and store tube on ice.

4. Forcibly vortex tube for 10 seconds. Remove contents of 50ml conical (intestine and PBS antibiotics solution) into a petri dish. Return tissue to 50ml conical and add more ice-cold PBS antibiotics solution. Repeat step 4 twice to wash the intestine tissue.

5. After final wash return intestine tissue to 50ml conical and add 10ml of warm pre-digestion solution.

6. Incubate tube for 20 min under slow rotation (250 RPM) at 37°C.

7. Forcibly vortex tube for 10 seconds. Collect pre-digestion solution, leave tissue undisturbed. Dispense the pre-digestion solution (which now contains isolated epithelial cells disrupted from the intestine mucosa) into 100um filter with a new 50ml conical tube.

8. To maximize cell count repeat steps 5-7 using the same tissue and 100um filter.

9. Centrifuge tubes at 1500 RPM for 10 minutes at 4°C.

10. Remove supernatant. Wash cells with 10ml cold 1X PBS. Repeat step 10.

11. Remove supernatant and resuspend cells in 1ml 1X PBS.

miR-150 Transfection and LPS Stimulation

Materials and Reagents.

- Young Adult Mouse Colonocytes
- Young Adult Mouse Colonocyte Media

  RPMI 1640 media

  2 mM glutamine

  1% Antibiotic-Antimycotic Solution

  1% ITS + Premix

  5% FBS
- 250ng of miR-150 expression plasmid
- PCMVMIR empty vector control
- Lipofectamine 2000
- Lipopolysaccharide
- Phosphate buffer solution
- Opti-Mem Reduced Serum Media

Protocol.

1. Plate $6x10^5$ YAMCs

2. One day after plating remove cells and prepare transfection solutions: Dilute 250 ng of miR-150 expression plasmid, PCMVMIR and Lipofectamine in Opti-Mem Medium. Incubate separately for five minutes. After the incubate mix miR-150 plasmid/Lipofectamine 2000 and PCMVMIR empty control/Lipofectamine mixture and incubate at room temperature for 20 minutes.

3. Add mixtures to the YAMCs cells and incubate the cells for 48 hours at 37°C.

4. Following the incubation, wash cells with PBS and then treat with LPS (100 ng/ml) for 6 h at 37°C.

5. After the 6 h LPS treatment, both the supernatant and cells are collected.

6. The cells were lysed and used for RNA isolation, total and enriched RNA were used for cDNA synthesis and subsequent qPCR. IL-6 and KC levels were measured from the supernatant were determined by ELISA kits according to the manufacturer's instructions.

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VITA

Niya Latrice Morris was born on the westside of Chicago, Illinois to Linda Morris. Her mother raised Niya and her brother Jermal with the constant support of her grandmother Bernice Morris and aunt Marsha Williams. Niya attended Lewis University where she received a Bachelor of Science in Biology and a Bachelor of Arts in Chemistry in May 2006. Several years later after working in both academia and in industry, Niya decided to continue her education receiving her Master of Science in Biology at the Illinois Institute of Technology in May 2012.

In August 2012, Niya matriculated to Loyola University Chicago Biomedical Sciences Program, joining their Integrative Cellular Biology Program. Niya joined the laboratory of Dr. Mashkoor Choudhry to study the role of microRNAs in impaired gut permeability following ethanol and burn injury. During her tenure in Dr. Choudhry's laboratory Niya was supported by a training fellowship from Loyola's Alcohol Research Program Training Grant funded by the National Institute on Alcohol Abuse and Alcoholism, before receiving a Research Supplements Grant to Promote Diversity in Health-Related Research. During her time at Loyola, Niya has authored and co-authored numerous research articles, and presented at both national and international meetings. After successful completion of her graduate studies, Niya will begin a post-doctoral fellowship at Emory University in the laboratory of Dr. Samantha Yeligar.