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MicroRNA-24 and Ulcerative Colitis: Expression, Functionality, and Therapeutic Potential

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

by

Artin Soroosh

2021

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ABSTRACT OF THE DISSERTATION

MicroRNA-24 and Ulcerative Colitis: Expression, Functionality, and Therapeutic Potential

by

Artin Soroosh

Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

University of California, Los Angeles, 2021

Professor Charalabos Pothoulakis, Chair

Ulcerative Colitis is a chronic inflammatory disease of the gastrointestinal tract that affects over 1 million people in the United States resulting in $2.7 billion in healthcare costs each year. Ulcerative Colitis is characterized by chronic, uncontrolled inflammation localized to the colon and rectum which leads to debilitating symptoms and significant complications. This disease has limited treatment options, with 20-25% of patients needing surgical intervention to alleviate symptoms. Many researchers are focused on finding new therapeutic targets for Ulcerative Colitis to aid those patients for whom the current treatments are not effective. One potential target, microRNAs, are small, single-stranded RNAs with the ability to repress gene expression. Many microRNAs are implicated in the pathophysiology of different diseases, including Ulcerative Colitis. MicroRNA-24 is a specific microRNA that is associated with multiple cancers, cardiovascular disease, and inflammatory activity. Here, we describe the expression, function, and therapeutic potential for microRNA-24 in Ulcerative Colitis. Our data reveal that microRNA-24 is elevated in Ulcerative Colitis and disrupts intestinal barrier function by targeting the tight junction protein cingulin. Furthermore, microRNA-24 inhibition in mouse models of colitis impaired epithelial restitution during the recovery period. We discovered that microRNA-24 robustly regulates apoptosis in the

intestinal epithelium. Consequently, microRNA-24 inhibition promoted cell death which hindered mucosal repair. Taken together, this work highlights microRNA-24 as a relevant factor in the pathogenesis of Ulcerative Colitis that deserves further study to unlock its utility as a therapeutic target.

The dissertation of Artin Soroosh is approved.

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2021

DEDICATION

I dedicate this to those that inspire me.

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ACKNOWLEDGEMENTS

I would like to acknowledge my advisor, Dr. Charalabos Pothoulakis. His mentorship was invaluable throughout my time as a PhD candidate. As a leader in digestive disease research, his impact is immeasurable. I consider myself lucky to have had him as a mentor and colleague. I also thank the members of my committee: Dr. Lin Chang, Dr. Joseph Pisegna, and Dr. Thomas Vallim. Their guidance during my graduate career was invaluable and their confidence in my abilities was a strong motivator. I would also like to acknowledge my previous mentors, Dr. David Padua and Dr. Dimitrios Iliopoulos. Both were instrumental for the progression of the projects and my personal development as a scientist.

I was part of a lab that was a vast group of experts in all types of research. My main colleague for much of this work was Dr. Carl Rankin, who was invaluable as a coworker. His experience in a wide variety of scientific concepts and experimental protocols was a significant reason for the completion of these projects. Further colleagues that contributed to my training and these data include Dr. Marina Koutsioumpa, Dr. Swapna Mahurkar-Joshi, Dr. Kai Fang, Dr. Ivy Ka Man Law, Dr. Jill Hoffman, Dr. Iordanis Karagiannidis, Dr. Hon Wai Koon, Dr. Elizabeth Videlock, and Dr. Emmanuelle Faure-Kumar. Additionally, the lab was fortunate to have multiple undergraduates that volunteered to work and contributed to the data shown here, including Zulfiqar Lokhandwala, Ami Patel, and Diane Bui.

Chapter 2 is a version of Soroosh A, Rankin CR, Polytarchou C, Lokhandwala ZA, Patel A, Chang L, Pothoulakis C, Iliopoulos D, and Padua, DM. miR-24 Is Elevated in Ulcerative Colitis Patients and Regulates Intestinal Epithelial Barrier Function. *Am J Pathol*. Copyright Elsevier 2019;189(9):1764-1774. The work was supported by NIH grants R01-DK11003-02 and DK47373, Center for Ulcer Research and Education – Digestive Disease Research Core Center grant DK41301, UCLA – Clinical and Translational Science Institute grant UL1TR0001881, and a

Crohn's and Colitis Foundation Career Development Award. CP, DI, and DMP conceived the study and edited the manuscript. AS, CRR, ZAL, AP, and CP performed experiments. LC obtained the clinical samples. AS, CRR, CP, and DMP wrote the manuscript.

Chapter 3 is adapted from a manuscript in preparation: Rankin CR, Soroosh A, Fang K, Hoffman JM, Law IKM, Lokhandwala ZA, Padua DM, Frey MR, and Pothoulakis C. "MicroRNA-24 inhibition promotes epithelial cell apoptosis and impairs the recovery from intestinal inflammation." This work was supported by NIDDK grants R01DK110003 and P30DK041301, the Blinder Foundation for Crohn's Disease, the Eli and Edythe Broad Foundation, the AGA-Takeda Research Scholar Award in IBD, and the Crohn's and Colitis Foundation Career Development Award. The work was also supported by NIH/NIDDK CURE DDRCC ISCB Core DK41301. DMP and CP conceived the study. CRR, AS, KF, JMH, IKML, and ZAL performed experiments. CRR, AS, MRF, and CP wrote the manuscript.

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- Soroosh A, et al. "KIAA1199, a novel Crohn's Disease-related protein: questioning the hyaluronan degradation paradigm"

Grants:

Ruth L. Kirschstein National Research Service Award (T32GM065823) "Training in Molecular, Cellular, and Integrative Physiology" October 2017 – September 2018

CHAPTER 1: INTRODUCTION

1.1 Ulcerative Colitis

Ulcerative Colitis (UC) is a chronic inflammatory disease of the gastrointestinal tract. First described in 1859[1], UC is one of the two major diseases considered an Inflammatory Bowel Disease (IBD). The other, Crohn's Disease (CD) is also a chronic inflammatory condition but has characteristics that distinguish it from UC. For example, while UC manifests itself only in the rectum and colon, CD can appear in any portion of the GI tract. Furthermore, UC-related inflammation is mainly restricted to the mucosal surface and appears in a continuous fashion, while lesions in the intestine of CD patients have a patchy distribution, infiltrating the bowel wall much deeper to cause complications including fibrosis and strictures[2]. Common symptoms of UC include bloody diarrhea, abdominal pain, and tenesmus, with long-term UC increasing the risk of colorectal cancer and dysplasia[3].

UC is generally a disease of the developed world but incidence is increasing in both developed and developing nations[4]. The highest incidence and prevalence values are in North America and Europe. In North America, the incidence is 19.2/100,000 persons and the prevalence is 249/100,000 persons. In Europe, the incidence is 24.3/100,000 persons and the prevalence is 505/100,000 persons[5]. Total economic burden in the United States is $8.1-14.9 billion annually, while in Europe the costs are €12.5-29.1 billion annually[6]. The goal of treatment for UC patients is management of symptoms, requiring therapeutic interventions that are successful in maintaining disease remission[3]. Its global burden is expected to increase in the near future with increasing populations of aging UC patients that are dealing with several side effects of their long-term treatment strategies[7]. Seeing these challenges, current research in UC is focused on finding new avenues of treatment while elucidating its pathogenesis and revealing clues to its etiology, all of which will help deal with the significant impact UC is having worldwide.

1.1.1 Ulcerative Colitis Etiology

Evidence strongly suggests that UC etiology is multifactorial. Though the exact cause of UC is unknown, the factors involved include genetics, environment, immune response, and microbiome, among others. Researchers have studied these factors individually and how they interact with one another to create the disease condition seen in UC.

The incidence of UC is equal between sexes until the age of 45, thereafter men have a higher incidence[8]. The average age of incidence for UC is between 30-40 years old[9]. UC has higher incidence amongst Jewish populations, particularly those in the United States and Europe[9,10]. There is a positive association between living in an urban environment and developing UC compared to living in a rural environment[11,12]. Though smoking cessation was once thought to worsen UC in patients, more recent studies have dispelled that notion[13]. Drugs that are associated with an increased risk of UC include hormone-replacement therapy[14], oral contraceptives[15], and non-steroidal anti-inflammatory drugs[16]. Above all environmental influences, dietary habits have been shown to have a significant impact on the development of UC because of their influence on gut health and the intestinal microbiome.

The fact that UC is a disease of the developed world suggests that western diets play a part. Many analyses have been done on this subject, pinpointing dietary habits that associate with UC risk. Consumption of sucrose[17], soft drinks[18], dietary arachidonic acid[19], and red meat along with high total fat levels are linked to higher risk of UC[20]. These foods are generally associated with a western diet, which has high-fat and high-sugar processed foods that are easily attainable. On the other hand, higher consumption of fruits and vegetables are associated with a decreased risk of UC[21]. There is evidence that dietary interventions can help with managing UC, although as individuals have a variety of tolerances to different foods, it is not a “one size fits all” strategy of

treatment[22]. Diet is heavily linked to gut health, including the gut microbiome, mucosal immunity, and intestinal barrier function. Animal studies have been done that have revealed that consumption of carbohydrates and fats at levels similar to western diets promote gut dysbiosis by changing the bacterial composition[23]. Sugar, salt, fats, and food additives have also been linked to changes in intestinal permeability[24]. Both the changes gut microbiome and intestinal barrier function have effects on mucosal immunity, as a leaky barrier allows bacteria to penetrate the intestinal wall and induce an immune response. Furthermore, research has shown that nutrient consumption can directly affect inflammation by both increasing detrimental immune cell activity and also weakening the mucous layers defensive abilities against the luminal contents of the colon[25,26]. Overall, improved dieting can help prevent the development of UC, but it is only one factor that plays a part in the etiology of UC.

The role of genetics in UC has been apparent since family studies have established some heritability of disease. Studies show between 7%-11% of UC patients have a family history of UC, while risk of UC increases 4-fold with a first-degree relative patient[27]. In multiple twin studies, the concordance rate of UC in monozygotic twins is 16% and 4% in dizygotic twins[28]. Large scale genome studies have expanded our knowledge of the genetic risk factors involved in UC. Several susceptibility loci have been found, including some on genes related to inflammatory activity and regulation, barrier function, and host-microbe interactions[29-31]. Even with the loci identified, genetics can only explain 19% of heritability[32]. Epigenetics, the modulation of gene expression absent of mutation, has emerged as an important factor in UC etiology and pathogenesis. Recent work has revealed changes to DNA methylation, histone modifications, and non-coding RNA activity in UC[33,34].

None of the factors described is able to single-handedly account for the development of UC. It is clear that there is a dynamic relationship between the environment, genetics, epigenetics, and

microbiome that results in the onset of UC. Considering the multifactorial etiology of this disease, it appears that each patient represents a separate entity, and thus the etiology of UC continues to be investigated by several research groups. However, research into etiology gives clues to pathophysiology, and eventually will lead to new targets for therapy to help those patients who do not respond to standard forms of treatment.

1.1.2 Ulcerative Colitis Pathophysiology

Ulcerative colitis manifests itself in the colon, resulting in significant inflammatory activity to the outer layer of the intestinal tract that, if untreated, leads to severe, unrelenting damage. The occurrence of UC involves dysfunction in intestinal homeostasis with changes in the symbiotic relationship between the luminal contents, mucosa, barrier integrity, and immune activity. During UC, some or all of these elements are awry. In understanding what occurs both at the intestinal and cellular level to disrupt homeostasis, researchers uncover new targets to focus on for therapeutic intervention.

The lumen of the intestine plays a major role both in the etiology and advancement of disease. The luminal components include a diverse population of bacteria that are a significant aspect of gut homeostasis. Studies have shown that UC patients have microbiome dysbiosis, with research pointing to changes in the population of specific species that may contribute to disease pathogenesis[35]. In one study, researchers found that UC patients had a decrease in butyrate-producing bacterium. Since butyrate is known to protect the intestinal barrier, reduced butyrate levels seen in UC patients can disrupt the intestinal epithelial barrier, leading to inflammation and damage[36]. Further evidence that bacterial dysbiosis plays a role in UC comes from studies that have used fecal transplants to induce remission of UC disease activity. Patients who received fecal transplants had increased microbial diversity, similar to the healthy patient microbiomes[37,38], suggesting that microbiome-associated therapeutic approaches may have a role in UC.

Normally, the intestinal physiological milieu is maintained by the intestinal mucosa and barrier. However, in UC, mucosal deficits allow luminal contents to cross the mucosa and stimulate inflammatory activity. The outermost layer of the gastrointestinal tract is the mucosa, which provides a layer of protection between the luminal components and the intestinal wall. The intestinal epithelial barrier consists of tight junctions, made up by transmembrane and cytosolic membrane protein complexes able to strictly control intestinal permeability. During UC, there are abnormalities in both of these intestinal components. A recent study found that uninflamed portions of UC patient colons have a depleted mucus layer, suggesting that the weakened mucosa precedes active inflammation[39]. Another study revealed that UC patients with active inflammation had more bacteria penetrating the mucosa, compared to UC patients in remission[40]. With increased bacterial mucosal penetration, it is up to the epithelial barrier to prevent microbial infiltration. However, many studies have suggested barrier dysfunction in UC patients. Genome-wide association studies have identified susceptibility loci on barrier-related genes[29]. An early electrophysiological study found a leaky epithelium in UC patients, potentially caused by increased apoptosis[41]. Further studies revealed elevated levels of pro-permeability proteins, such as myosin light chain kinase[42] and claudin-2[43], in the colon of UC patients. Moreover, two tight junction proteins important for mucosal integrity, claudin-4 and claudin-7, are found decreased in UC colon tissue[44]. In addition, there are many microRNAs dysregulated in UC that lead to intestinal barrier dysfunction which may also play a role in disease activity[45]. Inflammatory activity also plays a role in intestinal barrier function, but a permeable epithelial barrier will stimulate more inflammation, so the origin of this detrimental cycle is unclear[46,47].

The damage caused by UC stems from uncontrolled inflammatory activity. The luminal changes and mucosal degradation stimulate and/or exacerbate inflammation, but at the cellular level immune cell activity is also altered during UC. Histological analysis of UC patient colons have

revealed inflammatory infiltration to be a prognostic indicator of relapse[48]. There are multiple types of immune cells residing in the lamina propria and submucosal layers of the gastrointestinal tract. During UC, neutrophils infiltrate the colonic mucosa and create extracellular "traps", which normally play a protective role against bacteria. However, during active disease, infiltrating neutrophils express high levels of interleukin-1 beta (IL-1B) that promote further inflammatory activity in the lamina propria[49,50]. Studies investigating the macrophages present in UC patient colonic tissue found subpopulations that shifted towards inflammatory subtypes populating both the subepithelial and epithelial layers. Furthermore, those macrophages were observed to negatively affect barrier function *in vitro*, pointing to a role in UC pathogenesis[51]. Another study found that colonic dendritic cells in UC express higher levels of toll-like receptor 2 and 4 during active inflammation, potentially leading to increased secretion of inflammatory cytokines and exacerbating inflammatory activity[52]. In terms of adaptive immunity, UC is thought to involve T-helper cell type 2 (Th2)-mediated inflammation[53,54]. Th2 immunity involves secretion of interleukin (IL)-13, IL-4, and IL-5, among others, all of which have been found elevated in various studies with UC patients[43,53,54]. More recent studies have identified IL-9 expression in UC patient tissue and disease activity, suggesting an additional pathway apart from Th2 involvement in UC[55]. However, therapies targeted towards Th2 cytokines have been ineffective in treating UC patients during clinical trials[56,57], further complicating the role of different immune networks in UC pathophysiology and treatment.

1.1.3 Treatments for Ulcerative Colitis

Extensive research into the pathogenesis of UC has uncovered complex networks and mechanisms that include changes in genes, proteins, cells, and bacteria. Studies have also

pointed towards specific molecules that can be targeted for therapeutic intervention. The short-term goals of UC treatment are to reduce symptoms and achieve endoscopic healing of intestinal damage. These therapeutic outcomes are also associated with normalization of biomarker levels including C-reactive protein and calprotectin[58]. In the long term, maintaining remission and preventing the need for surgery is a priority, along with avoiding colorectal cancer[59]. Treatment depends on the severity of the disease at that moment, which can range from mild to moderate all the way to severe acute disease. The end result if treatments fail is surgical colectomy, in which a portion or all of the colon is removed.

For mild to moderate UC, patients may receive treatment with 5-aminosalicylic acid (5-ASA), a non-steroidal anti-inflammatory drug, that can be administered orally, topically, or in combination[60]. After monitoring patients for a number of weeks, if remission is not achieved, the next step is adding corticosteroids. Topical steroids are safer compared to systemic steroids and should be considered first when trying to achieve remission[59]. If remission is achieved, then steroids can be tapered off to prevent unwanted side effects, while 5-ASA continues to maintain remission[2]. Thiopurines also can be added to maintain remission following steroid treatments. Thiopurines are immunosuppressive drugs that help achieve remission, but they also have adverse effects that can be serious[2,61]. If the none of the aforementioned treatments lead to remission, more advanced therapeutics, in particular biologics, should be administered[59].

Biologic therapies involve the use of antibodies targeting a specific protein to prevent its activity. Biologics generally are injected or infused intravenously. One of the most commonly used biologics for UC is anti-tumor necrosis factor-alpha (TNFa) monoclonal antibodies. There are various types of anti-TNFa monoclonal antibody preparations available that are effective either alone or in combination with thiopurines both in achieving and maintaining remission[62-64]. Another biologic targeting a cytokine is anti-IL-12/IL-23 antibody therapy, which recently showed

effectiveness in achieving and maintaining remission in UC patients[65]. A different type of biologic used in UC targets integrins, which can prevent the trafficking of T-cells from the blood into inflamed intestinal tissues[66]. These anti-integrin receptors have been shown in clinical trials to achieve and maintain remission, even at better levels that anti-TNFa therapy[67-69]. However, even with these many types of treatments, each of which has many subtypes, 20-25% of UC patients require surgical intervention[2].

When UC patients do not respond to treatment and/or suffer from serious complications like toxic megacolon, surgery becomes necessary. Because UC is limited to the colon and rectum, surgical resection of the colon can be used as a cure. Surgical resection of the colon also reduces the possibility of colorectal cancer development, which is elevated in UC patients[70]. Surgical options include proctocolectomy, which removes the entire colon and rectum, colectomy, which removes just the colon, and subtotal colectomy, which removes only a part of the colon. These surgeries also have different end results for the reconnection of the digestive tract. A common strategy is to connect the two ends left after the colonic resection, which is called an anastomosis, and create a pouch just before the anus. This method allows waste to naturally flow through your digestive system, be stored in the pouch, and then expelled out of your anus[71]. These surgeries are very invasive and are associated with many side effects, including inflammation of the pouch, leaks from the anastomosis, dyssynergic defecation, metabolic abnormalities and dysplasia in the surgical sites, among many others[2,72].

Even with the advancements of UC therapy in the last 20 years, there are still 20-25% of UC patients requiring surgical interventions of their disease, further supporting the notion for the need of new therapies for these patients. Due to research into the pathogenesis of UC, many drugs targeting different pathways and mechanisms are in clinical trials. These include drugs targeting the janus kinase pathway[73], different integrin subunits[74,75], and T-cell migration[76] along with

targeting other cytokines and biosimilars to the current class of drugs that target cytokines[2,59]. Research into the role of the microbiome in UC has created the emergence and the potential of fecal transplant therapies, which have shown positive early results[37,38]. However, further research into disease pathophysiology is needed to uncover new and potential important cellular and molecular factors implicated in UC pathophysiology that may provide new forms of treatment for UC.

1.2 MicroRNAs

MicroRNAs are small non-coding RNAs that are 22 base pairs in length. The first identified gene to code for a microRNA was *lin-4*, discovered in *C. elegans* by Victor Ambros in 1993[77]. Since then, microRNAs have been an emerging area of study for their significant influence on multiple and diverse cellular functions. MicroRNAs are not translated into a protein. Instead, they are transcribed and processed to eventually remain as single-stranded RNAs with the ability to repress gene expression by binding to mRNAs that have sequence complementarity. As a result of this function, microRNA activity has been implicated on a variety of disease conditions, connecting the dysregulation of a specific microRNA to corresponding changes in expression of multiple genes. This activity often results in malfunctioning cell processes that contribute to the pathogenesis of different disease states. As such, there have been substantial investigations in the last 30 years examining the biogenesis, regulation, and functionality of microRNAs that have greatly expanded our knowledge of non-coding RNAs, cell biology, and disease.

1.2.1 MicroRNA Biogenesis

MicroRNA sequences are located in different genomic contexts. MicroRNAs can be either transcribed from their own DNA sequence, or derived from an intronic or exonic region of protein-coding or non-coding genes[78,79]. Additionally, microRNAs can be expressed as polycistronic transcription units, characterizing what are known as microRNA clusters. These clusters consist

of multiple microRNAs from nearby loci that are often transcribed from one promoter region and have similar expression patterns and functions[80,81]. Similarly, microRNAs on intronic or exonic portions of protein-coding DNA are coordinately expressed with their host gene mRNA[82].

MicroRNA genes are transcribed by RNA Polymerase II, as evidenced by their regulation from RNA Polymerase II-associated transcription factors and epigenetic modulators[83]. Further evidence to confirm the involvement of RNA Polymerase II is the existence of a 5' cap and a 3' polyadenylated tail following transcription[84]. The structure following transcription is known as a primary precursor for microRNA (pri-miRNA)[80]. Pri-miRNAs can be multiple kilobases long and have double-stranded portions with terminal loops, known as the stem-loop structure, which consists of the eventual active microRNA strand[85].

The pri-miRNA remains in the nucleus to be processed by an RNA-polymerase III like protein named Drosha in association with the nuclear protein DiGeorge Syndrome Critical Region 8 (DRGC8)[86,87]. Both Drosha and DRGC8 have double stranded RNA binding domains which contribute to the specificity of the binding and cleavage of the pri-miRNA, recognizing the junction of the stem-loop and the single-stranded tails along with the terminal loop [88-90]. The cleavage occurs between the terminal loop and the junction between the stem-loop and the tails, approximately 11 base-pairs from the junction and 22 base-pairs from the terminal loop[89,91]. This creates a hairpin structure called a precursor-microRNA (pre-miRNA) that is approximately 65 nucleotides and contains the microRNA strands.

Following the cleavage by Drosha/GRGC8, the pre-miRNA is exported into the cytoplasm for further processing. A transport complex is formed by the protein exportin 5 (Exp5) complexed with a GTP nuclear binding protein called RAN-GTP. Exp5 requires RAN-GTP for this transport to occur, as it is a karyopherin. Karyopherins are a family of nucleocytoplasmic factors that require

RAN-GTP to bind to its cargo[92,93]. Exp5 has the ability to bind the double-stranded RNA of a pre-miRNA in a RAN-GTP dependent manner[94]. After translocation to the cytoplasm, the RAN-GTP is hydrolyzed which leads to its disassociation from Exp5 and the release of the pre-miRNA for cytoplasmic processing[95].

In the cytoplasm, pre-miRNA goes through cleavage by the RNAse III endonuclease Dicer. This enzyme cleaves off the terminal loop of the pre-miRNA, leaving a double-stranded RNA duplex. Dicer recognizes the terminal loop by binding to the 3' end and measuring out the strand until the terminal loop begins and ends, separating out the double-stranded microRNA[96,97]. This measurement is approximately 22 nucleotides from the 3' end of the pre-miRNA[98]. Dicer also binds to the 5' end of the pre-miRNA for more specificity and efficiency of the cleavage[99]. The RNA duplex resulting from this cleavage is contains the mature microRNA and within it are the strands that can participate in mRNA suppression.

Directly following processing by Dicer, the RNA duplex is loaded onto an argonaute (AGO) protein. This RNA duplex-AGO complex is called a pre-RNA Induced Silencing Complex (RISC)[85]. There are eight AGO proteins in humans, four of which are widely expressed in human tissues and are involved in microRNA-mediated gene silencing (AGO1-AGO4)[100,101]. The next step in the process is the unwinding of the duplex, which allows for subsequent strand selection in order to end up with a single-stranded microRNA within the complex[102]. Of the two strands of the duplex, thermodynamic instability determines which one remains in the RISC as the "guide" strand that is then active in gene suppression. The strand with a relatively unstable 5' end is selected, particularly if it has an uracil at the first nucleotide position[103,104]. The other strand is known as the "passenger" strand. This strand is generally released and degraded, though it also has the potential to be active at a less potent level[105,106]. Of the four AGO proteins, AGO2 is the only one to have cleavage ability[102,107]. Pre-RISC with AGO2 go through unwinding and passenger strand

separation through a cleavage dependent mechanism, similar to how the final RISC ends up degrading target mRNAs[108]. Pre-RISC with the other AGO proteins use cleavage-independent mechanisms that rely on mismatches between the two strands of the duplex to promote a passive unwinding and separation of the passenger strand[109]. In the end, a RISC complex with a single-stranded microRNA is in the cytoplasm, where it can perform its function of mRNA silencing.

1.2.2 MicroRNA Function

At the end of the biogenesis process, the single strand of microRNA is incorporated with an AGO protein in the cytoplasm, forming the RISC. The process of gene regulation is centered around targeting mRNAs via sequence complementarity. Specifically, the RISC targets the 3' untranslated region (UTR) of mRNAs that have Watson-Crick base pairing with nucleotides 2-8 (called the seed sequence) from the 5' end of the microRNA[110,111]. When the seed sequence of the microRNA binds to the 3' UTR of a mRNA, the repression occurs through a number of avenues.

One example of mRNA suppression is mRNA cleavage, if AGO2 is part of the RISC. AGO2 is the only human AGO protein with substrate cleavage capability[107], which allows it to be responsible for both assembly of the RISC and facilitating microRNA-mediated mRNA degradation[112]. AGO2 obtains its catalytic activity through its PIWI domain, which shares similarities with the catalytic domain of RNAse H[113]. The microRNA and the complementary 3'UTR form a duplex, which causes a conformation change in the AGO2 that leads to cleavage of the phosphodiester bond between residues 10 and 11 on the target mRNA, as measured from the 5' end of the guide strand[114]. This cleavage renders the mRNA unable to be translated into a protein, thereby repressing expression of that gene.

The cleavage-independent method of mRNA suppression is translational repression. One mechanism involves the deadenylation of the 3' poly-A tail on the target mRNA. The 3' tail is important for initiation of translation, as it interacts with multiple proteins that create a complex involved in the process[115]. The microRNA-mediated deadenylation leads to mRNA instability that hampers the translation process and thus, represses gene expression[116].

Another cleavage-independent example of microRNA-mediated mRNA suppression involves the recruitment of eukaryotic translation initiation factor 6 (eIF6). eIF6 inhibits the formation of the 80S ribosome, which is necessary for translation[117]. In some cases, the RISC includes eIF6, where it can act to prevent the formation of the 80S ribosome and prevent translation of the target mRNA[118]. There are also mechanisms of translational inhibition that occur after initiation of translation, including reduction of elongation rates and ribosomal drop-off[119]. These functions all ensure the translational repression that prevents the gene from being expressed.

With the potent ability of microRNAs to suppress the expression of mRNAs solely based on the sequence complementarity of 7 nucleotides, there are deep implications for cell functionality. The overexpression or downregulation of a **single** microRNA has the potential to discombobulate multiple cell functions, leading to many downstream consequences. In a disease state, there could be multiple microRNAs that are dysregulated, causing the downregulation and upregulation of a wide variety of genes, leading to the homeostatic issues that result in the cellular and physiological symptoms that occur in patients

1.2.3 MicroRNAs in Disease

Much of the research involving microRNAs follows a similar template. Investigators will use high throughput methods to identify the dysregulated microRNAs in a disease, usually using patient tissue. With the list of elevated or downregulated microRNAs, they will then run sequence

complementarity programs to find targets of those microRNAs. These programs allow investigators to hone down specific microRNAs to pursue by identifying those with gene targets relevant to the condition being studied. Researchers then will transition to *in vitro* methods to manipulate the specific microRNAs in cell culture models. This approach, coupled with results from animal experiments in disease models, will determine what effect the dysregulation of specific microRNAs in a disease state has on cellular functions that could give clues to the role the microRNAs play in the pathogenesis or progression of the disease. In the end, the investigations of microRNAs in disease result in novel biomarkers, mechanistic clues, and potential therapeutic opportunities.

Biomarkers can be considered as biological observations that can substitute for and/or predict a clinical endpoint or intermediate outcome that is difficult to observe otherwise[120]. MicroRNAs are useful as biomarkers because they are detectable both inside and outside of the cells. In fact, a study in 2010 profiled the microRNA expression in 12 different bodily fluids including plasma, saliva, and urine and found ubiquitous expression[121]. This allows physicians and researchers to measure microRNA levels in specific diseased tissue and/or an easily attainable bodily fluid. These measurements can assist with disease screening, diagnosis, and monitoring with prognostic capability and personalized therapeutic potential[120]. Facilitating these high throughput measurements is the emergence of newer technology that reports the expression level of every known microRNA in a large number of samples[122]. With this, microRNAs have been profiled in a wide range of diseases, including many types of cancer[123], cardiovascular disease[124], aging[125], Alzheimer's disease[126,127], diabetes and other metabolic diseases[128,129], autoimmune diseases[130], and IBD[131].

Particularly in IBD, microRNA profiling has contributed greatly. Studies have revealed that differential microRNA expression can distinguish between CD and UC[132,133], associate with

therapeutic response[134,135], and act as prognostic markers[136,137]. Studies that have identified dysregulated microRNAs in IBD have been expansive, looking at tissues and fluids to create a vast characterization of the microRNA profile in IBD patients[138]. With the profiling in IBD and other diseases, researchers can then look at those microRNAs to determine the functional role they may play in the disease process.

When profiling microRNAs of diseased tissue, researchers can identify individual microRNAs that are significantly upregulated or downregulated in cells. Based on the simple fact that microRNA activity requires sequence complementarity, investigators can identify potential mRNA downstream targets of those microRNAs using databases and computer software[139]. If the microRNA is downregulated in the disease, its targets may be upregulated. If the microRNA is upregulated in the disease, its targets may be downregulated. Due to this, researchers have been able to elucidate specific consequences of dysregulated microRNAs in diseased cells, which has given clues to disease pathogenesis. Disease-relevant cellular processes that are affected by dysregulated microRNAs include apoptosis[140], cell cycle[141], proliferation[142], angiogenesis[143], epithelial to mesenchymal transition[144], and metastasis[145]. With the particular functions of specific dysregulated microRNAs established in disease states, researchers can then look to utilizing microRNA-based therapies to amend the dysregulation and potentially treat patients.

In IBD, microRNA-focused research has expanded our knowledge of mechanisms that may contribute to the disease process. Roles have been established for both cell-specific[146] and exosomal microRNAs in IBD[147]. There is evidence that microRNAs have a significant role in IBD-related processes, including mucosal immunity[148], barrier function[45], and the gut microbiome[149,150]. The vast number of studies that have uncovered specific roles of unique microRNAs suggest a strong potential for these microRNAs to be targeted for therapeutic intervention, with either *in vivo* overexpression or inhibition[151]. More research is needed to

elucidate the specific mechanisms by which microRNAs function and the molecular details of the microRNA-gene networks in play to help avoid potential side effects. Nevertheless, microRNA therapeutics could provide a new avenue for treatment for IBD patients that have not had success with other common therapies.

1.2.4 MicroRNA Therapeutics

In the last 20 years, research into the role microRNAs play in disease has uncovered very influential microRNAs for different disease states. Studies have revealed detailed microRNA/gene networks that exist in cells to control specific functions and processes. With that knowledge, many labs have been able to show that modifying the expression certain microRNAs in animal models may ameliorate disease status. Thus, specific microRNAs emerge as viable candidates for targeted therapeutic intervention in particular diseases. MicroRNA manipulation centers around inhibiting or overexpressing a specific microRNA. Each type of treatment has its own unique biostructure, method of application/delivery, and challenges. With recent advancements in oligonucleotide technology, manipulation of microRNAs in humans is a feasible goal for therapeutic development.

Targeting microRNAs for inhibition is a commonly used manipulation strategy because of advancements in technology that have made microRNA inhibition reliable and specific. There are three broad methods of microRNA inhibition currently employed: small molecule inhibition, microRNA sponges, and antisense oligonucleotides[152]. Small molecule inhibition targets the transcription or microRNA processing, not the microRNA itself. In turn, the identification of a small molecule that targets a specific microRNA is difficult, as transcriptional targeting and/or affecting the processing pathway may affect other microRNAs as well. Furthermore, identifying an accurate small molecule requires high throughput screens using luciferase reporters transfected into the specific cell line of interest due to the heterogeneity of microRNA expression between cell lines[153].

MicroRNA sponges are long RNA sequences that have multiple binding sites for a specific microRNA, which are very similar to naturally occurring long non-coding RNAs[154]. The theory is that many microRNA/RISC complexes will bind to the sponge instead of mRNA target sequences, and thus the activity of the microRNA will be sequestered. To prevent RISC-induced cleavage of the sponge after binding, there is a bulge at nucleotides 9-12 (where the cleavage normally would take place) that prevents degradation[155]. Sponges can be artificially synthesized for transfection, or transgenically expressed for i*n vitro* and *in vivo* application[156]. However, the transfection of microRNA sponges is somewhat more difficult than antisense oligonucleotides, often requiring viral vectors[157]. Furthermore, *in vivo* experiments with microRNA sponges have issues with cytotoxicity and unwanted side effects relating to the delivery of the material[152,157]. The most commonly used microRNA inhibitor for experimentation is the antisense oligonucleotide (ASO), which is modifiable, easily transfectable, and potent without cytotoxicity.

ASOs are single-stranded oligonucleotides that can directly bind to a target nucleotide sequence for inactivation. The first use of an ASO targeting microRNAs was a simple complementary DNA sequence[158]. Since then, research has uncovered the ability of modifications to the nucleotide chemical structure to greatly enhance the effectiveness of the ASO *in vitro* and *in vivo*[159]. The first major modification was to add a methyl group to the 2' position, called 2'-OMe. This modification increases the melting temperature of the duplex that is formed when the ASO binds to the microRNA[160]. The 2'-OMe modification also confers resistance to some nuclease activity, but not all, so this adjustment alone is not enough to protect the oligonucleotide from degradation[161]. Another approach was the substitution of a sulfur atom for the non-bridging oxygen in the phosphate backbone, called a phosphorothioate (PS) modification. This variation reduces the ability of many nucleases from being able to cut the phosphate backbone of the oligonucleotide, but decreases the melting temperature for each substitution thus lowering binding affinity to the target microRNA[162]. Therefore, PS modifications are only placed in certain locations of the ASO

backbone to keep its binding affinity sufficient. Another change was the addition of a cholesterol group to the end of the ASO with 2'-OMe/PS modifications. An ASO with these alterations is called an antagomir, which was coined Markus Stoffel in 2005[163]. The addition of the cholesterol group assisted in uptake of the ASO into cells without a delivery vehicle, both *in vitro* and *in vivo.* Antagomirs are commonly used in microRNA inhibition studies for a wide variety of research focuses[164]. Another widely used modification is the locked nucleic acid (LNA). LNA modifications involve tethering the 2' oxygen with the 4' carbon with a methylene bridge, locking the nucleotide into a C3-*endo* sugar conformation. This alteration greatly increases biding affinity by increasing the melting temperature of the ASO/microRNA duplex[165]. Researchers have been able to compare modifications both individually and in combination and have observed that combining modifications is very effective[161]. With these tools, researchers can optimize an ASO depending on the application that is needed, and inhibition of microRNAs can be studied extensively.

MicroRNA mimics provide researchers with the ability to overexpress microRNAs in cells and tissues. MicroRNA mimics are a duplex, with the guide strand being the sequence of the microRNA of interest. When a mimic enters the cell, it can be incorporated into a RISC by AGO2 and perform its functions as if it was endogenously created. However, the incorporation into the RISC along with the fact that the microRNA needs to maintain its binding affinity with its targets means that it cannot have as many modifications compared to ASOs. Thus, microRNA mimics are very susceptible to nuclease degradation[166] and this makes delivery of the microRNA difficult. One adjustment that protects against nucleases but doesn't interfere with RISC loading is replacing the 2'-O with a fluoride atom[167]. Another strategy is using the passenger strand of the mimic duplex to add modifications. Since the passenger strand is not to be used for mRNA repression, it can be used to help the effectiveness of the mimic. A cholesterol group can be added to the end of the passenger strand to enhance uptake of the duplex into cells, while further alterations can ensure that it doesn't get incorporated into the RISC complex over the guide

strand[168]. Even with these variations, artificial microRNA overexpression *in vivo* remains very difficult to achieve. For the overexpression and inhibition *in vivo*, the delivery method of the molecule is vital in ensuring survival of the nucleotide and uptake into the desired cells.

Delivery systems for microRNA modulation oligonucleotides can determine both specificity and efficiency of the end result. The difficulties reside with *in vivo* applications in particular, where needing larger concentrations leads to toxicity. Furthermore, exonucleases in serum can quickly degrade an unprotected oligonucleotide, while processing through the liver and kidneys is very rapid and effective. For ASOs, the extensive modifications protect against nuclease degradation without sacrificing specificity. For microRNA mimic delivery, the adjustments are much more limited, so delivery method is vital. In both situations, viral-mediated delivery is a viable option due to the ability to confer target cell specificity, but for *in vivo* application, addition of viruses may be associated with several side effects, including immune responses and toxicity[169]. Lipid-based delivery vectors are a very common method used *in vitro* and *in vivo*, particularly because of the simple protocol and relatively low cytotoxicity. However, when considering *in vivo* application, issues with target specificity and uptake may occur since systemic lipid-based delivery may target unwanted cell types, and, in addition, can easily be filtered out by the liver. However, optimization of the lipid technology, like using neutral lipid emulsions, has led to promising results for microRNA mimic delivery *in vivo*[170]. Other delivery methods, including nanoparticles and polymer conjugates, have shown encouraging results for both microRNA and small interfering RNA preclinical studies, but drawbacks include low loading efficiency, cytotoxicity, and inefficient uptake by the target cells[171]. Researchers have diligently worked in recent years to greatly optimize both the modifications and delivery methods that have led us to the emergence of clinical trials involving microRNA manipulation.

There are a few studies currently in clinical trials that involve manipulation of microRNAs. The first involved treating hepatitis C with an LNA-modified ASO targeting microRNA-122 for inhibition. In its phase IIa trial, results showed safety of the drug along with a dose-dependent reduction in virus levels[172]. While these were encouraging results, further work is needed to determine long-term efficacy, as some patients had a return of increased viral levels in a follow-up study[172]. Another clinical trial with microRNA inhibition involves microRNA-103 (miR-103) and microRNA-107 (miR-107) in type-2 diabetes. Pre-clinical research revealed a comprehensive role of miR-103 and miR-107 in insulin sensitivity, with inhibition improving insulin signaling, glucose uptake, and decreasing adipocyte size[173]. The clinical trial involves an ASO conjugated with a GalNac sugar and targets non-alcoholic steatohepatitis in type-2 diabetes patients[171]. A clinical trial with microRNA overexpression involves targeting microRNA-16 in mesothelioma. This trial uses the miR-16 mimic encased in bacteria derived 400nm nanocells that can be coated to imbue cell uptake specificity[174]. Preliminary results showed that while there was an increase in circulating cytokines, the treatment was well tolerated over an 8-week period[175]. Another trial involving a miR-34 mimic encapsulated in a lipid-base vehicle had some promising responses in treated multiple types of cancer[176], but ended up being terminated due to immune-related toxicity[171].

In viewing microRNA therapeutic potential as a whole, there are two main barriers in their development. Firstly, clarifying the specific function of a microRNA including its gene networks is extraordinarily difficult. Second, the delivery method can be difficult to develop. Thirdly, clinical trials frequently will not confirm pre-clinical evidence. Nevertheless, clinical trials involving microRNAs are at its infancy, and as these trials progress, they will inevitably optimize the best delivery methods for human treatment and allow for more success with microRNA-based therapies.

1.3 MicroRNA-24-3p

MicroRNA-24-3p (miR-24) is a 22 base pair microRNA originating from two separate precursors, miR24-1 and miR24-2. Each is transcribed at a different chromosomal position, with miR24-1 transcribed at chromosome 9 and miR24-2 transcribed at chromosome 19. miR-24 is highly conserved across species, including in *mus musculus*. Being that miR-24 has the same sequence among species, research on this microRNA is very translatable from *in vitro* to *in vivo*.

MicroRNA-24-3p is the active strand of the precursor microRNA processed from each chromosome, with the passenger strand of miR24-1 being miR-24-1-5p and the passenger strand of miR24-2 being miR-24-2-5p. The focus of most research about the miR-24 family is on microRNA-24-3p, but its passenger strands have shown evidence of some functionality[177,178]. Moreover, miR-24 is also studied as part of a cluster, the miR-23/miR-24/miR-27 cluster which exists on both chromosomes[179,180]. The existence of this cluster suggests that the targets and regulation of miR-24 may have some overlap with miR-23 and miR-27. Nonetheless, each of those microRNAs must be studied separately, as they have different seed sequences that will have unique mRNA targets for each of them.

Since miR-24 is transcribed at two separate locations on the genome, this microRNA may have quite different regulation and activity depending on the cell type and location. This creates a difficulty in studying this microRNA, for there may be contradictory data describing the function of miR-24 between different organs. However, the ubiquitous expression and activity of this microRNA, regardless of its specific actions, makes it an important gene for its biomarker, mechanistic, pathogenic, and therapeutic potential.

1.3.1 MicroRNA-24-3p in Cancer

Of the many studies published relating to miR-24, the most prevalent are those pertaining to its role in cancer. A meta-analysis done in 2020 revealed over 500 publications relating to miR-24 and cancer[181]. In the various studies, miR-24 has been shown as a diagnostic and prognostic biomarker and involved in various cancer-related cellular responses. However, the role it plays in cancer can contrast greatly[182]. In some studies, miR-24 is a tumor suppressor while in others, it is an oncogenic microRNA. For example, MiR-24 increases tumor sensitivity to treatments for some cancers, while in others it enhances resistance to therapies. Its biomarker status also varies between cancer types, with some showing upregulation and others showing downregulation of miR-24. Either way, the presence of miR-24 in cancer is strong and has major biomarker and therapeutic potential for the many cancer subtypes.

MiR-24 has been part of many profilings in cancer patients with both diagnostic and prognostic potential. A study from 2014 revealed that elevated serum miR-24 was associated with hepatocellular carcinoma (HCC) and was able to distinguish HCC patients from patients with chronic liver disease. Within the HCC cohort, patients with higher serum miR-24 had a lower overall survival rate[183]. In a breast cancer study, patients with metastases had higher levels of miR-24 in both plasma and tissue along with lower survival rates compared to patients without metastases[184]. In a study comparing colon cancer tissues to adjacent non-cancerous tissue, researchers found that miR-24 was significantly downregulated in the cancerous tissue. They observed decreased miR-24 correlated with increased local invasion, increased lymph node metastasis, advanced stages, and poorer prognosis[185]. In an investigation of oral squamous cell carcinoma (OSCC), researchers found that the salivary miR-24 was significantly elevated in the OSCC patients compared to the saliva of healthy patients. Furthermore, the OSCC tumor tissue had higher levels of miR-24 compared to non-cancerous peritumoral tissue[186]. These data

strongly suggest the potential of miR-24 as a diagnostic and prognostic biomarker for different cancer types.

Another major area of study within miR-24 and cancer is its potential to influence sensitivity of the cancer to treatments. These investigations are generally done using cell lines to model the tumor in order to manipulate the microRNA expression within them. In one study using a nasopharyngeal cancer cell line, researchers found that miR-24 overexpression sensitized those cells to ionizing radiation, correlating with the finding that miR-24 is decreased in tissue from recurring nasopharyngeal cancer[187]. In another study focused on prostate cancer, miR-24 elevation was shown to increase the sensitivity of a prostate cancer cell line to paclitaxel, an anticancer drug used with chemotherapy[188]. In studies of breast cancer chemoresistance, miR-24 overexpression in multiple breast cancer cell lines was observed to increase resistance to both tamoxifen[189] and cisplatin[190] while miR-24 inhibition increased sensitivity to both. In a study of the anticancer therapy doxorubicin in osteosarcoma, miR-24 was found significantly increased in patient tissue, serum, and osteosarcoma cell lines. This elevation was determined to suppress the effects of doxorubicin when studied *in vitro*[191]. Taken together, these data along with many others clearly outline a role for miR-24 in the treatment of multiple cancers.

Many investigators have described the role of miR-24 in a variety of cellular processes related to cancer. However, the studies contrast greatly when experimenting with different subtypes of cancer and even have contradictory data when studying miR-24 within the same type of cancer. Nevertheless, the large number of publications that exist suggests that miR-24 is an influential microRNA that is centrally involved in many cancer-related mechanisms. Without any contradictory evidence, miR-24 was observed to be an “oncomiR” in breast cancer[192,193], liver cancer[194,195], tongue cancer[196,197], and Hodgkin’s lymphoma[198,199]. MiR-24 was found to be only tumor-suppressive in nasopharyngeal cancer[200,201] and esophageal cancer[202]. In lung cancer,

overexpression of miR-24 was found to promote proliferation, migration, and metastasis with decreased apoptosis while inhibition of miR-24 reduced tumorigenic properties of lung cancer cells. This correlates with data that shows miR-24 is elevated in lung cancer tumor tissue and patient serum compared to healthy controls[203-207]. Taken together, this suggests that miR-24 plays the role of an oncogenic microRNA in the context of lung cancer, although some research suggests the opposite[208,209]. In multiple gastric cancer studies, inhibition of miR-24 was found to be oncogenic while overexpression of miR-24 had tumor suppressive effects within the cells. This correlates with data showing decreased miR-24 in gastric cancer tissues compared to adjacent non-cancerous tissue[210-213]. These multiple studies clearly indicate that miR-24 acts as a tumor suppressor during gastric cancer, although some studies did not confirm this response[214,215]. This contradiction is seen in many other cancers, including colon[185,216], bladder[217,218], and prostate cancer[219,220].

There are several explanations for these contradictory results, as cancers have different stages and subtypes within a single organ. MiR-24 could act differently depending on the stage of cancer, the type of cells involved, or the type of treatment the patient receives. Furthermore, a single microRNA can affect multiple downstream target mRNAs, and multiple microRNAs can affect a single mRNA. Nonetheless, miR-24 has proven to be dysregulated in a vast number of cancers and has shown to affect a variety of phenotypes within the cancer cells. Alongside its diagnostic and prognostic potential, once these mechanisms are clarified, miR-24 can be obvious candidate for targeted cancer treatments.

1.3.2 MicroRNA-24-3p in Cardiovascular Physiology

MiR-24 has also been vastly studied in cardiovascular physiology. Both in the context of homeostatic function and disease, miR-24 has been implicated as a microRNA influencing cardiovascular function. Strikingly, miR-24 dysregulation has been pronounced during different

types of cardiac events and their recoveries. While the experimental procedures to address the mechanisms of these events are challenging, these results point to several possibilities for the development of miR-24 based therapies for cardiovascular health.

As a biomarker, miR-24 has diagnostic potential in a few different contexts. In a recent study, serum from patients with atherosclerosis showed decreased miR-24 expression when compared to serum from healthy patients[221]. In patients with abdominal aortic aneurysm, miR-24 was decreased in both aortic tissue and plasma in diseased patients compared to healthy controls[222]. In a study focused on coronary heart disease and type-2 diabetes, researchers found circulating miR-24 to be significantly decreased in diseased compared to healthy patients, and further decreased in the patients with both coronary heart disease and type-2 diabetes[223]. This correlates well with results from prior studies[224,225] suggesting the possibility for plasma miR-24 levels to predict coronary heart disease in type-2 diabetes patients.

Many studies have elucidated roles for miR-24 in the vasculature, which heavily influences the progression of cardiovascular diseases and diabetes. Following vascular injury or stress, miR-24 is decreased. Multiple studies showed that miR-24 overexpression following vascular smooth muscle stress *in vitro* or vascular injury *in vivo* repressed pathogenic vascular remodeling and promoted homeostatic endothelization and reduced oxidative stress, inflammation, collagen deposition, and proliferation in vascular cells[221,226-228]. Based on these data, miR-24 has significant potential to prevent serious vascular complications stemming from diabetes, including atherosclerosis.

MiR-24 has also been extensively studied in the context of myocardial infarction (MI). Researchers have aimed to understand the role of miR-24 in cardiomyocytes and their response following infarct. Studies using a mouse MI model observed a significant decrease in miR-24 at

the ischemic zone of the heart[229,230]. Another study using rat MI models found a similar miR-24 expression pattern[231]. Correlating with these data was the improvement of heart function following targeted overexpression of miR-24 in infarcted hearts, both decreasing fibrosis[229] and apoptosis of cardiomyocytes[230]. Furthermore, a transgenic mouse model with miR-24 specifically overexpressed in cardiomyocytes revealed decreased apoptosis and improved heart function after MI[232]. These data taken together suggest a protective role for miR-24 in cardiac function that has therapeutic potential for recovering MI patients.

Several studies investigating a role of miR-24 in cardiac function have demonstrated an involvement of this microRNA in cardiovascular diseases. While most of the data points to a protective role for miR-24, some data suggests differently[233,234]. As with the research involving miR-24 in cancer[182], there are many variables that could explain this contrasting data, including different gene and microRNA activity in different cell types and a larger network of miR-24/gene relationships than is currently understood. Nonetheless, miR-24 is undoubtedly involved in cardiac and vascular function and further research into this area will likely enhance our understanding of cardiovascular disease pathogenesis and a potential new therapeutic application.

1.3.3 MicroRNA-24-3p in Ulcerative Colitis

While not as greatly studied compared to the aforementioned diseases, research into miR-24 in UC has uncovered data that suggest a role for miR-24 as a biomarker[235]. While investigations into the function of miR-24 related to UC have been lacking, there has been data published about the role miR-24 plays in other relevant processes that could affect gut homeostasis. Together, these studies all indicate a major role for miR-24 in UC that starts with its diagnostic capability and may end with a targeted therapy.

A major area of study for microRNAs and IBD is the emergence of biomarkers for disease. The ability to determine the type of IBD in different patients (CD vs UC or active disease vs inactive disease) using microRNA profiling is potentially invaluable. In the studies that profile microRNAs in IBD, particularly in UC, miR-24 has emerged as a possible biomarker. In an early study profiling microRNAs in UC colon tissue, researchers found miR-24 elevated in the UC colonic tissue compared to healthy control colon tissue and also found an increase of miR-24 in active vs inactive disease[235]. This was confirmed by two later studies that also showed elevated miR-24 when comparing UC to CD patient colon tissues and suggested that miR-24 expression could distinguish between the two IBD subtypes[236,237]. In three separate studies, researchers investigating circulating microRNA expression profiles in IBD observed an elevation of miR-24 in UC vs healthy control patient blood[238-240]. In a recent study looking at fecal microRNA, researchers found a distinct expression profile that included upregulation of miR-24 in CD patient feces along with feces from two mouse IBD models compared to controls[241]. These data point to a significant role for miR-24 as a biomarker to distinguish UC from CD and healthy subjects, particularly when measuring microRNAs from colonic tissue.

There are limited studies that directly connect miR-24 with UC, but these studies together with research that associates miR-24 with other relevant inflammatory processes indicated that miR-24 could be influential in UC pathogenesis. A recent study investigating miR-24 in UC found that the decrease of the long noncoding RNA PMS2L2 is correlated with the increase of miR-24 in UC patients. Using an intestinal cell line *in vitro, t*hey revealed that miR-24 overexpression increases LPS-induced inflammation and apoptosis, while PMS2L2 decreases miR-24 expression and also suppresses LPS-induced inflammation and apoptosis[240]. This suggests that inflammation in UC is induced by upregulation of miR-24, which is caused by downregulation of PMS2L2.

While not studied in the context of UC, other inflammation-related research involving miR-24 reveals a role in different inflammatory phenotypes. There are multiple studies investigating the role of miR-24 in monocyte activity. One study observed an attenuation of phagocytosis by macrophages and dendritic cells when overexpressing miR-24, which furthermore modulated the secretion of multiple inflammatory cytokines[242]. In another study, miR-24 overexpression repressed macrophage invasion by decreasing expression of matrix metalloproteinase-14. Concurrently, miR-24 inhibition increased macrophage invasion, phagocytosis, and plaque formation *in vivo*[243]. This correlates with a study showing circulating miR-24 decreased in patients with atherosclerosis. In the same study, miR-24 overexpression was observed to decrease endothelial cell migration and proliferation along with cytokine production[221]. Another group revealed that miR-24 overexpression elevates M2 macrophage polarization and expression of M2 markers and cytokines in *S. aureus* stimulated macrophages[244]. In a study of microglia during spinal cord injury, researchers observed a decrease in activation and cytokine secretion in LPS-induced microglia following overexpression of miR-24[245]. Similarly, miR-24 overexpression repressed LPS-induced lung inflammation and cytokine expression in a neonatal rat model of acute lung injury[246].

Several other studies involving miR-24 indirectly support a role for this microRNA in UC pathophysiology. One study on hepatocellular oncogenesis revealed that miR-24 regulates hepatocyte nuclear factor-alpha[247], which has been identified as a susceptibility locus for UC[29] and is implicated in inflammatory processes[248]. Two separate investigations implicated miR-24 to the modulation of intestinal barrier function by regulating the tight junction proteins cingulin, zona occludens-1, and E-cadherin as well as by affecting RhoA activity[249,250]. Other studies related to wound healing and fibrosis show a role for miR-24 in these responses, particularly during cardiovascular[229,251] and liver diseases[252].

These data together suggest a role for miR-24 in inflammatory activity and other related processes. Further studies could potentially uncover more mechanistic information for the involvement of miR-24 in UC pathogenesis, and may eventually reveal miR-24-based therapeutic strategies in this disease.

CHAPTER 2: MICRORNA-24 IS ELEVATED IN ULCERATIVE COLITIS PATIENTS AND REGULATES INTESTINAL EPITHELIAL BARRIER FUNCTION.

2.1 Abstract

Inflammatory bowel disease is characterized by high levels of inflammation and loss of barrier integrity in the colon. The intestinal barrier is a dynamic network of proteins that encircle intestinal epithelial cells. MicroRNAs regulate protein-coding genes. In this study, miR-24 was found to be elevated in colonic biopsies and blood samples from ulcerative colitis (UC) patients compared to healthy controls. In the colon of UC patients, miR-24 is localized to intestinal epithelial cells, which prompted an investigation of intestinal epithelial barrier function. Two intestinal epithelial cell lines were used to study the effect of miR-24 overexpression on barrier integrity. Overexpression of microRNA-24 in both cell lines led to diminished transepithelial electrical resistance and increased dextran flux, suggesting an effect on barrier integrity. Overexpression of miR-24 did not induce apoptosis or affect cell proliferation, suggesting that the effect of miR-24 on barrier function was due to an effect on cell-cell junctions. Although the tight junctions in cells overexpressing miR-24 appeared normal, miR-24 overexpression led to a decrease in the tight junction-associated protein cingulin. Loss of cingulin compromised barrier formation; cingulin levels negatively correlated with disease severity in UC patients. Together, these data suggest that miR-24 is a significant regulator of intestinal barrier that may be important in the pathogenesis of UC.

2.2 Introduction

Inflammatory bowel disease (IBD) is comprised broadly of two subcategories: Crohn's disease (CD) and ulcerative colitis (UC). CD and UC are chronic inflammatory diseases of the gastrointestinal tract, currently affecting >1.6 million Americans [253]. With a complex etiology and limited lasting medical treatments, IBD patients may experience a lifetime of significant symptoms, which include severe diarrhea, bleeding, and vomiting. One pathophysiological feature of IBD

patients is a loss of the intestinal barrier, even in areas that contain an intact epithelium [254,255]. The intestinal barrier comprises a single layer of epithelial cells bound together by proteins that traverse the plasma membrane. This selectively-permeable barrier compartmentalizes bacteria and other toxins to the lumen while allowing ions, nutrients and water to be absorbed. Loss of the intestinal barrier leads to the exposure of luminal components to the underlying tissue. A dysfunctional barrier combined with an aberrant immune response are thought to be major risk factors for IBD [256].

The protein networks enabling intestinal barrier function are known as cell-cell junctions, which include the tight junction and adherens junction. Cell-cell junctions can confer strength or pore forming abilities to the barrier [257]. A major transmembrane protein in the adherens junction is E-cadherin, whereas tight junction transmembrane proteins include the junctional adhesion molecule family and claudin family of proteins [257]. These transmembrane proteins are connected to cytosolic adaptor proteins, which include the zona occludens protein family and cingulin, which, in turn, connect to the actin cytoskeleton of the cell [258]. This connection between cells to the underlying cellular cytoskeleton confers rigidity and strength to the junction [259]. However, junctional complexes are also highly dynamic [260]. To remove damaged proteins, these complexes are continually internalized and recycled back to the membrane or degraded [261]. To replace degraded proteins, junctional proteins are constantly synthesized by intestinal epithelial cells. When any of these aspects of the junctional dynamics are dysregulated, barrier dysfunction is likely to occur.

One of the pathways the intestinal epithelium uses to regulate the levels of tight junction proteins is by using microRNAs[262]. MicroRNAs are small non-coding RNAs that repress gene expression by binding to complementary sequences on mRNAs. Multiple studies have assayed alterations in microRNAs in IBD patients using unbiased methods [263]. A subset of these microRNAs including

miR-223 and miR-301a regulate intestinal epithelial barrier function [264,265]. One microRNA consistently shown to be elevated in IBD patients is miR-24, yet the possible role for miR-24 in IBD is unclear. Two studies have demonstrated 2- and 17-fold changes in miR-24 in colonic biopsies from actively inflamed UC patients compared with controls [235,236]. In addition, two studies have observed a twofold increase in blood-associated miR-24 in active UC patients compared with controls [238,239]. In the results presented herein, using semi-quantitative real time PCR, miR-24 levels were found to be elevated in biopsies and whole blood from actively inflamed UC patients. To better define a possible role for miR-24 in the pathogenesis of IBD, it was determined that miR-24 was expressed by intestinal epithelial cells in UC patients. When overexpressed *in vitro*, intestinal barriers in target epithelial cells failed to establish. Although cells overexpressing miR-24 grew normally, the protein levels of the tight junction adaptor protein cingulin were significantly reduced. When cingulin was downregulated in intestinal epithelial cells, barrier formation was impaired. Therefore, targeting the miR-24/cingulin axis could represent one pathway to strengthen the intestinal barrier and reduce inflammation in UC patients.

2.3 Materials and Methods

2.3.1 Clinical Samples

Colonic tissue biopsies and blood used in the microRNA analyses were obtained from the University of California, Los Angeles (UCLA) Center for Inflammatory Bowel Diseases and the G Oppenheimer Center for Neurobiology of Stress and Resilience under the following institutional review board (IRB)-approved protocols: 12-00420, 13-000537, and #11-000199. Colon tissue biopsies from patients who met diagnostic Rome III criteria [266] for irritable bowel syndrome (IBS) and healthy control subjects were obtained during flexible sigmoidoscopy (at 30cm) after tap water enemas. Colonic tissue used for the mild and severe inflammation, on the basis of pathology report analysis, was obtained from the UCLA Center for Inflammatory Bowel Diseases under IRB-approved protocol 18-000209. Specimens were flash frozen in liquid nitrogen and RNA was

extracted with TRIzol reagent (ThermoFisher Scientific, Waltham, MA). A Ficoll gradient (Roche, Basel, Switzerland) was used to isolate peripheral blood mononuclear cells from whole blood, according to the manufacturer's instructions. All UCLA samples were obtained after a written informed consent was provided. Human UC patient colonic tissue used for the microarray analysis was obtained from Origene (Rockville, MD). Diagnoses were confirmed by an independent set of pathologists associated with Origene. Microarray analysis of Origene samples was conducted as previously described [267]. Samples were obtained through IRB protocols and with documented patient consent, all from accredited U.S.-based medical institutions. The microarray data are accessible through the Gene Expression Omnibus (*https://www.ncbi.nlm.nih.gov/geo*; accession number GSE77013).

2.3.2 Cell Culture

Caco-2 and T84 cells (ATCC, Manassas, VA) were grown at 37°C with 5% CO_2. The cells were grown in Dulbecco's Modified Eagle's Medium (Corning, Corning, NY) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% penicillin-streptomycin (Corning). To subculture cells, confluent monolayers were washed with phosphate-buffered saline (PBS) and incubated with 0.25% trypsin with 1 mmol/L EDTA (Thermo Fisher Scientific). Suspended cells were centrifuged at 233 x g for 5 minutes. Both Caco-2 and T84 cells were split at a 1:5 ratio.

2.3.3 Barrier Assays

Transepithelial electrical resistance (TEER) was used as a measurement of barrier integrity. A total of 100,000 cells were plated on 6.5-mm transwell inserts that had 0.4-μm pores (Thermo Fisher Scientific). A TC20 Automated cell counter was used to determine cell numbers (Bio-Rad Laboratories, Hercules, CA). Each subsequent day, TEER was measured using a dual electrode connected to an epithelial volt/ohm meter (World Precision Instruments, Sarasota, FL). On last

day of the experiment, dextran flux was measured. A solution containing 50 µg fluorescein isothiocyanate (FITC)-labeled 4-kilodalton (kDa) dextran and 50 µg of AlexaFluor 555-labeled 10-kDa dextran (Thermo Fisher Scientific), diluted in Hank's balanced salt solution, was used (Corning). The dextran solution (100 µL) was added to the top of the transwell; and every 2 hours, for 6 total hours, 50 µL of the media in the bottom of the transwell was sampled. A Synergy HT plate reader was used to measure fluorescence (BioTek Instruments, Winooski, VT). To obtain absolute amount of dextran flux, a standard curve was used.

2.3.4 Transfections

Cells were transfected during plating, and all transfection reactions were performed in Opti-MEM media (Gibco). Seventy micromolar lipofectamine RNAiMax reagent was used according the manufacturer (Invitrogen, Carlsbad, CA). For overexpression experiments, both a miR-24 precursor and a microRNA precursor negative control were used at the concentration of 50 nmol/L (PM10737 & AM17110, respectively; Ambion, Austin, TX). For inhibition experiments, a chemically modified [151] miR-24 antisense oligonucleotide and negative control were used at the concentration of 50 nmol/L (YC10201383-FZA and YC10202119-FZA; Qiagen, Hilden, Germany). For siRNA experiments, both the cingulin siRNA and negative control were used at the concentration of 50 nmol/L (S33237 & 4390843, respectively; Ambion). To produce a miR-24 over-expression of 15-fold, 50 pmol/L control precursor and miR-24 precursors were used.

2.3.5 RNA Extraction, cDNA Synthesis, and Real-Time Quantitative PCR

A miRNeasy kit (Qiagen) was used to extract and purify mRNA and microRNA from tissue or cells grown on transwells, according to the manufacturer's instructions. To harvest, transwell cells were first washed twice with PBS and then the membrane was excised. The membrane was then submerged in Qiazol before storing lysed cells at -80 ^{o}C (Qiagen). All other purification steps were

performed according to the manufacturer's instructions. To create cDNA from mRNA, an iScript cDNA synthesis kit was used (Bio-Rad Laboratories). mRNA (500 ng) was reverse transcribed into cDNA, according to manufacturer's instructions. A miRCURY LNA RT kit (Qiagen) was used to create cDNA from 100 ng of microRNA, according the manufacturer's instructions. A CFX384 real time PCR system was used to amplify and detect SYBR Green-mediated signal (Bio-Rad Laboratories). To measure miR-24, a miRCURY LNA microRNA PCR assay primer was used (YP00204260; Qiagen). The housekeeping primers for microRNA experiments were U6 small nuclear RNA and 5S rRNA (YP00203907 & YP00203906, respectively; Qiagen). Cingulin (*CGN*), E-cadherin (*CDH1*) and claudin-7 (*CLDN7*) primers were purchased from Integrated DNA Technologies (Coralville, IA). Beta-actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were used as housekeeping genes for mRNA analysis. The primer sequences are as follows: *CGN*, 5'-GCAACAAGGAGCTCCAGAAC-3' (forward) and 5'-CCCTGGACATGTTTCAGCTT-3' (reverse); *CDH1*, 5'-GGATTGCAAATTCCTGCCATTC-3' (forward) and 5'-AACGTTGTCCCGGGTGTCA-3' (reverse); *CLDN7*, 5'-GGATGATGAGCTGCAAAATG-3' (forward) and 5'-CACCAGGGAGACCACCATTA-3' (reverse); *ACTB* 5'-CCCAGCACAATGAAGATCAA-3' (forward) and 5'-ACATCTGCTGGAAGGTGGAC-3' (reverse); *GAPDH* 5'-ATGTTCGTCATGGGTGTGAA-3' (forward) and 5'-GGTGCTAAGCAGTTGGTGGT-3' (reverse); *PLEKHA7,* 5'-TAAAGACAGCCGAGAAGAAG-3' (forward) and 5'-TGTCGGCACTGAAGTAGTAG-3' (reverse); *CLDN2* 5'-TGGCCTCTCTTGGCCTCCAACTTGT-3' (forward) and 5'-TTGACCAGGCCTTGGAGAGCTC-3' (reverse); *CLDN3* 5'-CATCACGTCGCAGAACATCT-3' (forward) and 5'-AGCAGCGAGTCGTACACCTT-3' (reverse); *KRT8* 5'-CGAGGATATTGCCAACCGCAG-3' (forward) and 5'-CCTCAATCTCAGCCTGGAGCC-3' (reverse). The comparative Ct method was used to calculate fold change relative to the housekeeping genes [268].

2.3.6 Western Blot Analysis

Confluent monolayers were washed twice with PBS and then the membrane was excised. Membranes were then submerged in 1x Lammeli sample buffer (Bioland Scientific, Paramount, CA) supplemented with 10% 2-mercaptoethanol (Sigma). Samples were left on ice for 30 minutes before slowly passing lysates through a 25-gauge needle. Lysates were then incubated at 95^{o}C for 5 minutes, and then 10 µL of each sample added to a 4-20% SDS-containing polyacrylamide gel (Bio-Rad Laboratories). A Trans-Blot Turbo system (Bio-Rad Laboratories) was used to transfer proteins onto a polyvinylidene difluoride membrane. Membranes were then blocked for 1 hour at room temperature in 5% milk in PBS with 0.01% Tween-20 (PBS-T). Membranes were then incubated with primary antibody diluted in 5% bovine serum albumin (BSA) overnight at 4^{o}C. After five 10-minute washes with PBS-T, the membrane was then incubated in secondary antibody diluted in 5% milk in PBS-T for 1 hour at room temperature. After five 10-minute washes with PBS-T, protein was visualized using a Clarity enhanced chemiluminesence kit (Bio-Rad Laboratories) and ChemiDoc Touch Imager (Bio-Rad Laboratories). The cingulin (117796) and claudin-2 (53032) antibodies were from Abcam (Cambridge, UK). The claudin-7 (349100) and E-cadherin (Clone 4A2C7) antibodies were from Invitrogen. The claudin-3 (SAB4500435) and PLEKHA7 (HPA038610) antibodies were from Sigma-Aldrich. The GAPDH antibody was from Cell Signaling Technologies (Danvers, MA; Clone 14C10). Horseradish peroxidase-conjugated secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA).

2.3.7 Cell Growth and Apoptosis Assays

To measure cell growth, 50,000 cells were plated in 24-well plates in duplicate. Each day after seeding, cells were trypsinized and a TC20 Automated Cell Counter (Bio-Rad Laboratories) was used to count the cells. To measure cell proliferation, 10,000 cells were plated in 96-well plates and a bromodeoxyuridine assay kit was performed according to the manufacturer (Cell Signaling). To measure apoptosis, a Caspase GLO 3/7 assay (Promega, Madison, WI) was employed using

the manufacturer's instructions. Briefly, cells grown on transwells were washed twice with PBS and the membrane was excised and submerged in the buffer provided by the kit. After 15 minutes of incubation with shaking, a BioTek plate reader was used to measure luminescence from 50 µL of lysate. As a positive control, staurosporine (Tocris, Bristol, UK) was added to the bottom of the transwell at a concentration of 2 µmol/L for 8 hours prior to conducting the assay. Lysis buffer was used to measure background luminescence. As an alternate method to measure apoptosis, a Click-it terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) kit (Invitrogen) was performed on cells at the endpoint of TEER experiments, according to the manufacturer. To stain the nuclei, 4', 6-diamidino-2-phenylindole (DAPI) was used (Invitrogen). Staurosporine was used as a positive control for TUNEL assays and was used at the concentration of 2 µmol/L for a total of 3 hours for Caco-2 cells or 16 hours for T84 cells.

2.3.8 Immunofluorescence

Cells grown on transwells were washed twice with PBS and then fixed in 100% cold methanol for 20 minutes at -20 ^{o}C. Following two subsequent washes with PBS, transwells were blocked in 5% BSA in PBS for 1 hour at room temperature. The transwell membranes were then excised from the transwell and incubated with mouse anti-ZO1 (339100; Invitrogen) overnight at 4^{o}C in a humidified container. Claudin-2, claudin-3, and claudin-7 antibodies were the same as used for Western blots analyses. The following day, after three washes in PBS, the transwell was incubated with a goat anti-mouse fluorescently labeled 488 antibody for 1 hour at room temperature (Invitrogen). After 3 washes with PBS, nuclei were stained with DAPI for 1 minute and Prolong Gold (Invitrogen) was used to mount membranes onto slides. Slides were imaged using an upright microscope (Zeiss, Oberkochen, Germany).

2.3.9 In Situ Hybridization

A double-DIG labeled miRCURY detection probe for miR-24 (YD00617308-BCG; Qiagen) was used as previously described [269].

2.3.10 Statistics

Statistical differences between groups were evaluated using an unpaired two-tailed *t*-test. Graphing was completed using Graphpad Prism version 6 (Graphpad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 miR-24 is Elevated in Biopsies and Blood from Ulcerative Colitis Patients

Multiple studies have seen that miR-24 is elevated in the serum and colon of active inflamed ulcerative colitis (UC-active) patients [235,236,238,239]. Although many studies have analyzed the effects of miR-24 on oncogenesis, few studies have profiled the function of miR-24 in the context of IBD. To validate these microarrays and RNA-sequencing studies, human colonic biopsies were obtained from patients undergoing colonoscopy procedures and real-time PCR was performed. Healthy controls were compared to UC-active patients, UC-inactive patients, irritable bowel syndrome (IBS) patients, and CD patients. It was observed that miR-24 levels were elevated sevenfold in UC-active patients ($P < 0.0001$) compared to the other groups, who had similar levels of miR-24 (**Figure 2-1A**). In UC-active patients, the elevated levels of miR-24 do not appear to be exclusive to colonic tissue as miR-24 was also elevated fourfold in the blood of UC-active patients compared to healthy controls and CD patients (**Figure 2-1B**). To determine the subset of cells that express miR-24, in situ hybridization was performed on colonic sections from UC patients and on healthy controls. It was observed that miR-24 localized to intestinal epithelial crypts in the

colon (**Figure 2-1C**). These results indicate that in UC patients, miR-24 is specifically elevated in the intestinal epithelium and this elevation persists in the bloodstream.

2.4.2 miR-24 Overexpression Significantly Disrupts Intestinal Barrier Function

Given the localization of miR-24 to the colonic epithelium, which functions to generate a selectively permeable barrier that lines the gut, the effects of miR-24 on barrier formation were tested. If miR-24 regulates the barrier and miR-24 is altered in IBD, this alteration could contribute to the pathogenesis of IBD. Two barrier-forming human cell lines of intestinal epithelial origin were used, Caco-2 and T84 cells. As miR-24 is elevated in UC-active patients (**Figure 2-1A, B**), a miR-24 mimic was transfected into Caco-2 and T84 cells. A 40-fold increase or greater in miR-24 was observed in both cell lines compared with control mimic treated cells (**Figure 2-2A**). After plating both control or miR-24-overexpressing cells in transwells, two methods to test barrier function were then performed. As tight junctions form a barrier against ion flow, an electrode can be used to measure TEER (**Figure 2-2B**). Another method to test barrier function is a dextran flux assay. In this assay, fluorescently labeled dextran is added to the upper chamber of the transwell and the amount of dextran in the lower chamber is measured bihourly (**Figure 2-2B**). On every experiment performed, control cells gained TEER at an exponential rate. However, cells overexpressing miR-24 had diminished TEER at all time points. (**Figure 2-2C**). To test if these alterations in barrier function occur at lower levels of over-expression, oligonucleotides were diluted to obtain a 15-fold overexpression of miR-24. Overexpression was still sufficient to impair barrier function **(Supplemental Figure 2-1A**, **B**). For both T84 and Caco-2 cells, miR-24 overexpression significantly increased the amount of 4- and 10-kilodaton (kDa) dextran flux at all time points (**Figure 2-2D**, **E**). In control Caco-2 cells, some dextran fluxed over time, whereas T84 cells were almost completely impermeable to dextran flux when a high TEER was established (**Figure 2-2D**, **E**). These data suggest that miR-24 regulates the formation of the intestinal epithelial barrier. To test if miR-24 inhibition could accelerate barrier formation, antisense miR-24

oligonucleotides were added to the cells during barrier formation. Although antisense miR-24 oligonucleotides caused a dramatic decrease in miR-24 in both Caco-2 and T84 cells, no alterations were observed in the establishment of barrier function, as measured by TEER (**Figure 2-2F**, **G**).

2.4.3 miR-24 Overexpression Does Not Increase Intestinal Epithelial Cell Apoptosis.

To understand the potential mechanism for the disruption of barrier function in cells with elevated miR-24, assays for apoptosis were performed. It would be detrimental to the establishment of a barrier if apoptosis is increased. As a first measurement of apoptosis, a caspase 3/7 activity assay was performed on Caco-2 and T84 cells with altered TEERs. Overexpression of miR-24 in Caco-2 cells reduced apoptosis, as demonstrated by decreased caspase 3/7 activity. In T84 cells, no difference in caspase 3/7 activity was observed between the two groups (**Figure 2-3A**). As a positive control, cells were treated with staurosporine, which induces apoptosis in caspase-independent and caspase-dependent mechanisms [270], as demonstrated by a dramatic increase caspase 3/7 activity (**Figure 2-3A**). As an alternate method to measure apoptosis, a TUNEL assay was performed on transwell inserts from Caco-2 and T84 cells. Both control microRNA- and miR-24 microRNA-treated cells displayed low levels of TUNEL positivity that are indicative of a healthy barrier (**Figure 2-3B**). Staurosporine treatment, similar to increased caspase activity, markedly increased TUNEL positivity (**Figure 2-3B**).

2.4.4 miR-24 Overexpression Does Not Reduce Intestinal Epithelial Cell Proliferation.

If cells overexpressing miR-24 were not able to proliferate then the establishment of barrier would be hindered. To measure cell proliferation, Caco-2 and T84 intestinal epithelial cells were plated sparsely and counted over the course of 4 days or were pulsed with bromodeoxyuridine. It was observed that miR-24-overexpressing cells had similar growth trajectories as control cells over

the period of 4 days (**Figure 2-4A**). Both control microRNA and miR-24 microRNA cells also had similar levels of bromodeoxyuridine uptake at 2 hours, and these levels increased at 4 hours; however, no differences were seen between the groups (**Figure 2-4B**).

2.4.5 miR-24 Overexpression Does Not Alter Junctional Morphology.

As apoptosis levels and proliferation rates were similar between control and miR-24 microRNA-treated cells but barrier function was hindered in miR-24 overexpressing cells, it was next sought to visualize the gross architecture of cell-cell junctions. On the final day of the transwell experiment, cells were fixed and junctions were labeled with fluorescently labeled antibodies. Similar to control cells, Caco-2 cells over-expressing miR-24 had intact tight junctions as observed by ZO-1, claudin-3, and claudin-7 localization and levels (**Figure 2-5**). In T84 cells, claudin-2 immunofluorescence labeling was similar between control and miR-24-overexpressing cells (**Supplemental Figure 2-2**). Thus, the effect of miR-24 negatively regulating the intestinal barrier appears to occur through specific barrier promoting proteins.

2.4.6 miR-24 Regulates the mRNA and Protein Levels of the Tight Junction-Associated Protein Cingulin.

As miR-24-overexpressing cells grew normally and overall junctional appearance seemed normal, western bot analyses were performed on a panel of established junctional proteins to determine how miR-24 regulates the intestinal barrier. The transmembrane proteins claudin-2, claudin-3 and claudin-7 were unaltered in both Caco-2 and T84 cells overexpressing miR-24 compared with controls (**Figure 2-6A**). Although the levels of the cell-cell adhesion protein E-cadherin were unaltered in Caco-2 cells overexpressing miR-24, in T84 cells overexpressing miR-24, a 30% loss in E-cadherin was observed as compared to controls (**Figure 2-6A**). However, the tight junction-associated protein and known direct target of miR-24 [249], cingulin, was significantly

decreased 60 to 70% in miR-24-overexpressing Caco-2 cells and 80 to 90% in T84 cells compared with controls (**Figure 2-6A**). The protein levels of a binding partner of cingulin, pleckstrin homology-domain-containing family A member 7 (PLEKHA7) [271], were not altered in miR-24-overexpressing Caco-2 cells compared with controls (**Figure 2-6A**). In T84 cells, PLEKHA7 protein levels were reduced significantly by 20%. To better understand how miR-24 might regulate cingulin protein levels, the mRNA levels in transfected cells were determined by real time PCR. Similar to the protein levels, cingulin mRNA was significantly decreased in miR-24-overexpressing cells (**Figure 2-6B**). The mRNA levels of PLEKHA7, claudin-2, claudin-3 and claudin-7 were not altered by miR-24 overexpression, whereas the levels of E-cadherin mRNA were significantly decreased in Caco-2 and T84 cells (**Figure 2-6B**). These results suggest that the effect of miR-24 on the intestinal barrier could be due to effects on cingulin mRNA.

2.4.7 Cingulin Is Necessary for Barrier Integrity and is Downregulated in Ulcerative Colitis

To test if cingulin is necessary for the establishment of an intestinal epithelial barrier, an siRNA was used to downregulate cingulin during barrier establishment in both Caco-2 and T84 cells (**Figure 2-7A**). Of interest, cingulin delayed the formation of an intestinal barrier as knockdown of cingulin in both cell lines resulted in an attenuated TEER (**Figure 2-7B**). Dextran flux was also altered in these cells. Both the 4- and 10-kDa dextran assays revealed increased paracellular permeability that almost doubles over the course of the measurement (**Figure 2-7C**, **D**). Given the role of cingulin in barrier function and the alteration of barrier function in IBD patients, a compelling question is whether cingulin is altered in IBD patients. Microarray analysis of human colonic biopsies from patients with active UC and healthy controls revealed a significant 3.2-fold decrease in cingulin levels in UC, even when normalized to an epithelial cell marker cytokeratin 8 ($P = 0.05$) (**Figure 2-7E**). However, the microarray analysis did not reveal a difference in cingulin levels in CD compared with controls (data not shown). Furthermore, cingulin expression was decreased in severely inflamed UC patient colonic biopsies compared with mildly inflamed UC

patient colonic biopsies (**Figure 2-7F**). Therefore, miR-24-mediated regulation of cingulin is likely important for intestinal epithelial barrier function, and this relationship is correlated with disease severity in UC patients.

2.5 Discussion

The intestinal epithelial barrier is vital for normal gut homeostasis and the prevention of inflammation. IBD patients exhibit a faulty epithelial barrier [255]. The establishment and maintenance of the epithelial barrier is regulated in part by microRNAs [272]. Therefore, aberrant expression of microRNAs may contribute to the pathogenesis of IBD. Multiple studies, using unbiased methods, have identified miR-24 as being elevated in the sera and colonic biopsies from IBD [235,236,238,239]. In this study, the gold standard for measuring microRNA expression, real-time quantitative PCR, was used to not only validate these large-scale studies but extend an understanding of colonic miR-24 expression in noninflamed UC, IBS, and CD patients. MiR-24 was specifically elevated in colonic biopsies from actively inflamed UC patients and not noninflamed UC, CD, or IBS patients compared with healthy controls. As miR-24 localized to the colonic epithelium, the alteration of miR-24 levels in UC appears to be specific to intestinal epithelial cells. The cause for upregulation of miR-24 in the colon remains unknown as intestinal epithelial cells treated with cytokines or bacterial antigens failed to show an upregulation in miR-24 levels (data not shown).

Although there is multitude of evidence for miR-24 alterations in UC patients, information was lacking about the function for miR-24 in intestinal epithelial cells. As a major function of intestinal epithelial cells is to establish a barrier, the role for miR-24 in barrier function was tested and miR-24 was found to have a significant detrimental effect. The intestinal epithelial barrier is a combination of cell growth, death and junctional dynamics. This study observed that miR-24 overexpression did not alter cell growth, death, or overall junctional architecture. To understand

how miR-24 might regulate barrier function even with seemingly normal tight junction architecture, the levels or localization of individual junctional proteins that have been previously shown to be directly or indirectly regulated by miR-24 were assessed [249,250]. Although overexpression did not affect ZO-1 localization or E-cadherin protein levels, miR-24 overexpression in intestinal epithelial cells resulted in a striking loss of cingulin protein expression. MiR-24 regulates cingulin through a single binding site on the 3' UTR of its mRNA [249]. Cingulin is a globular protein located in the cytoplasm that can interact with many components of the adherens and tight junctions [273]. A previous study tested if cingulin participated in barrier function; however, those experiments were performed on renal epithelial cells [274]. Therefore, it was tested if cingulin regulates intestinal barrier function, and it was determined that cingulin participates in the formation of a tight barrier.

Although miR-24 inhibits apoptosis, it remains unclear how it regulates apoptosis in intestinal epithelial cells. It has previously been reported in heart, liver and bladder epithelial cells that miR-24 overexpression also reduces apoptosis [218,230,275]. The mechanisms for miR-24 regulating apoptosis have been proposed to be direct regulation of BIM and Caspase 3/8. Further experimentation testing these known targets of miR-24 in intestinal epithelial cells also represent important questions to be addressed in future studies. As the lack of increased apoptosis was evident when miR-24 was altered, it was clear that miR-24 regulated cell-cell junctions. Previous evidence indicates that TEER measures a pore pathway, whereas dextran flux measures a leak pathway [276]; and it has been shown that there can be alterations to the pore pathway without alterations to the leak pathway [277]. In this study, it was observed that miR-24 and cingulin both regulated TEER formation and dextran flux, suggesting that miR-24 and cingulin are important players in intestinal barrier function. Although these observations are novel for miR-24, cingulin has been linked to both the pore and leak pathways. The pore pathway is established by the binding of the claudin family of proteins between cells and depends on the composition of tight and leaky claudins [260]. The leak pathway occurs when the peripheral actomyosin ring contracts,

and this contraction is in part regulated by the small GTPase RhoA. Cingulin has been shown to negatively regulate RhoA and a leaky claudin, claudin-2 [274,278]. However, it was observed that increased claudin-2 occurred in the kidney/duodenum and not the ileum or colon of cingulin knockout mice [279]. In this study, although miR-24 overexpression resulted in a drastic loss of cingulin, there were no alterations in claudin-2 mRNA or protein levels. Further studies on downstream targets for miR-24 and cingulin should include an in-depth characterization of the localization and levels of claudins expressed as well as the role for actomyosin contraction in intestinal epithelial cells.

The elevation of miR-24 levels in both the colon and blood could be a mechanism to open cell-cell junctions allowing for transmigration of immune cells to sites of inflammation or infection. The increased levels of miR-24 in UC patients appear to be secondary to inflammation as UC patients in remission do not display this increase and genetic alterations in miR-24 have not been observed in UC patients [280]. Regardless of the cause for miR-24 upregulation, the data presented in this article suggest that reducing miR-24 levels in actively inflamed UC patients could strengthen the intestinal barrier. This strategy could be used to prevent the vicious cycle of immune cell hyperactivation and further barrier breakdown.

Through this work, strong evidence has been provided that a specific microRNA, miR-24, is related to a specific issue in IBD, intestinal barrier dysfunction. MicroRNAs have been widely studied in IBD, with implications for biomarkers, pathogenesis, and therapeutics. In this study, a role for miR-24 in barrier integrity was established, which lays the foundation for a variety of future studies investigating miR-24 not only in IBD, but also in other barrier associated diseases [281]. Mechanistically, it was established that in colonic epithelial cells, cingulin is a target for miR-24 and this interaction could be involved in the pathogenesis of UC. Future directions will involve

various animal models of colitis and barrier dysfunction, using a miR-24 inhibitor or miR-24 knockout mice to attempt to improve disease outcome.

2.6 Figures

Figure 2-1: miR-24 is elevated in biopsies and blood from ulcerative colitis patients.

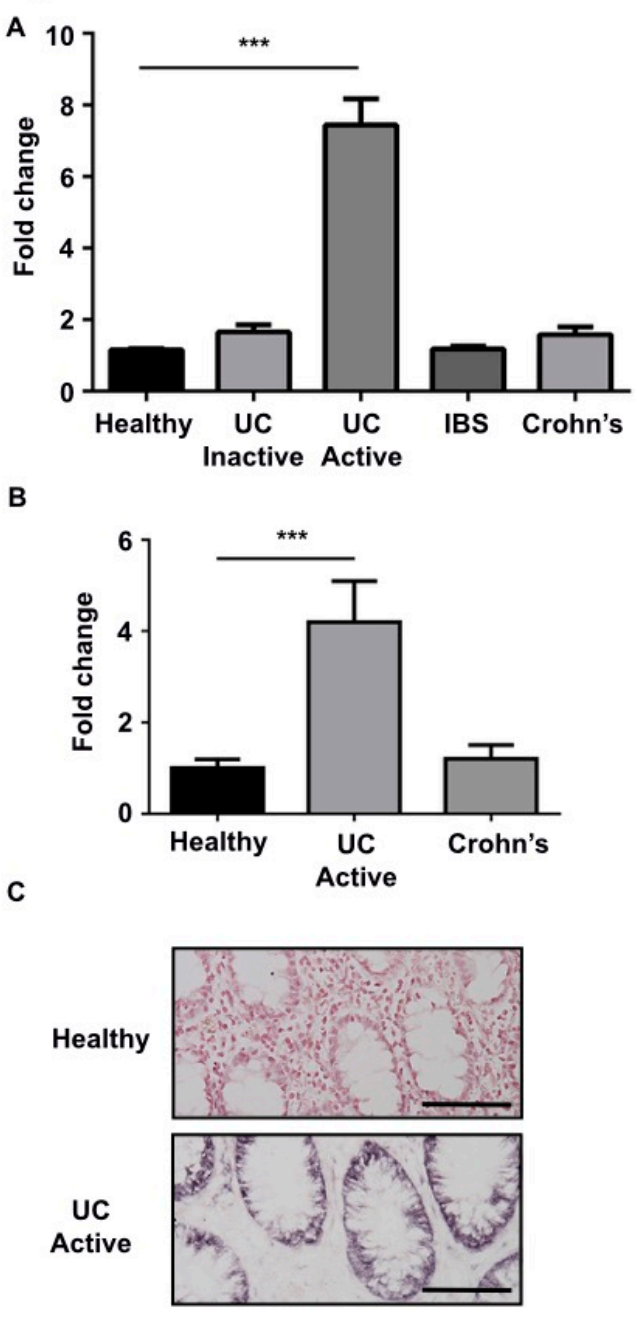


Figure 2-1: **miR-24 is elevated in biopsies and blood from ulcerative colitis patients.**

A: Real-time PCR was used to assess miR-24 levels in colonic biopsies from healthy controls, actively inflamed ulcerative colitis (UC) patients, noninflamed (inactive) UC patients, irritable bowel syndrome (IBS) patients, or Crohn's disease (CD) patients. **B:** Real-time quantitative PCR measurements of miR-24 from human blood collected from patients with active UC, CD, or healthy controls. **C:** *In situ* hybridization of miR-24 in colon tissue from an actively inflamed UC patient or healthy control. Data are expressed as means ± SEM. n = 26 (**A**, healthy controls); n = 48 (**A**, actively inflamed UC patients); n = 9 (**A**, noninflamed (inactive) UC patients); n = 18 (**A**, IBS patients); n = 22 (**A**, CD patients); n = 8 (**B**, patients with active UC); n = 6 (**B**, patients with active CD); n = 7 (**B**, healthy controls). *** $p < 0.001$. Scale bars = 50 μm (**C**).

Figure 2-2: miR-24 overexpression inhibits barrier formation.

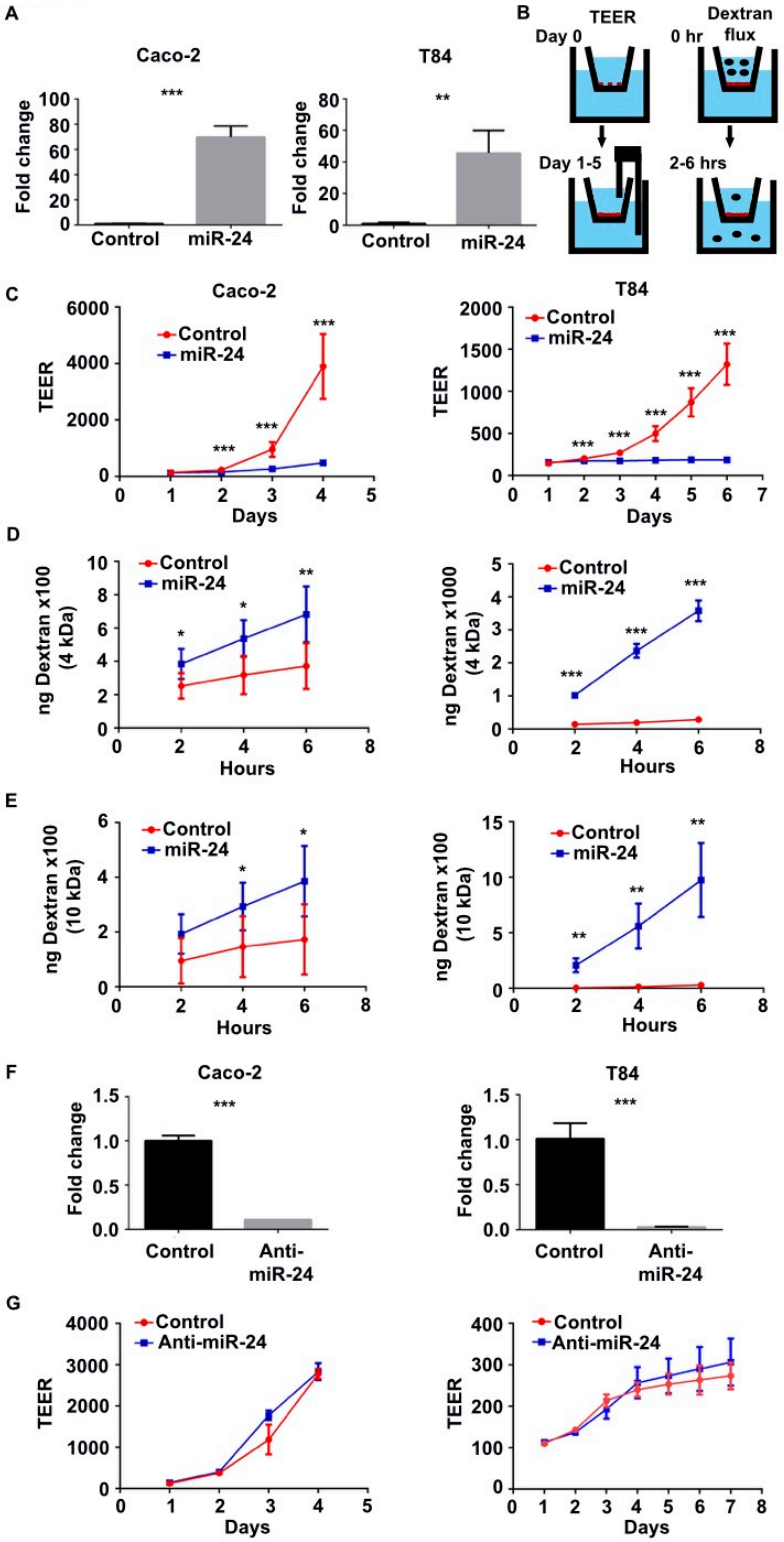


Figure 2-2: miR-24 overexpression inhibits barrier formation.

A: Real-time PCR was used to determine miR-24 overexpression in Caco-2 and T84 cells after transfection. **B:** Illustration showing two different methods to measure barrier function *in vitro*. **C:** Measurements of transepithelial electrical resistance (TEER) over time in Caco-2 or T84 cells treated with a control microRNA or miR-24 microRNA. **D** and **E:** 4-kDA (**D**) or 10-kDa (**E**) dextran flux in Caco-2 or T84 cells was measured at the end point of the experiment. **F:** At the end point of the experiment, real-time quantitative PCR was used to measure miR-24 expression in control microRNA inhibitor-treated cells or miR-24 inhibitor-treated cells. **G:** TEER was measured in control or miR-24 inhibitor-treated cells during barrier establishment. Each graph is representative of three separate experiments with significant results conducted in experimental triplicate. Data are expressed as means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2-3: miR-24 overexpression does not increase intestinal epithelial cell apoptosis.

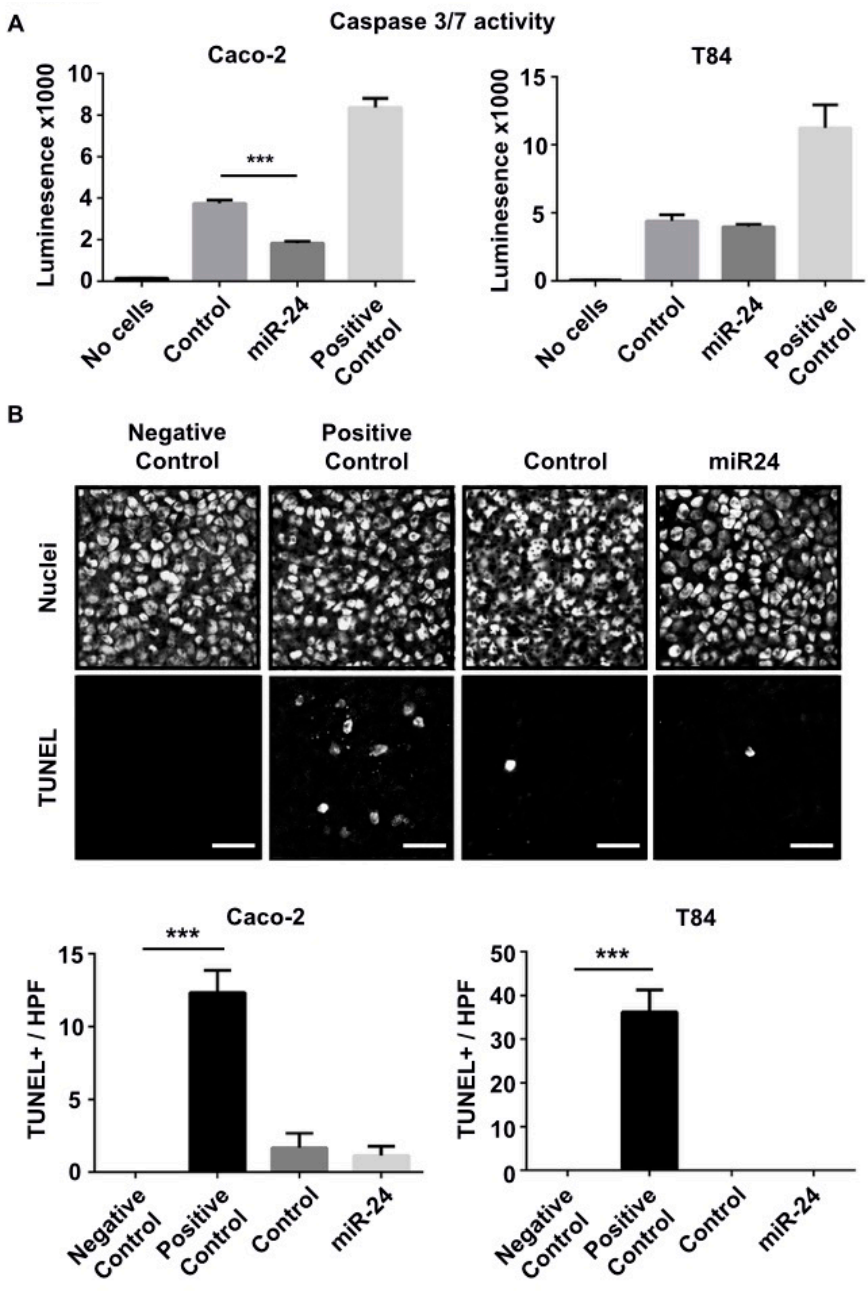


Figure 2-3: miR-24 overexpression does not increase intestinal epithelial cell apoptosis.

A: Caspase 3/7 activity, a measurement of apoptosis, on control or miR-24-treated Caco-2 or T84 cells at the endpoint of the TEER experiment. **B:** A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was used to determine the presence of cells undergoing apoptosis after control microRNA or miR-24 microRNA treatment. Staurosporine was used as a positive control for apoptosis experiments. Each graph is representative of two (T84) or three (Caco-2) separate experiments conducted in experimental triplicate. Data are expressed as means ± SD. *** $p < 0.001$. Scale bars = 50 μm (**B**).

Figure 2-4: miR-24 overexpression does not reduce intestinal epithelial cell proliferation.

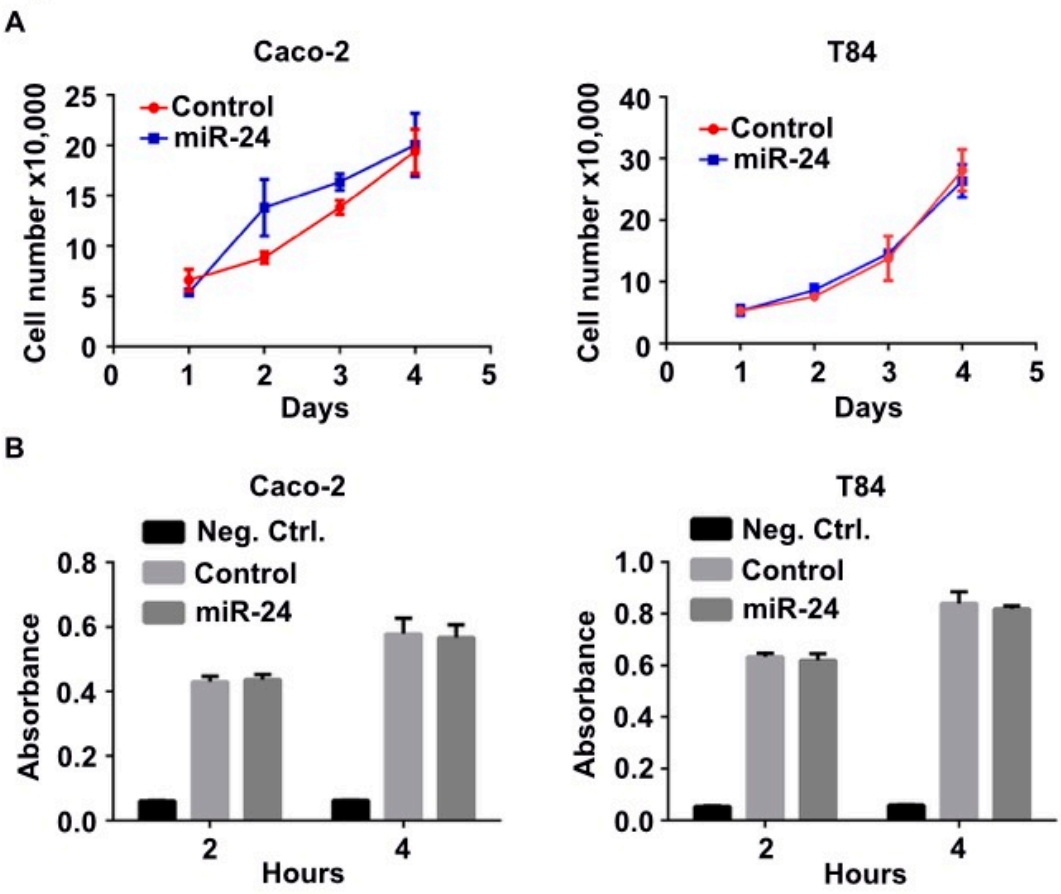


Figure 2-4: miR-24 overexpression does not reduce intestinal epithelial cell proliferation.

A: A quantification of Caco-2 or T84 cell numbers over the period of 4 days after transfection with either a control microRNA or miR-24 microRNA. **B:** To measure cell proliferation, cells were pulsed with BrdU for either 2 or 4 hours before measurement of BrdU incorporation with anti-BrdU antibodies. Cells plated in the absence of BrdU were used as a negative control. Each graph is representative of three separate experiments conducted in experimental triplicate. Data are expressed as means ± SD.

Figure 2-5: miR-24 overexpression does not alter junctional morphology.

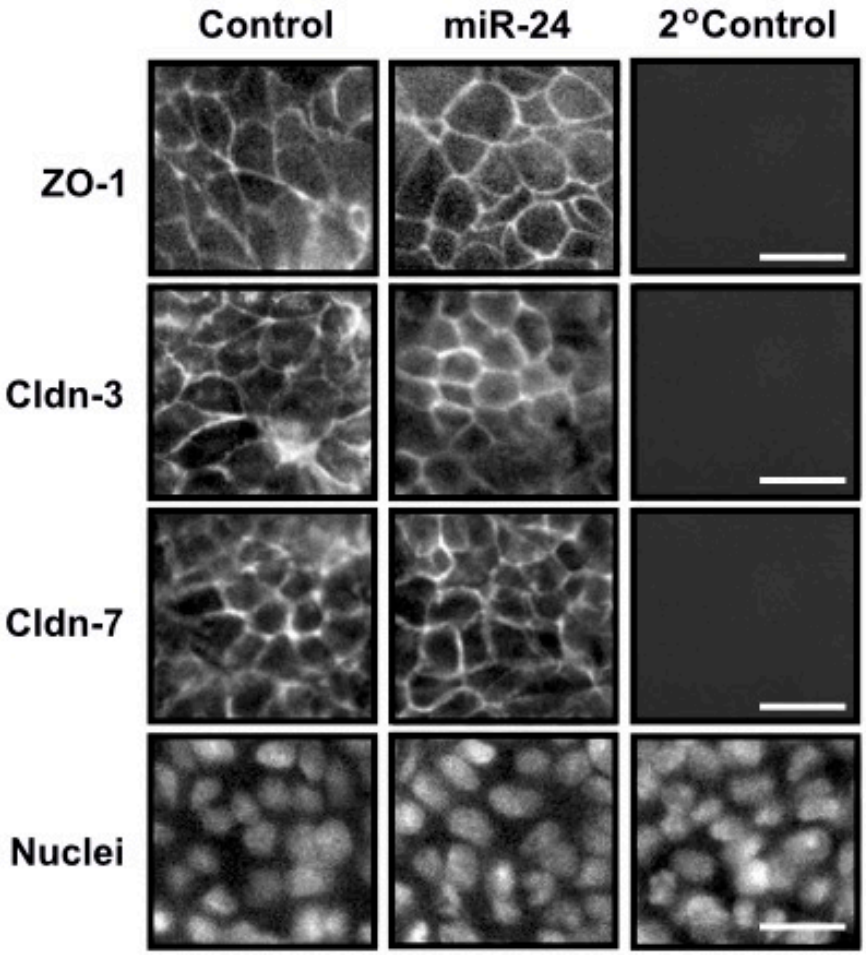


Figure 2-5: miR-24 overexpression does not alter junctional morphology.

Caco-2 cells were fixed at the end point of the experiment, and junctions were labeled with ZO-1, claudin-3 (Cldn-3), and claudin-7 (Cldn-7) antibodies. Nuclei were labeled with a DNA dye. To control for non-specific secondary antibody binding, a control was included that only contained the secondary antibody (secondary control). Scale bar = 25 μm.

Figure 2-6: miR-24 regulates the mRNA and protein levels of the tight junction-associated protein cingulin.

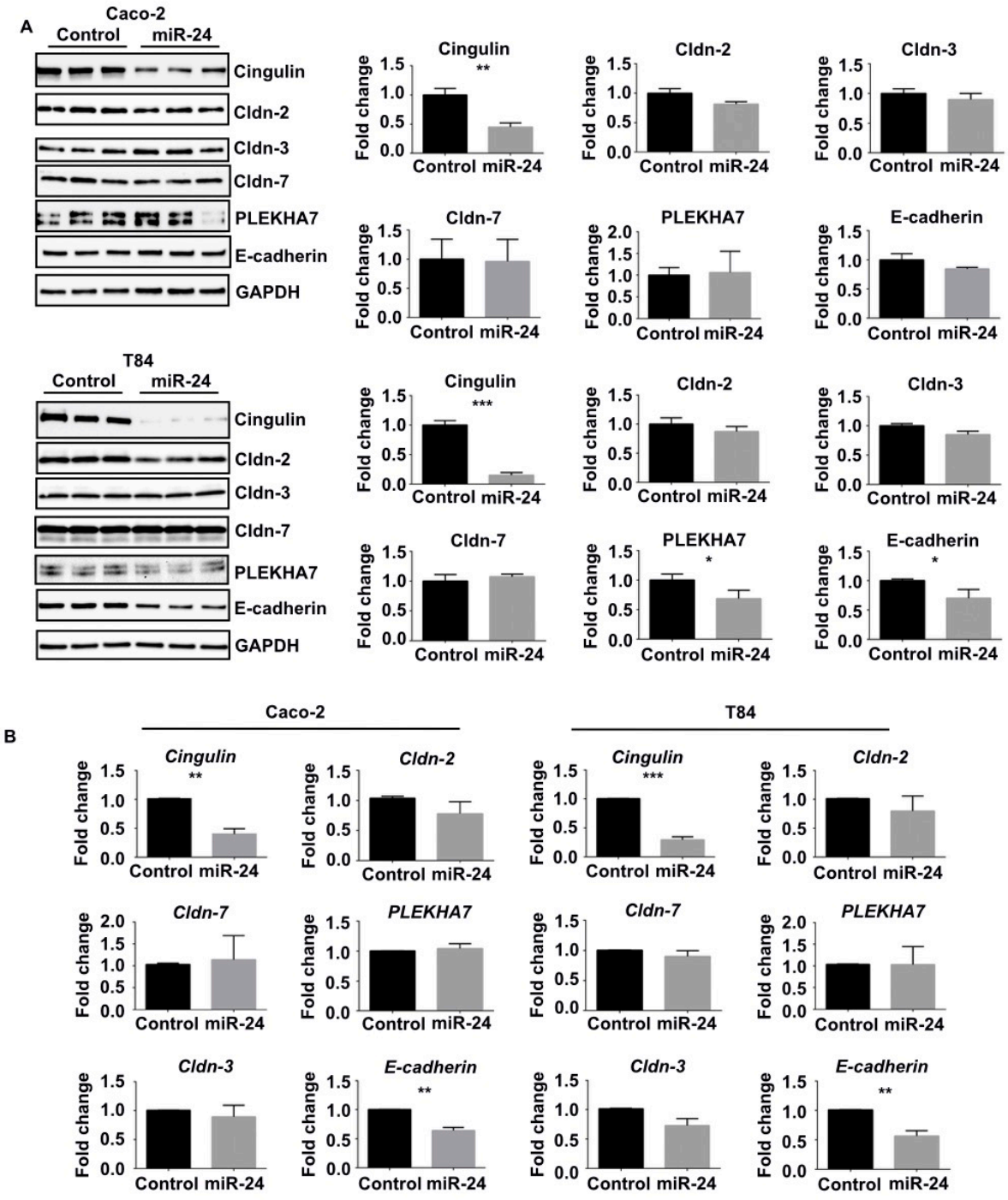


Figure 2-6: miR-24 regulates the mRNA and protein levels of the tight junction-associated protein cingulin.

A: After treatment of Caco-2 or T84 cells with either a control microRNA or a miR-24 microRNA, western blot analyses were performed against cingulin, claudin-2 (Cldn-2), claudin-3 (Cldn-3), claudin-7 (Cldn-7), PLEKHA7 and E-cadherin. GAPDH was used as the loading control. Each graph is representative of three separate experiments conducted in experimental triplicate. **B:** Real-time PCR was used to quantify *Cgn*, *Cldn2*, *Cldn3*, *Cldn7*, *PLEKHA7*, and *E-cadherin* mRNA levels in control microRNA- or miR-24 microRNA-treated Caco-2 or T84 cells. Three independent experiments were performed (**A**). Data are expressed as means ± SD (**A**); data are expressed as means ± SEM (**B**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2-7: Cingulin is decreased in UC patients and downregulation of cingulin impairs barrier formation.

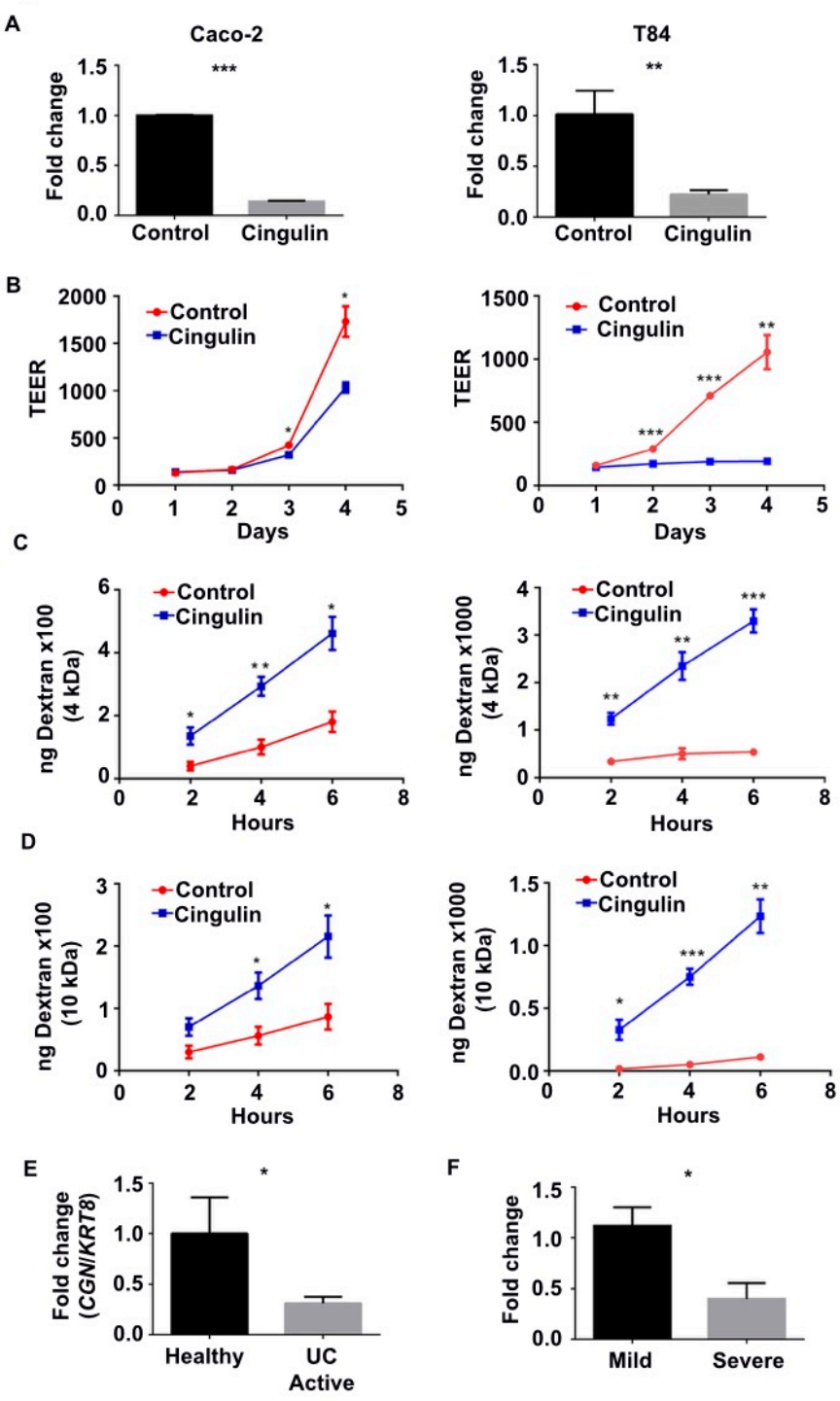
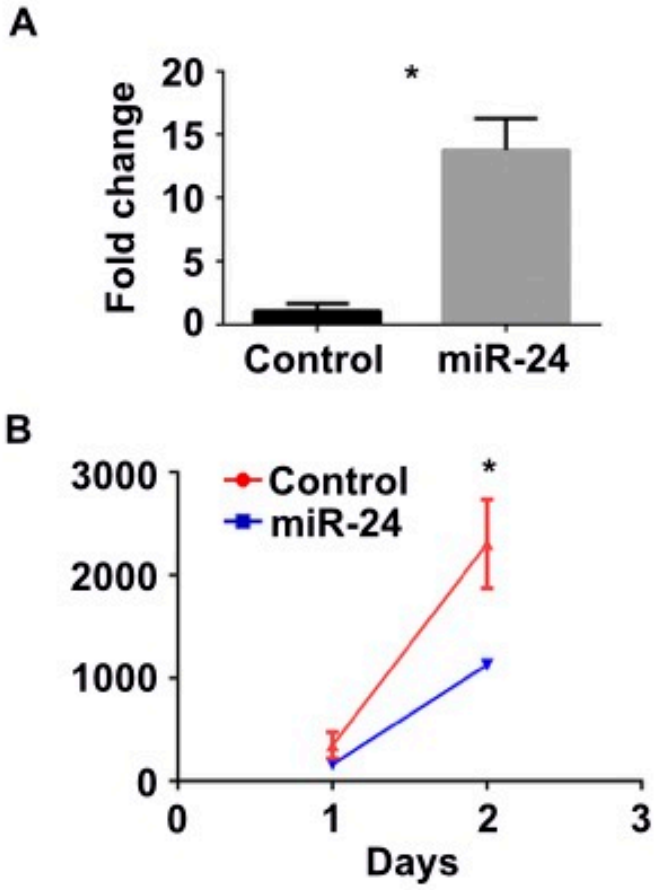


Figure 2-7: Cingulin is decreased in UC patients and downregulation of cingulin impairs barrier formation.

A: Real-time quantitative PCR was used to measure cingulin expression in Caco-2 and T84 cells in control or cingulin siRNA-treated cells. Results are representative of three independent experiments. **B-D:** TEER (**B**) or dextran flux (**C** and **D**) was measured over time in cingulin siRNA-treated Caco-2 and T84 cells. Results are representative of three independent experiments. **E:** Cingulin mRNA levels in healthy controls or UC-active patients, as determined by microarray analysis. To adjust for differences in epithelium, cingulin levels were normalized to cytokeratin 8 (Krt8). **F:** Real-time PCR measurement of cingulin in either mildly inflamed or severely inflamed UC patients. Data are expressed as means ± SEM (**A**-**D**). $n = 7$ (**E**, healthy controls, and **F**, mildly inflamed UC patients); $n = 8$ (**E**, UC-active patients); $n = 4$ (**F**, severely inflamed UC patients). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

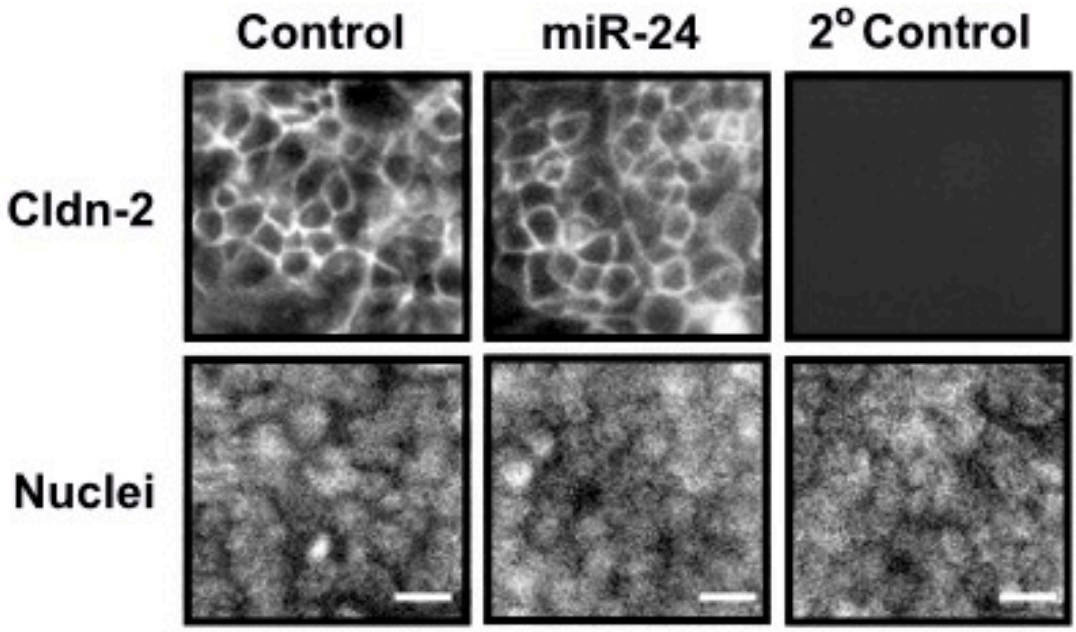
Supplemental Figure 2-1: Low levels of miR-24 overexpression disrupt barrier function.



Supplemental Figure 2-1: Low levels of miR-24 overexpression disrupt barrier function.

A: Cells were transfected with a low concentration of miR-24 or control oligonucleotides and the levels of miR-24 were measured by PCR. **B:** In cells with low levels of miR-24 overexpression, the establishment of TEER was measured of the course of 2 days. Data are expressed as means ± SD. * $p < 0.05$.

Supplemental Figure 2-2: Claudin-2 localization and levels are similar between control and miR-24-overexpressing T84 cells.



Supplemental Figure 2-2: Claudin-2 localization and levels are similar between control and miR-24-overexpressing T84 cells.

Immunofluorescence against claudin-2 was performed on transwell monolayers transfected with a miR-24 oligonucleotide or control oligonucleotide. Scale bar = 25 μm.

CHAPTER 3: MICRORNA-24 INHIBITION PROMOTES EPITHELIAL CELL APOPTOSIS AND IMPAIRS THE RECOVERY FROM INTESTINAL INFLAMMATION

3.1 Abstract

Epithelial restitution following a period of active inflammation is an important sign of remission for Ulcerative Colitis patients. While apoptosis plays a significant role in intestinal homeostasis, it can also be pathogenic if overactive during the period of recovery from inflammation. We recently reported that microRNA-24 is elevated in the colonic epithelium of Ulcerative Colitis patients during active inflammation and regulated cell survival *in vitro*. However, its function during intestinal restitution following inflammation had not been examined. In this study, mice that were recovering from colitis were treated with an antisense oligonucleotide targeting microRNA-24 to determine the influence of microRNA-24 on mucosal repair. We observed that mice treated with the microRNA-24 inhibitor had significantly worsened recovery based on weight loss, colon length, and double-blinded histological scoring. *In vitro* analysis of microRNA-24 inhibition in colonic epithelial cells revealed that inhibition promotes apoptosis and increases levels of the proapoptotic protein BIM. Further experiments determined that silencing of BIM reversed the proapoptotic effects of microRNA-24 inhibition. Taken together, these data suggest that microRNA-24 robustly controls intestinal epithelial cell apoptosis through BIM and its inhibition is detrimental to epithelial restitution following intestinal inflammation.

3.2 Introduction

Ulcerative Colitis (UC) is a chronic inflammatory disease of the colon and rectum. For patients with UC, achieving and maintaining remission is a significant endpoint. UC includes many risk factors that, when combined, result in chronic gastrointestinal inflammation[12]. These factors include immune cell activation, the intestinal microbiome, environmental effects, and epithelial

cell responses[12]. Following a flare of intestinal inflammation, mucosal repair requires intestinal epithelial cells to proliferate, migrate, and resist apoptosis [282-284]. Molecular pathways such as Wnt and EGFR signaling regulate proliferative events [285,286] while integrins regulate migration into the wound [287,288]. Apoptosis is regulated by families of proteins such as Bcl-2 that can either be activated by extrinsic or intrinsic apoptotic signals, initiating a cascade of events that result in caspase activation, PARP cleavage, and DNA fragmentation [289].

Various studies over the past 15 years have profiled UC patient tissue for alterations in microRNA expression[138]. These experiments have uncovered many specific microRNAs with functional significance for the pathophysiology UC. In a recent study, we revealed that microRNA-24-3p (miR-24) is expressed in the intestinal epithelium and is elevated in UC patients[290]. We also observed that overexpression of miR-24 was able to reduce caspase activity in intestinal epithelial cells. Therefore, we hypothesized that *in vivo* inhibition of miR-24, through dysregulation of apoptosis, alters mucosal repair responses in a mouse model of colitis.

In this manuscript, we first tested the efficacy of a chemically modified[151] antisense oligonucleotide targeting miR-24 *in vivo*. We then observed the effect of miR-24 inhibition on recovery from experimental colitis. As miR-24 inhibition dramatically impaired recovery from colitis, further experiments were done to uncover the molecular mechanism enabling miR-24 regulation of intestinal cell apoptosis. Our data show a robust network wherein miR-24 regulates cell survival to affect epithelial restitution following colitis.

3.3 Materials and Methods

3.3.1 In vivo inhibitor treatment and colitis recovery model

Mouse-based experiments were done in accordance with the UCLA IACUC under protocol #2013-030. Custom large scale *in vivo* miRCURY LNA inhibitors were used (Qiagen, Hilden, Germany).

The catalog numbers for the control inhibitor and miR-24 inhibitor were 339203 YCI0201861-FZA & 339203 YCI0201383-FZA, respectively. The drugs were diluted in sterile 0.9% saline solution and 100 µl of drug was injected per mouse at the concentrations stated in the figure legends. For intravenous injections, intraocular administration was performed. For colitis experiments, mice were injected intraperitoneally with 2.5 mg/kg of the inhibitor on days -1, 3 and 7 after DSS. Male 9-week-old C57BL/6J mice were used (Jackson Laboratory, Bar Harbor, ME). Mice were housed 4 per cage, maintained on a 12:12 hour light-dark cycle, and given access to food and water ad libitum. To induce colitis, mice were given 5% dextran sulfate sodium (DSS) (w/v; MP Biomedical, Irvine, CA) dissolved in drinking water. The DSS solution was changed on day 3. Mice received DSS for 5 days and were then switched to water alone and harvested on day 10.

3.3.2 Histology

After mice were euthanized via carbon dioxide and cervical dislocation, colons were dissected, flushed with phosphate buffered saline (PBS) and cut longitudinally. Filter paper was used to create Swiss rolls of the colons. After overnight fixation in 4% paraformaldehyde, colons were transferred to 70% ethanol and the Translational Pathology Core Laboratory at UCLA embedded them in paraffin, cut 5-micron sections, and performed hematoxylin and eosin (H&E) staining. A Versa scanner was used to digitize the slides and ImageScope software was used to view the H&E slides (Leica Camera, Wetzlar, Germany). Three independent reviewers performed double-blinded histology scoring based on immune cell infiltration and epithelial ulceration with the scores of 0-3 corresponding to none, mild, moderate and severe, respectively.

3.3.3 RNA isolation and RT-PCR

A miRNeasy kit (Qiagen) was used to extract and purify microRNA from the mouse distal colon or cultured cells according to the manufacturer's instructions. An LNA RT kit (Qiagen) was used

to generate cDNA from microRNA, according to the manufacturer's instructions. A CFX384 real-time PCR system was used to amplify and detect SYBR Green mediated signal (Bio-Rad Laboratories, Hercules, CA). To measure miR-24, a miRCURY LNA microRNA PCR assay primer was used (YP00204260; Qiagen). The housekeeping primers were RNU1A1 and RNU5G (YP00203909 and YP00203908, respectively; Qiagen).

3.3.4 Cell culture and Transfections

SW480 cells were grown according to instructions (ATCC, Manassas, VA). To facilitate transfection, 70 μM lipofectamine RNAiMax was used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). For overexpression experiments, both a miR-24 mimic and a microRNA mimic negative control were used at a concentration of 50 nM (PM10737 and AM17110, respectively; Ambion, Austin, TX). For inhibition experiments, the miR-24 antisense oligonucleotide and control inhibitor were used at a concentration of 50nM. The control (4390843) and BIM (4390824-s195011) Silencer Select siRNAs were also used at 50 nM (Invitrogen).

3.3.5 Western Blotting

All general western blot reagents were from Bio-Rad Laboratories. Semi-confluent cells were harvested in reducing Laemmlli sample buffer as previously described[290]. Equal volumes of lysates were loaded onto 4-20% gradient denaturing polyacrylamide gels. To transfer proteins to PVDF membranes, a TransBlot Turbo RTA transfer kit was used. Membranes were blocked in 5% blotting-grade blocker in PBS with 0.05% Tween-20. Clarity enhanced chemiluminescent reagent and a Chemidoc Touch imaging system were used to develop and image the blots. The following primary antibodies were used: BIM (1:1000, Cell Signaling Technology, Danvers, MA, #2933), Cleaved PARP (1:1000, Cell Signaling Technology, #9541), and Tubulin (1:5000, Sigma-

Aldrich, St. Louis, MO, #T5168). Horseradish peroxidase-labeled secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

3.3.6 Apoptosis Assays

Caspase 3/7*:* 100,000 cells were plated in transfection mix on 96 well plates. The cells were incubated overnight before dimethyl sulfoxide (DMSO) (1:1000) or 2 μM staurosporine (Tocris Bioscience, Bristol, UK) treatment for 4 hours. After removing the media, the cells were washed once in PBS and 50 μl of Caspase 3/7 GLO buffer was added per well (Promega, Madison, WI). After 15-30 minutes of lysis, a Synergy HT plate reader was used to measure luminescence (BioTek Instruments, Winooski, VT).

TUNEL*:* 180,000 cells were plated in transfection mix on chambered slides. After 24 hours, cells were treated with DMSO (1:1000) or 2 μM staurosporine for 4 hours. Cells were then fixed with PFA and a Click-it TUNEL plus kit was used according the manufacturer's instructions (Invitrogen). Nuclei were counterstained with DAPI and slides were mounted in Prolong Gold (Invitrogen). The cell counter function in ImageJ (National Institutes of Health, Bethesda, MD) was used to individually label and count cells. A 710 confocal microscope was used for imaging the slides (Carl Zeiss AG, Oberkochen, Germany).

3.3.7 Statistical analysis

Statistical differences between groups were evaluated using an unpaired t-test. Graphing was completed using GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

3.4 Results

3.4.1 A cell-permeable miR-24 inhibitor reduces colonic miR-24 levels in vivo

To identify whether miR-24 regulates the recovery from colitis, we first tested the route of administration and efficacy of a cell-permeable antisense oligonucleotide against miR-24. We started with a high dose, 12.5 mg/kg, and analyzed its ability to inhibit miR-24 three days after either intravenous (IV) or intraperitoneal (IP) injections. We observed that both methods of administration resulted in near 100% inhibition of miR-24 as measured by qPCR (**Figure 3-1A**). We next determined that an IP injection with a dose as low as 2.5 mg/kg resulted in full inhibition, and at that dose, this effect persisted for 5 days but not 7 (**Figure 3-1B**, **C**). From these experiments, we concluded that a 4-day, 2.5 mg/kg dosing interval would be sufficient to inhibit miR-24 expression in our *in vivo* recovery model.

3.4.2 Inhibition of miR-24 reduces mucosal repair after colitis

To test the effects of miR-24 inhibition on colitis, we injected mice on day -1, day 3, and day 7 with either a control inhibitor or the miR-24 inhibitor (**Figure 3-2A**). On day 0, mice received dextran sulfate sodium (DSS), a molecule that induces colitis by causing distal colonic ulcerations[291]. Starting on day 5, mice were placed on water for an additional 5 days to enable the onset of mucosal repair mechanisms that result in near total recovery from colitis. Similar to what was observed in **Figure 3-1**, we confirmed near complete downregulation of miR-24 expression at the end of our 10-day protocol (**Figure 3-2B**). Mice treated with either the control inhibitor or the miR-24 inhibitor reached peak weight loss levels by day 8 (**Figure 3-2C**). However, after peak weight loss, control inhibitor-treated mice began to recover whereas miR-24-inhibited mice continued to lose weight. This resulted in a statistically significant 8% difference in body weight between the two groups on day 10 (**Figure 3-2C**). Another indicator of the severity of intestinal inflammation is colon shortening. We observed that miR-24-inhibited colons were almost

a full centimeter shorter than control inhibitor-treated colons (**Figure 3-2D**). To analyze histological damage, we H&E stained Swiss-rolled colons, with the distal colon on the outside and the proximal colon on the inside. In control inhibitor-treated mice, only the most distal region of the colon was ulcerated on day 10; however, miR-24-inhibited mice were significantly ulcerated throughout the colon (**Figure 3-2E**). When observing magnified segments of the central-distal region of the colons, crypt regeneration was apparent in control inhibitor-treated mice on day 10 (**Figure 3-2F**). However, there was little crypt regeneration in miR-24-inhibited mice (**Figure 3-2F**). These effects were quantified by double-blinded reviewers who scored the amount of immune cell infiltrate and epithelial ulceration, with the sum of the two being the total histology score. All three independent scores were significantly elevated in the miR-24 inhibitor-treated DSS recovery group as compared to controls, confirming impaired intestinal restitution (**Figure 3-2G**). We did not observe any differences in the histology of miR-24 inhibitor-treated mice without DSS treatment as compared to controls (**Figure 3-2F**). These results suggest that miR-24 inhibition weakens the recovery from acute colitis.

3.4.3 miR-24 inhibition promotes intestinal epithelial cell apoptosis in vitro

To determine the mechanism of miR-24 inhibition's impairment of intestinal restitution, we transitioned to *in vitro* studies. Based on previous data[290], we hypothesized that miR-24 regulated intestinal epithelial cell apoptosis. We conducted two apoptosis assays on intestinal epithelial cells treated with either the miR-24 inhibitor or a miR-24 mimic, in the presence or absence of the cell death inducer staurosporine. We chose to add a cell death inducer as an *in vitro* method to mimic the proapoptotic inflammatory environment observed during active UC. We chose two apoptosis assays based on the step in the apoptotic cascade: a caspase 3/7 activity assay, an intermediary event, and TUNEL staining, which measures DNA cleavage, a final event. Cells treated with the miR-24 inhibitor had increased caspase activity at baseline compared to control inhibitor treated cells (**Figure 3-3A**). Increased caspase activity was also observed in cells treated

with staurosporine plus the miR-24 inhibitor compared to cells treated with staurosporine plus the control inhibitor (**Figure 3-3A**). Conversely, cells overexpressing miR-24 at baseline had reduced caspase activity compared to vehicle controls and miR-24 overexpression also reduced caspase activity induced by staurosporine (**Figure 3-3B**). With TUNEL staining, we were able to identify that miR-24 inhibition increased the number of dying cells per total cells by approximately 2-fold compared to controls (**Figure 3-3C**). Furthermore, miR-24-inhibited cells co-treated staurosporine had a 2-fold increase in TUNEL positive cells compared to control inhibitor cells co-treated staurosporine (**Figure 3-3C**). While overexpression of miR-24 at baseline resulted in no significant effects on TUNEL staining, there was a large repression of TUNEL positive cells induced by staurosporine following miR-24 elevation (**Figure 3-3D**). These results suggest that inhibition of miR-24 can further promote intestinal epithelial cell death caused by inflammation, which likely contributes to the weakened recovery observed in our murine model of mucosal repair after colitis.

3.4.4 miR-24 regulates the proapoptotic protein BIM in intestinal epithelial cells

When comparing the seed sequence of miR-24 to the 3'UTR of the Bcl-2 family of apoptosis-associated proteins, BIM (Bcl-2 interacting mediator of cell death), a proapoptotic mediator, has perfect complementarity. We therefore tested if miR-24 regulated BIM in intestinal epithelial cells. Western blots against BIM and cleaved PARP (Poly ADP-ribose polymerase), a downstream target of BIM, demonstrated that miR-24 inhibition alone can increase BIM and cleaved PARP levels compared to cells treated with the control inhibitor (**Figure 3-4A**). In staurosporine-treated cells, miR-24 inhibition also increased the levels of both BIM and cleaved PARP compared to control inhibitor-treated cells (**Figure 3-4A**). When miR-24 was overexpressed, BIM levels dramatically decreased compared to control cells (**Figure 3-4B**). Additionally, miR-24 overexpression was able to almost entirely revert the increases in BIM and cleaved PARP induced by staurosporine, demonstrating the potent anti-apoptotic abilities of a miR-24 analog in intestinal epithelial cells.

3.4.5 Downregulation of BIM significantly reduces the induction of apoptosis caused by miR-24 inhibition

In order to further elucidate the microRNA/gene network involving miR-24 and BIM, we sought to determine if the proapoptotic effects of miR-24 inhibition requires BIM. To achieve this, we employed an siRNA targeting BIM in the presence of staurosporine. We observed that treatment with BIM siRNA resulted in a robust downregulation of BIM protein via western blot (**Figure 3-5A**). After establishing the ability to successfully silence BIM in intestinal epithelial cells, we next determined caspase 3/7 activity with and without BIM downregulation in the presence of the miR-24 inhibitor. In cells treated with the mir-24 inhibitor and BIM siRNA, caspase activity was significantly reduced compared to cells treated with the miR-24 inhibitor and control siRNA, demonstrating that BIM mediates some of the effects on apoptosis downstream of miR-24 inhibition (**Figure 3-5B**). Lastly, BIM siRNA treatment was able to reduce the increase in the number of TUNEL positive cells seen after miR-24 inhibition to a level similar to control inhibitor plus control siRNA treatment (**Figure 3-5C**). These experiments demonstrate that BIM is partly required for the effects of miR-24 inhibition on apoptosis and is a likely player in miR-24-mediated regulation of mucosal repair after colitis (**Figure 3-6**).

3.5 Conclusions

Treatments directly targeting the intestinal epithelium to treat UC remain an important focus of ongoing therapeutic development [283]. Such efforts have been largely focused on proinflammatory cytokines, immune cell function, intestinal permeability, and apoptosis. Given our previous findings that miR-24 is increased in the colonic epithelium of UC patients, we sought to evaluate the potential protective actions of miR-24 manipulation in mice recovering from colitis [290]. While it was at first surprising that miR-24 inhibition worsened the recovery from colitis, when analyzing the literature, miR-24 has been shown to regulate apoptosis in many other cell types and diseases

not related to colitis [189,230,232,292-295]. Thus, inhibition of miR-24 promoting apoptosis during intestinal recovery would be consistent with the literature and reinforce the circumstantial importance of miR-24 function.

The heterogeneity of miR-24 between different conditions is well established[182]. Our previous study demonstrated that miR-24 is elevated during active UC and that overexpression diminished intestinal epithelial barrier integrity. In this study, we reveal that during recovery from colitis, inhibition of miR-24 is detrimental. Therefore, our data supports the significance of spatiotemporal differences when studying miR-24 function. Future directions would determine miR-24 expression levels during induction, peak, and resolution of inflammation in both UC patients and mouse models of colitis. Knowledge of the situational expression of miR-24 would shed light on the function of this microRNA during these varying conditions.

These results suggest that using an *in vivo* miR-24 mimic only during restitution would promote epithelial recovery and remission of disease. While it is currently technically difficult to overexpress a microRNA *in vivo*, improving the stability and uptake of microRNA mimics, perhaps using newly developed techniques involving lipid nanoparticles [296], could overcome these obstacles. These results could also be confirmed with a miR-24 knockout mouse that currently does not exist. This approach is particularly difficult because miR-24 is produced at two separate genomic loci. However, we have recently created mice that will allow us to breed double miR-24 knockout mice for future studies.

Our results also shed light on important aspects of the biology of BIM. Interestingly, the downregulation of BIM almost completely abrogated the increased TUNEL staining caused by miR-24 inhibition, yet the downregulation of BIM only had modest effects on the increased caspase 3/7 activity caused by miR-24 inhibition. These results suggest that miR-24 inhibition

may regulate apoptotic pathways other than BIM to promote caspase activity. These results connecting miR-24 to BIM also have implications on intestinal permeability, as we have shown that miR-24 overexpression diminishes intestinal barrier function. While it is obvious that elevated apoptosis of the epithelium would increase permeability, it would be interesting to see if a baseline level of apoptosis is also required for proper barrier function and if BIM restoration could rescue the effects of miR-24 overexpression on permeability. Lastly, contrary to the hypothesis that inhibition of apoptosis would result in increased mucosal repair via regenerative epithelial effects, BIM knockout mice display worse histology scores during colitis [297]. However, these knockout mice have global BIM deficiency, so the negative effects on colitis could be due to a lack of cell death in activated immune cells. As we have previously observed that miR-24 colonic expression to be specific to the epithelium [290], the possibility of cell-specific targeting of BIM through miR-24 could be key to future therapies.

3.6 Figures

Figure 3-1: A cell-permeable miR-24 inhibitor efficiently reduces colonic miR-24 levels.

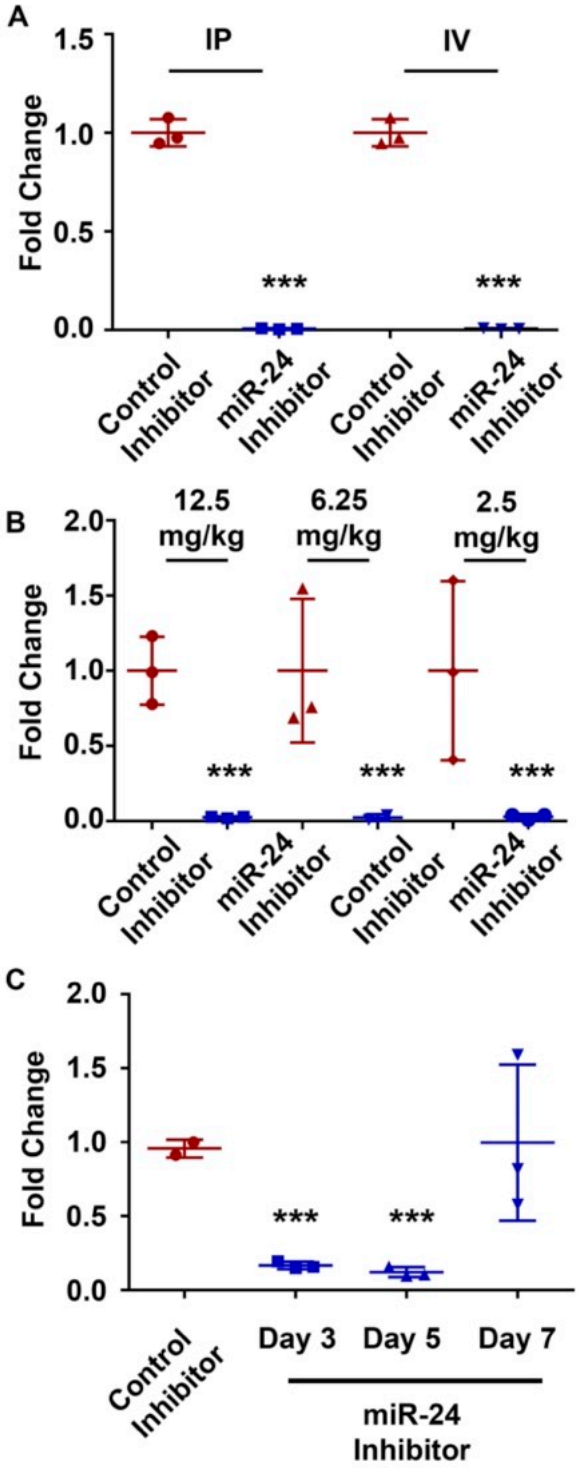


Figure 3-1: A cell-permeable miR-24 inhibitor efficiently reduces colonic miR-24 levels.

A: Mice received an intraperitoneal (IP) or intravenous (IV) injection of 12.5 mg/kg of a control inhibitor or miR-24 inhibitor and colonic RNA was extracted 4 days later. n=3 mice per group, for IP representative of 2 independent experiments. Mean±SD. **B:** IP injections of 12.5, 6.25 and 2.5 mg/kg of a miR-24 inhibitor were tested for their efficacy at 4 days post injection. n=3 mice per group, representative of 2 independent experiments. Mean±SD. **C:** Mice were IP injected with 2.5 mg/kg of a miR-24 inhibitor and were sacrificed 3, 5, or 7 days later. n=3 mice per group. Mean±SD. *** $p<0.001$.

Figure 3-2: Inhibition of miR-24 reduces mucosal repair after colitis.

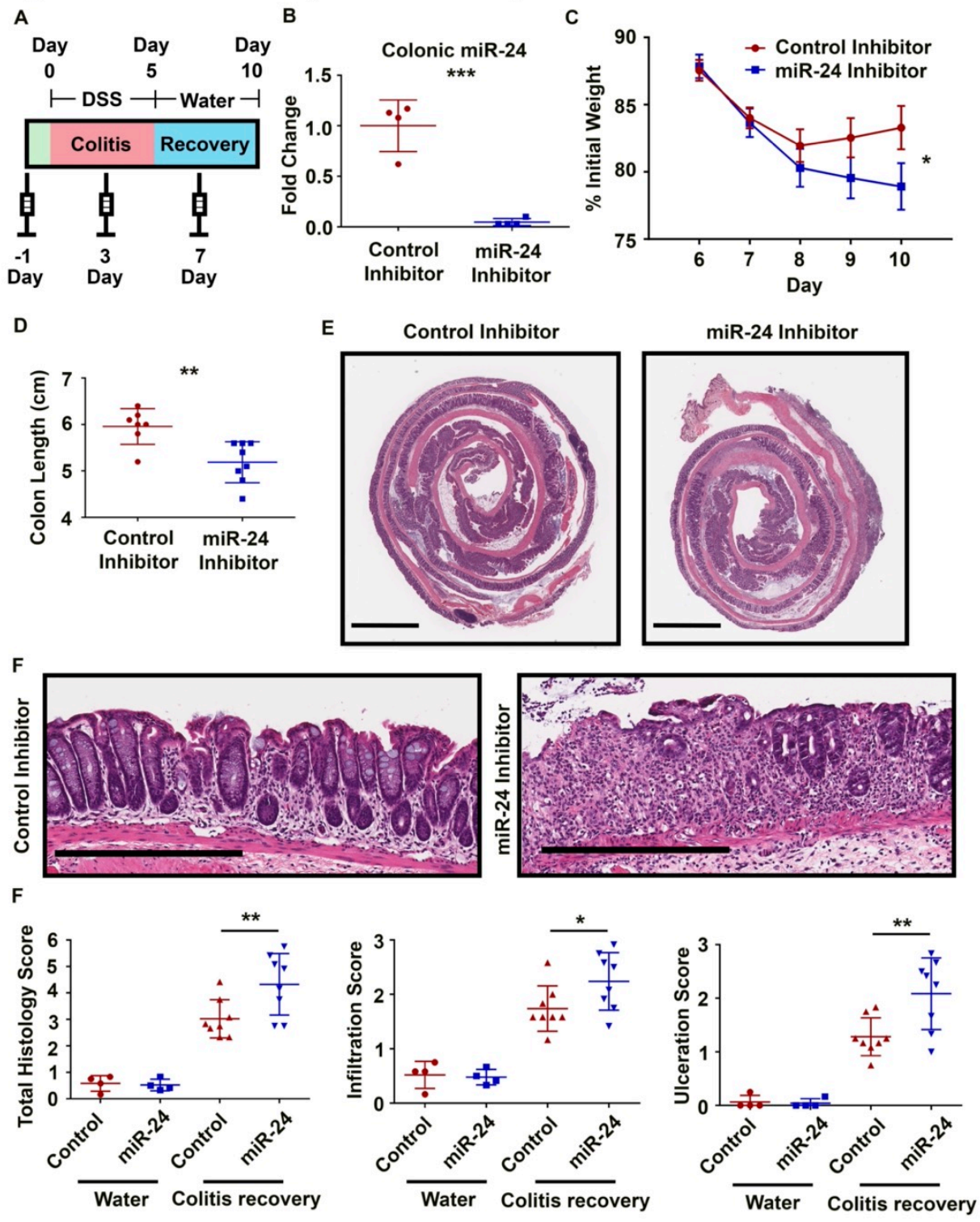


Figure 3-2: Inhibition of miR-24 reduces mucosal repair after colitis.

A: A schematic describing the time course for *in vivo* experiments. **B:** After colitis and recovery, the level of miR-24 in the distal colon was measured by RT-qPCR. n=4 mice/group. Mean±SD. **C:** A graph of percent weight change over the course of recovery after colitis. n= 24 mice / group. Mean±SEM. 2 independent experiments. **D:** On day 10 of the protocol, mice were euthanized and colon lengths were measured from the rectum to the cecum. n= 8 mice per group. Mean±SD. 2 Independent experiments. **E:** Representative H&E stained Swiss-rolls used for histology scoring. Scale bars = 2 mm. **F:** A magnified image of the central colons of control inhibitor or miR-24 inhibitor treated mice. Scale bars = 0.4 mm **G:** Graphs of double-blind histology scores. n=8 mice per group for water treated, n=16 mice per group for DSS treated. Mean±SD. 2 independent experiments * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3-3: miR-24 suppresses intestinal epithelial apoptosis *in vitro*.

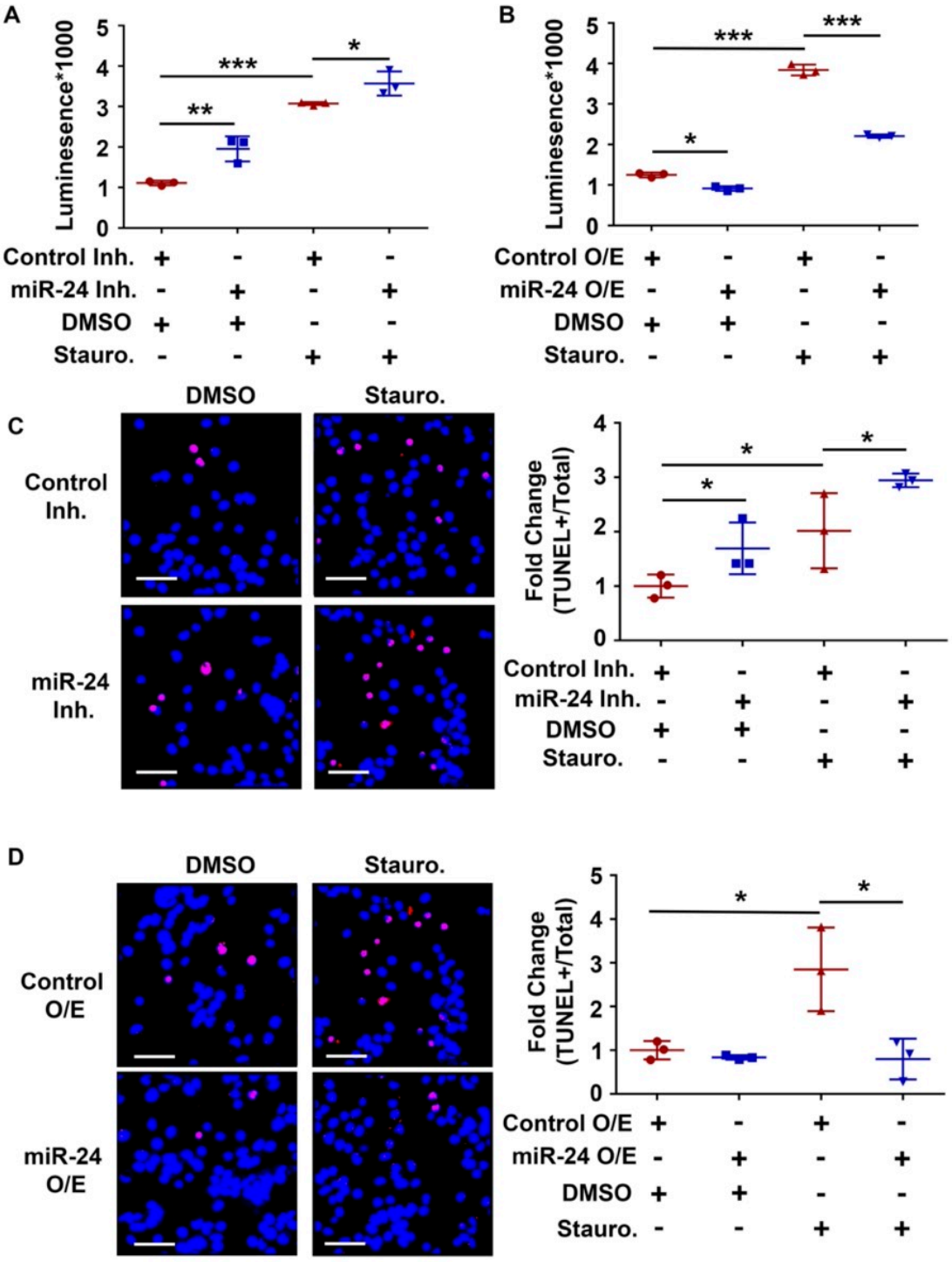


Figure 3-3: miR-24 suppresses intestinal epithelial apoptosis *in vitro*.

A, B: Intestinal epithelial cells transfected with either control or miR-24 inhibitor (Inh) (**A**) or mimic (O/E) (**B**), in the presence or absence of staurosporine (Stauro), were lysed in a buffer containing a caspase 3/7 reactive luminescent buffer. n=3 replicates per group. Results are representative of 2 independent experiments. Mean±SD. **C, D:** Intestinal epithelial cells transfected with either inhibitors (**C**) or mimics (**D**), in the presence or absence of staurosporine, were fixed and TUNEL stained. Magenta cells are TUNEL positive and non-TUNEL positive cell nuclei are labeled in blue. Three 250 μm x 250 μm fields were selected for quantification and are depicted in the graph. Results are representative of 2 independent experiments. Scale bars = 50 μm. Mean±SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3-4: miR-24 regulates the proapoptotic protein BIM in intestinal epithelial cells.

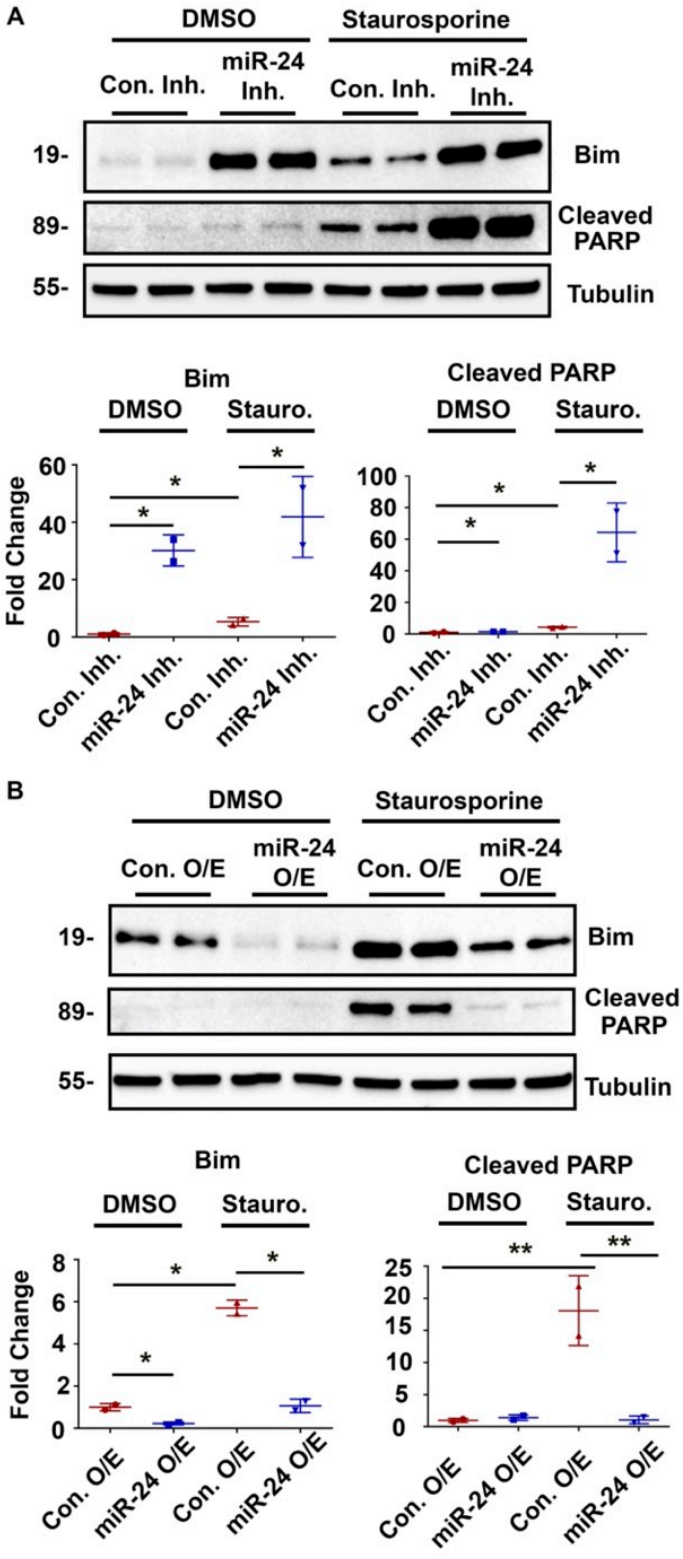


Figure 3-4: miR-24 regulates the proapoptotic protein BIM in intestinal epithelial cells.

A: Western blots and densitometric analysis of BIM, Cleaved PARP and Tubulin from cells treated with control or miR-24 inhibitor in the presence or absence of staurosporine (Stauro). n=2 replicates per group. Results are representative of 2 independent experiments. Mean±SD. **B:** Western blots and densitometric analysis of BIM, Cleaved PARP and Tubulin from cells treated with control or miR-24 mimic in the presence or absence of staurosporine. n=2 replicates per group. Results are representative of 2 independent experiments. Mean±SD. ** $p<0.01$; *** $p<0.001$.

Figure 3-5: Downregulation of BIM significantly reduces the induction of apoptosis caused by miR-24 inhibition.

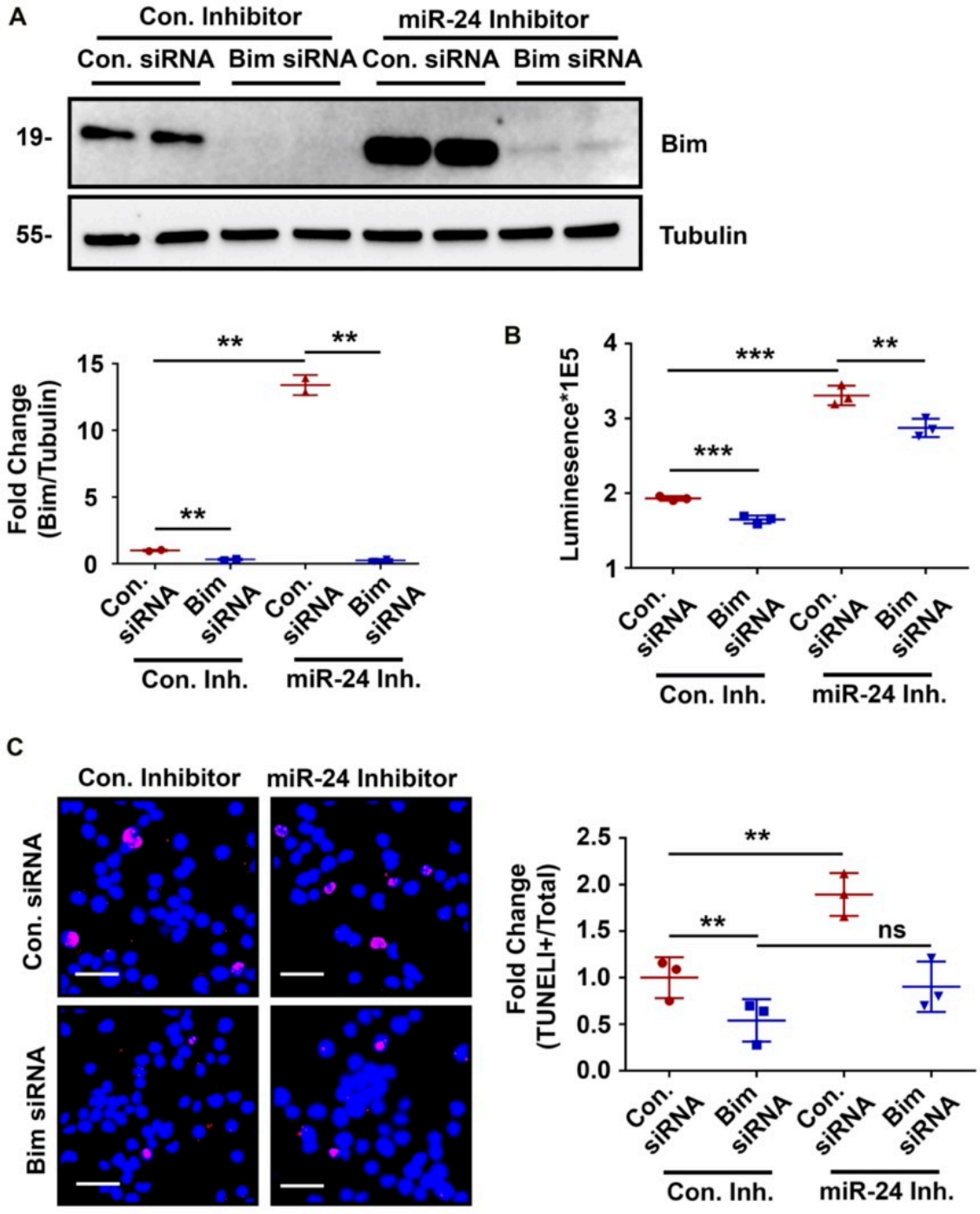


Figure 3-5: Downregulation of BIM significantly reduces the induction of apoptosis caused by miR-24 inhibition.

A: Western blots and densitometric analysis of BIM and Tubulin from cells treated with staurosporine concomitantly with either a control or BIM siRNA and a control inhibitor or miR-24 inhibitor. n=2 replicates per group. Results are representative of 2 independent experiments. Mean±SD. ** $p<0.01$. **B:** Caspase 3/7 GLO assays were performed on siRNA and inhibitor treated cells in the presence of staurosporine. n=2 replicates per group. Results are representative of 2 independent experiments. Mean±SD. **, $p<0.01$; ***, $p<0.001$. **C:** TUNEL based fluorescence microscopy was used to measure the number of apoptotic cells. Magenta cells are TUNEL positive and non-TUNEL positive cell nuclei are labeled in blue. Three 250 µm x 250 µm fields were selected for quantification and are depicted in the graph. Results are representative of 2 independent experiments. Scale bars = 50 µm. Mean±SD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure 3-6: A schematic outlining the molecular mechanisms that enable miR-24 regulation of mucosal repair.

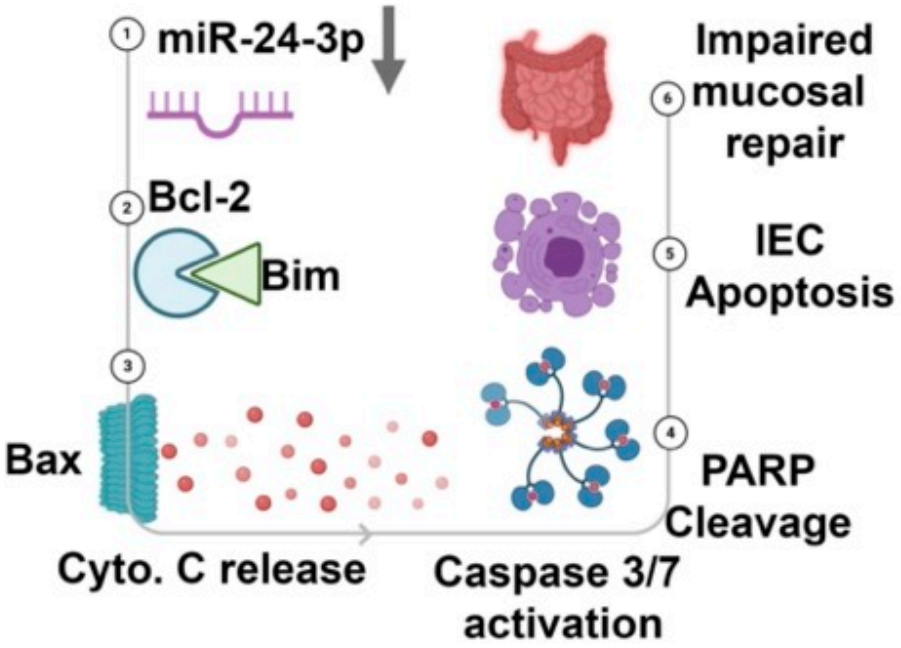


Figure 3-6: A schematic outlining the molecular mechanisms that enable miR-24 regulation of mucosal repair.

We observed that when miR-24 is inhibited (**1**) the protein levels of BIM increase (**2**). BIM then stabilizes Bcl-2 enabling Bax-mediated cytochrome C release from the mitochondria (**3**). Cytochrome C activates caspases which result in events such as PARP cleavage (**4**). The activated proapoptotic enzymes then induce apoptosis in the intestinal epithelium (**5**). Induction of apoptosis in during mucosal repair after colitis worsens outcomes (**6**).

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The human genome encodes ~2,600 mature microRNAs[298]. For each mature microRNA, there is a unique sequence that may have complementarity with one or more mRNAs. Inevitably, there are multiple microRNAs that target the same gene, and multiple mRNAs that a microRNA can bind to[299,300]. While elucidating the activity of a given microRNA in order to define microRNA/gene networks in different cells and organs is difficult, results from such studies could be significant for human physiology and pathophysiology. Research focused on microRNA activity has the potential to establish expression patterns in disease, clarify molecular mechanisms of important cellular functions, and introduce targets for therapeutic intervention. The work presented here has described two unique functional roles for a specific microRNA, miR-24, while outlining its expression in UC and the specific genes and processes it regulates.

Already greatly studied in cancer and cardiovascular physiology[182,226,229], there was a gap in knowledge for the role of miR-24 in IBD. The data presented here reveal two separate but related roles for miR-24 in UC, intestinal barrier damage during active disease and impaired wound healing during recovery (**Figure 2-2**, **Figure 3-2**). With a specific gene target determined for each functional role of miR-24, the potential network of regulation and consequence has been established. While these data provide strong evidence to pursue miR-24-related therapies for UC, it also illustrates the obstacles that must be overcome to optimize its usage. Nevertheless, these data describe roles for miR-24 that have far-reaching implications beyond UC and suggest multiple paths forward in future directions for a role of miR-24 in inflammation and mucosal healing.

4.1 miR-24 and Barrier Function

Our data revealed that miR-24 overexpression hampered barrier function through direct downregulation of the tight junction protein cingulin (**Figure 2-2**, **Figure 2-6**). The implications of these data are important, as intestinal barrier dysfunction is linked to a variety of other diseases. Furthermore, dysregulation of cingulin has been studied in the context of other cell permeability-related conditions. Taken together, these data support further studies of miR-24's influence on cellular processes and diseases linked to cingulin and/or intestinal barrier function.

4.1.1 Implications of miR-24-mediated intestinal barrier dysfunction for other diseases

The gastrointestinal epithelial barrier plays an important role in gut homeostasis. The luminal components of the intestinal tract comprise of bacteria, toxins, and other pathogens that illicit an immune response if able to infiltrate the intestinal wall. One defense against these luminal components is the epithelial barrier, which consists of protein complexes that make up the space between the cells of the first layer of the gastrointestinal tract. These complexes, which include the tight junction, adherens junction, and desmosome, take part in regulation of paracellular transport, cell-cell and cell-matrix communication, and structural stability[46]. Studies have shown that UC patient colons have impaired barrier function[254], which could be caused by dysregulation of important tight junction proteins and/or inflammatory cytokines[43,44]. *In vivo* modeling of barrier dysfunction showed that a leaky barrier promotes immune cell activation and cytokine secretion[301]. Supplementing these studies is our data revealing elevated miR-24 in UC, which could be a major mechanistic component in the barrier dysfunction seen during disease. At the very least, miR-24's upregulation combined with the other specific tight junction defects during UC is contributing to the perpetuating disease state.

Intestinal barrier dysfunction has also been implicated in a variety of other human diseases. The expanding role of the gut microbiota and systemic inflammation on the organs outside of the

intestinal tract has emphasized the importance of the epithelial barrier. One major example is the gut/liver axis. It is well known that alcohol increases gut permeability while patients with alcoholic liver disease have a more diminished intestinal barrier[302,303]. Bacterial infiltration caused by a leaky barrier significantly affects the liver, where the bacteria will translocate after successful infiltration into the vascular system. While the liver can clear these microbes, chronic bacterial infiltration will lead to constant hepatic inflammation that may cause significant damage to hepatocytes[304,305]. The emergence of miR-24 as a significant disruptor of the intestinal epithelial barrier suggests that further research should be done into the levels of miR-24 in the colons of patients or animal models of liver disease. As the intestinal barrier is becoming a focus in the treatment of liver diseases[306], miR-24 could arise as a potential therapeutic target. Other barrier-related diseases for which to study intestinal miR-24 levels include various cardiovascular diseases[307], coeliac disease[308], and graft-versus-host disease[309]. MiR-24 could unlock new mechanistic insights to the pathogenesis of these diseases and provide researchers new avenues of investigation.

4.1.2 miR-24-mediated repression of cingulin – mechanistic relevance in other contexts

Our data demonstrate the direct targeting of miR-24 to cingulin mRNA, leading to downregulation of both gene expression and protein levels (**Figure 2-6**). While our investigation focused on the consequences of this miR-24/cingulin axis in intestinal permeability, miR-24 mediated downregulation of cingulin could have significant consequences beyond intestinal barrier function.

First identified in 1988 by Sandra Citi[310], cingulin is a cytosolic membrane protein that connects structural junction proteins to the actomyosin cytoskeleton[273]. In epithelial cells, cingulin regulates the expression of junctional proteins including claudin-2 and zona occludens-3 through multiple mechanisms involving distinct signaling pathways[274,311]. A study of cingulin knockout mice revealed that while tight junctions were still intact without cingulin, expression of various junctional proteins were changed and the mice were more sensitive to duodenal ulcerations[279]. The

emergence of miR-24 as a repressor of cingulin links miR-24 to any conditions involving cingulin modulation.

Multiple investigations have been done that uncovered a role for cingulin in specific disease models. One such study determined that miR-24 upregulation in mesothelioma promotes migration and suppresses cingulin levels, which was correlated with diminished survival in patients[249]. In studies of endothelial barrier function, cingulin was found to be important for the endothelial tight junction structure and for maintaining the blood-brain barrier[312]. Further research revealed that cingulin knockout exacerbated thrombin-induced lung endothelial cell permeability[313]. A recent investigation demonstrated that cingulin-knockout vasculature led to a greater non-perfused area and edema following burn injury, suggesting cingulin-dependent increased vascular leakage[314]. For diseases of increased paracellular permeability and/or a disrupted tight junction, miR-24 levels should be assessed to see if its pathophysiological mechanism involves miR-24 repression of cingulin. The ability to clarify this mechanism will aid in future investigations of therapeutic assessments for these various conditions.

4.1.3 Using in vivo overexpression of miR-24 to further study the mechanisms of intestinal barrier dysfunction

Our data involving miR-24's effect on barrier function is focused on *in vitro* models. We therefore next determined the *in vivo* relevance of miR-24 in models of barrier function and UC. As demonstrated, *in vivo* miR-24 inhibition can be achieved (**Figure 3-1**), but miR-24 overexpression is far more challenging. Due to the mechanism of their function, synthetic microRNA mimics are not able to have as many modifications and thus are more susceptible to nuclease degradation[166]. Nevertheless, further studies are needed to optimize both the mimic alterations and delivery method in order to achieve *in vivo* miR-24 overexpression.

Our *in vitro* models used the barrier-forming intestinal epithelial cell lines Caco-2 and T84 (**Figure 2-2**). The miR-24 mediated barrier defects were seen exclusively in these cells, so a follow up experiment would target barrier-forming intestinal epithelial cells *in vivo* for miR-24 overexpression. Villin-specific transgenic overexpression of miR-24 is one method to increase miR-24 levels in intestinal epithelial cells[315], but the generation of this model is costly and time consuming. The emergence of viral vectors with strategies to confer cell specificity[316] makes delivery of a synthetic miR-24 mimic possible. Following the optimization of both the mimic and the delivery method, intestinal epithelial cell-specific miR-24 overexpression would ensure that the results of subsequent experiments wouldn't be convoluted with miR-24 elevation in non-epithelial cells. Primarily, the investigation would aim to confirm a depletion of barrier function *in vivo* following miR-24 overexpression. Furthermore, miR-24 elevation in the intestinal epithelium would reveal its role in the pathogenesis of UC by determining what occurs to a healthy animal with miR-24-mediated barrier dysfunction. Simultaneously, researchers would increase miR-24 expression during models of UC, including DSS. Based on our *in vitro* evidence, we would hypothesize that the miR-24 overexpression would worsen disease by allowing the luminal components to infiltrate the intestinal tract at a faster rate than with the colitis model alone. With this, downstream consequences of epithelial miR-24 elevation in both healthy and inflamed mice could be elucidated, potentially connecting miR-24 to microbiome changes, modulation of immune activity, and organ function.

4.1.4 Current and future directions for targeting barrier as a treatment for UC

The importance of a healthy intestinal barrier has led researchers develop barrier-targeting treatments. While details of the structure and function of intestinal barrier function have been described, therapeutically restoring barrier represents an important target for IBD treatment. As previously mentioned, a faulty barrier is not just prevalent in UC, so improving intestinal barrier function may have implications for a variety of immune-related diseases. Repairing a damaged

barrier can theoretically be pursued from different angles, including targeting cytokines, immune cells, the microbiome, and components of tight junctions[317]. Indirectly, the biologic treatments currently used for UC may improve barrier function. While this hasn't been directly studied for UC, studies done with CD patients showed that the anti-TNFa therapy infliximab promoted barrier function both in patients along with *ex vivo* and *in vitro* studies[318,319]. Other types of treatments for UC in the therapeutic pipeline that may indirectly affect barrier include those targeting the mucus layer[320] and the microbiome[321]. Drugs that directly target barrier function, however, are rarer.

One of the first treatments that directly targeted barrier function was the small molecule larazotide acetate, aimed to treat celiac's disease. Even though preclinical studies found barrier strengthening effects of larazotide[322], the subsequent clinical trials did not show the same intestinal barrier restoring capability in patients[323,324]. Current research into barrier restoring therapies has found difficulty due to the multifactorial influences on barrier integrity. One idea involved attempting to repair damaged intestinal epithelium with engrafted stem cells, but the technique mechanistically proved difficult and harbored malignant potential[325,326]. Other avenues to pursue include targeting the tight junction components that are changed during UC and promote permeability, which include claudin-2[43]. However, molecules that target these components are not currently available and manipulation of certain members of the tight junctional complex may disrupt the homeostatic mechanisms involved in ion and water transport in the colon[326].

Targeting microRNAs provide a new avenue for treatment with a lot of potential to amend intestinal barrier malfunction. The fact that microRNAs can only target specific genes promotes specificity in the exact portion of the complex that is altered. The next step would be to identify genes or proteins critical for barrier dysfunction that are altered during UC, and researchers could use a complementary microRNA to overexpress and repress the responsible downstream target

gene. Alternatively, a microRNA that is elevated during UC and targets a junctional protein could be targeted for inhibition in order to restore the repressed gene. While these studies would require specificity in delivery along with precise definition of all potential side-effects, the end result could be substantial. Repairing barrier function could reduce inflammation, restore the mucosal lining, and reestablish the microbiome to a homeostatic population[327]. Though it may not work by itself, treatment of barrier dysfunction along with other successful UC treatments may help enhance healing and maintain remission in patients. As UC is a multifactorial disease, the future of its treatment will likely be multifaceted.

4.2 miR-24 and wound healing

Mucosal wound healing is considered an important endpoint for the management of UC[328]. Determined by endoscopic analysis, mucosal healing has been associated with reduced risk of relapse and low risk of future colectomy[63,329,330]. During our investigation of the therapeutic potential of miR-24 inhibition during murine colitis, we observed a detrimental effect on mucosal wound healing following intestinal inflammation. Our previous data showing that miR-24 overexpression decreases apoptosis (**Figure 2-3**) suggested that miR-24 inhibition increases apoptosis through elevation of the proapoptotic protein BIM, which we confirmed (**Figure 3-3**, **Figure 3-4**). While this may be an obstacle to developing the miR-24 inhibitor as a barrier-protective therapy, it opens the door for further investigation into miR-24 inhibition's potential for any apoptosis-related pathologies, particularly those related to mucosal wound healing. The regulation of BIM by miR-24 also suggests that this microRNA is related to BIM dysregulation and BIM-induced apoptosis in several disease processes. With these data, future investigations can elucidate mechanisms of BIM-related and apoptosis-related diseases and advance the study of miR-24 manipulation as a therapy for these conditions.

4.2.1 miR-24 inhibition and the dynamics of wound healing in the intestinal mucosa

The intestinal mucosa can be severely damaged due to the inflammatory activity during UC flares. The process of mucosal wounding includes apoptosis and anoikis of intestinal epithelial cells that is promoted by severe immune cell activity in the gastrointestinal tract[331]. During remission, intestinal wound healing is vital to maintain the non-inflamed state, as a damaged mucosa increases barrier permeability allowing infiltration of the luminal components of the gut. Mucosal restitution involves differentiation, proliferation, and migration of intestinal epithelial cells, processes which overlap to restore the dynamic functions of the epithelial barrier and mucosa [332]. Promoting the effort are a variety of secreted growth factors and cytokines from the various cells of the gastrointestinal lining. Several activated signaling cascades regulate the cellular processes required of the epithelium to properly restore the mucosa[333]. Therefore, the interruption of these elements would have a detrimental effect to the wound healing capability of the intestine.

Our *in vivo* data showing miR-24 inhibition diminishing mucosal healing following murine colitis (**Figure 3-2**) suggests that the lack of miR-24 is affecting part of the wound healing process. Our employment of systemic miR-24 inhibition suggests that the complex cell population of the intestinal wall may have decreased miR-24. This creates a difficulty in identifying the specific cell type(s) being affected by the miR-24 inhibition that is causing the attenuated wound healing. When manipulating miR-24 in SW480 colon cells, we found that miR-24 was a tight regulator of BIM expression, and thus apoptosis (**Figure 3-3**, **Figure 3-4**). While our cell line is derived from colon adenocarcinoma and does not represent every cellular subtype in the intestinal wall, it suggests that increased apoptosis is occurring in at least some of the intestinal epithelial cells. The harmful effect of pathological apoptosis to intestinal epithelial cells is well-known[334], so increasing the cell death due to miR-24 inhibition exacerbates the damage. Furthermore, cells of the other layers of the intestine are vital to wound healing[333], so elevated apoptosis would further impair this process.

To accurately and mechanistically explain how miR-24 inhibition is affecting the intestinal mucosal restitution *in vivo*, methods of cell separation should be considered. To be able to assess exactly which cells of the intestine are undergoing apoptosis during murine colitis would reveal the mechanism behind how miR-24 inhibition prevents wound healing. Several investigative groups have discovered how to isolate murine intestinal epithelial cells[335], epithelial stem cells[336], lamina propria leukocytes[337], and myofibroblasts[338]. A possible future experiment would be to repeat the wound healing model with the miR-24 inhibitor and isolate subpopulations of intestinal cells from the inhibitor-treated and control mice during the healing period. Using the isolated cells as previously mentioned, investigators would be able to determine the effects of miR-24 depletion on each subtype, particularly in regards to apoptosis and BIM regulation. Describing the functional consequence of miR-24 inhibition in each of these cell types would provide valuable data for future studies of targeted miR-24 restoration therapy.

It would be imperative that any attempt at miR-24 overexpression as a therapy for diminished would healing be specifically targeted. As mentioned previously, miR-24 overexpression diminishes intestinal barrier function *in vitro* (**Figure 2-2**). However, if the miR-24 inhibitor was discovered to promote apoptosis in non-barrier forming cells, then reversing apoptosis could be beneficial to the mucosal restoration process. For example, leukocytes in the intestinal tract secrete factors during wound healing that promote epithelial restitution following inflammatory damage[339]. If miR-24 inhibition were to promote apoptosis in those cells, it may explain the resulting diminished wound healing. Thus, a leukocyte specific miR-24 restoration therapy would prevent apoptosis in those cells and help promote the cellular processes necessary for epithelial barrier restoration. It should also be noted that miR-24 overexpression should only be employed during the recovery period following inflammatory damage. For mouse studies, this would occur following the stabilization of mouse weight after the period of DSS exposure (**Figure 3-2C**).

Increased survival due to miR-24 overexpression may help the recovery process, but only if employed properly.

4.2.2 The extensive implications of miR-24's regulation of BIM

First discovered in 1998[340], BIM is an ubiquitously expressed proapoptotic protein that is part of the intrinsic apoptosis pathway[341]. BIM is part of the BH3-only protein family and can interact with both anti-apoptotic BCL2 proteins and proapoptotic proteins to induce cell death[342,343]. BIM is activated in response to a variety of apoptotic stimuli and this cascade ends in caspase-induced cell death[344]. Since BIM represents significant proapoptotic protein, its dysregulation has been associated with a variety of diseases including autoimmune disorders[345], diabetes[346], neurological diseases[347,348], and cancer[349].

The BIM/miR-24 relationship has been previously identified in various other conditions. MiR-24 was identified as an oncomiR through its antiapoptotic repression of BIM in pancreatic cancer[350], breast cancer[189], gastric cancer[214], and osteosarcoma[191]. MiR-24 was also found to protect against apoptosis during acute liver failure[295] and chronic obstructive pulmonary disease[351]. Multiple studies determined that exosomal miR-24 was protective against ischemic injury and promoted repair through BIM repression[352,353]. However, it must be mentioned that miR-24 overexpression has also been found to be proapoptotic in some conditions such as bladder cancer and laryngeal cancer[217,354], reinforcing the heterogeneity of this microRNA in humans. Thus, miR-24 function should always be considered within the specific cell type and tissue it is studied in.

Our data is consistent with some of the studies discussed above [189,214,295,350], showing potent downregulation of BIM by miR-24 overexpression (**Figure 3-4B**). We further showed that miR-24 inhibition resulted in the elevation of BIM, suggesting that at homeostasis, miR-24 expression

levels in colonic SW480 cells are at a level where it represses BIM (**Figure 3-4A**). As expected, the miR-24 manipulation affects apoptosis (**Figure 3-3**) and BIM knockout reverses the apoptosis induced by miR-24 inhibition (**Figure 3-5**). Such a robust regulation of BIM by miR-24 is likely to have repercussions during miR-24 dysregulation, particularly in the colon. MiR-24 overexpression should be examined for any diseases where BIM downregulation is implicated, possibly profiling clinical samples and transitioning to *in vitro* studies to determine the exact molecular pathways involved. If the miR-24/BIM network can be properly defined, miR-24 inhibition treatment could be studied as a start to preclinical trials for the various diseases where BIM is downregulated. On the other hand, conditions that show an increase of BIM expression should be studied for the involvement of miR-24 downregulation. For multiple conditions in which increased apoptosis is the culprit, increased BIM levels in the tissues are evident[344]. Researchers could measure clinical samples for miR-24 levels to see if the BIM elevation is associated with miR-24 repression. In turn, miR-24 overexpression could be attempted *in vitro* and *in vivo* to clarify the mechanisms and potential side effects leading to the development of miR-24 restoration therapy for the BIM-related condition.

4.3 miR-24 and the challenges of microRNA-based therapeutic interventions

Multiple pharmaceutical companies are in clinical trials for microRNA manipulation-based therapies[355]. If successful, these drugs will target diseases with limited treatment options and poor prognoses. As more research is conducted into microRNA activity, more will emerge as therapeutic targets and the diseases covered by microRNA therapies will expand. Clearly, there is a prosperous future with microRNA research and therapeutically targeting miR-24 has great potential.

MiR-24 has been implicated in a wide range of diseases both as a biomarker and pathophysiologically[182,229]. In our studies of UC, we describe miR-24 playing a role in tight junction

structure and epithelial apoptosis. However, we found detrimental results where we thought would be beneficial. As our *in vitro* work showed that miR-24 overexpression disrupted the intestinal barrier (**Figure 2-2**), we thought that a miR-24 inhibitor would protect against colitis. In our *in vivo* experimentation, we found that the inhibitor did not make a difference in acute colitis, and actually worsened recovery following colitis (**Figure 3-2**). At first glance, this suggests a significant flaw of microRNA research, but it is simply an example of the potent influence microRNAs may have in multiple and sometimes diverse cellular functions and points towards more investigations needed in this matter. There are many obstacles to microRNA-based therapeutics, but the identification of successful microRNA therapies and elucidation on the mechanisms governing their applications could provide new treatments for a wide variety of diseases.

4.3.1 The heterogeneity of miR-24 in UC

The heterogeneity of microRNAs, and in particular miR-24, is impressive, but based on our knowledge of microRNA biology, not surprising. Different cells may have different levels of miR-24, while they also may not have the same expression of miR-24 target genes. The difficulties of clarifying cell-specific mechanisms for miR-24 is further complicated by the simple fact that any given organ has many subtypes of cells, some even including stem cells. The literature suggesting opposite roles of miR-24 in cancer depending on the cancer subtype[182] insinuates that targeted therapies for any disease involving miR-24 must be site specific, potentially all the way down to the cellular level. Additional research is needed to confirm the putative downstream targets of miR-24 in different cells and tissues.

Our data described two roles for miR-24 that could define the therapeutic potential of miR-24 manipulation. On the one hand, miR-24 overexpression promotes intestinal barrier dysfunction (**Figure 2-2**). While our studies of miR-24 inhibition did not promote barrier function *in vitro* (**Figure 2-2G**) nor did it prevent acute colitis *in vivo* (**Figure 3-2C**), a more cell-specific, cell-targeted

approach of miR-24 repression may protect against barrier dysfunction. On the other hand, systemic miR-24 inhibition prevented mucosal wound healing following acute colitis (**Figure 3-2**). While our data points to apoptosis as the culprit (**Figure 3-3**), it is unclear exactly which cells have increased cell death following miR-24 depletion. Revealing this information would then allow researchers to employ a cell-specific miR-24 restoration that could prevent apoptosis and foster wound healing. Without these further experiments, the employment of miR-24 manipulation may limit its effectiveness as a potential UC therapy.

These realities of the future of UC-focused miR-24 research are indicative of the challenges facing many other microRNA investigations. However, researchers must embrace these complications in order to fully clarify the mechanisms and effects involving specific microRNA pathways.

4.3.2 Downstream consequences of microRNA manipulation – redundancy and compensatory reactions

Canonically, microRNAs exert their influence through downregulation of mRNAs. In turn, the gene downregulation should lead to protein downregulation. However, the story does not end with just a simple microRNA/gene relationship. It is always possible that unpredictable mechanisms can be involved, as we observed when the tight junction protein ZO-1 was elevated following miR-24 overexpression in intestinal epithelial cells (data not shown). Although we did not determine what directly caused the ZO-1 elevation, this upregulation did not prevent miR-24-mediated barrier dysfunction. Nevertheless, this observation epitomizes the unexpected outcomes that occur with microRNA manipulation. All consequences of microRNA alterations *in vitro* and *in vivo* must be defined, for changes in a microRNA in either direction could have its own unforeseen effects due to downstream reactions arising. In the end, these unanticipated side-effects could prove more influential on the tissue than the intended microRNA/gene pathway.

Related to this issue is the redundancy of many pathways involved in important cellular functions[356-358]. The issue with multiple pathways involved in the same cellular function is that a microRNA may only affect a gene in one of the pathways. Often, a cell may respond by upregulating another pathway as a compensatory response. This may weaken the influence of a specific microRNA as a therapy targeting a particular cellular process by focusing on one gene. While this caveat should be considered when applying a microRNA manipulation treatment, it does not necessarily completely diminish the microRNA's impact. Researchers may just need to consider new specific treatments combination with others, as is common for treatment of many diseases.

4.3.3 Caveats of artificial microRNA manipulation – overexpression and inhibition

An issue that relates to microRNA therapeutics is artificial effects of forced overexpression. When cells *in vitro* are overexpressing a microRNA, the simple existence of exponential levels of the microRNA will force many complementary mRNAs to be downregulated. This means that studies of microRNAs using artificial mimics may show unrealistic outcomes. This highlights the importance of *in vitro* research using microRNA manipulation to a level that is seen in clinical samples, as we illustrated in our data. By controlling the amount of overexpression and seeing the same end result, we ensured that the barrier dysfunction is caused by an amount of miR-24 that was seen in patient UC colon tissue (**Figure 2-1**, **Supplemental Figure 2-1**). In both preclinical and clinical stages of microRNA therapeutic research, this nuance must be considered to ensure that the treatment will actually reverse a situation seen in patients and not risk having unforeseen side effects.

Another complication of microRNA therapeutics is the idea that a given microRNA may not be expressed to high enough levels in normal circumstances to have a functional consequence. For

example, our experiment using a miR-24 inhibitor aimed to improve barrier *in vitro* was unsuccessful (**Figure 2-2G**), even though miR-24 overexpression caused barrier dysfunction. This suggests that homeostatically, the miR-24 in the relevant cells were not at a high enough level where the downregulation would remove any repression it has on cingulin or another unknown junctional gene. In contrast, our *in vitro* studies of miR-24 in the SW480 cells showed that inhibition increased apoptosis and increased levels of BIM (**Figure 3-3**, **Figure 3-4**). This suggests that in normal conditions, miR-24 is at a high enough level to be constantly repressing BIM mRNA, and thus inhibiting the miR-24 removed the BIM downregulation and led to increased apoptosis. This relates to the important task of cell-specific profiling of the microRNA of interest, for its expression may be varied enough to cause inconsistent responses during therapeutic application.

4.4 Final Thoughts

In all, the data presented here have filled a gap in the knowledge pertaining to miR-24 and UC. We revealed roles for miR-24 in barrier function, wound healing, and apoptosis. These results described the mechanisms involved and provide a foundation for many follow-up experiments and future avenues of study. MicroRNAs are extraordinarily influential in the human body and if researchers can harness their potential, the outcome will be significant for the future of therapeutics in many diseases and conditions.

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