

**The impact of miR-21 on epithelial intercellular junctions
influencing gut inflammation and barrier integrity**



**Thesis submitted for the degree of
Doctor of Philosophy**

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, with the exception of the following figures and tables:

- Figure 1.3 – was drawn by Sarah Stiegeler and used in our joint review paper (*Cells* 2021, 10(12), 3358; <https://doi.org/10.3390/cells10123358>).
- Table 1.1 and Table 1.2 were used in the joint review paper mentioned above.
- Figure 3.5 + Figure 3.6 – Elaine Dempsey supported in scoring and culling the mice used in this DSS recovery trial.
- Figure 3.12 + 3.13 – Elaine Dempsey and Kate Sheehan supported in scoring and culling the mice used in this DSS recovery trial.
- Figure 5.9 – Claire Fergus performed the rederivation process in this breeding step.
- Figure 5.11 + Figure 5.13 – Mark Travers supported by setting up breeding pairs for these breeding steps.

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Kevin Jay Belarmino Mercurio

6 January 2026

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Love,

Kev

Abbreviations

ABX	antibiotic
AGO	Argonaute
AHR	aryl hydrocarbon receptor
AJ	adherens junction
AJC	apical junction complex
AOM	azoxymethane
AP-1	activator protein 1
APC	antigen presenting cell
ASO	antisense oligonucleotides
AREC	Animal Research Ethics Committee
ARF	ADP-ribosylation factor
AUC	area under the curve
BCA	bicinchoninic acid
BMP	bone morphogenic protein
BSA	bovine serum albumin
C1GalT1	core 1 β 1,3-galactosyltransferase
CAR	coxsackievirus and adenovirus receptor
CBC	crypt base columnar
CD	Crohn's disease
CD(3/4)+	cluster of differentiation (3/4) positive
CDH	cadherin
cDNA	complimentary deoxyribonucleic acid
CMU	Comparative Medicinal Unit
CMV	cytomegalovirus
Co-Smad	common partner Smad
CO ₂	carbon dioxide
COPI	coatomer
CRB3	crumbs homologue 3
CT	cycle threshold
CTNN	catenin
CXCL	C-X-C motif ligand
DAI	disease activity index
DAMP	damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DMBA	7,12-dimethylbenz(a)anthracene
DMEM	Dulbecco's Modified Eagle Medium
DSC	desmocollin
DSG	desmoglein
Dsh	Dishevelled (protein)
DSS	dextran sulfate sodium
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
EHEC	enterohemorrhagic <i>Escherichia coli</i>
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EPEC	enteropathogenic <i>Escherichia coli</i>

ERK	extracellular signal-regulated kinase
ES	embryonic stem
FAE	follicle-associated epithelium
FCS	foetal calf serum
FCGBP	Fc-gamma binding protein
FD4	FITC-dextran (4 kDa)
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FLP	flippase
FLPo	mouse-codon optimized FLP recombinase
FMT	faecal matter transplant
GAP	GTPase-activating proteins
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCK	ginsenoside compound K
GDP	guanosine diphosphate
GEF	guanine exchange factor
GFI1	growth factor independence-1
GI	gastrointestinal tract
GJ	gap junction
GLP-2	glucagon-like peptide-2
GTP	guanosine triphosphate
GWAS	genome-wide association study
H&E	hematoxylin and eosin
H ₂ O ₂	hydrogen peroxide
HCl	hydrochloric acid
HEMI	hemizygous
HET	heterozygous
HGF	hepatocyte growth factor
HIF-1 α	hypoxia inducible factor 1 alpha
HMC-1	human mast cell 1
HOM	homozygous
HPRA	Health Products Research Authority
HRP	horseradish peroxidase
HTAB	hexadecyltrimethylammonium bromide
I-Smad	inhibitory Smad
IBD	inflammatory bowel disease
IEC	intestinal epithelial cell
IFN(γ)	Interferon (gamma)
IGF(-I/II)	insulin-like growth factor (1/2)
IHB	miR-21 inhibitor
IL	interleukin
ILC	innate lymphoid cells
(r)ISC	(reserve) intestinal stem cell
ISG	interferon-stimulated gene
IVF	<i>in vitro</i> fertilization
JAM	junction adhesion molecule
JNK	Jun N-terminal kinase
KGF	keratinocyte growth factor
KO	knockout
LCN2	lipocalin-2

LId	distal colon (large intestine distal)
LIp	proximal colon (large intestine proximal)
LM	littermate
LNA	locked nucleic acid
LPS	lipopolysaccharide
LRRK2	leucine-rich repeat kinase 2
MAGI	membrane-associated guanylate kinase inverted
MAL	myelin and lymphocyte
MAPK	mitogen-activated protein kinase
MARVEL	MAL and related proteins for vesicle trafficking and membrane link
MCFA	medium chain fatty acid
MDP	muramyl dipeptide
miR-21	microRNA-21
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MLC	myosin light chain
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MPO	myeloperoxidase
MPR	mannose-6-phosphate receptor
NaOH	sodium hydroxide
NEO	neomycin
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
NOD	nucleotide-binding and oligomerization domain
NOS2	nitric oxide synthase 2
OCN	occludin
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffer saline
PDCD4	programmed cell death protein 4
PFKM	phosphofructokinase
PGE2	prostaglandin-E2
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PRR	pattern recognition receptor
PS	penicillin-streptomycin
PSF	point spread function
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real-time polymerase chain reaction
R-Smad	receptor-regulated Smad
RIPA	radioimmunoprecipitation assay
RhoB	Ras homolog family member B
RNA	ribonucleic acid
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
SARA	Smad anchor for receptor activation
SCFA	short chain fatty acid
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SId	ileum (small intestine distal)

SIp	duodenum/jejunum (small intestine proximal)
siRNA	silencing RNA
SLE	systemic lupus erythematosus
SMAD	mothers against decapentaplegic homolog
SPDEF	SAM pointed domain-containing ETS transcription factor
SPF	specific pathogen-free
SRPK	serine/arginine-rich splicing factor protein kinase
TBS	Tris buffer saline
TCD	Trinity College Dublin
TCR(- α)	T-cell receptor (alpha)
TEER	transepithelial electrical resistance
TGF(- α/β)	transforming growth factor (alpha/beta)
TGFBR	transforming growth factor beta receptor
TGN	trans-Golgi network
Th(1/2/17)	type-(1/2/17) helper T-cell
THP-1	Tohoku Hospital Pediatrics-1
TJ	tight junction
TLR	toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF(- α)	tumor necrosis factor (alpha)
T _{reg}	regulatory T-cell
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
UC	ulcerative colitis
UTR	untranslated region
WT	wild type
ZG16	zymogen granule protein-16
ZO	zonula occludens
ZONAB	zonula occludens 1 (ZO-1)-associated nucleic acid binding protein

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Summary

MicroRNAs (miRNAs) are short nucleotides that bind to seed regions in target mRNA and impact protein synthesis. These molecules have emerged as a diverse set of regulators for the immune response during inflammation. One such category of immune dysregulation studied in conjunction with miRNAs is inflammatory bowel disease (IBD) like Crohn's disease or ulcerative colitis, characterized by debilitating chronic inflammation along regions of the gastrointestinal tract. During the inflammatory response, intestinal epithelial cells (IECs) react to changes in the gut environment which can impact gut permeability via intercellular junctions. These responses can be regulated by miRNAs such as miRNA-21 (miR-21). Previous work from the Corr group identified a novel role for miR-21, whereby loss of miR-21 protects against development of colitis in mice by inducing health-promoting microbiota. However, the molecular mechanisms underlying this phenotype, particularly the mechanisms by which miR-21 negatively regulates mucosal barrier permeability, remain to be elaborated. Therefore, this thesis addressed the knowledge gap by which miR-21 influences gut barrier integrity and susceptibility to intestinal inflammation. Among the major barrier components, intercellular junctions govern integrity by dictating transport, adhesion and communication between IECs. Most research focuses on associations between these junctions and IBD activity, which lack mechanistic understanding of their roles during inflammation. Here, *in vivo* and *in vitro* approaches were used to expand on the mechanistic impacts miR-21 has on intercellular junctions and cellular processes connected to these entities. This work demonstrated that miR-21 deficiency results in differential expression of adherens junction (AJ) and tight junction (TJ) components within whole colon tissue and colon IECs from miR-21^{-/-} mice compared to wild type (WT) under healthy conditions. Furthermore, the DSS-induced colitis model for acute IBD as well as a DSS recovery model using miR-21^{-/-} mice were utilized to investigate the impact of miR-21 expression on the mucosal barrier, particularly the regulation and integrity of junctional complexes. This work found that gene expression of the major epithelial AJ component E-cadherin showed a consistent negative association with miR-21 levels in the DSS recovery model, along with the increase of fragmented E-cadherin protein and localization to the top of distal colon crypts during miR-21 deficiency. With the regulation of E-cadherin involved in epithelial wound healing, further investigations using the human adenocarcinoma cell line Caco-2 transfected with miR-21 modulators were conducted during TNF- α -induced inflammation. This work showed that

miR-21 directly targets E-cadherin and acts as a fine-tuner for regulating both activators and inhibitors of epithelial wound healing processes like TGF- β signalling. Another integral component of gut barrier permeability is the TJ protein claudin-4, also showed induced expression during miR-21 deficiency. Here, this work showed that expression of claudin-4 was consistently altered *in vivo* at the mRNA and protein level in miR-21^{-/-} mice. These observations suggest a novel link between miR-21 and shared TJ regulators among foundational cellular processes such as vesicular trafficking via the Golgi. Indeed, miR-21 levels were shown to positively associate with components of the retrograde transport pathway in miR-21^{-/-} tissue of the DSS recovery model, and their localization to the Golgi *in vitro* during TNF- α -induced inflammation. Lastly, this work highlighted the importance of epithelial-specific expression of miR-21. Regulation of these pathways and the resulting mucosal barrier for disease susceptibility was assessed through the in-house creation of an IEC-specific conditional knockout of miR-21 (miR-21^{IEC-KO}). This strain has initiated the discovery of novel IEC-specific pathways by which miR-21 impacts overall gut homeostasis, contributing to the pathophysiology of intestinal inflammation. Notably, this work will enhance our understanding of mucosal barrier regulation and contribute to the development of new therapeutic strategies for the treatment of chronic diseases like IBD.

Chapter 1

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General Introduction

1. General Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a debilitating disease characterized by chronic inflammation along the gastrointestinal (GI) tract. In Europe, it is estimated that 2.5-3 million people are living with IBD.¹ Patients diagnosed with IBD are symptomatic for recurrent intestinal inflammation, diarrhea, abdominal pain, rectal bleeding, weight loss and anaemia. IBD is often considered a multifactorial disease, as host genetics, gut microbiome, lifestyle and external environment associate with impaired barrier integrity and enhanced exposure to immune cells.²

Research on IBD has highlighted changes in gut immunity and the overall functioning of cells that participate in the characteristic overactive immune response. Immune cells can be a part of innate immunity, which serves as the initial rapid defence upon the recognition of foreign pathogens, or adaptive immunity, which leads to slow but long-lasting defensive measures.³ An effective inflammatory response to any pathogenic invasion is conducted mainly by innate immune cells, which include neutrophils, dendritic cells, monocytes, macrophages and natural killer cells. For an immunological response to specific pathogens, adaptive immune cells are more critical and include effector T-cells, regulatory T-cells (T_{regs}) and B-cells.³ The function within, and communication between, the two arms of immunity contribute to chronic inflammation associated with IBD, leading to tissue damage through reactive oxygen species (ROS) production, fibrosis and continuous feedback loops of pro-inflammatory cytokine signalling.⁴

In the case of IBD, the activation of central immune cell populations leads to the recruitment of non-specific mediators of the inflammatory response, such as the formation of metabolites like prostaglandins and leukotrienes.⁵ All these factors can compromise the gut epithelial barrier where the majority of host and gut microbiota interactions occur. In such a disease state, luminal antigens gain access to the lamina propria, triggering a response by innate and adaptive immune cells via pattern recognition receptors (PRRs), causing antigen presenting cells (APCs) to further activate central effector immune cells as well as other pro-inflammatory mediators. This cascade thereby perpetuates a positive feedback loop of leukocyte recruitment and increasing tissue damage in IBD.⁴

Clinically, IBD is segregated into two distinct types known as Crohn's disease (CD) and ulcerative colitis (UC). CD can impact any part of the GI tract with patchy regions of

inflammation, whereas UC has inflammation typically localized to the colon or rectum.⁶ Incidents of CD or UC can also lead to increased risk of other diseases like colorectal cancer.⁷ Previously accepted notions were that CD typically has a major CD4⁺ lymphocyte population with a type-1 helper T-cell (Th1) phenotype, while UC has a type-2 helper T-cell (Th2) phenotype.⁸ Due to these differences in lymphocyte populations, CD is driven by interferon- γ (IFN- γ) and interleukin (IL)-12 expression, while UC is driven by transforming growth factor (TGF)- β , IL-4, IL-5 and IL-13 expression. More recently, this paradigm has been expanded to incorporate the IL-23/Th17 axis to further distinguish the two types.⁸ The interactions between immune cells and other cells that constitute the gut epithelial barrier are paramount in understanding permeability within CD and UC disease states.

The pathogenesis of IBD can be further categorized into the two major types of CD and UC. Excluding IL-12 dysfunction, innate immunity genes encoding *NOD2*, *ATG16L1* and *IRGM* have been associated with CD.⁹ Additionally, mutations in *NOD2* have been observed in CD patients which help the immune system recognize microbes across the epithelial barrier.⁹ Moreover, leucine-rich repeat kinase-2 (LRRK2), a negative regulator of T-cell functionality and supports lysozyme secretion in lysosomes, was shown to be associated with the manifestation of CD.¹⁰ The microbial community in CD patients is also specifically altered, where the more systemic inflammatory environment leads to the promotion of sugar degradation and quinone biosynthesis, along with the downregulation of fermentation pathways.¹¹ In contrast, excluding IL-13 dysfunction, mutations in *TLR4* and *NOD1* have been associated with UC, which encode receptors important for microbial detection.⁹ There is a stronger association between UC and dysfunction with the gut mucosa, along with specific changes to the gut microbiota leading to an increase in lactate production along with a decrease in butyrate and acetate production, exacerbating inflammation in the intestines.¹¹

The archetypical method of diagnosis for IBD is endoscopy in conjunction with biopsies.¹² Despite the established belief that CD is mainly attributed to the overactivation of the inflammatory response throughout the GI tract while UC is mainly confined to the rectum and colon, there remain issues in the proper diagnosis of the two conditions. Serological biomarkers include the presence of perinuclear-staining anti-neutrophil cytoplasmic antibodies in 70% of UC patients, or the presence of anti-*Saccharomyces cerevisiae* antibodies in 50% of CD patients.¹³ Other biomarkers include C-reactive protein in serum and granulocyte proteins lactoferrin and calprotectin in feces.¹⁴ Moreover, a panel

of 51 protein biomarkers in serum was determined to be predictive of CD within up to five years before diagnosis that included associated changes to the complement cascade, lysosomes, innate immune responses and glycosaminoglycan metabolism.¹⁵ Further development on IBD biomarkers is still required for differentiation between CD and UC, along with indication of their severity and active state.

Treatments for IBD include pharmaceuticals, antibody therapies and full surgical procedures. One frequently prescribed treatment is 5-aminosalicylate, which was suggested to block the production of prostaglandins and leukotrienes, inhibit bacterial peptide-induced neutrophil chemotaxis, scavenge circulating ROS and further inhibit the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B).⁵ Others include the use of corticosteroids, though it is important to note that continuous monitoring of prevalent side effects such as corticosteroid-induced osteoporosis, hypertension and diabetes must be performed. Immunosuppressants such as azathioprine are also prescribed, however this often puts patients at risk of opportunistic infections and toxic side effects such as neutropenia, pancreatitis and drug-induced hepatotoxicity.⁵ Distinguishing between types of IBD is crucial as immunoregulatory treatments could sometimes be beneficial for one and not the other. This is indeed the case for cyclosporin in treating UC, or anti-tumour necrosis factor (TNF) therapy via infliximab combined with thiopurines for CD.^{5,12} Even appendectomies have been associated with an increased risk of developing strictures in CD patients while having improved effects in UC patients.⁴ Thus, along with innovations in diagnosis, the development of treatments are equally as important for patients suffering from the various types of IBD and stages of severity.

1.2 Gut microbiome and physiology

One of the primary functions of the gut is to digest and absorb nutrients. Most nutrients are absorbed in the small intestine, passing on pellets to the colon that contain nutrients exceeding the absorption capacity of the small intestine along with indigestible fibres. Importantly, complex carbohydrate structures are one of the main exogenous energy sources for the colon microbiota. The variety of fibres from different sources can support the diversity within the gut microbiota and thereby support gut health.¹⁶

Since the human microbiome project¹⁷, a major focus to understand the pathogenesis of IBD has been centred on the gut microbiome. Gut microbiota and their genetic makeup play a substantial role in gut physiology, metabolism and immunological

functioning.¹⁸ Most research to date has concentrated on the bacterial microbiome, as anaerobes and facultative anaerobes demonstrated the ability to adhere to the outer mucus layer and aid in the digestion of essential nutrients by fermenting complex carbohydrates consumed by the host.¹⁹ Additionally, commensal bacteria have the ability to regulate the pathogenicity of invaders through the production of intestinal metabolites that downregulate the expression of virulence factors.²⁰ Thus, IBD can be described as a dysregulated immune response in individuals genetically susceptible to an altered gut microbiome.

Forming the gut lining, intestinal epithelial cells (IECs) are organized into macrostructures called villi (restricted only to the small intestine) and crypts (found in both small and large intestines). Every 4-5 days, the epithelial lining of the gut is completely renewed.²¹ Intestinal stem cells (ISCs) are found in the crypts, pushing newly differentiated cells upwards and thereby maintaining the epithelium. The gut epithelium consists of highly specialized cells, such as enterocytes, enteroendocrine cells, Tuft cells, goblet cells and Paneth cells. Enterocytes are involved in cell-cell communication, the absorption of nutrients and the sampling of luminal antigens along the GI tract.²² Enterocytes also produce membrane-anchored mucins at the apical site, creating a protective cover called the glycocalyx.²³ Paneth cells are mostly found in the small intestine and with a reduced cell number in the proximal colon that give rise to antimicrobial agents. These antimicrobial peptides counteract bacterial growth near IECs and are crucial to limit bacterial exposure in order to maintain the gut barrier.²⁴ In contrast, intestinal Tuft cells are important for parasitic defence.²⁵ While enteroendocrine cells secrete hormones, goblet cells are known to secrete gel-forming mucins covering the epithelium in the GI tract.²⁶

In IBD, dysbiosis of the gut microbiome and permeability of the gut barrier occur, but it is unclear which is the consequence of the other. Symptoms of IBD are linked to the impact of inflammation on IECs at the gut barrier. For example, IECs at the gut barrier can be dysfunctional in cell polarization and cell-cell adhesion, which are essential for proper barrier function.²⁷ Each type of IEC contributes to homeostasis, reacting to changes in the gut environment, whether that be influenced by therapeutic interventions or impact by microbiota. Their major roles include segregating microbiota and host immune cells to prevent undesired inflammation, while also mediate interactions between these groups by activation of host immune pathways via release of specialized compounds like mucins, hormones, antimicrobial peptides, cytokines and chemokines.²⁸ Goblet cells in particular are imperative for establishing the mucosal barrier between the gut lumen and epithelial

membrane. In the colon, these secreted mucins compose the inner and outer dual layer system, the latter of which resident microbes can colonize. An overview of the gut barrier can be seen in **Figure 1.1**.

Under normal circumstances, permeability of the gut epithelial barrier is warranted since it is at this interface that essential nutrients can be absorbed and taken up into the human body. Impaired or increased permeability has been associated with IBD pathogenesis. There are several external factors that can contribute to impaired permeability of the gut epithelial barrier including dietary regimen, pharmaceuticals, smoking, as well as physical cellular factors such as the immune system, presence of microbiota, the mucus layer, as well as IEC adherence and communication via intercellular junctions.^{29–31} Additionally, cellular processes such as autophagy and the epithelial-mesenchymal transition (EMT) have also been implicated in IBD pathogenesis.^{32–34} Importantly, all these cellular factors can be regulated by microRNAs (miRNAs). In this thesis, we focus on a major component comprising the initial physical barrier governing permeability during IBD, intercellular junctions of IECs, and the how miRNAs regulate their functions.

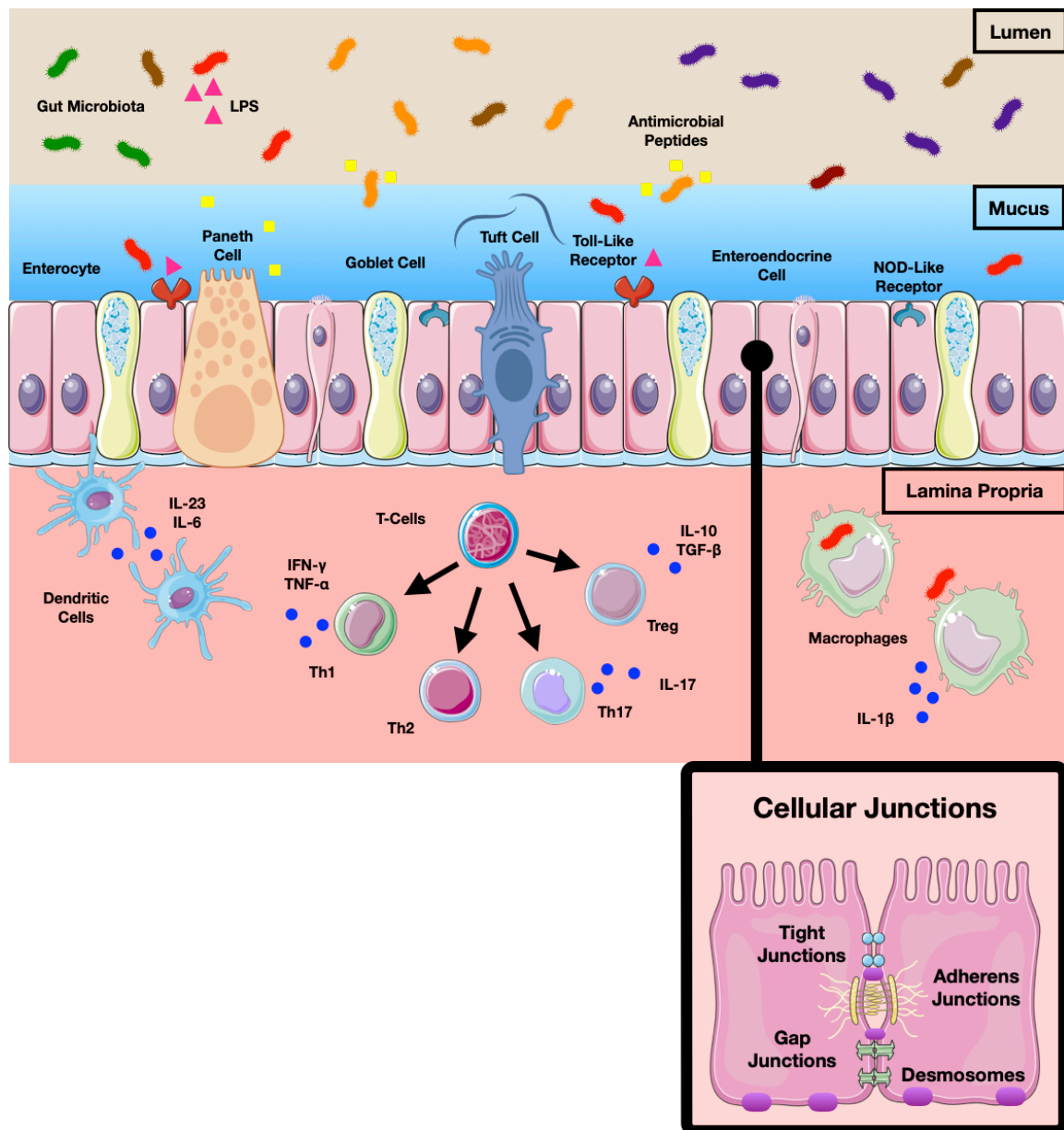


Figure 1.1 – Schematic overview of the gut barrier. Layers of the colon include the lumen, mucus layer, epithelium and lamina propria. At the epithelium are intestinal epithelial cells (IECs) like enterocytes instilling structure and the passage of luminal contents, enteroendocrine cells which are involved in hormone production, and goblet cells which produce glycoproteins that create the mucus layer where most interactions between the host and gut microbiota occur. IECs possess receptors such as toll-like receptors (TLRs) and NOD-like receptors (NLRs) that respond against foreign invasion. Antimicrobial peptides produced by Paneth cells are also found at this layer, while Tuft cells are important for parasitic defence. Common immune cells are included in the lamina propria, like dendritic cells, naive and activated T-cells, and macrophages. Importantly, the integrity of the epithelial barrier is governed by intercellular junctions: tight junctions close to the apical membrane used for paracellular transport, adherens junctions and gap junctions used for cell binding and transfer of compounds, as well as desmosomes that further anchor cells to each other. (Mercurio, K.; created with Servier Medical Art).

1.3 microRNAs and IBD

Research has further pushed our understanding on gene regulation within the gut. One area of prominence brought forth regarding IBD is the regulation of host genes via miRNAs.^{33,34} MiRNAs are approximately 20 nucleotides in length after maturity and can bind primarily to complimentary portions of untranslated regions (UTRs) of target mRNA. The synthesis of miRNAs is summarized in **Figure 1.2**. Briefly, the majority of miRNAs are encoded within intronic regions of both protein-coding and non-coding genes but can also be found within exons.³⁵ Their biogenesis starts with a primary (pri)-miRNA molecule of nuclear, hairpin-structure. The pri-miRNA matures through sequential steps of enzymatic cleavage, first in the nucleus by Drosha/DGCR8 and then in the cytoplasm by Dicer/TRBP, leaving a processed miRNA duplex in the cytoplasm. Finally, the guidance strand of the miRNA duplex is loaded into an Argonaute (AGO) protein, forming the miRNA-induced silencing complex (miRISC).³⁵ MiRISC-targeted RNA molecules are recognized by sequence-specific binding and typically lead to the silencing of mRNA through translational repression or stimulation of degradation.²⁹ In addition, miRNA binding motifs are found in protein-coding sequences and at the 5'-UTR, but miRNA-mediated mRNA repression seems to be most efficient when binding to the 3'-UTR.^{36,37} Overall, there are several ways in which host genes are regulated by miRNAs, and these genes are involved in multiple cellular processes including cell differentiation, proliferation and apoptosis that can contribute to IBD pathogenesis.³⁸

There are multiple factors that help determine the functional relevance of miRNA binding to target mRNA seed regions. Seed regions (6-8 nucleotides long) within mRNA molecules are categorized into two major groups³⁹: 1) canonical sites that have perfect complementary Watson-Crick pairing with the target mRNA and 2) non-canonical sites that have imperfect sequence matching with the target mRNA. The primary determinant of miRNA binding is the presence of a canonical site allowing for complimentary base pairing with the mRNA at position 2-7 of the 5' end of the miRNA.⁴⁰ While miRNA binding to canonical sites often leads to mRNA degradation by the cell, imperfect matching in non-canonical sites typically lead to translational repression, blocking the ribosome from accessing the mRNA within the RISC complex.³⁹ Within these seed groups, there are also further characteristics that can impact mRNA regulation and protein synthesis. Fang *et al.* (2011)⁴¹ compared a list of 45 miRNAs to an assortment of randomized miRNA sequences to determine computationally that the presence of extended (base pairing of seven

nucleotides or more), conserved seed regions showed the greatest indication of binding across 11 different species. Furthermore, seed regions can be found in various locations along the mRNA molecule. Generally, seed regions found in the 3'-UTR are the most impactful for mRNA regulation, with multiple seed regions in the 3'UTR conferring stronger regulation than singular sites.⁴¹ Seed regions can also be found in mRNA coding regions and have been shown to act synergistically with 3'-UTR sequences to further strengthen mRNA regulation.^{41,42} However, as mentioned by Mohr *et al.* (2015)⁴³, an inherent problem in miRNA research is understanding the functional relevance within different cellular contexts, since a single miRNA can have opposing functions in different systems. Therefore, it is highly valuable for IBD researchers to confirm not only binding of miRNAs to novel mRNA targets but determine downstream consequences in relation to the environment the cell resides in, particularly in disease contexts like the epithelial gut barrier of IBD patients.

An additional challenging aspect in studying miRNA relevance in IBD and innate immunity are the species-specific and cell-specific features. For instance, toll-like receptors (TLRs) are key for IECs to identify pathogen-associated molecular pathways (PAMPs). TLR4-activated NF- κ B induction of miR-9 occurs primarily in human monocytes and neutrophils, while miR-210 plays a regulatory role in the NF- κ B feedback pathway typically in murine macrophages.^{44,45} Additionally, nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are important in the surveillance of the intracellular environment for signs of possible infection. Modulations by miR-29 in general dendritic cells, miR-122 in HT-29 cells, miR-146a in muramyl dipeptide (MDP)-activated macrophages, and miR-192/miR-495/miR-512/miR-671 in HCT116 cells were shown to play critical roles in the regulation of NLRs.^{46–49} It is essential that researchers understand and explore all contexts to study miRNA relevance in IBD.

Numerous other miRNAs demonstrated relevance to IBD and adaptive immunity. T-cells have significant roles in the genesis and development of IBD. The loss of miR-155 tends to decrease Th1/Th17, suggesting it is a key regulator in T_{reg} homeostasis.⁵⁰ Continued work has demonstrated other miRNAs in T_{reg} regulation such as miR-10a, miR-17-92 cluster, miR-146a and miR-212/132.^{51–54} MiRNAs implicated in Th1 and Th2 differentiation include miR-17-92 cluster, miR-27b, miR-29, miR-128, miR-146a, miR-155 and miR-340.^{53,55–57} For Th17 differentiation, miRNAs that show impact when imbalanced are miR-10a, miR-155 and miR-326, as well as miR-301a as an indirect inducer.^{58–61} Overexpression of miR-210 may negatively impact Th17 differentiation due

to targeting hypoxia-induced inhibitor (HIF)-1 α .⁶² Finally, B-cell maturation was shown to be regulated by miR-10a, miR-17-92 cluster and miR-181a.^{58,63,64} The coordinated interplay between immune regulation and IECs is essential in controlling barrier permeability.

Another hallmark factor in those afflicted by IBD is the presence of oxygen, either as increased levels of ROS through continuous activation of macrophages or the sensing of oxygen in the gut environment. Several miRNAs were shown to be involved in regulating nitric oxide synthase-2 (NOS2) in IBD tissues. Induction of the nitric oxide pathway by miR-126, miR-146a, miR-221 and miR-223 led to senescence among adjacent epithelial cells via the upregulation of HP1 γ .⁶⁵ Regarding the sensing of environmental oxygen in the gut, HIF was demonstrated to be a key regulator of barrier integrity and induced expression of miR-320a to improve barrier function in T84 cells.⁶⁶ Developing methods for measuring levels of oxygen as damaging free radicals and in its gaseous state within the inflamed gut may help researchers track the progression of the disease.

1.4 microRNA-21 and IBD

One of the most widely studied miRNAs in regards to health and disease is miR-21. Among the most abundantly expressed miRNAs in various mammalian cell types, miR-21 is considered an oncomiR within the intronic region of the protein coding gene *TMEM49*.⁶⁷ Regulation of miR-21 is still not fully understood, as there are multiple layers towards maturity that can be regulated, including a number of transcription factors which bind to its promotor region and to its pri-miR-21 form⁶⁸, independent of *TMEM49* regulation. Additional cofactors support the maturation of pri-miR-21, such as in the case of TGF- β -induced miR-21 expression, where activation of TGF- β signalling permits the adaptor protein Smad6 to bind to pri-miR-21 for the recruitment of RNA helicase p68 and cleavage by Drosha.⁶⁹ Moreover, induction of miR-21 in tumorigenic cells demonstrated an anti-inflammatory response within macrophages, yet conversely, miR-21 has also been shown to promote inflammatory mediators in non-hematopoietic cells.⁶⁸

In fact, elevated levels of miR-21 are suggested to be pathological in IBD.^{70,71} Epigenome-wide whole blood DNA methylation profiles of pediatric CD treatment-naïve patients showed that hypomethylation of the miR-21 locus correlated with increased expression in leukocytes and inflamed intestinal tissue.⁷² For UC patients in remission, miR-21 was found to be downregulated while known target programmed cell death protein

(PDCD)-4 was upregulated in CD3⁺ T-cells compared to active disease and healthy controls.⁷³ Strikingly, miR-21 regulation has been suggested to affect the gut mucosa.³⁵ Mo *et al.* (2016)⁷⁴ demonstrated loss of mucus integrity was miR-21-dependent. Myristoylated alanine-rich C-kinase substrate (MARCKS), a direct target of miR-21, is known to regulate function of mucus barriers.⁷⁵ Additionally, predicted targets of miR-21 also include intestinal mucus components like MUC2, Fc-gamma binding protein (FCGBP), SLC26A3 and zymogen granule protein 16 (ZG16); as well as goblet cell differentiation markers growth factor independence-1 (GFI1) and SAM pointed domain-containing ETS transcription factor (SPDEF).³⁵ Though these require further investigation, the evidence of upregulated miR-21 in IBD patients may cause detrimental changes to the protective mucus layer and thus interfere with mucus function and gut permeability.

Murine models have been used successfully to advance anti-miR-21 therapy in the treatment of various diseases. For example, locked nucleic acids (LNAs) were used to silence miR-21 in systemic lupus erythematosus (SLE) to reduce inflammation in T-cell and B-cell function.⁷⁶ LNAs that antagonized miR-21 ameliorated symptoms in patient-derived psoriatic skin xenotransplants in mice⁷⁷, while in a separate study also versatile in inducing apoptosis within melanoma cell lines.⁷⁸ Other technologies based on antisense oligonucleotides (ASOs) against miR-21 has also been used to treat cardiac hypertrophy⁷⁹ and renal fibrosis⁸⁰, which showed decreased levels of tissue damage in mice. Furthermore, clinical trials are underway that use anti-miR-21 ASOs to treat Alport nephrology through increasing PPAR α /retinoid X receptor (PPAR α /RXR) activity and improved mitochondrial function.⁸¹

Full knockout (KO) miR-21 mice (miR-21^{-/-}) have been used to study several inflammatory diseases. Researchers have used miR-21^{-/-} to study neuropathy in dorsal root ganglia after nerve damage that can in part be exacerbated by secreted miR-21 in neuronal-derived exosomes.⁸² One study utilized miR-21^{-/-} mice to assess cardiac dysfunction and showed lower levels of inflammation in post-myocardial infarction due to alleviating damage associated molecular patterns (DAMPs) that would typically lead to macrophage release of pro-inflammatory cytokines.⁸³ Using miR-21^{-/-} mice in the 7,12-dimethylbenz(a)anthracene (DMBA) / 12-O-tetradecanoylphorbol-13-acetate mouse model for skin carcinogenesis resulted in a reduction in papilloma formation through a reduction in extracellular signal-regulated kinase (ERK), AKT and c-Jun N-terminal kinase (JNK) phosphorylation for Ras pathway activation.⁸⁴ Several studies showed that ablation of miR-21 in mice led to protection against dextran sulfate sodium (DSS)-induced colitis.

Shi *et al.* (2013)⁸⁵ discovered that miR-21^{-/-} mice have reduced disease-associated indices compared to wild type (WT) counterparts and suggest intestinal barrier function via RhoB and Cdc42 is regulated in a miR-21-dependent manner. Further, Johnston *et al.* (2018)⁸⁶ attributed this protection to miR-21-associated changes to gut microbiota. In that study, it was observed that in miR-21^{-/-} mice, there were significant differences in various Actinobacteria, Firmicutes and Bacteroidetes compared to WT mice. Additionally, co-housed WT with miR-21^{-/-} mice led to significant amelioration of disease in WT, while protection against DSS-induced colitis was lost upon pre-treatment of mice with antibiotics. Changes in gut microbiota were also observed when using miR-21^{-/-} with a focus on liver injury in cholestasis, where increased *Lactobacillus* in KO mice reduced liver fibrosis.⁸⁷ Interestingly, from infection models, miR-21^{-/-} mice were protected from *Listeria monocytogenes* challenge⁸⁸, while macrophages isolated from miR-21^{-/-} showed the dependency of *Mycobacterium tuberculosis* infection on miR-21 induction for an anti-inflammatory response and evade immune detection.⁸⁹ Furthermore, miR-21^{-/-} mice have been utilized in other colitis models such as the 2,4,6-Trinitrobenzene sulfonic acid (TNBS) and CD4⁺ T-cell transfer in *Rag2*^{-/-} mice. Although miR-21 is upregulated in both DSS and TNBS models, ablation of miR-21 resulted in greater disease severity for TNBS and T-cell transfer models, showing an importance of miR-21 for inflammation through CD4⁺ T-lymphocytes.⁹⁰ T-cell effects due to miR-21 modulation comes to no surprise, as an earlier study demonstrated miR-21 ablation impacts Th1 polarization through targeting IL-12, causing an increase in IFN- γ levels and reduction in IL-4 during induced hypersensitivity in lungs of miR-21^{-/-} mice.⁹¹ However, exacerbation of pathogenesis was also observed when using miR-21^{-/-} mice. Researchers showed that deletion of miR-21 had greater development of hepatocellular carcinoma due in part to negative alterations of immune anti-tumoral responses.⁹² In general, complete removal of miR-21 in mice has shed light on the many ways miR-21 can regulate the mammalian immune response.

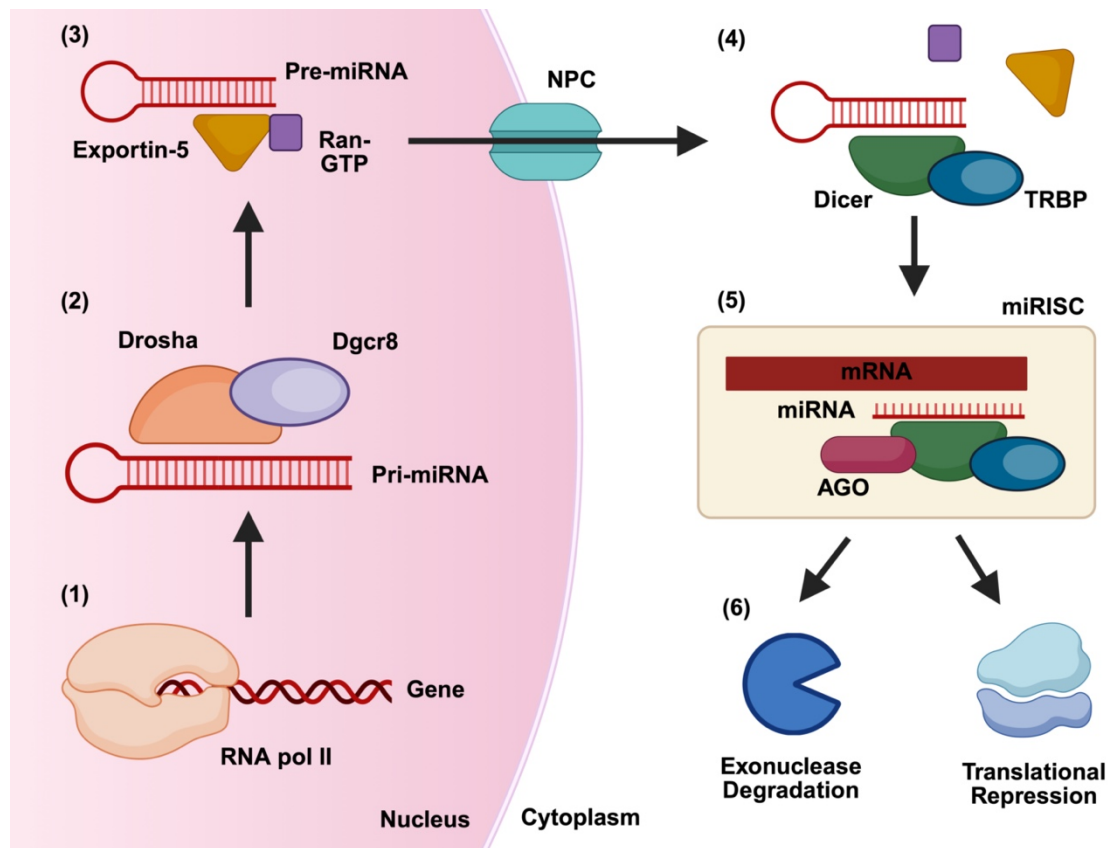


Figure 1.2 – Schematic overview of microRNA synthesis. (1) Biogenesis begins via binding of RNA polymerase II to miRNA transcription site in the nucleus. (2) Formation of the primary (pri)-miRNA transcript and enzymatic cleavage of Drosha/Dgcr8 to form the precursor (pre)-miRNA. (3) Pre-miRNA interacts with exportin-5 and is exported into the cytoplasm in a RanGTP-dependent manner. (4) Binding of Dicer and Tar RNA binding protein (TRBP) create the mature form miRNA. (5) Loading the guidance strand of the miRNA into an Argonaute (AGO) protein creates the miRNA-induced silencing complex (miRISC). (6) The miRISC recognizes seed regions in target mRNA and typically lead to the silencing of mRNA through stimulation of degradation or translational repression. (Mercurio, K.; created with BioRender.com).

1.5 Intercellular junctions and microRNA regulation

Along the epithelial barrier are connection points between various IECs called intercellular junctions, which dictate paracellular transport, cell adhesion and cell–cell communication. The four types of intercellular junctions are tight junctions (TJs), adherens junctions (AJs), desmosomes/hemidesmosomes and gap junctions (GJs), all composed of distinct protein complexes used for specific roles. Of the four, TJs, AJs and desmosomes comprise the apical junctional complex (AJC) of polarized IECs that associates with the dense network of actin and myosin filaments²⁷, while hemidesmosomes are typically located towards the basolateral end⁹³, and GJs have no specific localization. Most of the literature on gut permeability in IBD focuses only on TJs or has conducted predictive scans for potential junction targets of interest.⁹⁴ A detailed schematic of known and predicted miRNA regulation regarding intercellular junctions can be seen in **Figure 1.3**, with miR-21 being a focus of this thesis.

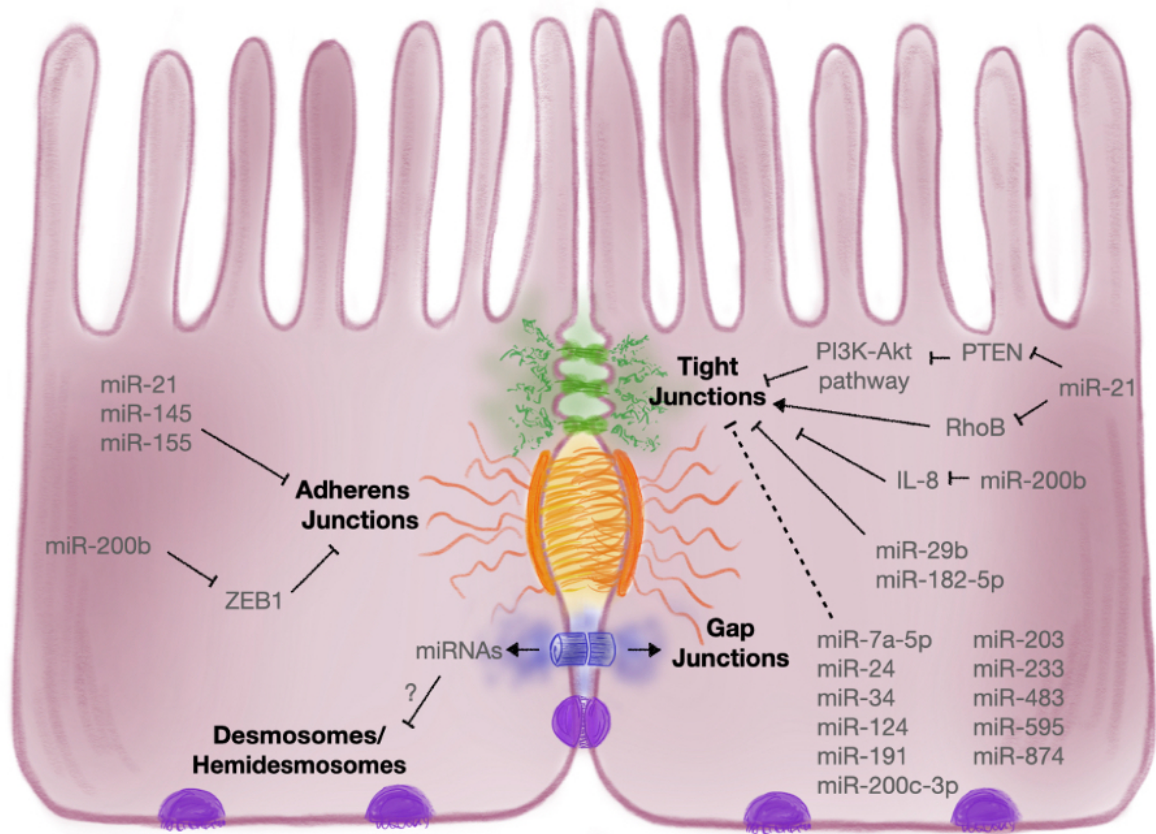


Figure 1.3 – Schematic overview of intercellular junctions regulated by miRNAs. The most well studied intercellular junction regarding miRNA regulation is the tight junction, with both direct and indirect targeting not only suggested but confirmed (miR-21, miR-200b, miR-29b, miR-182-5p). Adherens junctions have also been studied for direct and indirect targeting of miRNAs with some confirmed targeting reported (miR-21, miR-145, miR-155, miR-200b). Less is known about the regulation of desmosomes / hemidesmosomes and gap junctions, which may prove to be an untapped area regarding miRNA regulation of the epithelial gut barrier. This figure was originally published in Stiegeler S., Mercurio K. *et al.* (2021).³⁵ Dashed line: indirect target, solid line: direct target.

1.5.1 Tight junctions

Regarded as essential components of the gut epithelial barrier, TJs are the selective gates that control the paracellular diffusion of ions, the passage of small soluble and macromolecular compounds, and the entry restriction of microbes.^{27,95} TJ proteins are often divided into three main categories: (1) transmembrane proteins, (2) cytosolic scaffolding proteins and (3) regulatory proteins. Among the transmembrane proteins, tetraspannin-like claudins (~26 subtypes) are important sealing or pore-forming proteins that allow for selective permeability.⁹⁵ Cytosolic scaffolding proteins are largely attributed to zonula occludens (ZO) family proteins that interact with claudins, junction adhesion molecules (JAMs) and MARVEL family proteins such as occludin and tricellulin, which all govern interactions with the actin cytoskeleton.²⁷ These complexes work together to serve in both the “pore” and “leak” pathways dependent on the claudins that comprise TJs, for either high-capacity size/charge selection or low-capacity limited-selectivity transport, respectively.⁹⁶ A simplified schematic overview of TJs and associated gene regulatory pathways can be seen in **Figure 1.4**.

In a disease state such as increased inflammation and damage to the gut epithelial barrier, TJ abundance, structure and composition are modulated, affecting the passage of microbiota and large proteins from the lumen.²⁷ Immunostaining for occludin and ZO-1 was severely reduced in IBD tissues, particularly in IECs at the luminal surface.⁹⁷ Upregulation and redistribution of occludin, claudin-2, claudin-5 and claudin-8 were associated specifically with CD.⁹⁸ There was also increased expression of myosin light chain kinase (MLCK), claudin-1 and claudin-2 within patients that had active IBD.^{99,100} Understanding the differences in the composition and function of TJs in healthy and diseased states could help discover underlying issues with gut permeability.

Direct and indirect targeting of occludin and several claudins were suggested with various miRNAs. Using colon tissues from both UC patients and colitis mouse models, IL-1 β -induced upregulation of miR-200c-3p decreased levels of occludin, which negatively impacted barrier function.¹⁰¹ Pro-inflammatory marker IL-8 and regulator of barrier function CDH11 were shown to be directly regulated by miR-200c-3p in inflamed mucosa biopsies obtained from UC patients.¹⁰² Additionally, TNF-induced miR-122 increased gut permeability *in vitro* for Caco-2 monolayers and *in vivo* for recycled perfused mouse intestine.¹⁰³ Moreover, miR-34 in combination with long non-coding RNA PlncRNA1 cooperatively regulated the expression of occludin and ZO-1 in Caco-2 monolayers

undergoing DSS-induced colitis.¹⁰⁴ Regarding relevant claudins, claudin-2 was recently demonstrated to be a target of miR-182-5p, whose inhibition led to increased claudin-2 and TGF- β 1 expression, as well as anti-inflammatory and anti-oxidative genes. The suppression of occludin and claudin-1 has been attributed to miR-874, while also targeted by miR-29b, which was shown to be sequestered via the addition of long non-coding RNA uc.173 as well as circular RNA CircHIPK3, rescuing barrier function.^{105–107} Likewise, downregulation of claudin-8 was observed by miR-233.¹⁰⁸ Interestingly, human mast cell (HMC-1)-derived exosomes enriched in miR-223 inhibited claudin-8 expression in various IEC lines, destroying barrier function.¹⁰⁹ Thus, TJ regulation by miRNAs is abundant and demonstrates the importance of further studying their scope in IBD.

Other components of TJs, such as cytosolic scaffolding/adaptor proteins, can also be targeted by miRNAs. In T84 monolayers, ZO-2 levels were impacted when inhibitors for miR-203, miR-483-3p and miR-595 were used.¹¹⁰ Overexpression of miR-24 led to decreased levels of cingulin, which negatively associates with disease severity in UC patients.¹¹¹ Additionally, TNF-induced miR-191a expression led to decreased levels of ZO-1 in IEC-6 cells.¹¹² The suppression of aryl hydrocarbon receptor (AHR) protein and TJ proteins by miR-124 was associated with intestinal barrier disruption in both the TNBS-induced colitis mouse model and CD patient mucosal biopsy samples.¹¹³ Interestingly, the injection of a miR-7a-5p antagomir within mice that had undergone TNBS-induced colitis increased ZO-1 expression and promoted barrier recovery, potentially through downregulation of the JNK pathway.¹¹⁴ MiRNA regulation of core proteins and secondary scaffolds of the TJ is frequent and thus further complicates the overall picture of the inflamed gut.

Some miRNAs have been identified to have positive roles in IBD. The expression of miRNAs that showed amelioration of disease symptoms include miR-200b, miR-320a, miR-93 and miR-1. Through direct targeting of MLCK and c-Jun protein of the JNK-activator protein-1 (AP-1) early response transcription factor that inhibit epithelial cell proliferation, miR-200b suppressed IL-8 secretion and attenuated TJ dysfunction *in vitro* by preventing morphological redistribution of claudin-1 and ZO-1.¹¹⁵ Transfection of T84 cells with miR-320a showed increased transepithelial resistance (TEER) and JAM-A expression.¹¹⁶ MiR-93 targeted protein tyrosine kinase-6 and attenuated TNF/IFN- γ -induced barrier dysfunction during IBD.¹¹⁷ Lastly, health benefits of salvianolic acid B, a traditional Chinese herb, were suggested to restore barrier function and TJ protein expression via the induction of miR-1 and downregulation of MLCK.¹¹⁸ Though much

research has already uncovered a lot of relevant miRNAs and their targets, the non-intuitive aspect of their direct and indirect effects through downstream targeting makes it difficult to ascertain their positive or negative roles in gut permeability.

Many TJ proteins are regulated by miR-21. It has been suggested that TNF- α -induced miR-21 increases intestinal permeability by decreasing levels of phosphatase and tensin homolog (PTEN), an inhibitor of the phosphatidylinositol 3-kinase (PI3K)-AKT signalling pathway.¹¹⁹ Interestingly, intestinal symbiotic flora can upregulate miR-21-5p to target inhibitors of the PI3K-AKT, AP-1 and ERK pathways, which upregulate ADP ribosylation factor (ARF)-4 and reduce claudin-4 and occludin, ultimately increasing barrier permeability.¹²⁰ Furthermore, miR-21 was shown to induce the degradation of RhoB mRNA, leading to the depletion of occludin.¹²¹ RhoB, part of TJ protein complexes and a target of miR-21, impacts various cellular pathways including cell cycle, apoptosis, actin organisation, cell migration and adhesion.¹²² Conversely, another study suggested that miR-21 inhibition of the Rho and Rho-associated protein kinase (ROCK) pathway, which can protect against inflammation by upregulating TJ proteins.¹²³ ZO-1 was also shown to be indirectly regulated by miR-21.¹²⁴ TJs targeted by miRNAs are summarised in **Table 1.1**. It is crucial that further research on the controversial nature of miR-21 and its influence in inflammation is conducted to elaborate on its full impact.

Table 1.1 – Summary of literature showcasing miRNA impact on tight junction proteins. This table was originally published in Stiegeler S., Mercurio K. *et al.* (2021)³⁵.

Target	miRNA	Description
Claudin-1	miR-29	Uc.173 and CircHIPK3 sequestered miR-29, which targets claudin-1 ¹⁰⁶
	miR-200b	MiR-200b suppressed IL-8 secretion and thereby attenuated TJ dysfunction via claudin-1 ¹¹⁶
	miR-874	Suppression of claudin-1 has been attributed to miR-874 ¹⁰⁵
Claudin-2	miR-182-5p	Claudin-2 was shown to be a target of miR-182-5p ¹²⁵
Claudin-4	miR-21-5p	Intestinal symbiotic flora upregulated miR-21-5p to target inhibitors of the PI3K-Akt, AP-1 and ERK pathways to upregulate ARF4 and reduce claudin-4 ¹²⁰
Claudin-8	miR-233	MiR-233 downregulated claudin-8; HMC-1 derived exosomes enriched in miR-233 inhibited claudin-8 in various intestinal epithelial cell lines ^{108,109}
Occludin	miR-21-5p	Intestinal symbiotic flora upregulated miR-21-5p to target inhibitors of the PI3K-Akt, AP-1 and ERK pathways to upregulate

		ARF4 and reduce occludin; also negatively affects occludin by targeting RhoB ^{120,121}
	miR-29b	Uc.173 and CircHIPK3 sequestered miR-29, which targets occludin ¹⁰⁶
	miR-34	PlncRNA1 and miR-34 cooperatively regulated expression of occludin in vitro during DSS-induced colitis ¹⁰⁴
	miR-200c-3p	IL-1 β -induced upregulation of miR-200c-3p in UC patients decreased levels of occludin ¹⁰¹
	miR-874	Suppression of occludin has been attributed to miR-874 ¹⁰⁵
Zonula Occludens-1	miR-7a-5p	MiR-7a-5p antagomir increased ZO-1 expression and promoted barrier recovery within TNBS-induced colitis models ¹¹⁴
	miR-21-5p	ZO-1 shown to be indirectly regulated by miR-21 ¹²⁴
	miR-34	PlncRNA1 and miR-34 cooperatively regulated expression of ZO-1 in vitro during DSS-induced colitis ¹⁰⁴
	miR-191a	TNF-induced miR-191a expression led to decreased levels of ZO-1 in IEC-6 cells ¹¹²
	miR-200b	MiR-200b suppressed IL-8 secretion and thereby attenuated TJ dysfunction via ZO-1 ¹¹⁵
Zonula Occludens-2	miR-203	ZO-2 levels were impacted when inhibitors for miR-203, miR-483-3p and miR-595 were used on T84 monolayers ¹¹⁰
	miR-483	
	miR-595	
Cingulin	miR-24	Overexpression of miR-24 led to decreased levels of cingulin, which negatively correlated with disease severity in UC patients ¹¹¹
JAM-A	miR-320a	Elevated miR-320a increased TEER and JAM-A expression in T84 cells ¹¹⁶
Other	miR-1	Health benefits of salvianolic acid B were suggested to restore barrier function and TJ protein expression via induction of miR-1 ¹¹⁸
	miR-93	MiR-93 targeted protein tyrosine kinase 6 and attenuated TNF/IFN γ -induced barrier dysfunction during IBD ¹¹⁷
	miR-122	TNF-induced miR-122 increased gut permeability ¹⁰³
	miR-124	Suppression of AHR protein and TJ proteins by miR-124 was associated with intestinal barrier disruption ¹¹³

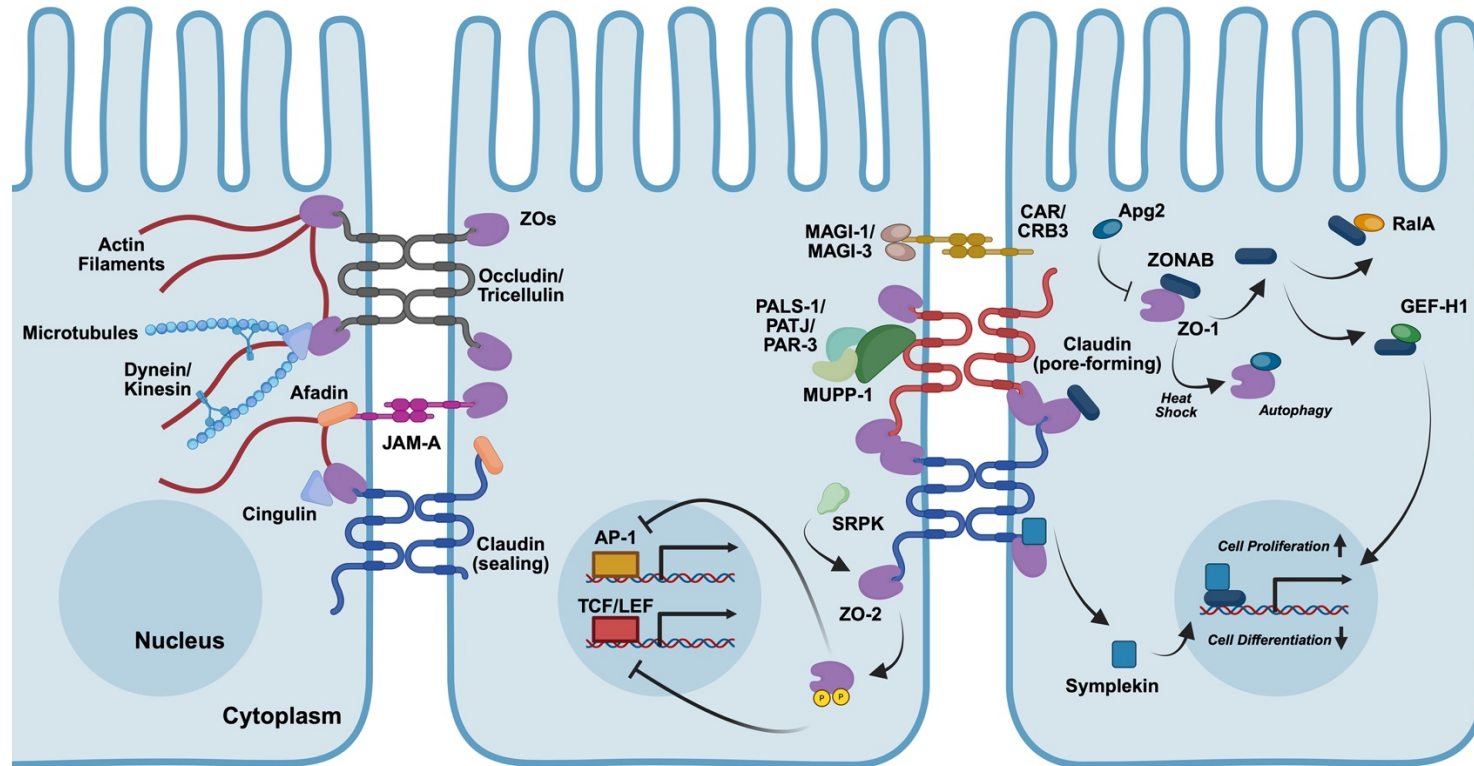


Figure 1.4 – Simplified schematic overview of the tight junction between intestinal epithelial cells. Depicted on the leftmost panel is a state of association between TJ components when the complex is functioning normally. Along the apical membrane are MARVEL family proteins such as occludin and tricelluin, as well as claudins (sealing and pore-forming). Cytosolic tails bind to scaffolding proteins such as zonula occludens (ZOs), cingulin and afadin, which themselves bind to cytoskeletal elements like actin filaments and microtubules. Other membrane associated TJ proteins include junction adhesion molecules (JAMs, ex. JAM-A) that interact with ZO-2 and afadin for further stability and control of paracellular transport with MARVEL family proteins. Other aspects of TJ components and their participation in gene regulation can be seen in the middle and rightmost panels. First, similar in structure to immunoglobulin family protein JAM, the coxsackievirus and adenovirus receptor (CAR) and crumbs homologue 3 (CRB3) helps stabilize the TJ points and binds with membrane-associated guanylate kinase inverted (MAGI) proteins that are involved in PI3K signalling. Scaffolding protein MUPP-1 helps bind JAMs/CARs with claudins, and its interaction with other adaptor proteins like PALS-1, PAT-J and PAR-3 support TJ assembly and cell polarization. Furthermore, many TJ proteins are associated with gene regulatory pathways. Examples here include ZO-2, which upon phosphorylation of SRPK, translocates into the nucleus and blocks transcription factors AP-1 and TCF/LEF binding to promoter regions. In the rightmost panel, ZO-1 participates in the sequestration of transcription factor ZONAB, which would normally activate cell proliferation and decrease cell differentiation with the nuclear translocation of another TJ protein symplekin. This interaction with ZO-1 and ZONAB can be inhibited by Apg2 particularly during heat shock stress leading to its degradation, while other modulating proteins like RalA and GEF-H1 can inhibit or activate ZONAB gene regulation, respectively. Note that due to picture size and amount of TJ and TJ-related proteins, some are seen to localize to the basolateral end when in fact TJs localize solely to the apical end. Information for this diagram was obtained from González-Mariscal *et al.* (2003)¹²⁶, González-Mariscal *et al.* (2014)¹²⁷, and Zihni *et al.* (2016)¹²⁸. (Mercurio, K.; created with BioRender.com).

1.5.2 Adherens junctions

The major functions of AJs at the gut epithelial barrier include the stabilization of cell-cell adhesion, the regulation of the structural actin cytoskeleton and interaction between transmembrane glycoproteins and cytosolic junctional proteins, along with linkage to intracellular signalling for transcriptional regulation.¹²⁹ The main component of AJs along the epithelial barrier is E-cadherin, which interacts with other AJ proteins such as p120-catenin and β -catenin to link with α -catenin and regulate regional strength via perijunctional actin assembly.¹³⁰ AJ protein complexes are also essential for the proper formation of TJs regulating the transport of molecules between IECs, which emphasises the importance of seamless regulation between intercellular junctions.²⁷ States of association for these AJ components and consequences of disassociation can be seen in **Figure 1.5**.

E-cadherin has a variety of functions in both healthy and diseased states. The dysregulation of E-cadherin leads to improper barrier function and the manifestation of IBD.¹³¹ Full KO of E-cadherin was lethal in mice, and conditional KO at the intestinal epithelium within mouse embryos led to death within 24 hours.^{132,133} Reduced immunoreactive E-cadherin and α -catenin were observed in IBD tissue samples via immunofluorescence and western blot analyses; however, no changes in mRNA levels or localization were noted.⁹⁷ Moreover, transgenic mice have been used to study AJ dysfunction, such as the conditional deletion of E-cadherin within the intestinal epithelium, but this is likely not a primary mechanism of IBD presentation.¹³⁴ Levels of *CDH1*, which encodes E-cadherin, were downregulated in colon samples from active IBD patients.¹³⁵ Further, polymorphisms in *CDH1* have been associated with UC.¹³⁶ β -catenin-induced Wnt signalling has been implicated in IBD and has been suggested as a key regulatory pathway for barrier homeostasis.^{137,138}

There is some research focusing on the regulation of AJ proteins by miRNAs. Studies on the most prominent AJ proteins with patients suffering from UC or fibrosis found a negative correlation between levels of E-cadherin and miR-21.^{139,140} Recently, one study showed that exosome-derived miR-21a-5p from abnormally polarized macrophages was taken up by IECs and decreased levels of E-cadherin, exacerbating DSS-induced colitis in mice.¹⁴¹ Upregulation of circRNA_102610 led to decreased levels of miR-130a-3p, promoting TGF- β 1-induced EMT in human IECs and NCM460 cells, and downregulating E-cadherin expression.¹⁴² Interestingly, research using early life inflammatory stressor rat

models found sustained epithelial injury through the suppression of E-cadherin via miR-155.¹⁴³ The introduction of TGF- β 1, commonly upregulated in IBD patients, to IEC-6 cells demonstrated direct targeting by miR-200b of Zeb1, a negative regulator of AJ formation and thus increased E-cadherin expression.⁴⁶ In addition to E-cadherin, other AJ proteins such as epithelial membrane protein-1 were shown to be targeted by miR-145, known to have another target cathepsin B that accumulates in IBD patients.^{144,145} AJs targeted by miRNAs are summarised in **Table 1.2**. More research is required to further elucidate modulations in AJ proteins during IBD pathogenesis.

Table 1.2 – Summary of literature showcasing miRNA impact on adherens junction proteins. This table was originally published in Stiegeler S., Mercurio K. *et al.* (2021)³⁵.

Target	miRNA	Description
E-cadherin	miR-21	A negative correlation between levels of E-cadherin and miR-21 was found in UC patients; exosome-derived miR-21a-5p from abnormally polarised macrophages decreased levels of E-cadherin in the epithelium, exacerbating DSS-induced colitis in mice ^{139–141}
	miR-130a-3p	Upregulated circRNA_102610 decreased miR-130a-3p, promoted TGF- β 1-induced EMT <i>in vitro</i> and downregulated E-cadherin expression ¹⁴²
	miR-155	E-cadherin was negatively affected by miR-155, leading to epithelial injury in early-life inflammatory stressor rat models ¹⁴³
	miR-200b	MiR-200b directly targets Zeb1, a negative regulator of AJ formation, and thus increased E-cadherin expression ¹⁴⁶
Epithelial Membrane Protein-1	miR-145	Other AJ proteins such as epithelial membrane protein 1 were shown to be targeted by miR-145 ¹⁴⁴

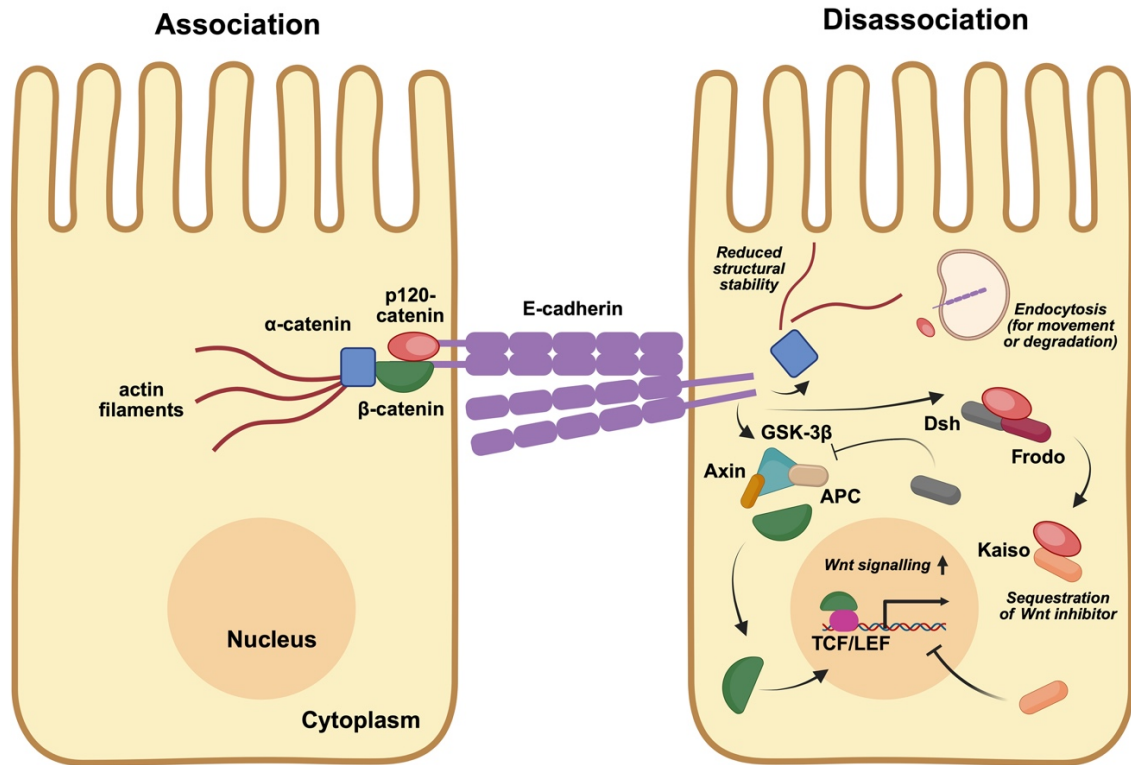


Figure 1.5 – Simplified schematic overview of the adherens junction between intestinal epithelial cells. Depicted on the left is a state of association between AJ components when the complex is functioning normally (adapted from Baum *et al.* (2011)¹⁴⁷). In the presence of Ca^{2+} ions, E-cadherin forms homophilic interactions via the extracellular domain. Binding sites along the intracellular domain allow recruitment of p120-catenin and β -catenin to form the cadherin-catenin complex. When associated, p120-catenin helps link the complex to microtubules and prevent endocytosis by the cell. β -catenin further stabilizes E-cadherin at the membrane and interacts with α -catenin and other AJ proteins that link the complex to actin filaments. Depicted on the right is a state of disassociation between AJ components. Loss of α -catenin reduces structural stability and connection with the epithelial cytoskeleton.¹⁴⁸ β -catenin disassociation can also destabilize the cadherin-catenin complex and can lead to its degradation by the GSK3-Axin-APC complex or migration to the nucleus by Beclin-1, all part of the canonical Wnt signalling pathway.¹⁴⁹ Finally, loss of p120-catenin can increase the level of AJ protein recycling through endocytosis¹⁵⁰, while also sequestering Wnt signalling inhibitor Kaiso after stabilization in the cytoplasm via interaction with Dishevelled (Dsh) and Frodo.¹⁵¹ (Mercurio, K.; created with BioRender.com).

1.5.3 Desmosomes/Hemidesmosomes

The primary role of desmosomes and hemidesmosomes at the gut epithelial barrier is to resist shearing forces, acting as anchoring points to the apical or basolateral membrane, and to establish a continuum layer of cells with membrane proteins (desmocollins, desmogleins, desmoplakins), armadillo repeat family proteins (plakoglobin and plakophilin), desmoplakin and intermediate filaments.¹⁵² Desmocollins and desmogleins are part of the larger cadherin family, and thus have similar characteristics to AJs. However, they possess the unique ability of calcium-independent hyper-adhesiveness.¹⁵³ For hemidesmosomes, proteins such as BP230 and plectin are the most studied and are implicated in the organisation of cytoskeletal elements as well as integrin $\alpha 6\beta 4$.^{93,154}

Although integral components for structural stability, desmosomes / hemidesmosomes have been largely overshadowed by TJs and AJs, particularly in their relevance to IBD.¹⁵² Full KO of desmoglein-2 led to decreased levels of claudin-1 and occludin, increasing intestinal permeability.¹⁵⁵ Epithelial-specific KO of desmocollin-2 showed no increase in barrier permeability during DSS-induced colitis; however, another study showed that an inducible KO of desmocollin-2 had impaired mucosal repair after recovery from DSS-induced colitis.¹⁵⁶ Additionally, desmoglein-2 regulated claudin-2 expression via the sequestration of PI3K in IECs of mice during DSS-induced colitis.¹⁵⁷ Desmosome staining of IECs within CD or UC patients was reduced in a manner dependent on the severity of inflammation, complemented by decreased protein abundances of desmoglein-2 and desmocollin-2 via western blot.⁹⁷ Other studies of CD patient cohorts of various levels of disease severity confirmed the decrease and abnormal distribution of desmoglein-2 and desmocollin-2 within inflamed tissue.¹⁵⁸ In a recent study by Kelm *et al.* (2025)¹⁵⁹, plakoglobin was reduced in IBD patients and in mice undergoing DSS-induced colitis. Furthermore, the study showed plakoglobin removed from the intestinal epithelium led to submucosal infiltration of immune cells like macrophages and neutrophils, along with activation of the inflammasome by p38MAPK phosphorylation¹⁵⁹.

For hemidesmosomes, full and conditional KO mice for integrin $\alpha 6\beta 4$ were generated and led to spontaneous colitis caused by detachment from the basal membrane and the underlying lamina propria that induces an IL-1 β /IL-18 pro-inflammatory response.¹⁶⁰ Despite their clear roles in maintaining barrier integrity, no studies on the regulation of desmosomes/hemidesmosomes by miRNAs could be found, and therefore more research should be conducted to provide knowledge on their function in IBD.

1.5.4 Gap junctions

Less structural but rather communicative components of the gut epithelial barrier are GJs. Prevalent between adjacent IECs are GJs made of intracellular plasma membrane channels allowing for cell-cell communication via the passage of biologically important ions and small metabolites.¹⁶¹ GJs are typically composed of homologous proteins called connexins (~21 in humans) that can form up to six homomeric/homotypic or heteromeric/heterotypic connexons, differing in content and spatial arrangement depending on their permeability roles when binding to adjacent cell connexons to form intercellular channels.¹⁶² Therefore, a core function of these junctions is to share metabolic demands across groups of cells and buffer gradients in space, nutrients and signalling molecules.

A major role of GJs is for effective crosstalk among different cell types at the gut barrier. Mouse macrophages were shown to communicate with IECs via the use of GJs.^{163,164} Additionally, TLR4-mediated crosstalk between macrophages and IECs results in IL-10 production, which could regulate barrier integrity, requiring the coordinated functioning of GJs.¹⁶⁵ Co-cultures with IECs and THP-1 macrophages demonstrated the importance of these communication channels between different cell types.¹⁶⁶ In Caco-2 cell monolayers, stable overexpression of connexin-26 increased claudin-4 expression and TEER measurements during monolayer disruption with oleic acid and taurocholic acid.¹⁶⁷

Regarding IBD, immunohistochemistry analysis of common connexins such as connexin-26 and connexin-43 demonstrated lower expression at the apical end of IECs with greater localization at the basolateral end in IBD tissue, suggesting a role in communication with infiltrating macrophages during a disease state.¹⁶⁶ Colocalization of the prominent connexin-43 with other intercellular junction proteins important at the epithelial barrier such as E-cadherin and ZO-1 is lost in IBD tissues.¹⁶⁶ TLR2-mediated mucosal healing after acute intestinal barrier damage modulated levels of connexin-43.¹⁶⁸ One study by Liu *et al.* (2024)¹⁶⁹ demonstrated a significant increase in connexin-43 mRNA of CD8⁺ T lymphocytes of UC patients, suggesting increased crosstalk between immune cells and cells within the inflamed intestine. Interestingly, miRNAs have been shown to pass through GJs of adjacent cells in a connexin-dependent manner that favoured connexons primarily made of connexin-43, further demonstrating a synchronised coordination of miRNA regulation at the gut epithelial barrier.¹⁷⁰ Therefore, furthering work on the coordination of TLRs and GJ proteins in IEC communication is necessary to determine the overall inflammatory environment of both healthy and diseased states.

1.6 Wound healing at the gut barrier

The gut barrier is the interface that separates host cells within the lamina propria from the microbial communities residing in the GI mucus layer and other luminal contents. Importantly, the barrier must be semi-permeable, since IECs must balance tight adherence to one another for structural integrity, as well as permittance for the paracellular passage of important compounds necessary for nutritional uptake or immune functionality. The gut epithelium must undergo a lifecycle to rid of old cells and also respond to environmental changes in a regulatory manner, including injurious circumstances due to toxic or harmful digestion, microbial attack or inflammation.¹⁷¹ In injurious circumstances, the gut epithelium undergoes the process of wound healing, which is governed by three major events: 1) restitution/migration, 2) proliferation and 3) differentiation. Although there exist three distinct events, these processes overlap and may not be separately observed *in vivo*.¹⁷²

Under normal conditions, the intestinal epithelium is renewed every 4-5 days.²¹ By regulating barrier elements of IECs like intercellular junctions or mucus secretions, mainly found at the apical end of polarized cells, dying cells can migrate towards the tops of villi or crypts and be replaced by proliferating epithelial cells at the bottom of crypts that spatially differentiate as they move upwards.¹⁷¹ Homeostasis for barrier preservation is tightly controlled by various regulatory peptides such as cytokines and growth factors, along with non-peptide compounds like phospholipids, nucleotides, polyamines, short chain fatty acids (SCFAs), microbial metabolites and pharmacological elements.¹⁷²

In the event of an injury that causes breakage in what is typically a monolayer barrier, the initial step of restitution begins within as little as several minutes to a few hours by nearby IECs that respond by losing their columnar polarity and rapidly migrate to the wound site.¹⁷² This complex process is regulated by multiple pathways. In mouse epithelial cell lines, it was shown that PI3K and Src signalling cascades cooperate with Rac and promote migration.¹⁷³ A reorganization of the actin cytoskeleton is required for migration of normally integral barrier cells to occur.¹⁷⁴ It is suggested that certain GTPases control this migration process, such as Rho, Rac and Cdc42.¹⁷⁵ These proteins act to regulate stress fibers, form lamellipodia protrusions and extend filopodia from the cell periphery.¹⁷¹ In both rat IEC-6 and human T84 monolayers, Rho-GTP and F-actin were localized to the leading edge of wounds through CXCL12, which itself is stimulated through the chemotaxis of CXCL4.¹⁷⁶ CXCL4-mediated localization of Rho may facilitate lamellae formation important for cell motility during wound healing. Additionally, the phospholipid

binding protein Annexin-2 demonstrated some regulatory control over actin cytoskeleton remodelling during cell migration through Rho-dependent signalling pathways.¹⁷⁷ Interestingly, DSS exacerbated colitis in vitamin-D receptor KO mice compared to WT counterparts, with severe disruption to epithelial junctions.¹⁷⁸ *In vitro* supplementation of 1,25-dihydroxy-vitamin D3 upregulated tight junctions and promoted epithelial cell migration.¹⁷⁹ Moreover, expression of fibroblast integrin B1 (ITGB1) through HIF-1 α -dependent activation was critical for cell restitution.¹⁸⁰ Thus, from the moment of barrier breakage, the gut epithelium responds in a manner that ensures a swift migration of non-polarized IECs to the wound site.

Once IECs take hold at the wound site, proliferation initiates to cover gaps in the barrier.¹⁷¹ This process is regulated mainly through the activation of the ERK, mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways.¹⁷¹ One important finding was that glutamine, the most abundant free amino acid in the bloodstream, activated the ERK signalling pathway, stimulated IEC growth, and prevented apoptosis in the small intestine.¹⁸¹ Moreover, glucagon-like-peptide-2 (GLP-2) was shown to be secreted from local neuroendocrine cells to promote epithelial proliferation by stimulating enteric neurons.¹⁸² These signalling cascades play a dual role in downregulating cell motility, which is no longer required in this stage.

During the proliferation phase, epithelial cell populations emerge that mature and differentiate as needed to maintain barrier integrity and function.¹⁷² IEC differentiation of intestinal stem cells (ISCs) is directed by signals released by sub-epithelial myofibroblasts lining intestinal crypts.¹⁸³ ISCs at the base of crypts are grouped into two populations: crypt base columnar (CBC) cells that actively proliferate, and reserve intestinal stem cells (rISCs) that are quiescent until injury.¹⁸⁴ Normally, Lgr5⁺ ISCs differentiate into transit-amplifying progenitors to absorptive (enterocytes) or secretory (mucin/hormone-secreting) progenitors via Wnt/Notch signalling. Secretory progenitors can further differentiate into enteroendocrine cells in a Neurog3-dependent manner or into goblet or Paneth cells by activation of Atoh1 with Klf4 or Sox9, respectively.¹⁸⁴ Interestingly, during DSS-induced colitis, prostaglandin-E2 (PGE2) was shown to play a major role in the regeneration of epithelial crypts and maintain epithelial cell proliferation, synthesized through the metabolic cyclogenesis 1 and 2 (COX-1, COX-2).¹⁷¹ In the HT-29 colon cell line, it was demonstrated that the neuropeptide neurotensin upregulated COX-2 mRNA to stimulate release of PGE2.¹⁸⁵ Thus, the final stage of cell proliferation reinstates the gut barrier back to normality.

While the process of intestinal wound healing particularly concerns IECs, it is important that other cells located in the gut are performing their duties to protect the host from microbial invasion. For example, signal transduction via the cell surface receptor Trem2 by IL-4 and IL-13 inhibits cytokines that enhance M1 macrophage activation, leading to the promotion of inflammation-resolving M2 macrophages.¹⁸⁶ IL-23 is also an important mediator of wound healing, mainly produced by macrophages.¹⁸⁴ Other immune cells like Th17 and Th22 produce mediators of cell proliferation and activation of ISC-like cells like IL-22 to activate STAT3 signalling.¹⁸⁴ Innate lymphoid cells (ILCs) can recruit inflammatory cells via the secretion of TNF- α and IFN- γ or produce amphiregulin which promoted intestinal epithelial degeneration in DSS-treated mice through activation by IL-33.¹⁸⁷ Amphiregulin production was also enhanced in IECs directly via the presence of neutrophils.¹⁸⁸ Moreover, TLRs expressed on both immune and IECs play substantial roles in recognizing microbes in the gut environment during injury.¹⁷¹ Chemokine ligand CCL20 binding to CCR6 was demonstrated to localize dendritic cells and circulating T-cells to the wound site.¹⁸⁹ In addition, CCL20 and human β -defensins stimulated the accumulation of F-actin, phosphorylation of the myosin light chain for cellular morphogenesis, and RhoA in IEC-6 cells to facilitate their migration.¹⁸⁹ Growth factors that have been shown to stimulate wound healing include TGF- α , TGF- β , epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), and insulin-like growth factor I and II (IGF-I, IGF-II).¹⁷¹ TGF- α has an additional role in promoting epithelial cell proliferation through activation of ERK1/ERK2 and MAPK.¹⁹⁰ IFN- γ demonstrated a 3.8-fold increase in epithelial cell restitution¹⁹¹, however this positive association has been challenged in a separate study that showed IFN- γ attenuated intestinal epithelial wound closure by inhibiting cell migration in a B1 integrin-dependent manner¹⁹², and in addition an inhibitory effect of IFN- γ on enterocyte migration through the prevention of GJ communication among IECs.¹⁹³ Importantly, the context regarding cell type and even type of injury should be considered when determining the overall impact of intestinal cell responses.

Notably, TGF- β is a major player in wound healing by promoting epithelial cell migration, while also inhibiting epithelial cell proliferation.¹⁹¹ Within the intestinal mucosa, TGF- β is produced by different cell types that include epithelial cells, stromal cells, T_{reg} cells, dendritic cells and macrophages.¹⁹⁴ Once latent TGF- β becomes activated via the presence of inflammatory cytokines or environmental changes, resulting target genes include the upregulation of matrix metalloproteinase 1 and 10 (MMP-1, MMP-10)

as well as other genes such as *Slc28a1*, *Tubb2a* and *Cpe*, along with the expression of growth factors that kickstart the restitution process.¹⁹⁵ Paradoxically, it is agreed that the impact of TGF- β on cellular growth is context-dependent and bifunctional, inducing anchorage-independent growth in both an EGF-dependent and EGF-independent manner, while also inhibiting growth through induction of cyclin-dependent kinase inhibitors and inhibiting proliferation drivers like c-Myc, Cdc25A and Id proteins.¹⁹⁶ Thus, it is important to acknowledge that TGF- β regulates a variety of events during the normal development of epithelia, and it is the dysregulation of TGF- β signalling that leads to pathogenesis.

Post-binding of TGF- β isoforms, activins and bone morphogenic proteins (BMPs) to Type I/Type II TGF- β heteromeric receptor complexes induces a signalling cascade mediated through ligands named mothers against decapentaplegic homolog (Smad) proteins. There consists of eight Smad proteins in the human genome categorized into receptor-regulated (R-Smad), common partner (co-Smad) and inhibitory (I-Smad) groups. Activated R-Smads colocalize to the nucleus with the only known co-Smad protein in humans, Smad4, to impact the expression of target genes regarding proliferation and differentiation, including I-Smad proteins Smad6 and Smad7 in a negative feedback loop.¹⁹⁷ Non-canonical TGF- β signalling pathways also occur, such as activation of MAPK signalling pathways ERK, JNK, and p38.¹⁹⁷ These canonical and non-canonical pathways of TGF- β signalling have been extensively studied during carcinogenesis, as a characteristic feature of tumour progression is reduced sensitivity to TGF- β growth inhibition, leading to loss of AJs/TJs, along with an induction of EMT.¹⁹⁷ The overall signalling cascade during wound healing is summarized in **Figure 1.6**.

There exists the TGF- β -dependent pathway for epithelial cell restitution, which growth factors TGF- α , EGF, FGF, HGF, along with IL-1 β and IL-2 are involved to induce bioactive TGF- β .¹⁷¹ Alternatively, there also exists the TGF- β -independent pathway for epithelial cell restitution, with trefoil peptides, and galectins 2 and 4.¹⁷¹ Upon wound creation, stored TGF- β 1 within platelets are released at the site of injury and progress outwards, acting as a chemoattractant for monocytes and fibroblasts.¹⁹⁶ In addition, TGF- β 1 was also shown to induce the expression of extracellular matrix (ECM) proteins at the site of injury, such as fibronectin and collagen, and the inhibition of their degradation.¹⁹⁶ In rat IEC-6 monolayers, anti-TGF- β 1 antibodies hindered the promotion of cell restitution by TGF- α , EGF, IL-1 β and IFN- γ .¹⁹¹ Increased TGF- β signalling at the start of wound healing results in epithelial cells undergoing partial or complete EMT to promote epithelial migration, leading to a loss of polarity and increased motility.¹⁹⁸ It is interesting to note that

despite TGF- β signalling supporting wound healing in the early stages, TGF- β isoforms can play different roles in the outcome of wound closure. Treatment with exogenous TGF- β 1 or TGF- β 2 does not enhance the quality of scarring, where it was shown that in the absence of Smad3 or through the blockage of TGF- β 1 and TGF- β 2, scarring was reduced.¹⁹⁹ Moreover, administration of TGF- β 3 reduced cutaneous scarring.¹⁹⁶ In addition, TGF- β receptors play significant yet differing roles during wound healing and overall health. One study demonstrated that truncation of TGF- β receptor II (TGFB2) in the intracytoplasmic tail halts the signalling cascade despite ligand binding, relieves the TGF- β inhibition of IEC-6 proliferation and reduces cell migration during wound healing.²⁰⁰ This same study also found that this was not replicated *in vivo* within transgenic mice where IECs expressed truncated TGFB2 and were indeed more susceptible to DSS-induced colitis with impaired tissue recovery.²⁰⁰ Understanding the holistic profile of TGF- β during wound healing would be extremely valuable for targeted therapeutics, and research is still ongoing particularly within non-hematopoietic cells.

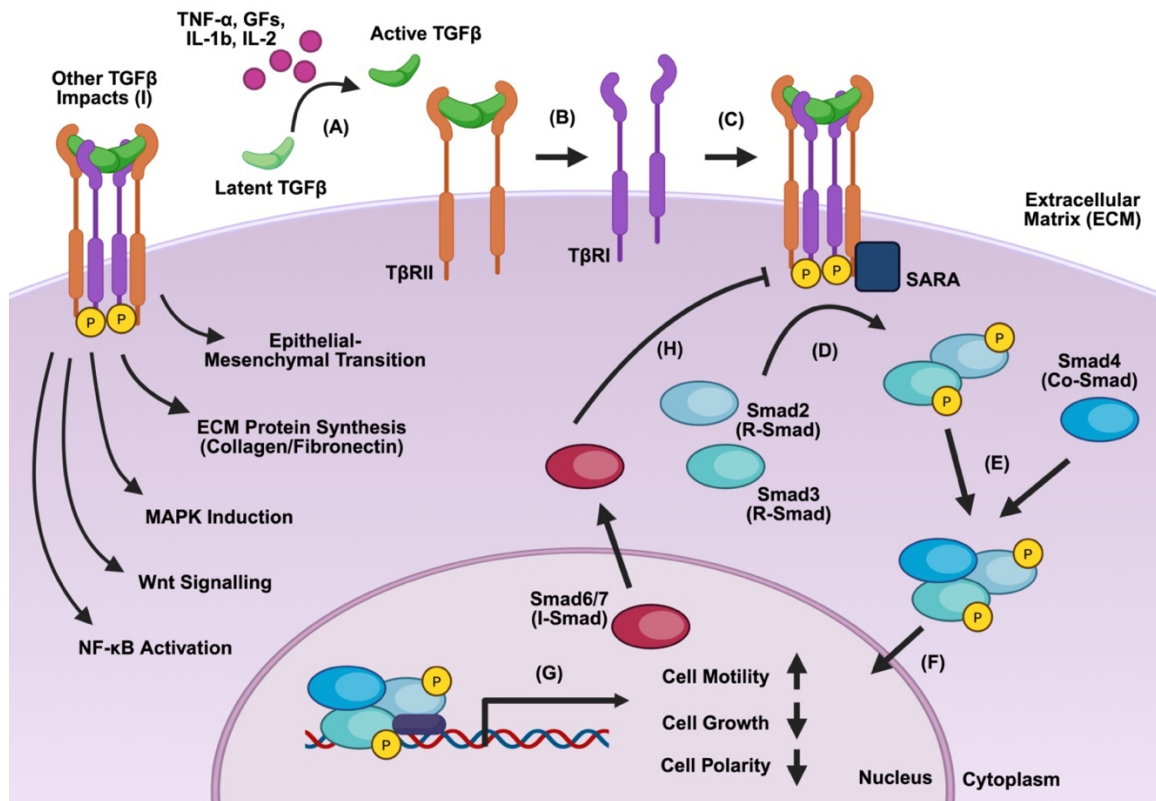


Figure 1.6 – Schematic overview of TGF- β signalling during wound healing. Canonical TGF- β Signalling Pathway: (A) Conversion of latent TGF- β isoforms to their active state via the presence of pro-inflammatory cytokines TNF- α , IL-1 β and IL-2, along with cellular growth factors (GFs). (B + C) Post-binding of TGF- β isoforms to Type I/Type II TGF- β receptors induce homomeric and heteromeric complex formation initiating a signalling cascade mediated through ligands named Smad proteins. (D) Binding of Smad Anchor for Receptor Activation (SARA) to TGF- β receptor complex recruits inactive receptor-regulated (R)-Smad proteins for phosphorylation by receptor intracellular domain. (E) Common partner (Co)-Smad protein Smad4 binds to R-Smads for their (F) localization into the nucleus. (G) Binding of specific transcription factors and cofactors leads to TGF- β regulated gene activation based on their protein combinations, which include FOX, HOX, RUNX, E2F, AP-1, CREB/ATF, Zinc-finger and other families. (H) Activation of TGF- β genes induces expression of inhibitory (I)-Smad proteins that compete with R-Smads for TGF- β receptor binding. Non-Canonical TGF- β signalling include Wnt/ β -catenin pathway, Notch pathway, NF- κ B pathway and GTP-binding protein signalling that are involved in epithelial-specific processes like proliferation, apoptosis, EMT and ECM protein synthesis. Information for this diagram was obtained from Chaudhury *et al.* (2009)¹⁹⁷, Kubiczкова *et al.* (2012)²⁰¹ and Morikawa *et al.* (2016)¹⁹⁶. (Mercurio, K.; created with BioRender.com).

1.7 Model organisms for the study of inflammatory bowel disease

There have been a number of animal models developed with the purpose of understanding IBD at the molecular level. These are incredible tools used to dissect the mechanisms underlying IBD pathogenesis and test therapeutics. Here, animal models of both spontaneous and induced colitis will be discussed to emphasize missing gaps in the literature for miRNA regulation in IBD.

Many mammalian models have been deployed regarding both acute and chronic inflammation for IBD studies. Rats have the advantage of being quite large for sampling purposes and similar to humans regarding the intestinal environment.²⁰² Pigs have been commonly used as a monogastric model due to their similar morphology and function to the human intestine.²⁰³ Non-human primates have also been used and provide the most comparable model system, however they have major drawbacks due to their ethical considerations, high costs and potential of carrying virulent zoonotic pathogens.²⁰⁴ Others include rabbits, guinea pigs and canine models, the latter of which has led to the discovery of various biomarkers of IBD.²⁰² Alternatively, invertebrates such as nematodes and *Drosophila* have also been used for intestinal studies mainly for the purposes of investigating mechanisms involved in innate immunity.²⁰⁵ These models have specific uses depending on where researchers need to focus attention on for the multifactorial inflammation responses seen in afflicted IBD individuals.

The most widely used animal to study IBD is the mouse (*Mus musculus*). Genetic backgrounds vary between research groups but often begin from C57BL/6J or BALB/c.²⁰⁶ Importantly, genetically engineered mice serve incredible value for the research community when elucidating mechanisms leading to inflammation in the intestine. Thus, certain genetic lines are more suited to investigate specific conditions of acute and chronic disease. Although there are many similarities between mice and humans including gene homology, immune and intestinal function, gut physiology and intestinal microbiota communities, there are some disadvantages to the use of mice in IBD research. Lesions observed in mice that have been treated with chemical agents to induce colitis are not completely reflective of those seen in afflicted human patients.^{202,206} In addition, different levels of expression for important TLRs are observed in mice when compared to humans.²⁰⁷ Moreover, the mouse behaviour of coprophagy, in which mice ingest their own stool for re-ingestion of nutrients impacting intestinal health which is obviously not reflected in normal human behaviour, should be considered when designing experiments.²⁰²

As a major symptom of IBD, colitis has been observed to develop spontaneously in a number of murine KO models, such as *Muc2*^{-/-}, *IL-10*^{-/-}, *IL-2*^{-/-} and *TCR-α*^{-/-} mice. *Muc2* is a secretory gel-forming mucin that serves as the main component of the intestinal mucus layer.²⁰⁸ *Muc2*-deficient mice have increased expression of pro-inflammatory cytokines and show increased severity to chemically induced colitis.²⁰⁹ *IL-10* is an anti-inflammatory cytokine that dampens the immune response after the initial influx of immune cells. With typical vivarium conditions, *IL-10*-deficient mice develop colitic symptoms including a reduction of mucin-producing goblet cells in the intestine.²¹⁰ Interestingly, *IL-10*^{-/-} mice of BALB/c background develop spontaneous colitis more frequently than in C57BL/6 background.²⁰² *IL-2*-deficient mice develop a progressive IBD with similarities to human UC, dependent on the presence of microflora but independent of TLR signalling.²¹¹ Interestingly, *TCR-α*-deficient mice also share similarities with human UC such as an upregulation of the Th2 pathway during colon inflammation.²¹² Importantly, spontaneous KO or transgenic mouse models are useful and simple tools to study colitis.

The most studied mouse model in the field of IBD is the use of DSS for inducing colitis.^{213,214} DSS is a sulfated polysaccharide colitogen with anticoagulant properties, administered into the drinking water of mice, resulting in what mostly resembles human UC. Though, despite wide usage, the mechanism of action for how DSS in fact causes colitis is not fully understood. It has been suggested that DSS forms nanolipocomplexes with medium chain fatty acids (MCFAs) in the colon to pass through epithelial cells and disrupt the barrier.^{213,214} DSS induces intestinal inflammation and damage along the large intestine, more centralized to the distal colon, leading to disruption in the barrier and interaction with gut microbiota and luminal elements with host tissue.²¹³ The exposure triggers a release of inflammatory cytokines like TNF-α, IL-1β, IL-6, IL-12 and IFN-γ.²¹⁵ Monitoring of animal weight, stool blood and diarrhea can track the progression of disease on a routine basis when compared to a non-treated control. Sex of mice used in a trial, among other factors such as age and specifics about the exact DSS compound used can be a key factor for onset of disease, while sex separation of mice during data analysis is essential since generally males develop severe disease more rapidly than females.²¹⁶ The simple nature of DSS administration and ability to control for acute and chronic severity is likely the reasons for its widespread usage.

Other chemical agents can induce intestinal inflammation similar to DSS. These include TNBS, oxazolone and azoxymethane (AOM). Through solubilizing in ethanol, TNBS causes colitis by altering host proteins in a way that creates self-antigens recognized

by the host immune system which follows a Th1 response.²⁰² It was first developed for use in rats but is now more widely used on mice and zebrafish.²⁰² In contrast to TNBS, oxazolone results in intestinal lesions that are associated with a Th2 immune response.²¹⁷ Likewise, it has been suggested that AOM induces intestinal inflammation by an upregulation of cyclooxygenases and overproduction of PGE2, as well as the induction of pro-mutagenic epithelial changes caused by O6-methylation of guanine during tumour formation.²¹⁸ AOM treatment is commonly used in conjunction with DSS to produce chronic intestinal inflammation that progresses towards what resembles colorectal cancer²¹⁸, though the combination also resembles intestinal lesions and cytokine responses similar to patients with UC.²⁰²

To no surprise, the microbiota of mouse models plays a substantial role in the progression of disease and the overall immune response. It is well known that microbiota of a standard mouse background from one facility can be different to the same mouse background in another facility.²¹³ In addition, depletion of the gut microbiota by broad spectrum antibiotics exacerbates DSS-induced colitis.²¹⁹ Interestingly, in the presence of ligands specific to TLR2 and TLR4, mice treated with antibiotics are protected against DSS-induced colitis.²¹⁹ Other compounds related to the recognition of gut microbiota by TLRs and NLRs such as poly (I:C), flagellin, CpG DNA and MDP offer protection against DSS-induced colitis when administered in a systemic manner.²¹³ Mice lacking any TLR signalling, such as Myd88-deficient mice exacerbated DSS-induced colitis for multiple reasons, including epithelial apoptosis and poor proliferation of epithelial cells lining the gut barrier.²¹⁹ Alternative to the baseline microflora that exists in mouse models, another microbiota-based mouse model used to study intestinal inflammation requires the infection of *Citrobacter rodentium*, a mechanism of infection related to that of enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *Escherichia coli* (EPEC).²²⁰

More recently, conditional KO mice have been implemented to understand how gene products from specific cellular sub-populations can influence pathogenesis. The most common methodology for conditional knockout creation is with the Cre-Lox system. Cre recombinase (Cre) is a protease that recognizes specific gene regions called loxP sites that flank the gene of interest. The gene for Cre is inserted into the mouse genome as a transgene regulated by a promotor of another gene that is cell type specific. During cell development, this cell-specific gene is expressed such that Cre can be transcribed within the specific cell sub-population.²²¹ In the case of IECs, the Vill promoter is often used to integrate upstream of the Cre gene. Researchers used this method to investigate the role of protein kinase A

(PKA) regulatory subunit *Prkar2a* and form a *Prkar2a*^{IEC-KO} mouse, which demonstrated protection from DSS-induced colitis due to changes in gut microbiota and increased interferon-stimulated gene (ISG) expression.²²² The IEC-KO of core 1 β 1,3-galactosyltransferase (*C1GalT1* ^{Δ IEC}) was shown to develop spontaneous colitis in the distal colon, leading to myeloid cell infiltration, crypt abscess, epithelial ulceration, goblet cell loss, disrupted mucus layer and increased epithelial-microbiota interactions.²²³ Deletion of *Lkb1* in IECs, a serine-threonine kinase involved in intestinal physiology and differentiation of epithelial secretory lineages, showcased greater susceptibility to DSS-induced colitis, which was ameliorated through exogenous IL-18 and restoration of antimicrobial peptide production.²²⁴ Similar results were seen in a p450 reductase conditional KO (IE-Cpr-null), where researchers observed more severe colitic inflammation and production of pro-inflammatory cytokines TNF- α , IL-6 and IL-1 β compared to WT mice.²²⁵ Another study used *Slc5a6*^{IEC-KO} mice to examine the role of the plasma membrane protein involved in biotin uptake in the intestine and showed structural abnormalities in KO intestinal tissue.²²⁶ Interestingly, researchers sought to understand the importance of A20, a target of NF- κ B, in intestinal inflammation since complete ablation of miR-21 lead to systemic inflammation of several organs.²²⁷ A20^{IEC-KO} showed increased susceptibility to colitis and isolated IECs were hypersensitive to TNF-induced apoptosis. Notably, 18 kinds of epithelial specific KO mice developed spontaneous colitis caused by impaired mucosal barriers, dysregulation of necroptosis/apoptosis, reduction in antibacterial peptides and an abnormal response to endoplasmic reticulum stress.²²⁸ These IEC KO models are becoming increasingly important to understand the specific role that epithelial cell populations play at the gut barrier that may be obscured by the presence of other cells types.

1.8 Objective and Aims

MiRNA regulation of intercellular junction complexes from a holistic perspective is an emerging field in IBD research. The associations of several pro-inflammatory miRNAs like miR-21 and their consequential role deserves further investigation, particularly the congruity of targeting on various intercellular junctions and adjacent cellular processes. Understanding whether there are impacts on miR-21 predicted gut barrier targets like intercellular junctions, coupled with the fact that miR-21 is an abundantly expressed miRNA in several mammalian cell types, will be extremely important in expanding the underlying mechanisms during inflammation. Thus, the pathophysiological role of miR-21 may be dependent on several factors during inflammation that will modulate available targets, leading to different responses within these contextualized cellular environments.

The overarching objective of this PhD thesis is to investigate the mechanisms by which miR-21 regulates gut barrier integrity and contributes to disease pathophysiology, with a specific focus on epithelial intercellular junctions and the consequence for development of inflammation. Furthermore, the importance of epithelial-specific expression of miR-21 at the gut barrier will be determined through creation of an IEC-specific miR-21 KO mouse. To that end, the project has two aims:

- **Aim 1:** Determine the influence of miR-21 on the gut barrier and susceptibility to intestinal inflammation.
- **Aim 2:** Resolve the importance of epithelial-expression of miR-21 for the development of intestinal inflammation.

Chapter 2

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Materials & Methods

2. Materials & Methods

2.1 Mice

For screening of miR-21 targets, 8-15 week old C57bl/6.NTac WT and miR-21^{-/-} mice (originally from Taconic Biosciences) were utilized. Mice were maintained in ventilated cages at 21 ± 1°C, humidity 50 ± 10% and with a 12 h-light/12 h-dark light cycle under specific pathogen-free (SPF) conditions, in line with Irish and European Union regulations. Food and water were available *ad libitum* throughout all experiments. At the end of trials, mice were sacrificed by CO₂ asphyxiation. Various parts of the intestinal tract (small and large intestine; proximal and distal) were removed, flushed with sterile phosphate buffer saline (PBS) solution (Fisher Scientific, Gibco, 10010023) and stored as 1 cm pieces at -80°C until further analyses. All experiments were subject to ethical approval by the Trinity College Dublin (TCD)'s Animal Research Ethics Committee (AREC) and Health Products Research Authority (HPRA) Ireland.

Two distinct groups of mice were used in these experiments. In the first group, non-littermate mice were used in which WT and miR-21^{-/-} litters were derived from genotypically separate parental breeding lineages. In the second group, littermate mice were used in which WT and miR-21^{-/-} litters were derived from the same heterozygous (HET) breeding pairs (miR-21^{+/-}). Details on the miR-21^{IEC-KO} mouse model will be discussed in Chapter 5.

2.2 DSS-induced colitis model

To evaluate the impact of colitis on miR-21 targets, non-littermate and littermate WT, heterozygous (HET) and miR-21^{-/-} mice were subjected to DSS-induced colitis as described in Iyer *et al.* (2022)²²⁹. Briefly, colitis was induced in 8-15 week old sex-matched mice by administration of 2.5% DSS (36–50 kDa, MP Biomedicals, 9011-18-1) *ad libitum* via drinking water for six days. For recovery trials, colitis was induced by administration of 2% DSS *ad libitum* via drinking water for four days, followed by five days of normal drinking water. Control mice in all trials received normal drinking water throughout. Mice were scored every day for disease progression by measuring percentage of their initial body weight, diarrhea and stool blood (via Hema-Screen™ Test; Immunostatics, HS-50) which were given a score from 0 to 4, as described in **Table 2.1**.

The sum of these three metrics provided the disease activity index (DAI) calculated every day for each mouse, and DAI was compared using area under the curve (AUC) analysis or DAI comparison on the final day of the trial. Water intake and food consumption per mouse were also weighed. At the end of each trial, mice were sacrificed by CO₂ asphyxiation. Intestinal tissues were removed, flushed with sterile PBS (Fisher Scientific, Gibco, 10010023) and 1 cm pieces of various parts of the colon (proximal and distal regions) were stored at -80°C until further analyses.

Table 2.1 – Scoring metrics for DSS-induced colitis progression in mice.

Score	Body Weight	Diarrhea	Stool Blood
0	No weight loss	Normal pellet	No blood
1	>0%, ≤5% weight loss	Soft pellet	Traces of blood in fecal occult test
2	>5%, ≤10% weight loss	Loose pellet	Strong positive fecal occult test
3	>10%, ≤15% weight loss	Shapeless pellet	Visible blood in stool
4	>15% weight loss	Liquid stool	Anal bleeding

2.3 Creation of an intestinal epithelial specific miR-21 conditional knockout mouse (miR-21^{IEC-KO})

To create an intestinal epithelial specific miR-21 conditional KO mouse (miR-21^{IEC-KO}), *in vitro* fertilization (IVF) with gametes of interest between the miR-21^{fl/fl} strain and the Villin-Cre strain was performed in collaboration with the Transgenics Facility lead by Professor Vincent Kelly, based at Trinity Biomedical Sciences Institute. Breeding stages were done in-house to remove unwanted alleles, with more details to be found in Chapter 5.

2.4 Genotyping of mice

Mice within the littermate miR-21^{-/-} and miR-21^{IEC-KO} mouse colonies were genotyped to separate mice into respective groups for DSS trials or further breeding stages. Two methods were used for gDNA extraction. In the first method, a crude gDNA extraction protocol was used for rapid and inexpensive breakdown of mouse ear tissue. Before weaning, mice were ear-clipped and tissue was placed into a 1.5 mL Eppendorf tube. Subsequently, 80 uL of 50 mM NaOH (Fisher Scientific) was added to each tube and tubes

were placed on a heat block at 95°C for 40 min. Tubes were vortexed every 10 min to ensure breakdown of tissue. After heat shock, 10 uL of 1 M Tris (pH 8; Fisher Scientific) was added to neutralize the sample, and tubes were vortexed for an additional 1 min. TaqPlus Master Mix (Vazyme, P211-03) was used for PCR amplification of the miR-21 locus within littermate mice using the following primers: F: 5'-CAGAGGACACTAGCAAGAAAGG-3', R: 5'-CTTTCTGGGCTCCTACTAGC-3'. A standard thermocycler was utilized with the following procedure: initial denaturation of 95°C for 3 min; followed by 30 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 3 min; and a final extension of 72°C for 5 min. In the second method, a clean gDNA extraction protocol was used to increase sample purity with REDExtract-N-Amp™ Tissue PCR Kit (Sigma, XNAT-100RXN). More details on the primers used for PCR amplification in the creation of the miR-21^{IEC-KO} strain can be found in Chapter 5. PCR amplicons were assessed via gel electrophoresis.

2.5 Intestinal permeability assay

Permeability of the gut mucosal barrier in mice following DSS-induced colitis was measured by determining the presence of fluorescein isothiocyanate (FITC)-dextran in blood at the end of the trial, measuring the mucosa-to-blood flux. On the final day of the trial, FITC-dextran (molecular mass 4 kDa, FD4; Sigma, 60842-46-8) was dissolved in sterile PBS (Fisher Scientific, Gibco, 10010023) and administered by oral gavage at 12 mg per mouse. After four hours, mice were euthanized with CO₂ and blood was collected by decapitation. Serum was separated by centrifuging blood samples at 4°C for 12 min at 1000 x g. FITC-dextran concentration was determined via a standard curve. Fluorescence was measured using a standard plate reader set to 485 nm (excitation) and 525 nm (emission).

2.6 Hematoxylin and eosin staining for histopathological scoring

Tissues were fixed in 4% formaldehyde (Fisher Scientific), dehydrated in ethanol and embedded in paraffin wax. Tissue sections (5 µm) were created using a Leica microtome and placed on polysine adhesion slides (Epremedia, Fisher Scientific, 12-545-78) to dry overnight. Histological analysis of mouse tissue was conducted via hematoxylin and eosin (H&E) staining, to visualize cell nuclei and cytoplasm/extracellular matrix, respectively. Slides were prepared using the Leica Autostainer with the following

procedure: submerged in xylene for 1 min, descending concentrations of ethanol for 1 min each, washed in water, hematoxylin for 5 min, eosin for 30 s, washed in water, and finally ascending concentrations of ethanol for 1 min each. Slides were air dried prior to the addition of cover slips, and slides were then imaged using an Olympus BX51 upright microscope. Histopathological blinded scoring of stained mouse intestinal tissue was done using the assessment scheme for tissue damage and cell infiltration described in **Table 2.2**, adapted from Wang *et al.* (2022)²³⁰.

Table 2.2 – Histopathological assessment scheme.

Score	Tissue Damage	Cell Infiltration
0	No mucosal damage	Few inflammatory cells in the lamina propria
1	Partial loss of crypts in some areas (less than 50%)	Increased cell infiltrate in the lamina propria, localized to the base of crypts
2	Partial to total loss of crypts in large areas; epithelium architecture still intact	Confluent cell infiltrate extending into the mucosa
3	Total loss of crypts in large areas; epithelium architecture lost	Transmural extension of cell infiltrate

2.7 Myeloperoxidase activity assay

Myeloperoxidase (MPO) is an enzyme found within lysosomal azurophilic granules of neutrophils and is often used as a biomarker for intestinal inflammation in mouse tissue.²³¹ Using mouse intestinal tissue, 1 cm pieces were homogenized in 100 µL of MPO Buffer I solution (100 mM sodium chloride [Fisher Scientific], 20 mM trisodium phosphate [Fisher Scientific], 15 mM disodium ethylenediaminetetraacetate [EDTA] dihydrate [pH 4.7; ThermoFisher]) with sterile metal ball bearings in a TissueLyser II (Qiagen) at 30 Hz for 1.5 min. Tubes were centrifuged at 4°C for 10 min at 10,000 x g. Pellets were resuspended in 1 mL of 0.2% sodium chloride solution and 1 mL of 1.6% sodium chloride / 5% glucose solution was added to the cell suspension. Tubes were further centrifuged at 4°C for 10 min at 10,000 x g. Pellets were resuspended in 100 µL of MPO Buffer II (50 mM trisodium phosphate [Fisher Scientific], 0.5% Hexadecyltrimethylammonium bromide [HTAB, pH 5.4; Sigma]). Samples underwent three cycles of freeze-thaw for complete cell lysis, and then tubes were centrifuged at 4°C for 15 min at 10,000 x g. Supernatants were transferred to Eppendorf tubes, and 30 µL of each sample was added to a 96-well plate in duplicate. Using OptEIA™ ELISA sets (BD Biosciences, 550534), 30 µL of substrate

reagent B was added to each well and after incubation at 37°C for 5 min, 30 µL of substrate reagent A was added to each well with a further incubation at 37°C for 5 min. Reactions were stopped by adding 30 µL of 1 M sulfuric acid (Fisher Scientific). Absorbance was measured using a standard plate reader set to 450 nm. Relative activity was measured by subtracting blank background absorbance and normalizing to mg of protein, with sample protein concentrations measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23225).

2.8 Enzyme-linked immunosorbent assay

Murine serum, protein lysates and stool samples were used to measure levels of pro-inflammatory and anti-inflammatory cytokines via enzyme-linked immunosorbent assays (ELISAs). At the end of disease trials, mice were sacrificed by CO₂ and blood was collected by decapitation in dark 1.5 mL Eppendorf tubes. Serum was prepared by spinning blood samples at 1000 x g for 12 min at 4°C and diluted 10-fold in PBS (Fisher Scientific, Gibco, 10010023) before use in ELISAs. Colon tissue was harvested and homogenized in radioimmunoprecipitation assay (RIPA) buffer (7.5 g/L Tris [Fisher Scientific], 10 g/L sodium chloride [Fisher Scientific], 1% NP-40 [Sigma], 0.25% sodium deoxycholate [Thermo Scientific], 1 mM EDTA [Thermo Scientific]; pH 7.4) supplemented with protease inhibitors (Roche, Sigma, 5892970001). Protein lysate concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, 23225) before use in ELISAs. Stool pellets were collected on the final day, and one pellet per mouse was homogenised in PBS using a pipette tip then diluted by 1000-fold prior to use in ELISAs. Markers included Lcn2 (R&D Systems, DY1857), IL-6 (Invitrogen, 88-7064-88), IL-1β (Invitrogen, 88-7013A-88), TNF-α (Invitrogen, 88-7324-88), IL-10 (Invitrogen, 88-7105-88) and TGF-β (ThermoFisher, 88-8350-22), and procedure was carried out as per manufacturer instructions. Briefly, coated Corning Costar 9018 ELISA plate with 100 uL/well of capture antibody (in coating buffer) overnight at 4°C. Sealed plate and incubated overnight at 4°C. Wells were washed and subsequently blocked with diluent for 1 h at room temperature. Incubated on a rocker at room temperature for 1 h. Samples were placed into plate with known standards and incubated overnight at 4°C. On the final day, wells were washed and incubated in Detection Antibody for 1 h at room temperature. This was followed by another wash and incubation with Avidin-HRP enzyme for 30 mins at room temperature. After washing, TMB solution was added to the wells and incubated for 15

mins at room temperature. Reaction was stopped with 1 M sulfuric acid (Fisher Scientific). Absorbance was read at 570 nm and 450 nm. For TGF- β ELISAs, media supernatants or cell lysates were acidified with 1 N HCl (Sigma) for 10 min then neutralised with 1 N NaOH (Fluka) to activate latent form TGF- β .

2.9 Primary intestinal epithelial cell isolation

Primary IECs were isolated from mouse colon tissues to determine the impact of miR-21 on this significant cell population. After removal of 2-3 cm of proximal and distal colon tissue for molecular analyses, the remaining colon was flushed with cold, sterile PBS (Fisher Scientific, Gibco, 10010023) and reversed inside-out with tweezers. Reversed colons were washed gently in PBS and placed into falcon tubes containing 3 mL of cell recovery solution (Corning) supplemented with protease inhibitors (Roche, Sigma, 5892970001). Tubes were placed horizontally and incubated at 4°C overnight. On the following day, tubes were mixed by inversion, and contents were placed overtop a nylon filter covering 50 mL falcon tubes. Samples were filtered by centrifuging tubes at 4°C for 10 min at 800 x g. Pellets were gently resuspended in 1 mL of PBS supplemented with protease inhibitors and used for further molecular analyses, along with remainder tissue lysates.

For *ex vivo* growth, primary colon IECs were isolated as described by Psichas *et al.* (2017)²³². Briefly, reversed colons were wash with sterile PBS and subsequently underwent multiple rounds of digestion in sterile collagenase solution (0.3 mg/mL collagenase XI [ThermoFisher, 17101015] in complete Dulbecco's Modified Eagle Medium [DMEM; Gibco, Fisher Scientific, 11965092]) incubated at 37°C for 10 min with vigorous shaking. Digested suspensions were combined and centrifuged for 3 min at 100 x g. Pellets were resuspended in pre-warmed complete DMEM supplemented with 10% foetal calf serum (FCS; Gibco, Sigma, F4135) and 10 μ M Y-27632 dihydrochloride (Sigma, Y0503) to prevent anoikis, then filtered through a 100 μ m filter (Fisher Scientific) to remove any undigested tissue. Tissue culture plates were prepared with Matrigel® basement membrane matrix (Corning, 354234), and suspension was plated overtop basement membrane. Plates were incubated at for at 37°C with 5% CO₂ for seven days prior to additional analyses.

2.10 Cell viability

Cell viability of isolated primary IECs was assessed in order to ensure *ex vivo* growth was successful for further molecular analyses. Briefly, using 96-well plates, negative control wells were treated with 1% Triton X-100 (Sigma) at 37°C for 2 h. Subsequently, 10X alamarBlue solution (Invitrogen, A50100) was added directly to sample wells and incubated at 37°C in darkness for 4 h. Absorbance was read at 570 nm and 600 nm. Cell viability differences were calculated using the following formula: $\frac{((O2 \times A1) - (O1 \times A2))}{((O2 \times P1) - (O1 \times P2))} \times 100$. O1 and O2 are the molar extinction coefficient of oxidized alamarBlue at 570 nm and 600 nm, respectively; A1 and A2 are the absorbances of the test well at 570 nm and 600 nm, respectively; and P1 and P2 were the average absorbances of basal or TNF- α (Peprotech, Fisher Scientific, 30001A50UG) treated WT primary IECs at 570 nm and 600 nm, respectively.

2.11 mRNA isolation and SYBR qRT-PCR gene expression analysis

Using mouse intestinal tissue, 1 cm pieces were homogenized and cell cultures were lysed in RNA lysis buffer, as described per PureLink RNA Mini Kit (Invitrogen, 12183018A) manufacturer's instructions. RNA yield and quality was quantified by nanodrop. Isolated RNA was normalized to the lowest concentrated sample and treated with DNase I (ThermoFisher, EN0521), and cDNA was prepared for qRT-PCR using the High Capacity cDNA Synthesis Kit (Applied Biosystems, 4374966) in a standard thermocycler. Expression of gene targets was analyzed using Power SYBR green reagents (Applied Biosystems, 4367659) relative to *GAPDH*. qRT-PCR was conducted on the Applied Biosystems StepOnePlus Real-Time PCR System using the standard two-step annealing procedure: 95°C for 2 min followed by 40 cycles of 95°C for 3 s, and 60°C for 30 s. Cycle threshold (CT) values were obtained and used for analysis. Fold changes were calculated using the $\Delta\Delta CT$ method. Primers (IDT) used for mouse (**Table 2.3**) and human cell lines (**Table 2.4**) samples are detailed below.

Table 2.3 – Primers used for SYBR qRT-PCR of mouse samples.

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
<i>Gapdh</i>	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT
<i>Pten</i>	AGGCACAAGAGGCCCTAGAT	AGTGCCACGGGTCTGTAATC

<i>Cdh1</i>	CCCAGAGACTGGTGCCATTT	TGGCAATGGGTGAACCATCA
<i>Ctnna1</i>	CCGAGGCAAAGGTCCACTCA	CCGAGTCTGGGCAATGGTCT
<i>Ctnnd1</i>	GCTGCCAGTTGCTTTGGAGC	AGTTCATAGCCTCGAGCAGGAC
<i>Ctnnb1</i>	AACCTTTCTCACCACCGCGA	TCCACTGGTGACCCAAGCAT
<i>Pdcd4</i>	AACCTATGATGACGACCAGGAGAAC	GCTAAGGACACTGCCAACACC
<i>Gjal</i>	ACAGGTCTGAGAGCCCGAAC	TGTCTGGGCACCTCTCTTTCA
<i>Gjb1</i>	ACACGCCTGCATACATTCCC	CATCCTGCCTCATTCACACCTCC
<i>Gjb2</i>	CCGCTTCAGACCTGCTCCTT	CCTCTGGATGGTTGGCACTGT
<i>Gjc1</i>	TCTGGTGAACAGGGCAAACC	ACAATCAGCACAGTGAGCCAGA
<i>Ocln</i>	TCCGTGAGGCCTTTTGAA	GGTGCATAATGATTGGGTTTG
<i>Mki67</i>	GTCGCTTTGGACAGTGAACC	ACTTCTTGGTGCATACAATGTCTT
<i>F11r</i>	TCCCGAGAACGAGTCCATCA	GTGCTGCCTTGGACGAACTT
<i>Tgfbr1</i>	AGGGTCTGGATCAGGTTTACCA	GCCTCGCCAAACTTCTCCAA
<i>Tgfbr2</i>	AGATGCCGCTTCTCCCAAGT	CTGCTGGTGGTGTATTCTTCCG
<i>Tgfbr3</i>	CCAGCCGCAGAGAGAGGTTA	ACACCAGCAGCCAGTCTCTC
<i>Smad7</i>	TTTCTCAAACCAACTGCAGGCT	AGTATGCCACCACGCACCA
<i>Tjp1</i>	CAAAGCCCACCAAGGTCA	TCTCTTTCCGAGGCATTA
<i>Cldn1</i>	ATGACCCCTTGACCCCCATC	GGAGCAGGAAAGTAGGACACC
<i>Cldn2</i>	CCTGGGATTGTGCTTGAGGT	TGACCCCCATCTACCACAGA
<i>Cldn3</i>	CCGCAAGGACTACGTCTGAG	CAAGCAGACTGTGTGTCGTCT
<i>Cldn4</i>	GTAGAGTGGATGGACGGGTT	CATTAGCAAGACAGTGCGGA
<i>Cldn7</i>	TTTTTCATTGTGGCAGGTCTTGCT	CCCTGCCCAGCCGATAAAGAT
<i>Cldn15</i>	GGACCCTCCACATACTTGCT	GCACTCCAGCCCAAGTAGAG
<i>Tjp2</i>	GCACCCTGACATCTATGCG	CACTGCCGTAGCTTCTCTCTG
<i>Tjp3</i>	GCCATCGCTGAACCAGAGTC	CAGCCGCAAGCCAATACTCG
<i>Dsc2</i>	GCCCCGACTTGGTGATAAGGT	AGCCGAGCCTTTTCCCTCAT
<i>Dsg2</i>	CAGGAACCATCGCTGCCAAC	AGGAGGCCGCTTTCTCTGTG
<i>Vil1</i>	CCCACCTTCACAGGCTGGTT	AACGTCCACTTTCGGGCTCA
<i>Arf4</i>	ACCAGACCCCCTCCTTTTGAC	CCAGCAGCATCCAATCCAACC
<i>Gbfl</i>	ACAGGTCAGTCAGGACTTGCTA	GGTGCGTGAGTCAGCTTCAA

Table 2.4 – Primers used for SYBR qRT-PCR of human cell line samples.

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
<i>GAPDH</i>	TCGACAGTCAGCCGCATCTT	GCCCAATACGACCAAATCCGT
<i>CDH1</i>	CTGGTGGTTCAAGCTGCTGAC	CACCTGACCCTTGACGTGGT
<i>PTEN</i>	GTGGCGGAACTTGCAATCCT	CCGTCGTGTGGGTCCTGAAT
<i>MARCKS</i>	TGCCCAGTTCTCCAAGACCG	GTGGCCATTCTCCTGTCCGTT
<i>F11R</i>	GGGCTTTTCTTCTCCCCGTG	CTTGGATGGAGGCACTGTGAT

<i>TGFB</i>	GTTGTGCGGCAGTGGTTGAG	GCCGGTAGTGAACCCGTTGAT
<i>OCN</i>	TCAGGGAATATCCACCTATCACTTCAG	CATCAGCAGCAGCCATGTACTCTTCAC
<i>CLDN2</i>	AAGACCATAGCACCAAGCGA	AGCAGTGCTATCACTCAG
<i>CLDN3</i>	ATCCAGCGTGCAGCCTT	TCACAGTCCATGCAGGTTGG
<i>CLDN4</i>	TCTCCTCTGTTCCGGGTAGG	CGTCCATCCACTCTGCACTT
<i>CLDN7</i>	GCATAATTTTCATCGTGGCAGGTCT	AGATGGCAGGGCCAAACTCA
<i>CLDN15</i>	CTCTCCAGGAAAGCCAAGCTG	TCGTACTTGGTTCCGGGGTA
<i>ARF4</i>	TCTGCCCATCACAAGTGCCA	CAGCAGCATCCAATCCAACCAT
<i>GBF1</i>	TAGGCCCCGCCATTTTGAT	TCCC GTTCTTCATCCAGTGGT

2.12 microRNA gene expression analysis

Using mouse intestinal tissue (1 cm) or Caco-2 cells, lysis was performed in RNA lysis buffer, as described previously per PureLink RNA Mini Kit (Invitrogen, 12183018A) manufacturer's instructions with a few modifications that were validated in previous studies.⁸⁸ Briefly, 100% ethanol was used to precipitate RNA after lysis and small size RNA molecules were retained by excluding Wash Buffer I (used for removal of organic contaminants). RNA yield and quality was quantified by nanodrop. Isolated RNA was normalized to 10 ng/uL, and cDNA was prepared for qRT-PCR using the High Capacity cDNA Synthesis Kit (Applied Biosystems, 4374966) using pri-miR-21, miR-21-5p and miR-21-3p TaqMan probes (Applied Biosystems) in a standard thermocycler. Expression of gene targets was analysed using TaqMan reagents (Invitrogen, N8080234) and represented as fold change in expression relative to snRNA U6. qRT-PCR was conducted on the Applied Biosystems StepOnePlus Real-Time PCR System using the standard two-step annealing procedure: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. CT values were obtained and used for analysis. Fold changes were calculated using the $\Delta\Delta CT$ method.

2.13 Protein lysates and immunoblotting

Using mouse intestinal tissue (1 cm) or Caco-2 cells, lysis was performed in RIPA buffer (7.5 g/L Tris (Fisher Scientific), 10 g/L sodium chloride [Fisher Scientific], 1% NP-40 [Sigma], 0.25% sodium deoxycholate [ThermoFisher], 1 mM EDTA [ThermoFisher]; pH 7.4) supplemented with protease inhibitors (Roche, Sigma, 5892970001). Protein concentrations were measured using the Pierce™ BCA Protein Assay Kit (Thermo

Scientific, 23225) and normalized to the lowest concentrated sample in Laemmli loading dye (50 mM Tris [pH 6.8; Fisher Scientific], 2% sodium dodecyl sulfate [SDS; Sigma], 0.1% bromophenol blue [Fisher Scientific], 10% glycerol [Sigma], 100 mM dithiothreitol [Sigma]). Samples were boiled for 5 min at 95°C, and protein lysates were separated by SDS-PAGE on 10% acrylamide (ProtoGel, National Diagnostics, EC-890) gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, IEVH85R). Total protein within lanes was visualized using 0.1% (w/v) Ponceau stain solution (ThermoFisher, A40000279). Blocking was performed with 5% (w/v) bovine serum albumin (BSA; Sigma, A4737) dissolved in Tris buffer saline (TBS; 7.5 g/L Tris [Fisher Scientific], 10 g/L sodium chloride [Fisher Scientific]) with 0.1% Tween20 (Acros) for 1 h. Following this, membranes were incubated overnight at 4°C with E-cadherin (Proteintech, 20874-1-AP), α -catenin (ThermoFisher, 139700), p120-catenin (Proteintech, 66208-1-Ig), β -catenin (Proteintech, 66379-1-Ig), occludin (Proteintech, 13409-1-AP), claudin-4 (Proteintech; 16195-1-AP) or Arf4 (Proteintech, 68204-1-Ig) primary antibody in blocking buffer. On the next day, membranes were washed with TBS-Tween (TBST) three times and incubated with anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology) for 1 h. Membranes were subsequently washed in TBST and were visualised using ECL horseradish peroxidase (HRP) substrate (ThermoFisher), the GelAS 4000 (Agilent) and ImageLab software (BioRad). For loading control, β -actin antibody (1:10000 dilution, Clone AC-74; Sigma) was used.

2.14 Immunofluorescence

Protein localization and abundance was determined using mouse intestinal tissue or Caco-2 monolayers seeded onto transwell plates (Costar, Corning) at a density of 3×10^4 cells/cm² for 21 days. For miR-21 modulation assays, Caco-2 monolayers seeded onto transwell plates (Costar, Corning) at a density of 5×10^5 cells/cm² for 1-2 days due to transfection protocols. Cells were subsequently starved in serum-free, antibiotic (ABX)-free DMEM for 24 h, and transwell membranes were cut out of holders and washed in TBS supplemented with 1 mM calcium chloride (Sigma) and 0.5 mM magnesium chloride (Sigma).

Tissues were fixed in 4% formaldehyde (Fisher Scientific), dehydrated in ethanol and embedded in paraffin wax. Tissue sections (10 μ m) were created using a Leica microtome and placed on polylysine adhesion slides (Epredia) to dry overnight. Slides were

deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol baths. An antigen retrieval step was performed by incubating slides in Tris-EDTA (pH 9.0; Fisher Scientific) just before boiling for 35 min. Slides were washed in TBST supplemented with 1 mM calcium chloride and 0.5 mM magnesium chloride and blocked in 1% BSA-TBST for 1 h. Following this, slides were incubated overnight at 4°C with E-cadherin (Proteintech, 20874-1-AP) or claudin-4 (Proteintech, 16195-1-AP) primary antibody in blocking buffer. On the next day, slides were washed with TBST three times and incubated with AlexaFluor™ secondary antibody (ThermoFisher) for 1 h. Slides were subsequently washed in TBST and then coverslips were added with the addition of a 1:1 mixture of Fluoroshield with DAPI (Sigma) and Mowiol (Sigma) mounting media. Tissue sections were visualized using the LSM 900 Confocal Laser Scanning Microscope (Zeiss) system and ZEN software (Zeiss). Images were analysed using ImageJ software and fluorescence was normalised to the number of nuclei per field of view.

Cell monolayers were fixed in 4% formaldehyde, washed in TBS, blocked in 1% BSA-TBST for 30 min, and subsequently incubated overnight at 4°C with E-cadherin (Proteintech, 20874-1-AP) primary antibody in a humidity chamber. On the next day, cells were washed three times in TBS supplemented with 1 mM calcium chloride and 0.5 mM magnesium chloride and further incubated with AlexaFluor™ secondary antibody (ThermoFisher) for 2 h in the dark. Membranes were placed onto microscope slides using 1:1 mixture of Fluoroshield with DAPI (Sigma) and Mowiol (Sigma) mounting media. Cells were visualised using the LSM 900 confocal microscope (Zeiss) and ZEN software (Zeiss). Images were analysed using ImageJ software and fluorescence was normalized to the number of nuclei per field of view.

For colocalization, GLG1 (R&D Systems, AF7879) and GBF1 (ThermoFisher, PA5-109757) primary antibodies were used. Merged z-stacks of each channel underwent deconvolution to reduce signal aberrations using the ImageJ plugins PSF Generator (with Born & Wolf 3D optical model) and DeconvolutionLab2 (with Bounded-Variable Least Squares model) as described in Sage *et al.* (2017)²³³. Colocalization analyses on deconvoluted z-stacks was performed through the identification of local maxima (ie. objects) using ImageJ plugin DiAna (with spot segmentation) as described in Gilles *et al.* (2016)²³⁴. Colocalized objects were normalized to the number of nuclei per field of view.

All images shown are a representation of one large field of view taken of at least three biological replicates.

2.15 Caco-2 cell culture, treatments and transfections

The human adenocarcinoma cell line Caco-2 (originally from the ATCC Cell Biology collection) was used to model the intestinal epithelium. Cells were grown at 37°C, 5% CO₂ and 95% relative humidity in complete DMEM (Gibco, 11965092) supplemented with 10% FCS (Gibco, Sigma, F4135) and 1% PS (Sigma, P4333).

Caco-2 cells were seeded onto tissue culture plates (Costar, Corning) at a density of 2.5×10^5 cells/cm² (undifferentiated, 5-day growth) or 3×10^4 cells/cm² (differentiated, 21-day growth). To induce the expression of miR-21, cells were starved in serum-free, ABX-free DMEM for 24 h and subsequently treated with 150 ng/mL TNF- α (Peprotech, Fisher Scientific, 30001A50UG) for an additional 24 h (RNA) or 48 h (protein).

Transfections with miR-21 inhibitor and pCMV-miR-21 overexpression plasmid were conducted in Caco-2 cell monolayers. Caco-2 cells were seeded onto 24-well plates at a density of 5×10^4 cells/cm² until a confluency of approximately 60-80% was reached. Cells were transfected with 50 nM miR-21 inhibitor (ThermoFisher, AM12979), along with respective negative controls for 24 h using RNAiMAX lipofectamine reagent (Invitrogen, 13778150). For plasmids, cells were transfected with 500 ng per well of pCMV-miR-21 (borrowed from the Sheedy group, TCD), along with respective empty vector for 24 h using lipofectamine reagent (Invitrogen, 18324012). For double transfections, inhibitor compounds were transfected first and incubated for 24 h, followed by pCMV vectors with an additional 24 h incubation. For TNF- α -induced inflammation assays, growth media was subsequently replaced with serum-free, ABX-free DMEM and TNF- α was added to designated wells for an additional 24-72 h depending on the assay.

2.16 Creation of a luciferase-reporter system for miR-21 regulation of E-cadherin

The 3'-UTR with the predicted seed region of miR-21 for *CDH1*, encoding E-cadherin, was amplified using the following primers: F: 5'-CAGGAGCTCGAAACCAATAAAATTCCCAT-3', R: 5'-CAGCTCGAGGATGAAAAAGTTCTAACACC-3'. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, 28104) and cloned into the pmirGLO Dual-Luciferase miRNA Target Vector (Promega, E1330) for subsequent luminescence assays. Plasmid sequence was partially sequenced via sanger sequencing (Eurofins).

For luciferase assays, transfections with a derivative of the human embryonic kidney cell line HEK293T (originally from the ATCC Cell Biology collection) was used due to their high transfection efficiency²³⁵ and grown in similar conditions to Caco-2 cells. Cells were seeded onto 96-well plates (Costar, Corning) at a density of 5×10^5 cells/cm². After 24 h, transfections with pCMV-miR-21 overexpression plasmid were conducted. Cells were transfected with 500 ng per well of pCMV-miR-21 for 24 h using lipofectamine reagent (Invitrogen, 18324012), with a subsequent treatment of 100 ng/mL LPS (Enzo Life Sciences, NC9241866) for 24 h. On the day of measurement, cells were washed in PBS (Gibco, 10010023) and lysed in 1X Passive Lysis Buffer from the Agilent Luciferase Assay Kit (Agilent, 219020). Lysates were split into two separate 96-well white polystyrene plates (Corning). To assay for Firefly luciferase activity, 40 μ L of luciferase assay buffer (Agilent, 219020) was added to each well of one plate and luminescence was measured via a luminometer. To assay for Renilla luciferase activity for transfection normalization, 40 μ L of coelenterazine (Sigma, 233900) was added to each well of the second plate instead.

2.17 Scratch test on Caco-2 monolayers

To assess the impact of miR-21 during wound healing *in vitro*, Caco-2 monolayers were seeded onto 96-well plates (Costar, Corning) at a density of 5×10^5 cells/cm² until cultures reached a confluency of 90-100%. Once reached, cells were subsequently starved in serum/ABX-free DMEM (Gibco, 11965092) for 24 h. Following this, 24 h transfections with miR-21 inhibitor and pCMV compounds were conducted in Caco-2 cell monolayers, after which cells were treated with 150 ng/mL of TNF- α (Peprotech, 30001A50UG) for an additional 24 h. On the day of wound creation, media was aspirated and replaced with PBS (Gibco, 10010023). Using the IncuCyte® Woundmaker pin system (Sartorius), scratches were produced in each well and were washed before replenishing with serum/ABX-free DMEM. Plates were incubated at 37°C in the Incucyte® SX5 Live-Cell Analysis System (Sartorius) and pictures were taken every 24 h to monitor wound closure.

2.18 Statistical analysis

Welch's t-tests were performed on all analyses comparing two independent groups. In comparisons with multiple independent groups, one-way or two-way ANOVA with Tukey test was used. Tests and the standard error of the mean (SEM) were calculated for experimental replicates using the GraphPad Prism 10 software (GraphPad Software Inc.).

Chapter 3
-
miR-21,
Adherens Junctions
& Intestinal
Wound Healing

3. miR-21 regulates epithelial adherens junctions and intestinal wound healing processes during inflammation

3.1 Introduction

One of the primary consequences of IBD is the dysfunction of the gut barrier, which maintains selective permeability of nutrients, water and electrolytes across the intestinal epithelium. As inflammation manifests, the mucus layer and epithelial barrier stop preventing the leakage of luminal contents that consequently lead to interaction with the host immune system. Luminal contents may include gut microbiota that possess PAMPs or DAMPs from host cells, which both contribute to inflammation.²³⁶ These molecules are recognized by IECs through TLRs, NLRs, and inflammasomes, where complete removal or loss of function polymorphisms in these elements have been implicated in intestinal inflammation.²³⁶

The breakdown of this barrier from the perspective of IECs leading to a leaky phenotype was of particular interest in this project. Studying aberrations in IEC function and cell adhesion at the gut barrier can build understanding on when normal dietary or microbial tolerance is disturbed, such as during active IBD. IECs are tightly connected through intercellular junctions; large protein complexes that have various roles in IEC function. These include TJs that control paracellular transport across the gut barrier, AJs that stabilize cell-cell adhesion, desmosomes and hemidesmosomes that provide strong anchorage and resistance to shearing forces, and GJs that serve as communication channels between adjacent cells. Importantly, gut epithelia are renewed every 4-5 days²¹, demonstrating that IECs are continuously shed and replaced to maintain barrier homeostasis, which require strict regulation of intercellular junctions. However, under intestinal injury that harms barrier integrity, gut epithelia undergo a cellular process of restitution, proliferation and differentiation known as wound healing, and this requires a large reorganization of intercellular junctions.³¹

Of particular interest in this chapter are AJs that play a crucial role in gut barrier integrity. E-cadherin, the most prominent AJ protein, has demonstrated a variety of different functions in intestinal tissue, and its decline or dysfunction has long been associated with IBD pathology.¹³¹ Several gastrointestinal pathogens such as *Helicobacter pylori*, *Campylobacter jejuni*, *Enterococcus faecalis* were shown to cleave E-cadherin during barrier dysfunction, causing AJ collapse.²³⁷ E-cadherin has also been implicated in

wound healing pathways and must be tightly regulated during the wound healing process. One study found that E-cadherin was essential during the restitution phase of wound healing for large epithelial sheet migration at the leading edge, and non-essential for isolated epithelial cell migration.²³⁸ A separate study concluded with similar findings, suggesting a CXCL12-dependent regulation mechanism for membrane localization of E-cadherin which decreases paracellular space between migrating IECs.²³⁹ Moreover, during skin wound repair, E-cadherin was significantly reduced in chronic wound fronts and was suggested as an intervention target to support chronic wound healing in skin disease.²⁴⁰ Considering the importance of E-cadherin and AJs during chronic intestinal inflammation, this chapter assessed their contribution to the development of colitis in relation to miR-21 expression.

The aim of this chapter was to elucidate the role of AJs at the gut barrier under regulation of miR-21 and highlight novel mechanisms these junctions have during epithelial wound healing. Using an *in vitro* epithelial cell model in which miR-21 expression was modulated, and furthermore a mouse model of DSS-induced colitis using WT and miR-21^{-/-} mice, this chapter investigated the impact of miR-21 expression on AJ regulation. The impact of miR-21 overexpression or inhibition on E-cadherin and intestinal wound healing was also assessed. Within this study, it was observed that at both the mRNA and protein level, AJ components E-cadherin, α -catenin, p120-Catenin and β -catenin were differentially expressed in the absence of miR-21 *in vivo*, and that overexpressing miR-21 *in vitro* caused a partial decrease in E-cadherin levels around cell peripheries. Further, it was observed that modulating miR-21 levels through overexpression or inhibition under TNF- α -induced inflammation showed greater wound healing capability in Caco-2 cells, demonstrating the novel tuning capacity of miR-21 in the wound healing process.

3.2 Results

3.2.1 miR-21 ablation increases gene expression of adherens junctions in mice under healthy conditions and DSS-induced colitis

To investigate the impact of miR-21 on the epithelial barrier, a preliminary search for predicted miR-21 targets in human intercellular junctions genes known to be important for maintenance of gut barrier homeostasis was performed using various miRNA prediction platforms: TargetScan³⁹, miRDB²⁴¹, miRWalk²⁴², DIANA-TarBase V9.0²⁴³ and Pictar²⁴⁴. Based on these tools, several known and predicted miR-21-5p and miR-21-3p targets were identified for AJs and GJs (**Table 3.1**).

Table 3.1 – miR-21 predicted targets related to adherens and gap junctions.

Type	Common Name	Gene Name	TargetScan (score*)	miRDB (score*)	miRWalk (score*)	DIANA-TarBasev9.0 (score*)	Pictar (score*)
Known Target	PDCD4**	<i>PDCD4</i>	5p (0.50)	5p (0.92)	3p (0.92)	5p (0.48)	Non-specific (0.16)
Adherens Junctions	E-cadherin	<i>CDH1</i>	3p (0.21)		3p (0.85)	5p (0.43)	
	α -catenin	<i>CTNNA1</i>			3p (0.92)	5p (0.49)	
	β -catenin	<i>CTNNB1</i>	3p (0.21)	3p (0.05)	5p (0.85)	5p (0.16), 3p (1.00)	
	p120-catenin	<i>CTNND1</i>			5p (1.00)	5p (0.40), 3p (0.43)	
Gap Junctions	Connexin-43	<i>GJA1</i>	3p (0.83)	3p (0.68)	3p (0.92)	5p (1.00), 3p (0.83)	
	Connexin-32	<i>GJB1</i>					
	Connexin-26	<i>GJB2</i>			5p (0.85)		
	Connexin-45	<i>GJC1</i>	3p (0.04)		3p (0.85)	3p (0.47)	

* - all scores are out of 1; note that the score from miRDB was divided by 100 and the score from Pictar was divided by 10 to equate to scores from other databases.

** - known direct target of miR-21; known negative association with miR-21 levels.

An initial screen was conducted using mouse homologs from the selection of these potential miR-21 targets in intestinal samples from non-littermate WT and miR-21^{-/-} male mice. As a preliminary step, miR-21 levels were measured in different healthy intestinal regions of WT mice to associate with potential changes in junction genes within the screen. These were categorized as distal colon / large intestine (LI_d), proximal colon / large intestine (LI_p), ileum / distal small intestine (SI_d) and duodenum-jejunum / proximal small intestine (SI_p). As seen in **Figure 3.1**, there were no significant differences in mature miR-21-5p levels among the various intestinal regions.

Subsequent RNA analyses used a known target of miR-21 *Pten*, important for cell growth, proliferation, survival, DNA repair and cell motility²⁴⁵, which demonstrated an increased trend in miR-21^{-/-} proximal tissue compared to WT, but not in distal tissue (**Figure 3.2A**). Among the AJ genes tested, there was increased expression in *Cdh1* and *Ctnd1* in miR-21^{-/-} proximal tissue compared to WT, but no major differences in *Cttna1* in any intestinal regions (**Figure 3.2B, 3.2C, 3.2D**). Interestingly, *Cttnb1* had a reduced trend in miR-21^{-/-} proximal colon tissue compared to WT mice despite being listed in the majority of prediction databases searched (**Figure 3.2E**).

Similar predicted miR-21 gene targets were evaluated in whole colon samples of non-littermate WT and miR-21^{-/-} female mice. Here, another known target of miR-21 *Pdcd4*, important for suppressing pro-inflammatory pathways through downregulating cytokine signalling, mTOR-mediated protein translation and inducing apoptosis²⁴⁶, was used and showed a similar induced trend within miR-21^{-/-} compared to WT mice (**Figure 3.3A**). For AJ genes, both *Cdh1* and *Cttna1* were significantly upregulated in miR-21^{-/-} colon tissue compared to WT, with no difference observed in *Cttnb1* (**Figure 3.3B, 3.3C, 3.3D**). GJ genes, showed no major changes in gene expression between groups (**Figure 3.3E, 3.3F, 3.3G, 3.3H**), suggesting specific influence of miR-21 on the structural integrity of the gut epithelium. However, *Gjal* did show a mild induction, which has a role in mucosal barrier homeostasis and GI motility.²⁴⁷

It is currently unknown to what extent miR-21 influences intercellular junction complexes during active inflammation. Since previous studies have demonstrated that miR-21 deletion protects against DSS-induced colitis^{85,86}, a separate screen for similar homologs in whole colon samples from DSS-treated WT and miR-21^{-/-} female mice was examined for the expression of similar junction genes. In this analysis, known miR-21 target *Pdcd4* lost the induction seen in healthy conditions (**Figure 3.4A**), and interestingly DSS treatment reduced expression of almost all AJ genes tested in both WT and miR-21^{-/-} colon

tissue when compared with expression in healthy groups, losing the previously seen induction phenotype of healthy groups (**Figure 3.4B, 3.4C, 3.4D**). This loss of induction in AJ genes may be due to the highly inflamed state of the tissue, which masked any differences in expression due to miR-21 regulation alone. There was one exception, the AJ gene *Cdh1*, which retained a trend of induced expression in miR-21^{-/-} colon samples compared to WT. GJ genes demonstrated no notable differences between WT and miR-21^{-/-} mice (**Figure 3.4E, 3.4F, 3.4G, 3.4H**), though there was a reduced trend for *Gjc1*, which is involved in GI motility and found differentially expressed in colorectal carcinomas.²⁴⁸

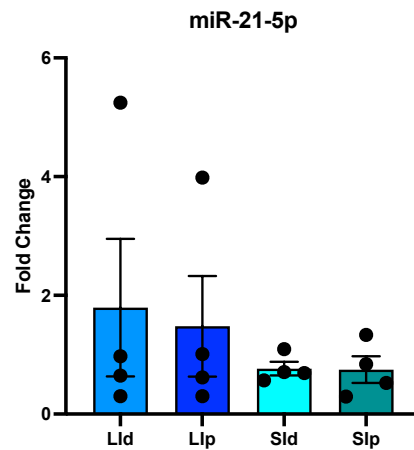


Figure 3.1 – No difference in expression of mature miR-21-5p in mouse intestinal regions under healthy conditions. RNA was isolated from healthy 10-week old WT male mice. qRT-PCR analyses of miR-21-5p in distal colon / large intestine (LId), proximal colon / large intestine (LIp), ileum / distal small intestine (SId) and duodenum-jejunum / proximal small intestine (SIp) tissue of WT mice. Denoted are $n = 4$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

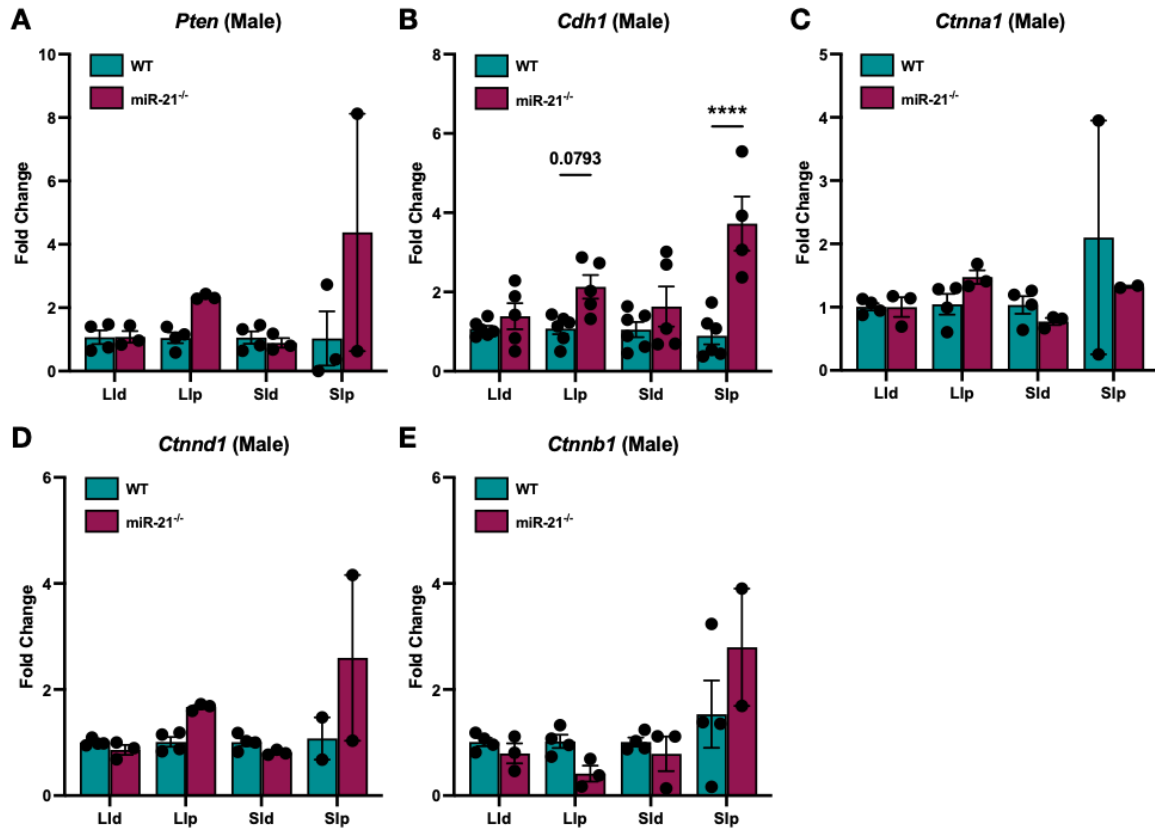


Figure 3.2 – Analysis of adherens junction gene expression under healthy conditions shows some junction genes are tied to miR-21 regulation in male mouse proximal tissue. RNA was isolated from healthy 10-week old WT and miR-21^{-/-} male mice. qRT-PCR analyses in distal colon / large intestine (LId), proximal colon / large intestine (LIp), ileum / distal small intestine (SIId) and duodenum-jejunum / proximal small intestine (SIp) tissue of WT and miR-21^{-/-} male mice. (A) Known target of miR-21: *Pten*. (B-E) Adherens junctions (AJs): cadherin (*Cdh*), catenin (*Ctnn*) alpha, delta, beta. Denoted are $n \geq 3$ mice per group excluding SIp groups specifically with $n \geq 2$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with **** - $p \leq 0.0001$.

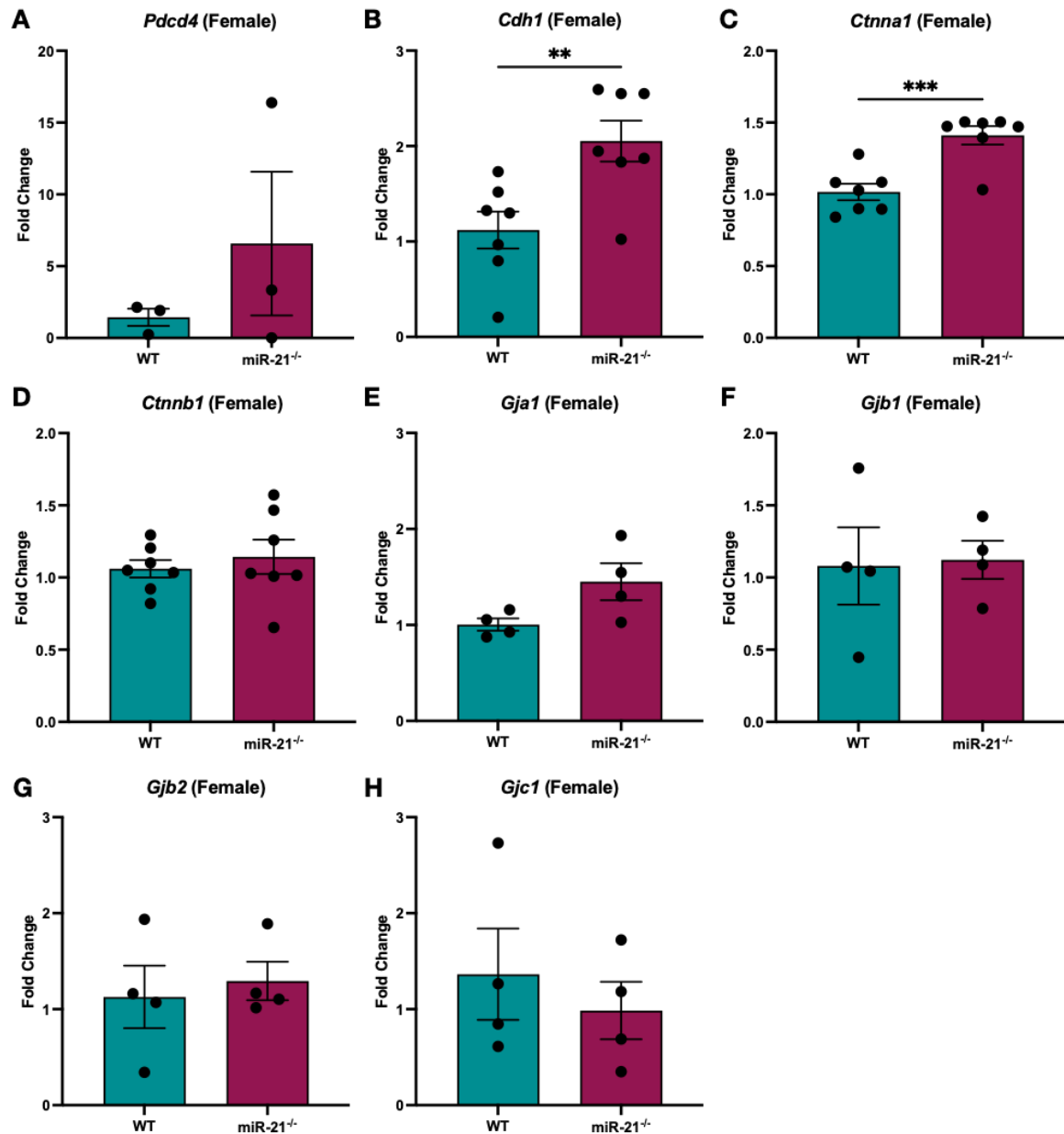


Figure 3.3 – Analysis of adherens junction gene expression under healthy conditions shows some junction genes remain tied to miR-21 regulation in female mouse whole colon tissue. RNA was isolated from healthy 10-week old WT and miR-21^{-/-} female mice. qRT-PCR analyses in whole colon tissue of WT and miR-21^{-/-} female mice. (A) Known target of miR-21: *Pcd4*. (B-D) Adherens junctions (AJs): cadherin (*Cdh*), catenin (*Ctnn*) alpha, beta. (E-H) Gap junctions (GJs): *Gj a-c*. Denoted are n ≥ 3 mice per group, mean values ± SEM, p value calculations using Welch's t-test with ** - p ≤ 0.01, *** - p ≤ 0.001.

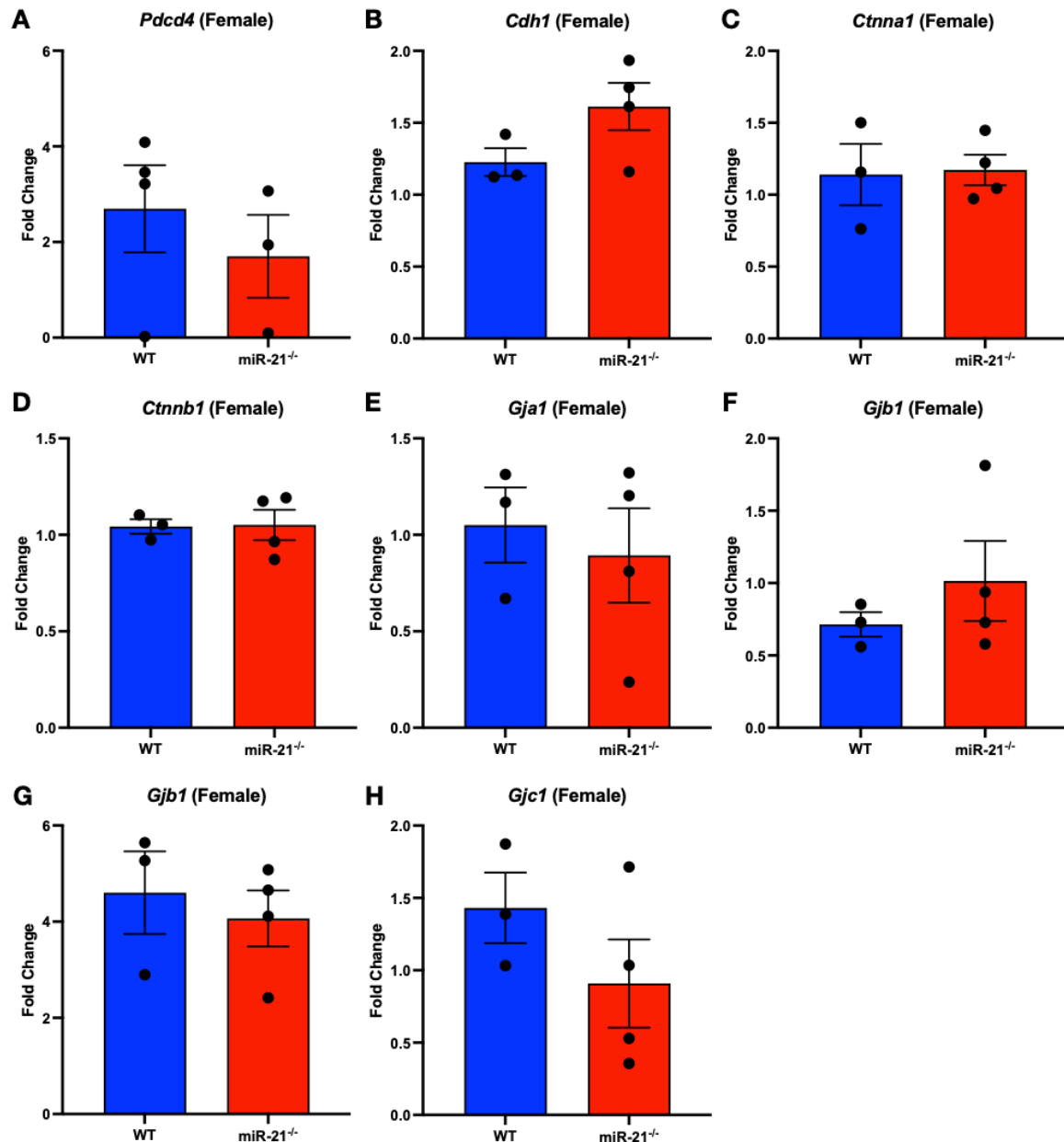


Figure 3.4 – Analysis of adherens junction gene expression during DSS-induced colitis in miR-21^{-/-} female whole colon tissue shows no differences to WT. RNA was isolated from DSS-treated 10-week old WT and miR-21^{-/-} female mice. qRT-PCR analyses in whole colon tissue of WT and miR-21^{-/-} female mice. (A) Known target of miR-21: *Pdcd4*. (B-D) Adherens junctions (AJs): cadherin (*Cdh*), catenin (*Ctnn*) alpha, beta. (E-H) Gap junctions (GJs): *Gj a-c*. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

3.2.2 Analysis of DSS recovery model shows retention in miR-21 regulation of adherens junctions

Previous mice had DSS administered for six days, leading to a strong downregulation in all junction genes tested in both WT and miR-21^{-/-}. This could potentially be due to the strong inflammatory response, inhibiting the integral function of intercellular junctions for the stability of the gut epithelial barrier in favour of effective immune cell infiltration and function. To mitigate this, a recovery model was performed in which non-littermate WT and miR-21^{-/-} mice were treated with 2% DSS for four days and subsequently given a five-day period of recovery post-treatment to re-establish gut barrier function. This concentration and duration of treatment was chosen due to previous trial optimization (data not shown).

For male mice, there was a significant difference in initial body weight between WT and miR-21^{-/-} male mice (**Figure 3.5A**). No notable differences in water and food consumption were observed throughout the trial (**Figure 3.5B, 3.5C**). As seen in previous studies, miR-21^{-/-} male mice were protected against DSS-induced colitis on all metrics. There was markedly reduced weight loss for miR-21^{-/-} male mice (**Figure 3.5D**) and lower diarrhea scores (**Figure 3.5E**) compared to WT, particularly during recovery phase. Stool blood scores were also lower in miR-21^{-/-} male mice throughout the trial (**Figure 3.5F**). These metrics culminated in a reduced DAI for miR-21^{-/-} male mice (**Figure 3.5G**), suggesting less susceptibility to DSS-induced colitis compared to WT mice, and quantified by a reduced AUC for DAI (**Figure 3.5H**). Colons of miR-21^{-/-} male mice were also significantly longer than WT (**Figure 3.5I**).

In female mice, there was no significant difference in initial body weight between WT and miR-21^{-/-} female mice (**Figure 3.6A**). Water consumption was observed to be slightly higher in miR-21^{-/-} female mice throughout the trial (**Figure 3.6B**). There was a notable drop in food consumption for WT mice between Day 5 and 7, but this reduction began to recover towards the end of the trial (**Figure 3.6C**). Overall, miR-21^{-/-} female mice were not protected from DSS-induced colitis as was observed in males, albeit disease symptoms were much less severe overall than male mice on all metrics (**Figure 3.6D, 3.6E, 3.6F**). These metrics culminated in a greater DAI peak for miR-21^{-/-} female mice during the recovery phase (**Figure 3.6G, 3.6H**), however miR-21^{-/-} recovered quicker in the final two days of the trial compared to WT mice. Colons of miR-21^{-/-} female mice were still longer than WT (**Figure 3.6I**).

Tissue integrity and inflammation was assessed through H&E staining and blind scoring between distal and proximal colon tissue of WT and miR-21^{-/-} mice in the recovery model. For male mice, tissue damage among intestinal crypts and infiltration of immune cells in the lamina propria were reduced in miR-21^{-/-} distal tissue compared to WT (**Figure 3.7A, 3.7B, 3.7C**), culminating in a lower cumulative histology score that suggests reduced inflammation in miR-21^{-/-} mice (**Figure 3.7D**). In proximal tissue, there was no significant differences in tissue damage or immune cell infiltration (**Figure 3.7E, 3.7F**), leading to similar cumulative histology scores between mouse groups (**Figure 3.7G**). Interestingly, the reverse phenotype was observed for female mice. There was a trend of greater tissue damage among intestinal crypts, but similarly low infiltration of immune cells in the lamina propria between WT and miR-21^{-/-} distal tissue (**Figure 3.8A, 3.8B, 3.8C**), culminating in a higher cumulative histology score that suggests greater inflammation in miR-21^{-/-} female mice (**Figure 3.8D**). In proximal tissue, there was no observable differences in tissue damage or cell infiltration between WT and miR-21^{-/-} female mice (**Figure 3.8E, 3.8F, 3.8G**). Therefore, this suggests differential impact due to miR-21 expression, as well as the effects of DSS by tissue region and sex of mice.

As another marker for inflammation, MPO activity was measured in distal and proximal colon tissue of WT and miR-21^{-/-} mice of the recovery model. MPO is an enzyme found within lysosomal azurophilic granules of neutrophils, directly tied to inflammatory damage caused by infiltrating immune cells within the gut mucosa.²³¹ There were no significant differences in MPO activity between WT and miR-21^{-/-} male mice in either distal tissue (**Figure 3.9A**), and neither in proximal tissue, though enzymatic activity was quite varied among proximal samples (**Figure 3.9B**). For female mice, there was also no observable differences in MPO activity between WT and miR-21^{-/-} mice within distal tissue (**Figure 3.9C**), with similar varied enzymatic activity in proximal tissue seen in miR-21^{-/-} mice (**Figure 3.9D**).

A screen for mouse homologs of AJ genes was carried out in distal and proximal colon samples from WT and miR-21^{-/-} mice of the DSS recovery model. Initially, levels of the mature form of miR-21 (miR-21-5p) was investigated in WT distal and proximal tissue samples to once again determine whether any differences in regional expression among the predicted targets can be due to associative differences in miR-21 levels at the end of recovery phase. There were no significant differences in expression of miR-21-5p between distal and proximal tissue, though levels remain slightly elevated in male and female DSS-treated distal tissue compared to untreated tissue post-recovery (**Figure 3.10A, 3.10B**).

In male mice, distal colon tissue showed a slight upregulation in *Pten* within miR-21^{-/-} compared to WT mice, while no observable changes were seen in proximal tissue (**Figure 3.11A**). Among the AJ genes tested, *Cdh1* had an induced trend in distal tissue of miR-21^{-/-} compared to WT mice (**Figure 3.11B**), but not proximal tissue. *Ctnna1* and *Ctnnd1* were notably upregulated in distal tissue of miR-21^{-/-} compared to WT mice, with no major differences observed in *Ctnnb1* expression (**Figure 3.11C, 3.11D, 3.11E**). In females, there was significantly higher expression of *Pten* in proximal colon tissue compared to distal tissue of female mice, but no differences observed between WT and miR-21^{-/-} mice (**Figure 3.12A**). There was an increased trend for *Ctnna1* and *Ctnnd1* in female miR-21^{-/-} distal tissue compared to WT, but no differences observed for *Cdh1* (**Figure 3.12B, 3.12C, 3.12D**). Moreover, *Ctnnb1* was elevated in both distal and proximal colon tissue of female miR-21^{-/-} mice compared to WT, although not significantly (**Figure 3.12E**). This suggests that, at least in male mice, a heightened inflammatory environment alludes to a more measurable impact of miR-21 gene regulation *in vivo*.

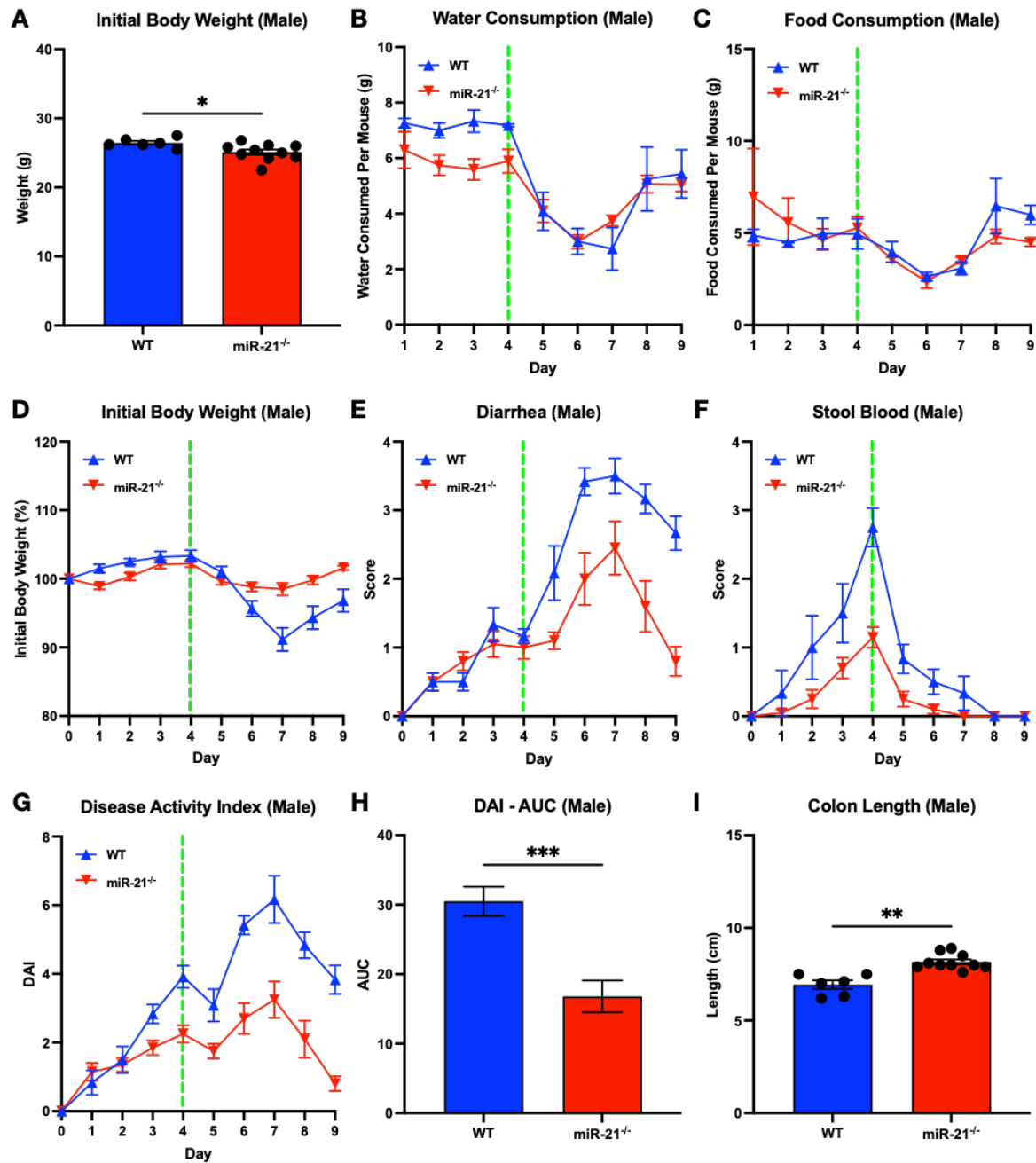


Figure 3.5 – Loss of miR-21 reduces disease susceptibility and increases recovery in non-littermate male mice to DSS-induced colitis. WT and miR-21^{-/-} male mice 8-15 weeks of age were subjected to 2% (w/v) DSS *ad libitum* via drinking water for four days, followed by five days of normal drinking water (time of switch indicated by green dotted line). (A) Initial body weights of WT and miR-21^{-/-} mice. (B + C) Water and food consumption of WT and miR-21^{-/-} mice throughout trial duration. Daily measurements and scoring were taken of mouse (D) body weight, (E) diarrhea and (F) stool blood, culminating in a (G) disease activity index (DAI). (H) Disease susceptibility was quantified as area under the curve (AUC) for DAI. (I) Mouse colon lengths were measured at the end of the trial. Denoted are $n \geq 6$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t -test with * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$.

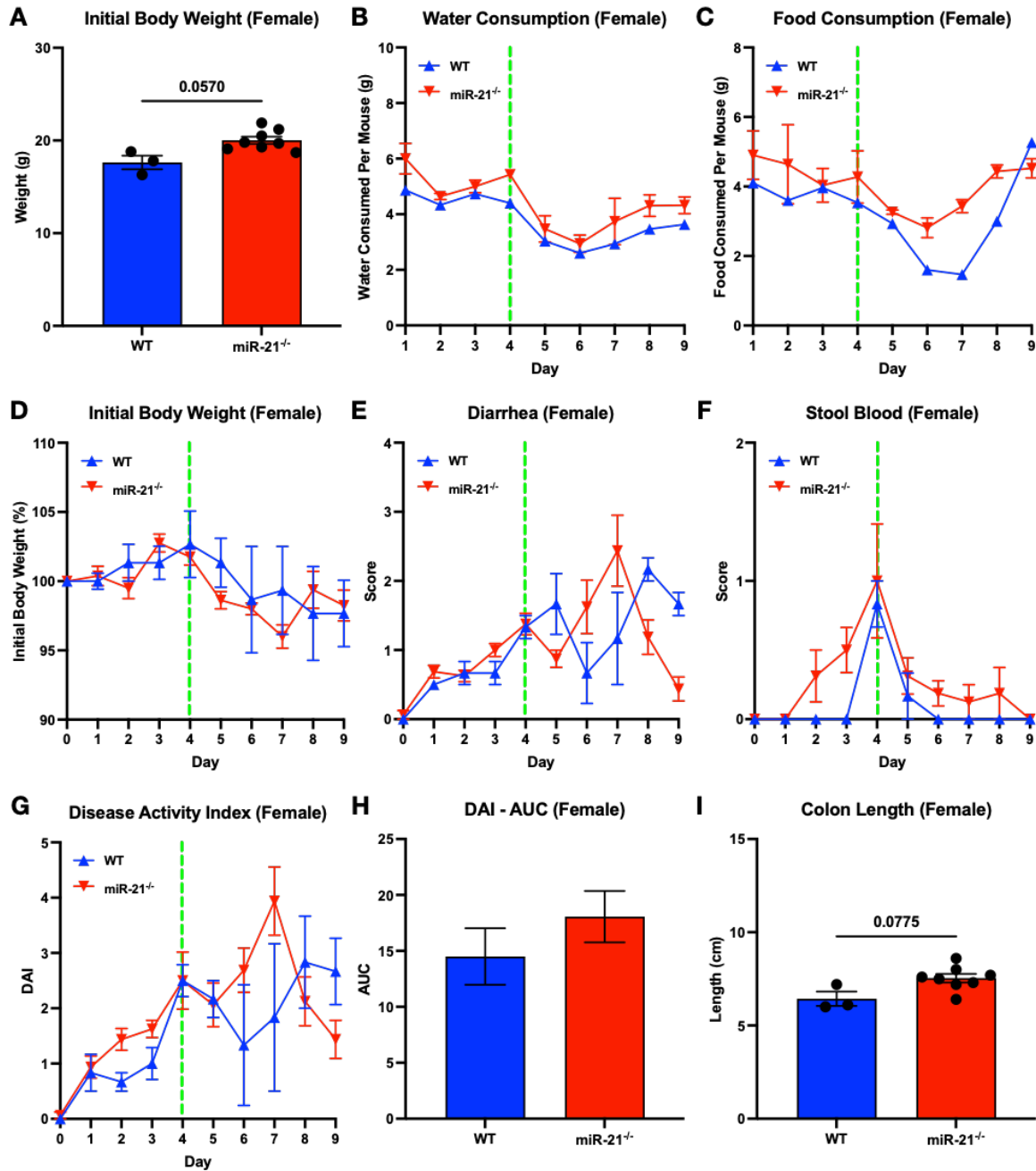


Figure 3.6 – Loss of miR-21 slightly increases disease susceptibility but induces recovery in non-littermate female mice to DSS-induced colitis. WT and miR-21^{-/-} female mice 8-15 weeks of age were subjected to 2% (w/v) DSS *ad libitum* via drinking water for four days, followed by five days of normal drinking water (time of switch indicated by green dotted line). (A) Initial body weights of WT and miR-21^{-/-} mice. (B + C) Water and food consumption of WT and miR-21^{-/-} mice throughout trial duration. Daily measurements and scoring were taken of mouse (D) body weight, (E) diarrhea and (F) stool blood, culminating in a (G) disease activity index (DAI). (H) Disease susceptibility was quantified as area under the curve (AUC) for DAI. (I) Mouse colon lengths were measured at the end of the trial. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

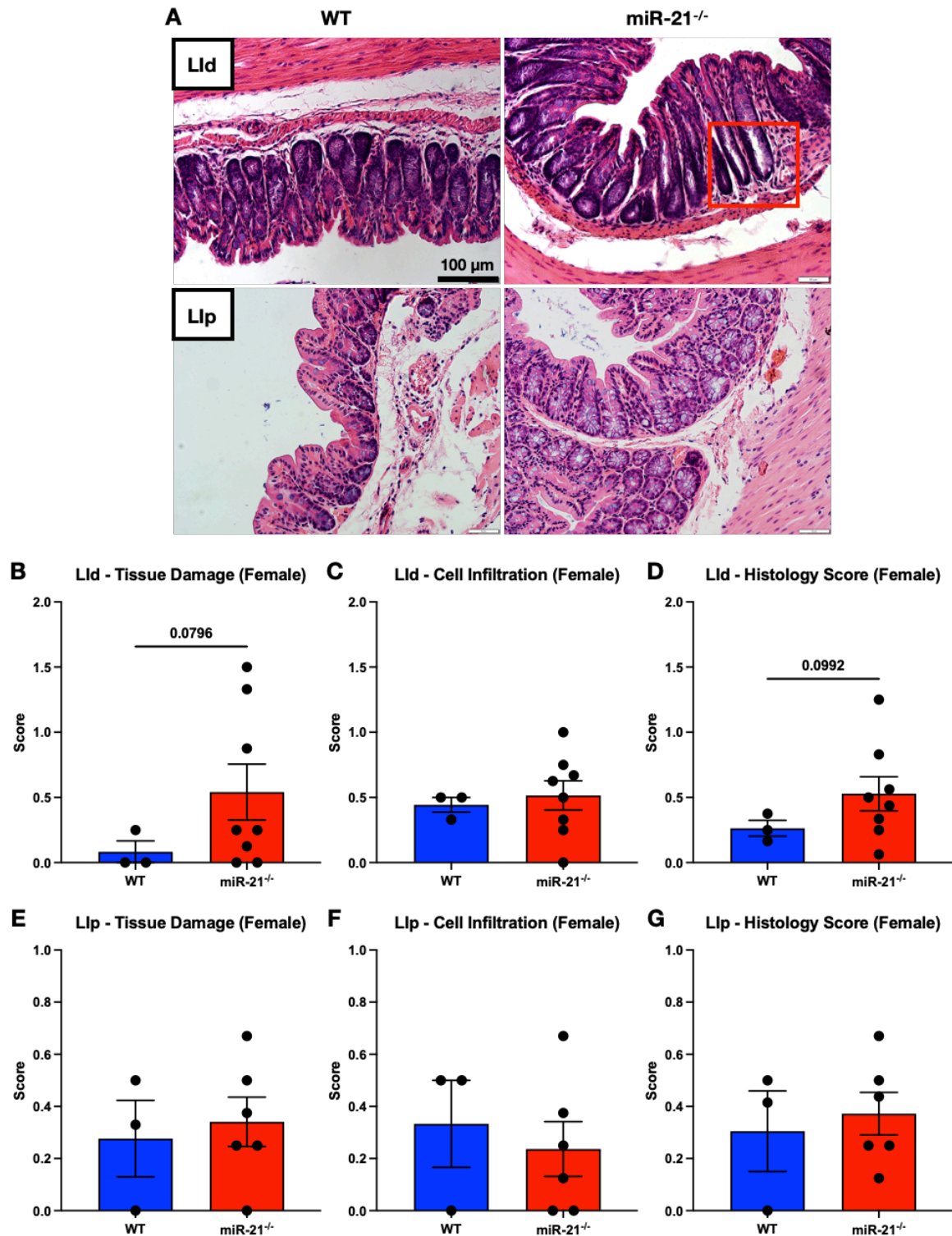


Figure 3.8 – Histological analysis shows loss of miR-21 increases tissue damage in distal colon tissue of non-littermate female mice in DSS recovery model. At the end of the trial, WT and miR-21^{-/-} mouse tissues were fixed in 4% formaldehyde, dehydrated in ethanol and embedded in paraffin wax. Histological analyses of (A) distal colon / large intestine (Lld) and proximal colon / large intestine (Llp) tissue sections (5 μ m) were conducted via H&E staining. Histopathological blinded scoring of stained mouse intestinal tissue was done for (B + E) tissue damage of intestinal crypts and (C + F) immune cell infiltration, adapted from Wang *et al.* (2022)²³⁰, culminating in a (D + G) histology score. Red box in miR-21^{-/-} - Lld denotes area of interest regarding tissue damage. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t -test.

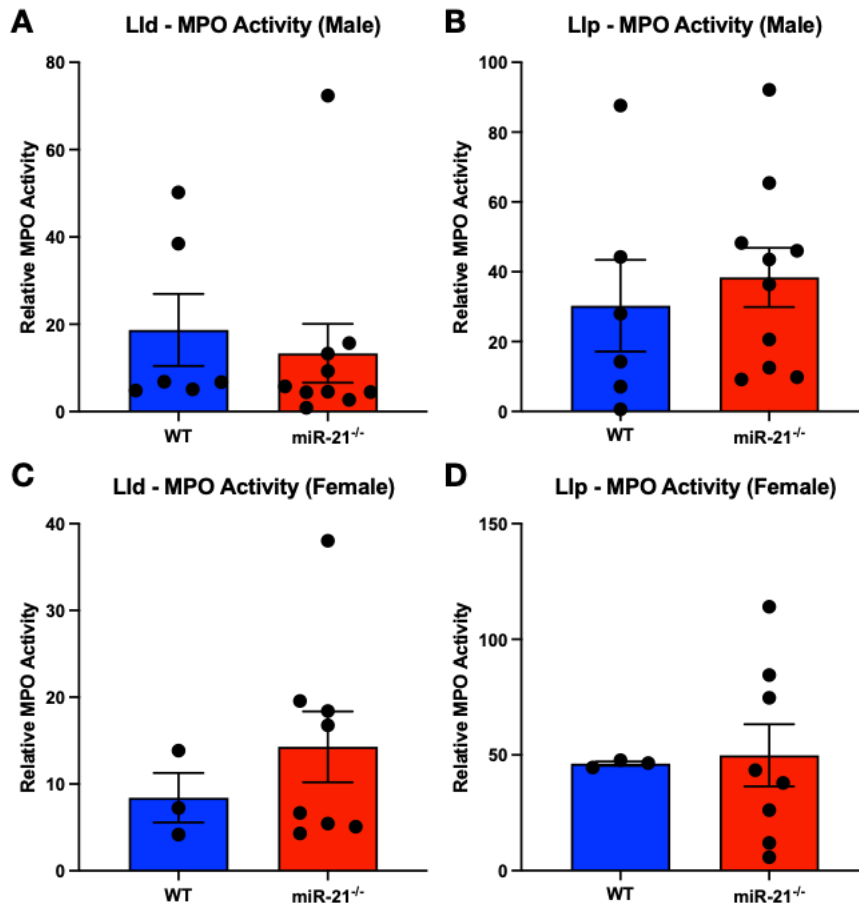


Figure 3.9 – No changes in myeloperoxidase activity between non-littermate WT and miR-21^{-/-} mice in DSS recovery model. At the end of the trial, WT and miR-21^{-/-} mouse tissues were homogenized in myeloperoxidase (MPO) Buffer I solution (100 mM sodium chloride, 20 mM trisodium phosphate, 15 mM disodium ethylenediaminetetraacetate [EDTA] dihydrate; pH 4.7), and centrifuged. Pellets were resuspended in 1 mL of 0.2% sodium chloride solution and 1 mL of 1.6% sodium chloride / 5% glucose solution. After another centrifugation, pellets were resuspended in MPO Buffer II (50 mM trisodium phosphate, 0.5% Hexadecyltrimethylammonium bromide [HTAB]; pH 5.4), underwent three cycles of freeze-thaw for complete cell lysis, and then centrifuged. Supernatants were transferred to 96-well plates in duplicate. MPO activity was induced using OptEIA™ ELISA sets and reactions were stopped with the addition of 1 M sulfuric acid. Absorbance was measured using a standard plate reader set to 450 nm. Relative activity for distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue of (A + B) male and (C + D) female mice were measured by subtracting blank background absorbance and normalizing to mg of protein. Denoted are $n \geq 6$ mice per male group or $n \geq 3$ mice per female group, mean values $\pm$ SEM, p value calculations using Welch's t -test.

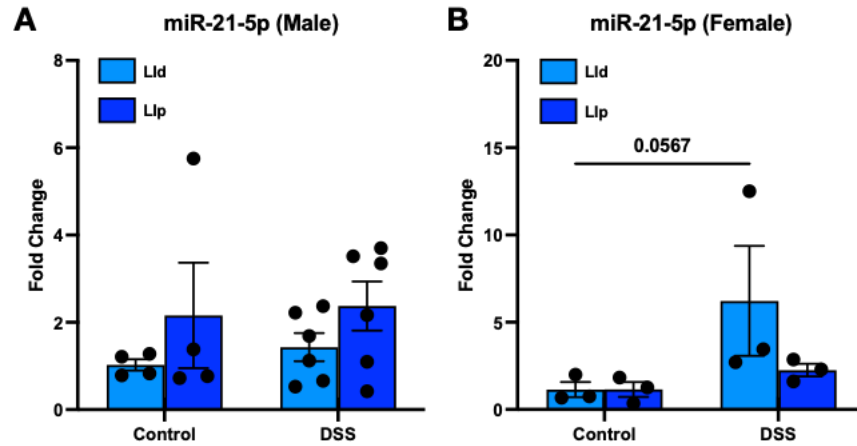


Figure 3.10 – No changes in miR-21-5p expression between colon regions, but levels remain elevated in DSS-treated WT male mice compared to untreated mice in DSS recovery model. RNA was isolated from 8-15 week old WT mice given normal drinking water or 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. qRT-PCR analyses of miR-21-5p in distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue of WT mice. Denoted are $n \geq 4$ mice per male group or $n \geq 3$ mice per female group, mean values $\pm$ SEM, p value calculations using two-way ANOVA.

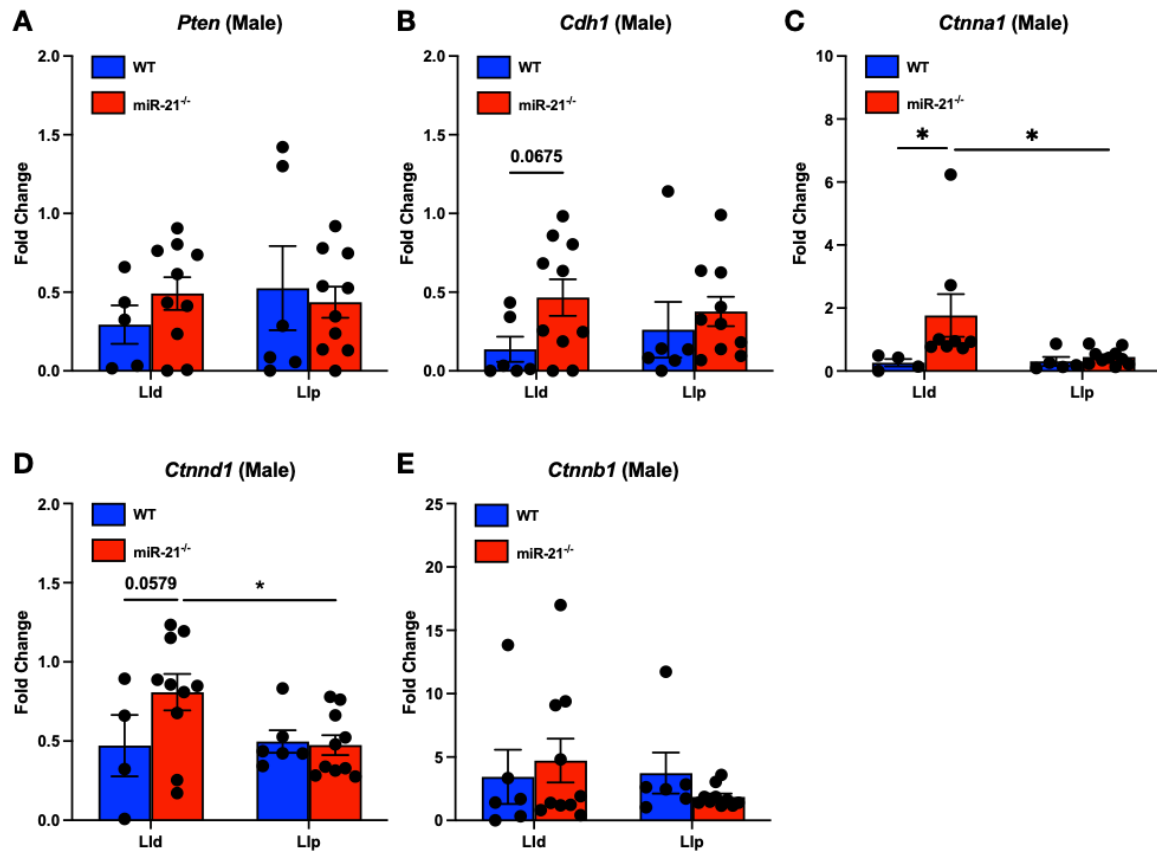


Figure 3.11 – Analysis of adherens junction gene expression shows some genes remain tied to miR-21 regulation in distal colon tissue of male mice during recovery from DSS-induced colitis. RNA was isolated from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. qRT-PCR analyses in distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue of WT and miR-21^{-/-} mice. (A-B) Known target of miR-21: *Pten*. (C-J) Adherens junctions (AJs): cadherin (*Cdh*), catenin (*Ctnn*) alpha, delta, beta. Denoted are $n \geq 4$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

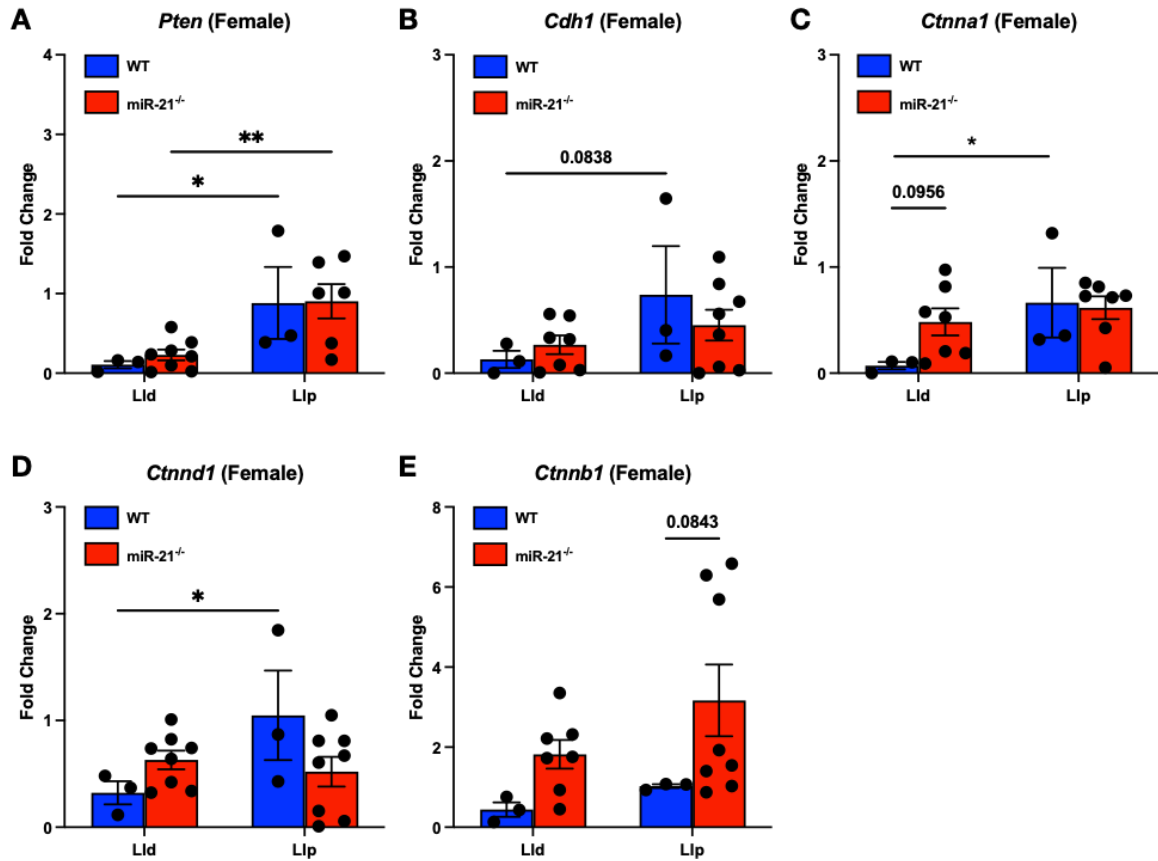


Figure 3.12 – Analysis of adherens junction gene expression shows some genes remain tied to miR-21 regulation in distal and proximal colon tissue of female mice during recovery from DSS-induced colitis. RNA was isolated from 8-15 week old WT and miR-21^{-/-} female mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. qRT-PCR analyses in distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue of WT and miR-21^{-/-} mice. (A-B) Known target of miR-21: *Pten*. (C-E) Adherens junctions (AJs): cadherin (*Cdh*), catenin (*Ctnn*) alpha, delta, beta. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$, ** - $p \leq 0.01$.

3.2.3 Loss of differential expression in intercellular junctions within miR-21^{-/-} littermate mice of the DSS recovery model

All previous studies used non-littermate mice, produced from separate lineages of their respective genotypes (WT [miR-21^{+/+}], miR-21^{-/-}). This separation of lineages has the consequence of producing different microbiomes which have been shown to affect phenotypes upon colitis induction, and thus makes it challenging to determine whether changes in phenotype were due to differences in specifically targeted genetic modifications alone.^{249,250} To address this, the recovery model was also performed using WT, homozygous miR-21^{-/-} and heterozygous (HET) miR-21^{+/-} mice. In this trial, control groups were added in which mice were given normal drinking water throughout the trial, but due to lack in genotype numbers, no female control groups were included.

For male mice, there was no significant difference in initial body weight (**Figure 3.13A**), and no notable differences in water and food consumption among control and DSS groupings throughout the trial (**Figure 3.13B, 3.13C**), though water consumption in DSS-treated groups were reduced upon initiation of the recovery phase. Interestingly, male littermate mice in general did not have as severe disease symptoms that were previously seen in non-littermate mice, though miR-21^{-/-} male mice were still mildly protected against DSS-induced colitis. Body weight was not notably different between DSS groups (**Figure 3.13D**). Some protection was kept, showcased by lower diarrhea (**Figure 3.13E**) and stool blood (**Figure 3.13F**) scores in miR-21^{-/-} mice throughout the majority of the trial, with miR-21^{-/-} mice showing a faster recovery post-treatment. These clinical symptom scores culminated in a significantly lower DAI score for miR-21^{-/-} male mice compared to WT towards the end of the recovery phase (**Figure 3.13G, 3.13H**), suggesting a more rapid recovery to DSS-induced colitis. Interestingly, the inclusion of HET mice showed greater weight loss and diarrhea scores than WT and miR-21^{-/-} mice during recovery, ultimately having similar disease severity to WT males. Colon lengths were not significantly different among any of the groups (**Figure 3.13I**).

For female mice, there was no significant difference in initial body weight (**Figure 3.14A**), and no notable differences in water and food consumption throughout the trial among any of the groups (**Figure 3.14B, 3.14C**). Indeed, all female littermate mice did not showcase much disease severity as was seen in female non-littermate mice, and miR-21^{-/-} female littermates were more symptomatic during DSS-induced colitis than other groups. There was a greater loss of body weight and slightly higher stool blood scores in miR-21^{-/-}

compared to WT mice, although some protection was observed such as lower diarrhea scores after two days of recovery (**Figure 3.14D, 3.14E, 3.14F**). Overall, higher DAI scores were observed for miR-21^{-/-} compared to WT mice, though there was a more rapid recovery post-treatment similar to males (**Figure 3.14G, 3.14H**). The inclusion of HET mice showed similar loss in body weight and diarrhea scores to WT, and even some protection with lower stool blood scores compared to WT and miR-21^{-/-} mice, ultimately having similar disease severity to WT females. Colon lengths were not significantly different among any of the groups (**Figure 3.14I**).

To assess gut permeability, the mucosa-to-blood flux of the permeability marker FD4 was compared in WT, HET and miR-21^{-/-} littermate mice. All control groups not given DSS had very low levels of detectable FITC in serum samples. In male mice, there was a reduced level of FITC in DSS-treated miR-21^{-/-} compared to WT and HET mice (**Figure 3.15A**), suggesting the absence of miR-21 decreased gut permeability *in vivo* within males, which supports prior observations of protection against DSS-induced colitis. In females, there was no major differences between any of the groups (**Figure 3.15B**), suggesting low gut permeability generally across female mice groups.

Following histological analysis, WT and miR-21^{-/-} mice were analyzed with a particular focus on assessing comparisons with non-littermate mice. In male mice, there were no significant differences in tissue damage or immune cell infiltration between WT and miR-21^{-/-} distal tissue (**Figure 3.16A, 3.16B, 3.16C, 3.16D**). However, in proximal tissue, there was a significant increase in tissue damage for miR-21^{-/-} compared to WT mice, but no notable changes in immune cell infiltration and overall cumulative histology scores (**Figure 3.16E, 3.16F, 3.16G**). In female mice, there were very low scores in general, but only immune cell infiltration was observed to be slightly reduced in miR-21^{-/-} distal colon tissue compared to WT (**Figure 3.17A, 3.17B, 3.17C, 3.17D**). There were no major changes in tissue damage or immune cell infiltration between groups in proximal tissue (**Figure 3.17E, 3.17F, 3.17G**). Furthermore, there were no significant differences in MPO activity in distal or proximal tissue in either sex (**Figure 3.18A, 3.18B, 3.18C, 3.18D**).

Since males were observed to have the most inflammation based on disease metrics measured during the trial, only male samples were utilized in molecular analyses. There were no differences in mature form miR-21-5p within distal or proximal colon tissue of control or DSS-treated WT male mice (**Figure 3.19**). Known miR-21 targets *Pdcd4* and *Pten* demonstrated an induced trend in distal tissue of miR-21^{-/-} compared to WT mice

(**Figure 3.20A, 3.20B**). Although occludin (an indirect target of miR-21) encoded by *Ocln* was also shown to be increased in miR-21^{-/-} distal tissue (**Figure 3.20C**), there were no major differences observed in *Cdhl* when compared to WT mice (**Figure 3.20D**). Therefore, these findings suggest that littermates did not experience similar severity of DSS-induced colitis as non-littermate mice, or that WT was indeed protected due to additional factors such as changes to the gut microbiome during upbringing in the pre-weaning phase.

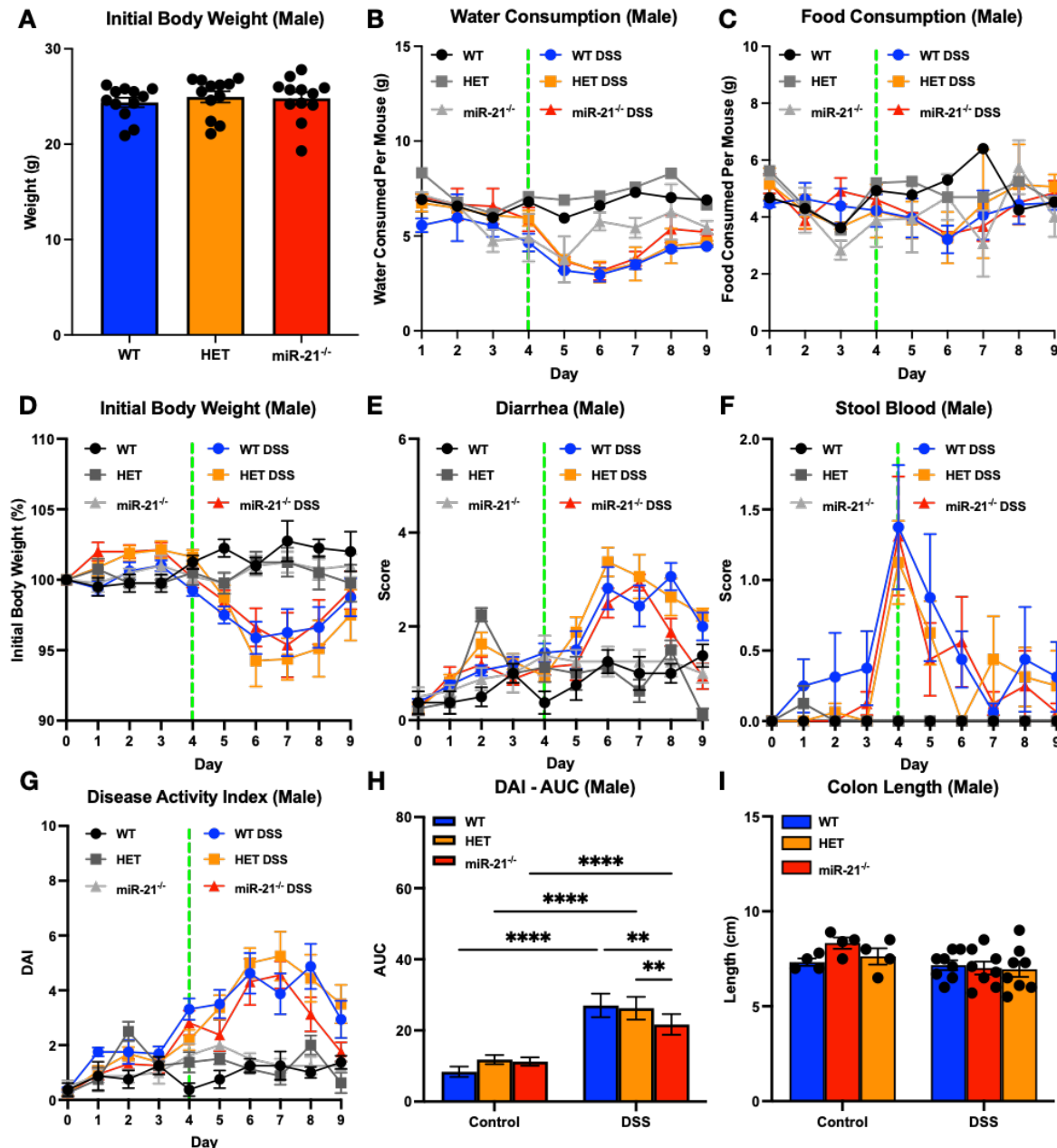


Figure 3.13 – Littermate $\text{miR-21}^{-/-}$ male mice lose some protection to DSS-induced colitis but recover more rapidly. Littermate WT, heterozygous (HET) and $\text{miR-21}^{-/-}$ male mice 8-15 weeks of age were subjected to 2% (w/v) DSS *ad libitum* via drinking water for four days, followed by five days of normal drinking water (time of switch indicated by green dotted line). (A) Initial body weights of WT, HET and $\text{miR-21}^{-/-}$ mice. (B + C) Water and food consumption of WT, HET and $\text{miR-21}^{-/-}$ mice throughout trial duration. Daily measurements and scoring were taken of mouse (D) body weight, (E) diarrhea and (F) stool blood, culminating in a (G) disease activity index (DAI). (H) Disease susceptibility was quantified as area under the curve (AUC) for DAI. (I) Mouse colons were measured at the end of the trial. Denoted are $n \geq 4$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with ** - $p \leq 0.01$, **** - $p \leq 0.0001$.

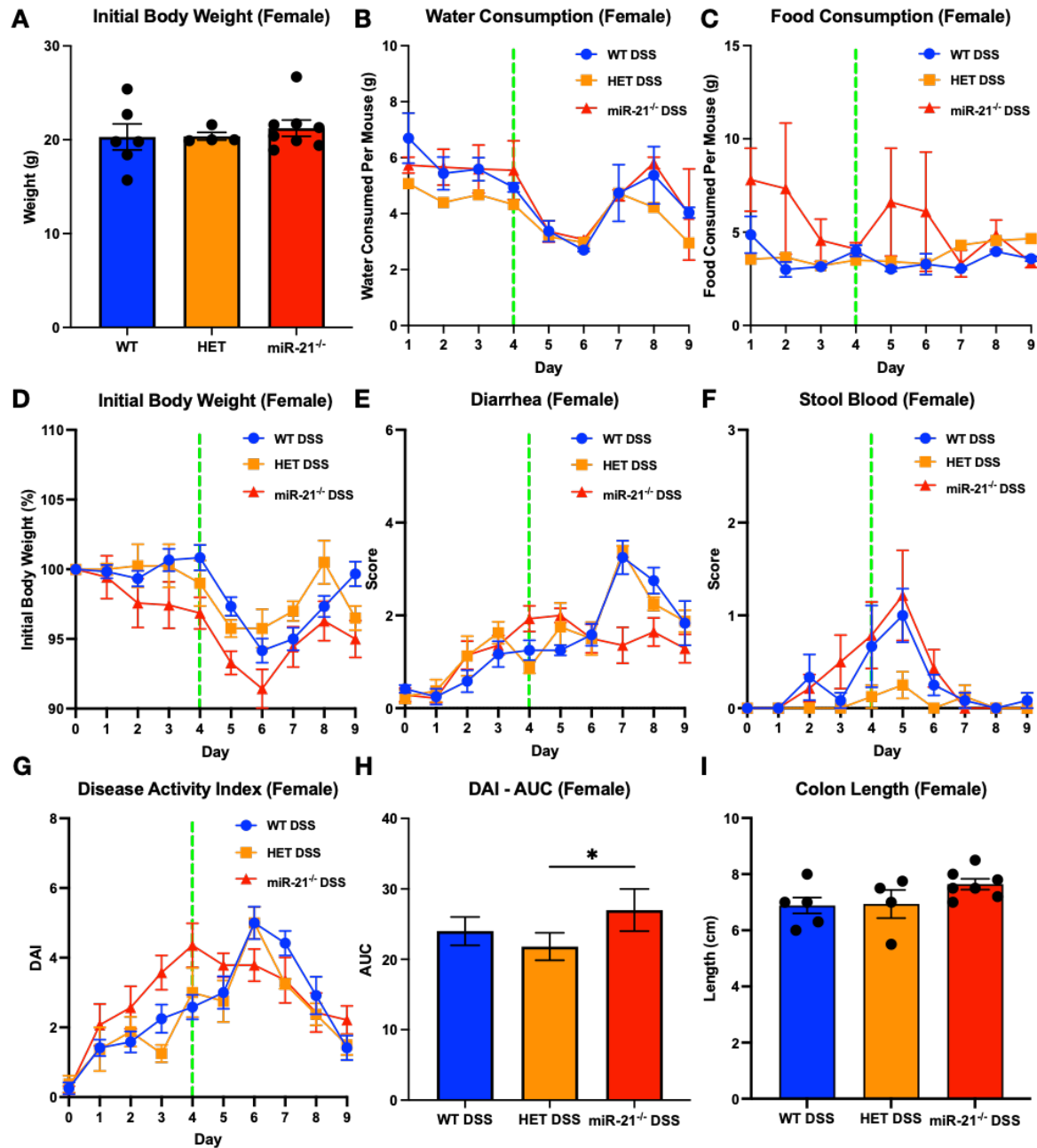


Figure 3.14 – Littermate miR-21^{-/-} female mice remain more susceptible to DSS-induced colitis but recover more rapidly. Littermate WT, heterozygous (HET) and miR-21^{-/-} female mice 8-15 weeks of age were subjected to 2% (w/v) DSS *ad libitum* via drinking water for four days, followed by five days of normal drinking water (time of switch indicated by green dotted line). (A) Initial body weights of WT, HET and miR-21^{-/-} mice. (B + C) Water and food consumption of WT, HET and miR-21^{-/-} mice throughout trial duration. Daily measurements and scoring were taken of mouse (D) body weight, (E) diarrhea and (F) stool blood, culminating in a (G) disease activity index (DAI). (H) Disease susceptibility was quantified as area under the curve (AUC) for DAI. (I) Mouse colons were measured at the end of the trial. Denoted are $n \geq 4$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with * - $p \leq 0.05$.

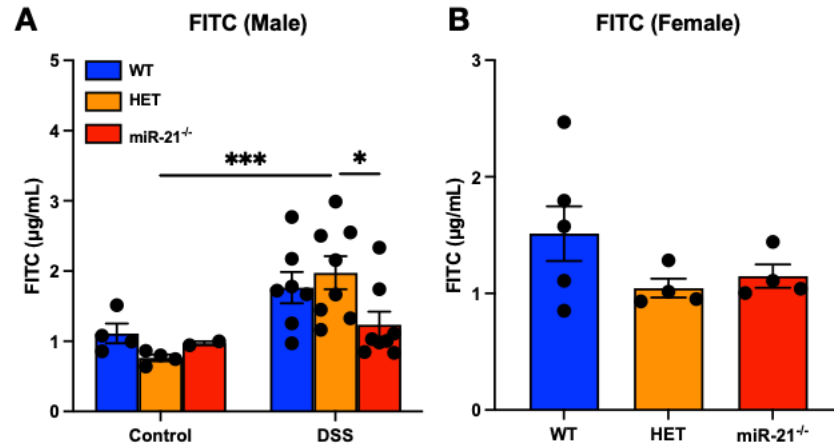


Figure 3.15 – Littermate miR-21^{-/-} male mice have reduced gut permeability in DSS recovery model. Fluorescein isothiocyanate (FITC)-dextran concentration in DSS-treated littermate WT, HET and miR-21^{-/-} (A) male and (B) female mice post-recovery. On the final day of the trial, FITC-dextran (molecular mass 4 kDa) was dissolved in sterile PBS and administered by oral gavage at 12 mg per mouse. After four hours, mice were euthanized with CO₂ and blood was collected by decapitation. Serum was separated by centrifuging blood samples at 4°C for 12 min at 1000 x g. FITC-dextran concentration was determined via a standard curve. Fluorescence was measured using a standard plate reader set to 485 nm (excitation) and 525 nm (emission). Denoted are $n \geq 4$ mice per group with the exception of miR-21^{-/-} male control mice that have $n \geq 2$, mean values $\pm$ SEM, p value calculations using two-way ANOVA for males or one-way ANOVA for females with * - $p \leq 0.05$, *** - $p \leq 0.001$.

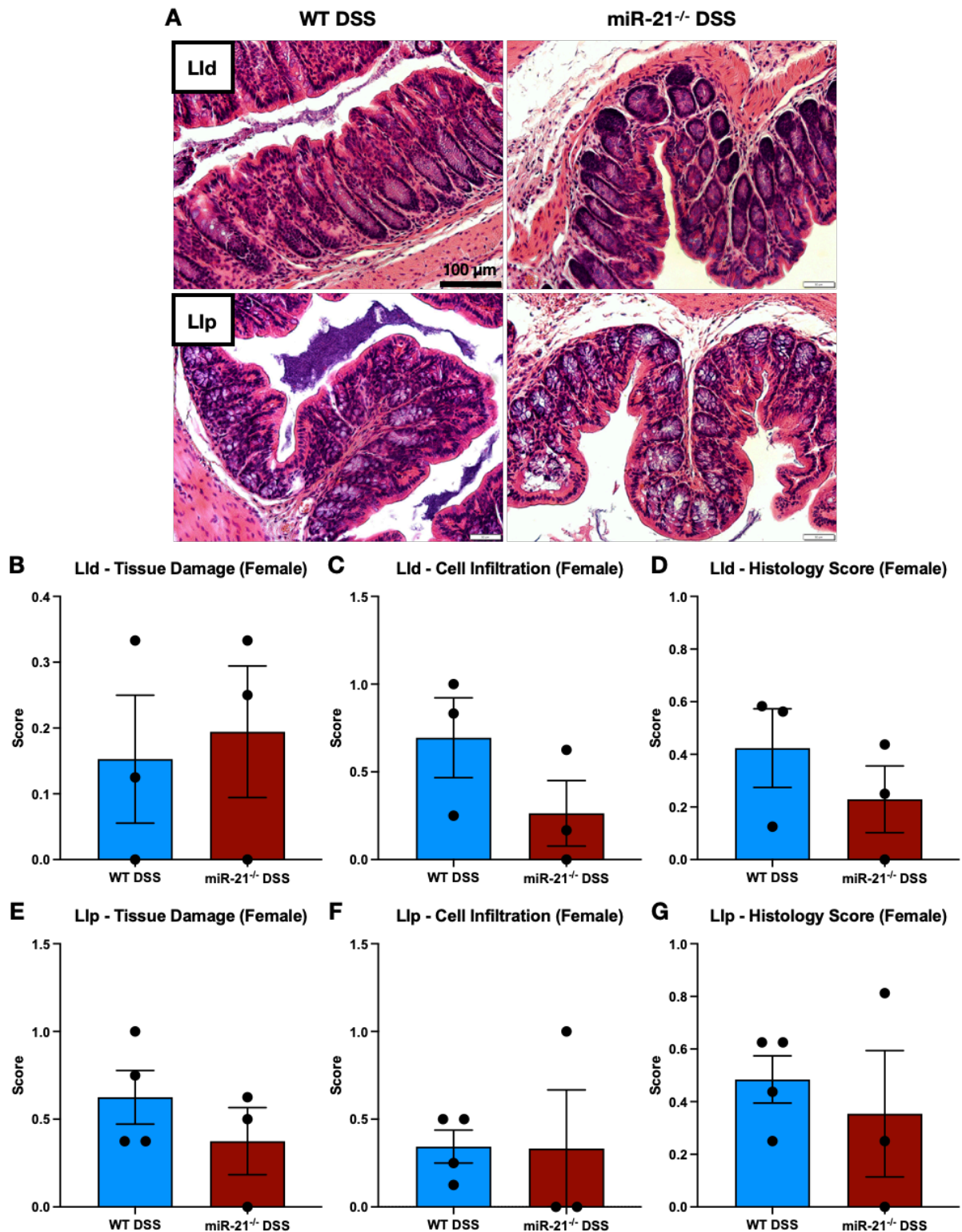


Figure 3.17 – Histological analysis shows a decrease in immune cell infiltration within distal colon of littermate miR-21^{-/-} female mice during recovery from DSS-induced colitis. At the end of the trial, littermate WT and miR-21^{-/-} mouse tissues were fixed in 4% formaldehyde, dehydrated in ethanol and embedded in paraffin wax. Histological analyses of (A) distal colon / large intestine (Lld) and proximal colon / large intestine (Llp) tissue sections (5 μ m) were conducted via H&E staining. Histopathological blinded scoring of stained mouse intestinal tissue was done for (B + E) tissue damage of intestinal crypts and (C + F) immune cell infiltration, adapted from Wang *et al.* (2022)²³⁰, culminating in a (D + G) histology score. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

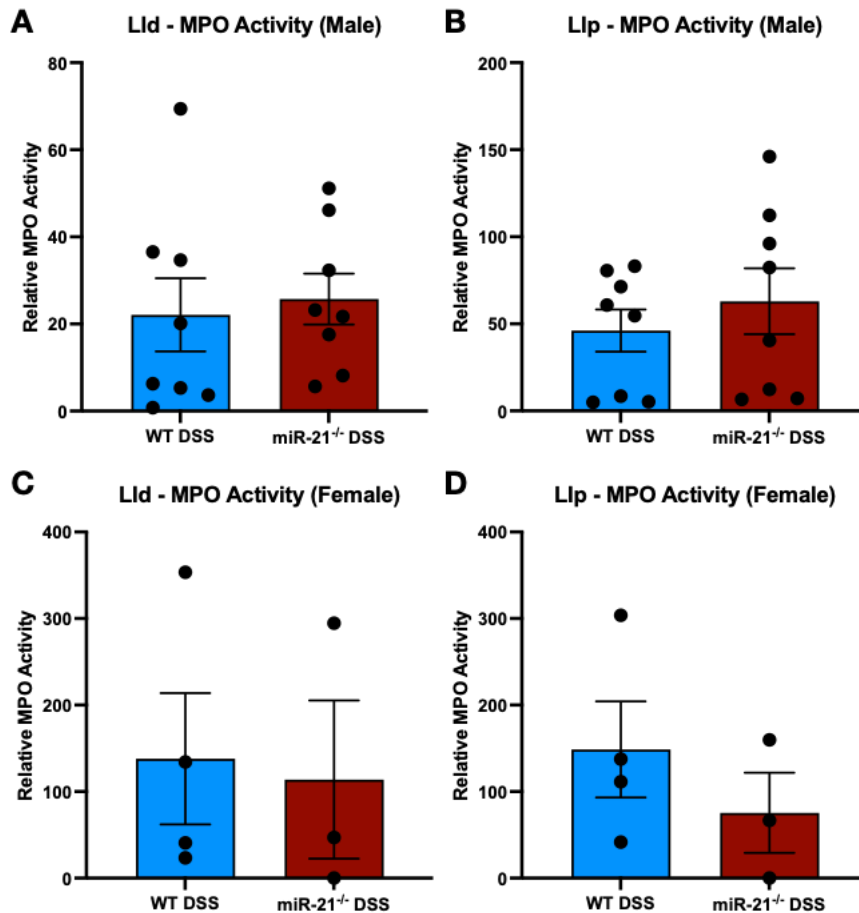


Figure 3.18 – No major changes in myeloperoxidase activity between littermate WT and miR-21^{-/-} mice in DSS recovery model. At the end of the trial, littermate WT and miR-21^{-/-} mouse tissues were homogenized in myeloperoxidase (MPO) Buffer I solution (100 mM sodium chloride, 20 mM trisodium phosphate, 15 mM disodium ethylenediaminetetraacetate [EDTA] dihydrate; pH 4.7), and centrifuged. Pellets were resuspended in 1 mL of 0.2% sodium chloride solution and 1 mL of 1.6% sodium chloride / 5% glucose solution. After another centrifugation, pellets were resuspended in MPO Buffer II (50 mM trisodium phosphate, 0.5% Hexadecyltrimethylammonium bromide [HTAB]; pH 5.4), underwent three cycles of freeze-thaw for complete cell lysis, and then centrifuged. Supernatants were transferred to 96-well plates in duplicate. MPO activity was induced using OptEIA™ ELISA sets and reactions were stopped with the addition of 1 M sulfuric acid. Absorbance was measured using a standard plate reader set to 450 nm. Relative activity for distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue of (A + B) male and (C + D) female mice were measured by subtracting blank background absorbance and normalizing to mg of protein. Denoted are $n \geq 8$ mice per male group or $n \geq 3$ mice per female group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

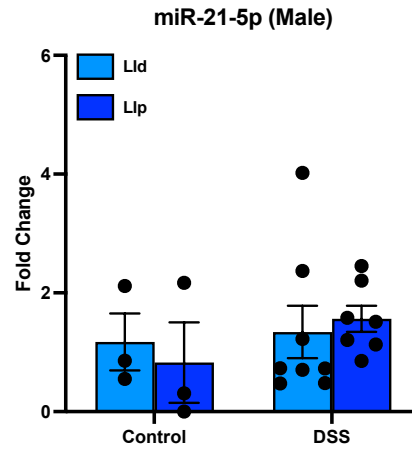


Figure 3.19 – No changes in miR-21-5p expression between colon regions in littermate mice during recovery from DSS-induced colitis. RNA was isolated from 8-15 week old littermate WT male mice given normal drinking water or 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. qRT-PCR analyses of miR-21-5p in distal colon / large intestine (Lld) and proximal colon / large intestine (Lip) tissue of WT mice. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA.

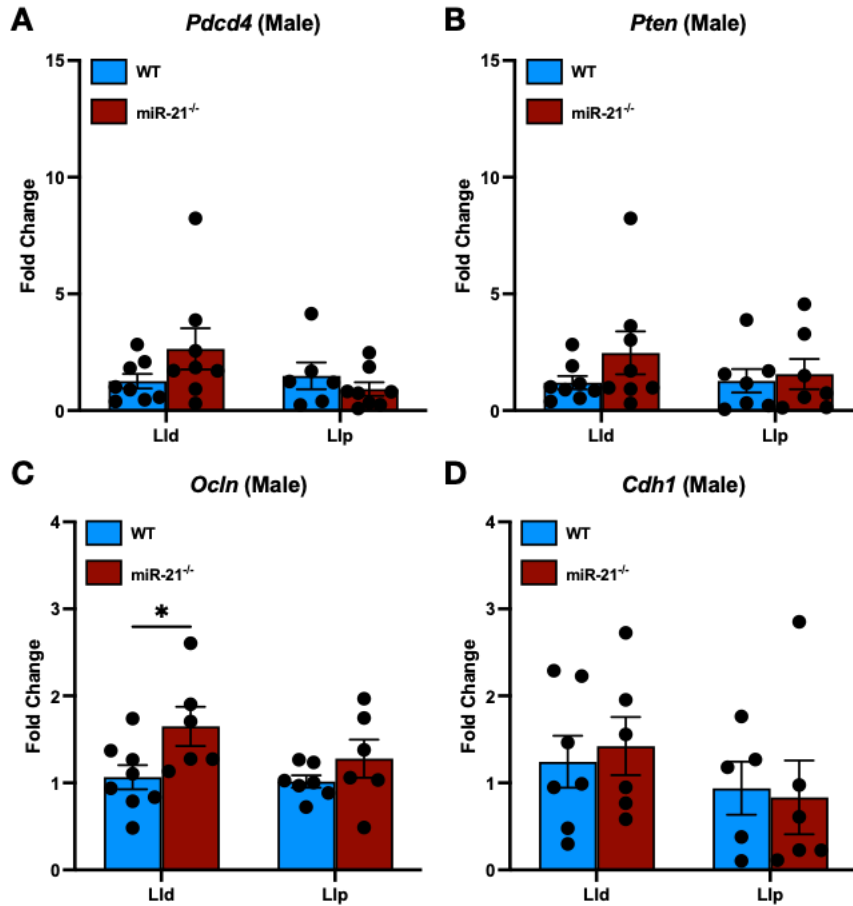


Figure 3.20 – *Cdh1* loses differential expression in littermate miR-21^{-/-} colon tissue of DSS recovery model. RNA was isolated from 8-15 week old littermate WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. qRT-PCR analyses in distal colon / large intestine (Lid) and proximal colon / large intestine (Lip) tissue of littermate WT and miR-21^{-/-} male mice. Known targets of miR-21: (A) *Pdc4* and (B) *Pten*. (C) Tight junctions (TJs): *Oc1n*. (D) Adherens junctions (AJs): cadherin (*Cdh*). Denoted are $n \geq 5$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

3.2.4 E-cadherin abundance, fragmentation and localization in colon tissue are altered in miR-21^{-/-} non-littermate mouse tissue, but not directly targeted

From the *in vivo* screening under basal and DSS-colitis conditions with non-littermate mice, a potential target emerged that is critical to the gut epithelial barrier, E-cadherin (encoded by *Cdh1*). Despite being major protein components of cell-to-cell contact points, AJ proteins are generally overshadowed by TJ proteins for their role during IBD. With *Cdh1* being one of the most consistently impacted, the abundance of E-cadherin protein was further investigated in healthy mouse tissue within miR-21^{-/-} mice. Distal colon samples from WT and miR-21^{-/-} male mice demonstrated no significant changes in overall E-cadherin protein levels (**Figure 3.21A, 3.21B**), however there was a slight induction among proximal colon tissue (**Figure 3.21F, 3.21G**). Interestingly, there were two distinct bands visualized on the blot, one at the predicted size at 120 kDa and another at approximately 100 kDa. When quantified individually, there were distinct trends among intestinal regions. In distal tissue, there were elevated levels of intact protein (upper band) in miR-21^{-/-} compared to WT mice (**Figure 3.21C, 3.21D**), and elevated levels of fragmented protein (lower band) in miR-21^{-/-} proximal colon samples compared to WT (**Figure 3.21H, 3.21I**), summarized by the upper/lower band fragmentation ratio (**Figure 3.21E, 3.21J**). These findings suggest differential distribution among size fragments between colon regions under healthy conditions.

To expand on protein abundance, distal and proximal colon sections were assessed for localization of E-cadherin in WT and miR-21^{-/-} male mice under healthy conditions through immunofluorescence. In distal tissue, E-cadherin was more localized to the tops of intestinal crypts in miR-21^{-/-} compared to WT mice (**Figure 3.22A**). Protein abundance was quantified by normalizing E-cadherin fluorescence to the number of nuclei per field of view, which was induced in miR-21^{-/-} compared to WT mice (**Figure 3.22B**). In proximal tissue, E-cadherin was similarly localized to the tops of intestinal crypts, observed more in miR-21^{-/-} tissue compared to WT (**Figure 3.23A**). However, there was no significant difference in E-cadherin abundance when quantifying fluorescence between WT and miR-21^{-/-} proximal tissue (**Figure 3.23B**).

It is possible that protein levels are more dramatically impacted in the inflamed state, and therefore E-cadherin levels were explored in whole colon tissue sections of control (untreated) and DSS-treated WT and miR-21^{-/-} female mice. Again, there were no significant changes to overall E-cadherin protein levels (**Figure 3.24A, 3.24B**). However,

when distinct band sizes were analysed individually, there was a reduction in intact protein (**Figure 3.24C**) but not the lower band fragment (**Figure 3.24D**) in DSS-treated miR-21^{-/-} compared to WT samples. In addition, there was a significant difference in the upper/lower fragmentation ratio between control and DSS-treated miR-21^{-/-} mice (**Figure 3.24E**). This suggests that miR-21^{-/-} female mice have more intact E-cadherin in healthy conditions but alternatively have more fragmented E-cadherin during DSS-induced colitis compared to WT mice.

Colon tissue sections were analyzed for localization of E-cadherin in control and DSS-treated WT and miR-21^{-/-} female mice through immunofluorescence. In colon tissue from control mice, E-cadherin was similarly more concentrated to the tops of intestinal crypts in miR-21^{-/-} samples compared to WT, while this spatial differentiation was more seen in WT compared to miR-21^{-/-} DSS samples (**Figure 3.25A**). Quantification of E-cadherin abundance showed no significant differences between WT and miR-21^{-/-} tissue (**Figure 3.25B**).

To further investigate a potential role for E-cadherin in an inflamed environment, levels of E-cadherin were evaluated in the DSS recovery model. E-cadherin protein levels were checked in distal and proximal colon tissue of DSS-treated WT and miR-21^{-/-} mice in recovery through immunoblot. For male mice, there was a clear, strong signal for the lower band fragment, but the only difference observed was a decrease in intact protein in miR-21^{-/-} compared to WT mice (**Figure 3.26A, 3.26B, 3.26C, 3.26D**), resulting in a reduced upper/lower fragmentation ratio (**Figure 3.26E**). In male proximal tissue, combined and individual band analysis showed no difference between WT and miR-21^{-/-} DSS-treated mice in recovery (**Figure 3.26F, 3.26G, 3.26H, 3.26I, 3.26J**). As DSS is known to primarily target the distal colon²¹³, localization of E-cadherin in distal tissue sections were assessed in DSS-treated WT and miR-21^{-/-} male mice in recovery through immunofluorescence. Compared to healthy tissue, E-cadherin was less concentrated to the tops of intestinal crypts and more evenly spread in miR-21^{-/-} tissue samples compared to WT (**Figure 3.27A**). There was no difference in quantified fluorescence among mouse groups (**Figure 3.27B**). This suggests that DSS-induced inflammation could stimulate different forms of E-cadherin with regional function along affected intestinal epithelia, depending on the severity of inflammation.

For female mice, overall levels of E-cadherin showed a reduced trend in miR-21^{-/-} distal colon tissue compared to WT mice (**Figure 3.28A, 3.28B**). There was a much weaker upper band signal, and quantification of individual bands showed an induction in intact

protein and reduced levels of the lower band fragment, resulting in a higher upper/lower fragmentation ratio in miR-21^{-/-} compared to WT mice (**Figure 3.28C, 3.28D, 3.28E**). In proximal tissue, there was a significant reduction in overall levels of E-cadherin, with a significantly weaker signal for intact protein in miR-21^{-/-} samples compared to WT, resulting in a reduced upper/lower fragmentation ratio (**Figure 3.28F, 3.28G, 3.28H, 3.28I, 3.28J**). Distal tissue sections were again evaluated for localization of E-cadherin in WT and miR-21^{-/-} female mice through immunofluorescence. E-cadherin was more evenly spread and less concentrated to the polar ends of intestinal crypts in miR-21^{-/-} tissue compared to WT, although there was notably reduced signal strength in all female DSS tissue (**Figure 3.29A**). Nonetheless, there was no difference in quantified fluorescence between WT and miR-21^{-/-} female tissue (**Figure 3.29B**). Similar to results seen in males, this suggests that E-cadherin may have different structural consequences that are regionally specific and dependent on the amount of inflammation.

AJs are large protein complexes of various barrier stabilizing components crucial for adherence between intestinal epithelial cells. Other AJ proteins such as α -catenin, p120-catenin and β -catenin were investigated for their abundance in WT and miR-21^{-/-} colon tissue of the recovery model, with a focus on male mice due to the stark difference in disease severity observed during DSS-induced colitis. Interestingly, there were notable reductions in all AJ proteins tested within miR-21^{-/-} male distal tissue compared to WT (**Figure 3.30A, 3.30B, 3.30C, 3.30D, 3.30E, 3.30F**). Moreover, there were no major differences in protein AJ protein levels between WT and miR-21^{-/-} male proximal tissue (**Figure 3.31A, 3.31B, 3.31C, 3.31D**). Thus, these findings point out that counterintuitively, there may be less significance for stable AJs post-DSS-induced colitis, leading to more plasticity along the recovering gut epithelial barrier.

To continue investigating the possibility that E-cadherin is a direct target of miR-21, HEK293T cells were used for a luciferase assay. These cells are frequently used for their high transfection efficiency.²⁵¹ Previous studies have conducted a treatment of LPS rather than TNF- α , along with a miR-21 modulating compound, to induce miR-21 expression.⁸⁹ Here, HEK293T cells were transfected with the pCMV-miR-21 overexpression plasmid and treated with 100 ng/mL LPS. First, levels of miR-21-5p and miR-21-3p were measured in transfected HEK293T cells under basal and LPS conditions. Both miR-21-5p and miR-21-3p were induced in cells transfected with pCMV-miR-21 overexpression plasmid compared to control groups, but more prevalent under LPS treatment (**Figure 3.32A, 3.32B, 3.32C, 3.32D**). Therefore, LPS conditions were used to

conduct the luciferase assay. Transfection efficiency of the luciferase vector was normalized to Renilla luciferase activity of the pmirGLO-*CDH1*-3'UTR construct. pmirGLO-*CDH1*-3'UTR had significantly reduced luminescence in LPS-treated cells transfected with pCMV-miR-21 compared to control, indicating that miR-21 directly targeted *CDH1*-3'UTR seed region (**Figure 3.32E**). In addition, there was much less luminescence overall compared to luciferase empty controls which could mean one of two possibilities: 1) other active regulators not studied here may have also targeted E-cadherin, or 2) the mere addition of the 3'-UTR of E-cadherin may have impacted luciferase function.

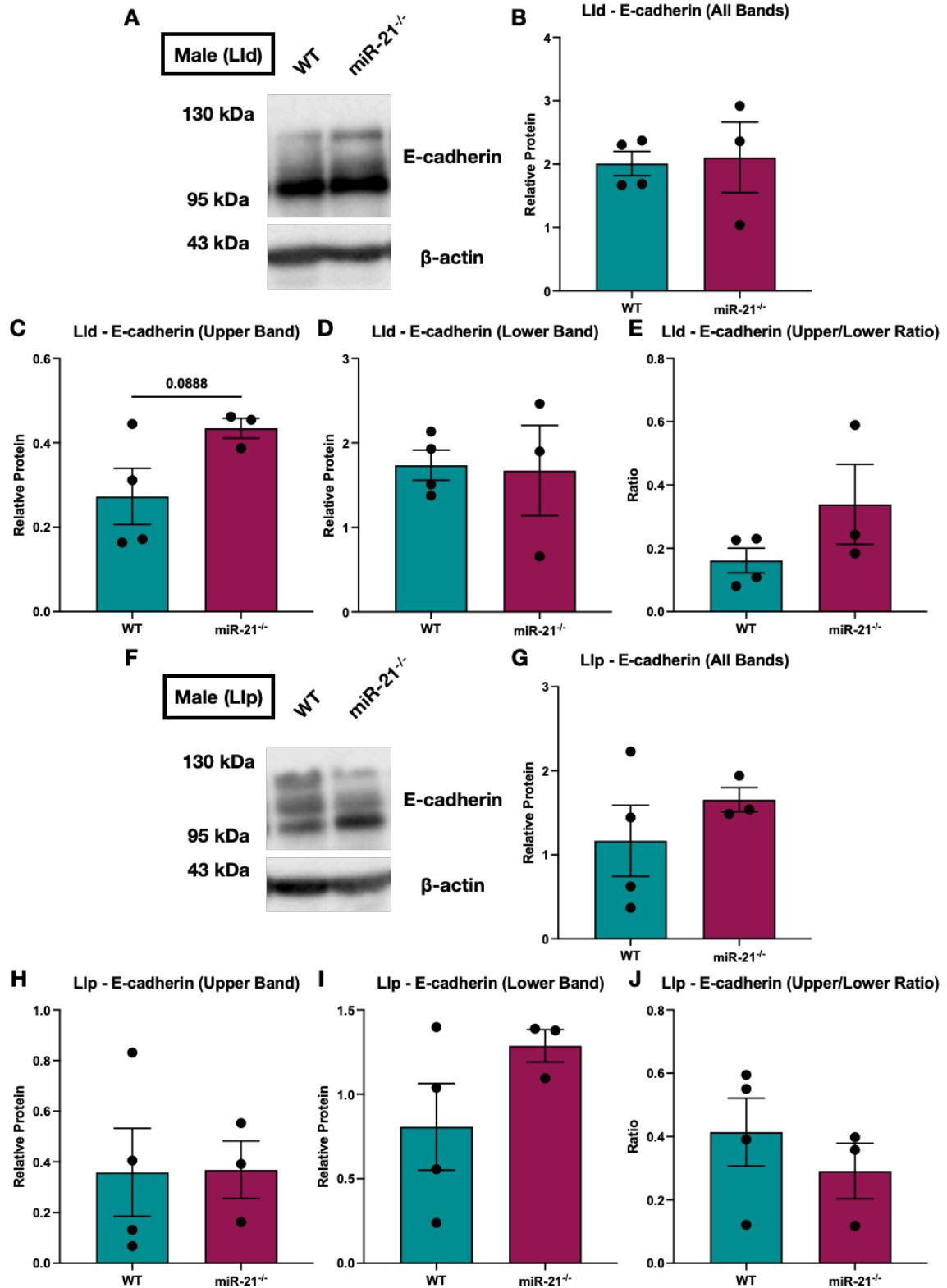


Figure 3.21 – Analysis of E-cadherin shows induction and potential differences in size fragments between distal and proximal colon tissue of WT and miR-21^{-/-} male non-littermate mice under healthy conditions. Protein lysates were obtained from healthy 10-week old WT and miR-21^{-/-} male mice. Immunoblots for E-cadherin from (A) distal colon / large intestine (LId) and (F) proximal colon / large intestine (Llp) tissue of WT and miR-21^{-/-} mice. Protein quantification of immunoblots combining (B + G) both bands, (C + H) upper band only, (D + I) lower band only and (E + J) upper/lower band fragmentation ratio. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

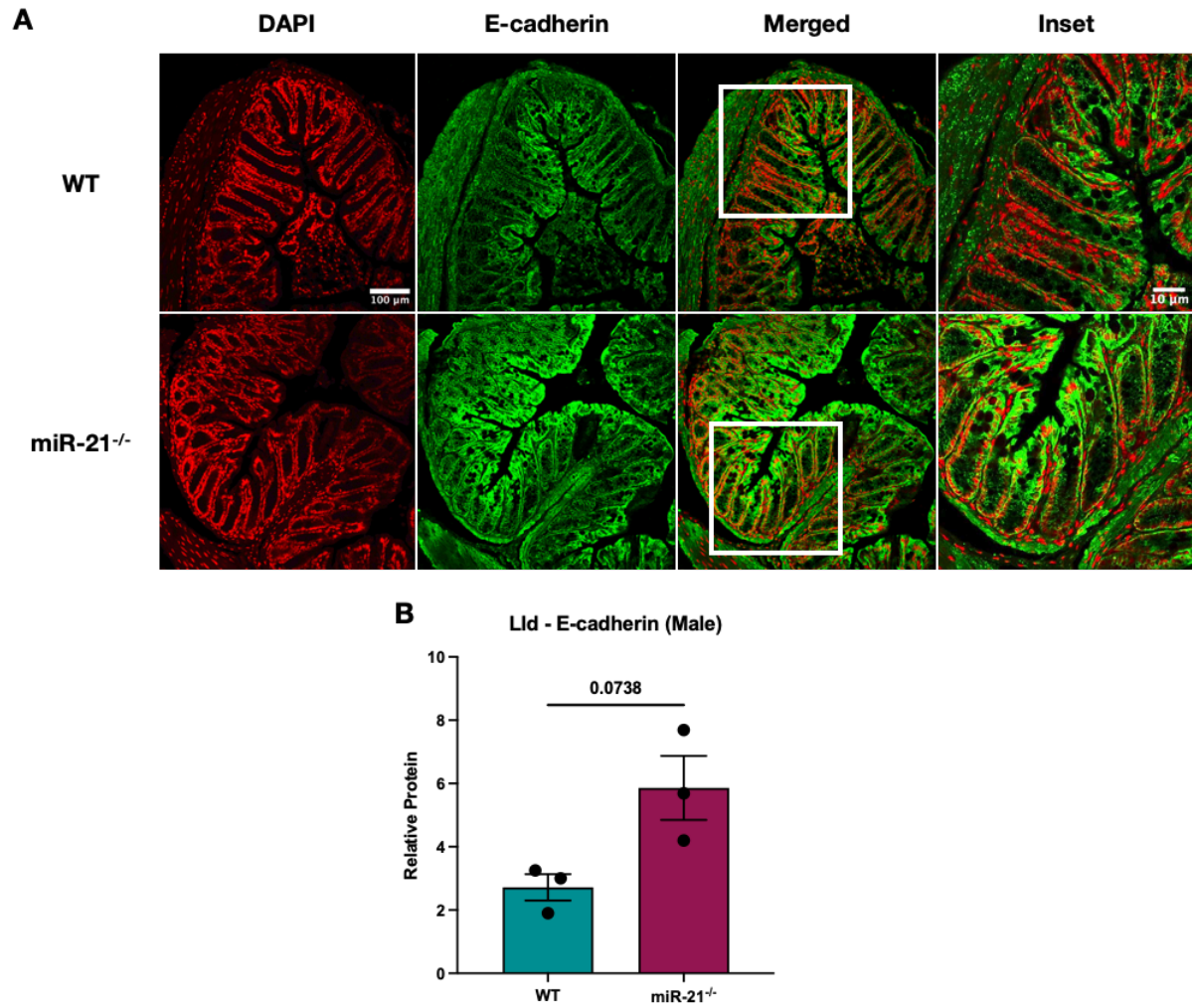


Figure 3.22 – E-cadherin is induced and localizes to the top of intestinal crypts in non-littermate miR-21^{-/-} male distal colon tissue under healthy conditions. (A) Distal colon / large intestine (LId) tissue sections of WT and miR-21^{-/-} mice were removed, flushed with PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (red) and anti-E-cadherin primary antibody (green). (B) Quantification of fluorescence was normalized to number of nuclei per field of view. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

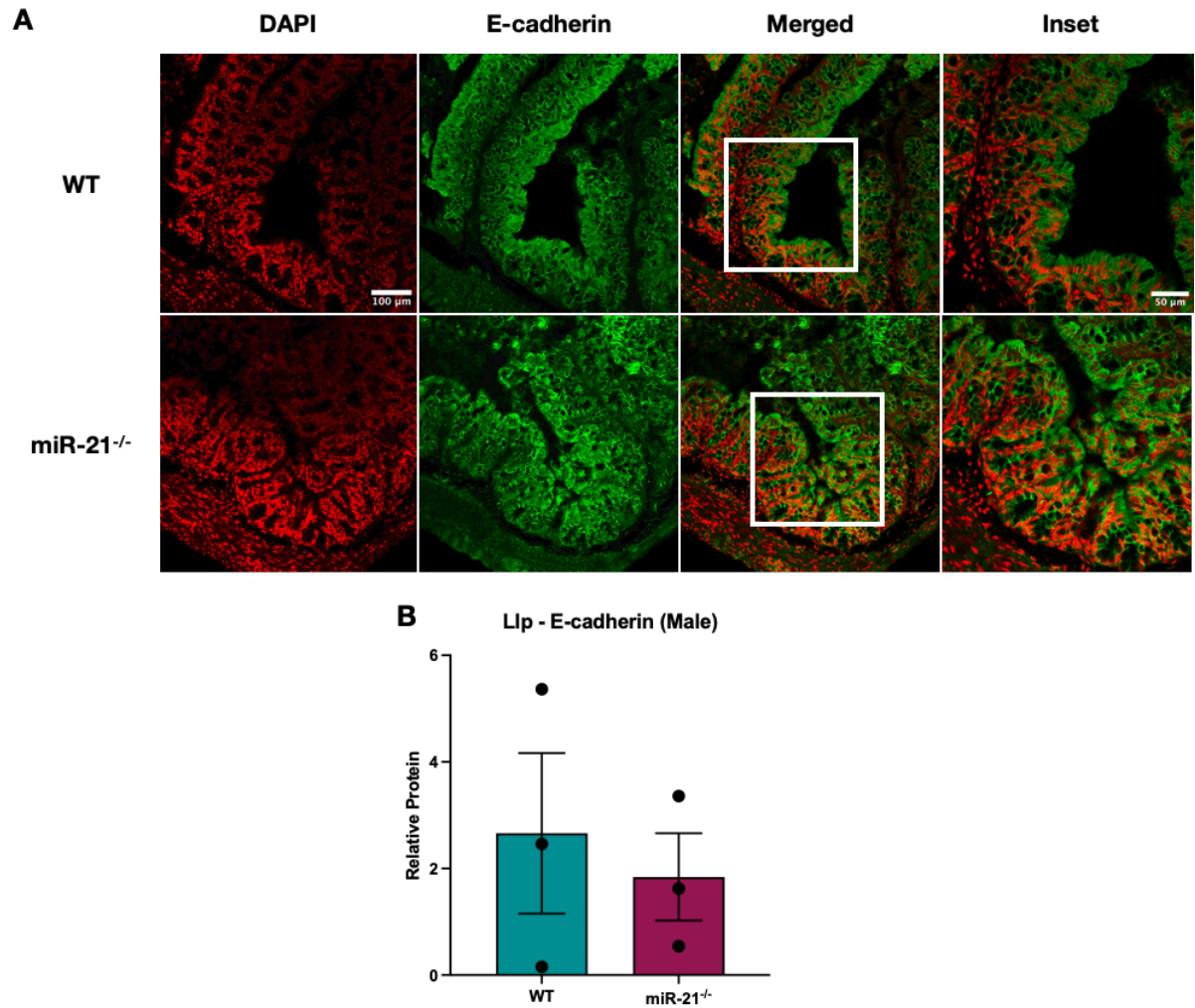


Figure 3.23 – E-cadherin levels are unchanged but still localize to the top of intestinal crypts in non-littermate miR-21^{-/-} male proximal colon tissue under healthy conditions. (A) Proximal colon / large intestine (Lip) tissue sections of WT and miR-21^{-/-} mice were removed, flushed with PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (red) and anti-E-cadherin primary antibody (green). (B) Quantification of fluorescence was normalized to number of nuclei per field of view. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

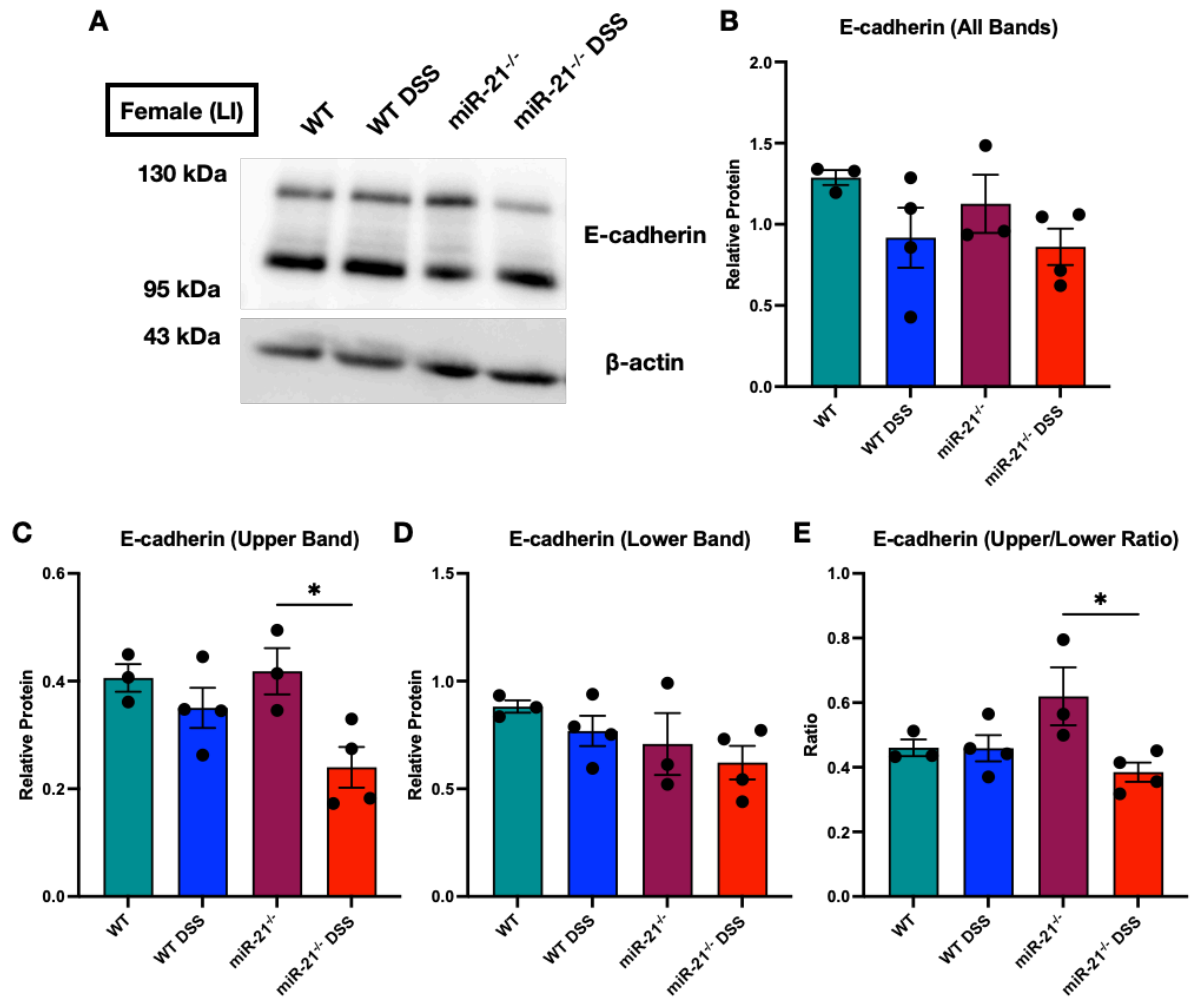


Figure 3.24 – Analysis of E-cadherin shows reduced levels of intact protein in whole colon tissue from miR-21^{-/-} female mice compared to WT. Protein lysates were obtained from healthy and DSS-treated 10-week old WT and miR-21^{-/-} female mice. (A) Immunoblot for E-cadherin from whole colons of WT and miR-21^{-/-} female mice. Protein quantification of immunoblots combining (B) both bands, (C) upper band only, (D) lower band only and (E) upper/lower band fragmentation ratio. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with * - $p \leq 0.05$.

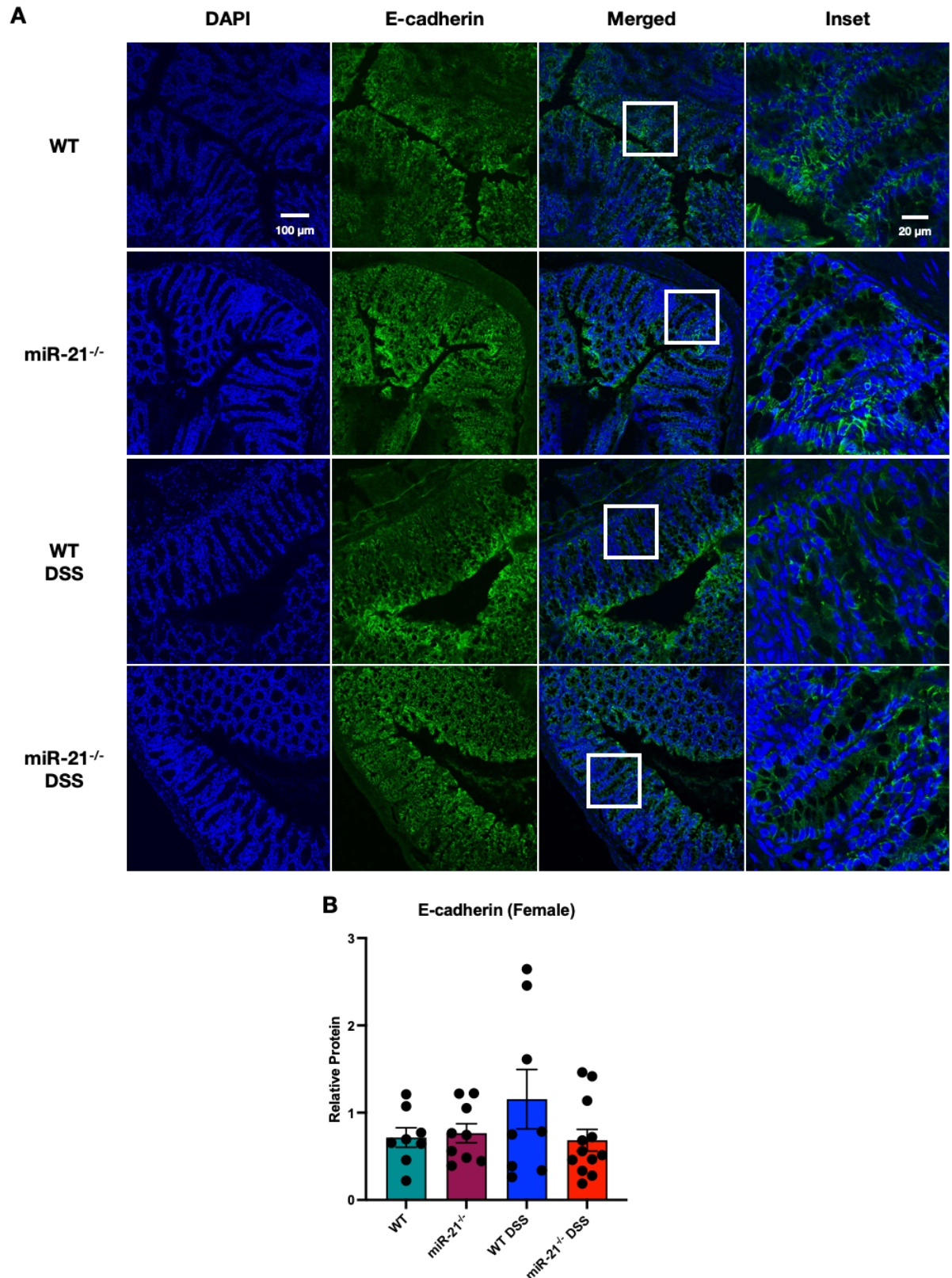


Figure 3.25 – Differential localization is lost as E-cadherin localizes to the top of intestinal crypts in non-littermate DSS-treated WT and miR-21^{-/-} female colon tissue. (A) Whole colon sections of healthy and DSS-treated WT and miR-21^{-/-} mice were removed, flushed with PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (B) Quantification of fluorescence was normalized to number of nuclei per field of view. Denoted are $n \geq 8$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

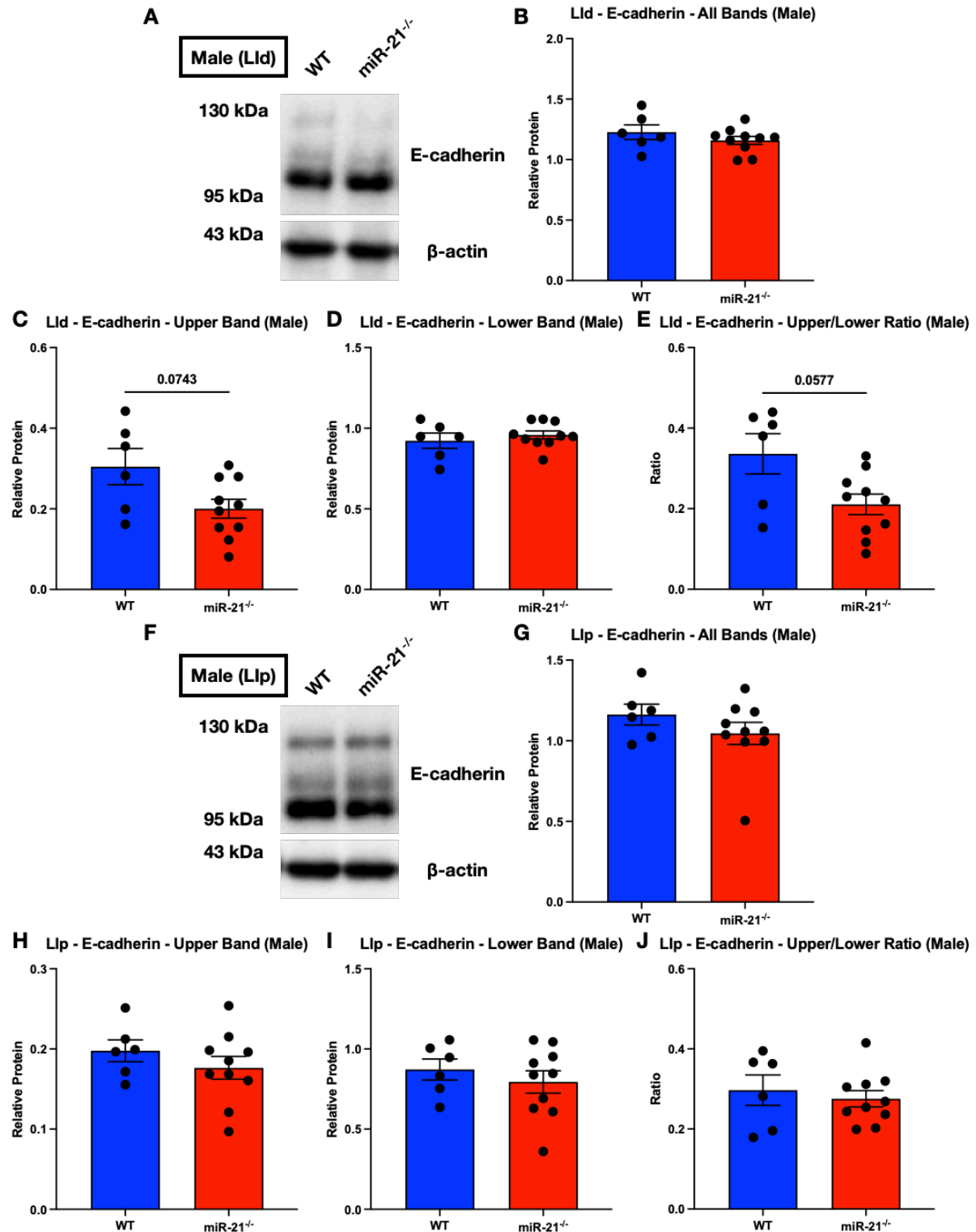


Figure 3.26 – Analysis of E-cadherin levels shows differences in size fragments between distal tissue of non-littermate WT and miR-21^{-/-} male mice of DSS recovery model. Protein lysates were obtained from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. Immunoblot for E-cadherin from (A) distal colon / large intestine (LId) and (F) proximal colon / large intestine (LIp) tissue of WT and miR-21^{-/-} mice. Protein quantification of immunoblots combining (B + G) both bands, (C + H) upper band only, (D + I) lower band only and (E + J) upper/lower band fragmentation ratio. Denoted are $n \geq 6$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

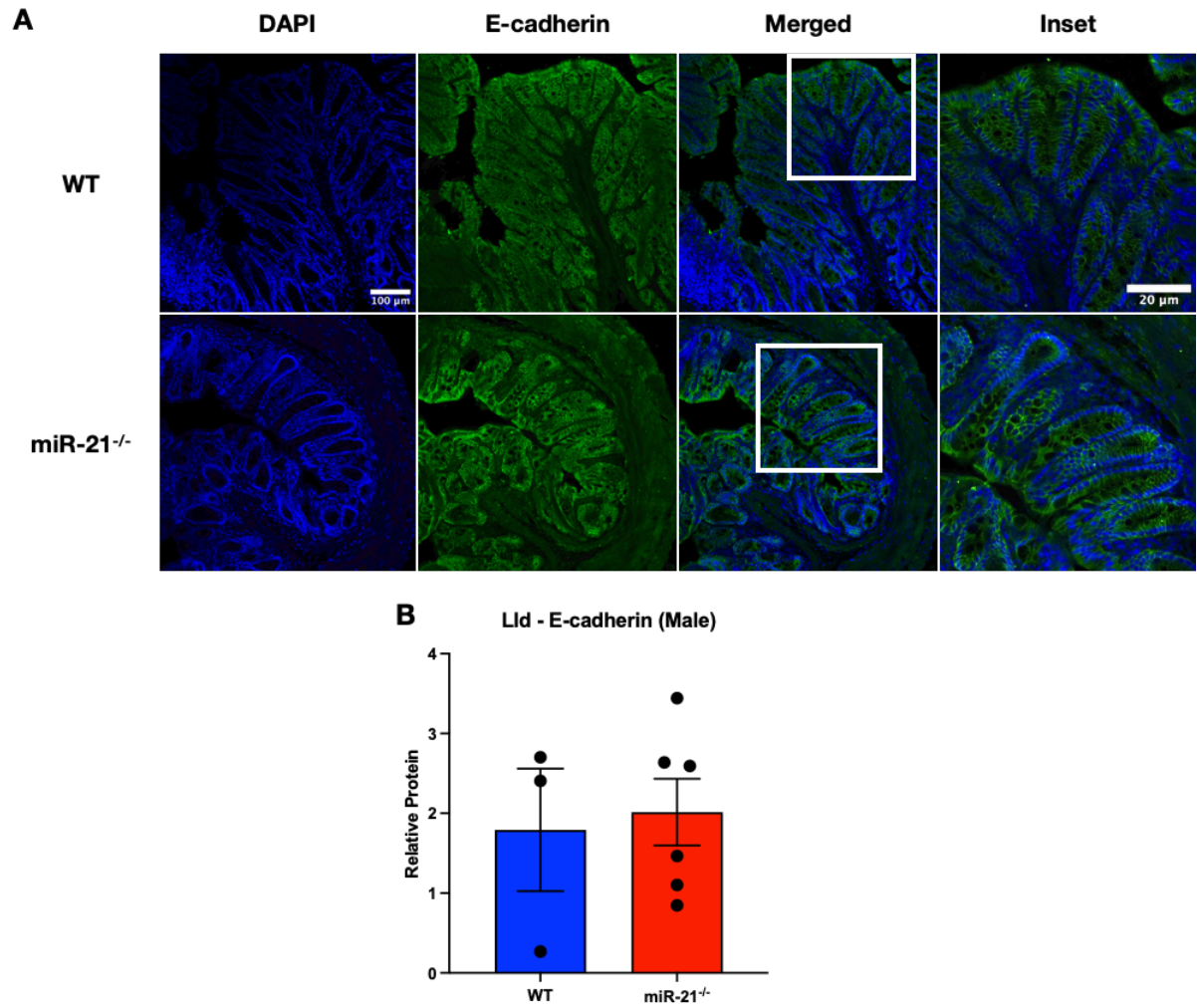


Figure 3.27 – E-cadherin is more spread along the crypt axis in non-littermate miR-21^{-/-} male distal colon tissue of DSS recovery model. (A) Distal colon / large intestine (LId) tissue sections of WT and miR-21^{-/-} mice were removed, flushed with sterile saline and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (B) Quantification of fluorescence was normalized to number of nuclei per field of view. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

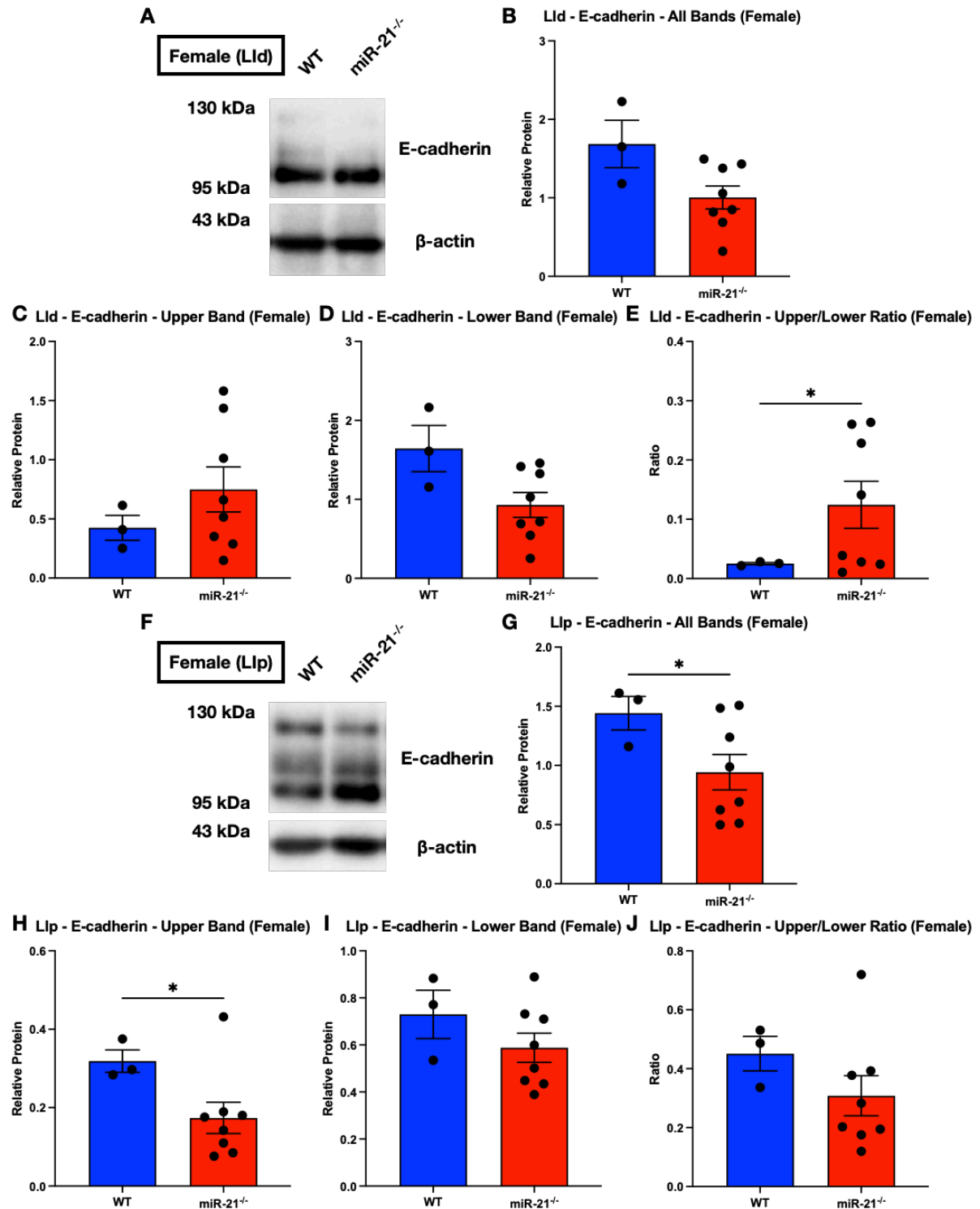


Figure 3.28 – Analysis of E-cadherin levels shows differences in size fragments between non-littermate WT and miR-21^{-/-} female distal and proximal tissue of DSS-induced colitis recovery model. Protein lysates were obtained from 8-15 week old WT and miR-21^{-/-} female mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. Immunoblot for E-cadherin from (A) distal colon / large intestine (LId) and (F) proximal colon / large intestine (LIp) tissue of WT and miR-21^{-/-} mice. Protein quantification of immunoblot (B + G) combining both bands, (C + H) upper band only, (D + I) lower band only and (E + J) upper/lower band fragmentation ratio. Denoted are n ≥ 3 mice per group, mean values ± SEM, *p* value calculations using Welch's *t*-test with * - *p* ≤ 0.05.

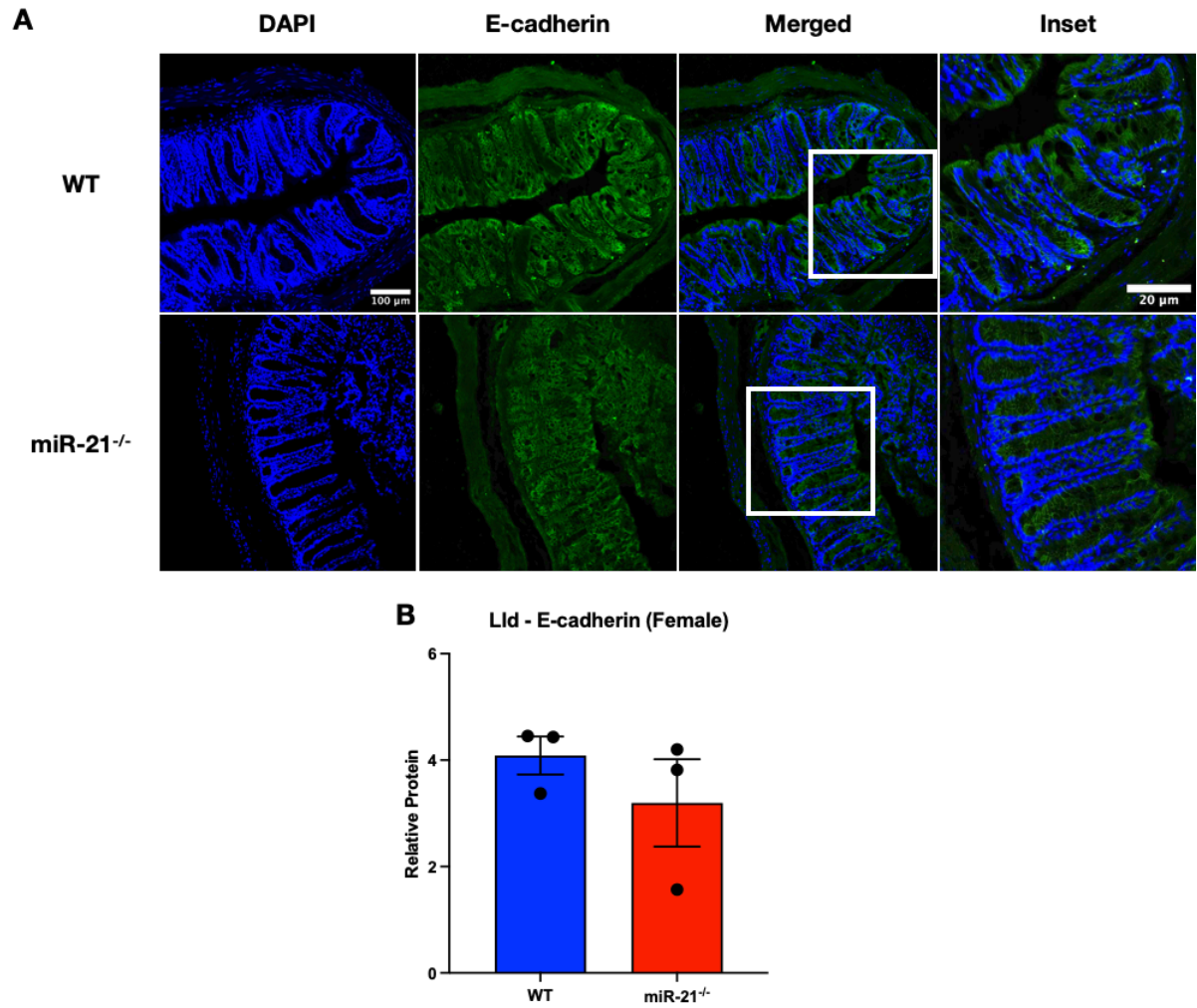


Figure 3.29 – E-cadherin is more evenly dispersed along crypt axis in non-littermate miR-21^{-/-} female distal colon tissue of DSS recovery model. (A) Distal colon / large intestine (LId) tissue sections of WT and miR-21^{-/-} mice were removed, flushed with sterile saline and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (B) Quantification of fluorescence was normalized to number of nuclei per field of view. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t -test.

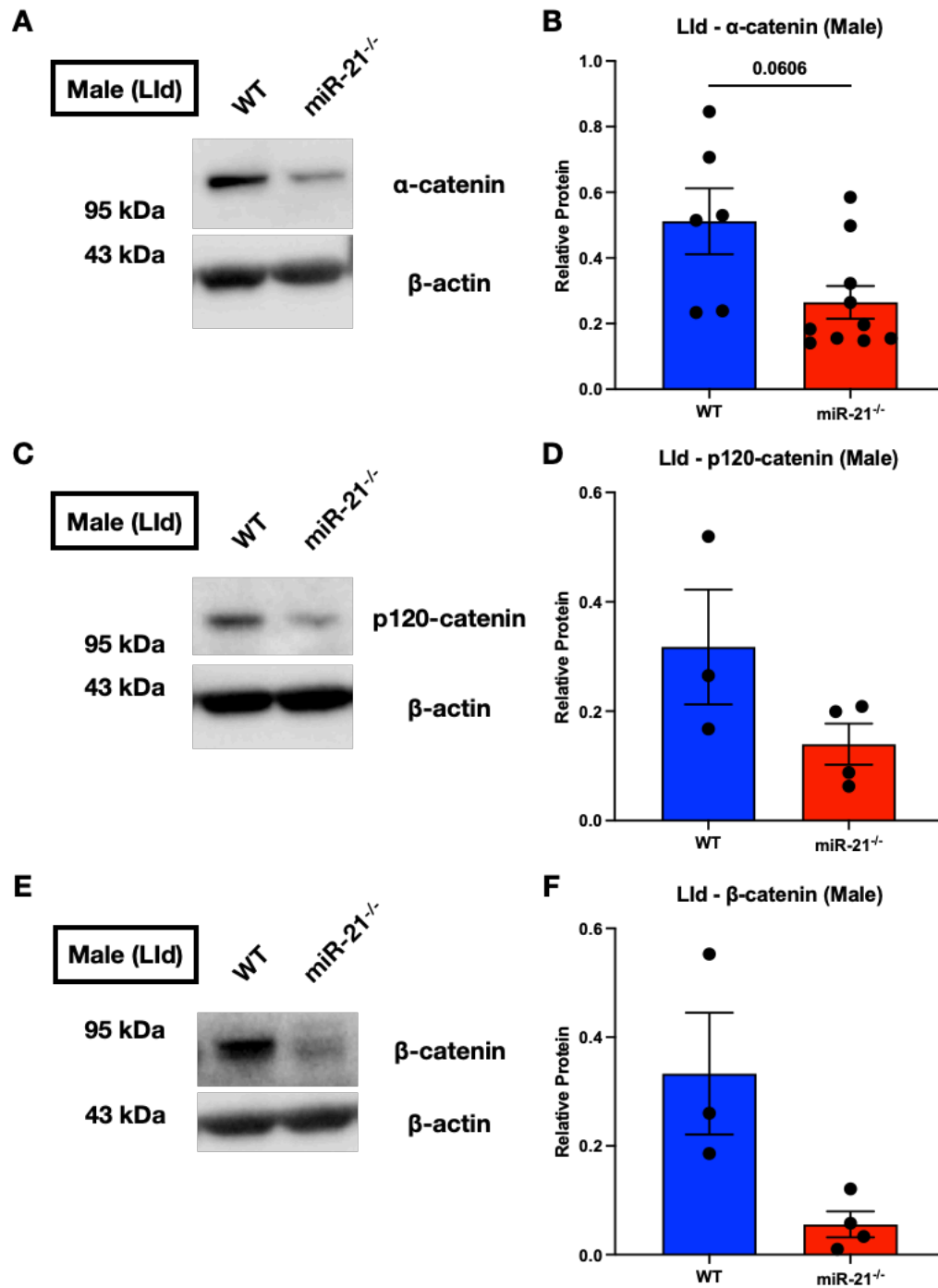


Figure 3.30 – Analysis of adherens junction protein levels shows a reduction in α -catenin, p120-catenin and β -catenin in distal colon tissue of non-littermate miR-21^{-/-} male mice of DSS recovery model. Protein lysates were obtained from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. Immunoblots and protein quantification for (A + B) α -catenin, (C + D) p120-catenin and (E + F) β -catenin from distal colon / large intestine (LId) tissue of WT and miR-21^{-/-} mice. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

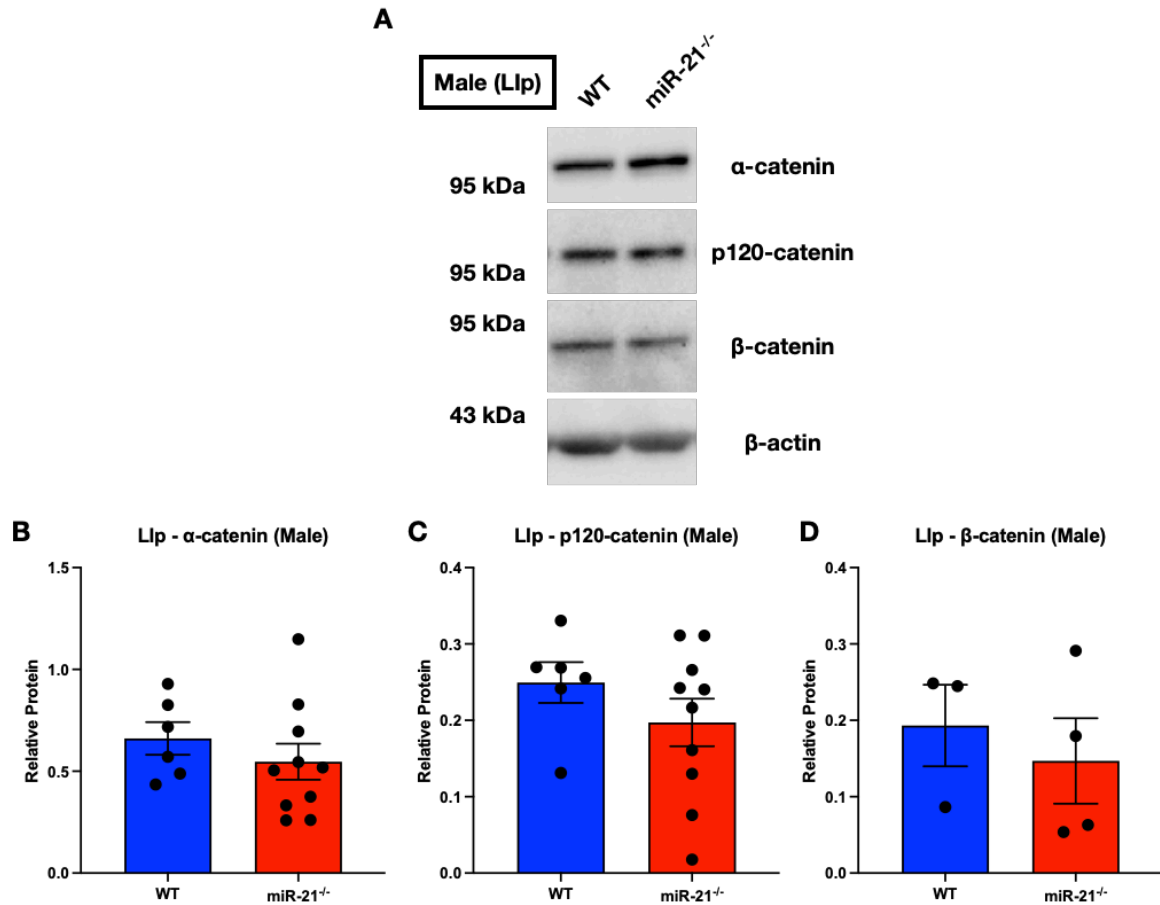


Figure 3.31 – Analysis of adherens junction protein levels shows no differences in proximal colon tissue of non-littermate WT and miR-21^{-/-} male mice of DSS recovery model. Protein lysates were obtained from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. (A) Immunoblots and protein quantifications for (B) α -catenin, (C) p120-catenin and (D) β -catenin from proximal colon / large intestine (Lip) tissue of WT and miR-21^{-/-} mice. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

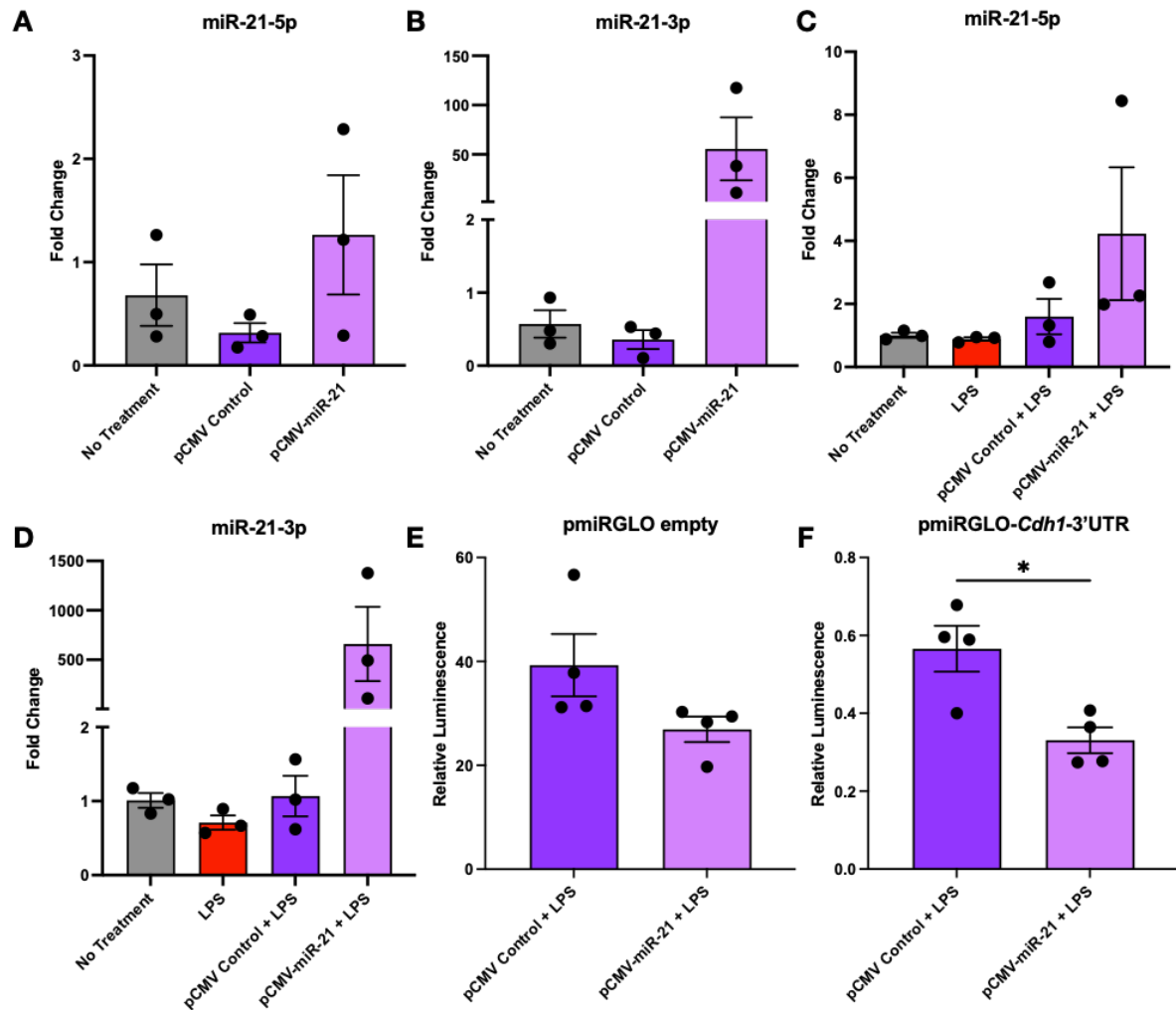


Figure 3.32 – E-cadherin was directly targeted by miR-21 in HEK293T cells under miR-21 overexpression conditions. qRT-PCR analyses of miR-21-5p and miR-21-3p in Caco-2 cells transfected with pCMV-miR-21 under (A + B) basal and (C + D) LPS-treated conditions. Luciferase assay of LPS-treated Caco-2 cells transfected with 25 ng (E) pmiRGLO empty vector and (F) pmiRGLO-*CDH1*-3'UTR. Denoted are $n \geq 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with * - $p \leq 0.05$.

3.2.5 miR-21 ablation increases wound healing gene expression through intercellular junctions and TGF- β pathway *in vivo*

Components of proliferation and wound healing processes in gut epithelia were checked in the *in vivo* miR-21 KO model. Namely, Ki-67 (encoded by *MKI67*) is a proliferation marker studied in Hodgkin lymphoma, but also involved in cell cycle regulation, heterochromatin maintenance, and assembly of the perichromosomal layer on mitotic chromosomes.²⁵² Regarding intercellular junctions, one study found that JAM-A (encoded by *F11R*) is essential for wound healing in the gut and is known to interact with E-cadherin at the apical end.²⁵³ Both *MKI67* and *F11R* are found in miR-21 prediction databases. Additionally, a large component of the wound healing apparatus at the cellular level can be attributed to proper functioning of the TGF- β signalling pathway. Studies on the TGF- β family of proteins demonstrated its significance in tissue repair through the attraction of monocytes and fibroblasts, upregulating ECM deposition and inhibiting metalloproteinases, as well as promoting epithelialization after EMT.¹⁹⁶ Genes for wound healing, TGF- β ligand and TGF- β receptors appeared in miR-21 prediction databases. A summary for these wound healing targets can be found in **Table 3.2** below.

Table 3.2 – miR-21 predicted target search related to wound healing.

Common Name	Gene Name	TargetScan (score*)	miRDB (score*)	miRWalk (score*)	DIANA-TarBasev9.0 (score*)	Pictar (score*)
Ki-67	<i>MKI67</i>			5p (0.85), 3p (0.92)	5p (1.00), 3p (0.41)	
JAM-A	<i>F11R</i>	3p (0.15)		5p (1.00), 3p (0.92)	5p (1.00)	
TGF- β	<i>TGFB1</i>					
TGF β R	<i>TGFBRI</i>			3p (0.92)	5p (0.62)	
TGF β R2	<i>TGFBR2</i>	5p (0.17), 3p (0.31)		3p (0.92)	5p (0.33)	
TGF β R3	<i>TGFBR3</i>	3p (0.02)		3p (1.00)	5p (0.03)	

* - all scores are out of 1; note that the score from miRDB was divided by 100 and the score from Pictar was divided by 10 to equate to scores from other databases.

Expression levels of mouse homologs *Mki67* and *F11r* were assessed in distal and proximal colon tissue of WT and miR-21^{-/-} mice of the recovery model for affects by miR-

21. In male mice, *Mki67* was significantly downregulated in miR-21^{-/-} distal tissue compared to WT, while *Fllr* was significantly upregulated in miR-21^{-/-} distal tissue (**Figure 3.33A, 3.33B**). In female mice, there was an induced trend for both *Mki67* and *Fllr* in miR-21^{-/-} distal tissue compared to WT (**Figure 3.33C, 3.33D**). There were no notable differences in proximal tissue between WT and miR-21^{-/-} mice for either sex. This suggests that cell proliferation, an important step in wound healing, may not be impacted by miR-21 regulation.

Due to the importance of TGF- β pathway activation in wound healing, TGF- β and TGF- β receptors were checked in remaining colon tissue of WT and miR-21^{-/-} DSS-treated mice of the recovery model. In male mice, ELISAs for TGF- β demonstrated a significant upregulation in miR-21^{-/-} mice compared to WT (**Figure 3.34A**), while there was no difference in ligand levels between groups in female mice (**Figure 3.34B**). Regarding expression levels of receptors, both *Tgfb1* and *Tgfb2* were induced in miR-21^{-/-} male mice compared to WT, with no notable difference observed with *Tgfb3* (**Figure 3.34C, 3.34D, 3.34E**). In female mice, only *Tgfb1* showed an induced trend in miR-21^{-/-} compared to WT (**Figure 3.34F, 3.34G, 3.34H**). These findings indicate that the presence of miR-21 may inhibit activation of the TGF- β pathway through receptor downregulation during recovery phase after DSS-induced colitis.

There is a complex network of different cell types that compose whole colon tissue that include IECs, hematopoietic cells, stem cells and fibroblasts.²⁵⁴ With a particular focus on the gut epithelial barrier, IECs were isolated from WT and miR-21^{-/-} non-littermate male mice in order to assess wound healing genes of interest. After separation from intestinal tissue, IEC isolates were grown *ex vivo* in Matrigel (Corning) with complete DMEM on 96-well plates for seven days. Images were taken on the final day that showed various clusters of IECs that adhered to the plate, with a density seemingly higher in miR-21^{-/-} isolate IECs compared to WT (**Figure 3.35A**). Cell viability was confirmed through the alamarBlue assay, demonstrating significantly greater viability compared to cells treated with Triton-X-100 for both WT and miR-21^{-/-} samples (**Figure 3.35B**). Subsequent analyses on known miR-21 targets, AJ-related wound healing genes and TGF- β receptors were carried out to determine any specific differences within IECs isolated from mouse groups. *Smad7*, encoding an I-Smad that downregulates TGF- β pathway activation and a known direct target of miR-21²⁵⁵, was also included. Both *Pdcd4* and *Pten* were induced in miR-21^{-/-} compared to WT IECs (**Figure 3.36A, 3.36B**). Furthermore, *Cdh1* and *Fllr* were also significantly upregulated in miR-21^{-/-} IECs compared to WT (**Figure 3.36C, 3.36D**).

For the TGF- β pathway, mRNA levels of the inhibiting *Smad7* were increased in miR-21^{-/-} IECs as expected (**Figure 3.36E**). Regarding TGF- β receptors, both *Tgfb1* and *Tgfb2* were significantly upregulated in miR-21^{-/-} compared to WT IECs (**Figure 3.36F, 3.36G**), while there was no difference in expression levels for *Tgfb3* (**Figure 3.36H**). Therefore, this suggests that stable AJs and the presence of a negative feedback loop in which TGF- β receptors are upregulated for pathway activation but tightly controlled via the presence of *Smad7*, are important for the homeostasis of gut epithelial barrier integrity.

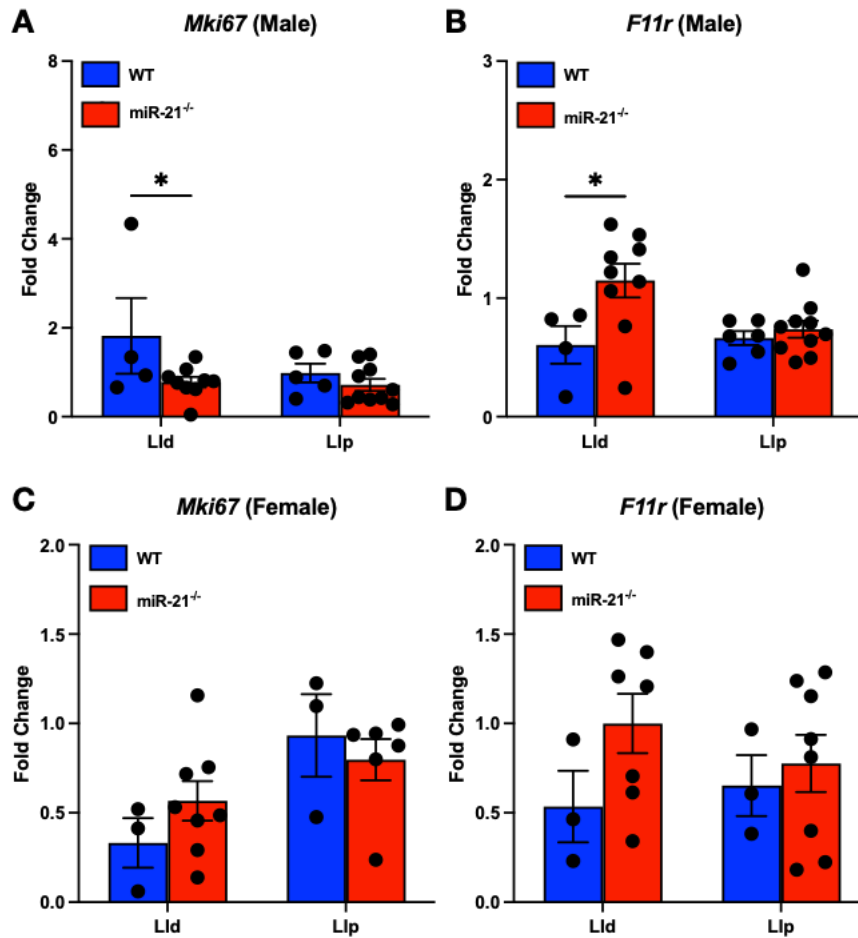


Figure 3.33 – *F11r* is induced in miR-21^{-/-} mice in DSS recovery model. RNA was isolated from 8-15 week old WT and miR-21^{-/-} mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. (A + B) qRT-PCR analyses of *Mki67* and *F11r* in distal colon / large intestine (Lld) and proximal colon / large intestine (Lip) tissue of WT and miR-21^{-/-} mice. (C + D) qRT-PCR analyses of *Mki67* and *F11r* in Lld and Lip tissue of WT and miR-21^{-/-} female mice. Denoted are $n \geq 4$ mice per male group or $n \geq 3$ mice per female group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

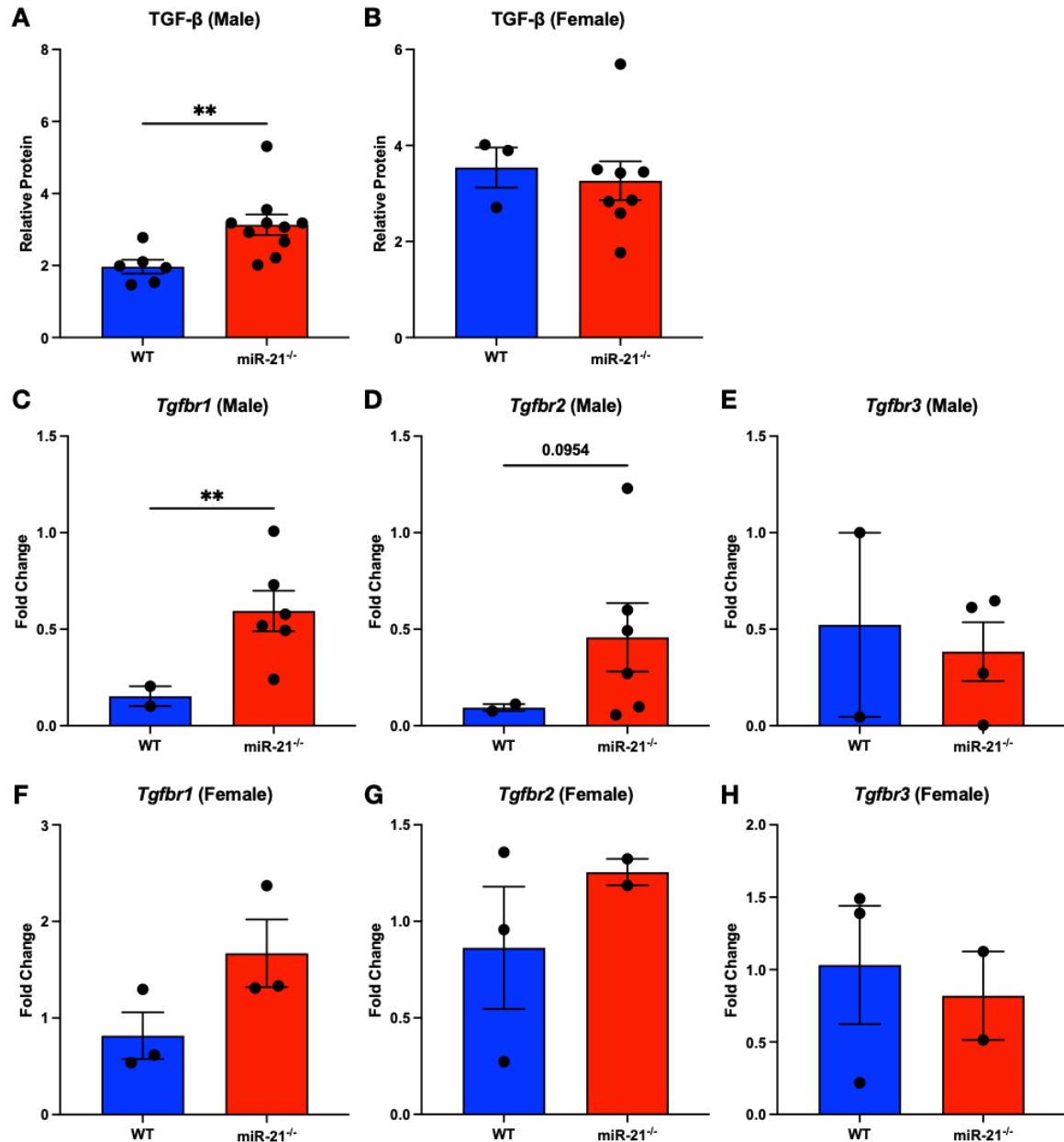


Figure 3.34 – TGF-β and TGF-β receptors were upregulated in miR-21^{-/-} colon tissue of DSS recovery model. Protein lysates and RNA were obtained from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. ELISA for TGF-β in (A) male and (B) female whole colon tissue of WT and miR-21^{-/-} of the DSS recovery model. qRT-PCR analyses of TGF-β receptors in (C-E) male and (F-H) female whole colon tissue of WT and miR-21^{-/-} of the DSS recovery model. Denoted are n ≥ 3 mice per group with the exception of male WT and female miR-21^{-/-} TGF-β receptor qPCRs that have n ≥ 2 mice per group, mean values ± SEM, p value calculations using Welch's t-test with ** - p ≤ 0.01.

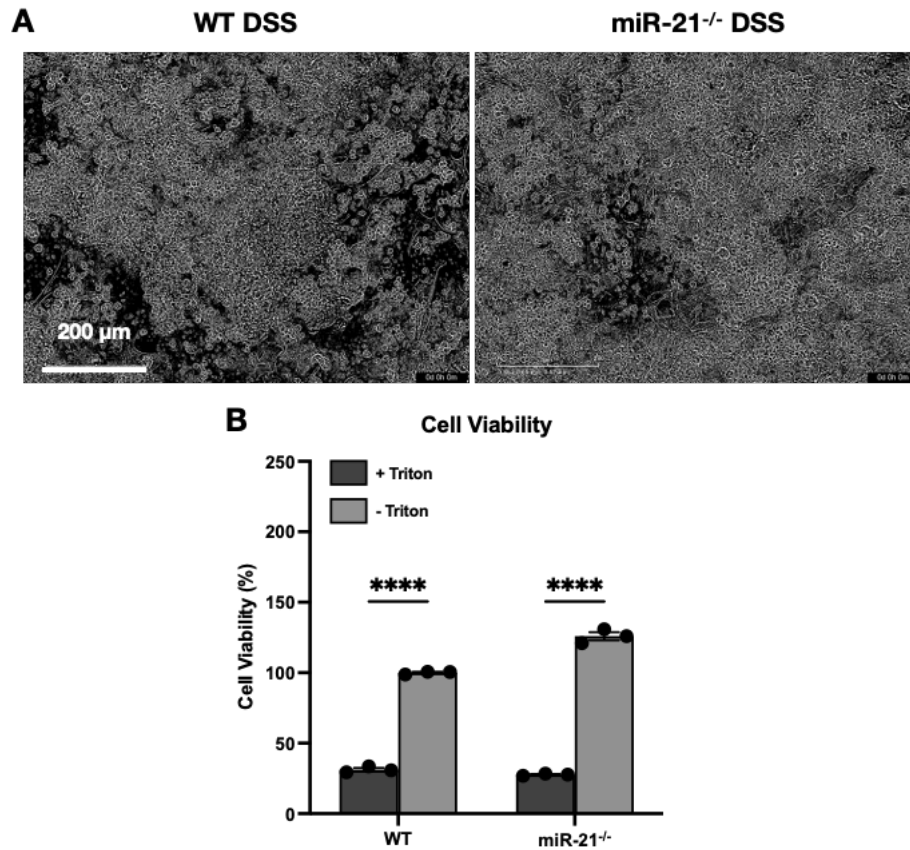


Figure 3.35 – Isolated intestinal epithelial cells from non-littermate WT and miR-21^{-/-} male mice are viable. Healthy 10-week old WT and miR-21^{-/-} male mice had colons digested in a digestion mixture of 0.3 mL collagenase dissolved in DMEM. Isolated IECs were plated on Matrigel (Corning) basement membrane with complete DMEM in 96-well plates for seven days. (A) Microscopy images of IECs from WT and miR-21^{-/-} mice taken seven days after plating. (B) alamarBlue assay measuring cell viability of WT and miR-21^{-/-} mice in the presence or absence of Triton X-100. Denoted are $n = 3$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with **** - $p \leq 0.0001$.

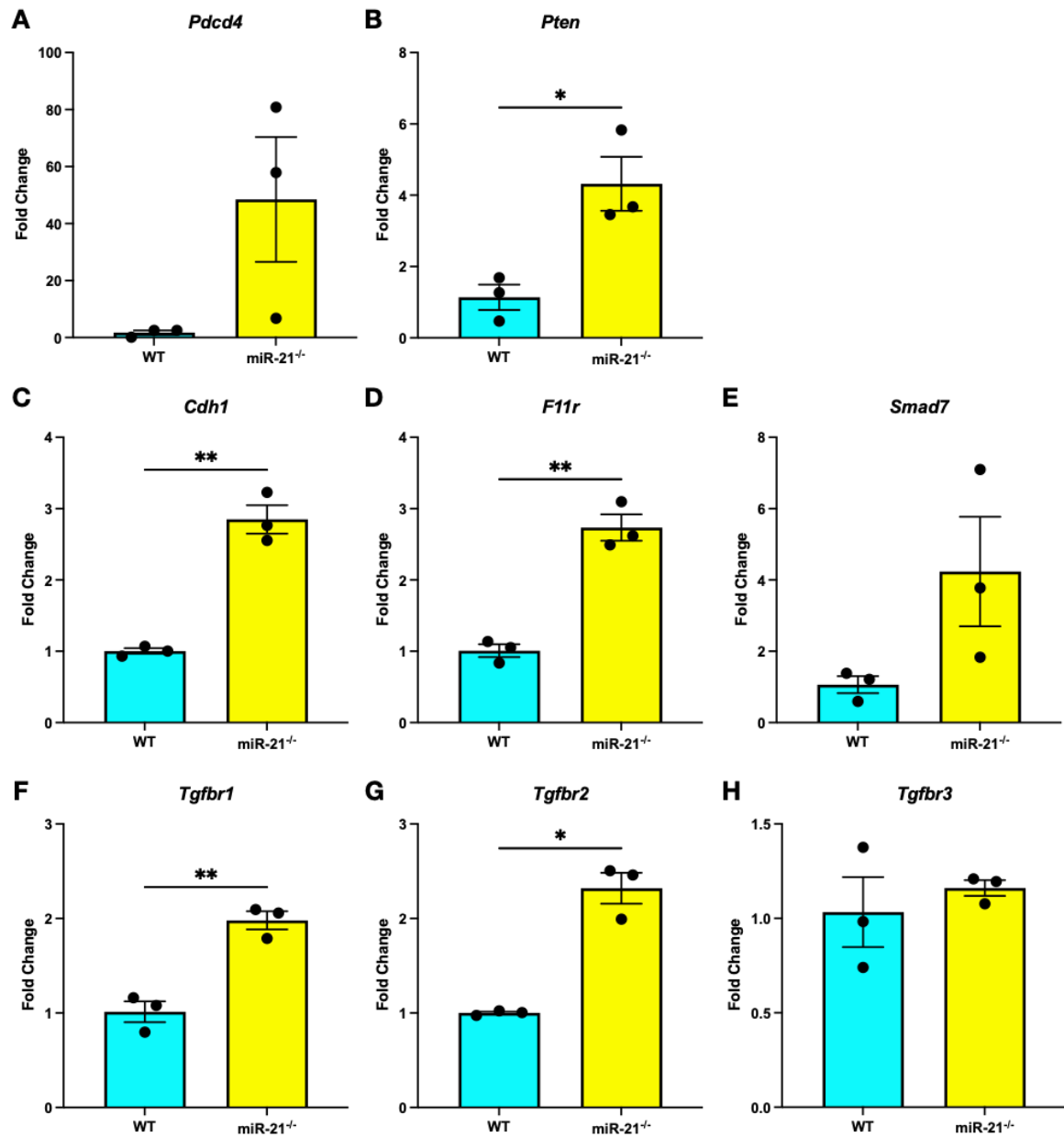


Figure 3.36 – AJ-related wound healing and TGF- β family genes were upregulated in IECs isolated from miR-21^{-/-} male mice under basal conditions. Healthy 10-week old WT and miR-21^{-/-} male mice had colons digested in a digestion mixture of 0.3 mL collagenase dissolved in DMEM. Isolated IECs were plated on Matrigel (Corning) basement membrane with complete DMEM in 96-well plates for seven days. RNA was isolated from IECs on the seventh day. qRT-PCR analyses from IECs isolated from WT and miR-21^{-/-} mice for (A + B) Known targets of miR-21: *Pdcd4*, *Pten*. (C + D) Junction related wound healing genes: *Cdh1*, *F11r*. (E-H) TGF- β family genes: *Smad7*, *Tgfr 1-3*. Denoted are n = 3 mice per group, mean values $\pm$ SEM, *p* value calculations using Welch's with * - $p \leq 0.05$, ** - $p \leq 0.01$.

3.2.6 E-cadherin is negatively associated to miR-21 levels in Caco-2 cells

It was of particular interest to investigate whether these negative associations between miR-21 and AJs in mice are transferrable to human models. To compliment the *in vivo* KO model with an *in vitro* system, modulating miR-21 levels would prove useful to observe direct consequences of miR-21 regulation. Here, to first replicate what was shown in previous studies using human cell lines²⁵⁶, the human adenocarcinoma cell line Caco-2 was used to model the intestinal epithelium during TNF- α inflammation, a state in which miR-21 is induced.

Expression of miR-21 and AJ targets of interest in response to TNF- α treatment was examined in Caco-2 cells. A dose response with TNF- α titrations (0, 50, 100, 150 ng/mL) and comparison among TNF- α treatment methods (ie. administration via apical, basal or both chambers) on epithelial monolayers differentiated to 21 days was performed in a 12-well transwell plate. Apical chamber addition of 150 ng/uL TNF- α had the most consistent upregulation of pri-miR-21, as well as mature forms miR-21-5p and miR-21-3p (**Figure 3.37A, 3.37B, 3.37C**). Using the consistent E-cadherin gene *CDH1* as a test of intercellular junction regulation, mRNA showed very little differences in expression levels after TNF- α treatment at any concentration or method of TNF- α administration despite the upregulation in miR-21 (**Figure 3.37D**).

To visualize the impact of TNF- α -induced miR-21 on epithelial cells, immunofluorescence was performed to stain E-cadherin in Caco-2 monolayers treated with 150 ng/mL TNF- α . In addition to TNF- α treatment, a mild treatment of 20 μ M hydrogen peroxide (H₂O₂) was used as a monolayer disrupter. A preliminary step was done to determine whether apical or basal administration of compounds could impact the stability of Caco-2 monolayers. TEER measurements showed no significant differences among groups when compounds were basally administered, and apical administration saw a significant decrease after hydrogen peroxide treatment only (**Figure 3.38A**). Nonetheless, apical administration was chosen to visualize Caco-2 monolayers post-treatment with TNF- α . Caco-2 monolayers were stained for DAPI and E-cadherin (**Figure 3.38B**). Among untreated cells, E-cadherin showed uniform distribution around the cell periphery, while cells treated with hydrogen peroxide showed a decrease in E-cadherin levels with rupture at junction points (**Figure 3.38C**). Cells treated with TNF- α demonstrated a modest decrease in E-cadherin levels at the cell periphery with some rupturing around contact

points, suggesting that induced expression of inflammatory regulators in the Caco-2 system could modulate major AJ components but keep barrier function intact.

TNF- α is a cytokine involved in inflammatory processes for immune regulation, most notably for activation of the NF- κ B pathway affecting proliferation and cell death.²⁵⁷ To ensure more specific miR-21 modulation in the *in vitro* system, the use of an overexpression method (pCMV-miR-21 overexpression plasmid) as well as an inhibitor system (miR-21 inhibitor) were transfected into TNF- α -treated Caco-2 cells and checked for levels of miR-21-5p and miR-21-3p. Cells transfected with pCMV-miR-21 showed much greater induction of both miR-21-5p and miR-21-3p compared to controls (**Figure 3.39C, 3.39D**). Alternatively, cells transfected with miR-21 inhibitor showed no difference for modulating miR-21-5p, though there was a reduction observed in miR-21-3p compared to controls (**Figure 3.39C, 3.39D**). TNF- α treatment in the inhibitor group on its own was not successful in inducing miR-21-3p expression. It is important to note that nucleic acid inhibitors may function to regulate of miR-21 levels by blocking function through sequestration as opposed to signalling the cell for degradation.²⁵⁸

To further confirm whether there is specific miR-21 impact on E-cadherin protein abundance and cellular localization, pCMV-miR-21 and miR-21 inhibitor were transfected into Caco-2 monolayers treated with TNF- α and visualized through immunofluorescence. Levels of E-cadherin were slightly reduced around the cell periphery of Caco-2 monolayers transfected with pCMV-miR-21 compared to control groups (**Figure 3.40A, 3.40B**). In cells transfected with miR-21 inhibitor, there was a slight rescue observed compared to inhibitor control group, though TNF- α treatment alone was not strong enough to reduce protein levels (**Figure 3.41A, 3.41B**). Overall, this suggests that there may be other mechanisms regulating this important AJ protein than miR-21, or there requires additional conditions not present in this *in vitro* model for miR-21 modulation.

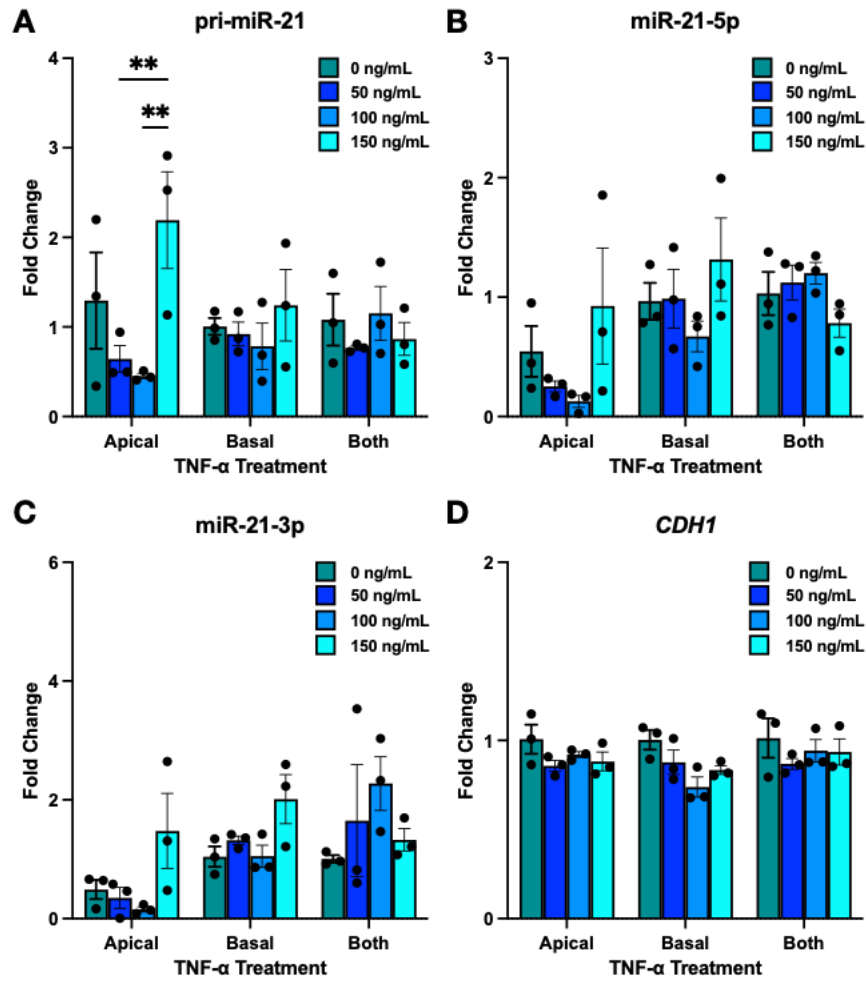


Figure 3.37 – TNF- α induces miR-21 expression in Caco-2 cells. RNA was isolated from differentiated (21-day) Caco-2 cells after 24 h of TNF- α treatment at various concentrations (0, 50, 100, 150 ng/mL TNF- α) and through different methods of administration in a transwell plate (apical chamber, basal chamber, both chambers). qRT-PCR analyses of (A) pri-miR-21, (B) miR-21-5p, (C) miR-21-3p and (D) AJ gene *CDH1* in Caco-2 cells. Denoted are $n = 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with ** - $p \leq 0.01$.

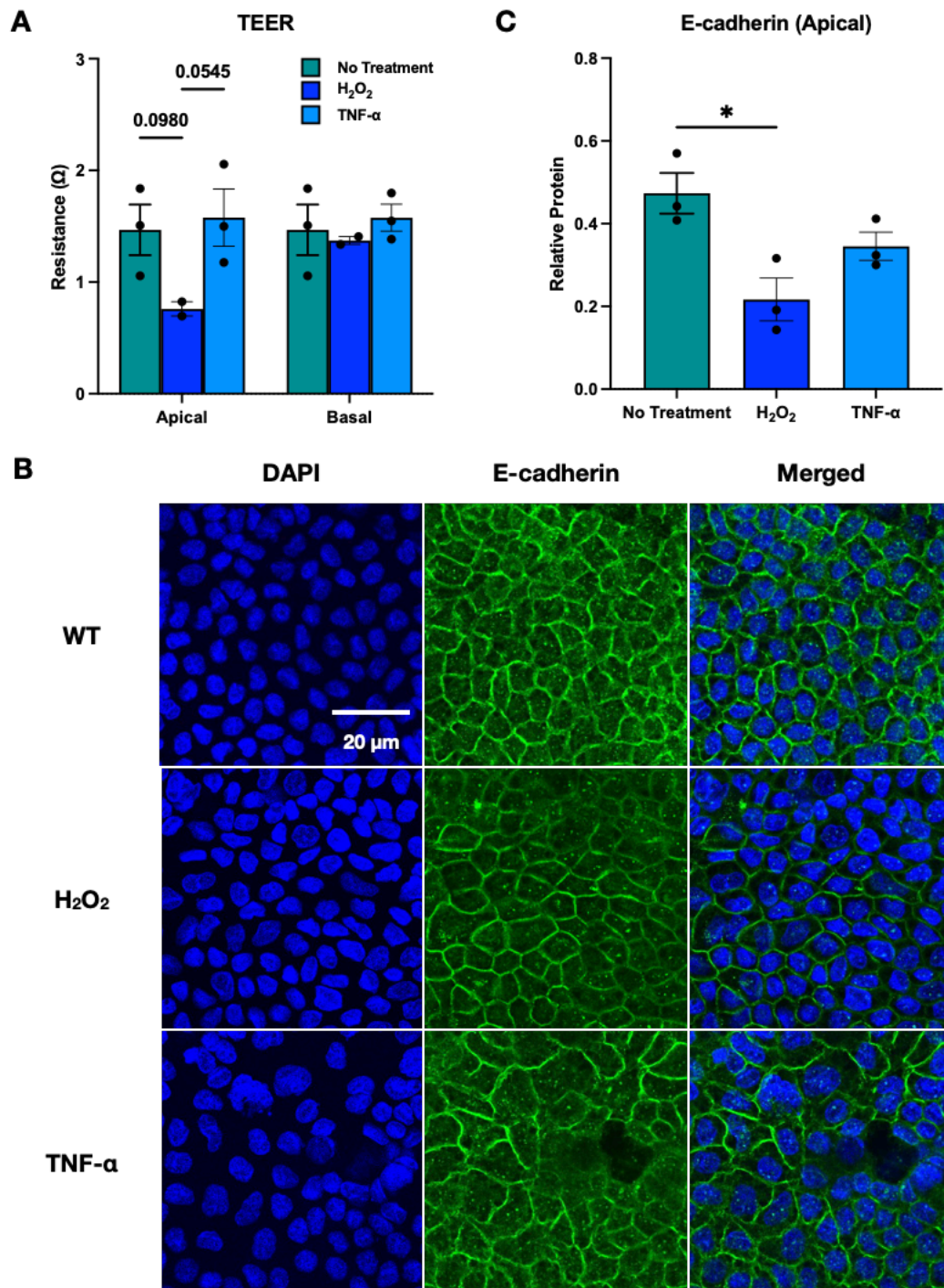


Figure 3.38 – E-cadherin levels are partially lost at cell peripheries in TNF- α -treated Caco-2 monolayers. (A) Transepithelial electrical resistance (TEER) assay for differentiated (21-day) Caco-2 cells treated with 20 nM H_2O_2 or 150 ng/mL TNF- α via apical or basal administration into the transwell plate for 24 h. Measurements were normalized to starting measurement at 0 h. (B) Apical administration of 20 nM H_2O_2 or 150 ng/mL TNF- α for treatment on Caco-2 cells and stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (C) Fluorescence was quantified by normalizing to the number of cell nuclei per field of view. Denoted are $n = 3$ biological replicates per group with the exception of TEER for H_2O_2 treated cells that have $n = 2$ per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA for Graph A or one-way ANOVA for Graph C with * - $p \leq 0.05$.

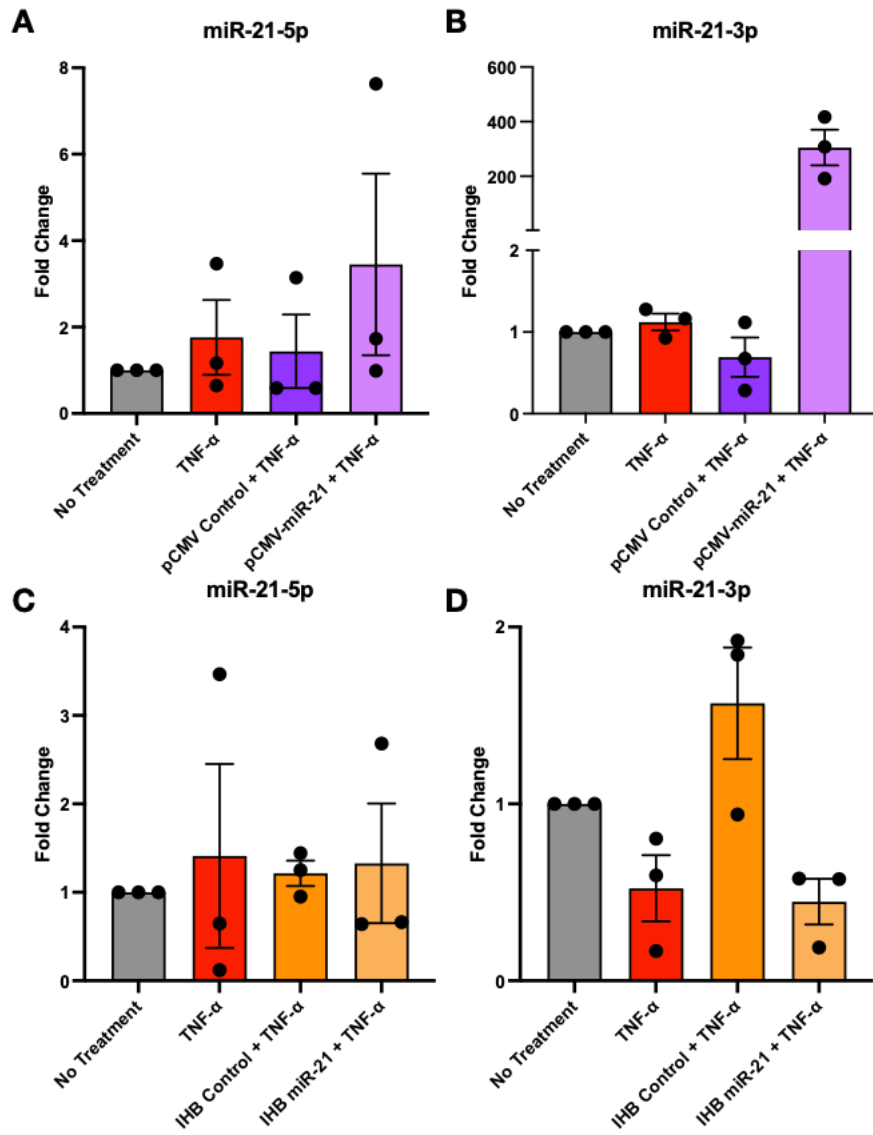


Figure 3.39 – Several methods for modulating mature forms of miR-21 in Caco-2 under TNF- α conditions. RNA was isolated from Caco-2 cells transfected with pCMV-miR-21 and miR-21 inhibitor (IHB) after 24 h of apical administrated 150 ng/mL TNF- α treatment. qRT-PCR analyses of (A + C) miR-21-5p and (B + D) miR-21-3p in Caco-2 cells. Denoted are $n = 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

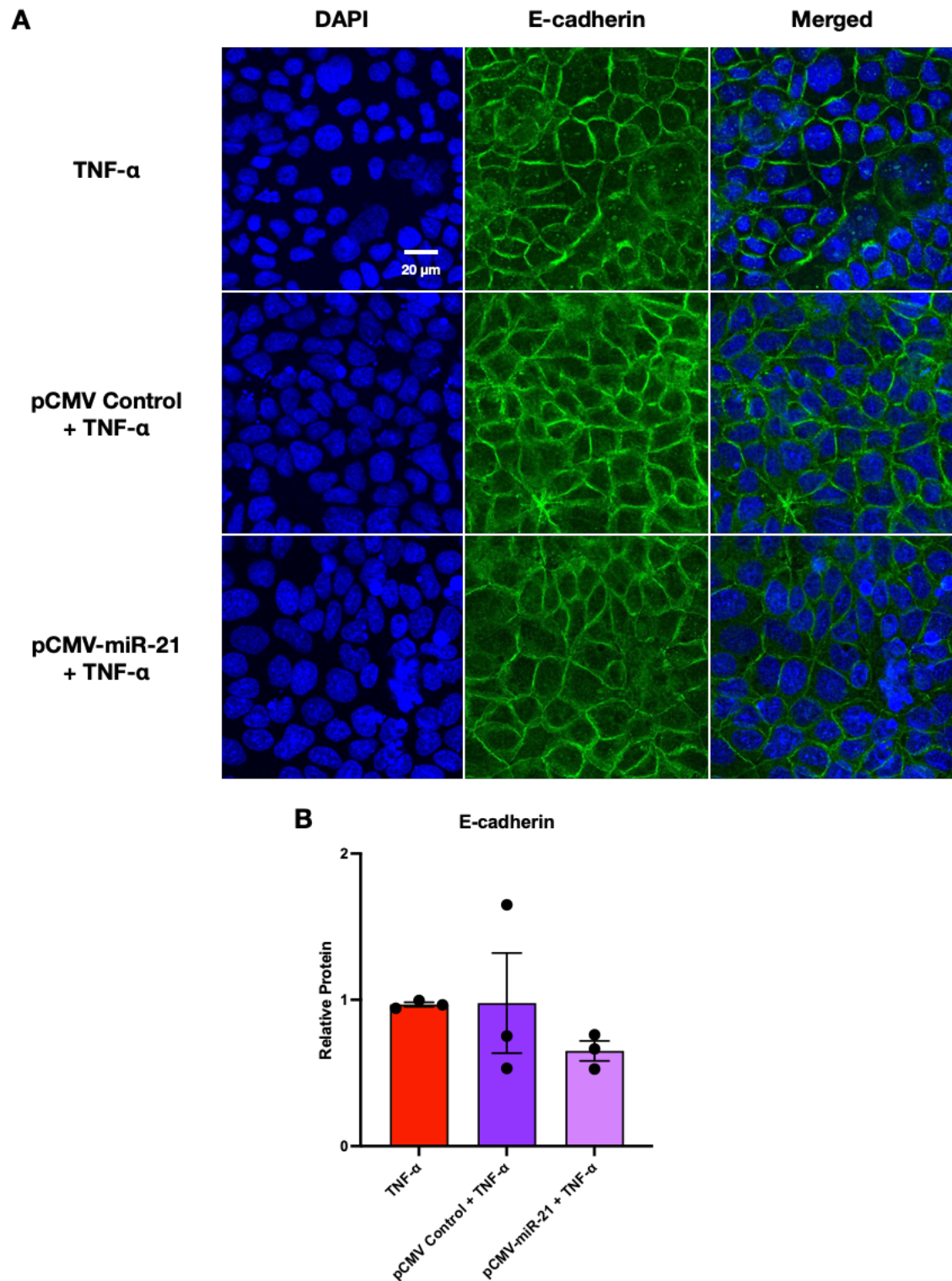


Figure 3.40 – E-cadherin levels are partially lost at cell peripheries in TNF α -treated Caco-2 monolayers transfected with pCMV-miR-21. (A) Caco-2 cells transfected with pCMV-miR-21 and controls were treated with 150 ng/mL TNF- α and after 24 h stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (B) Fluorescence was quantified by normalizing to the number of cell nuclei per field of view. Denoted are $n = 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

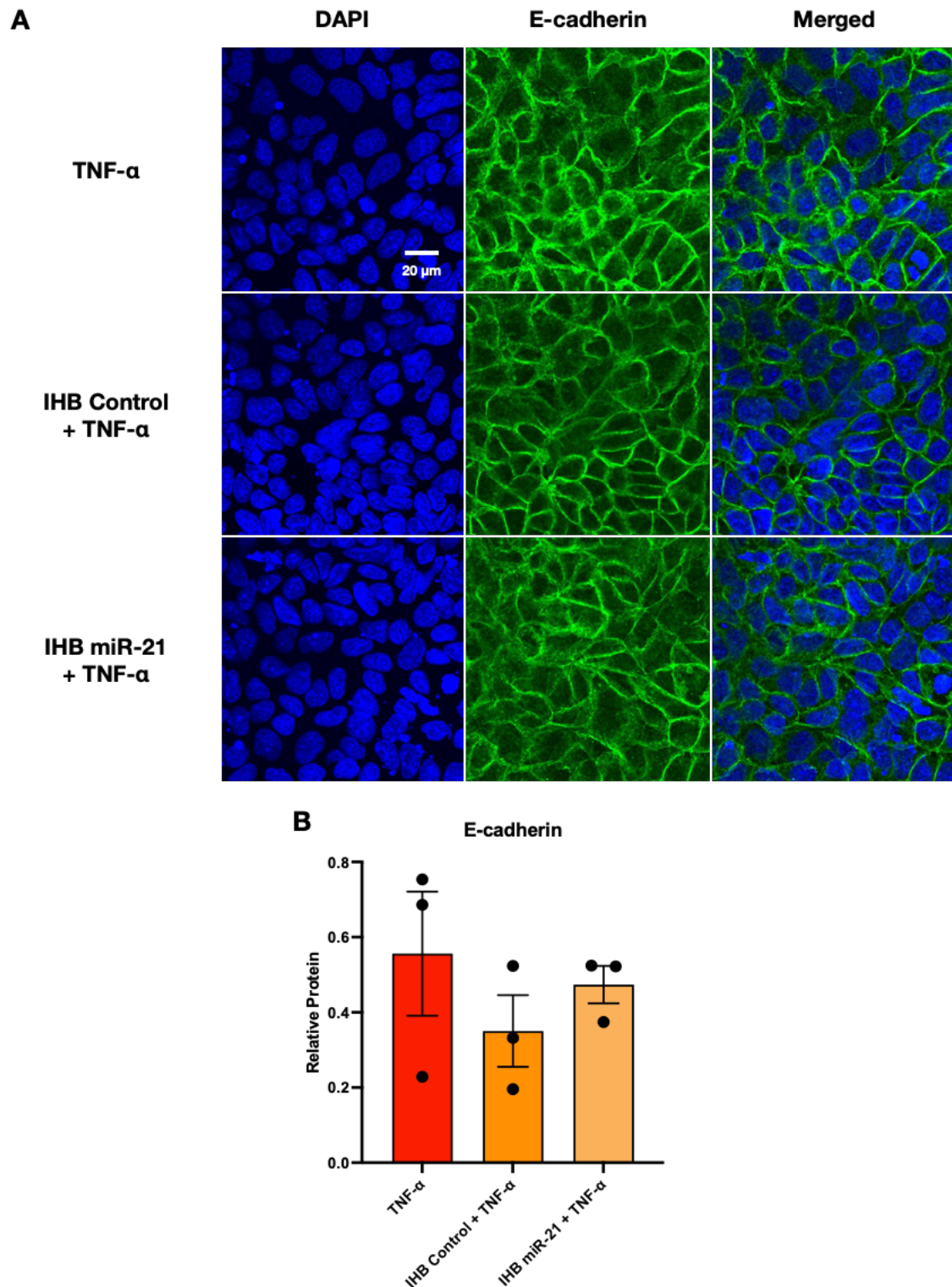


Figure 3.41 – E-cadherin levels are partially rescued at cell peripheries in TNF- α -treated Caco-2 monolayers transfected with miR-21 inhibitor. (A) Caco-2 cells transfected with miR-21 inhibitor (IHB) and controls were treated with 150 ng/mL TNF- α and after 24 h stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (B) Fluorescence was quantified by normalizing to the number of cell nuclei per field of view. Denoted are $n = 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

3.2.7 miR-21 regulates wound closure in Caco-2 cells

Using the *in vitro* system of miR-21 inhibition and overexpression, confluent Caco-2 monolayers were subjected to mechanical stress via a scratch assay to investigate wound healing capability. Caco-2 cells were seeded at high concentration (approximately 5×10^5 cells/cm²) onto 96-well plates, followed by subsequent transfections of miR-21 inhibitor and pCMV-miR-21 overexpression plasmid. Under basal conditions, monolayers were “scratched” using the Incucyte® Woundmaker 24 h after the final transfection, and plates were imaged each day for a maximum of three days. Transfections were designed whereby there would be scenarios of miR-21 inhibition, miR-21 overexpression and a combination of both. Daily images were analyzed by measuring the area of the wound, and areas were normalized to the total area of the wound directly following the scratch. As seen in **Figure 3.42A**, cells transfected with pCMV-miR-21 demonstrated the greatest wound healing capacity after 72 h. Groups that had any transfection with miR-21 inhibitor reduced wound healing capacity to levels similar to untreated control (**Figure 3.42B**). On the final day, there was significant reduction in wound area in cells transfected with pCMV-miR-21 overexpression plasmid compared to all groups (**Figure 3.42C**).

As this was a surprising result, transfected Caco-2 cells were also treated with TNF- α to determine whether additional inflammatory circumstances would impact wound healing capability during miR-21 modulation. Remarkably, TNF- α treatment was observed to strengthen wound healing capacity in all groups compared to basal conditions (**Figure 3.43A**). As seen in basal conditions, TNF- α treated cells transfected with pCMV-miR-21 showed the highest capacity for wound healing, while any groups transfected with miR-21 inhibitor had a wound healing deficiency similar to transfection control (**Figure 3.43B**). Quantification of wound closure on the final day showed again a significant decrease in wound area for TNF- α treated cells transfected with pCMV-miR-21 compared to all groups (**Figure 3.43C**). These findings suggest that inflammation can induce wound healing and miR-21 may ameliorate wound healing capability in IECs.

Further molecular analyses determined the expression levels of miR-21 forms and known miR-21 targets within tested groups of the scratch tests. MiR-21-5p showed minimal expression levels in the presence of pCMV-miR-21 and little reduction in transfections with miR-21 inhibitor under basal and TNF- α conditions (**Figure 3.44A, 3.44B**). Interestingly, miR-21-3p demonstrated a large upregulation in cells transfected with pCMV-miR-21 overexpression plasmid, which was modestly reduced when combined

with miR-21 inhibitor under basal conditions (**Figure 3.44C**). Although high induction of miR-21-3p was also observed under TNF- α conditions, there was high variability in the combination group (**Figure 3.44D**).

Previous AJ-related wound healing genes, along with *TGFB*, were assessed in scratch test groups to determine associations with wound healing closure. Under basal conditions, there was an induced trend for *CDH1* in cells transfected with pCMV-miR-21, with no change compared to non-transfected groups or cells transfected with miR-21 inhibitor, as well as no difference observed in TNF- α conditions between groups (**Figure 3.45A, 3.45B**). Expression levels of *F11R* were only significantly different under TNF- α conditions, where groups transfected with miR-21 inhibitor had a significant reduction in *F11R* mRNA compared to pCMV-miR-21 on its own and non-transfected cells (**Figure 3.45C, 3.45D**). When checking the expression profile of TGF- β ligand, *TGFB* exhibited higher mRNA levels in cells transfected with pCMV-miR-21 under basal conditions, but much lower expression overall in TNF- α conditions (**Figure 3.45E, 3.45F**). This suggests that there may be changes in the initial activation of the TGF- β pathway that is impacted by miR-21 which support wound healing capabilities in IECs.

Based on earlier *in vivo* results from the recovery model and *in vitro* Caco-2 transfected monolayers, it was expected that miR-21 would exacerbate wound healing capabilities after barrier disruption. Perhaps there was an excess of miR-21 during overexpression in which miR-21 inhibitor molecules were already saturated, reducing effectiveness of inhibition. Thus, a subsequent scratch test was performed in which the concentration of miR-21 inhibitor was increased by 4-fold (200 nM). Scratch tests in which the concentration of miR-21 inhibitor was doubled was also performed, however there was no difference compared to initial assays (data not shown). Under basal conditions, results were similar to the previous scratch test where cells transfected with pCMV-miR-21 showed a reduced trend in wound closure compared with other groups, with some reduction seen with miR-21 inhibitor group (**Figure 3.46A, 3.46B, 3.46C**). However, under TNF- α conditions, cells transfected with miR-21 inhibitor further demonstrated a reduced trend in wound closure compared to cells transfected with pCMV-miR-21 (**Figure 3.47A, 3.47B, 3.47C**). Interestingly, the combination group under TNF- α conditions remained similar to non-transfected cells despite the individual compounds strengthening wound healing capacity, potentially due to compounds antagonizing the functions of the other. Overall, this suggests novel TNF- α -dependent mechanisms by which fluctuations in miR-21 in either shortage or surplus can support wound healing capabilities in IECs.

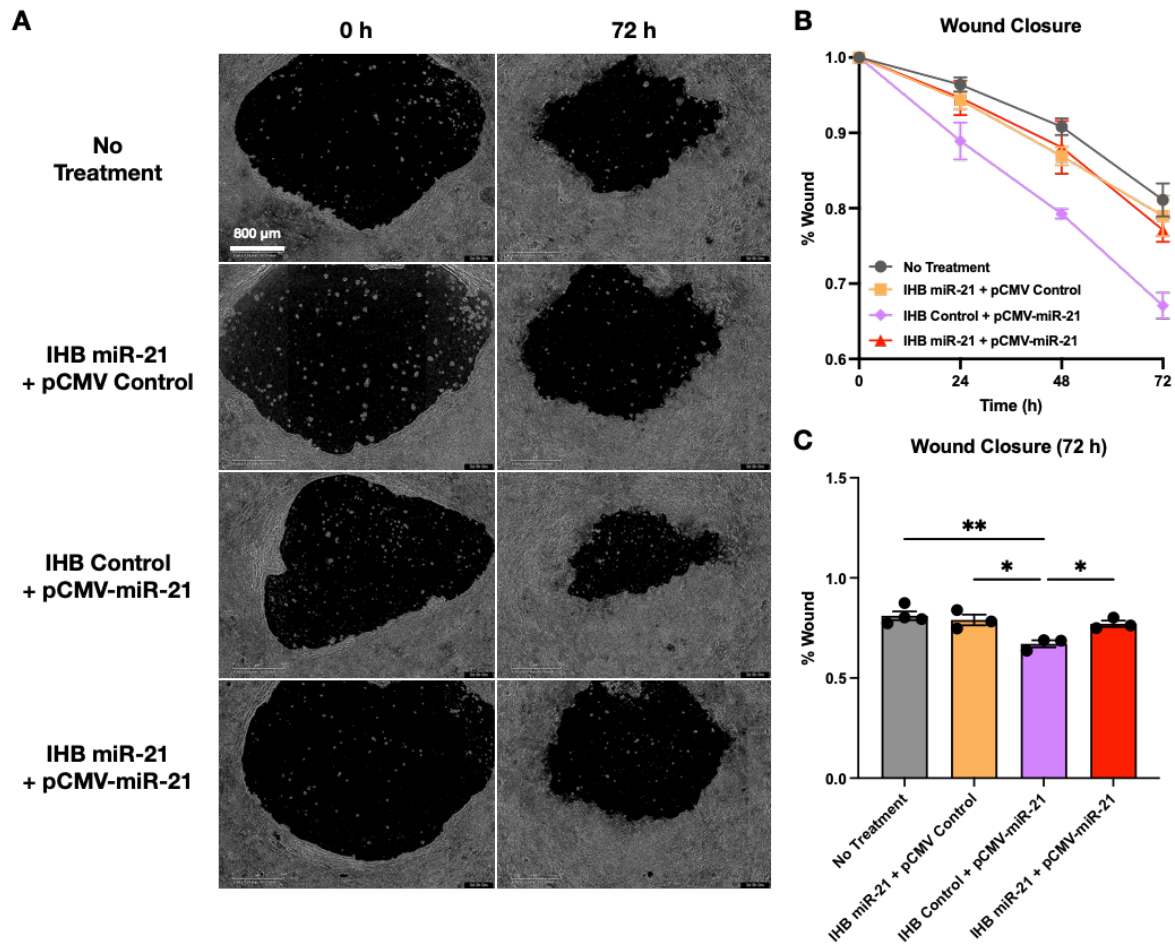


Figure 3.42 – Caco-2 monolayers improve wound healing capability when transfected with pCMV-miR-21 under basal conditions. Caco-2 cells were transfected with 50 nM miR-21 inhibitor (IHB), 200 ng/well pCMV-miR-21 or a combination of the two, and monolayers were scratched with the Incucyte® Woundmaker in 96-well plates. (A) Scratched monolayers were kept in DMEM and imaged each day for three days (presented images taken at 0 h and 72 h). (B) Wound closure was monitored by measuring the area of the wound each day using ImageJ as a fraction of wound area at 0 h. (C) Comparison of wound closure taken at 72 h. Denoted are $n = 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with * - $p \leq 0.05$, ** - $p \leq 0.01$.

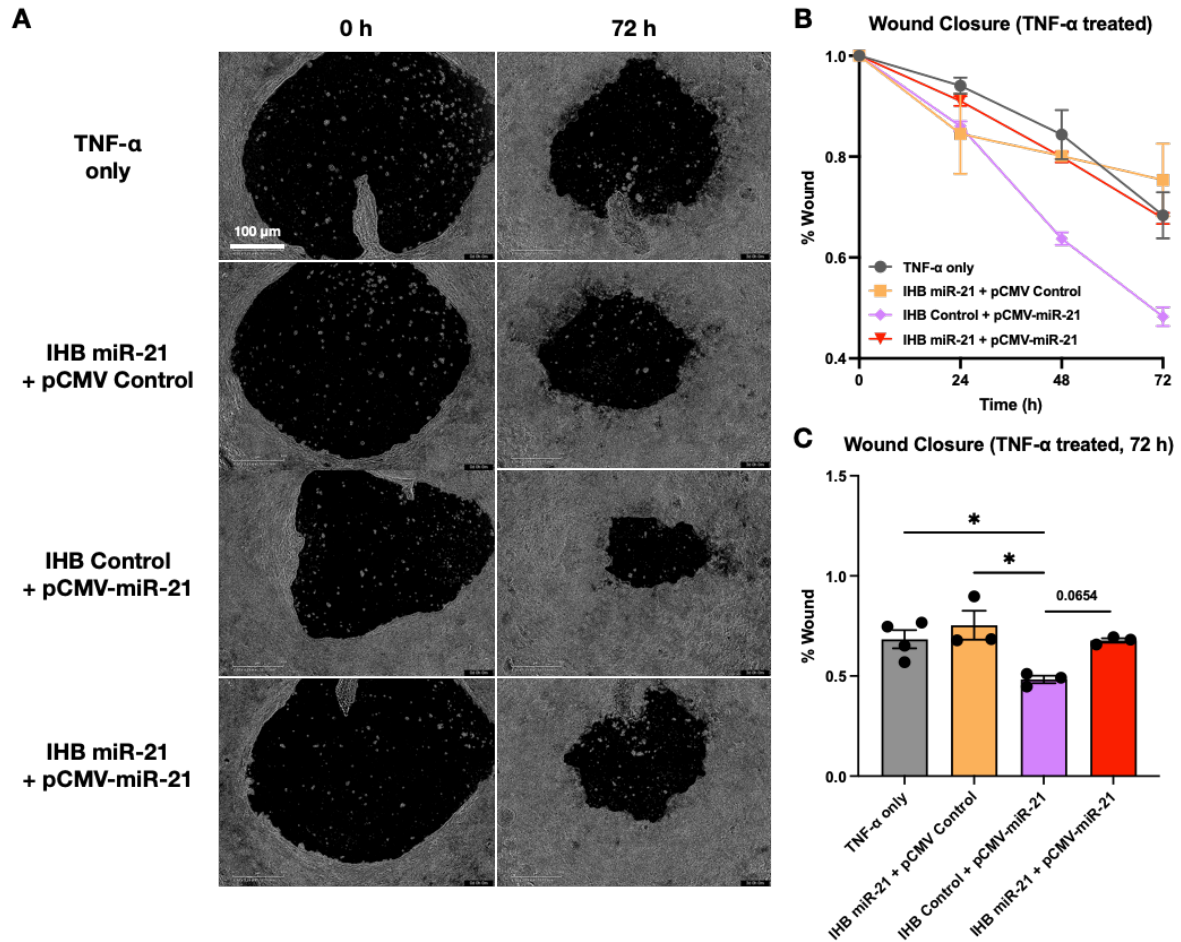


Figure 3.43 – Caco-2 monolayers improve wound healing capability when transfected with pCMV-miR-21 under TNF- α conditions. Caco-2 cells were transfected with 50 nM miR-21 inhibitor (IHB), 200 ng/well pCMV-miR-21 or a combination of the two, and treated with TNF- α for 24 h in 96-well plates. Monolayers were scratched with the Incucyte® Woundmaker. (A) Scratched monolayers were kept in DMEM and imaged each day for three days (presented images taken at 0 h and 72 h). (B) Wound closure was monitored by measuring the area of the wound each day using ImageJ as a fraction of wound area at 0 h. (C) Comparison of wound closure taken at 72 h. Denoted are $n = 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with * - $p \leq 0.05$.

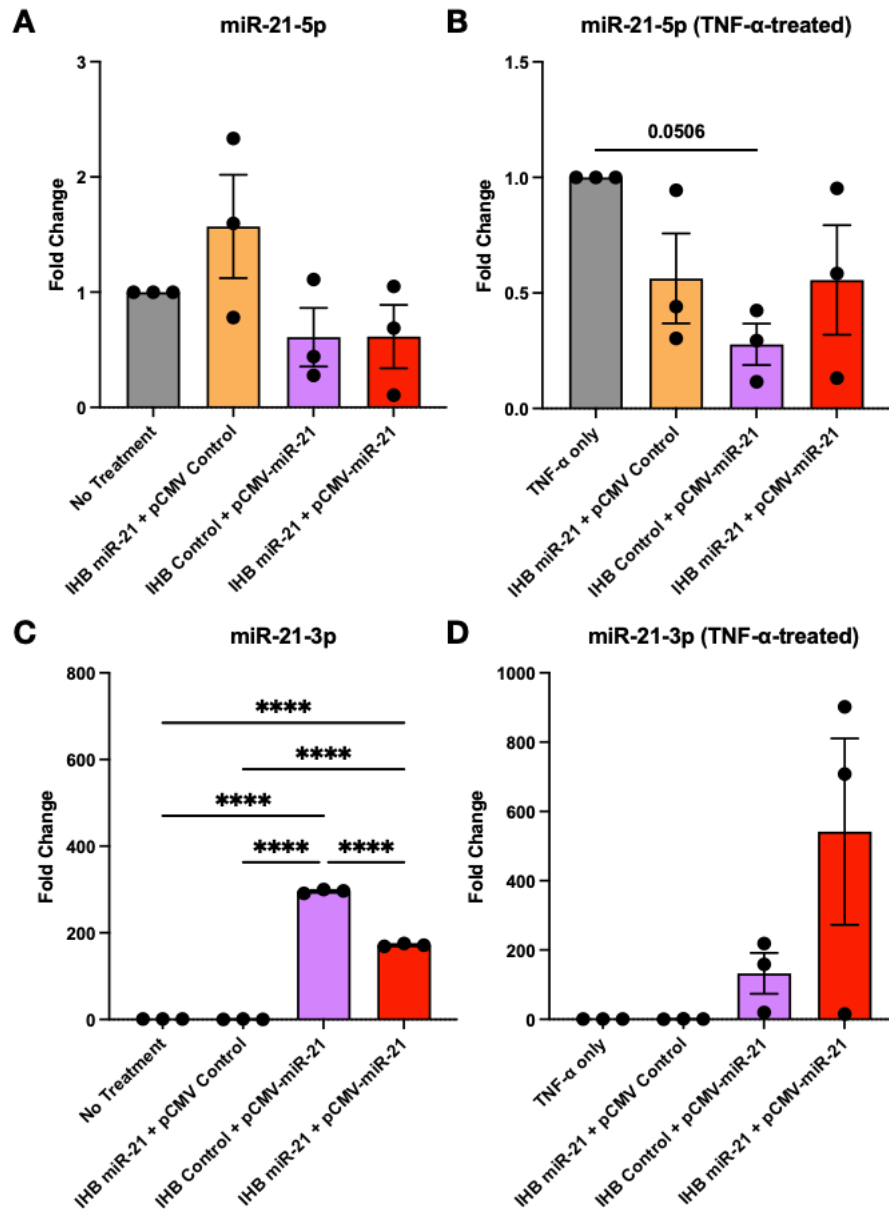


Figure 3.44 - miR-21-3p is induced in wounded Caco-2 monolayers transfected with pCMV-miR-21. RNA was isolated from basal and TNF- α -treated Caco-2 cells transfected with 50 ng miR-21 inhibitor (IHB), 200 ng/well pCMV-miR-21 or both at 72 h after scratch. qRT-PCR analyses for (A + B) miR-21-5p and (C + D) miR-21-3p in scratched Caco-2 cell monolayers. Denoted are $n = 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with **** - $p \leq 0.0001$.

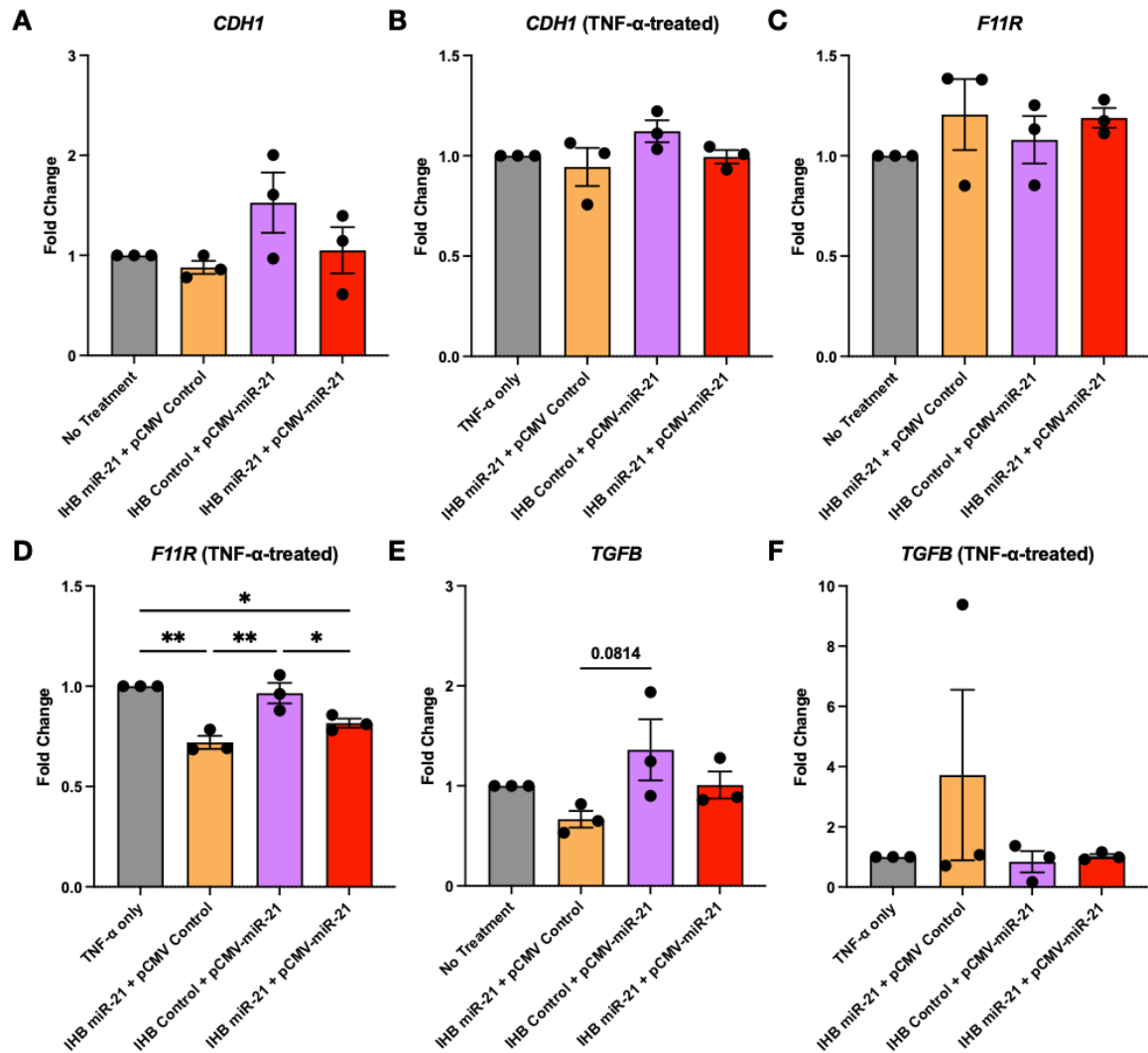


Figure 3.45 – *F11R* significantly decreased in the presence of miR-21 inhibitor under TNF- α conditions, while *TGFB* was induced in the presence of pCMV-miR-21 under basal conditions in wounded Caco-2 monolayers. RNA was isolated from basal and TNF- α -treated Caco-2 cells transfected with 50 ng miR-21 inhibitor (IHB), 200 ng/well pCMV-miR-21 or both at 72 h after scratch. qRT-PCR analyses for (A-D) Junction related wound healing genes: *CDH1*, *F11R*. (E + F) TGF- β family genes: *TGFB*. Denoted are n = 3 biological replicates per group, mean values $\pm$ SEM, *p* value calculations using one-way ANOVA with * - $p \leq 0.05$, ** - $p \leq 0.01$.

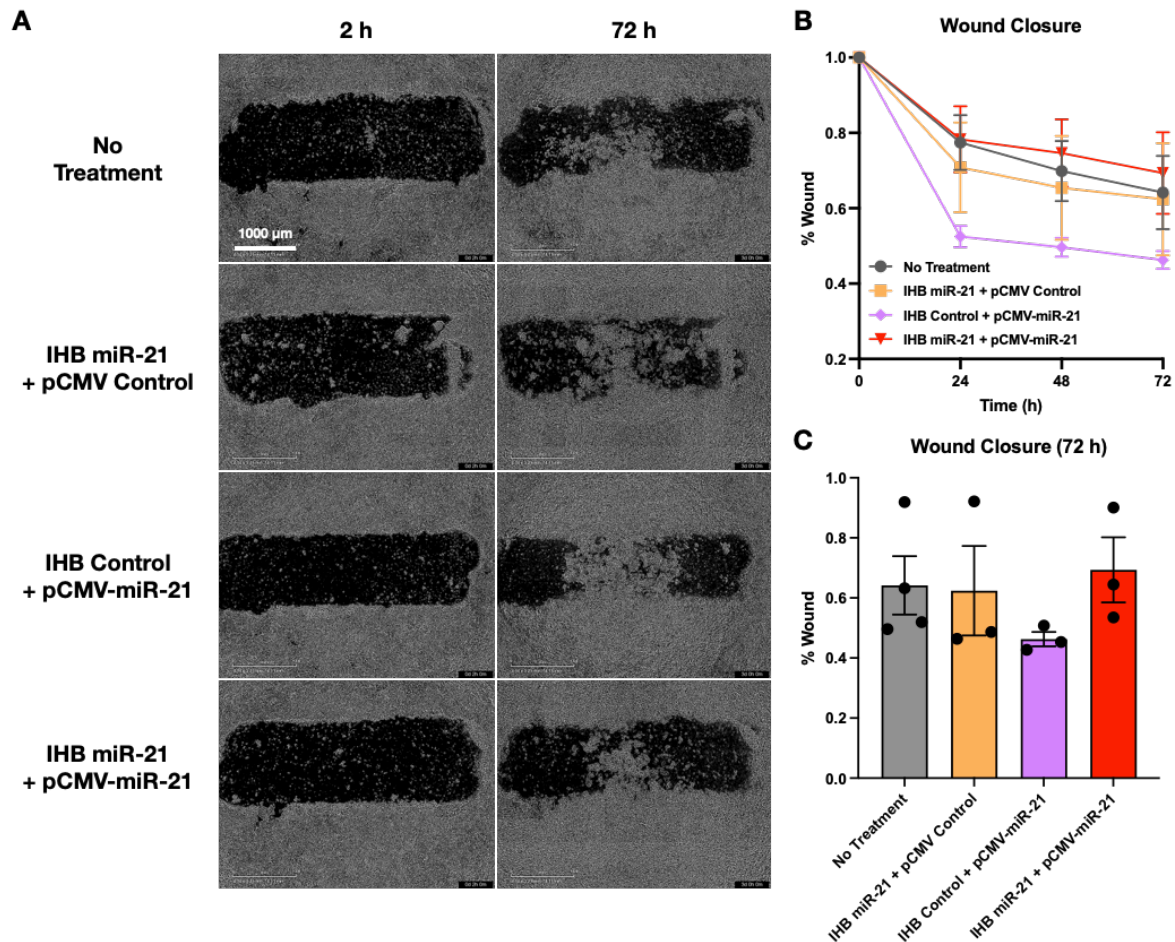


Figure 3.46 – Wounded Caco-2 monolayers transfected with high concentration of miR-21 inhibitor have some impact on wound healing capability under basal conditions. Caco-2 cells were transfected with 200 nM miR-21 inhibitor (IHB), 50 ng/well pCMV-miR-21 or a combination of the two, and monolayers were scratched with the Incucyte® Woundmaker in 96-well plates. (A) Scratched monolayers were kept in DMEM and imaged each day for three days (presented images taken at 0 h and 72 h). (B) Wound closure was monitored by measuring the area of the wound each day using ImageJ as a fraction of wound area at 0 h. (C) Comparison of wound closure taken at 72 h. Denoted are $n \geq 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

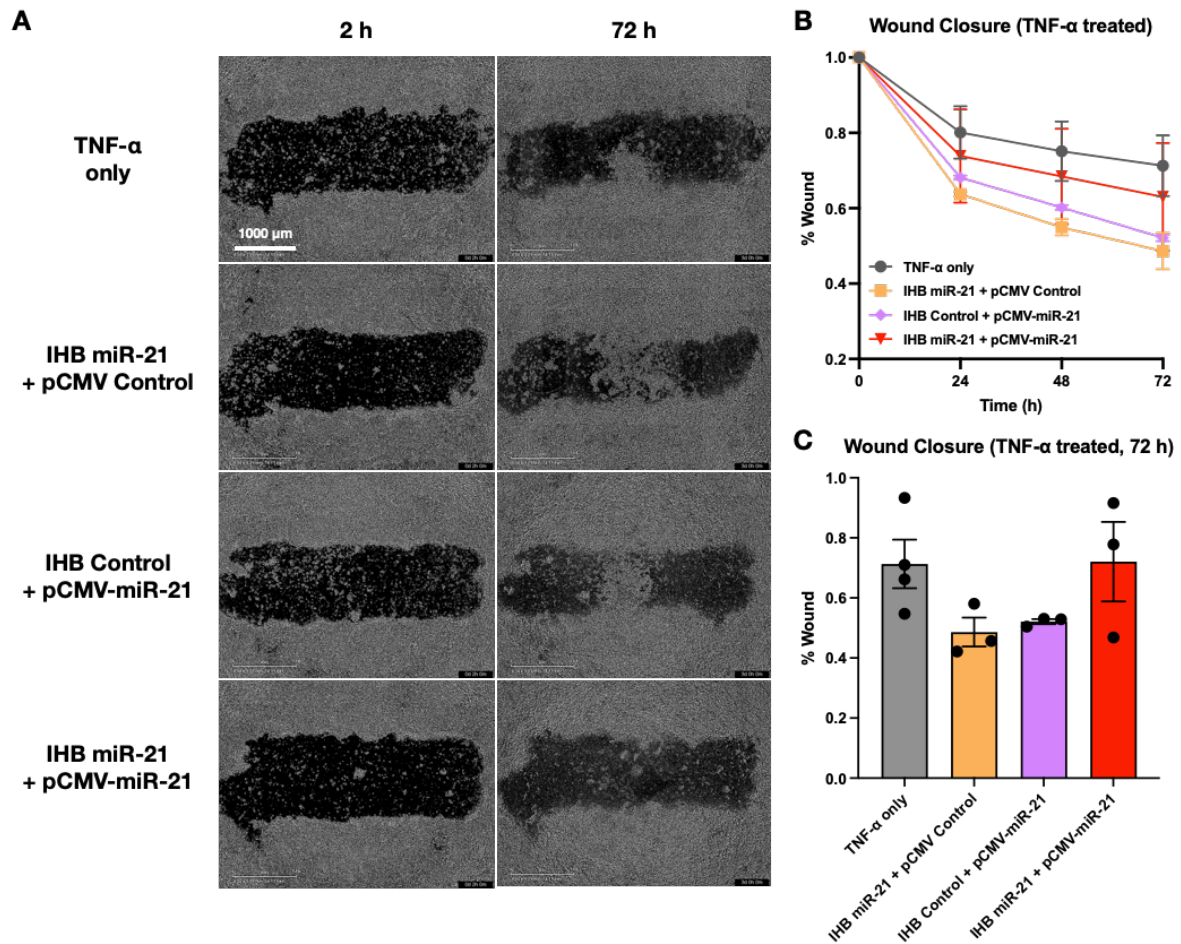


Figure 3.47 – Wounded Caco-2 monolayers transfected with high concentration of miR-21 inhibitor have improved wound healing capability under TNF- α conditions. Caco-2 cells were transfected with 200 nM miR-21 inhibitor (IHB), 50 ng/well pCMV-miR-21 or a combination of the two, and treated with TNF- α for 24 h in 96-well plates. Monolayers were scratched with the Incucyte® Woundmaker. (A) Scratched monolayers were kept in DMEM and imaged each day for three days (presented images taken at 0 h and 72 h). (B) Wound closure was monitored by measuring the area of the wound each day using ImageJ as a fraction of wound area at 0 h. (C) Comparison of wound closure taken at 72 h. Denoted are $n \geq 3$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

3.3 Discussion

Due to the large amount of predicted miR-21 targets within various prediction databases, this chapter began with a targeted approach to investigate the impact of miR-21 on cellular processes affected in IBD pathology. Within intestinal inflammation, a focus on intercellular junctions was natural in determining the impact of miR-21 on the gut epithelial barrier, both in healthy and disease states. Several prominent junction components were observed to be predicted targets of both mature forms of miR-21 (miR-21-5p and miR-21-3p), and therefore this indicated that there may exist a general regulatory model between miR-21 and vital intercellular junctions. In this chapter, AJs were chosen due to the lack of knowledge surrounding the involvement of miR-21 in AJ regulation.

It was important to establish differences in miR-21 expression along the GI tract. Regarding IBD types, CD typically impacts the terminal ileum, perianal area and colon in a discontinuous pattern, while UC primarily affects the rectum and colon in a continuous manner.⁶ Other regions of the small intestine can also be affected in CD, along with earlier regions of the entire GI tract. With a focus on the intestines, each intestinal region serves a purpose for helping mammals digest food effectively. When broken down food leaves the stomach (called chyme) and enters the small intestine, it first starts at the duodenum where it is met with digestive enzymes and bile salts that cause further breakdown.²⁵⁹ Moving forward through the jejunum, mucosal folds containing columnar epithelial cells and mucin-secreting goblet cells create plica and villi that allow for slow and efficient nutrient absorption, which are transported through connected circulatory channels. As chyme moves towards the terminal ileum, it encounters antibacterial enzymes from Paneth cells, along with gastric hormones from enteroendocrine cells, all regulated in part by food consumption. From there, chyme passes into the proximal colon. The main purpose of traversing the colon is for IECs to both absorb water and lubricate solid feces, eventually ending in the distal colon and passage through the rectum.²⁵⁹ These digestive processes and how they are impacted by miR-21 regulation are of particular interest to our group.

Initial *in vivo* work performed early in the project was conducted on tissue sections from WT and miR-21^{-/-} mice under healthy conditions. As various types of IBD can impact different regions of the GI tract, the first step sought out to investigate differential expression of miR-21 in the colon and small intestine. Previous global analyses on miRNA profiles in mice demonstrated as much as a 10-fold increase in colon IECs compared to IECs from the small intestine.²⁶⁰ However, there was no significant differences in the

expression of mature miR-21, and thus the global ablation of miR-21 should affect each region equally. Subsequent molecular analyses used the comprehensive screen for AJ genes that were predicted targets of miR-21, comparing levels of expression between genotypic groups and colon regions under healthy conditions. In male mice, distal intestinal tissue (from large or small intestine) had very little difference in expression, while proximal tissue showed upregulated trends in *Cdh1* and *Ctnnd1* within miR-21^{-/-} compared to WT mice. This trend was also reproduced in female mice, where in addition to *Cdh1*, *Cttna1* was significantly upregulated in miR-21^{-/-} whole colon tissue compared to WT. As mentioned, the role E-cadherin plays at the gut barrier is significant as not only the major AJ protein of intestinal epithelia, but also the primary anchor point for proteins in the AJC to link with the IEC cytoskeleton.²⁶¹ Notable junction interaction partners with E-cadherin include TJ protein Zo-1, desmosome protein desmoglein-2 and other AJ proteins like p120-catenin and β -catenin. p120-catenin, a cytoplasmic binding AJ protein, has also been shown to be important in E-cadherin stability, inhibiting AJ protein endocytosis and recycling, as levels correlate with proper E-cadherin function and reduced neutrophil infiltration.¹⁵⁰ p120-catenin also increases the area of adherence between contacting cells in a Rac1-dependent manner, involving the PI3K pathway and guanine exchange factors (GEFs)^{262,263}, thus leading to gut barrier stability through strong IEC adherence. There was a mild induction of α -catenin mRNA in proximal colon tissue as well, which encodes another prominent protein regulating gut permeability and important for binding AJs to the actin cytoskeleton.¹⁴⁸ This would indicate that miR-21^{-/-} IECs are more in tune with cytoskeletal dynamics under healthy conditions, providing added rigidity for stable barrier function. More complicated is the role that β -catenin plays, which was found reduced in proximal colon tissue of miR-21^{-/-} compared to WT mice. β -catenin holds a dual position at AJs in the intestinal barrier. Firstly, β -catenin bound to E-cadherin increases the stability of epithelial cell adhesion to one another.²⁶⁴ Secondly, unbound β -catenin and accumulation in the cytoplasm can lead to apoptosis or EMT through migration to the nucleus by Beclin-1, if not otherwise degraded by the GSK3-Axin-APC complex, all part of the canonical Wnt signalling pathway.¹⁴⁹ Therefore, less β -catenin does not necessitate a weaker AJ. Finally, GJ genes, serving as communicative components among IECs, were notably similar among groups and suggests that miR-21 may have a specific regulatory role for structural stability in gut epithelia.

In DSS-treated female mice, expression levels of AJ genes were markedly reduced compared to healthy conditions. Even the known target of miR-21, *Pdcd4*, lost upregulation in DSS-treated miR-21^{-/-} whole colon tissue. Although DSS is widely used, its mechanism of action is not fully understood. The chemical is known to create pores along the intestinal wall, primarily impacting the distal end of the colon, causing barrier leakage and an increase in immune cell infiltration and activation.²¹³ It was possible that the level of inflammation caused by DSS-induced colitis destroyed junction complexes in these colon tissue samples, since wounds along the intestinal barrier would be devoid of IEC connection points for junctions to regenerate. Nevertheless, the only gene that reproduced an induction in DSS-treated miR-21^{-/-} mice was *Cdh1*, which indicates that E-cadherin may indeed be regulated by miR-21 in a direct or indirect manner. Future investigations on how dysregulation in AJs may lead to the exhibition of a more CD or UC disease state will elucidate more on the differences among IBD phenotypes.

Furthering on previous literature that showed a protective effect in the miR-21^{-/-} murine model during DSS-induced colitis^{85,86}, this project looked at a recovery model using WT and miR-21^{-/-} mice. This was performed to allow the re-establishment of barrier integrity pathways after post-DSS treatment for investigating intercellular junctions recovering from an inflamed state. For males, miR-21^{-/-} mice remained protected against DSS-induced colitis. This protection was not observed as strongly in female mice, though miR-21^{-/-} females also had quicker rates of recovery towards the end of the trial, reinstating the importance for the inclusion of sex information in trials and separation within data analyses. Indeed, though utilized in the study of colorectal cancer, the AOM-DSS murine model was shown to have significantly different transcriptomes between males and females.²¹⁶ Additionally, hormonal differences like the induction of estrogen receptor (ER)- β were shown to decrease risk of DSS-induced colitis in females.²⁶⁵ Males can develop more severe symptoms in the onset of colitis, and removal of pro-inflammatory regulators like miR-21 may manifest more of an improved phenotype. In particular, the reverse in histological scoring between male and female mice among distal colon tissue could reflect sex-dependent susceptibility to types of IBD resulting in localized inflammation like UC that have a higher frequency in men, as opposed to more ubiquitous inflammation like CD that have a higher frequency in women.¹⁴⁸

As was seen in healthy samples, there were no significant differences in levels of mature miR-21 within WT DSS recovery model samples, but an induction within DSS

samples compared to untreated tissue was observed. Regarding AJ genes, differential gene expression between WT and miR-21^{-/-} mice were more prominent in distal tissue compared to proximal tissue. This is likely due the yet unknown mechanism of how DSS impacts the distal colon more than the proximal colon²¹³, highlighting the importance of noting intestinal region and disease state regarding gene regulation. *Cdh1* once again retained consistency, being induced in miR-21^{-/-} males and females compared to WT in distal colon samples. In both sexes, structural AJ genes like *Cttna1* and *Ctnd1* were also notably induced in miR-21^{-/-} distal tissue compared to WT. Although no changes were observed in male mice, *Cttnb1* was induced in both distal and proximal tissue of miR-21^{-/-} female mice, emphasizing another sex-dependent difference in gene regulation during DSS-induced colitis. Future work will look at the localization of induced β -catenin *in vivo* and *in vitro* to determine whether the protein is co-localizing to E-cadherin in AJs, or whether there is another subcellular localization that can be seen during inflammation. Since translocation into the nucleus may switch on oncogenic cellular processes seen in IBD pathology, this may in part be an explanation to the differences in disease severity between sexes.

All *in vivo* experiments thus far have been conducted on non-littermate mice. Littermate mice can be more rigorous at comparing genotypically different animal groups, particularly when considering microbiome effects in separated lineages.²⁵⁰ Thus, littermate WT and miR-21^{-/-} mice were also evaluated in their responses to DSS-induced colitis in the recovery model. Interestingly, the protective phenotype was not as prominent in either sex, though generally littermate mice did not reach the same disease severity as non-littermate mice under the same conditions. Some protection remained, such as reduced diarrhea scores in all miR-21^{-/-} compared to WT and HET groups. In males, there were significantly lower cumulative DAI scores for miR-21^{-/-} mice towards the end of the trial, indicating a faster recovery to DSS-induced colitis that was largely driven by the reduction in diarrhea prevalence. It is possible that in less severe colitis, WT littermates could gain protection from colitic symptoms, based on metabolic exposure in the pre-weaning phase and changes in gut microbiota abundances. Indeed, these findings are similar to phenotypes observed in co-housed mice where WT became protective against DSS-induced colitis in the traditional non-recovery model.⁸⁶ This protection was largely attributed to differences in gut microbiota that play a critical role on DSS-colitis pathogenicity. Future work determining differences between the gut bacterial populations of non-littermate and littermate miR-21^{-/-} mice of both sexes will aid in the understanding of miR-21 at the gut barrier.

At a functional level, littermate miR-21^{-/-} males had lower gut permeability to FITC despite having higher tissue damage in the proximal colon through histological analysis. This damage may have served to prime the immune system for pathological invasion, but otherwise this could be an artifact of tissue section quality. In females, the reverse was observed, albeit females displayed even milder disease symptoms than males. Littermate miR-21^{-/-} female mice had higher cumulative DAI scores during DSS treatment compared to WT that was largely driven by the reduction in weight loss, as they had lower diarrhea scores during the majority of the recovery phase and similar stool blood scores throughout the trial. There was a trend for reduced FITC gut permeability as seen in males, as well as a reduction in immune cell infiltration in distal tissue for miR-21^{-/-} compared to WT mice resulting in lower histology scores for the distal colon. These results indicate that changes at the tissue level were not sufficient to explain changes in disease severity observed between littermate WT and miR-21^{-/-} mice during recovery from DSS-induced colitis, and indeed future investigations on identifying differences in gut microbiota between non-littermates and littermates could support differences in the development of disease.

Due to the mild but notable protection retained in males, additional molecular analyses were performed to elucidate possible differences in expression profiles between littermate WT and miR-21^{-/-} male mice. Although no differences in mature miR-21 were observed in intestinal regions, there was a significant induction in TJ gene *Ocln* for miR-21^{-/-} distal colon tissue compared to WT. However, the induction seen in non-littermate miR-21 males for *Cdhl* was not evident in littermate counterparts. Again, this may be due to two reasons: 1) achieved disease severity was not high enough to elicit a regulatory response for AJs, or 2) littermate WT mice gained protective attributes that were not present in non-littermate WT mice. Indeed, littermate controls have been of growing interest in the field as a way to control for genetic background and environment.²⁶⁶ For example, despite being a proposed regulator of gut microbiota composition, the use of littermate controls demonstrated that Nlrp6/ASC inflammasomes do not alter gut bacteria, but rather changes were dependent on non-genetic confounders like maternal inheritance and long-term separate housing.²⁶⁷ There is, of course, the possibility that microbiota differences in non-littermate WT and miR-21^{-/-} mice are more prevalent than those in littermate WT and miR-21^{-/-} mice. In fact, it is highly likely that intestinal microbiota would be distinctly different between groups based solely on separating lineages for multiple generations. Robertson *et al.* (2019)²⁵⁰ showed that F2 littermates had robust microbiota standardization across fecal, colon and ileum samples, while another common practice of co-housing was not able to

normalize microbiota at the proximal colon and terminal ileum. As mentioned, future work using shotgun sequencing comparing the differences between gut microbiota composition in DSS-treated WT and miR-21^{-/-} non-littermate and littermate mice could prove useful in determining microbes that exacerbated pathogenesis in non-littermate WT, or those that ameliorated pathogenesis in littermate WT.

Focusing more on E-cadherin became apparent for its consistency with *in vivo* non-littermate gene expression analyses, but it was not certain whether this expanded to protein levels as well. Under healthy conditions, E-cadherin immunofluorescence was upregulated in distal colon tissue of miR-21^{-/-} mice compared to WT, and in all colon regions, E-cadherin seemed to localize to the tops of crypts. Female mice showed less differences in regional localization in both untreated and DSS-treated samples. This induction of E-cadherin is in line with results obtained from multiple studies, in which *Cdh1* conditional KO mice (*Cdh1*^{fl/fl}) demonstrated significant dysfunction in gut barrier integrity and gut morphology.^{133,134} Interestingly, *Cdh1*^{fl/fl} mice had a similar phenotype of E-cadherin apical localization to non-littermate miR-21^{-/-} mice¹³⁴, which could suggest faster turnover of the epithelial layer. Reduced expression of E-cadherin in intestinal epithelia also led to changes in TJ protein levels such as claudin-1, claudin-3 and claudin-4.¹³³

Most intriguing were findings seen through western blotting, as changes in protein levels were not greater in miR-21^{-/-} male or female mice, but there were distinct size fragments indicating the intact and a fragmented version of E-cadherin. Healthy miR-21^{-/-} males had higher levels of intact E-cadherin in distal colon tissue compared to WT, while in proximal tissue there were elevated levels of fragmented E-cadherin. DSS-treated miR-21^{-/-} female mice had significantly reduced levels of intact E-cadherin in whole colon tissue compared to WT. In the DSS recovery model, sex differences were prevalent in the abundances of intact and fragmented protein. Immunoblots for male mice showed a decrease in intact E-cadherin in distal colon tissue, while immunoblots for females had an increase in intact E-cadherin for distal colon tissue and an overall decrease in E-cadherin levels for proximal tissue. Interestingly, one study found that peptides from E-cadherin fragments aided in wound healing of IBD patients, and *in vitro* models showed inclusion of these peptides in growth media increased the rate of wound closure through scratch tests²⁶⁸, which could explain some protective effects observed in miR-21^{-/-} mice. These fragmented E-cadherin peptides did not produce mitogenic effects in Caco-2 cells and were observed to transmigrate across the plasma membrane and into the cytosol, impacting migratory capacity of IECs. It is possible that the fragmented version is a result of the

cleavage of the extracellular domain of E-cadherin, as this was found to occur in colorectal carcinomas leading to a protein size at approximately 100 kDa.²⁶⁹ Altogether, these results indicate the potential of a novel miR-21-dependent mechanism by which E-cadherin can be cleaved, with resulting peptides taken up by neighbouring IECs to facilitate epithelial turnover or migration during wound healing.

The general purpose of the AJ is to provide connection points for adjacent epithelial cells to adhere through binding with adjacent cell actin cytoskeletons. Other AJ proteins were also evaluated for their abundance in colon tissue of WT and miR-21^{-/-} male mice of the DSS recovery model. Although there was no difference in protein abundance in proximal colon tissue, there were notable decreases in α -catenin, p120-catenin and β -catenin in distal colons of miR-21^{-/-} compared to WT mice. This was surprising as results go against mRNA data that showed an upregulation in AJ genes within distal tissue of DSS-treated miR-21^{-/-} mice during recovery, suggesting miR-21-dependent post-transcriptional changes. With the decrease in intact E-cadherin levels in distal colons of miR-21^{-/-} mice, perhaps AJs are not necessary for recovery from DSS-induced colitis, as the presence of stable AJs would result in a very rigid epithelial layer that is difficult to adapt.

Since association of p120-catenin with E-cadherin is proposed to stabilize E-cadherin at the plasma membrane, it is likely that connection between adjacent IECs is less stable in the recovery phase of DSS-treated miR-21^{-/-}. In fact, the loss of p120-catenin-dependent stabilization of E-cadherin was linked to tumour progression, allowing for greater motility of cancerous cells.¹²⁹ In a wound healing scenario, IECs would also need destabilized AJs for better migration into the wound site. Interestingly, interaction of the E-cadherin-p120-catenin AJ complex with PLEKHA7 was shown to recruit miRNA synthesis machinery such as DROSHA and Ago2²⁶⁴, increasing the likelihood of producing pro-inflammatory miRNAs in WT mice. Moreover, β -catenin was shown to decrease in patients with active UC compared to other IBD types and controls.⁹⁷ As mentioned, β -catenin does not only localize to E-cadherin and AJs, but can translocate into the nucleus to mediate genes involved in cell proliferation through binding with transcription factor TCF/LEF.¹²⁹ Previous studies identified transcription factor BCL9-2 as a regulator for this β -catenin switch between cell adhesion and Wnt signalling activation.²⁷⁰ But with its mitogenic properties outside of AJs and its reduction in DSS-treated miR-21^{-/-} tissue during recovery, β -catenin is likely degraded in the cytosol by the proteasome. Nonetheless, future work on the subcellular localization of β -catenin in IECs will highlight the fate of β -catenin for transcriptional activation or degradation. Furthermore, its reduction may explain the

decrease in α -catenin levels as well, since α -catenin requires β -catenin interaction for linking the actin cytoskeleton to AJs.¹²⁹ However, it is unclear whether the reduction in AJ proteins is a consequence of the reduction in intact E-cadherin or caused by a separate mechanism. Altogether, more plasticity along intestinal epithelia may benefit re-establishing gut barrier integrity after colitis

Since there was evidence of some miR-21 regulation of E-cadherin, the final step was to determine if the regulation was due to direct binding to the 3'-UTR of the *CDH1* gene. Previous studies looking at direct targeting by miR-21 had used HEK293T cells as models for good transfection efficiency with LPS as an inducer of miR-21 expression.⁸⁹ This method was utilized, along with transfecting cells with the pCMV overexpression system. There was a notable decrease in luminescence observed for cells transfected with the pmiRGLO-*CDH1*-3'UTR construct compared to the empty luciferase reporter plasmid, suggesting there may be some hindrance of luciferase transcription due to the insertion of the UTR sequence. Nonetheless, there was a significant decrease in luminescence observed between pCMV-miR-21 and pCMV controls, indicating that miR-21 inhibited expression of the luciferase reporter, and that E-cadherin is directly impacted by miR-21 regulation.

As mentioned, the role of E-cadherin in gut barrier integrity is multi-faceted, and thus with fragmented forms of the protein seen in mouse tissue, a focus was put on epithelial wound healing. Regarding chosen targets to pursue, one study found that JAM-A (encoded by *F11R*) is essential for wound healing in the gut through interaction with E-cadherin and by establishing focal adhesion points between IECs.²⁵³ Additionally, several pathways that initiate wound healing closure were found to be TGF- β -dependent, with multiple pathway receptors that form homomeric or heteromeric complexes with one another and themselves.²⁷¹ *In vivo* gene expression analyses demonstrated *F11R* was negatively associated with the presence of miR-21, and in fact there was a partial rescue observed in TNF- α -treated Caco-2 cells transfected with miR-21 inhibitor (data not shown). TGF- β was also induced in miR-21^{-/-} male colon tissue of the DSS recovery model, and predicted targets of the TGF- β receptors were also upregulated within DSS-treated miR-21^{-/-} mice during recovery compared to WT. This was further confirmed in isolated IECs collected from non-littermate miR-21^{-/-} male mice, showing induction of AJ-related wound healing genes and TGF- β receptors. TGF- β is known to induce the expression of miR-21 in EMT models of HaCat keratinocytes, and is associated with an anti-proliferative, pro-migratory phenotype.²⁷² Smad7, an I-Smad that moderates TGF- β pathway activation via competitive

binding to activated TGF- β receptors, also showed an induction in miR-21^{-/-} IECs compared to WT. Lin *et al.* (2014)²⁵⁵ demonstrated that Smad7 was shown to be a direct target of miR-21, which was further confirmed via reduction of Smad7 protein levels in rat renal tubular epithelial cells transfected with lentivirus overexpressing miR-21 compared to empty controls. This negative feedback loop may be required for IECs to only undergo partial EMT, thereby alleviating the TGF- β -induced repression of their epithelial phenotype after migration into the wound. Therefore, miR-21 acts in both ways as a “fine tuner” of the TGF- β pathway mechanism.

Expanding to the *in vitro* system aided in determining whether miR-21 specifically impacted E-cadherin and AJs in general. TNF- α was shown to induce expression of miR-21 in Caco-2 cells^{119,256}, and therefore assay conditions were replicated to reproduce these effects. TNF- α is known to induce expression of miR-21, though this may be an indirect consequence of TNF- α -induced IL-6²⁷³, which was shown to upregulate miR-21 levels.²⁷⁴ Despite increasing levels of pri-miR-21 and mature forms, TNF- α treatments did not produce a reduction in E-cadherin gene expression. Nonetheless, there was a modest reduction around the cell peripheries of Caco-2 monolayers observed through immunofluorescence. It is possible that TNF- α -induced inflammation alone does not necessitate a negative impact on AJs, but rather an infiltration of opportunistic pathogens that would occur during colitis *in vivo*. Intracellular pathogens not only heighten cell pathways for altered cell growth/metabolism, but also commonly attack cytoskeletal elements bound to AJ complexes.²⁷⁵ While TNF- α treatment does mimic that environment to a certain extent with the activation of NF- κ B signalling, the additive presence of gut microbiota may be required to amplify deterioration, such as seen in studies assessing the impact of *M. tuberculosis* on macrophages.⁸⁹

Creation of an *in vitro* system that can induce expression of both mature forms of miR-21 would greatly benefit understanding general impact on barrier components like intercellular junctions. Multiple methods were explored, and in the end, transfection of the pCMV plasmid that constitutively expressed miR-21 through the CMV promotor was the most reliable method of modulating miR-21 levels in TNF- α -treated Caco-2 cells. The miR-21 inhibitor was also utilized despite its unknown mechanism of action for miR-21 degradation or sequestration.²⁵⁸ TNF- α was used to facilitate action of these miR-21 modulators as done by previous researchers^{119,256}, showcased by notable changes in mature miR-21 expression. Although no major changes were seen at the mRNA level (data not

shown), E-cadherin protein levels were partially lost at cell peripheries when transfected with pCMV-miR-21 and partially rescued when transfected with miR-21 inhibitor. This likely indicates that there are other regulators that influence E-cadherin synthesis in IECs. Although multiple transcription factors known to regulate E-cadherin such as Zeb1/2 and Rab11A²⁷⁶ were found in miR-21 prediction databases, others like snail family transcription factors did not appear in our search.

In the final part of this chapter, determining the link between these signalling pathways and AJ stability through artificial wounds in the Caco-2 system further elucidated the impact miR-21 has on wound healing. Interestingly, Caco-2 cells transfected with pCMV-miR-21 showed a decrease in wound area 72 hours post-scratch compared to groups transfected with miR-21 inhibitor. Previous studies have marked miR-21 as both an inducer and suppressor of inflammation in response to wound healing. One study showed that the presence of miR-21 upon wound injury is important for both activation of pro-inflammatory M1 macrophages to engulf damaged or apoptotic cells²⁷⁷, while a separate study demonstrated miR-21 aided in reducing TLR4-induced inflammation by targeting *PDCD4*.²⁷⁸ The effects observed in our scratch assays could be attributed to the specific induction of miR-21-3p during overexpression from analysed RNA lysates of test groups, and thus hindering this mature form could prevent wound healing pathways to take effect. Indeed, miR-21-3p agonist injected into dorsal wounds of mice demonstrated a significantly higher wound closure rate compared to control mice, associated with direct targeting of *SPRY1* mRNA.²⁷⁹ Therefore, specific forms of miR-21 may play different roles in the wound healing process, and their origin may be cell type specific.

In both basal and TNF- α -treated Caco-2 cells of the scratch test, junction-related genes *CDH1* and *F11R* showed a surprising stable expression in pCMV-miR-21 transfected cells compared to miR-21 inhibitor, which is similar to results seen in the miR-21^{-/-} mouse model during recovery from DSS-induced colitis. Therefore, excluding all other stimuli other than those produced by IECs, an increase in miR-21 *in vitro* led to the same result as ablation of miR-21 during DSS-induced recovery *in vivo*. This result may be explained by the several known regulatory pathways for these junction-related genes. In scratch tests, migration of large sheets of epithelial cells were observed during wound closure. Hwang *et al.* (2012)²³⁹ demonstrated that E-cadherin was shown to be upregulated at the leading edge of epithelial sheet migration in a CXCL12-dependent manner. Restitution is also impaired in the absence of JAM-A, shown to regulate migration distance and velocity of the leading edge of primary IEC cultures under wound conditions, leading to a reduction

in Rap1a activity for focal adhesion structure stability.²⁵³ Furthermore, tissues from our DSS-treated mice were taken five days post-treatment, where recovery is likely in its final stages of proliferation and cell differentiation, and re-establishment of strong connection between polarized IECs has already taken effect. Moreover, in the initial scratch tests, not only was *TGFB* induced in cells transfected with pCMV-miR-21 under basal conditions, but *TGFBR2* and *TGFBR3* were also reduced in overexpression groups (data not shown). This potentially increases the amount of TGFBR1 binding with TGF- β ligand for regulating signal transduction leading to specific downstream outcomes, since the formation of receptor homomeric or heteromeric complexes can increase affinity for different TGF- β family ligands and even downstream signal transduction in other signalling pathways.²⁸⁰ For example, activation of ERK MAP kinase signalling pathway through activated TGFBR1 interaction with p52ShcA is accomplished by competitive binding to TGFBR1 with SMAD3.²⁸¹ In addition, TGF- β -induced activation of the PI3K-mTOR-AKT signalling requires TGFBR1.²⁸² Both pathways were shown to be impacted by miR-21 and regulate intercellular junction gene expression and gut permeability.

With the current methodology for conducting the scratch test, it was uncertain whether miR-21 in IECs is truly important for wound healing, or whether miR-21 inhibitor molecules were saturated and thus ineffective at hindering miR-21 from binding to targets. Increasing the concentration of miR-21 inhibitor improved wound closure similar to transfection with pCMV-miR-21 in the presence of TNF- α , with some improvement under basal conditions. This suggests that miR-21 may play dual roles in the regulation of wound healing, and by overexpressing or inhibiting its function under an inflamed state, IECs can increase rate of wound closure. In this case, miR-21 inhibitor can act similar to DSS-treated miR-21^{-/-} mice during recovery, whereby increasing levels of TGF- β family genes for pathway activation. Indeed, one study using miR-21 inhibitors demonstrated a reduction of pro-inflammatory cytokine secretion by TNF- α -treated Caco-2 cells.¹¹⁹ Another study using miR-21 inhibitors showed a downregulation of Wnt signalling through derepressed Wnt pathway inhibitor MDTH and reduced levels of β -catenin.²⁵⁶ These findings suggest the function of miR-21 in wound healing is temporal. Thus, although inhibition of miR-21 may enhance the initial step of epithelial cell migration, the overexpression of miR-21 could relieve pathway inhibition through targeting Smad7, or enhance the subsequent steps of proliferation and differentiation once epithelial cells reach the wound site, indicating yet another novel fine-tuning aspect of miR-21 regulation during wound healing.

Chapter 4

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miR-21, Tight Junctions & Golgi Remodelling

4. miR-21 regulates tight junctions and Golgi remodelling for vesicular trafficking

4.1 Introduction

Under homeostatic conditions, specialized IECs are tightly bound to one another through connection points called intercellular junctions, ensuring gut barrier stability. Localization of intercellular junctions is based on the polarization of IECs, where AJs, TJs and desmosomes are found at the apical end, while GJs and hemidesmosomes are located on the lateral or basolateral sides.²⁷ Indeed, each type possesses unique roles that govern gut barrier homeostasis, and aberrations in their expression or general function have often been linked to gut permeability during intestinal inflammation.

The most frequently studied intercellular junction is the TJ, controlling paracellular diffusion of ions and small molecules through the barrier, as well as restricting entry of gut microbiota.²⁷ Within the TJ is a variety of proteins important for localization to the plasma membrane and regulation of their functional roles. Occludin, a transmembrane protein and one of the most prominent within the TJ complex, has been negatively associated with the development of IBD. Downregulation and redistribution of occludin have been observed in IBD tissues, while scaffolding protein ZO-1 was also reduced in IBD tissue samples.²⁷ Furthermore, full KO mouse models of various claudin proteins like claudin-3, claudin-7 and claudin-15 showed increased gut permeability and disruption of absorptive activity.^{179,283,284} Thus, this project sought to examine TJ expression and adjacent pathways under miR-21 regulation in both healthy and inflamed conditions.

The aim of this chapter was to expand on the role of TJs at the gut barrier under regulation of miR-21 with particular focus on a novel connection these junctions have to Golgi remodelling for vesicular trafficking. We investigate the impact of miR-21 expression on TJ regulation by performing an in-depth assessment on IECs and TJs using *in vivo* mouse samples from healthy and DSS-treated miR-21^{-/-} mice, as well as the *in vitro* Caco-2 system with miR-21 modulators. In the absence of miR-21, TJ components claudin-3, claudin-4 and claudin-15 were upregulated during recovery from DSS-induced colitis, along with an increase in claudin-4 protein and a decrease in Arf4 protein levels. Finally, colocalization analyses were performed and showed that Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor-1 (GBF1) localized to the Golgi under miR-21 overexpression during TNF- α -induced inflammation, providing novel evidence of miR-21 impact on Golgi remodelling processes for vesicular trafficking.

4.2 Results

4.2.1 miR-21 ablation increases tight junctions in intestinal epithelial cell isolates under healthy conditions

To fully understand the importance of miR-21 expression on intercellular junctions and general gut barrier integrity, a preliminary search was conducted to identify miR-21 predicted targets related to TJs and desmosomes using various miRNA prediction platforms.^{39,241–244} Based on these databases, multiple known and predicted miR-21-5p and miR-21-3p targets were collected and summarized in **Table 4.1**.

Table 4.1 – miR-21 predicted target search related to tight junctions and desmosomes.

Type	Common Name	Gene Name	TargetScan (score*)	miRDB (score*)	miRWalk (score*)	DIANA-TarBasev9.0 (score*)	Pictar (score*)
Tight Junctions	Occludin**	<i>OCN</i>	3p (0.10)		5p (0.92), 3p (0.92)	5p (1.00), 3p (0.79)	
	ZO-1**	<i>TJP1</i>	3p (0.02)		5p (0.92), 3p (1.00)	5p (1.00), 3p (0.56)	
	ZO-2	<i>TJP2</i>			3p (0.85)		
	ZO-3	<i>TJP3</i>			3p (0.92)		
	Claudin-1	<i>CLDN1</i>	3p (0.20)			5p (0.49)	
	Claudin-2	<i>CLDN2</i>			3p (0.85)		
	Claudin-3	<i>CLDN3</i>				5p (1.00)	
	Claudin-4	<i>CLDN4</i>			3p (0.92)	5p (1.00)	
	Claudin-7	<i>CDLN7</i>			5p (0.92), 3p (0.85)	5p (1.00)	
	Claudin-8**	<i>CDLN8</i>		5p (0.87)			
	Claudin-15	<i>CLDN15</i>			3p (0.92)		
Desmosomes	Desmocollin-2	<i>DSC2</i>		5p (0.54)	5p (0.85), 3p (0.85)	5p (0.70)	
	Desmoglein-2	<i>DSG2</i>			3p (0.85)	5p (0.51)	

* - all scores are out of 1; note that the score from miRDB was divided by 100 and the score from Pictar was divided by 10 to equate to scores from other databases

** - known direct target of miR-21; known negative association with miR-21 levels.

A comprehensive screen for mouse homologs of TJ and desmosome genes that were identified was performed using various intestinal samples from non-littermate WT and miR-21^{-/-} male mice under healthy conditions. Previous studies have shown that occludin (encoded by *Ocln*), a major component of gut epithelial TJs for paracellular transport¹²¹, and ZO-1 (encoded by *Tjp1*), a major scaffolding protein important for linking TJs to the actin cytoskeleton²⁸⁵, were both negatively associated to miR-21 levels¹²², and were included to assess the impact of miR-21 on intercellular junctions. As done previously, intestinal tissue samples obtained from four geographical locations were analysed. RNA analyses revealed induction of *Ocln* in various intestinal regions of miR-21^{-/-} compared to WT mice, while *Tjp1* was significantly upregulated in proximal colon tissue only (**Figure 4.1A, 4.1B**). Moreover, claudins play an important role in regulating gut permeability along the epithelial barrier.⁹⁵ Analyses on different claudins and zonula occludens showed little impact from miR-21 ablation for *Cldn1*, *Cldn3*, *Cldn4*, *Cldn7* and *Cldn15*, however there were notable increases for *Cldn2*, *Tjp2* and *Tjp3* in specific tissue regions of miR-21^{-/-} mice compared to WT (**Figure 4.1C, 4.1D, 4.1E, 4.1F, 4.1G, 4.1H, 4.1I, 4.1J**). There were no differences in the expression of desmosome genes *Dsc2* and *Dsg2* between WT and miR-21^{-/-} mice in any intestinal region (**Figure 4.1K, 4.1L**). Furthermore, expression levels of selected TJ genes were also examined in whole colon samples of non-littermate WT and miR-21^{-/-} female mice under healthy conditions. While there were no notable differences observed for *Cldn1* and *Cldn2* (**Figure 4.2A, 4.2B**), there was a significant upregulation of *Cldn3*, *Cldn4*, *Cldn7* and *Cldn15* in miR-21^{-/-} female mice compared to WT (**Figure 4.2C, 4.2D, 4.2E, 4.2F**). This suggests miR-21 expression can also have sex-dependent differences on TJ regulation.

An overarching goal of this project was to determine the contribution of miR-21 expressed by epithelial cells to the pathogenesis of IBD. Although it is generally accepted that there is an importance for IECs as both a functional element for processes like nutritional absorption and a structural obstacle for microbial protection, little is known on IEC-specific regulatory mechanisms affected by miR-21. Using whole colon tissue from WT and miR-21^{-/-} male and female colon tissue under healthy conditions, IEC isolate samples were investigated for differences in gene expression. As a preliminary step, levels of *Vill* were measured between both IEC isolates and remainder fractions. Vill is an actin-binding protein that organizes and stabilizes F-actin at the brush border and is primarily expressed by IECs.²⁸⁶ In both WT and miR-21^{-/-} colon tissue, IEC isolates had higher levels of *Vill* compared to the remainder fractions (**Supplementary Figure 8.1.1A, 8.1.1B**).

A screen was conducted comparing TJ gene expression between WT and miR-21^{-/-} male and female IECs. For male mice, there were no notable differences observed for *Cldn2*, *Cldn3* and *Cldn7* between WT and miR-21^{-/-} IEC isolate samples, though there was a significant induction in *Cldn3* and *Cldn7* IECs compared to remainder fractions, while *Cldn4* and *Cldn15* showed a significant upregulation in miR-21^{-/-} IEC isolate samples compared to WT (**Supplementary Figure 8.1.2A, 8.1.2B, 8.1.2C, 8.1.2D, 8.1.2E**). For female mice, there were no notable differences in claudin gene expression between WT and miR-21^{-/-} IEC isolate samples, though there was a significant upregulation in *Cldn2*, *Cldn3*, *Cldn7* and *Cldn15* IECs compared to remainder fractions (**Supplementary Figure 8.1.2F, 8.1.2G, 8.1.2H, 8.1.2I, 8.1.2J**). This further indicates that there exist sex-dependent differences in how miR-21 targets specific intercellular junction components in the colon epithelium.

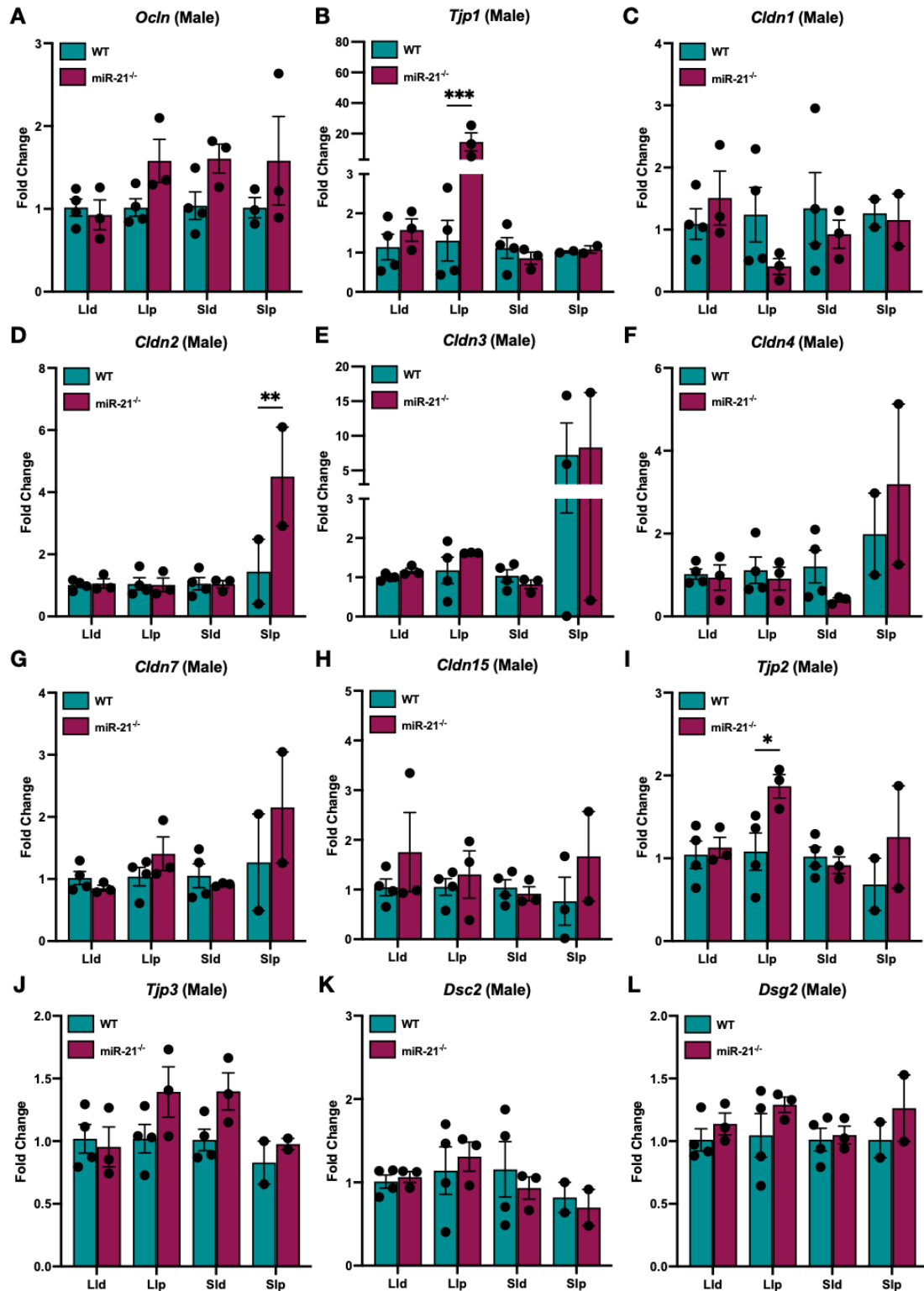


Figure 4.1 – Analysis of tight junction gene expression under healthy conditions shows few junction genes are tied to miR-21 regulation in male mouse intestinal tissue. RNA was isolated from healthy 10-week old WT and miR-21^{-/-} male mice. qRT-PCR analyses in distal colon / large intestine (LId), proximal colon / large intestine (LIp), ileum / distal small intestine (SIId) and duodenum-jejunum / proximal small intestine (SIp) tissue of WT and miR-21^{-/-} male mice. (A-J) Tight junctions (TJs): occludin (*Ocln*), zonula occludin (*Tjp*) 1-3, claudin (*Cldn*) 1, 2, 3, 4, 7, 15. (K + L) Desmosomes: desmocollin (*Dsc*) 2, desmoglein (*Dsg*) 2. Denoted are n ≥ 3 mice per group excluding SIp group specifically with n = 2, mean values ± SEM, p value calculations using two-way ANOVA with * - p ≤ 0.05, ** - p ≤ 0.01, *** - p ≤ 0.001.

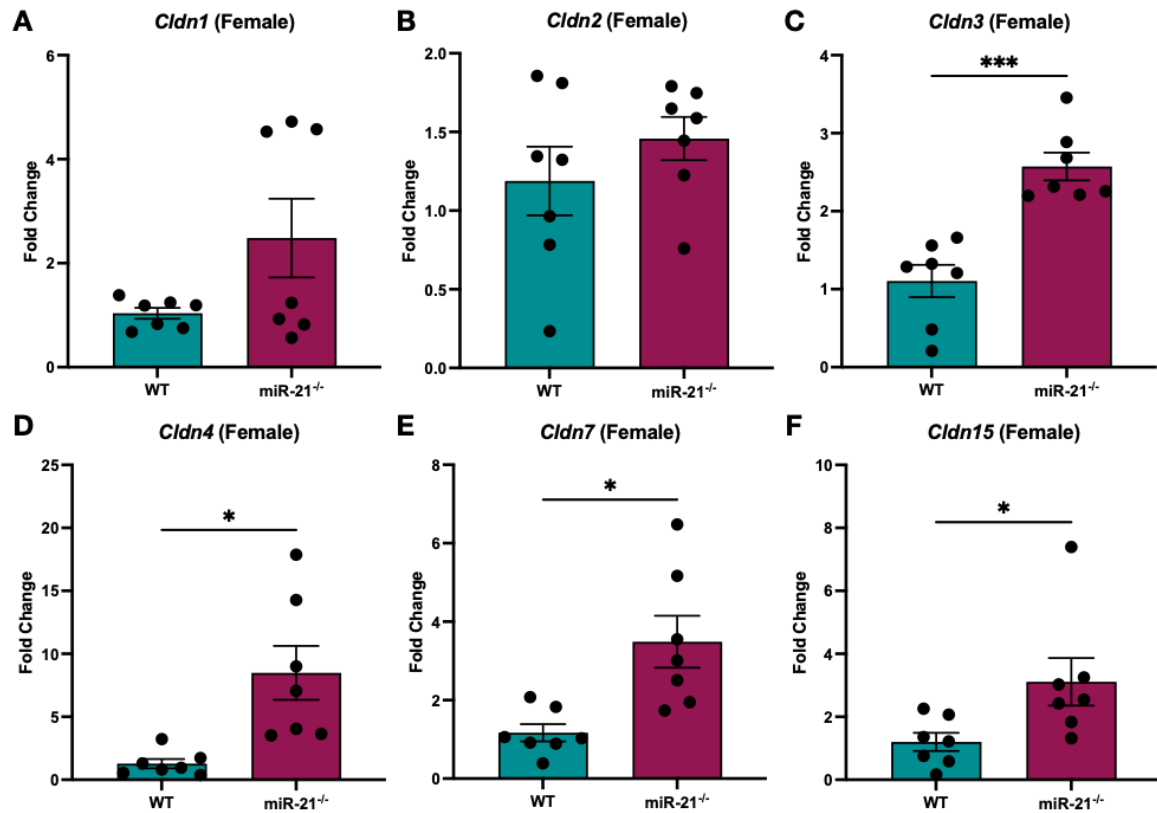


Figure 4.2 – Analysis of claudin gene expression under healthy conditions shows some genes are tied to miR-21 regulation in female mouse colon tissue. RNA was isolated from healthy 10-week old WT and miR-21^{-/-} female mice. qRT-PCR analyses in whole colon tissue of WT and miR-21^{-/-} female mice. (A-F) Tight junctions (TJs): claudin (*Cldn*) 1, 2, 3, 4, 7, 15. Denoted are n = 7 mice per group, mean values ± SEM, *p* value calculations using Welch's t-test with * - *p* ≤ 0.05. *** - *p* ≤ 0.001.

4.2.2 Analysis of DSS recovery model with non-littermate mice demonstrates miR-21 ablation increases tight junctions and desmosomes

To further expand on previous research that revealed a protective effect against inflammation due to miR-21 ablation^{85,86}, a subsequent screen for claudin gene expression was conducted on whole colon tissue of WT and miR-21^{-/-} female mice during DSS-induced colitis. There were induced trends observed for the expression of *Cldn2* and *Cldn3* in miR-21^{-/-} compared to WT mice, but there were no major differences in *Cldn1* and *Cldn4* expression (**Figure 4.3A, 4.3B, 4.3C, 4.3D**). Interestingly, there were notable reduced trends for the expression of *Cldn7* and *Cldn15* in miR-21^{-/-} colon tissue compared to WT (**Figure 4.3E, 4.3F**). Claudin-7 is a sealing claudin known for reducing microbiota dysbiosis in the gut²⁸⁴ but has significant roles in signalling pathways for cell proliferation via interaction with integrin $\beta 1$ ²⁸⁷ and cell differentiation through regulating Notch/Hippo signalling²⁸⁸, which are reduced during inflammation. Conversely, claudin-15 is a pore-forming claudin that plays a role in paracellular uptake of luminal ions⁹⁵, which can potentially increase disease severity during intestinal colitis through increased paracellular transport of water molecules and ions, leading to diarrhea. Therefore, miR-21 may act on specific signalling mechanisms during intestinal inflammation.

In WT and miR-21^{-/-} non-littermate mice undergoing recovery from DSS-induced colitis, TJ and desmosome gene expression were assessed in distal and proximal colon samples of male and female mice. For males, *Ocln* showed induction in both distal and proximal tissue samples of miR-21^{-/-} compared to WT mice (**Figure 4.4A**). For other TJ genes, there were no notable differences in the expression of *Cldn1* and *Cldn7* between WT and miR-21^{-/-} mice (**Figure 4.4B, 4.4F**). Expression levels of *Cldn2*, *Cldn3*, *Cldn4*, *Cldn15* and *Tjp3* showed an induced trend in distal tissue of miR-21^{-/-} compared to WT mice (**Figure 4.4C, 4.4D, 4.4E, 4.4G, 4.4H**). Moreover, for desmosome genes, only *Dsg2*, and not *Dsc2*, demonstrated a significant upregulation in distal tissue of miR-21^{-/-} mice (**Figure 4.4I, 4.4J**). For females, *Ocln* showed a similar induced trend in distal and proximal tissue of miR-21^{-/-} compared to WT mice (**Figure 4.5A**). Interestingly, there was an upregulated trend for *Cldn1* in both distal and proximal tissue of miR-21^{-/-} mice (**Figure 4.5B**). TJ genes *Cldn2*, *Cldn7*, *Cldn15*, showed no discernible differences between WT and miR-21^{-/-} mice in either colon region (**Figure 4.5C, 4.5F, 4.5G**). Moreover, there were notable increases in the levels of *Cldn3*, *Cldn4* and *Tjp3* in distal tissue of miR-21^{-/-} mice compared to WT, but not proximal tissue (**Figure 4.5D, 4.5E, 4.5H**). In fact, excluding *Cldn1* in female

tissue, there were no major differences observed between WT and miR-21^{-/-} mice within proximal colon tissue for any genes tested. Lastly, for desmosomes, no discernible trends were observed in distal or proximal colon tissue of miR-21^{-/-} compared to WT (**Figure 4.5I, 4.5J**). It should be noted that there were no detectable levels of *Tjp1* and *Tjp2* observed in tissue samples and therefore these were excluded from the analysis. These findings show the importance of re-establishing intercellular junctions for highlighting differences in miR-21 regulation after DSS-induced colitis.

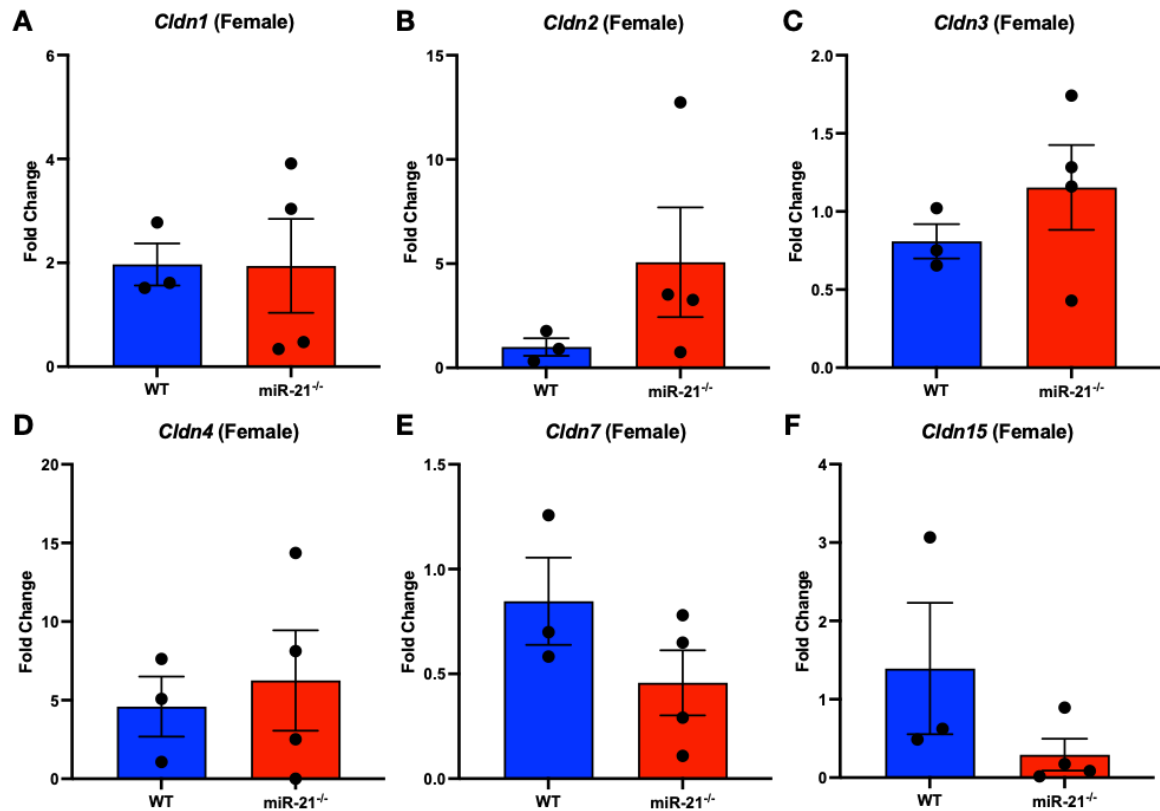


Figure 4.3 – Analysis of claudin gene expression from DSS-induced colitis in miR-21^{-/-} female colon tissue shows few differences to WT. RNA was isolated from DSS-treated 10-week old WT and miR-21^{-/-} female mice. qRT-PCR analyses in whole colon tissue of WT and miR-21^{-/-} female mice. (A-F) Tight junctions (TJs): claudin (*Cldn*) 1, 2, 3, 4, 7, 15. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

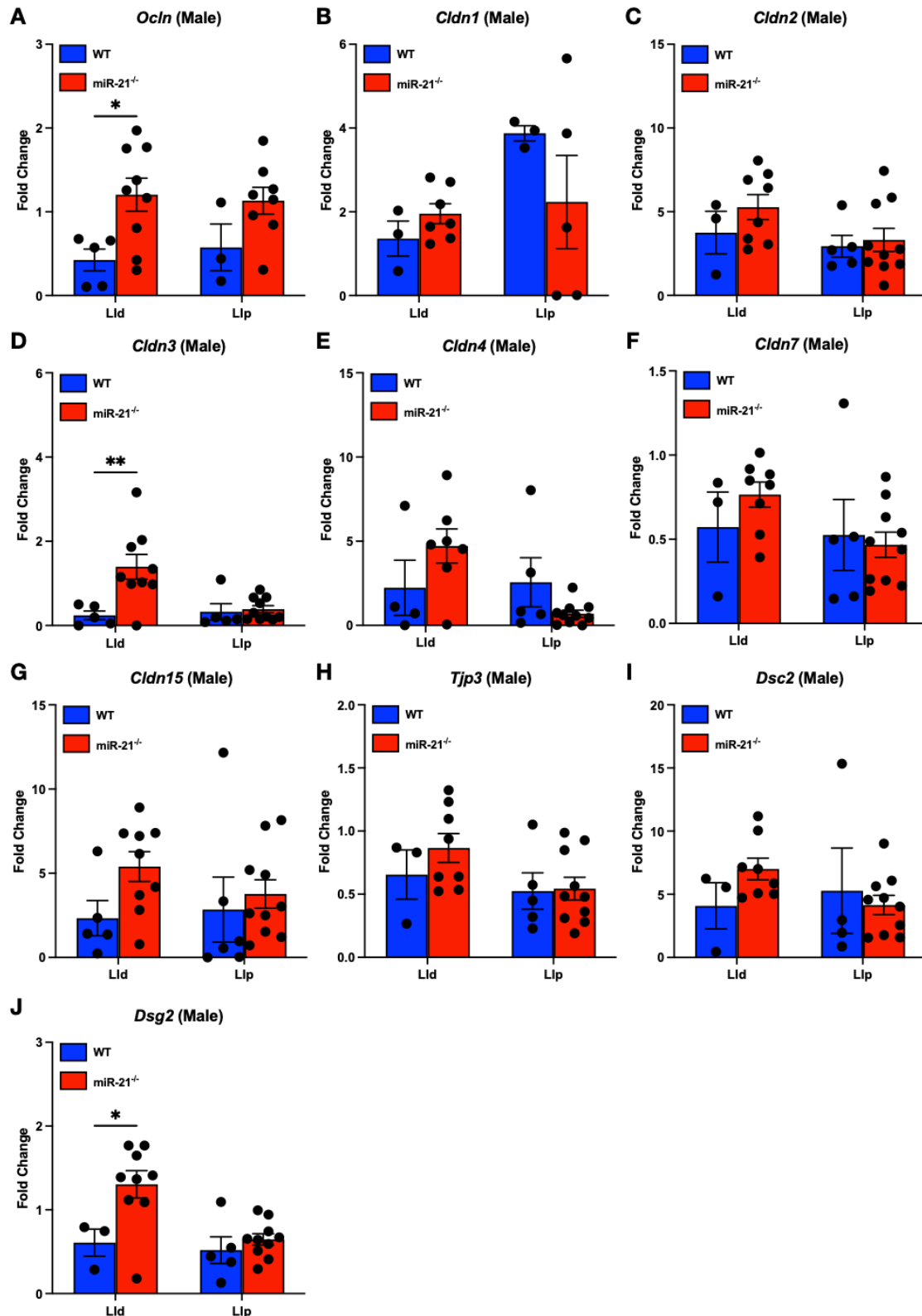


Figure 4.4 – Analysis of junction gene expression of male mice in DSS recovery model shows some tight junction and desmosome genes remain tied to miR-21 regulation. RNA was isolated from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. qRT-PCR analyses of intercellular junction genes in distal colon / large intestine (LId) and proximal colon / large intestine (Lip) tissue of WT and miR-21^{-/-} male mice. (A-H) Tight junctions (TJs): occludin (*Ocln*), claudin (*Cldn*) 1, 2, 3, 4, 7, 15; zonula occludens (*Tjp*) 3. (I-J) Desmosomes: desmocollin (*Dsc*) 2, desmoglein (*Dsg*) 2. Denoted are n ≥ 3 mice per group, mean values ± SEM, *p* value calculations using two-way ANOVA with * - *p* ≤ 0.05, ** - *p* ≤ 0.01.

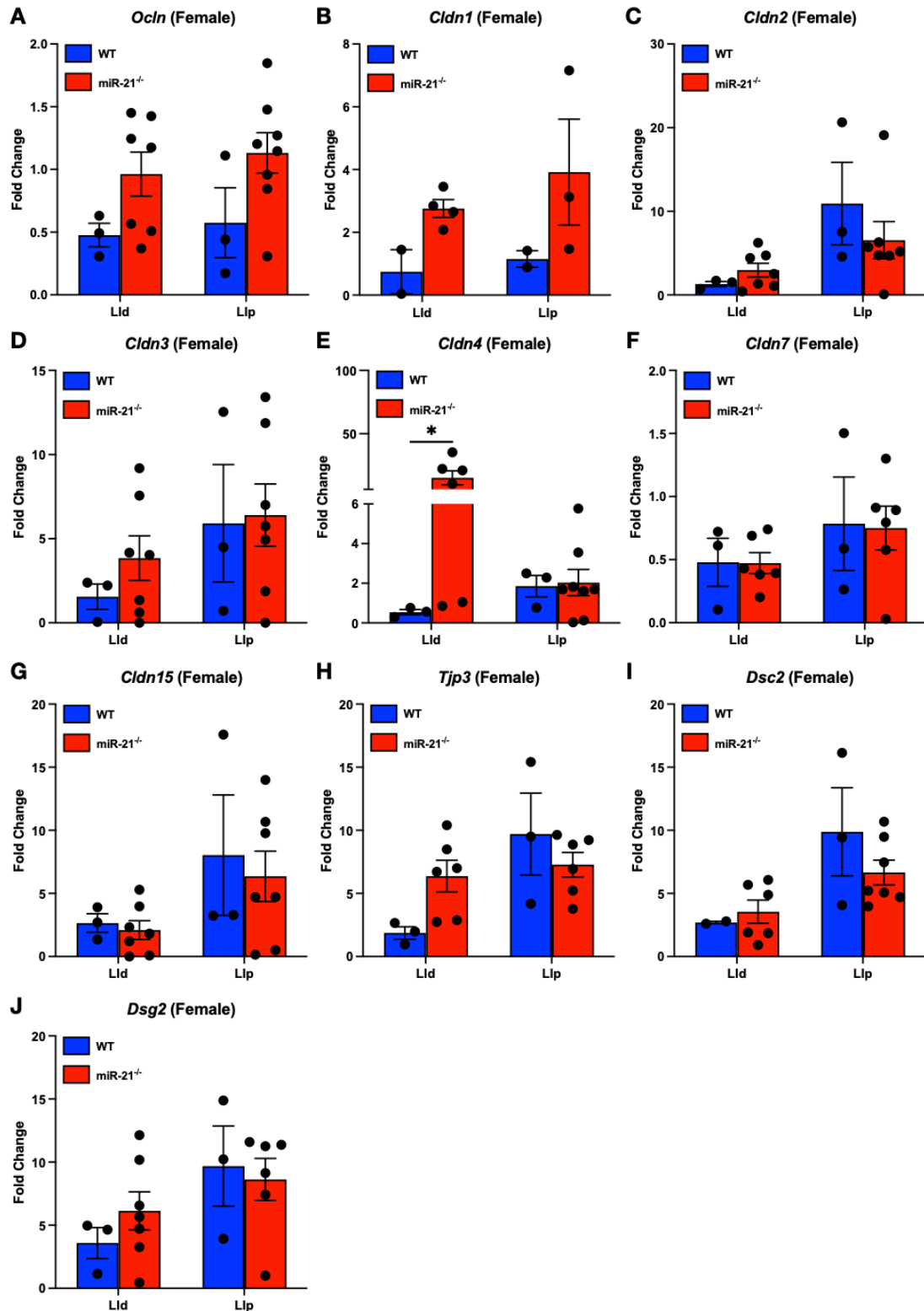


Figure 4.5 – Analysis of junction gene expression of female mice in DSS recovery model shows some tight junction genes remain tied to miR-21 regulation. RNA was isolated from 8-15 week old WT and miR-21^{-/-} female mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. qRT-PCR analyses of intercellular junction genes in distal colon / large intestine (LId) and proximal colon / large intestine (Lip) tissue of WT and miR-21^{-/-} female mice. (A-H) Tight junctions (TJs): occludin (*Ocln*), claudin (*Cldn*) 1, 2, 3, 4, 7, 15; zonula occludens (*Tjp*) 3. (I-J) Desmosomes: desmocollin (*Dsc*) 2, desmoglein (*Dsg*) 2. Denoted are $n \geq 3$ mice per group with the exception of WT *Cldn1* qPCR group that has $n = 2$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

4.2.3 Claudin-4 abundance and localization in colon tissue are altered in miR-21^{-/-} non-littermate mice in DSS recovery model

Due to its relevance for gut barrier homeostasis, along with notable differences in mRNA levels between WT and miR-21^{-/-} mice in healthy and DSS conditions, claudin-4 protein was further evaluated *in vivo*. Interestingly, immunoblots of whole cell lysates from IEC isolate samples of male mice under healthy conditions showed a downregulation in miR-21^{-/-} compared to WT (**Supplementary Figure 8.1.3A, 8.1.3B**), but no difference in IECs from female mice (**Supplementary Figure 8.1.3C, 8.1.3D**).

Additional tests assessed claudin-4 protein abundance during DSS-induced colitis. DSS-treated whole colon tissue of WT and miR-21^{-/-} female mice showed no difference in claudin-4 levels (**Figure 4.6A, 4.6B**). Using DSS recovery model samples with a focus on male mice, there was a notable induction of claudin-4 in distal colon tissue of miR-21^{-/-} compared to WT mice (**Figure 4.6C, 4.6D**). However, there was no difference observed between mouse groups in proximal colon tissue (**Figure 4.6E, 4.6F**). This is likely due to the unknown mechanism by which DSS targets the distal colon.²¹³ Furthermore, localization and abundance were examined in distal and proximal colon tissue of WT and miR-21^{-/-} male mice of the DSS recovery model through immunofluorescence. In distal tissue, claudin-4 was localized to the tops of intestinal crypts in both mouse groups, and there was a notable induction of claudin-4 fluorescence in miR-21^{-/-} compared to WT mice (**Figure 4.7A, 4.7B**). In proximal tissue, claudin-4 was more evenly spread along the crypt axis in miR-21^{-/-} mice compared to WT, with an increased trend of quantified fluorescence (**Figure 4.8A, 4.8B**). Therefore, this suggests that miR-21 regulation of claudin-4 may be more relevant post-intestinal injury, causing changes in protein levels and localization that is specific to intestinal region or severity of inflammation.

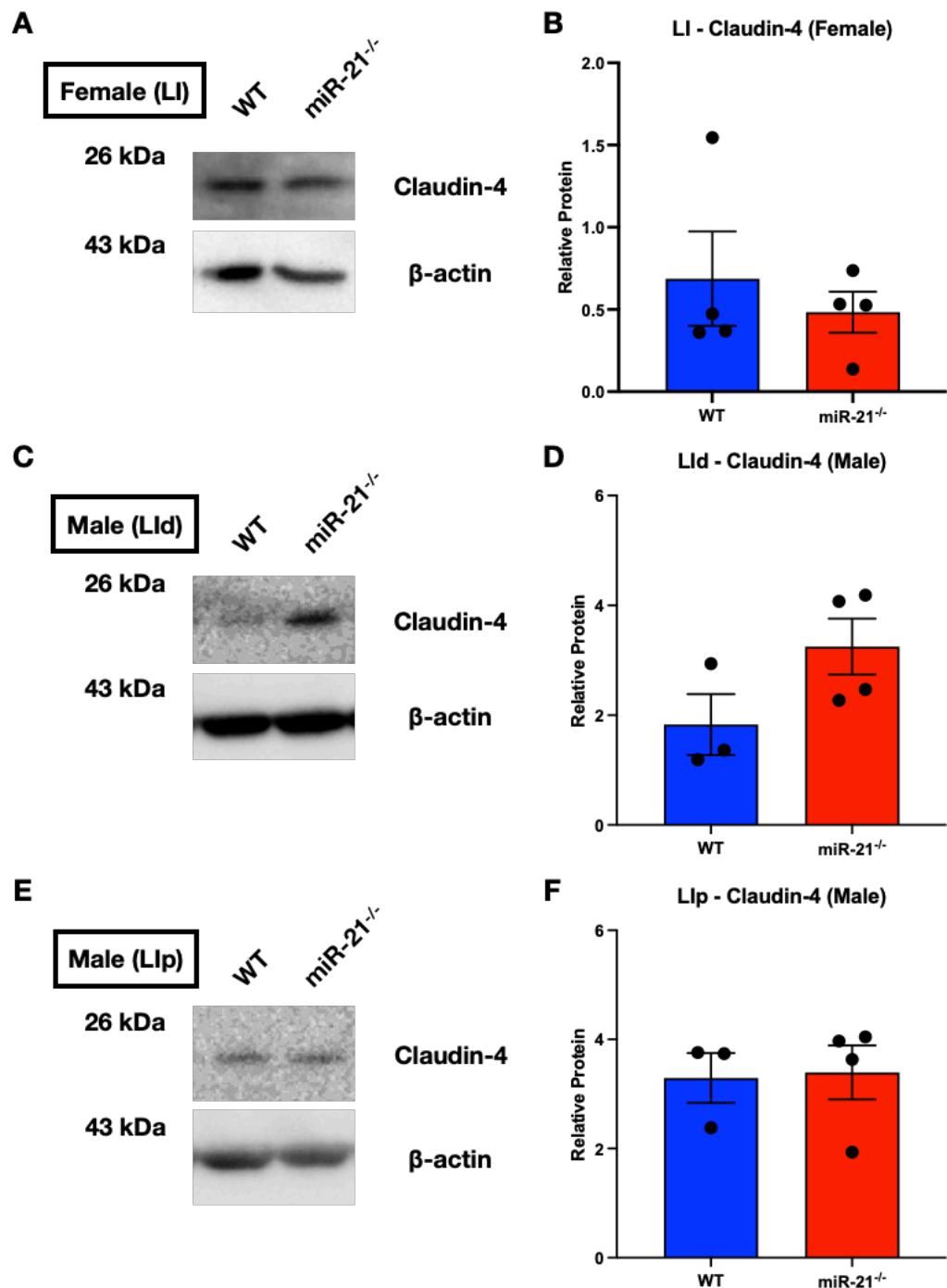


Figure 4.6 – Claudin-4 is induced in distal colon tissue of non-littermate miR-21^{-/-} male mice of DSS recovery model. Protein lysates were obtained from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. Immunoblots and quantification for claudin-4 from (A + B) whole colons of DSS-treated WT and miR-21^{-/-} female mice, as well as (C + D) distal colon / large intestine (LId) and (E + F) proximal colon / large intestine (LIp) tissue of WT and miR-21^{-/-} male mice. Note: β-actin loading control blots are the same as those used in Chapter 1, Figure 3.29 (p120-catenin figure for distal and same loading control for proximal blot). Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t -test.

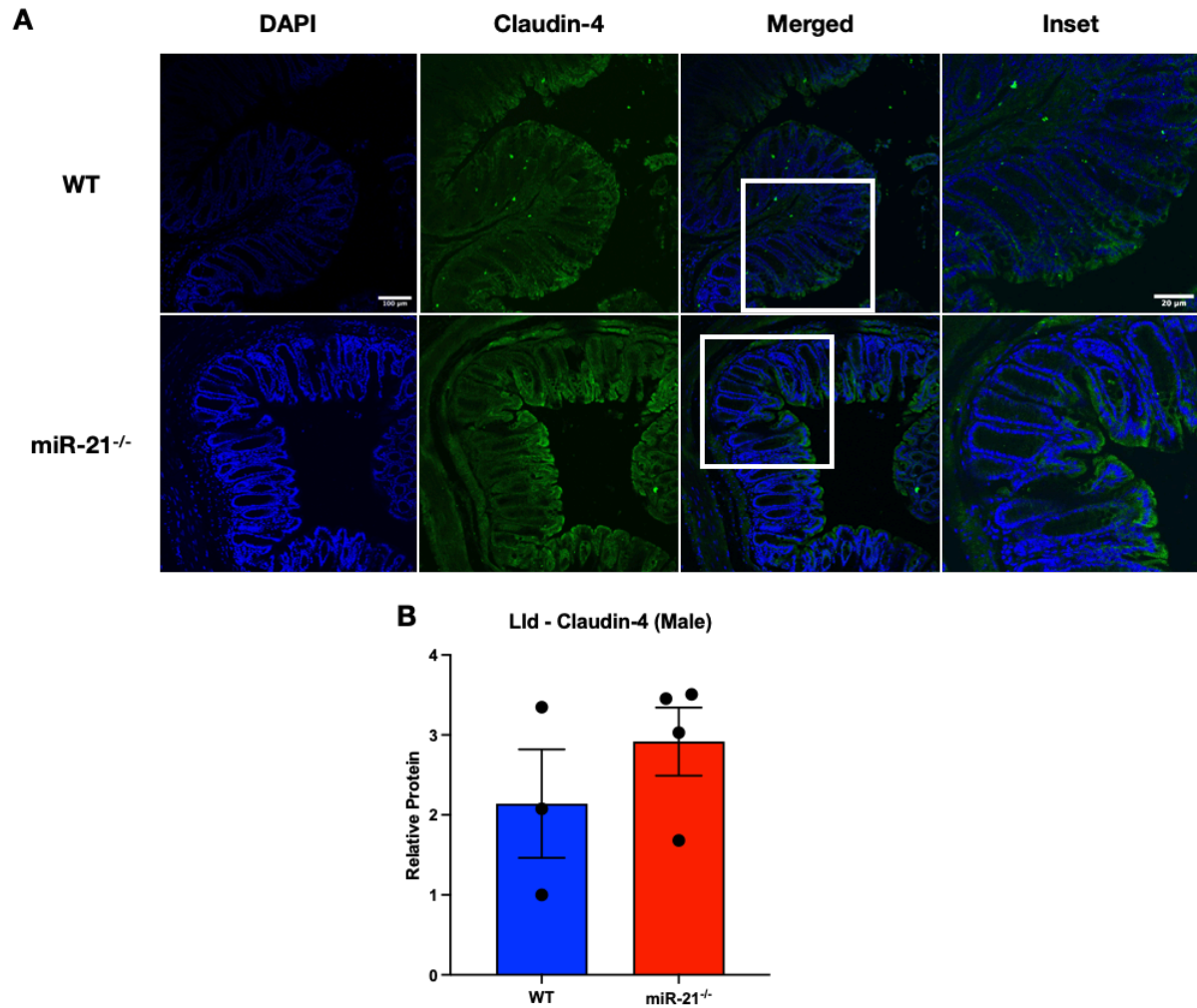


Figure 4.7 – Claudin-4 is induced in non-littermate miR-21^{-/-} male distal colon tissue and localized to the top of intestinal crypts of the DSS recovery model. (A) Distal colon / large intestine (LId) tissue sections of WT and miR-21^{-/-} male mice were removed, flushed with sterile PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-claudin-4 primary antibody (green). (B) Quantification of fluorescence was normalized to number of nuclei per field of view. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t -test.

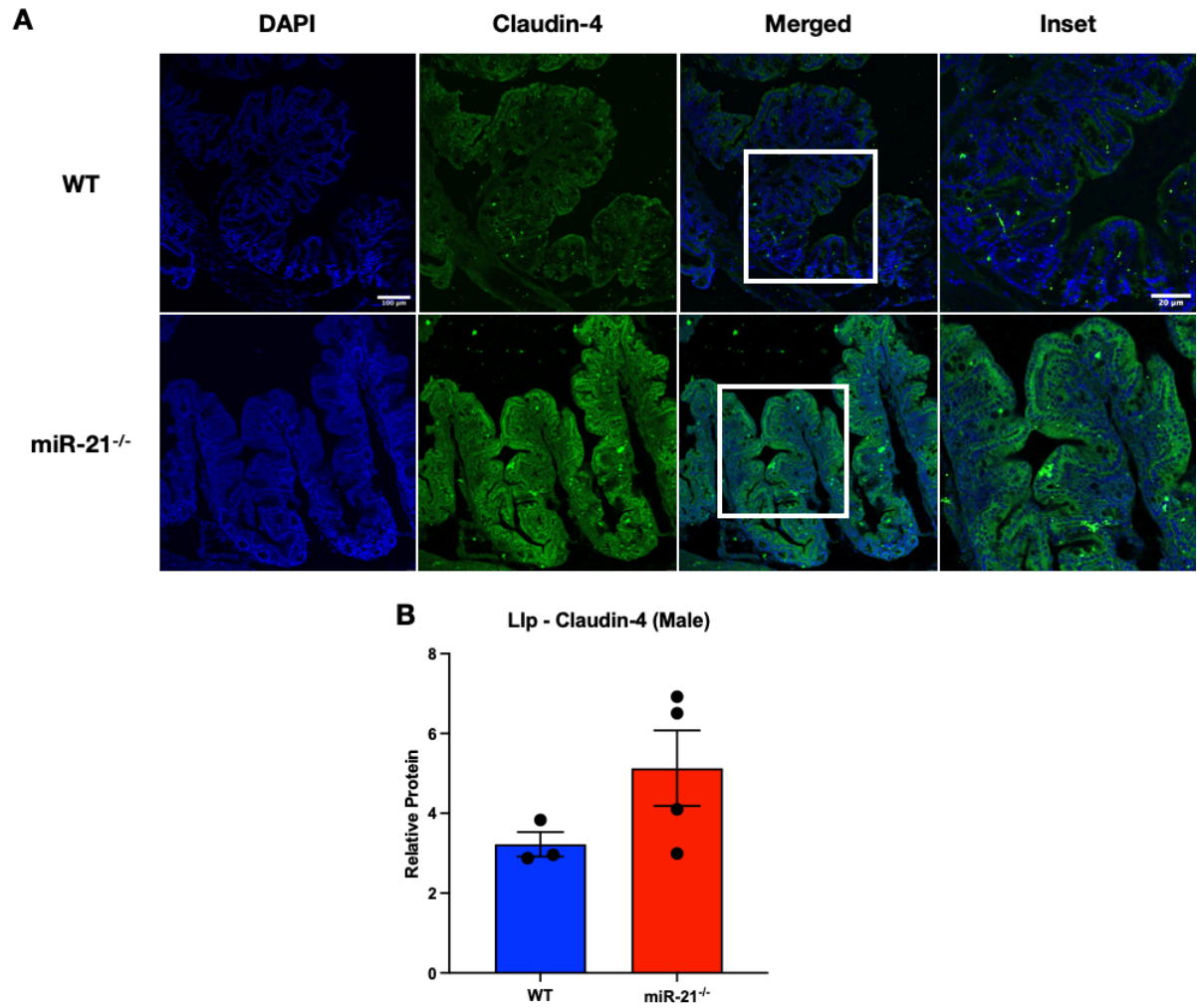


Figure 4.8 – Claudin-4 is induced in non-littermate miR-21^{-/-} male proximal colon tissue and more dispersed along crypt axes of the DSS recovery model. (A) Proximal colon / large intestine (Lip) tissue sections of WT and miR-21^{-/-} male mice were removed, flushed with sterile PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-claudin-4 primary antibody (green). (B) Quantification of fluorescence was normalized to number of nuclei per field of view. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

4.2.5 miR-21 regulation of claudin-4 and link to activation of Arf4 at the Golgi

To further explore the regulation of TJs and claudin-4, we questioned whether miR-21 directly or indirectly acts on these targets. A prior published study suggested an indirect link between miR-21 and claudin-4, through the regulation of the GTPase ARF4. Researchers found that through inhibition of *ARF4*, mRNA levels of claudin-4 were significantly upregulated in Caco-2 cells.¹²⁰ However, any mechanistic link or downstream consequences on cellular processes has yet to be established. Thus, we investigated the downstream impact of miR-21 on ARF4 and its related functions. ARF4 requires activation by GEFs like GBF1 and phosphoinositide (PIP) signalling molecules, found within the PI3K-Akt pathway for general cellular processes.²⁸⁹ Furthermore, these two Golgi-related genes appeared in miR-21 prediction databases, summarized in **Table 4.2** below.

Table 4.2 – miR-21 predicted target search related to Golgi processes.

Common Name	Gene Name	TargetScan (score*)	miRDB (score*)	miRWalk (score*)	DIANA-TarBasev9.0 (score*)	Pictar (score*)
Arf4	<i>Arf4</i>			3p (0.85)	5p (1.00)	
Gbfl	<i>Gbfl</i>			5p (1.00), 3p (0.85)	5p (1.00)	

* - all scores are out of 1; note that the score from miRDB was divided by 100 and the score from Pictar was divided by 10 to equate to scores from other databases

To expand on our observation that *Cldn4* is more highly expressed in IEC isolates from miR-21^{-/-} mice compared to WT, the expression of Golgi related genes *Arf4* and *Gbfl* were assessed. In male IECs, there was no major differences observed between mouse groups or fractions for *Arf4* (**Supplementary Figure 8.1.4A**). There was a notable reduction in *Gbfl* mRNA for miR-21^{-/-} IEC isolate samples compared to WT, with levels in WT IECs significantly greater than in the WT remainder fraction (**Supplementary Figure 8.1.4B**). In female IECs, there were no differences between WT and miR-21^{-/-} samples for either *Arf4* or *Gbfl*, though both genes demonstrated induced expression in WT IECs compared to the WT remainder fraction (**Supplementary Figure 8.1.4C, 8.1.4D**). These findings indicate the significance of these genes for the intestinal barrier and perhaps an impact on Arf4-Gbfl shuttling to the Golgi.

To determine the importance of TJs and these Golgi-related genes during intestinal barrier formation, primary IECs of non-littermate WT and miR-21^{-/-} male mice were grown *ex vivo* for seven days and subsequent RNA analyses were carried out. In addition to basal conditions, IECs were also treated with TNF- α for 24 hours to replicate an inflamed state. *Ocln* was observed to have a modest induction in miR-21^{-/-} IECs compared to WT in both basal and TNF- α -treated cells (**Figure 4.9A**). There was a significant upregulation of *Cldn4* in miR-21^{-/-} compared to WT IECs in both basal and TNF- α conditions (**Figure 4.9B**). Interestingly, there was also a mild but notable induction of *Arf4* and *Gbfl* in miR-21^{-/-} IECs compared to WT for both basal and TNF- α conditions (**Figure 4.9C, 4.9D**). Thus, *Arf4* and *Gbfl* may have important miR-21 dependent roles in barrier integrity.

Continuing the investigation into Golgi-related genes, expression levels were also examined in distal and proximal colon tissue of WT and miR-21^{-/-} male and female mice of the DSS recovery model. In males, there was an induction for *Arf4* and *Gbfl* in distal tissue of miR-21^{-/-} compared to WT mice, but not proximal tissue (**Figure 4.10A, 4.10B**), though there was high variability in *Arf4* levels within miR-21^{-/-} distal tissue. Similar results were also observed in females, with the addition of a mild induction of *Gbfl* seen in miR-21^{-/-} female proximal tissue (**Figure 4.10C, 4.10D**).

Arf4 protein abundance was evaluated in distal and proximal colon tissue of WT and miR-21^{-/-} mice used in the DSS recovery model, again focusing on male mice that showed differences in disease severity during DSS treatment. Despite the upregulation of *Arf4* mRNA in distal tissue, *Arf4* protein was reduced in miR-21^{-/-} distal tissue compared to WT (**Figure 4.11A, 4.11B**). Immunoblot for proximal tissue showed no major differences in *Arf4* abundance between WT and miR-21^{-/-} male mice (**Figure 4.11C, 4.11D**). Thus, this indicates that *Arf4* may have less impact on the Golgi during recovery from DSS-induced colitis in the absence of miR-21.

In conjunction with the reduction of *Arf4* protein within distal tissue of DSS-treated miR-21^{-/-} male mice, it was apparent that *Gbfl* protein abundance and localization in the cell was also important to connect phenotypic consequences with the Golgi, and thus overall function of the Golgi in IECs during inflammation. Despite *in vitro* RNA levels for *ARF4* and *GBF1* showing only a mild impact in response to miR-21 modulation during basal or TNF- α conditions in Caco-2 cells (data not shown), perhaps abundance or localization of GBF1 to the Golgi is impacted by miR-21 modulation. Here, the *in vitro* system with TNF- α -treated Caco-2 cells transfected with miR-21 modulators was utilized to investigate GBF1 through immunofluorescence. Similar to the scratch test in the

previous chapter, conditions were designed such that there were cells undergoing miR-21 inhibition, miR-21 overexpression and a combination of both. As a colocalization marker for the Golgi, cells were stained for GLG1, a glycoprotein and receptor for FGFs.²⁹⁰ Briefly, colocalization was measured by analyzing deconvoluted z-stack images with the DiAna ImageJ plugin.²³⁴ Through segmentation, local maxima (ie. high intensity fluorescent signals for GLG1 and GBF1) were designated as “objects”, and objects in each z-stack were assessed for overlap between GLG1 and GBF1 channels. Only objects that surpassed a strict threshold for radial size and seed intensity were used in image analyses. Representative images for each test group can be seen in **Figure 4.12A**. A visual representation of colocalized objects between GLG1 and GBF1 channels can be seen in the rightmost panel. Quantified GBF1 fluorescence showed no significant differences between test groups (**Figure 4.12B**). Colocalized objects were normalized to the number of nuclei per field of view, where cells transfected with pCMV-miR-21 demonstrated a significant increase compared to groups transfected with miR-21 inhibitor (**Figure 4.12C**). Lastly, to confirm whether the colocalized objects have differences in proximity, thereby potentially inflating the number of colocalized objects, volumes of overlapping objects were compared and showed no difference between test groups (**Figure 4.12D**). Overall, these findings indicate that overexpression of miR-21 leads to GBF1 localization to the Golgi, which can activate ARF4 and impact vesicular trafficking.

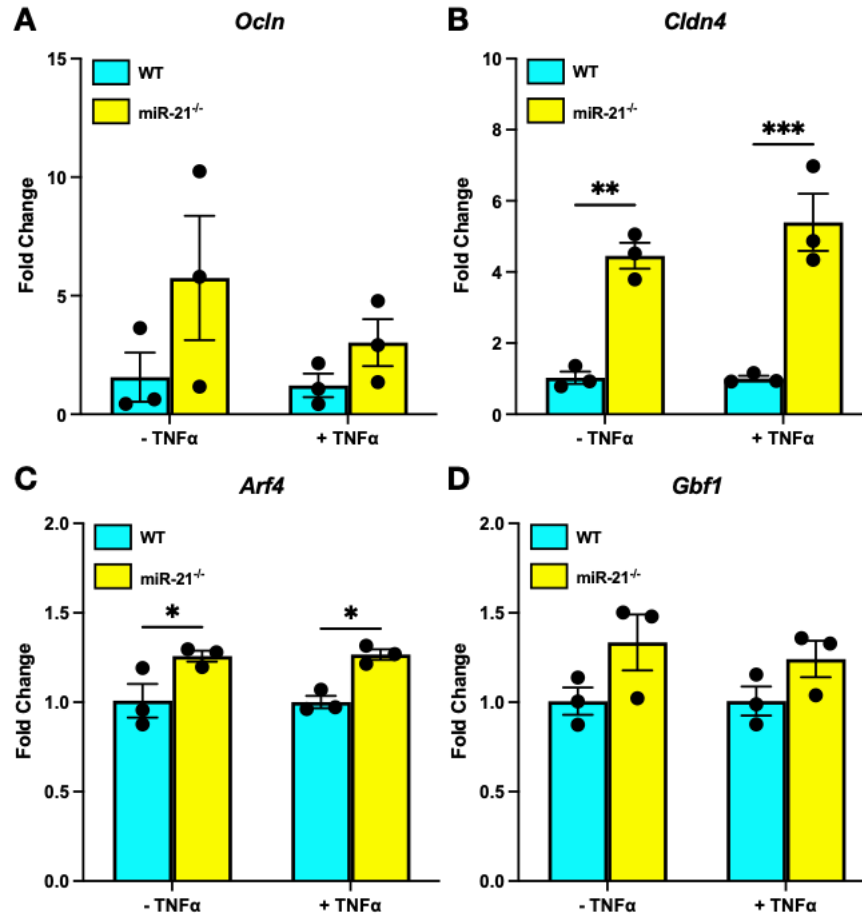


Figure 4.9 – Tight junction and Golgi-related genes are significantly upregulated in IECs isolated from miR-21^{-/-} male mice *ex vivo* grown under basal and TNF- α conditions. Healthy 10-week old WT and miR-21^{-/-} male mice had colons digested in a digestion mixture of 0.3 mL collagenase dissolved in DMEM. Isolated IECs were plated on Matrigel (Corning) basement membrane with complete DMEM in 96-well plates for seven days. RNA was isolated from basal IECs on the seventh day, while other IECs were treated with 150 ng/mL TNF- α and RNA isolated 24 h post-treatment. (A-D) qRT-PCR analyses from IECs isolated from WT and miR-21^{-/-} mice for tight junctions (TJs): occludin (*Ocln*), claudin (*Cldn*) 4; Golgi-related: *Arf4*, *Gbfl*. Denoted are n = 3 mice per group, mean values $\pm$ SEM, *p* value calculations using two-way ANOVA with * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$.

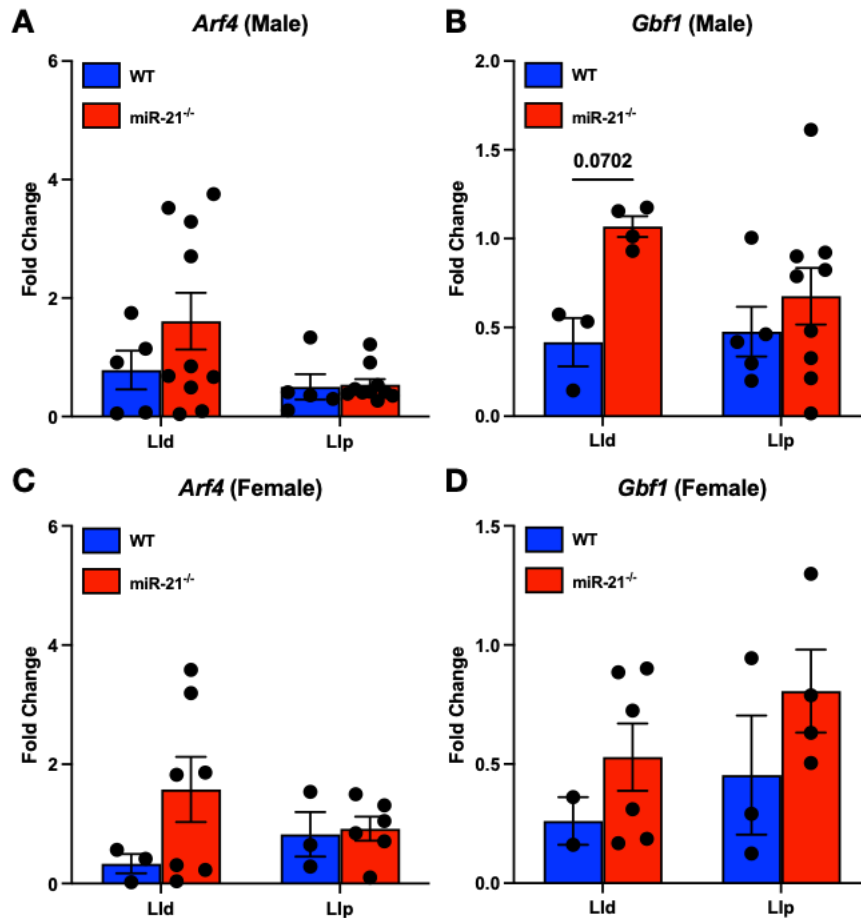


Figure 4.10 – *Arf4* and *Gbf1* are induced in miR-21^{-/-} mouse intestinal tissue from the DSS recovery model. RNA was isolated from 8-15 week old WT and miR-21^{-/-} female mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. (A-F) qRT-PCR analyses of Golgi-related genes *Arf4* and *Gbf1* in in distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue of WT and miR-21^{-/-} male and female mice. Denoted are $n \geq 3$ mice per group with the exception of female WT *Gbf1* – LId qPCR group that has $n = 2$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA.

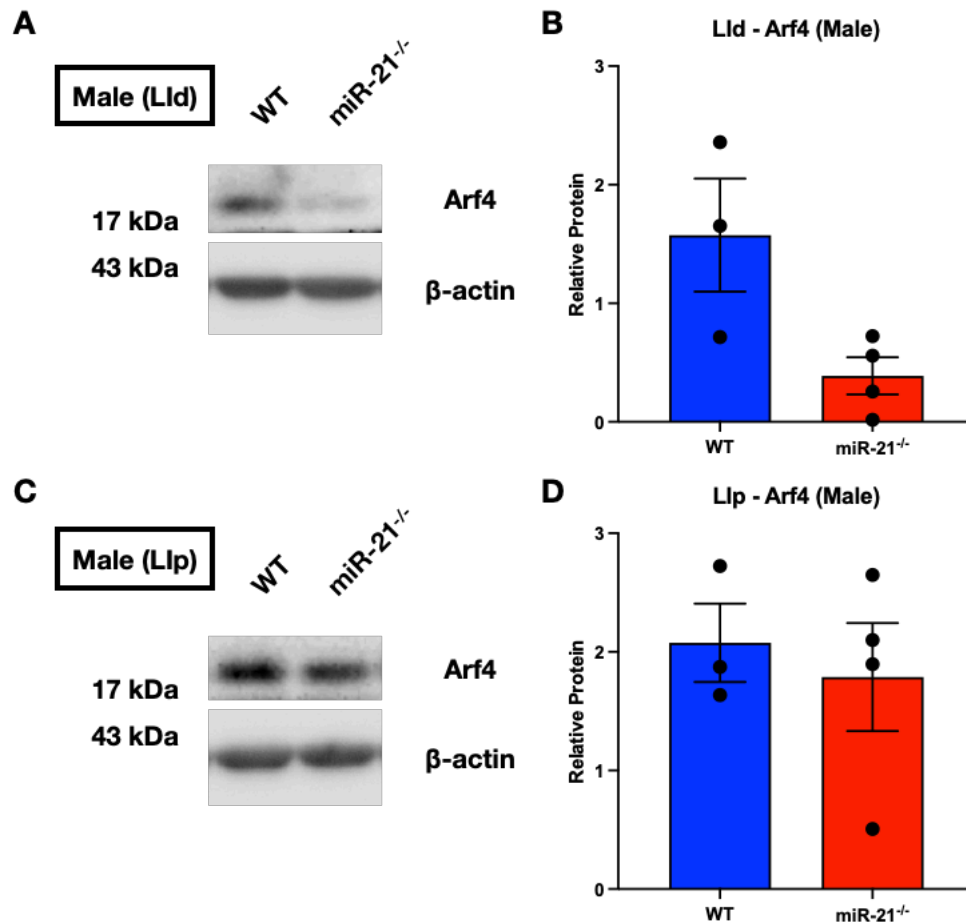


Figure 4.11 – Arf4 is reduced in distal colon tissue of non-littermate miR-21^{-/-} male mice of the DSS recovery model. Protein lysates were obtained from 8-15 week old WT and miR-21^{-/-} male mice given 2% DSS (w/v) water *ad libitum* for four days, followed by a recovery period of five days. Immunoblots and quantification for Arf4 from (A + B) distal colon / large intestine (LId) and (C + D) proximal colon / large intestine (LIp) tissue of WT and miR-21^{-/-} male mice. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

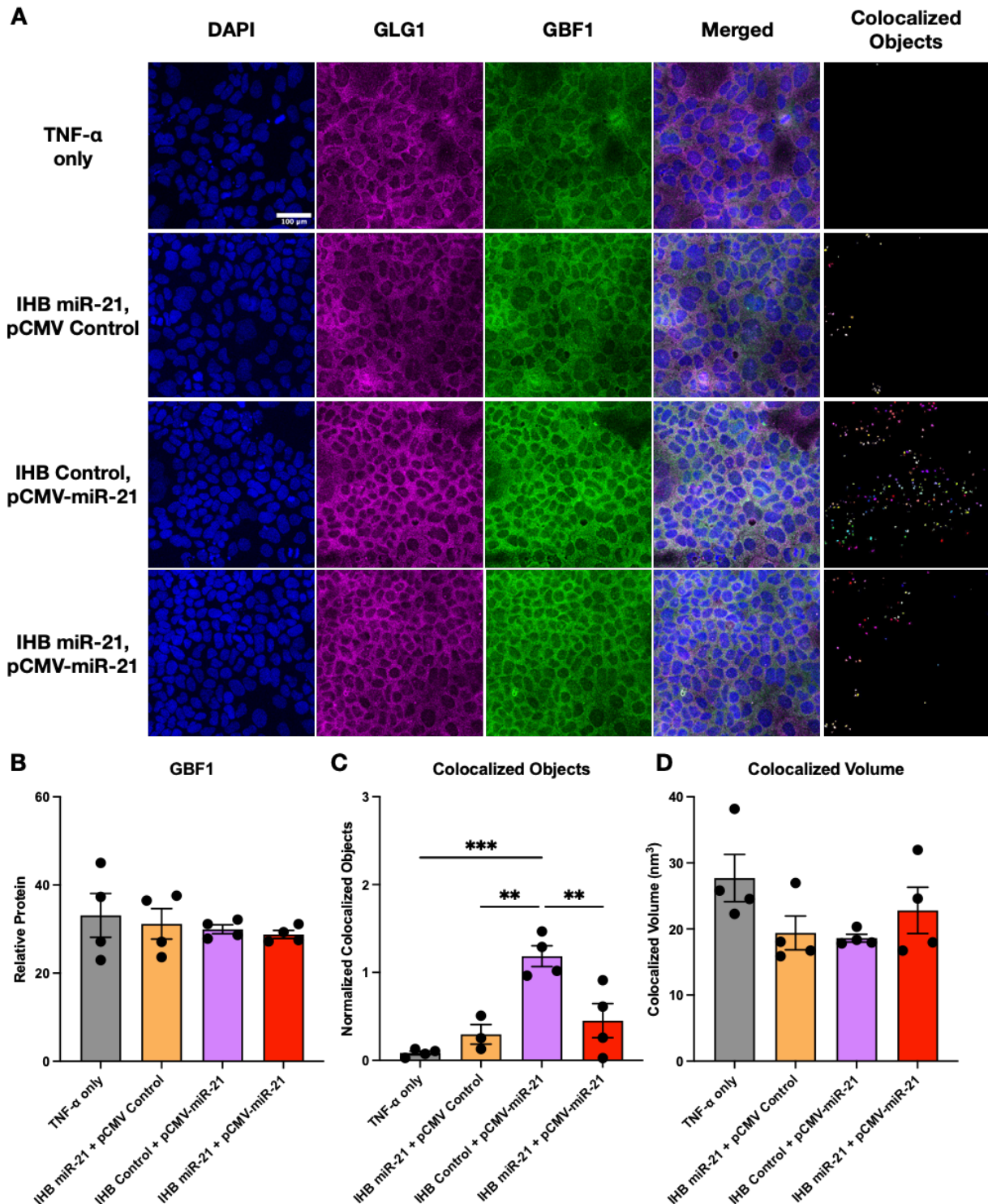


Figure 4.12 – GBF1 colocalizes to the Golgi in TNF- α -treated Caco-2 cells transfected with pCMV-miR-21. (A) Caco-2 cells were transfected with 200 nM miR-21 inhibitor (IHB), 200 ng/well pCMV-miR-21 or a combination of the two, and were then treated with 150 ng/mL TNF- α . After 24 h, cells were stained via DAPI (blue), anti-GLG1 primary antibody (magenta) and anti-GBF1 primary antibody (green). Visual representation of colocalized objects between GLG1 and GBF1 channels can be seen in the rightmost panel, obtained through the DiAna ImageJ plugin for colocalization analysis.²³⁴ (B) GBF1 fluorescence was quantified by normalizing to the number of cell nuclei per field of view. (C) Quantification for the number of colocalized objects in the GLG1 and GBF1 channels normalized to the number of cell nuclei per field of view. (D) Quantification of the overlapping volumes of colocalized objects in the GLG1 and GBF1 channels. Denoted are $n = 4$ biological replicates per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with ** - $p \leq 0.01$, *** - $p \leq 0.001$.

4.3 Discussion

An undeniable contributing factor or consequence of IBD is an increase in intestinal permeability. This chapter investigated the impact of miR-21 on the gut epithelial barrier during IBD, focusing on IECs that provide both functional and structural stability across the intestinal tract. IECs utilize various forms of specialized proteins called intercellular junctions, with TJs directing passage of luminal contents through the barrier. Dysfunction in TJ stability often occurs during IBD and can initiate the pro-inflammatory feedback loop. In this chapter, the central goal was to build on the understanding of TJs in healthy and inflamed states, while highlighting novel ways in which miR-21 can control IEC dynamics through regulation of the TJ and adjacent functions.

TJs are frequently studied for their critical role in barrier integrity and paracellular transport through the gut epithelial layer.³⁵ Despite this, several TJs significant to IBD pathogenesis have no mechanistic link to miR-21 regulation. Comprising the TJ complex includes several types of proteins broadly categorized into transmembrane proteins, scaffolding proteins and regulatory proteins.²⁷ We focused our investigations on several transmembrane claudins and scaffolding ZO, identified to have predicted miR-21 seed regions. Less studied are the roles of desmosomes that mediate mechanical stress at the apical end of polarized IECs.¹⁵² The main desmosome subtypes of proteins are desmocollins and desmogleins, of which only desmocollin-2 and desmoglein-2 are expressed in intestinal epithelia.¹⁵³ Thus, in some *in vivo* analyses, desmosome genes *Dsc2* and *Dsg2* were included to provide a more holistic understanding of IEC dynamics in healthy and diseased tissue.

Initial investigations analysed the expression profiles of TJ and desmosome genes in healthy tissue sections from non-littermate WT and miR-21^{-/-} male mice. Only *Cldn2* and *Tjp2* had notable differential expression in miR-21^{-/-} proximal tissue compared to WT. With claudin-2 being an important pore-forming channel that enhances paracellular uptake of water²⁹¹, the significant upregulation of *Cldn2* in the duodenum/jejunum may support absorption of water molecules and cations at the early stages of digestion. *Tjp2* (encoding Zo-2) is known for its role in barrier stability by binding occludin and AJ protein α -catenin²⁹², with a full Zo-2 KO leading to embryonic lethality.²⁹³ Zo-2 can also translocate to the nucleus and interact with several transcription factors for cell proliferation like Myc, Ap1 and Yap1.¹²⁷ *Tjp3* also had an induced trend in proximal colon and ileum tissue of miR-21^{-/-} compared to WT. *Tjp3* (encoding for Zo-3) is poorly characterized but is involved

in the recruitment of proteins to TJ complexes as well as the stabilization of cyclin D1 during mitosis.²⁹⁴ It is likely that Zo-3 has redundant functions to Zo-2 since previous studies have shown no observable phenotypic changes in Zo-3 KO mice.²⁹³ Nonetheless, presence of these scaffolding proteins may support barrier homeostasis and be beneficial in the event of intestinal damage caused by external stress or inflammation. Subsequent RNA analyses in whole colon tissue of healthy miR-21^{-/-} female mice showed a significant increase in *Cldn3*, *Cldn4*, *Cldn7* and *Cldn15* compared to WT. These genes were also induced in IEC isolates obtained from both male and female miR-21^{-/-} mice compared to WT. Claudin-3 and claudin-4 are sealing claudins similar to claudin-1 that have primarily been studied in kidney and lung epithelia, though their downregulation has been observed under conditions of increased permeability.⁹⁵ Claudin-7 and claudin-15 are pore-forming claudins similar to claudin-2, involved in paracellular flux of small organic solutes²⁹⁵, and vitamin D receptor signalling and Na⁺ cotransport across the intestinal membrane^{179,296}, respectively. Under healthy conditions, these TJ elements could support IEC homeostasis and build strong intestinal barriers.

Following studies on healthy tissue, TJ genes were also investigated in whole colon tissue of non-littermate WT and miR-21^{-/-} female mice during DSS-induced colitis. Similarly to AJ genes, there was a global downregulation in TJ genes for both mouse groups, but there was a reversed trend for downregulation of *Cldn7*. This may be due to the role claudin-7 has in cell survival, proliferation and differentiation through control of Wnt/ β -catenin signalling. Xing *et al.* (2020)²⁹⁷ demonstrated that a full murine KO of claudin-7 led to decreases in Wnt target genes *Axin2*, *Ascl2*, *Fzd5*, *Lef1*, and *Tcf4*, as well as an increase in phosphorylated β -catenin, suggesting destabilization in the absence of claudin-7. Cellular control of proliferation through Wnt signalling is important for the initiation of TGF- β wound repair.¹⁹⁶ Nonetheless, in addition to the traditional DSS treatment, samples were evaluated for differential gene expression between WT and miR-21^{-/-} male and female non-littermate mice during recovery from DSS-induced colitis. For both sexes, *Cldn3* and *Cldn4* had strong upregulation in miR-21^{-/-} distal colon tissue compared to WT. Previous research has shown that IBD patients had decreased levels of claudin-3 in tissue biopsies, while *Cldn3*^{-/-} mice exhibited a hyper-permeable gut phenotype and immune cell infiltration into the lamina propria during colitis induction compared to WT counterparts.^{283,298} Although there was little difference in immune cell infiltration based on earlier H&E staining of DSS recovery tissue samples in the previous chapter, claudin-3 may help miR-21^{-/-} mice reduce the immunological response during DSS

treatment and hinder tissue damage caused by activated immune cells. Regarding claudin-4, the sealing claudin was already shown to have a negative association with miR-21 levels *in vitro*, due in part to the induction of Akt and Jnk pathways.¹²⁰ Moreover, in female mice, *Cldn1* demonstrated an upregulated trend in both distal and proximal colon tissue of miR-21^{-/-} mice compared to WT. Interestingly, despite claudin-1 structural role at the TJ, *Cldn1* was found to be positively correlated with inflammatory activity in IBD patient biopsies and has been implicated in the activation of β -catenin and nuclear translocation.¹⁰⁰ This would suggest transcriptional induction of Wnt genes for cell survival and proliferation and can cause aberrations in wound healing and dysplasia, potentially leading to a loss of protection observed during DSS-induced colitis. Interestingly, differential expression of *Cldn7* was lost in recovery model samples. Ding *et al.* (2022)²⁸⁴ demonstrated that *Cldn7*^{IEC-KO} mice had increased susceptibility to DSS-induced colitis and promoted the migration of *Escherichia coli* through the intestinal epithelium to some extent by damaging the intestinal mucus layer. In that case, claudin-7 may only be significant in the initial response to intestinal inflammation to prevent translocation of bacteria or major alterations in gut microbiota. Full KO mouse models of pore-forming claudins demonstrate differing results, where ablation of claudin-2 reduces inflammation, while removal of claudin-7 and claudin-15 have demonstrated gut microbiota dysbiosis and impairment of cation colon absorption. Previous studies have selectively targeted expression of pore-forming claudins in full KO mouse models, as well as in intestinal cell lines and 3D organoids during induced inflammation^{288,299,300}, but little is known about the specific effects miR-21 has on these claudins.

For desmosome genes, at least in males where disease severity was measurably different, there was a significant upregulation of *Dsg2*, but not *Dsc2*, in distal colon tissue of miR-21^{-/-} compared to WT mice. Both desmosome components are connected to the cytoskeleton via interactions with armadillo proteins plakoglobin, plakophilin and desmoplakin.¹⁵³ These findings are in line with work by Gross *et al.* (2018)¹⁵⁵ that demonstrated *Dsg2*^{IEC-KO} mice had increased intestinal permeability even during healthy conditions, with alterations in other desmosome and TJ components. Researchers also showed that *Dsg2*^{IEC-KO} mice had lower cytoprotective chaperone Hsp70 in the plasma membrane, whereas *Dsc2*^{IEC-KO} mice compensated with recruitment of junction-tightening protein galectin-3.¹⁵⁵ Furthermore, *in vitro* analyses using human desmosome-deficient cells or *DSG2* inhibitors showed impaired barrier integrity which was restored by targeted induction of p38MAPK activity.³⁰¹ Researchers saw no rescue in barrier integrity or kinase

activity by re-expression of *DSC2*, indicating p38MAPK activity was DSG2-dependent.³⁰¹ Interestingly, desmoglein-2 is also known to regulate claudin-2 expression by sequestering PI3K at cell peripheries during TNF- α -induced inflammation on Caco-2 cells.¹⁵⁷ Alternative functions of desmoglein-2 other than barrier integrity during inflammation have been proposed. For example, proinflammatory cytokines like TNF- α were shown to induce cleavage of desmoglein-2, leading to apoptosis, which was proposed as a mechanism to remove damaged cells.³⁰² It would be beneficial to obtain a more holistic picture of desmoglein-2 during miR-21 induction under intestinal inflammation for a better understanding of intercellular junction dynamics during IBD, since to our knowledge there has been no link between desmoglein-2 and miR-21 regulation. Thus, future work will explore the abundance and localization of desmoglein-2 and desmosome integrity in intestinal tissue of miR-21^{-/-} mice as well as in the *in vitro* system where miR-21 levels can be modulated.

Outside of occludin, through RNA analyses of *in vivo* results under healthy and DSS conditions, it became apparent to examine the potential regulation of claudin-4 by miR-21. Dysregulation of TJs like claudin-4 has been associated to many outcomes of IBD. These often involve removing them from cellular membranes by internalization into the cytoplasm and eventual degradation, leading to aberrations in paracellular transport.³⁰³ Indeed, it has been suggested that downregulation of critical claudins like claudin-4 in inflamed epithelia is most likely due to TNF- α /IFN- γ induced MLCK activation, which is then translocated to the cytoplasm via clathrin-mediated redistribution.³⁰⁴ It has also been suggested that there exists an indirect link to miR-21 regulation via ARF4, an ADP-ribosylation factor protein that positively associates to levels of miR-21 in IECs.¹²⁰ Therefore, it was natural to focus on claudin-4 and define the relationship between these regulatory elements.

Claudin-4 protein levels were assessed in colon tissue and IEC lysates of WT and miR-21^{-/-} mice under healthy conditions. However, it was only in IECs isolated from miR-21^{-/-} male mice that had differential expression in claudin-4 protein, having a significant decrease compared to WT IECs. Despite the upregulated trend observed in RNA analyses, there may be different miR-21-dependent regulatory elements not studied here that are contributing to claudin-4 protein synthesis within epithelial cells under healthy conditions between males and females. Claudin-4 may not be as significant in male IECs compared to female IECs. One study showed that claudin-4 expression is closely tied to expression of

the estrogen receptor³⁰⁵, and important for cell motility in mammary epithelia.³⁰⁶ Future work will look at the impact of miR-21 ablation on the expression of estrogen and the estrogen receptor between male and female IECs. Interestingly, in DSS recovery samples, there was a notable induction of claudin-4 abundance in distal colon tissue of miR-21^{-/-} male mice compared to WT. Fluorescence images of claudin-4 also showed differences in localization between distal and proximal tissue of miR-21^{-/-} male mice of the DSS recovery model. This further indicates that additional stimuli of intestinal inflammation, which may include healing processes from wounds created by DSS treatment, can induce the expression of claudin-4 in a miR-21-dependent manner. Domazetovic *et al.* (2020)³⁰⁷ demonstrated that in *ex vivo* cultures of UC patient intestinal biopsies, addition of 1,25-dihydroxyvitamin D₃ (vitamin D derivative) increased levels of claudin-4 by blocking Smad7 activity. Smad7 is a key inhibitor of the TGF- β wound healing pathway and direct target of miR-21.²⁵⁵ Thus, claudin-4 plays a significant role in establishing gut barrier stability after DSS-induced colitis.

Other indirect consequences of miR-21 regulation in TJs during intestinal inflammation, particularly on claudin-4 expression, have been proposed to be caused by targeting of ARF4, part of a subset of the RAS superfamily of small GTPases. ARFs are involved in a variety of different cellular processes, most notably proliferation, motility, differentiation and membrane trafficking.³⁰⁸ ARF4, a predicted target of miR-21, has previously been implicated for cancer cell migration, invasion and metastasis.³⁰⁹ The exact functions for ARF4 are unclear, but localization to the Golgi and plasma membrane indicate relevance for vesicular trafficking and the recruitment of coat proteins for vesicle formation.³¹⁰ ARFs are activated by GEFs and are inactivated by GTPase-activating proteins (GAPs). All GEFs localized to the cytosol are inactive, and it is only when they are recruited to membranous organelles that they can act upon ARFs, representing a compartmentalized regulatory step.³¹⁰ It has been suggested that GEF GBF1 activates ARF4 at the Golgi, and with ARF1, is required for COPI vesicle formation.³¹¹ Recruitment of COPI coat protein complexes to cytosolic endosomes is important for retrograde transport to the trans-Golgi network (TGN)³¹², but overactivation of this can cause reduction in normal Golgi tubulation for directing cargo through Golgi stacks from the Golgi to the endoplasmic reticulum. ARF4 requires activation by GBF1 and PIP signalling molecules, found within the miR-21-regulated PI3K-Akt pathway for general cellular processes.³¹⁰ During stress, ARF4 has been shown to negatively regulate the functioning of the Golgi in packaging protein into endosomes and transport for recycling.³¹³ It remains

to be determined if ARF4 activity directly regulates claudin-4, or if ARF4 activation has a specific connection with claudin-4 internalization and recycling. Nonetheless, to further expand into new targets regulated by miR-21, investigations were carried out along the ARF4 pathway.

To determine their relevance in miR-21-specific dynamics of IECs, these Golgi-related predicted targets of miR-21 were initially examined in IEC isolate samples of WT and miR-21^{-/-} mice under healthy conditions. There was a notable reduction of *Gbfl* in miR-21^{-/-} male IECs compared to WT. This suggests that there may be less activation of Arf4-Gbfl in miR-21^{-/-} IECs. Subsequent *ex vivo* experiments in the absence or presence of TNF- α showed an induction of *Cldn4*, *Arf4* and, to lesser extent, *Gbfl* in miR-21^{-/-} IECs compared to WT. This molecular difference compared with IEC isolate mouse samples may likely be caused by the functions of ARFs in the IEC life cycle, particularly when establishing a confluent monolayer, as Arf4 is important for cell proliferation and motility.³⁰⁸ Indeed, in DSS recovery samples, *Arf4* and *Gbfl* also demonstrated trends of induction in distal colon tissue of miR-21^{-/-} mice compared to WT. Despite this induced trend, Arf4 protein levels decreased in miR-21^{-/-} distal tissue lysates. Little is known about the role Arf4 plays in mouse IECs, neither in healthy nor inflamed states, therefore juxtapositions to previous studies using human cell lines can provide useful insight into ARF4-related Golgi processes. Using HeLa cells, Nakai *et al.* (2013)³¹² revealed that singular deletion of *ARF4* does not result in notable morphological changes to the Golgi or recycling endosomes from the retrograde transport pathway, but when paired with *ARF1* deletion inhibit the retrograde transport of early endosomes marked by TGN38/46 or mannose-6-phosphate receptor (MPR). More striking was work done by Nakata *et al.* (2017)¹²⁰ that showed inhibition of ARF4 in Caco-2 monolayers led to increased TEER, associated with increased expression of occludin and claudin-4. The results obtained in miR-21^{-/-} DSS recovery samples provides further evidence that miR-21 does not directly target Arf4, but a separate regulatory protein. Of course, the mismatch between RNA and protein expression here and in some intercellular junction targets of interest in general seems to indicate that translation of targets is being hindered. This suggests that miR-21 may be regulating Arf4 through translational repression rather than exonuclease degradation. Future work on whether this is done through mRNA sequestration, blockage of ribosome binding or targeting separate regulatory elements would be useful in understanding this type of miR-21-specific regulation observed for Arf4.

Investigations on GBF1 protein abundance and localization was performed using the *in vitro* system during TNF- α -induced inflammation. Using the ImageJ plugin DiAna for colocalization of objects through segmentation²³⁴, TNF- α -treated cells transfected with pCMV-miR-21 showed greater colocalization of GBF1 to Golgi marker GLG1 than cell groups transfected with miR-21 inhibitor. This colocalization did not coincide with an increase in GBF1 fluorescence, nor were there differences in the size of overlapping volumes for assigned objects in cells transfected with pCMV-miR-21 alone compared to other test groups. Previous research using HeLa cells has shown a cytosol-to-Golgi localization ratio of 9:1.²⁸⁹ This large Golgi localization of detected GBF1 in Caco-2 transfected with pCMV-miR-21 suggests higher activation of ARFs such as Arf4. Recruitment of GBF1 to the Golgi is done through interaction of their N-terminal regions with small GTPase Rab1b³¹⁴, while the HDS domain in their C-terminal region is important for membrane association.³¹⁰ It is through interaction of the HDS1 region and PIPs that stimulates catalytic activity of GBF1 for ARF4 activation at membranes.³¹⁵ This translocation to the Golgi may be facilitated via induction of PIP₃ caused by miR-21 targeting of *PTEN* mRNA, in which GBF1 was shown to preferentially bind among other phosphoinositide molecules in the PI3K-Akt pathway.³¹⁵ Indeed, the combination of pCMV-miR-21 and miR-21 inhibitor reduced levels of GBF1 colocalization to GLG1 back to levels of miR-21 inhibitor alone. This indicates that in the absence of miR-21, not only can there be a repression of ARF4 translation, but its activation can also be hindered through loss of miR-21-specific Golgi localization of GBF1. Future work will look at the localization of ARF4 in IECs for clarity on functional aspects that ARF4 is known for during homeostasis, and in addition, assess the localization of GBF1 during re-expression of PTEN.

Taken together, this work shows that the ablation of miR-21 *in vivo* leads to increases in TJ gene expression, particularly those with a function for increasing gut epithelial barrier stability such as claudin-4. Further analyses on miR-21-dependent regulation of claudin-4 seems to be linked to Golgi-related processes in regard to vesicular trafficking. We introduce a novel mechanism by which miR-21 regulates levels of the retrograde transport pathway for protein recycling during inflammation, which could be activated in a miR-21-dependent manner through Golgi-specific localization of ARF activator GBF1.

Chapter 5

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miR-21 epithelial-specific knockout

5. Creation of an epithelial cell specific miR-21 knockout mouse to assess the contribution of miR-21 to intestinal barrier dysfunction and inflammation

5.1 Introduction

miR-21 is one of the most abundantly expressed miRNAs in the mammalian body. Although it is often attributed to immunity and inflammation, many non-immune cell types produce miR-21 for general gene regulation and even secretion into the extracellular environment. For non-hematopoietic cells, miR-21 is associated with gene regulation in epithelial cells, hepatocytes and glial cells for oncolytic activity such as cell growth, migration and invasion.⁶⁸ Stromal cells were also shown to highly express miR-21 during gastric cancer *in vivo*.³¹⁶ Even in neuropathy, researchers found that exosomes from sensory neurons during nerve injury in mice had significant levels of miR-21-5p, which are taken up by surrounding macrophages for pro-inflammatory activation leading to neuropathic hypersensitivity.⁸² In kidney cells, the importance of miR-21 for renal regeneration shows another cell type that benefits from the miRNA and has been reviewed extensively elsewhere.³¹⁷ Moreover, for hematopoietic cells, miR-21 can be expressed in B-cells, T-cells, monocytes, macrophages and dendritic cells.⁶⁸ Thus, cell type influences the functional impact of miR-21.

Generally, as immune cells mature, expression of miR-21 increases substantially and thus serves as a good marker for immune cell activation. Indeed, hematopoietic precursors that underwent targeted differentiation towards macrophages, neutrophils, dendritic cells and B-cells all had elevated levels of miR-21.⁶⁸ Ando *et al.* (2016)⁷³ showed that CD3⁺ T-cells from UC patients in remission had decreased levels of miR-21 compared to patients with active disease. Further focusing on lymphoid lineages, exosomes derived from immune cells demonstrate its function as a secreted gene regulator and biomarker of disease, particularly when take-up is primarily by IECs near the gut barrier.³¹⁸ The difficulty in ascertaining the exact contribution of miR-21 to disease pathogenesis or resolution may be the net result of many factors, ultimately depending on the exact conditions certain cells encounter that permit interaction between miR-21 with its many known and predicted mRNA targets.

To date, work investigating miR-21 pathogenesis have used full KO mouse models which can shroud cell-specific effects during intestinal inflammation. The aim of this chapter was to build on work done in the previous chapters to explore the importance of

epithelial-specific miR-21 expression to influence barrier stability. Here, we detail the breeding setups in the creation of the epithelial deficient miR-21 conditional KO mouse model (miR-21^{IEC-KO}). Once established with genotypes confirmed, we repeat the DSS-induced colitis model in miR-21^{IEC-KO} mice to determine gut barrier consequences for the loss of miR-21 specifically in epithelial cells on disease pathogenesis. This work shows differences in barrier stability between non-littermate and littermate WT mice when compared to miR-21^{IEC-KO} regarding disease severity and expression of intercellular junctions during DSS-induced colitis.

5.2 Results

5.2.1 Stage 1 – Hemizygous Vil-Cre mice x homozygous miR-21^{fl/fl} conditional mice

Hemizygous Vil-Cre transgenic mice were obtained from Jackson Laboratories (B6.Cg-Tg(Vil1-cre)997Gum/J; strain #004586). These mice have confirmed expression of Cre recombinase in villus and crypt epithelial cells of the small and large intestines. Creators of this strain have also reported very low levels of Cre recombinase in mouse testes, and in addition a small amount of mosaicism observed in the colon, but otherwise expression seems continuous.³¹⁹

Insertion of the 12.4 kb pair Vil:Cre transgene was carried out by injection of the segment into the pronuclei of fertilized C57BL/6J x SJL/J mouse eggs. These resulting “founder animals” were crossed with background C57BL/6J for germline transmission, in which the transgene integrated approximately 119 kb upstream of *St6gal2* on chromosome 17, where creators determined a 14.7 kb deletion at this integration site.³¹⁹ After five generations of backcrossing with background C57BL/6J, the resulting litter was provided to Jackson Laboratories, containing 16 copies of this transgene at the integration site mentioned. Upon receiving these mice, a litter of 19 female mice was ear-clipped and checked for the presence of the Vil:Cre transgene using the following primers suggested on the JAX strain page: F: 5'- GCCTTCTCCTCTAGGCTCGT - 3', R: 5'- AGGCAAATTTTGGTGTACGG - 3'. As seen in **Figure 5.1**, all 19 female mice possessed the Vil:Cre transgene. Subsequently, homozygous male miR-21^{fl/fl} conditional mice were provided by the Malcangio group in King's College. Originally sourced from Jackson Laboratories as well (Mir21a^{tm1Mtm}/Mmjax; strain # 036060-JAX), these mice are a conditional mutant strain designed to generate a null allele or a *lacZ*-tagged null allele when combined with Flp or Cre recombinase expressing strains. The strain had been previously crossed with an Flp deleter to create the conditional mouse and was used for the study of neuron-macrophage communication and the impact of miR-21 exosomal cargo on this crosstalk during neuropathic pain.⁸²

Creators of the strain required the construction of a targeting vector that contained an FRT site for recombination in further Flp breeding, followed by a *lacZ* gene for driving expression, a loxP site for Cre recombinase binding, a neomycin cassette (NEO) for selection, the second FRT site and another loxP site upstream of the miR-21 stem loop; finally followed by a third loxP site downstream of the miR-21 stem loop.³²⁰ This construct

was electroporated into 129P2/OlaHsd-derived E14 embryonic stem (ES) cells, through which derived chimeric mice were then crossed to a mouse with mixed genetic background, and finally crossed with C57BL/6 for two generations. The resulting litter was provided to Jackson Laboratories.

Male mice were crossed with randomly chosen Vil-Cre mice that were previously confirmed. The resulting litter of 22 mice (10 male, 12 female) was once again ear-clipped and checked for the presence of the Vil:Cre transgene, along with the presence of the miR-21 floxed allele using the following primers used by Park *et al.* (2012)³²⁰: F: 5'-GATGTTGACTGTTGAATCTCATGG - 3', R: 5' - AGTAAGGTGGGAATTCTGATGCTA - 3'. As seen in **Figure 5.2A** and **Figure 5.2B**, male mice #1.4, 1.6 and female mice #1.12, 1.13, 1.15, 1.17, 1.18, 1.19, 1.22 possessed the desired genotype of +Vil:Cre, HET miR-21^{fl/wt}, while remaining mice were -Vil:Cre, HET miR-21^{fl/wt}.

To ensure complete removal of antibiotic resistance cassettes used in early selection steps, the litter was also checked for the presence of NEO using the following primers provided by the TCD Transgenics Unit: F: 5' - AGGATCTCCTGTCATCTCACCTTGCTCCTG - 3', R: 5' - AAGAACTCGTCAAGAAGGCGATAGAAGGCG - 3'. In addition to this, mice that were -Vil:Cre, HET miR-21^{fl/wt} were checked for general Cre recombinase gene, to confirm that there was no other recombinase transgene in the genome of desired +Vil:Cre, HET miR-21^{fl/wt} mice, using the following primers provided by the TCD Transgenics Unit: F: 5' -CGATGCAACGAGTGATGAGG - 3', R: 5' - GCATTGCTGTCACTTGGTCGT - 3'. As seen in **Figure 5.2C** and **Figure 5.2D**, not only was the presence of NEO in all mice, but mice #1.3, 1.5, 1.7, 1.8, 1.9, 1.10, 1.11, 1.16 were positive for Cre recombinase.

Due to the nature of the mice provided by King's College, there was a possibility that the mice had not been crossed with a Flp deleter mouse and still possessed the germline-transmitted targeted allele (ie. FRT-*lacZ*-loxP-NEO-FRT-loxP-miR21-loxP) instead of the shortened conditional allele (ex. FRT-loxP-miR21-loxP). Additionally, in the study provided by the collaborators, mice were bred with Advillin-Cre driver mice for conditional ablation in dorsal root ganglia where monocytes/macrophages can infiltrate after injury in the peripheral

nerve causing neuropathic pain.⁸² This litter was further tested for the presence of Advillin:Cre using the following primers provided by the TCD Transgenics Unit: F: 5' - TGGCTCCCTGTTCACCTGTGAGTAGG - 3', R: 5' - GCATTGCTGTCACTTGGTCGT

- 3'. As seen in **Figure 5.2E**, mice #1.3, 1.5, 1.6, 1.7, 1.8, 1.9, 1.10, 1.11, 1.13, 1.16 were positive for Advillin:Cre allele. Therefore, this breeding resulted in the formation of four distinct genotypic groups:

- 1) +Vil:Cre, HET miR-21^{fl/wt}, +NEO cassette, +General Cre, +Advillin:Cre [3 mice]
- 2) +Vil:Cre, HET miR-21^{fl/wt}, +NEO cassette, +General Cre, -Advillin:Cre [6 mice]
- 3) -Vil:Cre, HET miR-21^{fl/wt}, +NEO cassette, +General Cre, +Advillin:Cre [8 mice]
- 4) -Vil:Cre, HET miR-21^{fl/wt}, +NEO cassette, -General Cre, -Advillin:Cre [5 mice]

To further breeding with desired genotypes and minimize passing on of undesired alleles, male and female mice of Group 4 were selected for the next stage of breeding (shown in **Figure 5.2F**).

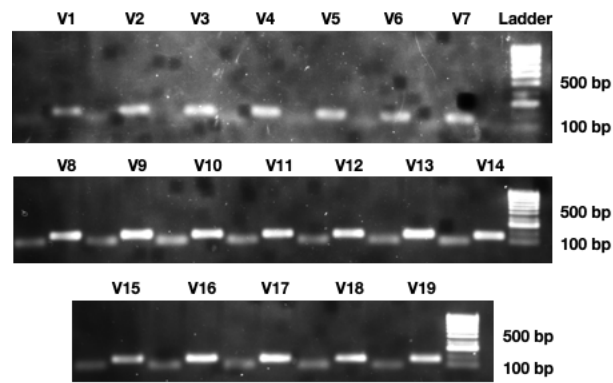


Figure 5.1 – Genotyping results to confirm Vil:Cre female mouse colony ($n = 19$). PCRs were conducted on gDNA extracted from earclip tissue. Expected product size = 150 bp.

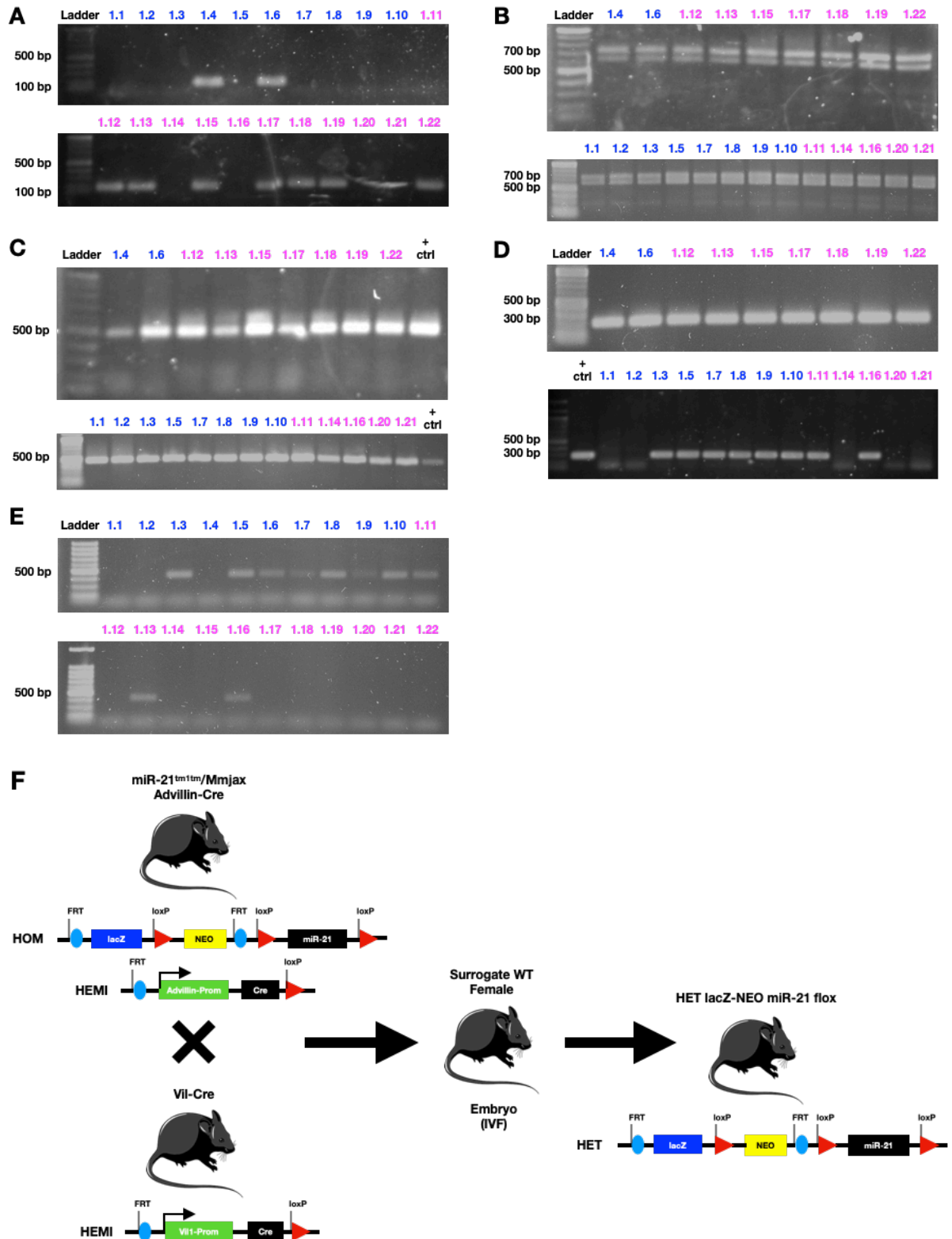


Figure 5.2 – Genotyping results of first cross between female hemizygous Vil-Cre x male homozygous miR-21^{fl/fl} mice (n = 22; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) Vil:Cre gene, expected product size = 150 bp. (B) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (C) Neomycin cassette, expected size = 493 bp. (D) Cre recombinase gene, expected size = 288 bp. (E) Advillin:Cre allele, expected product size = 450 bp. (F) True crossing of Stage 1 breeding with confirmed genotypes performed via *in vitro* fertilization.

5.2.2 Stage 2 – Heterozygous miR-21^{fl/wt} conditional mice

Mice from Group 4 of Stage 1 breeding were chosen to create a HOM miR-21^{fl/fl} strain without any Cre recombinase and subsequently crossed with an Flp deleter mouse. An overview of this breeding stage can be seen in **Figure 5.3**.

The resulting litter of 47 mice (19 male, 28 female) were ear-clipped and checked for the presence of the miR-21 floxed region, the NEO cassette, and general Cre recombinase. As seen in **Figure 5.4**, this led to the formation of three distinct genotypic groups:

- 1) miR-21^{wt/wt}, +NEO cassette, -General Cre [11 mice]
- 2) HET miR-21^{fl/wt}, +NEO cassette, -General Cre [14 mice]
- 3) HOM miR-21^{fl/fl}, + NEO cassette, - General Cre [11 mice]

There were 11 mice whose genotype was inconclusive despite repeated genotyping PCR attempts. Thus, to further breed out the targeted allele selection cassette, mice from Group 3 (#2.8, 2.14, 2.30, 2.33, 2.35, 2.44) were selected for the next stage of breeding.

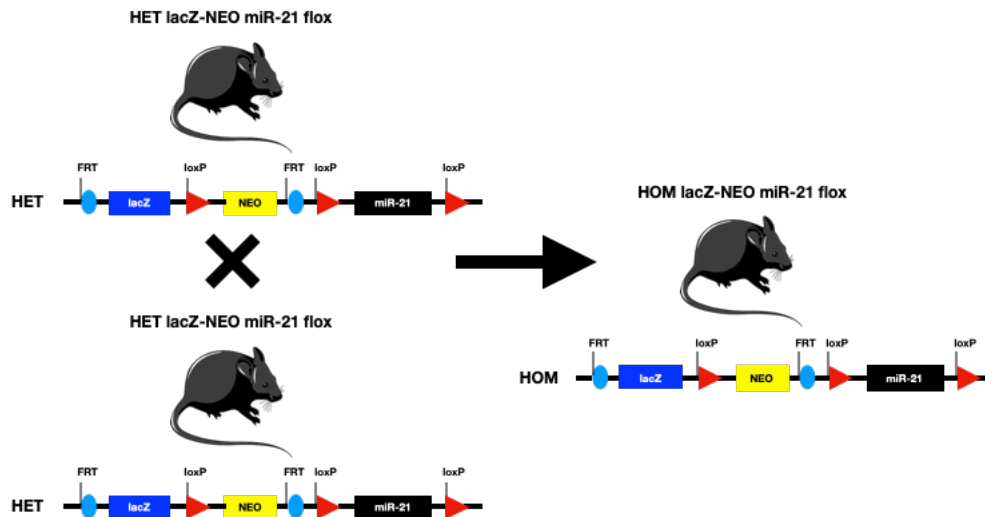


Figure 5.3 – Stage 2 breeding workflow, crossing heterozygous *miR-21*^{fl/wt} mice.

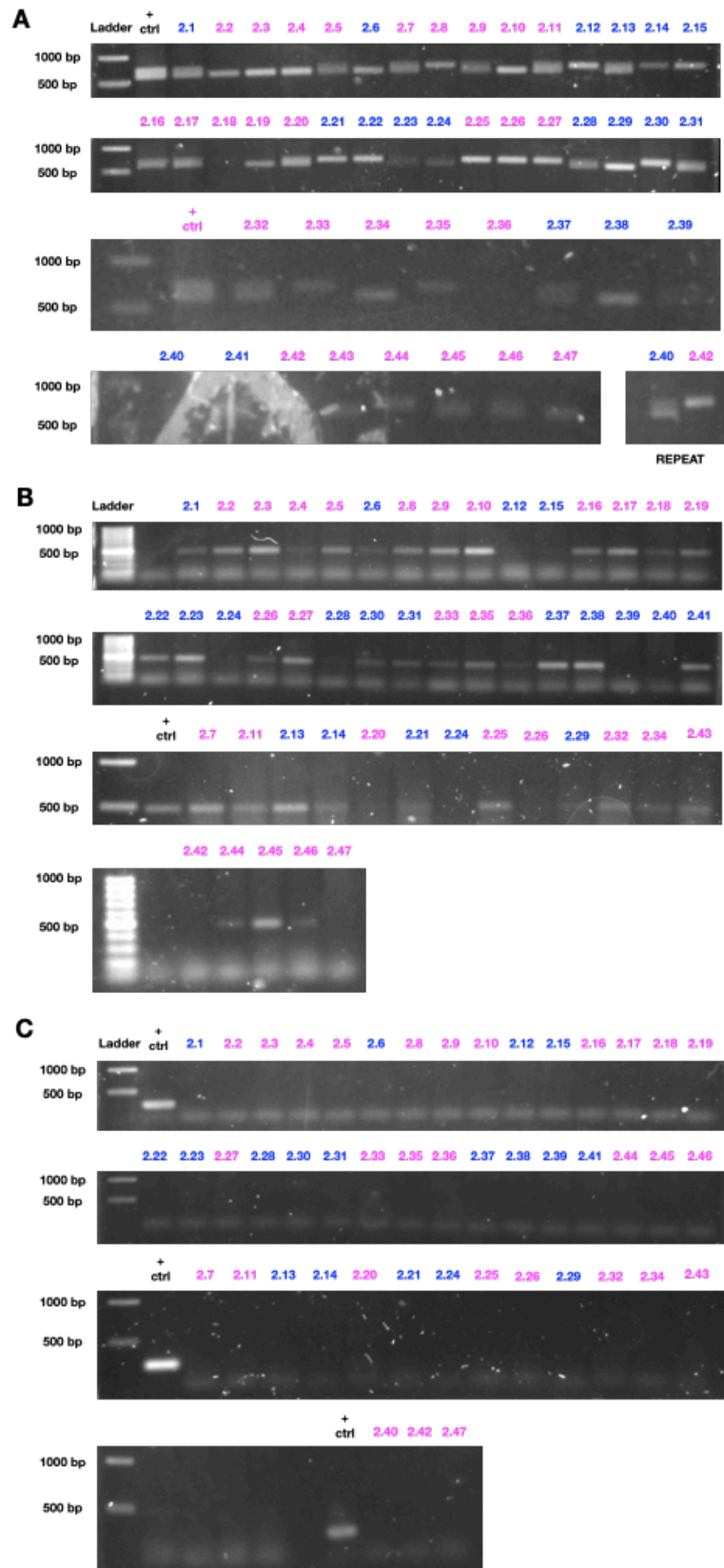


Figure 5.4 – Genotyping results of Stage 2 breeding between heterozygous miR-21^{fl/wt} conditional mice (n = 47; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (B) Neomycin cassette, expected size = 493 bp. (C) Cre recombinase gene, expected size = 288 bp. Note: 2.40 and 2.42 had repeated PCRs for confirming miR-21 floxed allele.

5.2.3 Stage 3 – Homozygous miR-21^{fl/fl} conditional mice x Flp-ROSA deleter mice

Mice from Group 3 of Stage 2 breeding were chosen to create the conditional allele genotype after crossing with an Flp deleter mouse. Specific male mice #2.14, 2.30 and female mice #2.8, 2.33, 2.35, 2.44 were chosen due to the confirmation of the selection cassette, as this should still be present in the genome, and thus excluded mice that although possessed the miR-21 floxed allele, was not able to confirm the NEO cassette through PCR (#2.12, 2.15, 2.26). Moreover, any remaining mice in Group 3 were kept as reserve in case breeding with Flp deleter mice was unsuccessful (#2.21, 2.22, 2.25, 2.26, 2.27). An overview of this breeding stage can be seen in **Figure 5.5**.

At this stage, a mouse-codon optimized FLP recombinase (FLPo) deleter strain was sourced from Jackson Laboratories (B6.129S4-*Gt(ROSA)26Sor^{tm2(FLP*)Sor}/J*; strain #012930). Creators of the strain required the construction of a targeting vector which contained a SA-Flpo-PGKneopA cassette combined with the FLPo coding sequence into a site located within intron 1 of the *Gt(ROSA)26Sor* locus.³²¹ This construct was electroporated into 129S4/SvJaeSor derived AK7 ES cells and after selection, desired ES cells were injected into recipient blastocytes. Chimeric animals were crossed with 129S4/SvJaeSor mice, and further breeding to create homozygous mice was provided to Jackson Laboratories. FLP variant is of the *Saccharomyces cerevisiae* *FLP1* recombinase gene, expression driven by the *Gt(ROSA)26Sor* promoter.

The resulting litter of 24 mice (11 male, 13 female) were ear-clipped and checked for the presence of the miR-21 floxed region as before. To confirm removal of the selection cassette at the miR-21 floxed region, primers for the *lacZ* gene were used instead of NEO due to the presence of NEO in the FLPo transgene, using the following primers: 5' - GGCGGTGATTTTGGCGATAC - 3', R: 5' - AATGCGGGTCGCTTCACTTA - 3'. In addition, due to difficulties in PCR amplification, a cleaner protocol for gDNA extraction was utilized here via the REDExtract-N-Amp™ Tissue PCR Kit (Merck). As seen in **Figure 5.6**, all mice were HET miR-21^{fl/wt}, while 17 mice were positive (#3.1, 3.2, 3.5, 3.6, 3.8, 3.10, 3.11, 3.13, 3.15, 3.16, 3.18, 3.19, 3.20, 3.21, 3.22, 3.23, 3.24) and 7 mice were negative (#3.3, 3.4, 3.7, 3.9, 3.12, 3.14, 3.17) for the *lacZ* gene. Therefore, to progress towards the tissue-specific conditional knockout, mice negative for the *lacZ* gene were selected for the next stage of breeding.

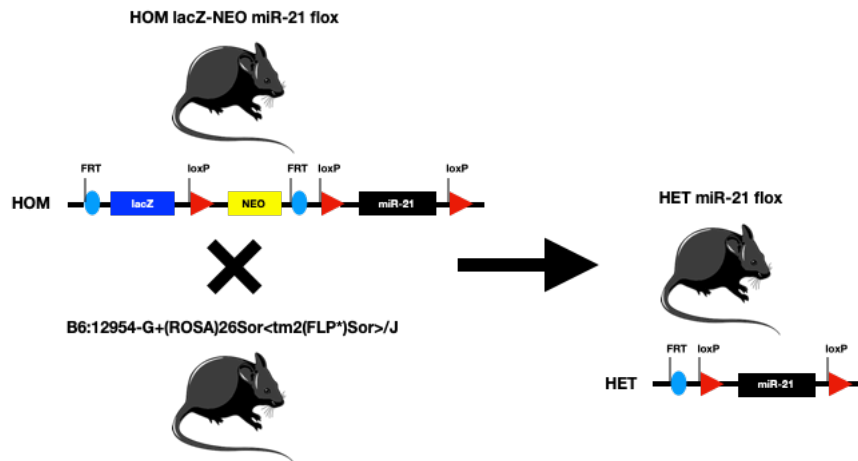


Figure 5.5 – Stage 3 breeding workflow, crossing homozygous miR-21^{fl/fl} mice and Flp-ROSA deleter mice.

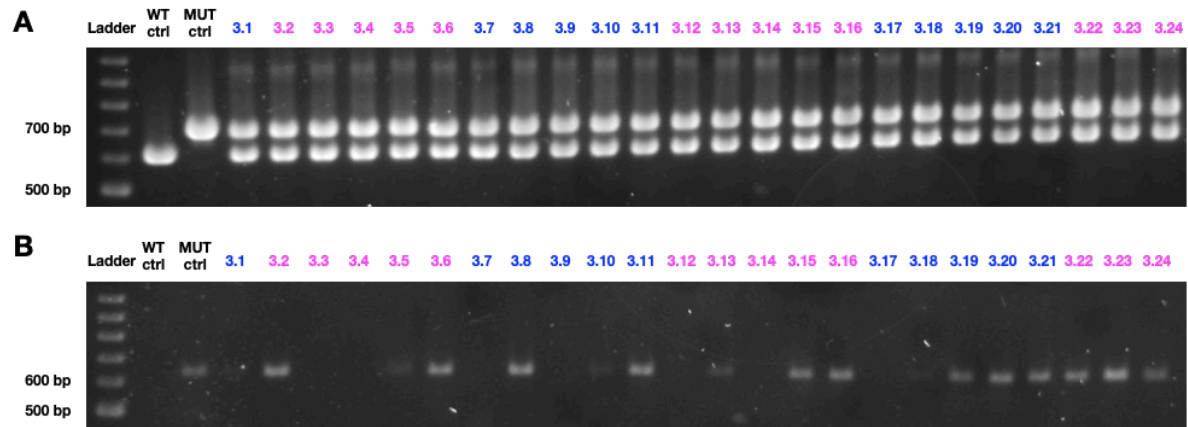


Figure 5.6 – Genotyping results of Stage 3 breeding between homozygous miR-21^{fl/fl} conditional mice and Flp-Rosa deleter mice (n = 24; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (B) lacZ gene (within Neomycin cassette), expected size = 639 bp.

5.2.4 Stage 4 – Heterozygous miR-21^{fl/wt} mice lacking lacZ-NEO selection cassette

In this stage, heterozygous miR-21^{fl/wt} conditional mice lacking the lacZ-NEO selection cassette were inbred to obtain HOM miR-21^{fl/fl} conditional female mice. With support from the TCD Transgenics Unit, eggs from these female mice will subsequently be used for rederivation with sperm samples of the original Vil-Cre mice and implantation of the embryos in surrogate female C57BL/6J mice through IVF. An overview of this breeding stage can be seen in **Figure 5.7**.

The resulting litter of 21 mice (6 male, 15 female) were ear-clipped and checked for the presence of the miR-21 floxed region and *lacZ* gene as before. Furthermore, it was recommended to track the heredity of the FLPo transgene which will be bred out in the final epithelial-specific miR-21 KO, using the following primers suggested on the JAX strain page: F: 5'-AAAGTCGCTCTGAGTTGTTAT-3', R(WT): 5'-GGAGCGGGAGAAATGGATATG-3' or R(FLPo): 5'-GCGAAGAGTTTGTCTCAACC-3' (data not shown). As seen in **Figure 5.8**, there were 3 females with miR-21^{wt/wt} (#4.15, 4.16, 4.21), 10 females with HET miR-21^{fl/wt} (#4.1, 4.2, 4.3, 4.11, 4.12, 4.13, 4.14, 4.17, 4.19, 4.20) and 2 females HOM miR-21^{fl/fl} (#4.10, 4.18). All mice were negative for the *lacZ* gene. These results were confirmed through Transnetyx genotyping services, including the presence or absence of the FLPo transgene (data not shown). Due to the lack of abundance for female homozygous miR-21^{fl/fl} mice, both homozygous and heterozygous females were used for subsequent rederivation.

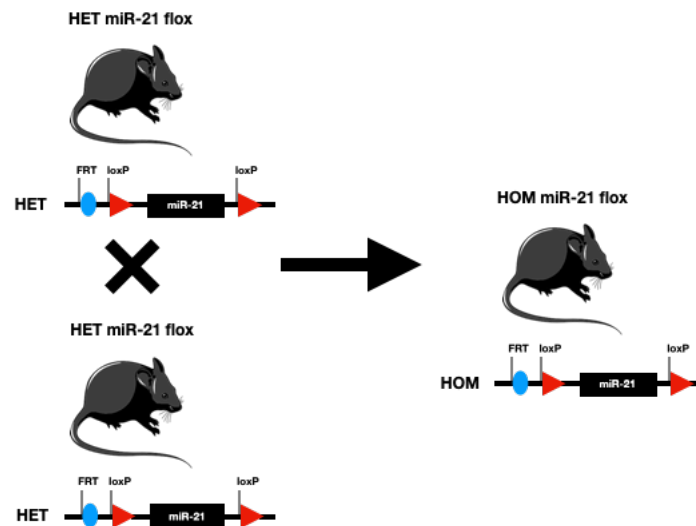


Figure 5.7 – Stage 4 breeding workflow, crossing heterozygous *miR-21*^{fl/wt} mice lacking lacZ-NEO selection cassette.

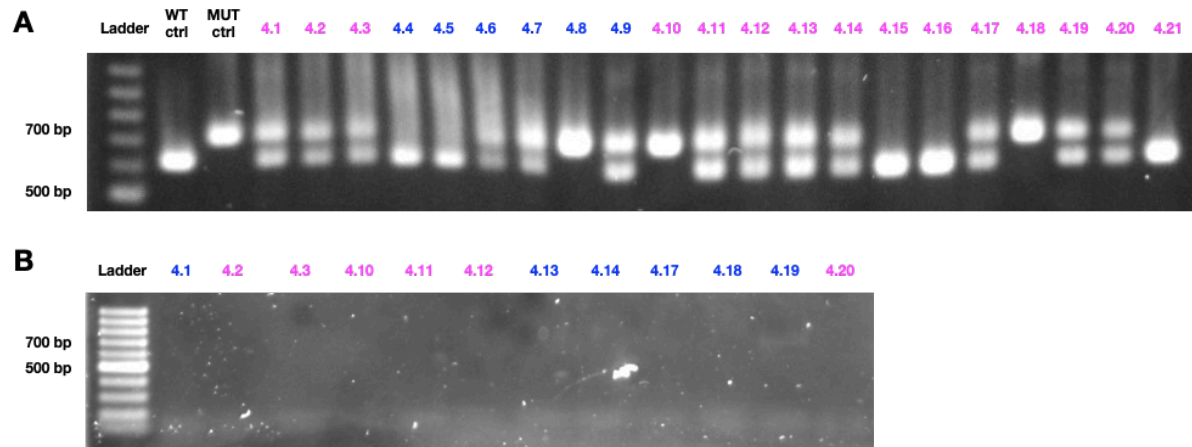


Figure 5.8 – Genotyping results of Stage 4 breeding between heterozygous miR-21^{fl/wt} conditional mice lacking lacZ-NEO selection cassette (n = 21; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (B) lacZ gene (within Neomycin cassette), expected size = 639 bp.

5.2.5 Stage 5 – Rederivation, miR-21^{fl/fl} and miR-21^{fl/wt} conditional female mice x Vil-Cre hemizygous male mice

Since mice were moved to the TCD Comparative Medicinal Unit (CMU) for Research, mice needed to be placed back into the TCD CMU for Production to establish the epithelial-specific miR-21 conditional KO colony. With support from Claire Fergus of the TCD Transgenics Unit, female mice from breeding Stage 4 were selected for rederivation. Rederivation is a process by which mice are brought into clean, pathogen-free facilities. Germplasm (eggs) were harvested from HOM miR-21^{fl/fl} and HET miR-21^{fl/wt} female mice and fertilized by sperm of male mice from the original Vil-Cre colony. Embryos were generated *in vitro* and underwent a strict pathogen removal process to be transferred into the transgenics unit, which were then surgically planted into foster female C57BL/6J mice. An overview of this breeding stage can be seen in **Figure 5.9**.

The resulting litter of 10 mice (6 males, 4 females) were ear-clipped and checked for the presence of the miR-21 floxed allele, along with the Vil:Cre and FLPo transgenes as before. Regarding the Vil:Cre transgene, a reverse primer for the typical Vill gene was also used to ensure hemizyosity using the following primers suggested on the original JAX strain page: R: 5'-TATAGGGCAGAGCTGGAGGA-3'. As seen in **Figure 5.10**, this led to the formation of four distinct groups:

- 1) miR-21 fl^{wt/wt}, HEMI Vil:Cre^{+/-}, FLPo^{-/-} [2 mice]
- 2) miR-21 fl^{wt/wt}, HEMI Vil:Cre^{+/-}, HEMI FLPo^{+/-} [4 mice]
- 3) HET miR-21 fl^{fl/wt}, HEMI Vil:Cre^{+/-}, HEMI FLPo^{+/-} [2 mice]
- 4) HET miR-21 fl^{fl/wt}, HEMI Vil:Cre^{+/-}, FLPo^{-/-} [2 mice]

Unfortunately, there were no mice homozygous for the miR-21 floxed allele. Due to the lack of mice in Group 4 that possessed the miR-21 floxed region and lacked the FLPo transgene, the death of mouse #5.7 due to hydrocephalus, and most importantly how all female mice were in either Groups 1 and 2 which lacked the miR-21 floxed allele, an additional round of breeding was required to obtain a more desired miR-21 floxed genotype among the sexes.

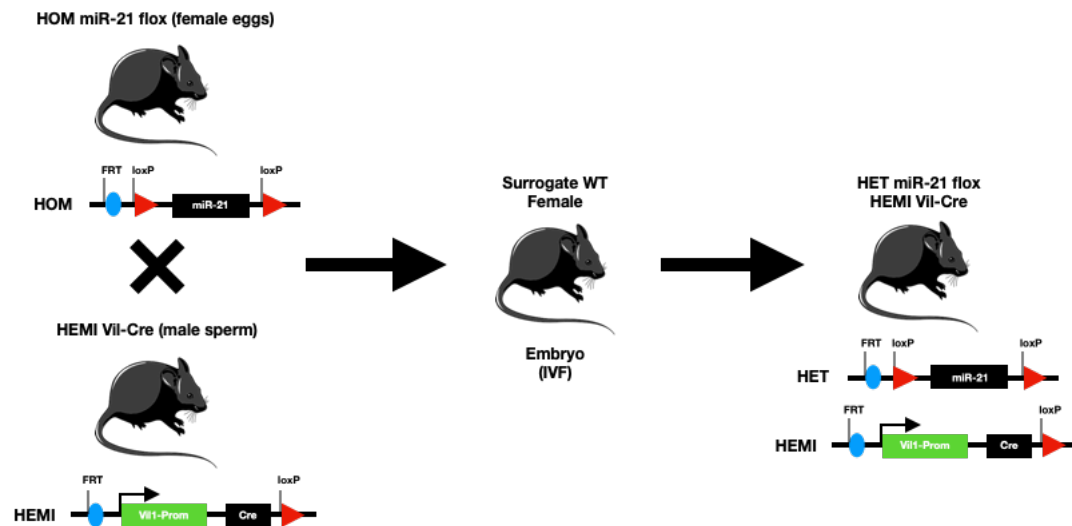


Figure 5.9 – Stage 5 breeding workflow, rederivation for crossing HOM *miR-21*^{fl/Δ} and HET *miR-21*^{fl/wt} female mice x *Vil-Cre* hemizygous male mice through *in vitro* fertilization.

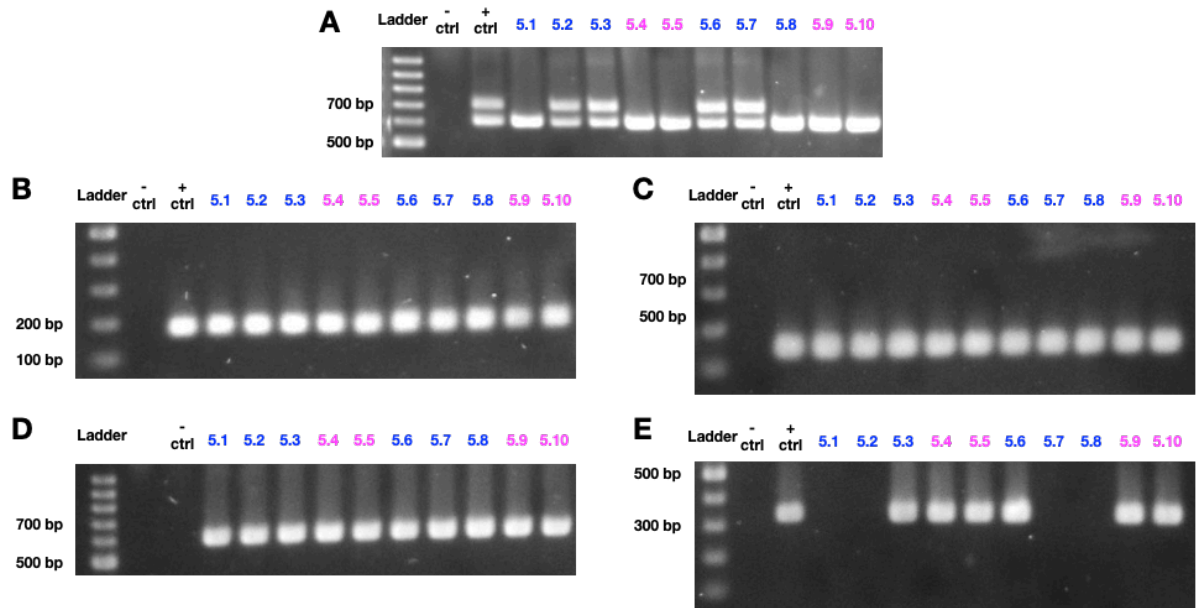


Figure 5.10 – Genotyping results of Stage 5 breeding involving rederivation for crossing $\text{miR-21}^{\text{fl/fl}}$ and HET $\text{miR-21}^{\text{fl/wt}}$ conditional female mice and hemizygous Vil:Cre male mice through *in vitro* fertilization ($n = 21$; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (B + C) Vil:Cre gene, expected size = 150 bp [mutant]. (D + E) FLPo gene, expected size = 603 bp [wt], 300 bp [mutant].

5.2.6 Stage 6 – HET miR-21^{fl/wt} KO x Vil-Cre mice lacking the miR-21 floxed allele

In this stage, HET miR-21^{fl/wt} conditional KO mice were inbred to obtain a more desired genotypical status among both sexes. Ideally, these ideal miR-21^{fl/fl} mice, would also be genotypically hemizygous for the Vil:Cre transgene and not possess the FLPo transgene. An overview of this breeding stage can be seen in **Figure 5.11**.

The resulting litter of 24 mice (14 males, 10 females) were again ear-clipped and checked for the presence of the miR-21 floxed region, along with the Vil:Cre and FLPo transgenes as before (but only in mice positive for the miR-21 floxed allele). As seen in **Figure 5.12**, this led to the formation of mixed genotypes. There were 3 mice (#6.9, 6.19, 6.24) that had the desired genotype of HET miR-21^{fl/wt}; HEMI Vil:Cre^{+/-}; FLPo^{-/-}. All Stage 6 genotyping results are summarized in **Table 5.1** below. Of these, 6 mice (#6.2, 6.5, 6.9, 6.15, 6.19, 6.24) were selected for the next stage of breeding to maximize the probability of HOM miR-21^{fl/fl} mice that are HEMI for Vil:Cre and lacked the FLPo transgene. Mouse #6.22, while having a useful genotype, was excluded for litter maintenance.

Table 5.1 – Stage 6 breeding litter summary.

Mouse	Sex	miR-21 flox	Vil:Cre	FLPo	Genotype
6.1	M	-/-	n/a	n/a	-
6.2	M	+/-	-/-	-/-	HET miR-21 ^{fl/wt} ; HEMI Vil:Cre ^{+/-} ; FLPo ^{-/-}
6.3	M	-/-	n/a	n/a	-
6.4	M	+/-	unknown	-/-	-
6.5	F	+/-	+/-	+/-	HET miR-21 ^{fl/wt} ; HEMI Vil:Cre ^{+/-} ; HET FLPo ^{+/-}
6.6	F	-/-	n/a	n/a	-
6.7	F	-/-	n/a	n/a	-
6.8	F	-/-	n/a	n/a	-
6.9	M	+/-	+/-	-/-	HET miR-21 ^{fl/wt} ; HEMI Vil:Cre ^{+/-} ; FLPo ^{-/-}
6.10	M	-/-	n/a	n/a	-
6.11	M	-/-	n/a	n/a	-
6.12	M	-/-	n/a	n/a	-
6.13	M	-/-	n/a	n/a	-
6.14	F	-/-	n/a	n/a	-
6.15	F	+/-	-/-	+/-	HET miR-21 ^{fl/wt} ; Vil:Cre ^{-/-} ; HET FLPo ^{+/-}
6.16	F	+/-	+/-	+/+	-
6.17	M	-/-	n/a	n/a	-
6.18	M	-/-	n/a	n/a	-
6.19	M	+/-	+/-	-/-	HET miR-21 ^{fl/wt} ; HEMI Vil:Cre ^{+/-} ; FLPo ^{-/-}
6.20	M	-/-	n/a	n/a	-
6.21	M	-/-	n/a	n/a	-
6.22	F	+/-	-/-	+/-	HET miR-21 ^{fl/wt} ; Vil:Cre ^{-/-} ; HET FLPo ^{+/-}
6.23	F	+/-	+/+	+/-	-
6.24	F	+/-	+/-	-/-	HET miR-21 ^{fl/wt} ; HEMI Vil:Cre ^{+/-} ; FLPo ^{-/-}

- Unwanted genotype (red), usable genotype (yellow), desired genotype (green)

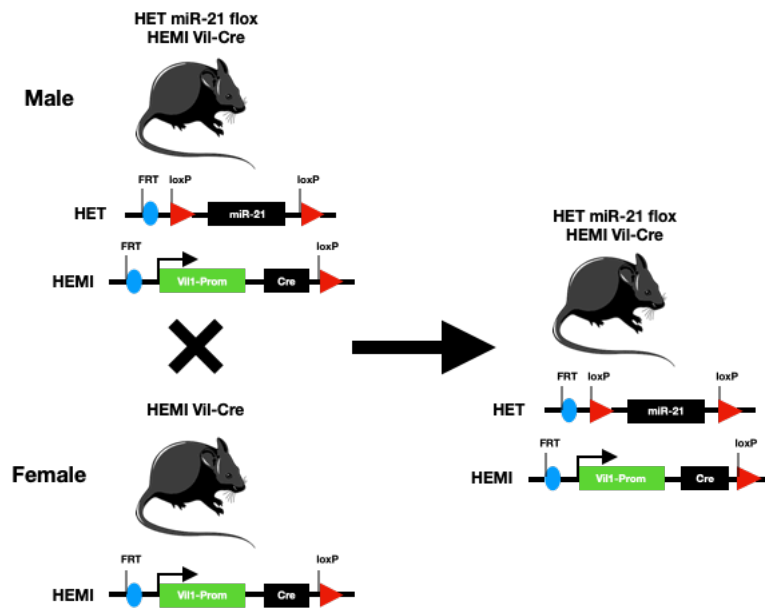


Figure 5.11 – Stage 6 breeding workflow, crossing heterozygous miR-21^{fl/wt} conditional knockout mice x Vil-Cre mice lacking the miR-21 floxed allele.

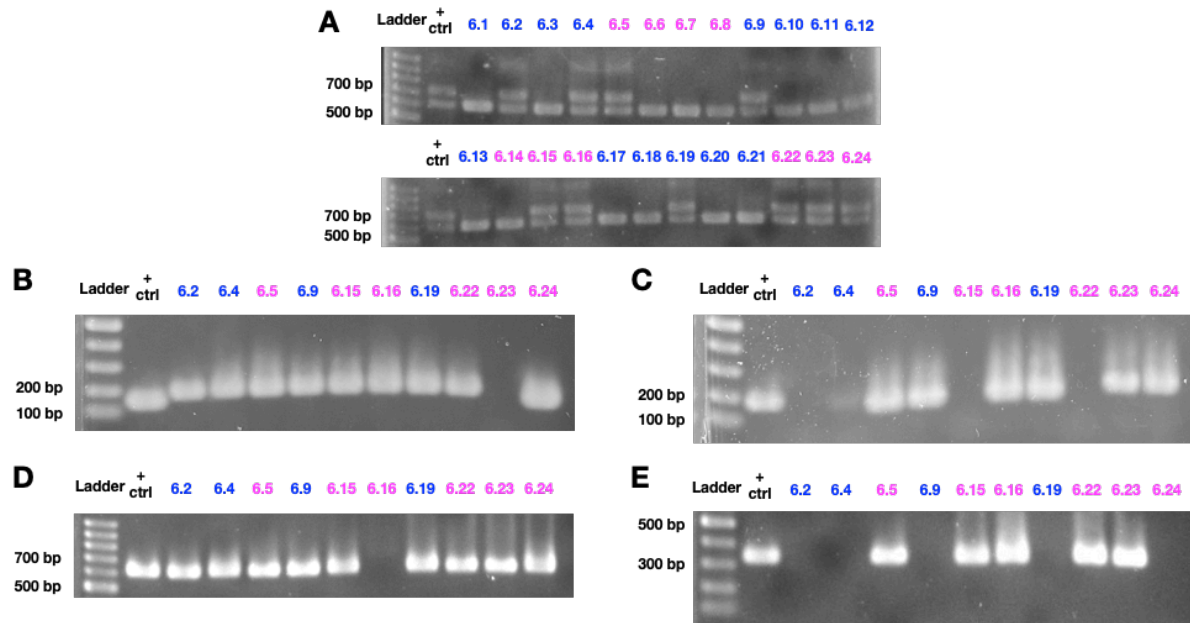


Figure 5.12 – Genotyping results of Stage 6 breeding between heterozygous miR-21^{fl/wt} conditional knockout x Vil-Cre mice lacking the miR-21 flox allele (n = 24; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (B + C) Vil:Cre gene, expected size = 150 bp [mutant]. (D + E) FLPo gene, expected size = 603 bp [wt], 300 bp [mutant].

5.2.7 Stage 7 – Heterozygous miR-21^{fl/wt} conditional knockout mice

In this stage, heterozygous miR-21^{fl/wt} mice were inbred to obtain homozygous miR-21^{fl/fl} mice among both sexes. As before, these mice would ideally be genotypically heterozygous for the Vil:Cre transgene and not possess the FLPo transgene. An overview of this breeding stage can be seen in **Figure 5.13**.

The resulting litter of 31 mice were ear-clipped and checked for the presence of the miR-21 floxed region, along with the Vil:Cre and FLPo transgenes as before for only the mice homozygous for the miR-21 floxed allele. As seen in **Figure 5.14**, only 3 male mice were HOM miR-21^{fl/fl} (#7.14, 7.18, 7.21). Of these 3 male mice, only one had the desired genotype of HOM miR-21^{fl/fl}, HET Vil:Cre^{+/-}, FLPo^{-/-}.

Multiple additional litters were genotyped to obtain males and females with the desired genotype for a subsequent round of breeding that would produce enough pups for characterization. A total of 60 pups were ear-clipped and checked for the presence of the miR-21 floxed allele, Vil:Cre and FLPo transgenes. As shown in **Figure 5.15**, 7 mice were HOM miR-21^{fl/fl} (#7.40, 7.41, 7.42, 7.57, 7.68, 7.71, 7.87), of which only 1 female had the desired genotype of HOM miR-21^{fl/fl}, HET Vil:Cre^{+/-}, FLPo^{-/-}. An additional litter of 8 pups was genotyped via Transnetyx genotyping services which had 3 mice with the desired genotype (#7.94, 7.95, 7.99; data not shown). It should be noted that all mice that were homozygous for the miR-21 floxed allele were also re-confirmed with Transnetyx genotyping services, along with the Vil:Cre and FLPo transgenes. A breeding trio using male #7.14 with females #7.87 and #7.99 was created for a subsequent round of breeding and establishment of the epithelial-deficient miR-21 conditional KO colony (HOM miR21^{fl/fl}, HEMI Vil:Cre^{+/-}, henceforth referred to as miR-21^{IEC-KO}). Another breeding trio using male #7.40 with females #7.68 and #7.71 was formed in addition to breed a separate colony of miR-21 flox mice lacking the Vil:Cre transgene (HOM miR21^{fl/fl}, Vil:Cre^{-/-}, henceforth referred to as miR-21^{fl/fl}).

From the final breeding stage, a total of 37 pups were ear-clipped and checked for the presence of the Vil:Cre transgene to determine whether mice had miR-21^{fl/fl} or miR-21^{IEC-KO} genotype. Since paternal and maternal mice all were miR-21^{fl/fl} and FLPo^{-/-}, these two allele confirmations were not necessary. As summarized in **Table 5.2**, there were 24 mice (13 male, 6 female) that were miR-21^{fl/fl} and 6 mice (3 male, 3 female) that were miR-21^{IEC-KO}. For male miR-21^{fl/fl}, due to the nature of the breeding, the 13 pups were further separated into two distinct categories of 8 non-littermates and 5 littermates. For female

miR-21^{fl/fl}, there were no littermates that survived post-weaning and therefore only had a non-littermate group. These mice were used in the DSS-induced colitis model where mice were treated with 2% DSS for six days followed by phenotypic and molecular analyses as done in previous chapters.

Table 5.2 – Final breeding litter summary.

Genotype	Sex	Litter Status	Amount
miR-21 ^{fl/fl}	M	non-littermate	8
	M	littermate	5
	F	non-littermate	6
miR-21 ^{IEC-KO}	M	-	3
	F	-	3

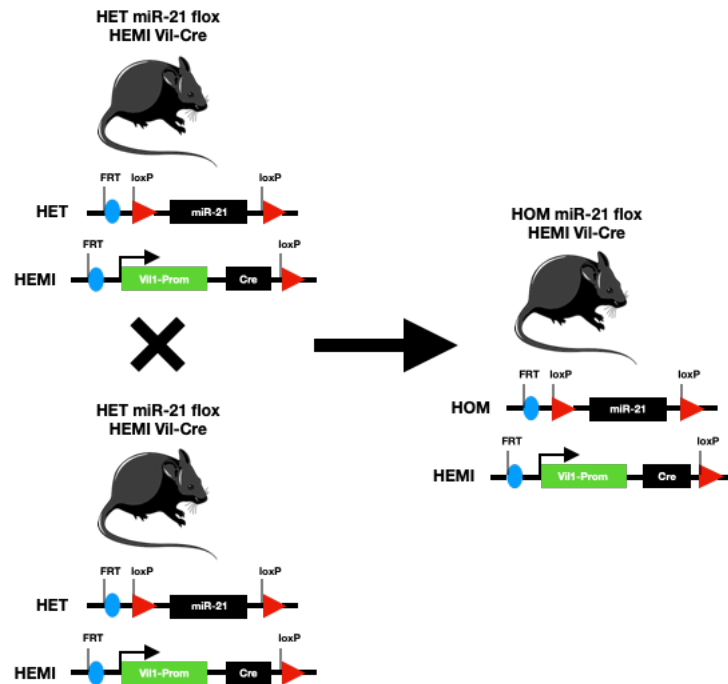


Figure 5.13 – Stage 7 breeding workflow, crossing heterozygous *miR-21*^{flox/wt} conditional knockout mice.

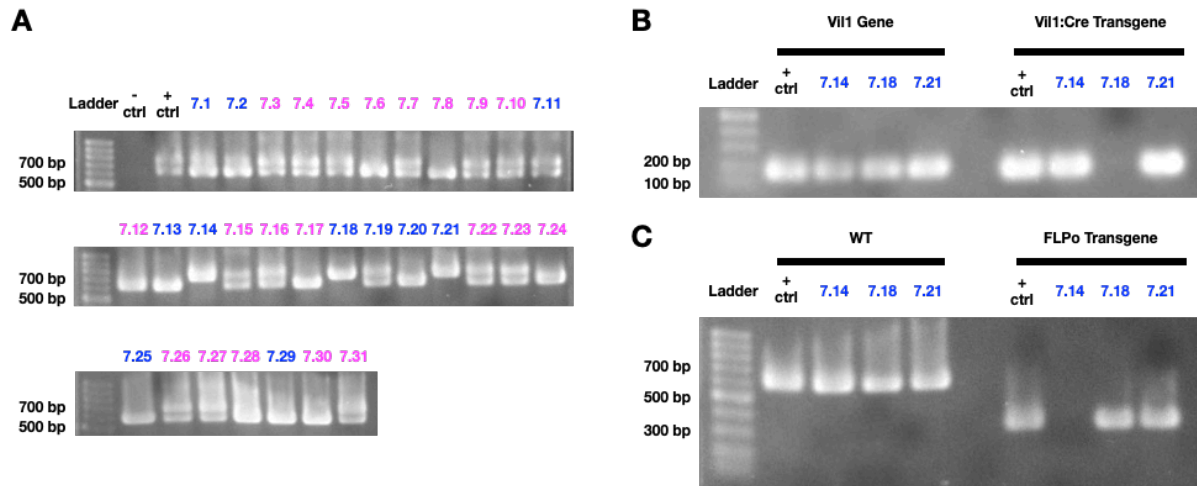
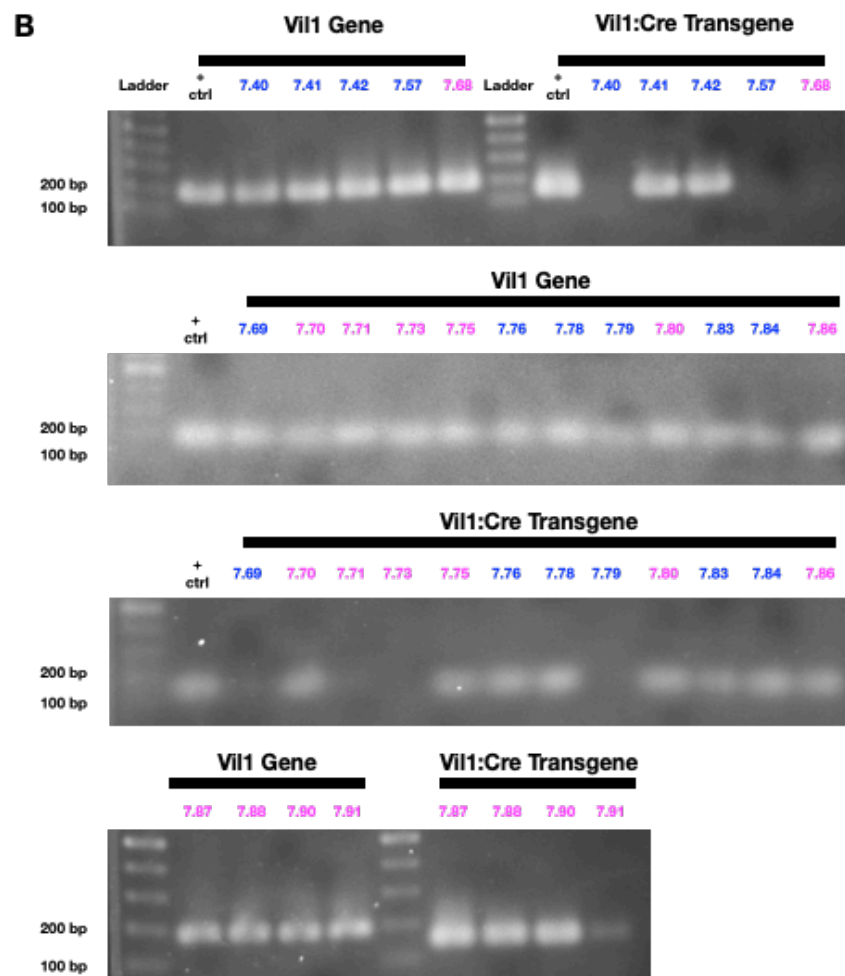
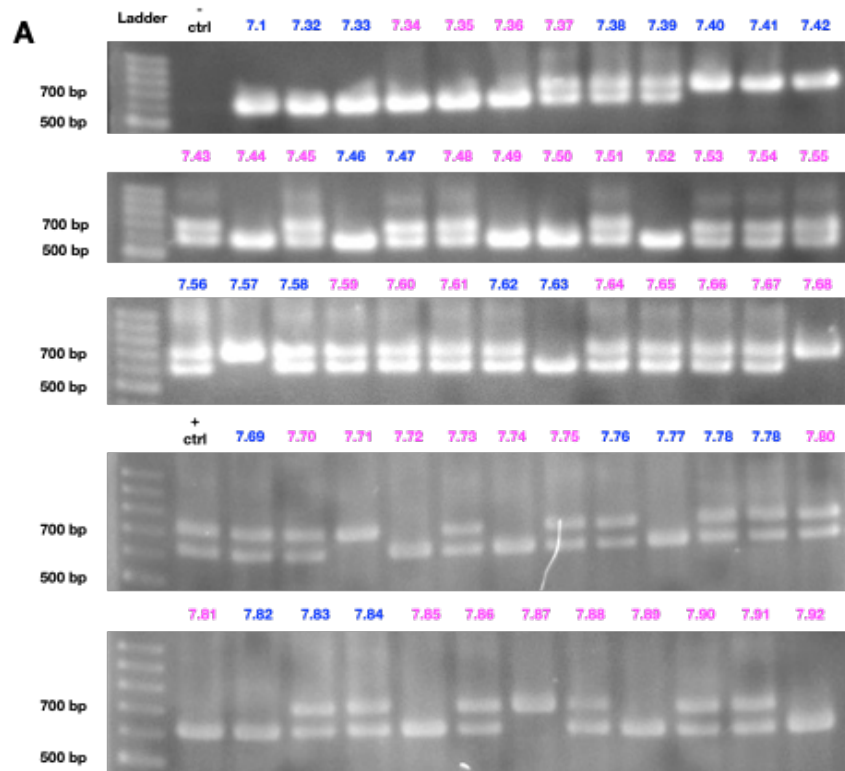


Figure 5.14 – Genotyping results of Stage 7 breeding between heterozygous $\text{miR-21}^{\text{fl/wt}}$ conditional knockout mice ($n = 31$; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (B) Vil:Cre gene, expected size = 150 bp [mutant]. (C) FLPo gene, expected size = 603 bp [wt], 300 bp [mutant].



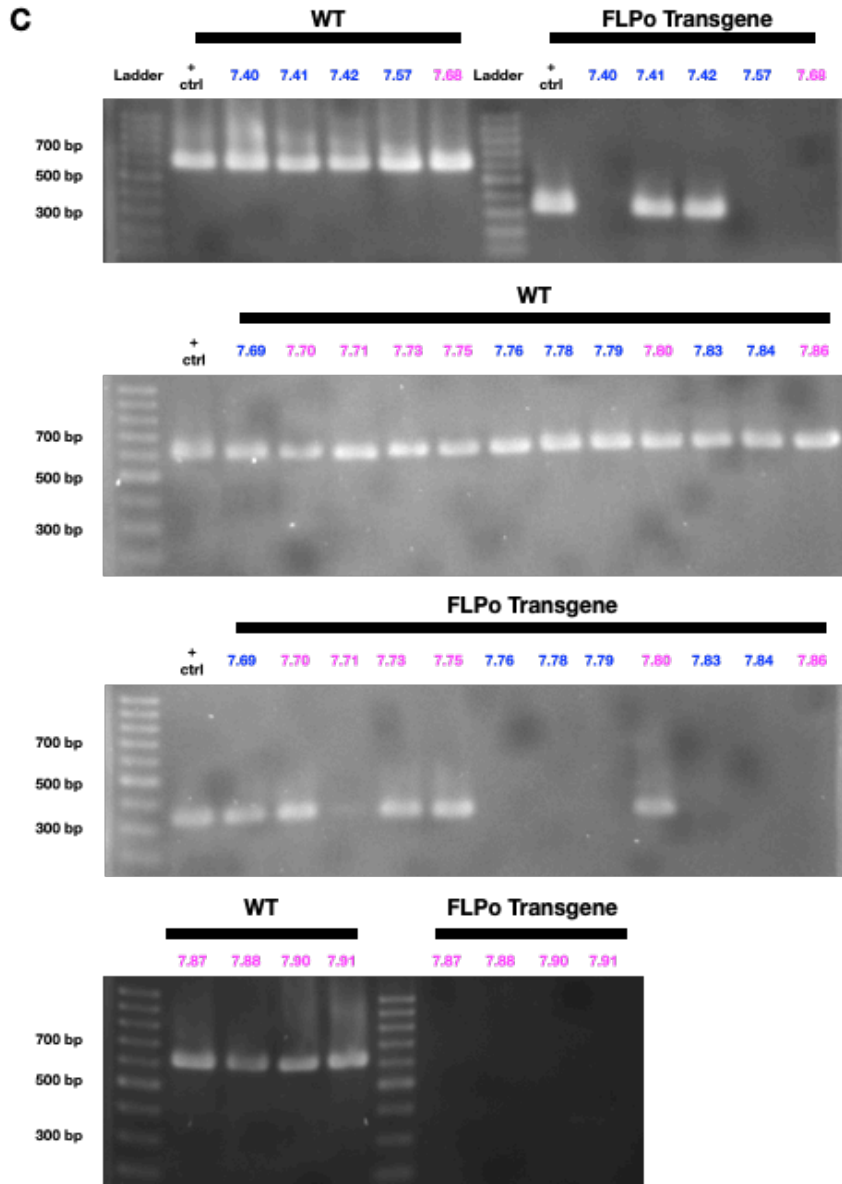


Figure 5.15 – Genotyping results of Stage 7 additional breeding between heterozygous miR-21^{fl/wt} conditional knockout mice (n = 68; male - blue, female - magenta). PCRs were conducted on gDNA extracted from earclip tissue. (A) miR-21 floxed allele, expected size = 613 bp [wt], 705 bp [mutant]. (B) Vil:Cre gene, expected size = 150 bp [mutant]. (C) FLPo gene, expected size = 603 bp [wt], 300 bp [mutant].

5.2.8 DSS-induced colitis in miR-21^{IEC-KO} strain and determination of disease susceptibility

Clinical scores following induction of DSS-colitis in male mice is shown in **Figure 5.16**. There was a significant difference in initial body weight between miR-21^{fl/fl} non-littermate and littermate groups, with no significant differences compared to miR-21^{IEC-KO} (**Figure 5.16A**). There were no notable differences in water or food consumption between groups (**Figure 5.16B, 5.16C**). MiR-21^{IEC-KO} mice were protected from DSS-induced colitis based on lower disease severity metrics such as reduced weight loss (**Figure 5.16D**), lower diarrhea scores (**Figure 5.16E**) and lower stool blood scores (**Figure 5.16F**). These metrics culminated in significantly lower DAI for miR-21^{IEC-KO} male mice compared to non-littermate miR-21^{fl/fl} (**Figure 5.16G**), particularly on the final day (**Figure 5.16H**). When comparing against littermate miR-21^{fl/fl} mice, there was less improvement in all metrics, albeit still lower disease scores and less symptomatic. There were no significant differences in colon length amongst the three male groups (**Figure 5.16I**).

Clinical scores following induction of DSS-colitis in female mice is shown in **Figure 5.17**. There were only two groups within this trial due to the lack of miR-21^{fl/fl} littermates in Stage 7 breeding litters. There was no significant difference in initial body weight between miR-21^{fl/fl} and miR-21^{IEC-KO} female mice (**Figure 5.17A**), nor were there any notable differences in water and food consumption (**Figure 5.17B, 5.17C**). In contrast to male mice, female miR-21^{IEC-KO} mice were not significantly different to non-littermate miR-21^{fl/fl} control mice in DSS-induced colitis. Regarding disease metrics, there were no significant differences between groups in body weight (**Figure 5.17D**), diarrhea scores (**Figure 5.17E**), stool blood scores (**Figure 5.17F**) and summarized DAI scores (**Figure 5.17G, 5.17H**) throughout the duration of the trial. Lastly, there were no significant differences in colon length between groups (**Figure 5.17I**).

Gut permeability was assessed between miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice through measuring leakage of FD4 in serum samples collected on Day 6. In male mice, there was a lower trend of FITC detected in the serum of miR-21^{IEC-KO} mice compared to non-littermate and littermate miR-21^{fl/fl} mice (**Figure 5.18A**), while in female mice there were no significant differences between groups (**Figure 5.18B**), albeit concentrations of FITC were generally low in both sexes.

Histological scoring of distal and proximal colon tissue between miR-21^{fl/fl} and miR-21^{IEC-KO} mice was conducted to assess levels of tissue damage and inflammation. For

male mice, there was reduced damage to the distal epithelium as well as infiltration of immune cells in miR-21^{IEC-KO} mice compared to miR-21^{fl/fl} groups (**Figure 5.19A, 5.19B, 5.19C**), leading to a lower cumulative histological score that suggests protection against DSS-induced colitis (**Figure 5.19D**). There were no differences in the histological scoring between male groups in proximal tissue (**Figure 5.19E, 5.19F, 5.19G**), likely due to the mechanism of action for DSS primarily targeting the distal colon.²¹³ This coincides with permeability data suggesting decreased gut barrier damage and leakage in the absence of miR-21 in gut epithelia. For female mice, there was a trend of lower tissue damage in both distal and proximal tissue (**Figure 5.20A, 5.20B, 5.20E**) in miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice. There were no significant differences in the levels of immune infiltration in either distal or proximal tissue between female groups (**Figure 5.20C, 5.20F**), thus leading to similar cumulative histological scores (**Figure 5.20D, 5.20G**). However, more replicates may be required to properly assess these differences. Further testing for inflammatory markers included MPO activity which was measured within distal and proximal tissue of miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice. In both males and females, there was a small decrease in MPO activity between miR-21^{IEC-KO} mice compared to non-littermate miR-21^{fl/fl} in distal (**Figure 5.21A, 5.21C**) but not proximal (**5.21B, 5.21D**) tissue, suggesting barrier damage can in part be attributed to neutrophil presence and activity.

Additional markers of inflammation were measured in stool, distal and proximal tissue, as well as serum samples, to compare the state of inflammation between miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice. For male mice, miR-21^{IEC-KO} mice had decreased levels of lipocalin-2 (Lcn2) in stool compared to both non-littermate and littermate miR-21^{fl/fl} groups, while in females there was a similar reduced trend in miR-21^{IEC-KO} mice (**Figure 5.22A, 5.22B**). Lcn2 (also known as neutrophil gelatinase-associated lipocalin (NGAL) in humans) is secreted by the innate immune system against infection primarily for iron sequestration and often used to determine the progression of colitis in murine models of IBD.³²² Pro-inflammatory markers IL-6, IL-1 β , and TNF- α were reduced within distal tissue of miR-21^{IEC-KO} compared to non-littermate miR-21^{fl/fl} mice (**Figure 5.23A, 5.23B, 5.23C**), however anti-inflammatory marker IL-10 was also reduced (**Figure 5.23D**). In proximal tissue, there were no major differences in levels of IL-6, TNF- α and IL-10 between groups, with the exception of IL-1 β having slightly lower levels in miR-21^{IEC-KO} compared to non-littermate miR-21^{fl/fl} mice (**Figure 5.23E, 5.23F, 5.23G, 5.23H**). Within serum, miR-21^{IEC-KO} had decreased levels of IL-6 compared to both non-littermate and littermate miR-21^{fl/fl} mice, with no major differences in IL-1 β , TNF- α or IL-10 between

groups (**Figure 5.23I, 5.23J, 5.23K, 5.23L**). These differences suggest more inflammation in non-littermate miR-21^{fl/fl} mice with some positive impact provided to littermate miR-21^{fl/fl} pups during DSS-induced colitis. For female mice, in distal and proximal colon tissue, there were no major differences observed between the two groups, though there were slightly elevated levels of pro-inflammatory TNF- α and anti-inflammatory IL-10 in distal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice (**Figure 5.24A, 5.24B, 5.24C, 5.24D, 5.24E, 5.24F, 5.24G, 5.24H**). Due to high variability in the ELISAs, there were no notable differences in inflammation markers within serum samples between female groups (**Figure 5.24I, 5.24J, 5.24K, 5.24L**).

Next, a comprehensive screen of intercellular junction gene expression was conducted in distal and proximal colon samples from male and female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. In contrast to the full KO (miR-21^{-/-}) model, whole colon tissue of miR-21^{IEC-KO} may still have low detectable levels of miR-21, but it would be valuable to know the holistic impact imbued upon whole tissue in the absence of miR-21 in intestinal epithelia. As a preliminary measure, mature miR-21-5p levels were measured in male and female mice to determine both success of miR-21 ablation and contribution of miR-21 of epithelial origin to levels detected in whole colon tissue (ie. baseline levels excluding epithelial miR-21). In male mice, miR-21^{IEC-KO} had reduced levels of miR-21-5p compared to non-littermate and littermate miR-21^{fl/fl} mice (**Figure 5.25A**). In females, there was a trend for lower levels of miR-21-5p in miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice, though more replicates may be required to fully assess impact of miR-21 deletion in IECs (**Figure 5.25B**).

With lower levels present in whole colon tissue of miR-21^{IEC-KO} mice, levels of known miR-21 targets were subsequently investigated in male and female colon tissue. distal and proximal tissue were separated, and expression levels were assessed independently. In male mice, known miR-21 targets *Pdcd4* and *Pten* were elevated in miR-21^{IEC-KO} colon tissue compared to non-littermate and littermate miR-21^{fl/fl} mice (**Figure 5.26A, 5.26B**), with *Pdcd4* significantly upregulated in proximal tissue. In female mice, there were also elevated levels of *Pdcd4* and *Pten* in miR-21^{IEC-KO} distal tissue compared to miR-21^{fl/fl} mice (**Figure 5.26C, 5.26D**), but not proximal tissue. Some biological replicates had undetectable levels of either gene, particularly in female proximal tissue and thus additional replicates may be necessary to conclusively assess miR-21 impact at the gut epithelium. Nonetheless, even in whole intestinal tissue, absence of miR-21 from IECs can increase the levels of known miR-21 targets.

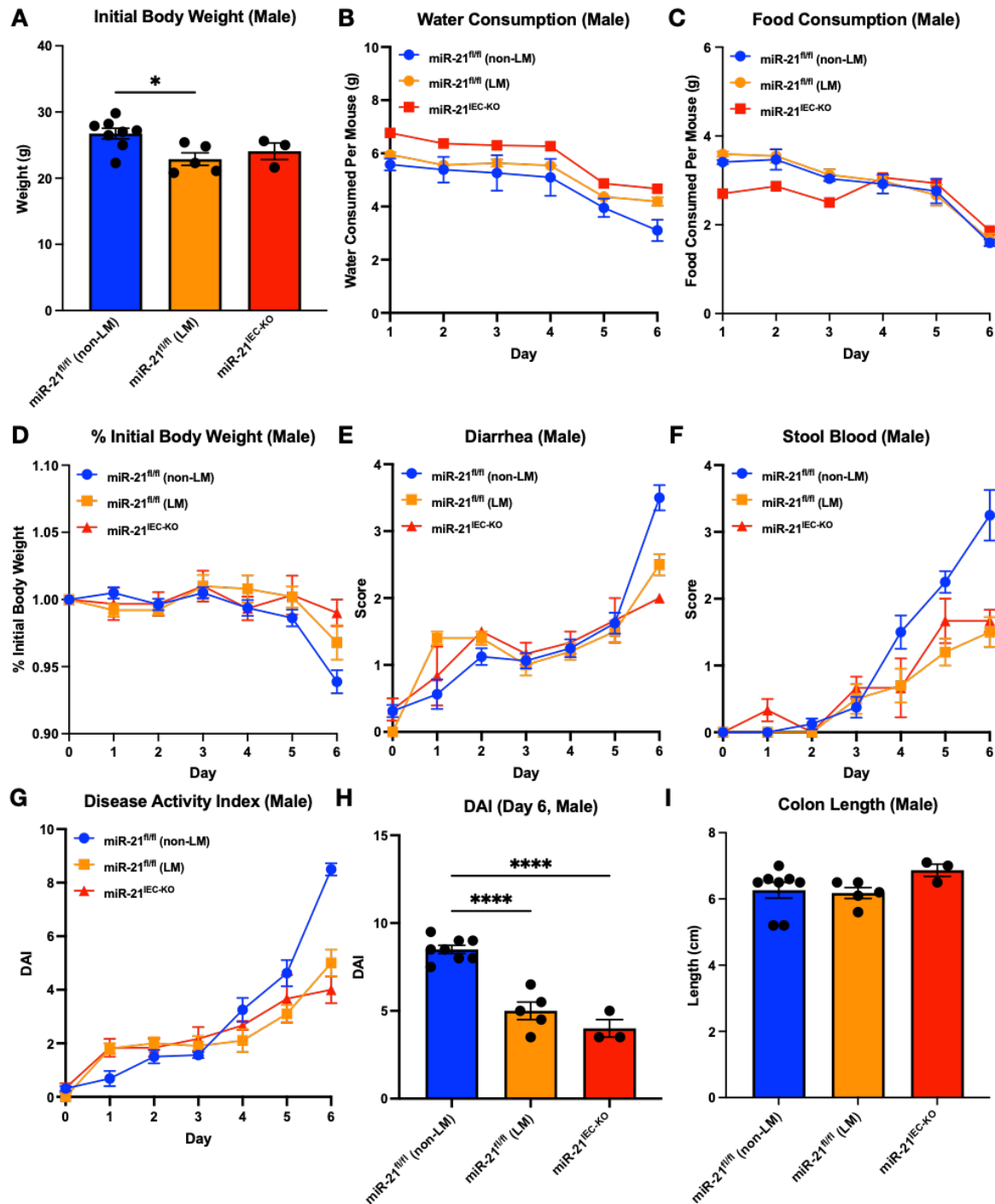


Figure 5.16 – Male miR-21^{IEC-KO} and littermate miR-21^{fl/fl} mice are protected against DSS-induced colitis. Non-littermate (non-LM) miR-21^{fl/fl}, littermate (LM) miR-21^{fl/fl} and miR-21^{IEC-KO} male mice 8-15 weeks of age were subjected to 2% (w/v) DSS *ad libitum* via drinking water for six days. (A) Initial body weights of miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice. (B + C) Water and food consumption of miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice throughout trial duration. Daily measurements and scoring were taken of mouse (D) body weight, (E) diarrhea and (F) stool blood, culminating in a (G) disease activity index (DAI) for whole trial and (H) DAI on Day 6 of trial. (I) Mouse colons were measured at the end of the trial. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with * - $p \leq 0.05$, **** - $p \leq 0.0001$.

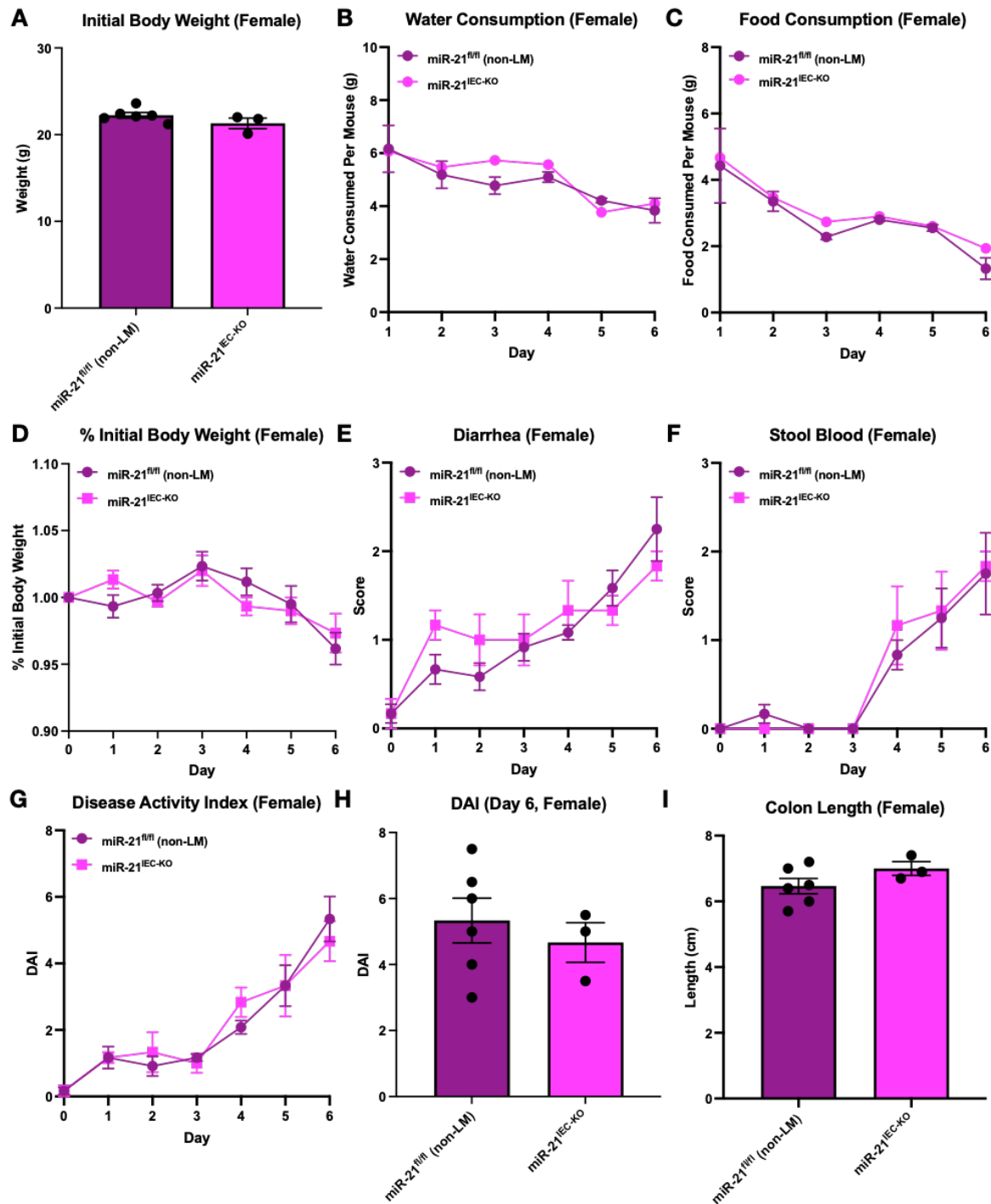


Figure 5.17 – Female miR-21^{IEC-KO} mice are not significantly different to non-littermate miR-21^{fl/fl} control mice during DSS-induced colitis. Non-littermate (non-LM) miR-21^{fl/fl} and miR-21^{IEC-KO} female mice 8-15 weeks of age were subjected to 2% (w/v) DSS *ad libitum* via drinking water for six days. (A) Initial body weights of miR-21^{fl/fl} and miR-21^{IEC-KO} mice. (B + C) Water and food consumption of miR-21^{fl/fl} and miR-21^{IEC-KO} mice throughout trial duration. Daily measurements and scoring were taken of mouse (D) body weight, (E) diarrhea and (F) stool blood, culminating in a (G) disease activity index (DAI) for whole trial and (H) DAI on Day 6 of trial. (I) Mouse colons were measured at the end of the trial. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

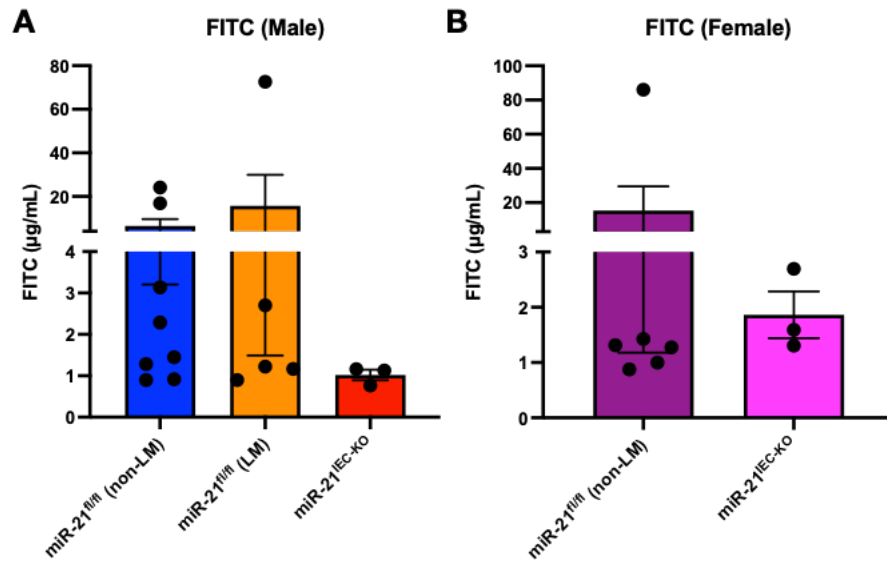


Figure 5.18 – Male miR-21^{IEC-KO} mice have slightly reduced gut permeability during DSS-induced colitis. FITC concentration in DSS-treated miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} (A) male and (B) female mice. On the final day of the trial, FITC-dextran (molecular mass 4 kDa) was dissolved in sterile PBS and administered by oral gavage at 12 mg per mouse. After four hours, mice were euthanized with CO₂ and blood was collected by decapitation. Serum was separated by centrifuging blood samples at 4°C for 12 min at 1000 x g. FITC concentration was determined via a standard curve. Fluorescence was measured using a standard plate reader set to 485 nm (excitation) and 525 nm (emission). Denoted are n ≥ 3 mice per group, mean values ± SEM, *p* value calculations using one-way ANOVA for male groups or Welch's *t*-test for female groups.

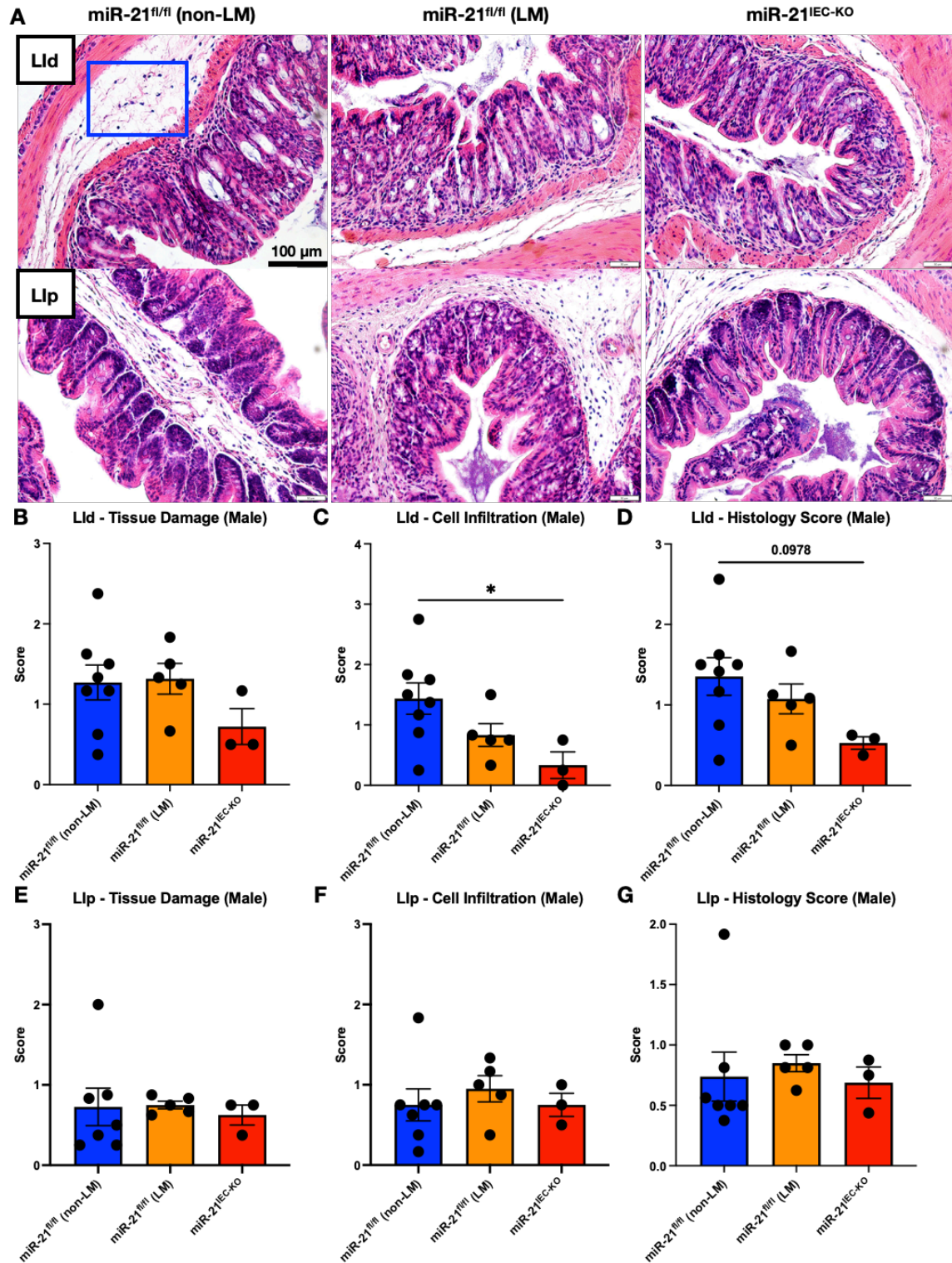


Figure 5.19 – Histological analysis shows loss of miR-21 in intestinal epithelium reduces tissue damage and immune cell infiltration in male distal colon tissue during DSS-induced colitis. At the end of the trial, miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mice tissue were fixed in 4% formaldehyde, dehydrated in ethanol and embedded in paraffin wax. (A) Histological analysis of distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue sections (5 μ m) was conducted via H&E staining. Histopathological blinded scoring of stained mouse intestinal tissue was done for (B + E) tissue damage of intestinal crypts and (C + F) immune cell infiltration, adapted from Wang *et al.* (2022)²³⁰, culminating in a (D + G) histology score. Blue box in miR-21^{fl/fl} (non-LM) - LId denotes area of interest regarding cell infiltration. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA with * - $p \leq 0.05$.

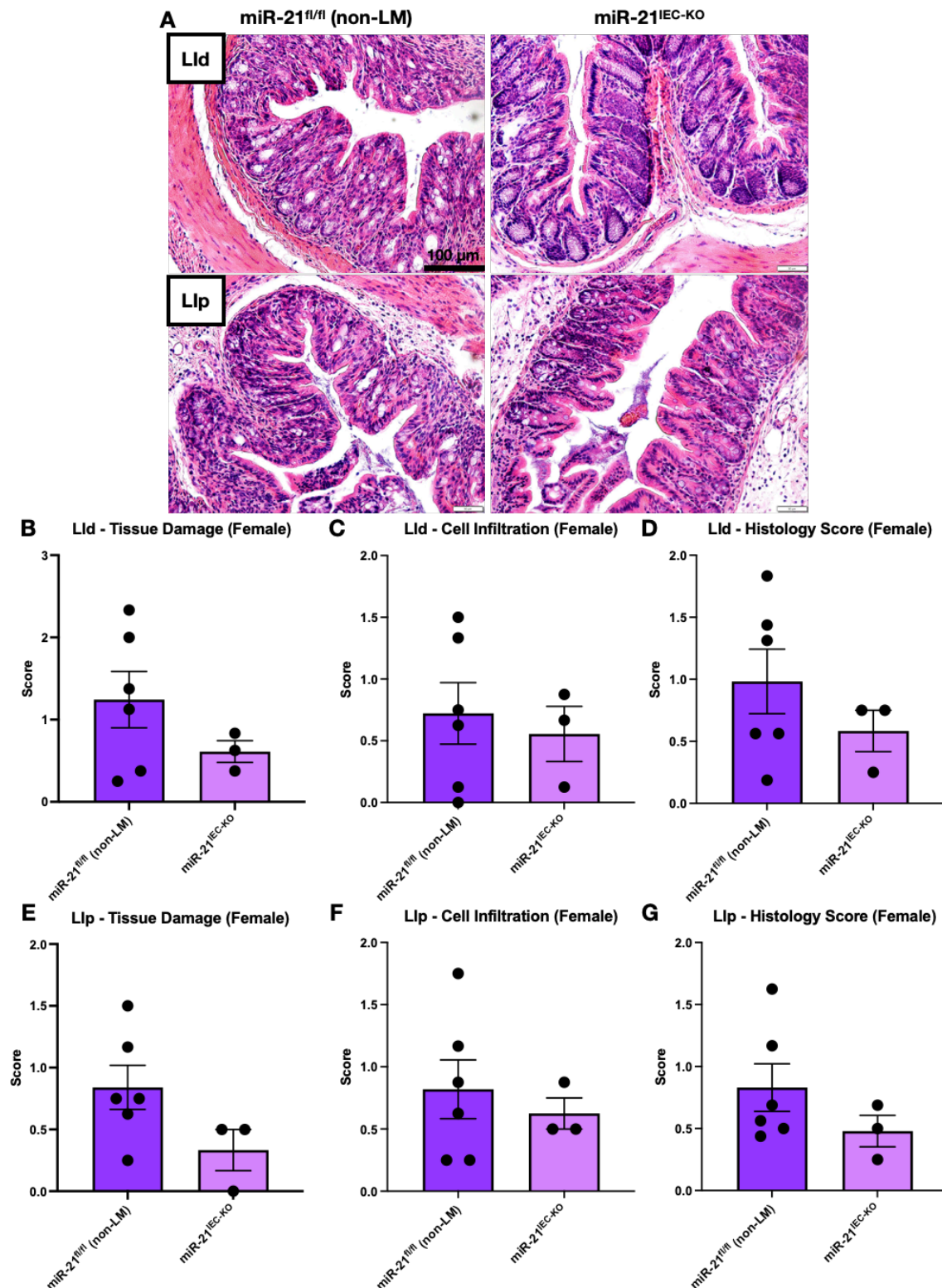


Figure 5.20 – Histological analysis shows loss of miR-21 in intestinal epithelium lowers tissue damage in female distal and proximal colon tissue during DSS-induced colitis. At the end of the trial, non-littermate (non-LM) miR-21^{fl/fl} and miR-21^{IEC-KO} mice tissue were fixed in 4% formaldehyde, dehydrated in ethanol and embedded in paraffin wax. (A) Histological analysis of distal colon / large intestine (Lld) and proximal colon / large intestine (Llp) tissue sections (5 μ m) was conducted via H&E staining. Histopathological blinded scoring of stained mouse intestinal tissue was done for (B + E) tissue damage of intestinal crypts and (C + F) immune cell infiltration, adapted from Wang *et al.* (2022)²³⁰, culminating in a (D + G) histology score. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

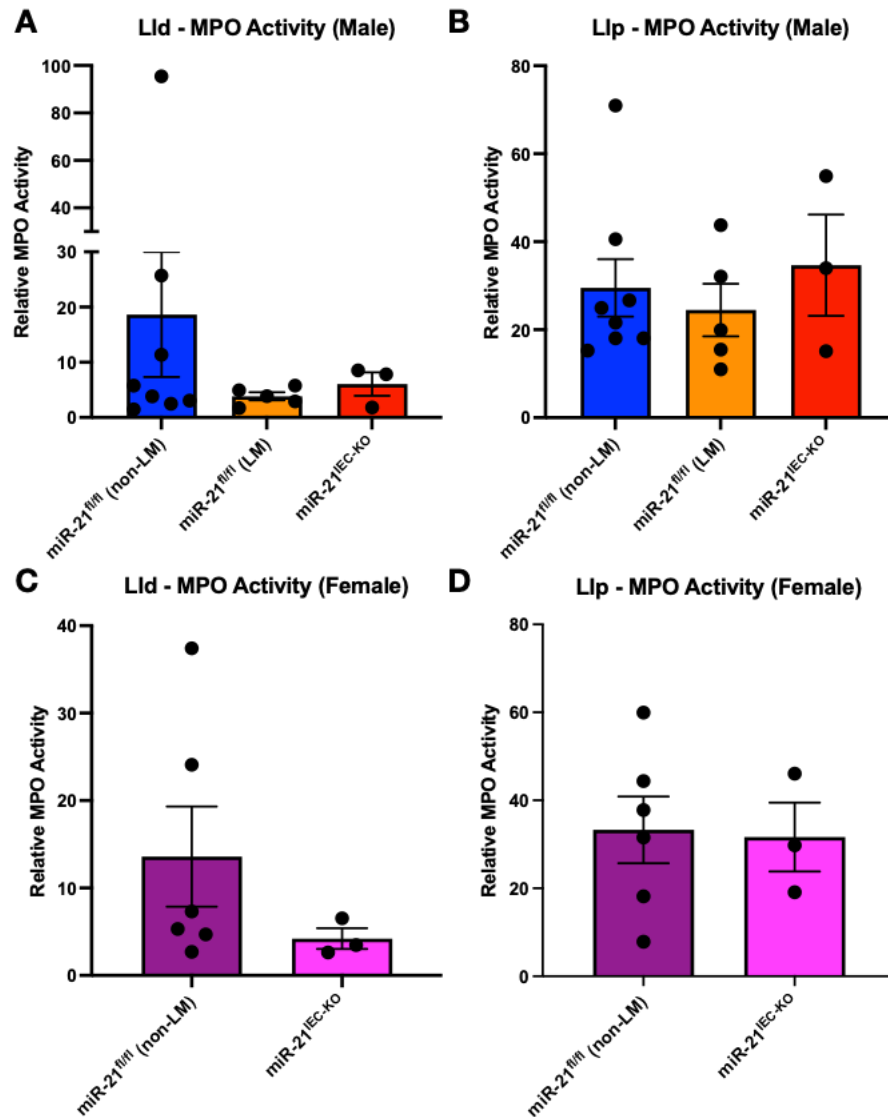


Figure 5.21 – miR-21^{IEC-KO} and littermate miR-21^{fl/fl} mice have a lower trend of myeloperoxidase activity than non-littermate miR-21^{fl/fl} in distal tissue during DSS-induced colitis. At the end of the trial, miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mouse tissue were homogenized in MPO Buffer I solution (100 mM sodium chloride, 20 mM trisodium phosphate, 15 mM disodium ethylenediaminetetraacetate [EDTA] dihydrate; pH 4.7), and centrifuged. Pellets were resuspended in 1 mL of 0.2% sodium chloride solution and 1 mL of 1.6% sodium chloride / 5% glucose solution. After another centrifugation, pellets were resuspended in MPO Buffer II (50 mM trisodium phosphate, 0.5% Hexadecyltrimethylammonium bromide [HTAB]; pH 5.4), underwent three cycles of freeze-thaw for complete cell lysis, and then centrifuged. Supernatants were transferred to 96-well plates in duplicate. MPO activity was induced using OptEIA™ ELISA sets, and reactions were stopped with the addition of 1 M sulfuric acid. Absorbance was measured using a standard plate reader set to 450 nm. Relative activity for distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue of (A + B) male and (C + D) female mice were measured by subtracting blank background absorbance and normalizing to mg of protein. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA for male groups and Welch's t -test for female groups.

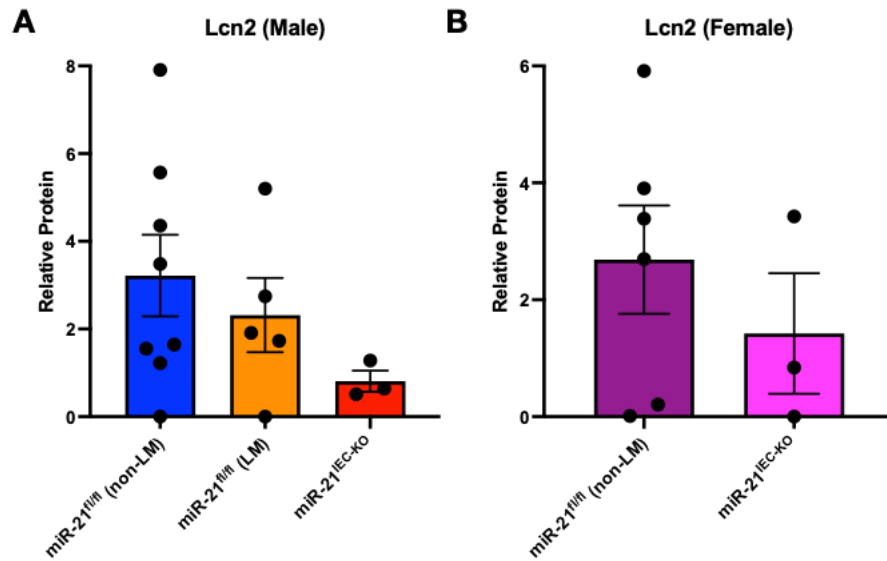


Figure 5.22 – miR-21^{IEC-KO} mice have reduced lipocalin-2 in stool than miR-21^{fl/fl} groups during DSS-induced colitis. At the end of the trial, miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mouse stool samples were homogenized in PBS and transferred to 96-well plates. ELISAs were conducted to detect pro-inflammatory marker Lcn2. Absorbance was measured using a standard plate reader set to 450 nm. Concentrations of inflammation markers in stool of (A) male and (B) female mice were determined via a standard curve, measured by subtracting blank background absorbance and normalizing to mg of protein. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA for male groups and Welch's t -test for female groups.

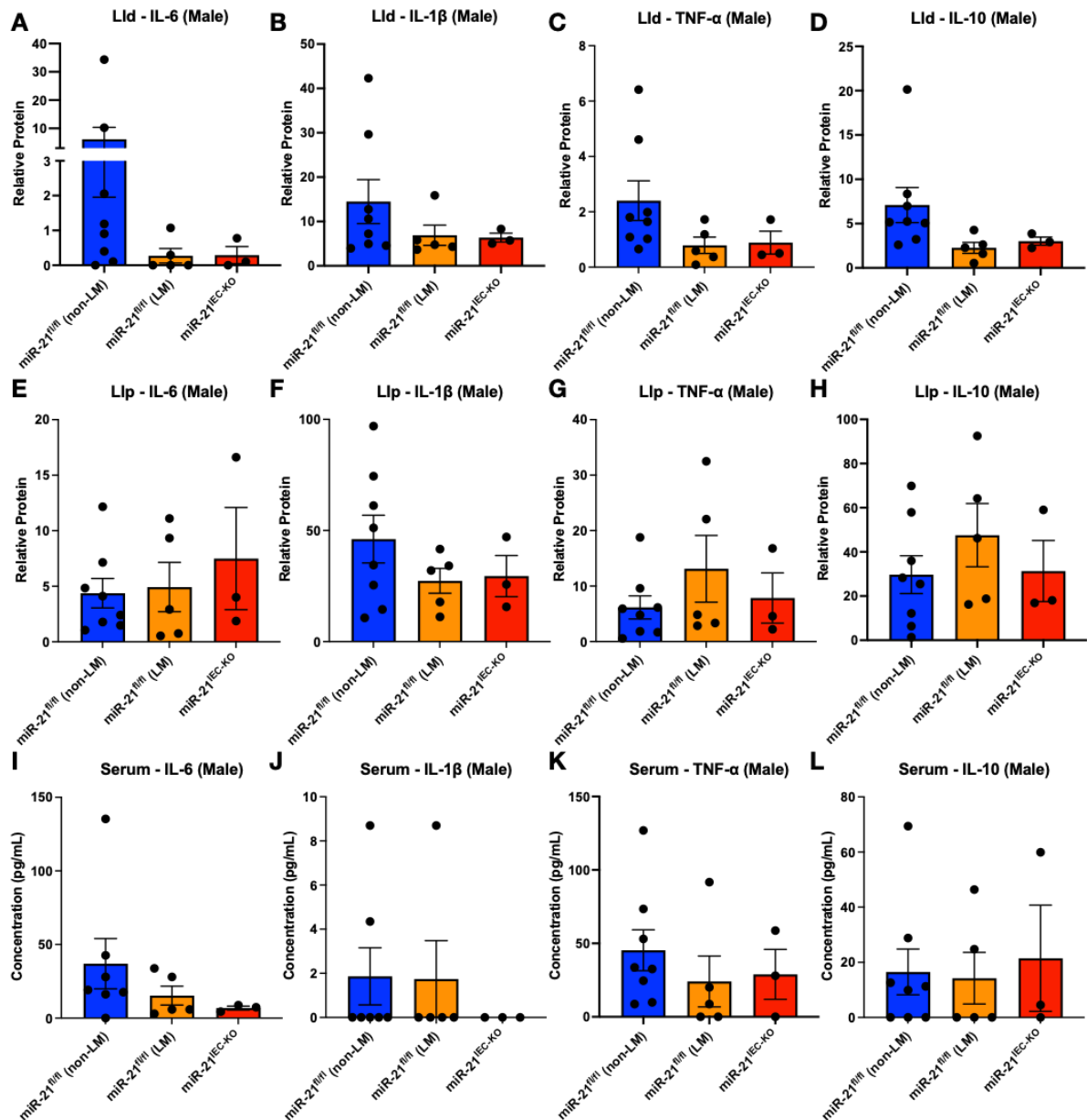


Figure 5.23 – miR-21^{IEC-KO} and littermate miR-21^{fl/fl} mice have lower inflammation markers in various sample types than non-littermate miR-21^{fl/fl} during DSS-induced colitis. At the end of the trial, miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mouse colon tissue were homogenized in lysis buffer. After centrifugation, cell lysates were transferred to 96-well plates. Blood was also collected at the end of the trial by decapitation. Serum was separated by centrifuging blood samples at 4°C for 12 min at 1000 x g and also transferred to 96-well plates. ELISAs were conducted to detect pro-inflammatory markers IL-6, IL-1 β and TNF- α , along with anti-inflammatory marker IL-10. Absorbance was measured using a standard plate reader set to 450 nm. Concentrations of inflammation markers in (A-D) distal colon / large intestine (Lid) and (E-H) proximal colon / large intestine (Lip) tissue, as well as (I-L) serum were determined via a standard curve, measured by subtracting blank background absorbance and normalizing to mg of protein. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

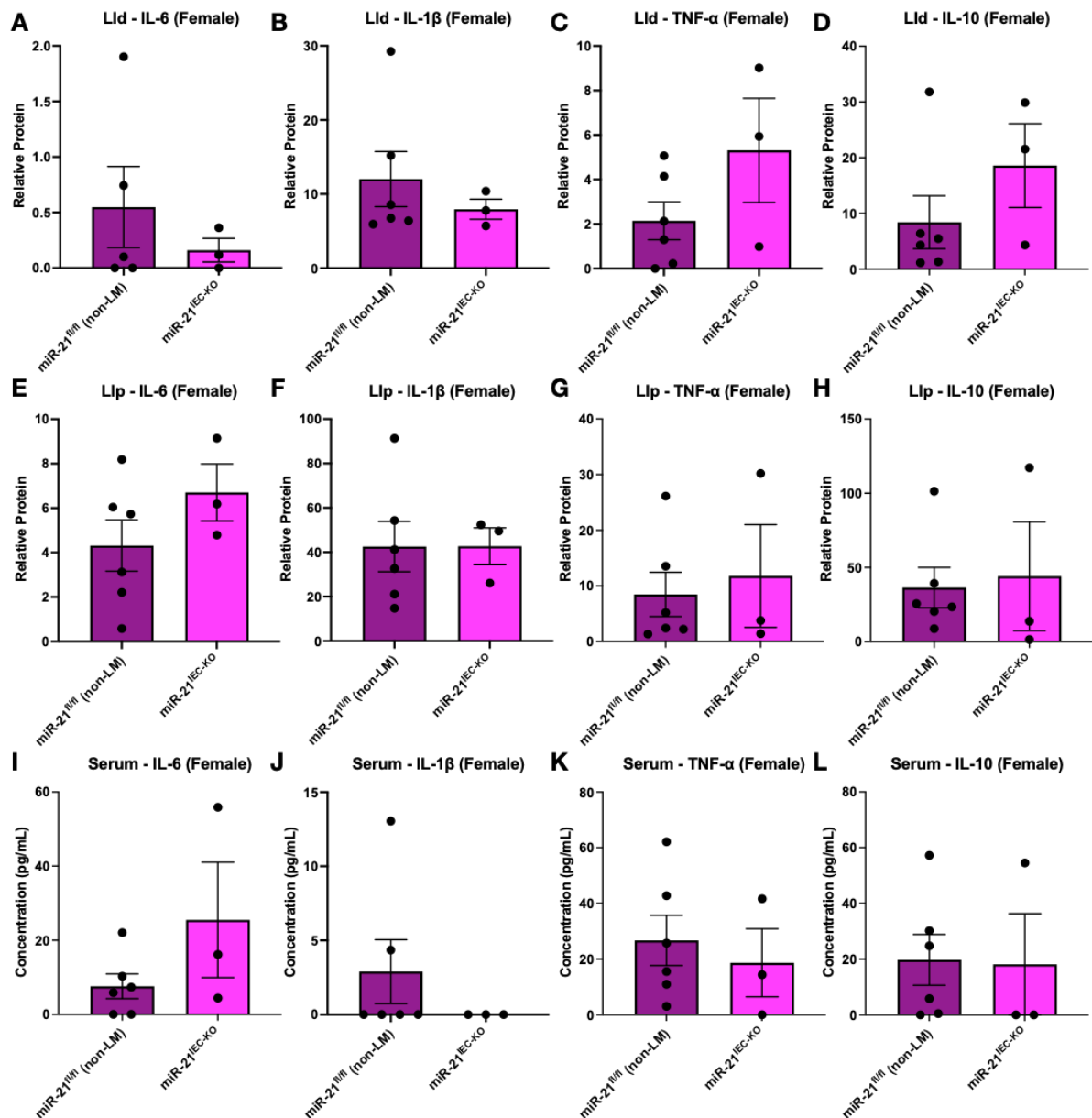


Figure 5.24 – No major differences in levels of pro-inflammatory or anti-inflammatory markers in various samples between non-littermate $\text{miR-21}^{\text{fl/fl}}$ and $\text{miR-21}^{\text{IEC-KO}}$ female mice during DSS-induced colitis. At the end of the trial, non-littermate (non-LM) $\text{miR-21}^{\text{fl/fl}}$ and $\text{miR-21}^{\text{IEC-KO}}$ mouse colon tissue were homogenized in lysis buffer. After centrifugation, cell lysates were transferred to 96-well plates. Blood was also collected at the end of the trial by decapitation. Serum was separated by centrifuging blood samples at 4°C for 12 min at $1000 \times g$ and also transferred to 96-well plates. ELISAs were conducted to detect pro-inflammatory markers IL-6, IL- 1β and TNF- α , along with anti-inflammatory marker IL-10. Absorbance was measured using a standard plate reader set to 450 nm. Concentrations of inflammation markers in (A-D) distal colon / large intestine (Lid) and (E-H) proximal colon / large intestine (Lip) tissue, as well as (I-L) serum were determined via a standard curve, measured by subtracting blank background absorbance and normalizing to mg of protein. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t -test.

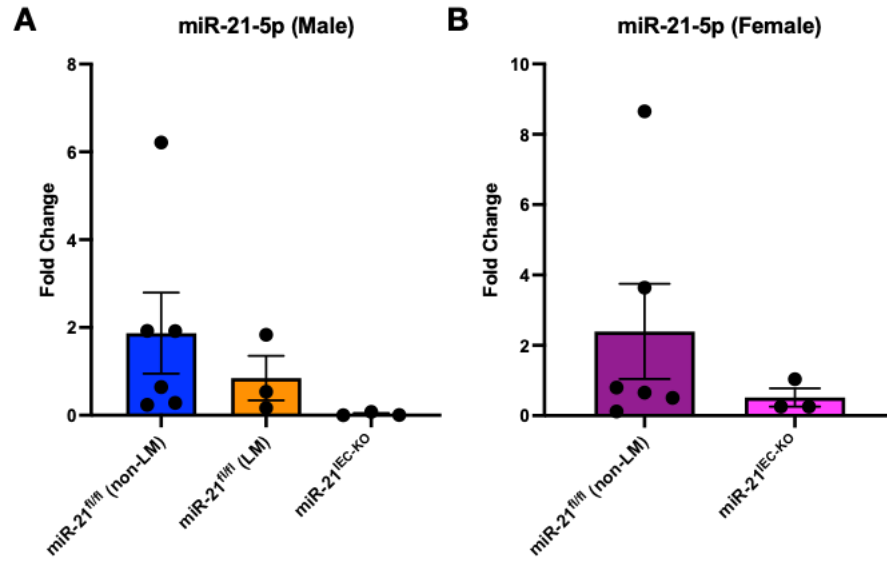


Figure 5.25 – miR-21^{IEC-KO} mice had reduced levels of miR-21-5p in whole colon tissue compared to miR-21^{fl/fl} controls in DSS-induced colitis model. RNA was isolated from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of miR-21-5p in whole colon tissue of (A) male and (B) female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA for male groups and Welch's t -test for female groups.

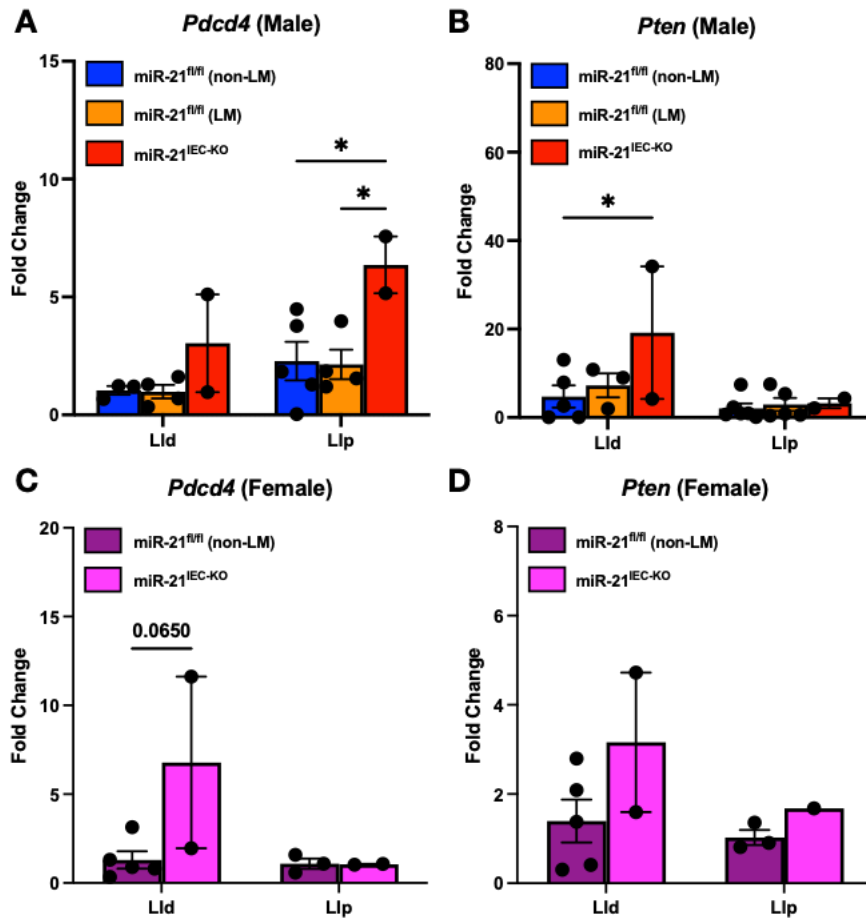


Figure 5.26 – miR-21^{IEC-KO} had elevated levels of known miR-21 targets *Pdc44* and *Pten* in colon tissue compared to miR-21^{fl/fl} controls in DSS-induced colitis model. RNA was isolated from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of *Pdc44* and *Pten* in distal colon / large intestine (Lid) and proximal colon / large intestine (Lip) tissue from (A-B) male and (C-D) female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. Denoted are $n \geq 3$ mice per group with the exception of miR-21^{IEC-KO} mice that have $n = 2$ per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

5.2.9 Adherens junction gene expression in miR-21^{IEC-KO} during DSS-induced colitis

The absence of miR-21 at the intestinal epithelium was further examined with a focus on gene expression of AJ components. In male mice, there were no significant differences in distal or proximal tissue between miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice for *Cdh1* or *Ctnna1* (**Figure 5.27A, 5.27B**), though a significant upregulation in *Ctnnd1* was observed in distal colon tissue for littermate miR-21^{fl/fl} compared to non-littermate miR-21^{fl/fl} (**Figure 5.27C**). Interestingly, there was a downregulation in *Ctnnb1* for miR-21^{IEC-KO} distal tissue compared to littermate miR-21^{fl/fl} mice (**Figure 5.27D**). Similarly in females, there were no significant differences in any AJ gene tested (**Figure 5.27E, 5.27F, 5.27G, 5.27H**), which may be attributed to the genotypic groups sharing similar disease severities during the trial. This suggests absence of miR-21 at the intestinal epithelium may not be sufficient to modify the AJ.

To continue exploring the role of E-cadherin during inflammation in relation to miR-21 expression at the intestinal epithelium, protein levels were assessed in miR-21^{fl/fl} and miR-21^{IEC-KO} mice. E-cadherin levels were examined in distal and proximal colon tissue through immunoblot. For male mice, there were no significant differences in protein abundance between miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice in distal tissue, however there seemed to be a slight reduction in fully intact protein (upper band) levels for miR-21^{IEC-KO} compared to non-littermate and littermate miR-21^{fl/fl} mice resulting in a reduced upper/lower band ratio (**Figure 5.28A, 5.28B, 5.28C, 5.28D**). In proximal tissue, there were no significant differences in E-cadherin levels (**Figure 5.28F, 5.28G, 5.28H, 5.28I, 5.28J**). This suggests that in male distal tissue, there may be less intact E-cadherin in the absence of miR-21 at the distal colon epithelium. For female mice, despite having a similar disease severity during the trial, there were higher levels of E-cadherin in distal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice, particularly in the levels of fully intact (upper band) protein (**Figure 5.29A, 5.29B, 5.29C, 5.29D**), resulting in a significantly higher upper/lower band ratio (**Figure 5.29E**). In proximal tissue, though not significantly different, there was a trend of increased levels in miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice, similar to distal tissue (**Figure 5.29F, 5.29G, 5.29H, 5.29I**). However, there were no notable differences in the upper/lower band ratio, suggesting that the amount of intact-to-fragmented E-cadherin were similar in both groups (**Figure 5.29J**). These findings in female mice, in contrast to males, demonstrates potential sex-specific differences in the

fate of E-cadherin regarding its regulation by miR-21 in the gut, potentially leading to a protective phenotype over time in the DSS-induced colitis mouse model.

In previous trials, the localization of E-cadherin in DSS tissue was observed to change between WT and miR-21^{-/-} male mice. To continue this analysis, E-cadherin was visualized through immunofluorescence to assess localization and abundance in distal and proximal tissue of miR-21^{fl/fl} groups and miR-21^{IEC-KO} male mice. As seen in **Figure 5.30A**, E-cadherin was evenly spread across the epithelial barrier, with more concentrated levels towards the top of intestinal crypts for both miR-21^{fl/fl} groups and miR-21^{IEC-KO} mice. There was a slight reduction in fluorescence for both littermate miR-21^{fl/fl} and miR-21^{IEC-KO} compared to non-littermate miR-21^{fl/fl} mice (**Figure 5.30B**), suggesting overall abundance of E-cadherin in the distal colon may not necessarily benefit during inflammation. In proximal tissue, there were no differences in E-cadherin localization or abundance between groups, as E-cadherin was evenly concentrated along the crypt axes (**Figure 5.31A, 5.31B**).

If E-cadherin and AJ components were not notably different between non-littermate miR-21^{fl/fl} compared with littermate miR-21^{fl/fl} and miR-21^{IEC-KO} mice at the RNA level, perhaps other adjacent pathways led to differences in disease severity during DSS-induced colitis. Gene expression levels of wound healing proponents were measured in distal and proximal tissue of male and female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. There were no significant differences observed in the expression of *Fllr* among male groups (**Figure 5.32A**). Among the TGF- β receptors, only *Tgfbri* was significantly upregulated in miR-21^{IEC-KO} compared to both non-littermate and littermate miR-21^{fl/fl} groups (**Figure 5.32B, 5.32C, 5.32D**). Similarly in female mice, there was only significant upregulation in *Tgfbri* within miR-21^{IEC-KO} distal tissue compared to miR-21^{fl/fl} mice (**Figure 5.32E, 5.32F, 5.32G, 5.32H**). There were no notable differences observed in proximal tissue for any wound healing genes tested. This suggests that, as DSS targets intestinal damage and inflammation to the distal colon, the TGF- β pathway may be activated through the specific induction of *Tgfbri*. Furthermore, levels of TGF- β were assessed through ELISA in both distal and proximal tissue, as well as serum of miR-21^{fl/fl} and miR-21^{IEC-KO} mice. For male mice, there were no significant differences in the levels of TGF- β detected in distal, proximal tissue or serum, though there were very low levels detected in tissue to make an accurate assessment (**Figure 5.33A, 5.33B, 5.33C**). For female mice, there was a trend of increased TGF- β levels for proximal tissue and serum in miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice, with similarly very low levels detected in distal tissue (**Figure 5.33D, 5.33E, 5.33F**).

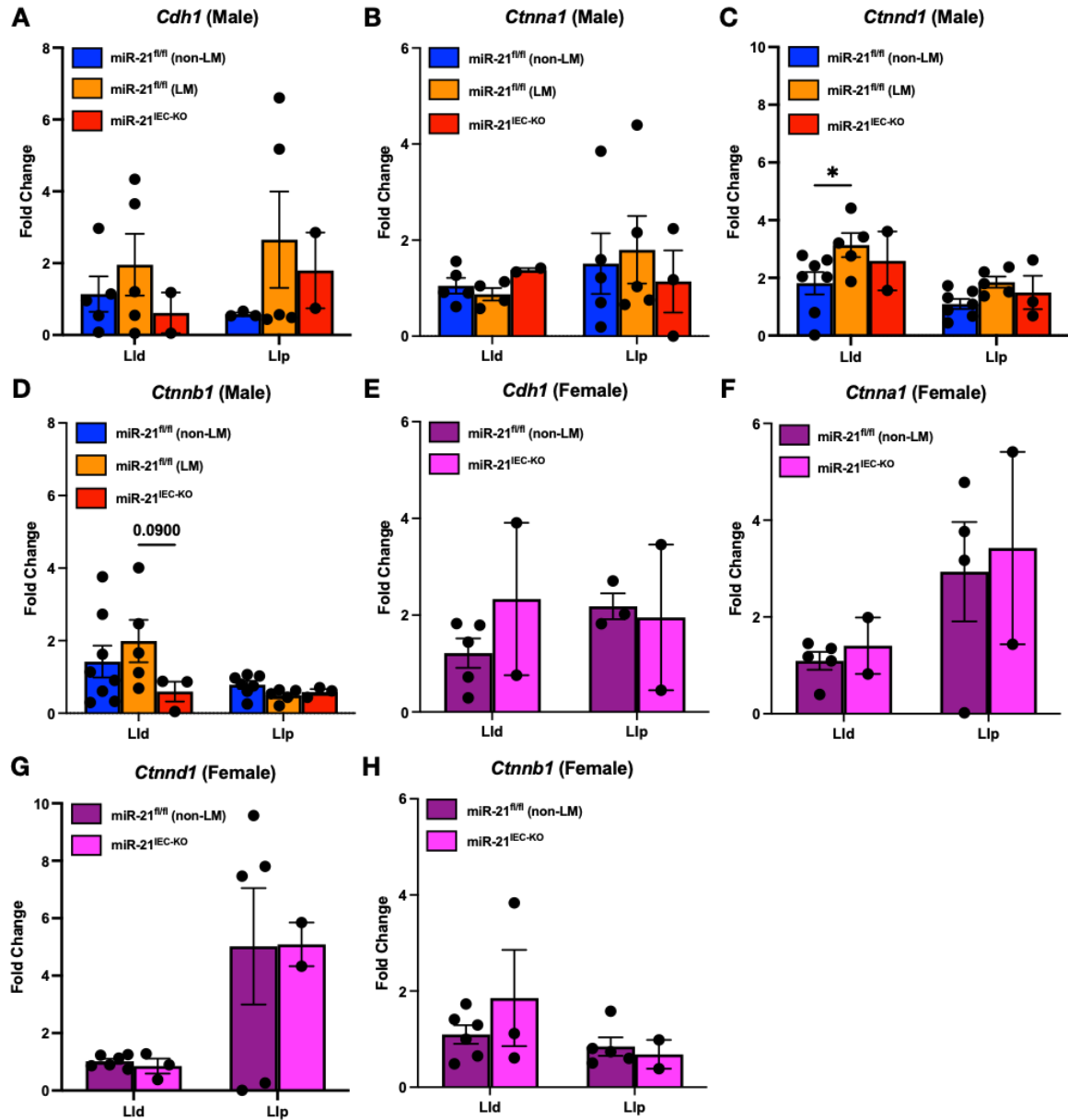


Figure 5.27 – No major differences in adherens junction gene expression between miR-21^{fl/fl} and miR-21^{IEC-KO} mice in DSS-induced colitis model. RNA was isolated from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of AJ genes *Cdhl*, *Ctnna1*, *Ctnnd1* and *Ctnnb1* in distal colon / large intestine (Lid) and proximal colon / large intestine (Lip) tissue from (A-D) male and (E-H) female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. Denoted are $n \geq 3$ mice per group with the exception of male *Cdhl* miR-21^{IEC-KO} qPCRs and all female miR-21^{IEC-KO} qPCRs that have $n = 2$ per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

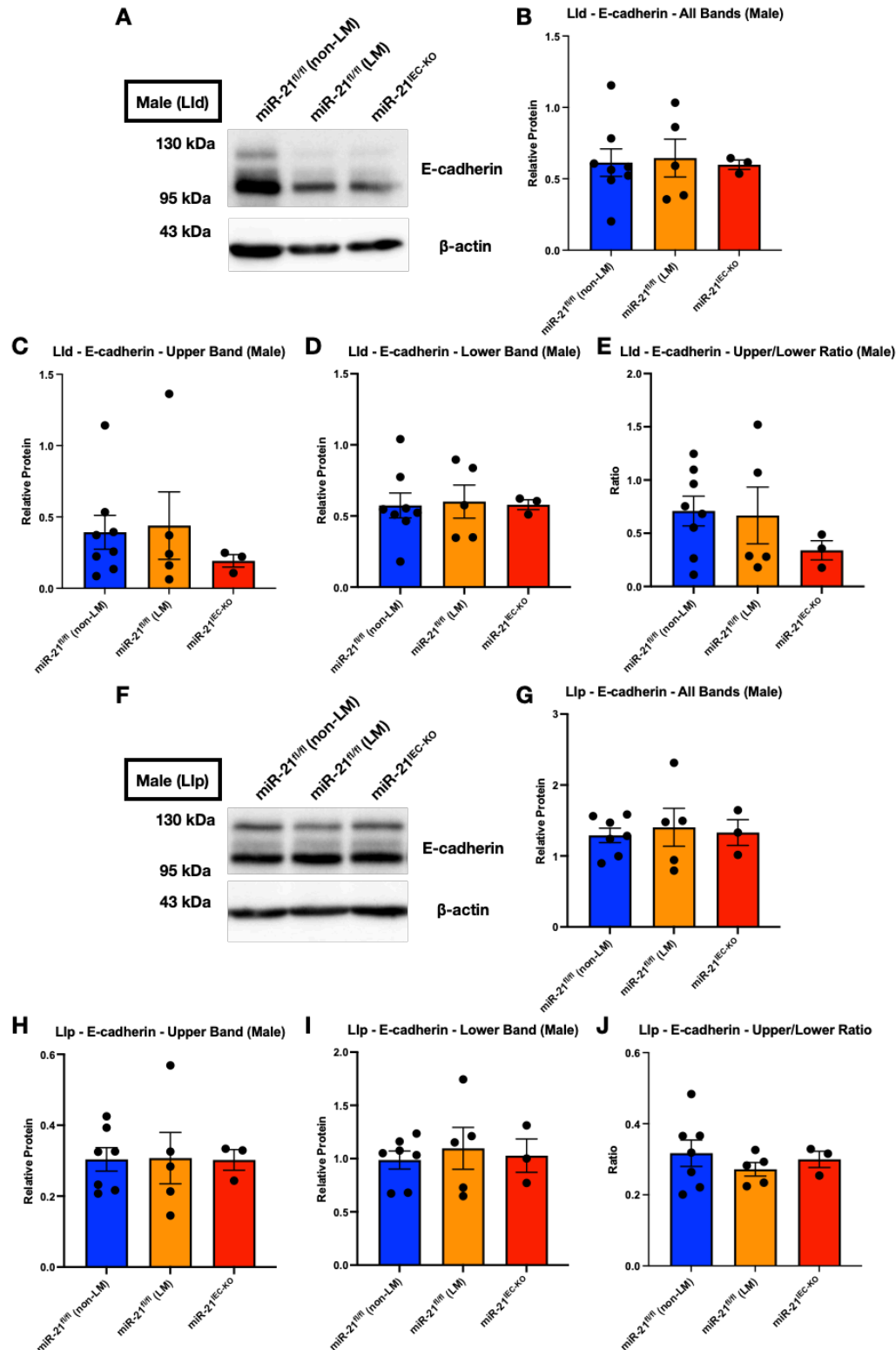


Figure 5.28 – Analysis of E-cadherin levels shows potential differences in size fragments between male distal, but not proximal, tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups in DSS-induced colitis model. Protein lysates were obtained from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} male mice given 2% DSS (w/v) water *ad libitum* for six days. Immunoblot for E-cadherin from (A) distal colon / large intestine (Lid) and (F) proximal colon / large intestine (Lip) tissue of miR-21^{fl/fl} and miR-21^{IEC-KO} male mice. Protein quantification of immunoblots combining (B + G) both bands, (C + H) upper band only, (D + I) lower band only and (E + J) upper/lower band fragmentation ratio. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

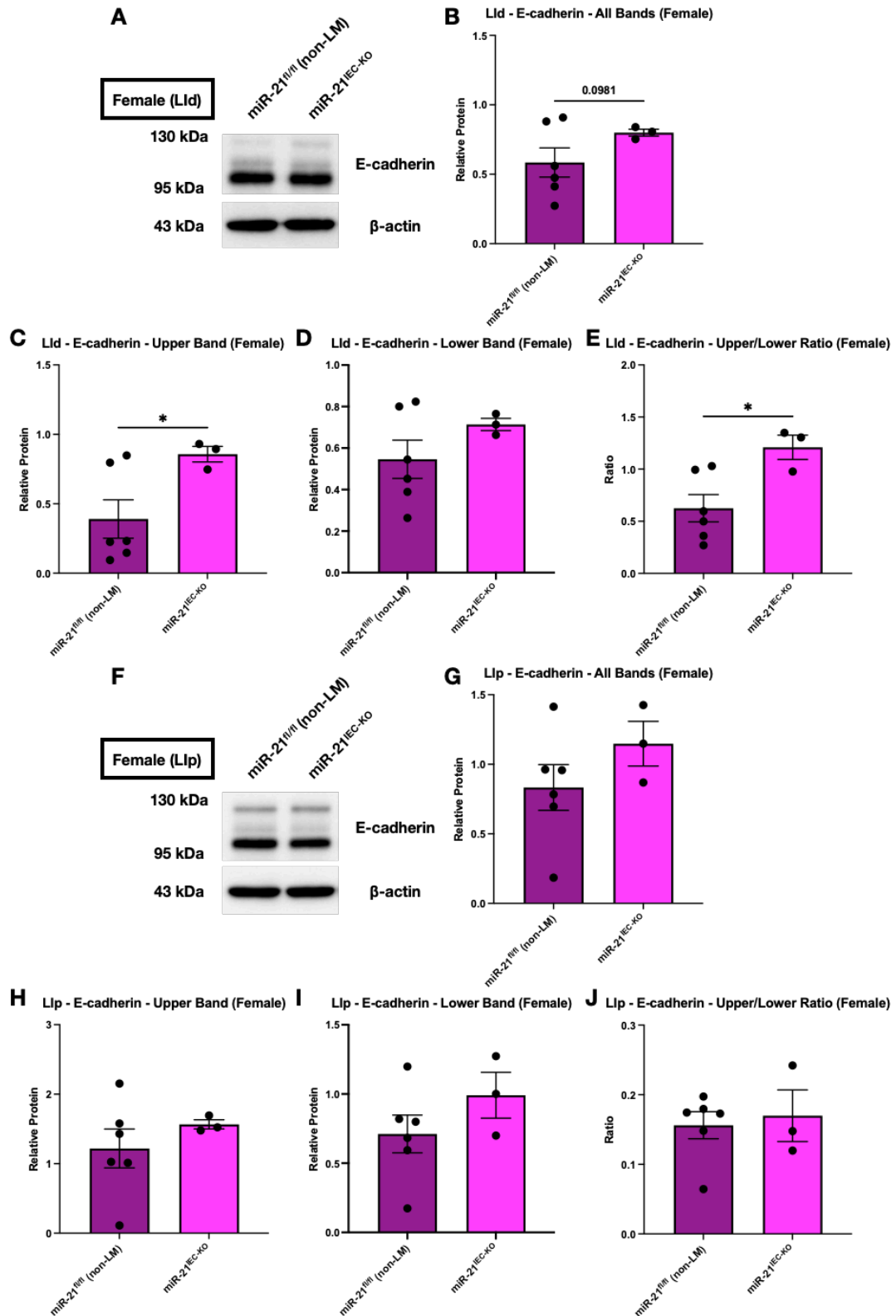


Figure 5.29 – Analysis of E-cadherin levels shows induced levels in colon tissue between female miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups in DSS-induced colitis model. Protein lysates were obtained from 8-15 week old non-littermate (non-LM) miR-21^{fl/fl} and miR-21^{IEC-KO} female mice given 2% DSS (w/v) water *ad libitum* for six days. Immunoblot for E-cadherin from (A) distal colon / large intestine (LId) and (F) proximal colon / large intestine (LIp) tissue of miR-21^{fl/fl} and miR-21^{IEC-KO} female mice. Protein quantification of immunoblots combining (B + G) both bands, (C + H) upper band only, (D + I) lower band only and (E + J) upper/lower band fragmentation ratio. Denoted are n ≥ 3 mice per group, mean values ± SEM, p value calculations using Welch's t-test with * - p ≤ 0.05.

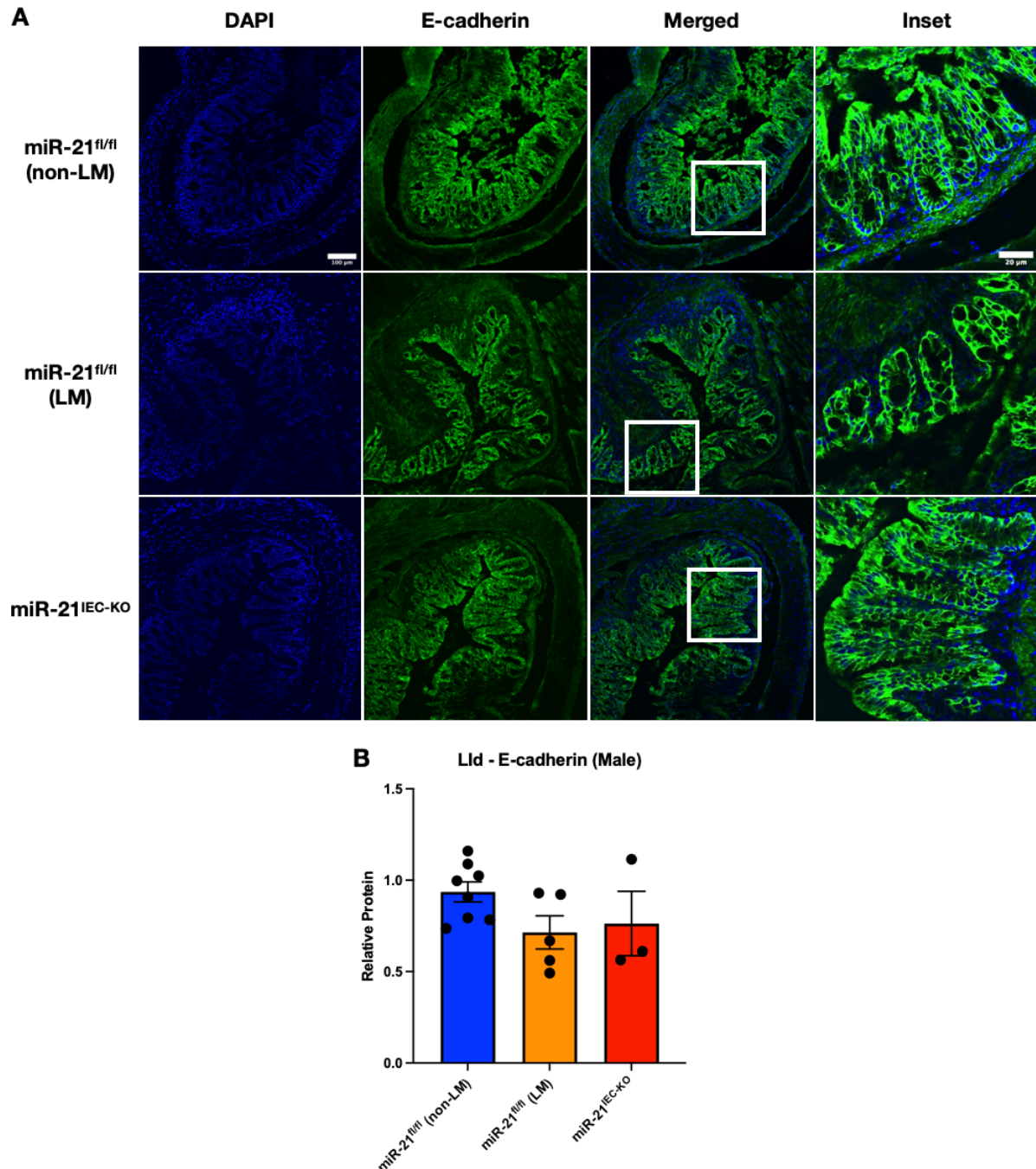


Figure 5.30 – E-cadherin localization and abundance are similar at the top of intestinal crypts between miR-21^{fl/fl} groups and miR-21^{IEC-KO} male distal colon tissue in DSS-induced colitis model. (A) Distal colon / large intestine (LId) sections of miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} male mice were removed, flushed with sterile PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (B) Quantification of fluorescence normalized to number of nuclei. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

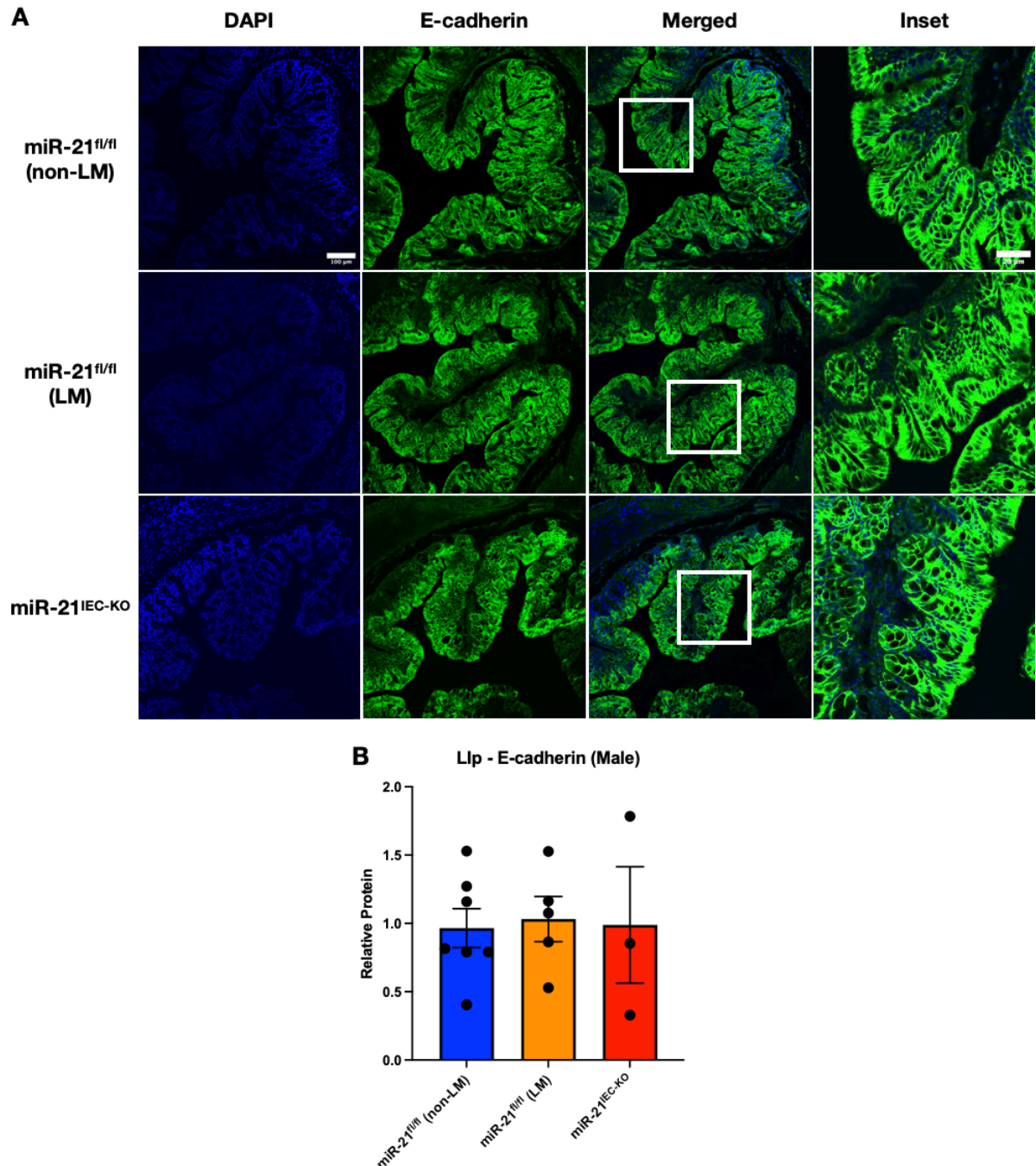


Figure 5.31 – E-cadherin localization and abundance are similarly evenly distributed along the gut epithelium between miR-21^{fl/fl} groups and miR-21^{IEC-KO} male proximal colon tissue in DSS-induced colitis model. (A) Proximal colon / large intestine (Llp) sections of miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} male mice were removed, flushed with sterile PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-E-cadherin primary antibody (green). (B) Quantification of fluorescence normalized to number of nuclei. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

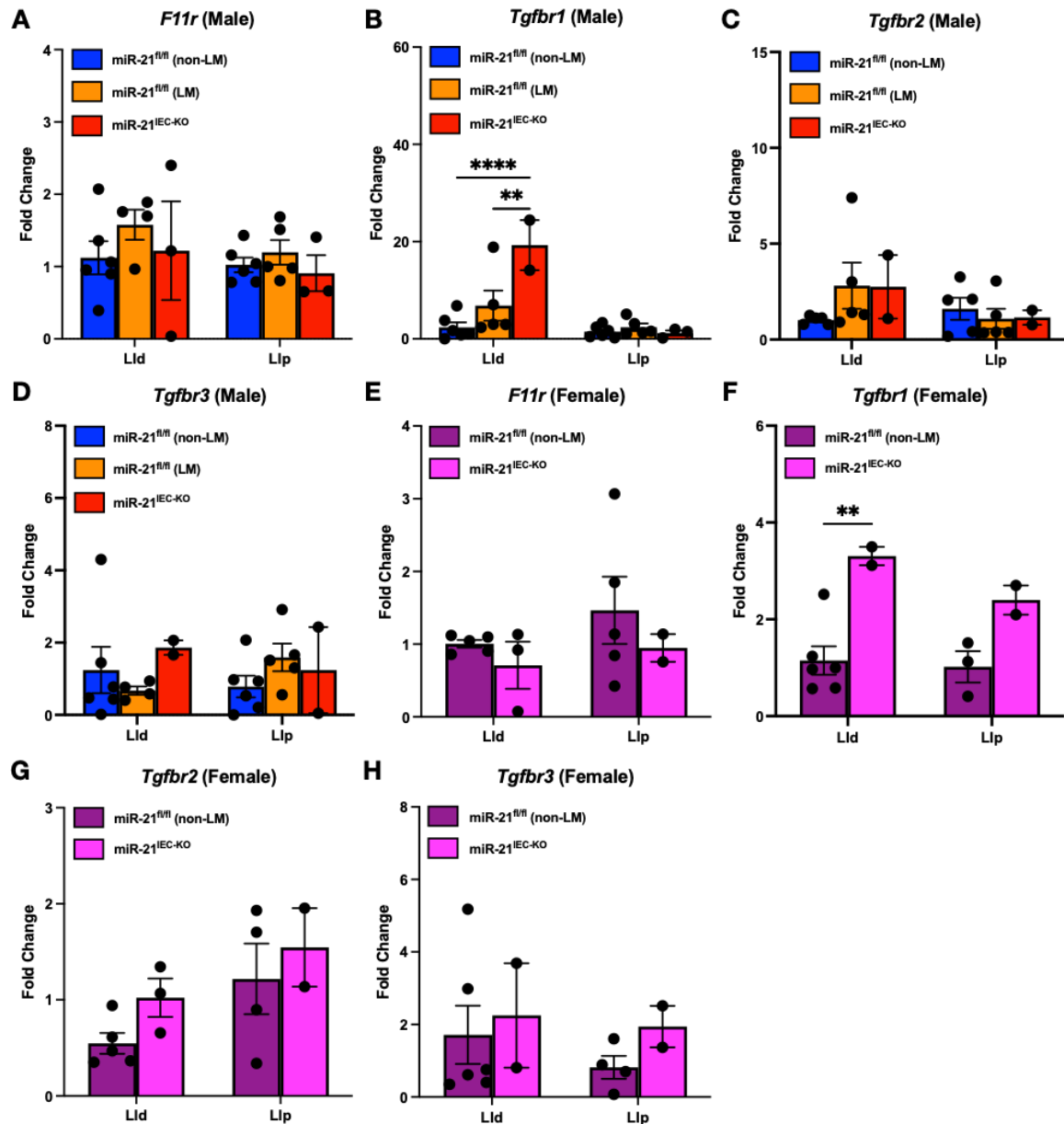


Figure 5.32 – *Tgfb1* was induced in miR-21^{IEC-KO} mice in DSS-induced colitis model. RNA was isolated from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of wound healing genes *F11r*, *Tgfb1*, *Tgfb2* and *Tgfb3* in distal colon / large intestine (Lid) and proximal colon / large intestine (Lip) tissue from (A-D) male and (E-H) female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. Denoted are n ≥ 3 mice per group with the exception of miR-21^{IEC-KO} qPCRs that have n = 2 mice per group, mean values ± SEM, *p* value calculations using two-way ANOVA with ** - *p* ≤ 0.01, **** - *p* ≤ 0.0001.

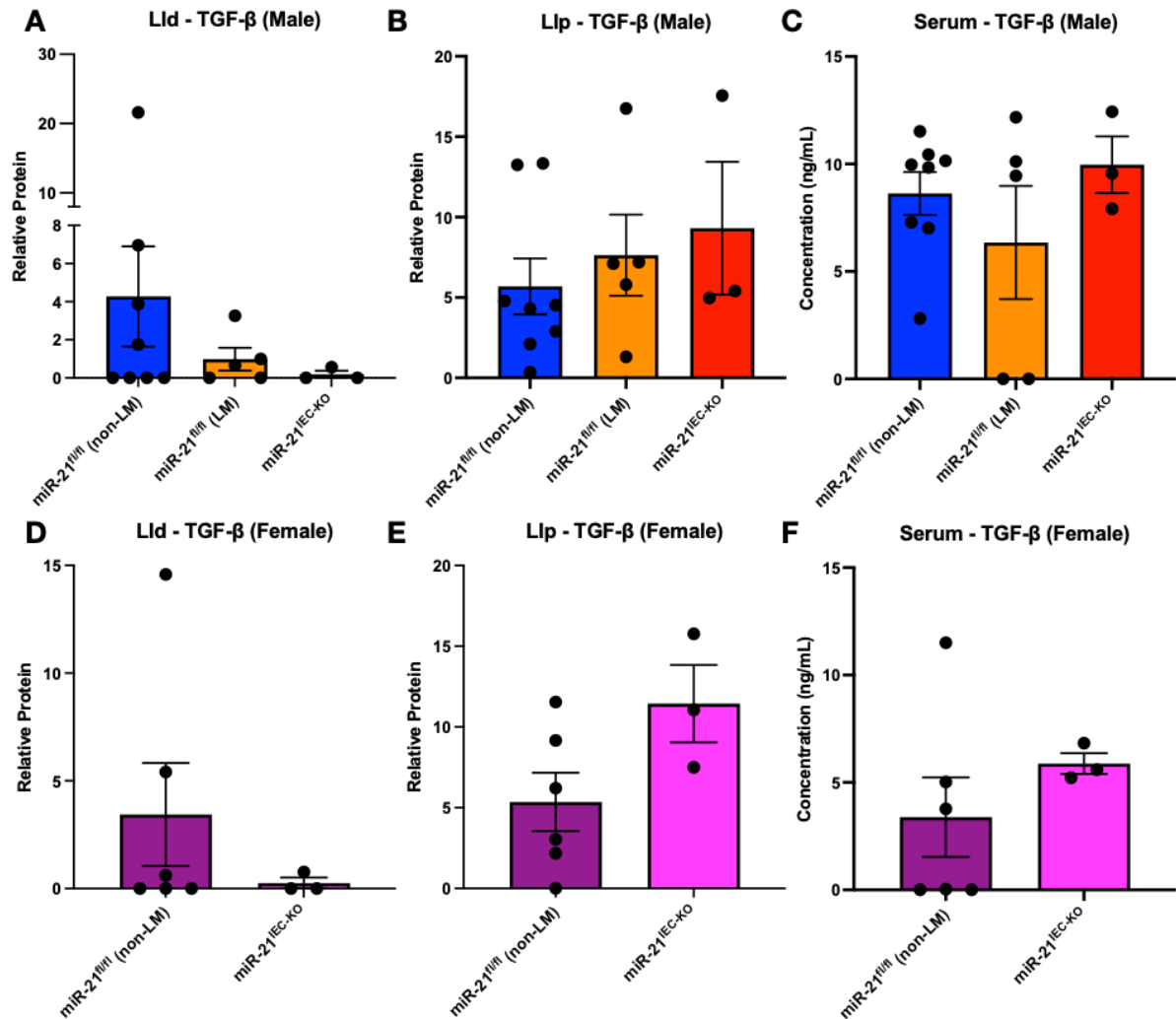


Figure 5.33 – miR-21^{IEC-KO} female mice have slightly elevated levels of TGF-β detected in proximal tissue and serum during DSS-induced colitis. At the end of the trial, miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mouse tissue were homogenized in lysis buffer. After centrifugation, cell lysates were transferred to 96-well plates. Blood was also collected at the end of the trial by decapitation. Serum was separated by centrifuging blood samples at 4°C for 12 min at 1000 x g and also transferred to 96-well plates. ELISAs were conducted to detect TGF-β cytokine levels. Absorbance was measured using a standard plate reader set to 450 nm. Concentrations in distal colon / large intestine (Lid) and proximal colon / large intestine (Lip) tissue, as well as serum in (A-C) male and (D-F) female mice were determined via a standard curve, measured by subtracting blank background absorbance and normalizing to mg of protein. Denoted are n ≥ 3 mice per group, mean values ± SEM, *p* value calculations using one-way ANOVA for male groups and Welch's *t*-test for female groups.

5.2.10 Tight junction and desmosome-related gene expression in miR-21^{IEC-KO} during DSS-induced colitis

TJs can also play an important role during intestinal inflammation as a reduction in protein expression has been associated with IBD severity.²⁷ In earlier chapters using miR-21^{-/-} for DSS-induced colitis, there were higher mRNA levels of occludin in distal tissue of miR-21^{-/-} compared to WT mice, along with several barrier-forming claudins such as *Cldn3*, *Cldn4*, *Cldn7* and *Cldn15*. Here, TJ genes were assessed in DSS tissue of male and female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. For males, there were elevated levels of *Ocln* in distal tissue of miR-21^{IEC-KO} mice compared to miR-21^{fl/fl} groups (**Figure 5.34A**). Other TJ genes that showed notable differential expression were pore-forming *Cldn2* which was elevated in miR-21^{IEC-KO} distal tissue compared to miR-21^{fl/fl} groups (**Figure 5.34B**), which could be a response to disease progression. Interestingly, despite having an induced trend in DSS-treated miR-21^{-/-} distal tissue, *Cldn4* seemed to have a slight reduction in levels in miR-21^{IEC-KO} distal tissue compared to miR-21^{fl/fl} groups (**Figure 5.34D**). There were no major differences in other claudin or ZO genes tested in either colon region, though more replicates may be required to properly assess their expression (**Figure 5.34C, 5.34E, 5.34F, 5.34G, 5.34H**). For female mice, in contrast to what was observed in males, there was a noticeable reduction in *Ocln* mRNA for both distal and proximal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice (**Figure 5.35A**). In distal tissue, *Cldn4* showed a reduced trend in miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice (**Figure 5.35D**), along with *Tjp3* (**Figure 5.35H**). For proximal tissue, *Cldn3* and *Cldn7* also had a reduced trend with miR-21^{IEC-KO} mice (**Figure 5.35C, 5.35E**). There were no major differences in TJ gene expression observed for *Cldn2*, *Cldn15* (**Figure 5.35B, 5.35F**), and only *Tjp2* showed an induced trend in distal tissue for miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice (**Group 5.35G**). These differences in expression of TJ components between miR-21^{IEC-KO} and previously shown miR-21^{-/-} mice demonstrates potential separate roles for the miRNAs depending on cell origin.

In addition to TJs, desmosomes also play a crucial role in gut barrier integrity, serving as both strong connection points between epithelial cells and anchoring IECs to the basal membrane.¹⁵² Intestinal desmosomes such as desmocollin-2 (encoded by *Dsc2*) and desmoglein-2 (encoded by *Dsg2*) were measured in DSS tissue of miR-21^{fl/fl} and miR-21^{IEC-KO} mice. In male mice, there were no observable differences for *Dsc2* between mouse groups (**Figure 5.36A**). However, in proximal tissue, there was an upregulation of *Dsg2* in

miR-21^{IEC-KO} compared to non-littermate and littermate miR-21^{fl/fl} mice (**Figure 5.36B**), suggesting a tighter gut epithelial barrier. For females, again there were no differences observed for *Dsc2* mRNA between mouse groups in either colon region (**Figure 5.36C**). Interestingly, there were notably lower levels of *Dsg2* expression in proximal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice (**Figure 5.36D**), which serves as another example of sex-dependent differential expression impacted by miR-21 in gut epithelia.

To further investigate TJs at the molecular level after removal of miR-21 from the intestinal epithelium, protein abundance of integral TJ protein occludin was examined through immunoblot. Distal and proximal colon tissue from male and female miR-21^{fl/fl} and miR-21^{IEC-KO} mice was used. In male mice, occludin was observed fragmented in both colon tissue samples of all mouse groups (**Figure 5.37A, 5.37F**). This occurs frequently for intercellular junction proteins alike, and in the case of TJ proteins like occludin, proteolytic cleavage by matrix metalloproteinases negatively impacts the epithelial barrier by increase permeability.³²³ Within distal tissue, both miR-21^{IEC-KO} and littermate miR-21^{fl/fl} mice had slightly elevated levels of overall occludin compared to non-littermate miR-21^{fl/fl} mice (**Figure 5.37B, 5.37C, 5.37D**), and also had higher intact/fragments ratios (**Figure 5.37E**), suggesting TJ greater stability which coincides with disease severity observed during DSS-induced colitis. In contrast, within proximal tissue, this phenotype seemed to be reversed. Both miR-21^{IEC-KO} and littermate miR-21^{fl/fl} mice had notably reduced levels of intact and fragmented occludin compared to non-littermate miR-21^{fl/fl} mice (**Figure 5.37G, 5.37H, 5.37I**), while also had slightly lower intact/fragments ratio (**Figure 5.37J**). Regional specificity of occludin expression and fragmentation has not been fully documented in IBD and may pose a novel role for miR-21 regarding intercellular junction functionality at the gut epithelial barrier. For female mice, the same fragmentation was also observed in both distal and proximal tissue for both groups (**Figure 5.38A, 5.38F**). miR-21^{IEC-KO} showed elevated levels of fragmented occludin in distal tissue compared to miR-21^{fl/fl} mice, but had similar levels of fully intact protein, while nonetheless had no notable differences in the intact/fragments ratio (**Figure 5.38B, 5.38C, 5.38D, 5.38E**). Moreover, these results were similar to measured occludin in proximal tissue (**Figure 5.38G, 5.38H, 5.38I, 5.38J**). These results suggest that fragmentation could be a marker of disease symptoms in mice during DSS-induced colitis.

Next, protein expression of claudin-4 was investigated to further explore its reduction in gene expression observed in distal tissue of DSS-treated miR-21^{IEC-KO} mice, Here, there was a focus on male tissue to associate differences in disease severity between

non-littermate miR-21^{fl/fl} compared to littermate miR-21^{fl/fl} and miR-21^{IEC-KO} mice at the molecular level. Protein localization and abundance for claudin-4 was assessed through immunofluorescence in both distal and proximal colon tissue. In distal tissue, there was more localized protein around the top of intestinal crypts in miR-21^{IEC-KO} samples, as opposed to an even distribution of claudin-4 along the crypt axis as seen in both non-littermate and littermate miR-21^{fl/fl} groups (**Figure 5.39A**). However, there were no significant differences in claudin-4 abundance among mouse groups (**Figure 5.39B**). In proximal tissue, the differences in localization were lost, or at least not as apparent, which showed an even distribution along the crypt axis in all mouse groups (**Figure 5.40A**). There were no differences in claudin-4 abundance between mouse groups (**Figure 5.40B**), which may be attributed to the longer crypts observed in histological samples. Therefore, similar to AJ proteins like E-cadherin, localization of TJ proteins may play an important factor in barrier integrity during intestinal inflammation.

The consequence of claudin-4 expression and localization due to the presence or absence of miR-21 at the gut epithelium remains an important focus of this research. Since it is known that claudin-4 negatively associates with Arf4, a protein that regulates Golgi functioning for protein trafficking, expression of Golgi-related genes previously studied were assessed in distal and proximal colon tissue of miR-21^{fl/fl} and miR-21^{IEC-KO} mice. In male mice, there were no major differences in mRNA levels for *Gbfl* in either colon region between mouse groups, however there were promising results of a reduction in *Arf4* in distal tissue of miR-21^{IEC-KO} mice compared to both non-littermate and littermate miR-21^{fl/fl} mice (**Figure 5.41A, 5.41B**). More replicates will be required to more accurately assess this difference. In female mice, there were also no notable differences in any Golgi-related genes tested between mouse groups (**Figure 5.41C, 5.41D**). Again, more replicates will be necessary to fully conclude whether epithelial miR-21 plays a role in Golgi functioning during intestinal inflammation.

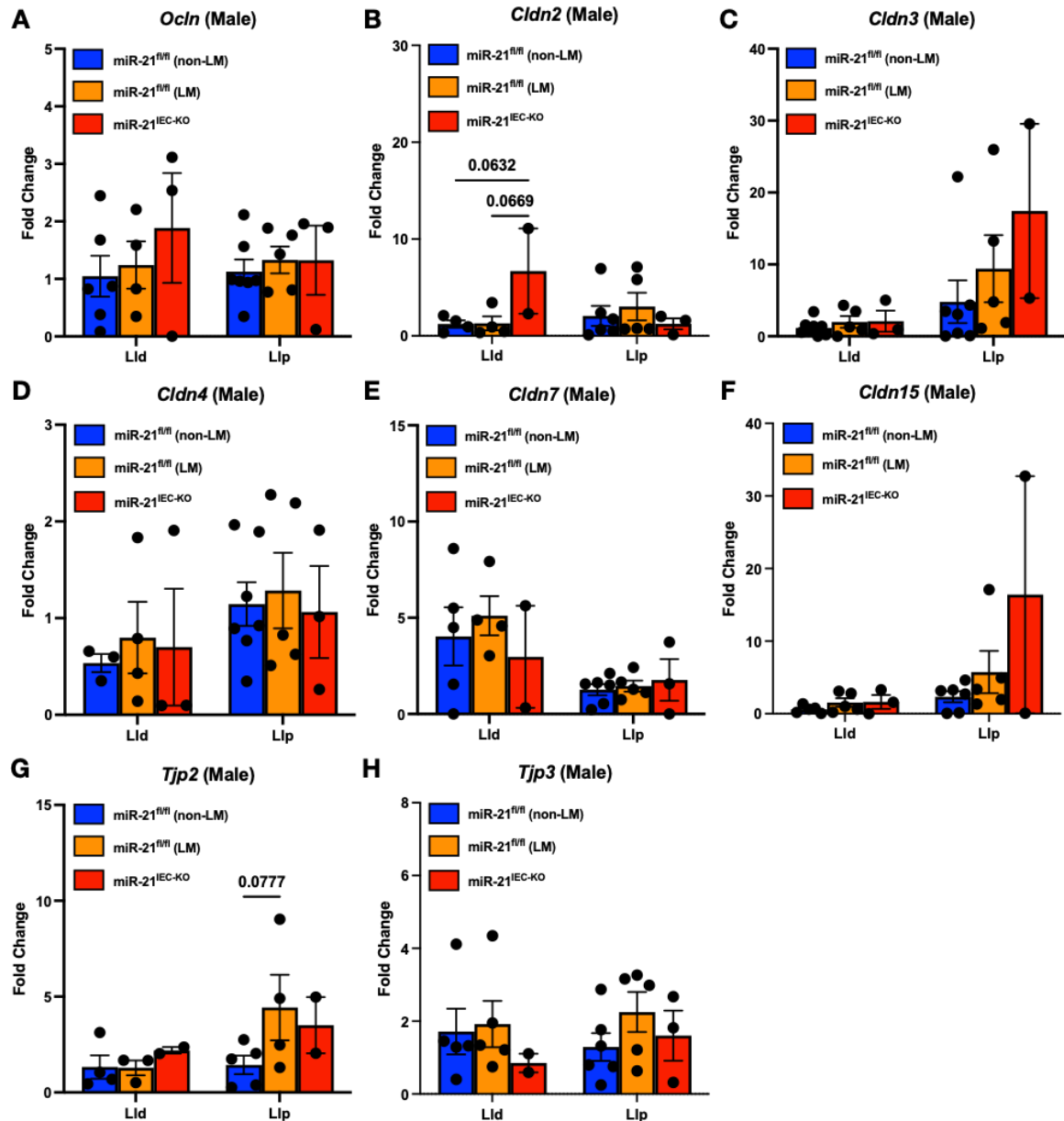


Figure 5.34 – Some tight junction genes were differentially expressed in miR-21^{IEC-KO} compared to miR-21^{fl/fl} male mice in DSS-induced colitis model. RNA was isolated from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} male mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of TJ genes *Ocln*, *Cldn2*, *Cldn3*, *Cldn4*, *Cldn7*, *Cldn15*, *Tjp2* and *Tjp3* in distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue from miR-21^{fl/fl} and miR-21^{IEC-KO} male mice. Denoted are $n \geq 2$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA.

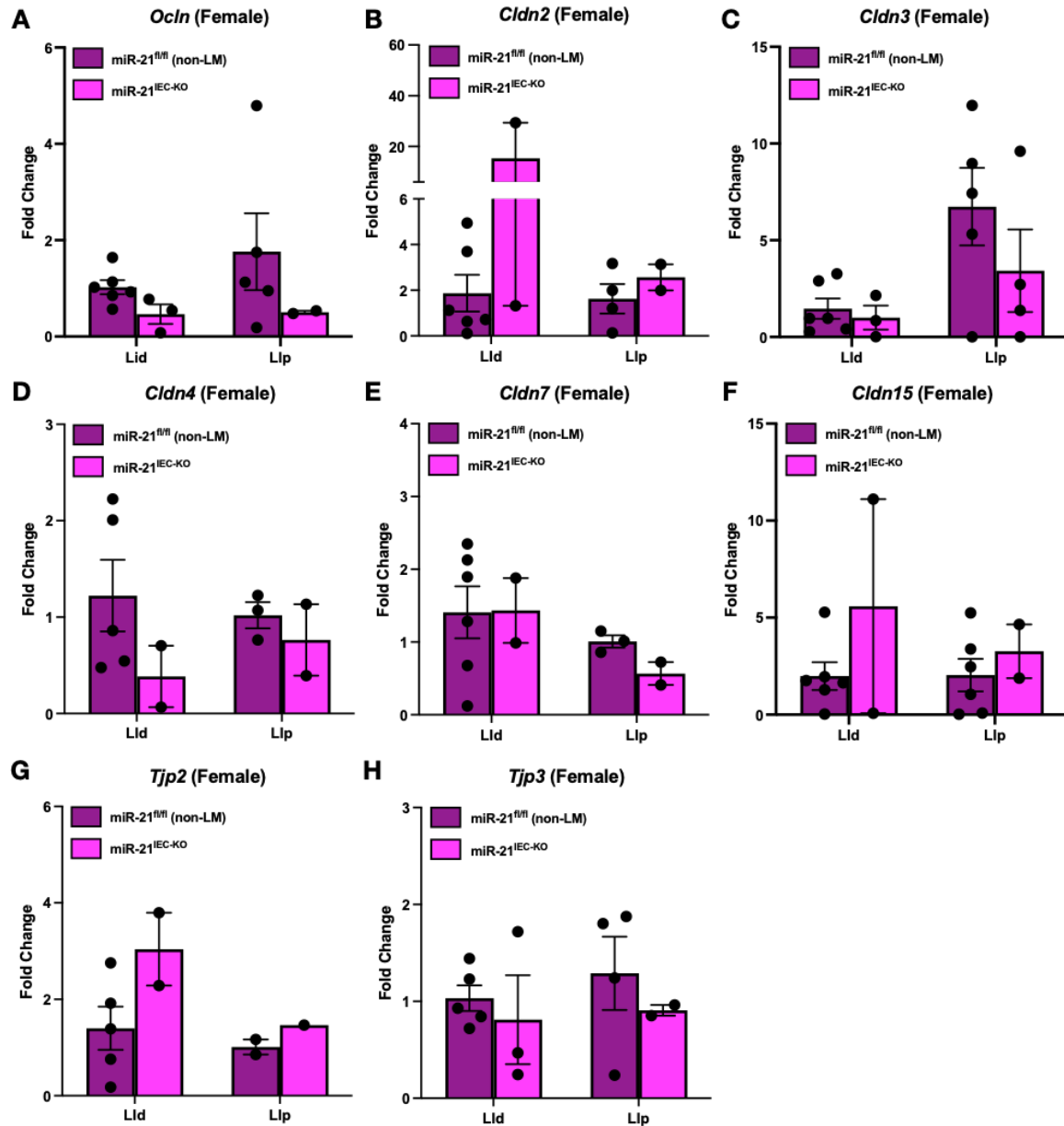


Figure 5.35 – Some tight junction genes were differentially expressed in miR-21^{IEC-KO} compared to miR-21^{fl/fl} female mice in DSS-induced colitis model. RNA was isolated from 8-15 week old non-littermate (non-LM) miR-21^{fl/fl} and miR-21^{IEC-KO} female mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of TJ genes *Ocln*, *Cldn2*, *Cldn3*, *Cldn4*, *Cldn7*, *Cldn15*, *Tjp2* and *Tjp3* in distal colon / large intestine (LId) and proximal colon / large intestine (Llp) tissue from miR-21^{fl/fl} and miR-21^{IEC-KO} female mice. Denoted are $n \geq 2$ mice per group with the exception of *Tjp2* – LId miR-21^{IEC-KO} qPCRs that have $n = 1$ mouse per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA.

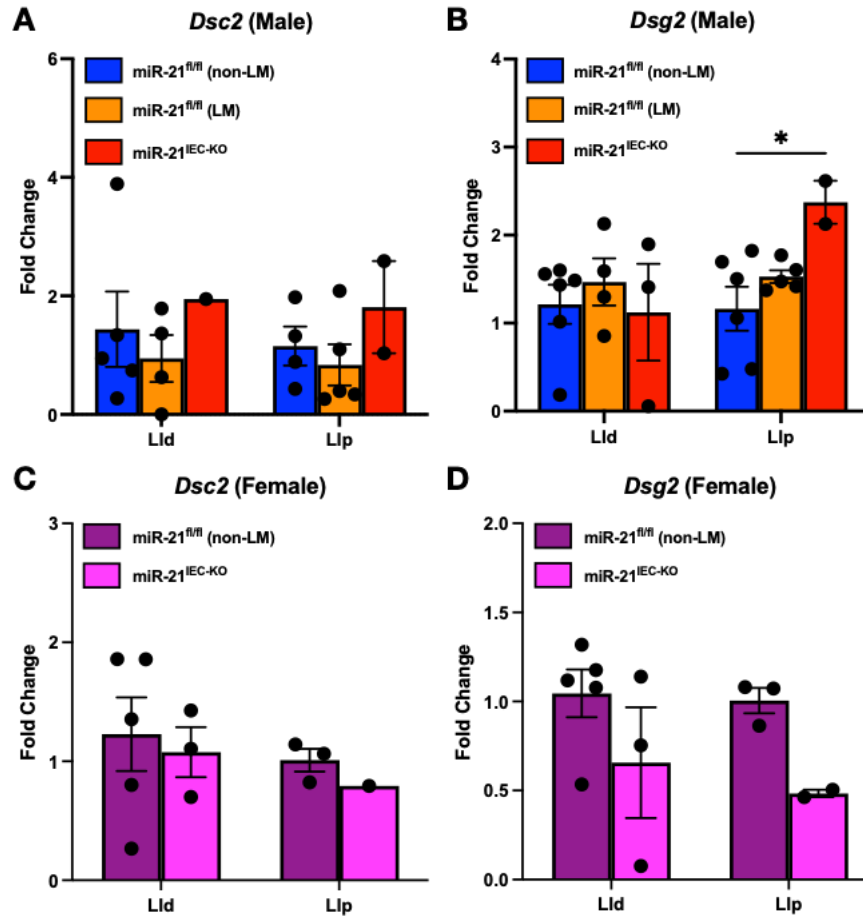


Figure 5.36 – Desmoglein-2 was differentially expressed in proximal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups in DSS-induced colitis model. RNA was isolated from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of desmosome genes *Dsc2* and *Dsg2* in distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue from (A + B) male and (C + D) female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. Denoted are $n \geq 2$ mice per group with the exception of *Dsc2* – LId miR-21^{IEC-KO} and *Dsc2* – LIp female miR-21^{IEC-KO} qPCRs that have $n = 1$ mouse per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

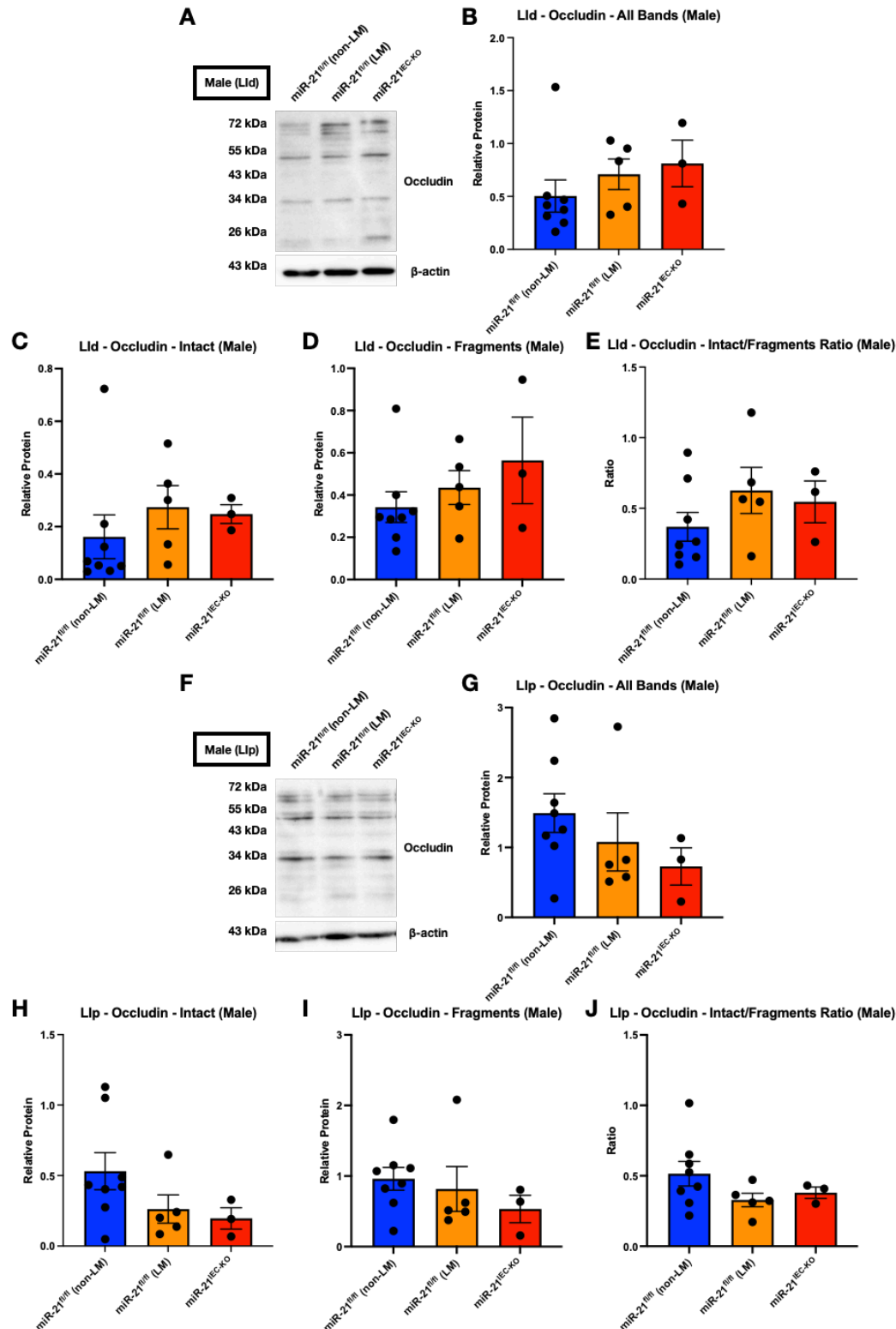


Figure 5.37 – Analysis of occludin levels shows mild induction in male distal tissue, but not proximal tissue of miR-21^{IEC-KO} and littermate miR-21^{fl/fl} mice compared to non-littermate miR-21^{fl/fl} mice in DSS-induced colitis model. Protein lysates were obtained from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} male mice given 2% DSS (w/v) water *ad libitum* for six days. Immunoblot for occludin from (A) distal colon / large intestine (Lid) and (F) proximal colon / large intestine (Lip) tissue of miR-21^{fl/fl} and miR-21^{IEC-KO} male mice. Protein quantification of immunoblots combining (B + G) all bands, (C + H) intact protein, (D + I) fragments and (E + J) intact/fragments ratio. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

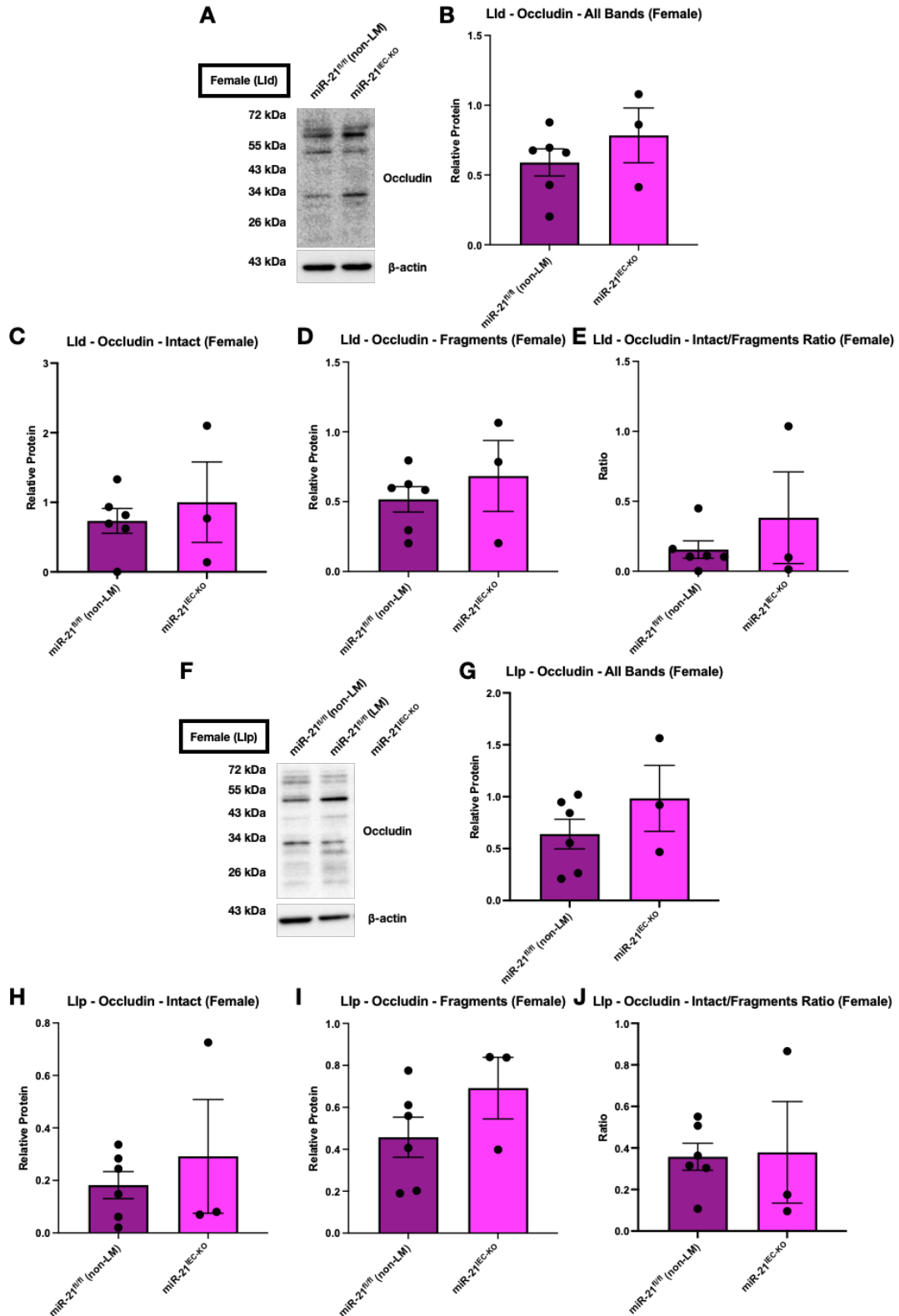


Figure 5.38 – Analysis of occludin levels shows fragmentation but slightly elevated levels in female colon tissue of miR-21^{IEC-KO} in DSS-induced colitis model. Protein lysates were obtained from 8-15 week old non-littermate (non-LM) miR-21^{fl/fl} and miR-21^{IEC-KO} female mice given 2% DSS (w/v) water *ad libitum* for six days. Immunoblot for occludin of protein lysates obtained from (A) distal colon / large intestine (LId) and (F) proximal colon / large intestine (Lip) tissue of miR-21^{fl/fl} and miR-21^{IEC-KO} female mice. Protein quantification of immunoblots combining (B + G) all bands, (C + H) intact protein, (D + I) fragments and (E + J) intact/fragments ratio. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using Welch's t-test.

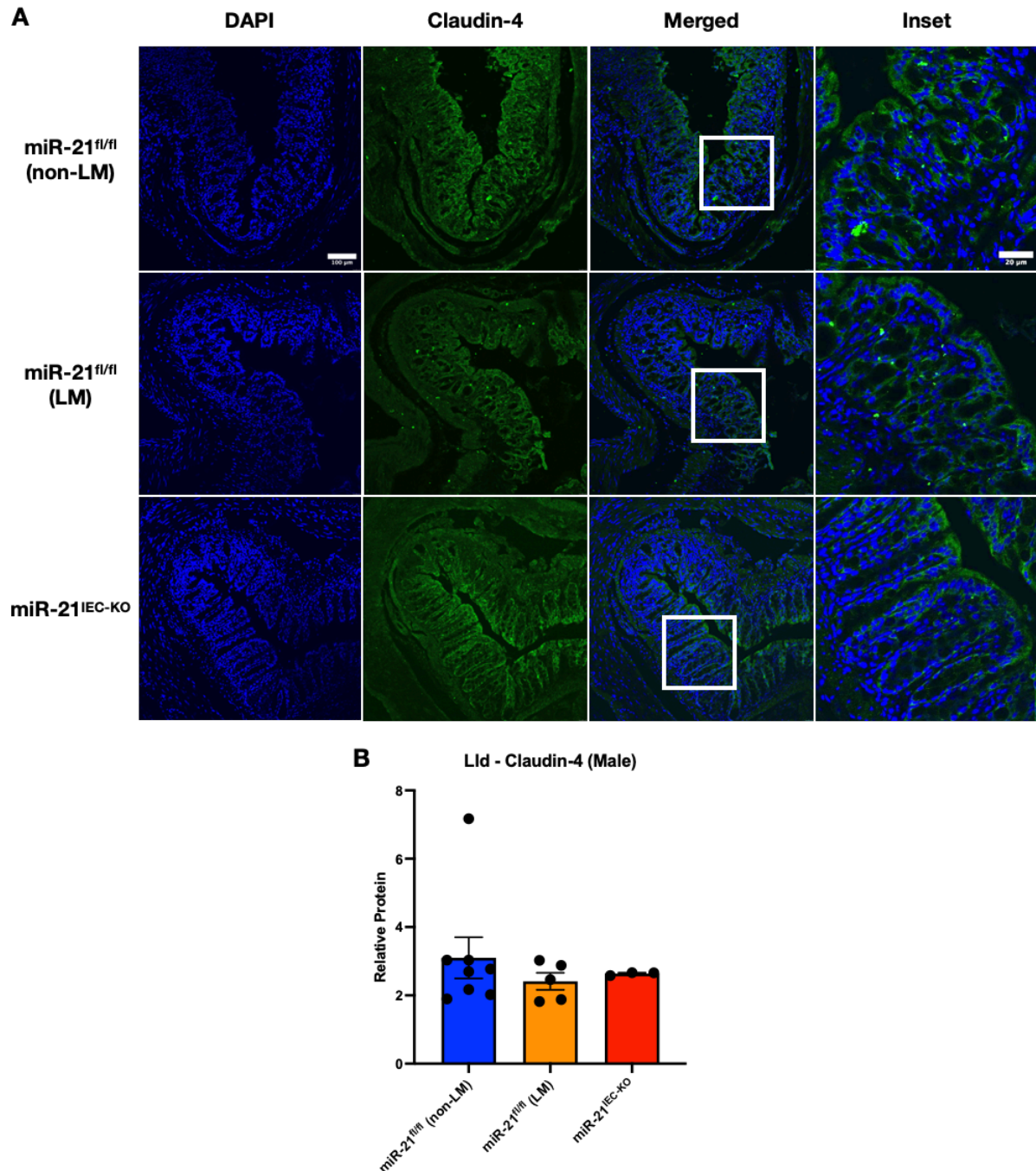


Figure 5.39 – Claudin-4 is more localized to the tops of intestinal crypts in miR-21^{IEC-KO} male distal colon tissue in DSS-induced colitis model. (A) distal colon / large intestine (LId) tissue sections of miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} male mice were removed, flushed with sterile PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-claudin-4 primary antibody (green). (B) Quantification of fluorescence normalized to number of nuclei. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

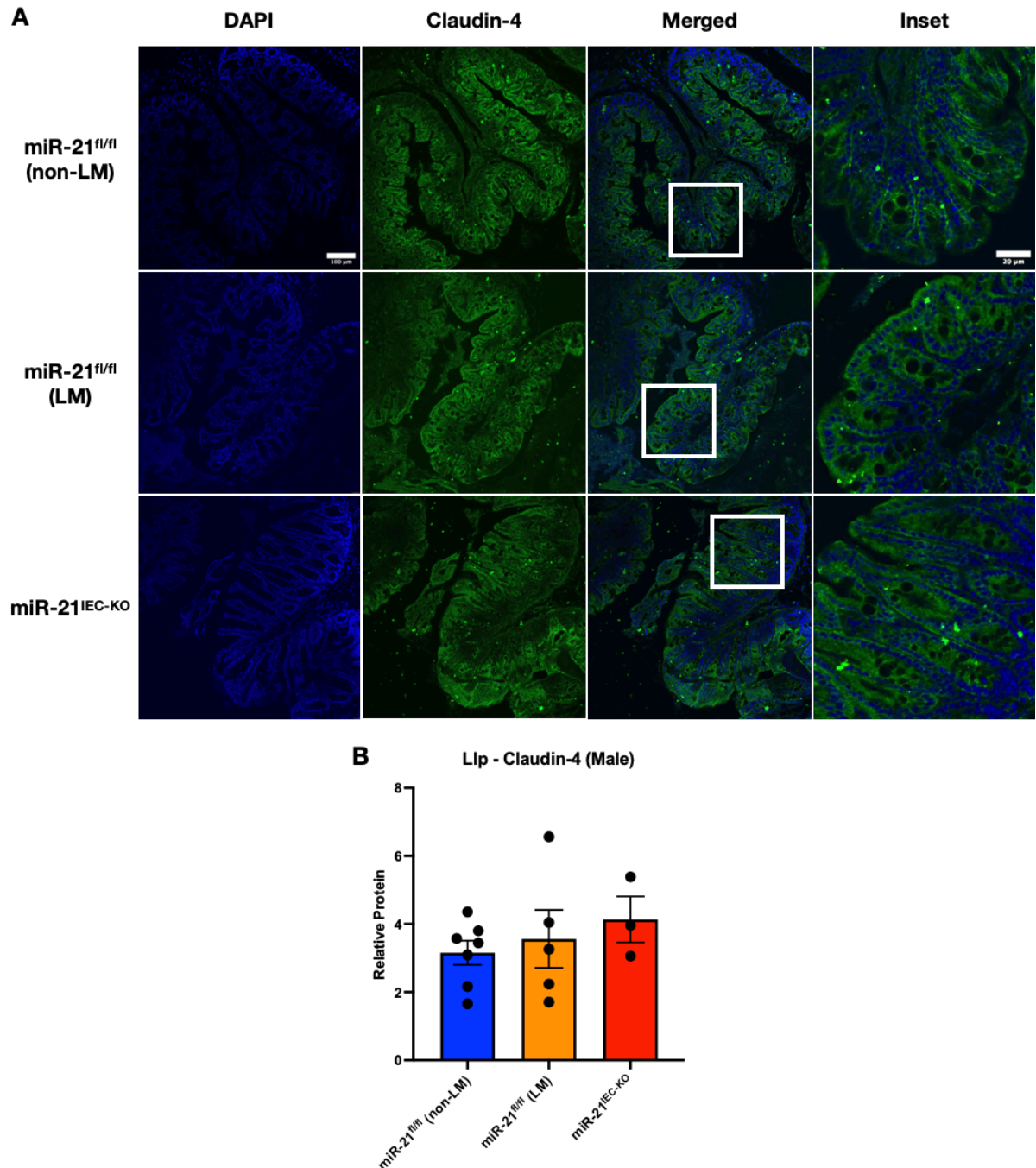


Figure 5.40 – Claudin-4 localization and abundance are similar between miR-21^{fl/fl} and miR-21^{IEC-KO} male proximal colon tissue in DSS-induced colitis model. (A) Proximal colon / large intestine (Llp) sections of miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} male mice were removed, flushed with sterile PBS and 1 cm sections were fixed in 4% formaldehyde. Tissue sections were stained via DAPI (blue) and anti-claudin-4 primary antibody (green). (B) Quantification of fluorescence normalized to number of nuclei. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using one-way ANOVA.

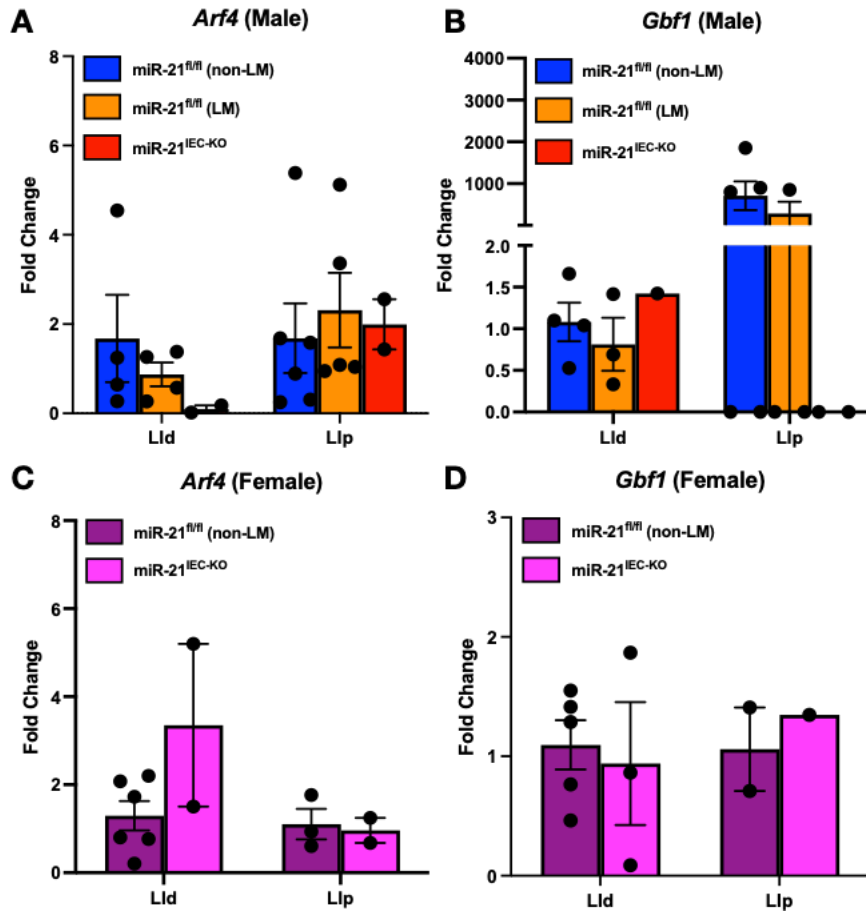


Figure 5.41 – *Arf4* is reduced in distal colon tissue of miR-21^{IEC-KO} male mice in DSS-induced colitis model. RNA was isolated from 8-15 week old miR-21^{fl/fl} groups (non-littermate [non-LM], littermate [LM]) and miR-21^{IEC-KO} mice given 2% DSS (w/v) water *ad libitum* for six days. qRT-PCR analyses of Golgi-related genes *Arf4* and *Gbfl* in distal colon / large intestine (LId) and proximal colon / large intestine (LIp) tissue from (A + B) male and (C + D) female miR-21^{fl/fl} and miR-21^{IEC-KO} mice. Denoted are $n \geq 2$ mice per group with the exception of male *Gbfl* – LId and female *Gbfl* – LIp qPCRs that have $n = 1$ mouse per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$.

5.3 Discussion

Being an autoimmune disorder arising from many potential causes, IBD requires various models to address key questions surrounding pre-disposition, severity and quality of life for afflicted individuals. For example, several gene KOs in mice have demonstrated higher susceptibility to colitis. In IL-10^{-/-} mice, spontaneous colitis occurs, due in large part to the absence of the anti-inflammatory cytokine to subdue the overactive immune response in the gut environment, and can be ameliorated of disease through anti-TNF- α therapy similar to CD.²¹⁰ In contrast, some genetic deletions can benefit the organism in regards to models in which colitis is induced. Yin *et al.* (2024)³²⁴ demonstrated that deletion of Fc ϵ RI, an IgE high-affinity receptor that activates mast cells, protected mice from DSS-induced colitis by increasing levels of commensal *Lactobacillus* and activating the NLRP6 inflammasome. These consequential genetic differences can also be found in IBD patients compared to healthy controls, which can then be followed up with in genetically manipulated animal models. With the use of genome-wide association studies (GWAS) on many ethnic and geographic locations, around 240 susceptibility loci have been identified for IBD.³²⁵ Addressing the mechanism of action that leads to differences in disease outcome can greatly support IBD patients and improve quality of life.

The particular focus of this chapter was to build on observations formed in Chapter 1 and 2 to further investigate the importance of epithelial expression of miR-21. The understanding of cellular context is invaluable, since researchers have shown that miR-21 from certain immune cells have direct impacts on the structural integrity of IECs. For example, exosomes containing miR-21 expelled from macrophages reduced E-cadherin, leading to barrier permeability.¹⁴¹ Interestingly, specific deletion of miR-21 in macrophages has been performed to study wound healing during inflammation or transplantation.^{326,327} Despite the prevalence of miR-21 in IBD research, less is known about the expression of miR-21 within non-hematopoietic cell types, and the impact of miR-21 of epithelial origin on gut barrier integrity. This chapter investigated the impact of miR-21 expressed from IECs, specifically through the creation of an IEC-specific miR-21 KO mouse. We developed a murine IEC conditional KO using the Cre-Lox system to further investigate the pathological contribution of IEC-specific miR-21 during inflammation. Targeting genetic modification to IECs was performed by attachment of the Cre recombinase gene to the *Vill* promotor, since the majority of Vill (an actin-binding protein) is expressed in intestinal epithelia.³²⁸ Upon confirmation of hemizygoty in

Vil:Cre female mice, these were bred with male miR-21 conditional KO mice as described in Park *et al.* (2012)³²⁰. Ultimately, it was discovered that male mice contained another Cre transgene in which the recombinase was attached to an Advillin promotor (Advillin:Cre), used by collaborators for a previous study on miR-21 ablation in sensory neurons.⁸²

Additional breeding was required for removal of the Advillin:Cre transgene as well as the shortening of the germline transmitted targeted allele (FRT-*lacZ*-loxP-Neo-FRT-loxP-miR21-loxP) to the conditional allele (FRT-loxP-miR21-loxP). Formation of homozygous mice possessing the miR-21^{fl} allele with the NEO cassette but lacking Cre recombinase were chosen for further breeding, to eliminate the chance of genomic contamination of unwanted Cre transgenes. Breeding with Flp-ROSA deleter mice leads to removal of the *lacZ* reporter and NEO through recognition and excision at integrated FRT sites.³²⁰ Here, mice that lacked the *lacZ*-NEO genetic region were selected and subsequently bred to form miR-21 flox conditional mice (miR-21^{fl/fl} or miR-21^{fl/wt}).

In animal breeding, it is best practice to maintain mouse colonies in a SPF environment. Up until Stage 4 breeding, mice were bred in non-SPF conditions as per the university standard for breeding mice ordered from industry. An additional rederivation step was required in which female miR-21^{fl/fl} mice were sacrificed and germplasm was extracted. Eggs were fertilized by male Vil-Cre sperm preserved from the initial colony and IVF performed using surrogate C57BL/6J female mice. Certain heterozygous miR-21^{fl/wt} that were also heterozygous for the Vil:Cre transgene and lacked any additional Cre recombinase transgenes were selected. Mice that also lacked the FLPO transgene necessary for previous crosses were also selected for in Stage 7 and the final breeding step to produce HOM miR-21^{fl/fl} mice that were genotypically either HEMI Vil:Cre^{+/-} or HOM Vil:Cre^{-/-}.

Interestingly, there was one genotype that was notably difficult to obtain or led to developmental complications post-weaning. This was littermate female miR-21^{fl/fl} mice. The reason there was a reasonable number of miR-21^{fl/fl} mice for use in the DSS-induced colitis model was due to the additional breeding of a separate non-littermate miR-21^{fl/fl} colony lacking the Vil:Cre transgene. Strangely, female miR-21^{fl/fl} mice were able to be produced in the separated non-littermate miR-21^{fl/fl} colony breeding. There were littermate miR-21^{fl/fl} mice born, but post-weaning and after separation into their own cages in preparation for the trial, all five mice died of advanced autolysis in all major organs. This finding had not been previously reported in the literature, and studies that have used the Cre-Lox system to create conditional miR-21 knockout mice either used males only⁸² or did not specify sex of mice.^{123,326,327} A number of factors could be contributing to this sex

ratio, but due to the differences observed in both non-littermate and littermate colonies, it likely has to do with ectopic expression of Cre that leads to changes in miR-21 levels during embryonic development in littermate mice, rather than potential genetic consequences of inbreeding. Although the Cre-Lox system is widely used, many caveats are apparent and are often not discussed, such as unexpected Cre activity in off-target tissues, inconsistent expression in littermates, lack of regulatory elements surrounding the cell-specific promotor, and even differential expression depending on maternal or paternal inheritance.³²⁹ Since dysregulation in miR-21 levels sourced through follicular fluid³³⁰ or extracellular vesicles secreted by pregnant mothers³³¹ can result in suppression of embryo development, this could explain the absence or abnormalities observed for littermate miR-21^{fl/fl} female mice. Future work that will deepen the characterization of the IEC-specific miR-21 conditional KO mouse strain will look at Cre and miR-21 expression across multiple tissues, as well as in embryos to measure abnormal localization and abundance.

To complement previous work done in our lab⁸⁶ and work in previous chapters that helped elaborate on the impact of non-specific miR-21 during colitis with the use of miR-21^{-/-} mice, miR-21^{IEC-KO} mice were used to determine the impact on the gut barrier due to epithelial-specific miR-21 during DSS-induced colitis. For male mice, non-littermate and littermate miR-21^{fl/fl} mice were used to compare the differences in mouse upbringing, as metabolite contribution of parental mice through weaning and the overall change in gut microbiome due to genotypically distinct parents are important factors to consider in any colitis model. Ultimately, mice of non-littermate and littermate status used in the trial were only separated by one generation. Prior to the beginning of the trial, there was a significantly greater initial body weight between non-littermate miR-21^{fl/fl} compared with littermate miR-21^{fl/fl} and miR-21^{IEC-KO} mice. This can be attributed to the age of distinct mouse groups, as 15-week old non-littermate mice were used, while 8-12 weeks old mice were used for the other two groups. Colitic symptoms such as weight loss, diarrhea and stool blood were present starting on Day 5, where there was strong separation of DAI scores on Day 6 between the non-littermate miR-21^{fl/fl} group with littermate miR-21^{fl/fl} and miR-21^{IEC-KO} mice. Although non-significant, there was a decrease in Day 6 DAI and increase in colon length for the miR-21^{IEC-KO} compared to littermate miR-21^{fl/fl} mice. Due to the severity observed in the non-littermate miR-21^{fl/fl} males, the trial ended on Day 6, and thus perhaps a longer duration of DSS treatment or more concentrated dose may lead to better separation among littermates. However, to some extent, protection was observed for both miR-21^{IEC-KO} and littermate miR-21^{fl/fl}. These are similar results to what was shown

previously in DSS-recovery trials, where protection was observed in non-littermate miR-21^{-/-} but was lost in littermate miR-21^{-/-} compared to WT mice during the DSS-treatment phase. These findings in non-littermates are also in line with previous studies by our group⁸⁶ and others⁸⁵. Therefore, this suggests a greater contribution of epithelial-specific miR-21 on the integrity of the gut barrier than previously thought, and perhaps more importantly, the absence of miR-21 during upbringing may strengthen the gut barrier in preparation to better handle induced inflammation by DSS.

Furthering on disease phenotypes observed in male mice during DSS-induced colitis, the functional aspect of the gut barrier was assessed in males for permeability, histological damage and neutrophil activity. Gut permeability was generally low for all groups when measuring FITC fluorescence in serum samples. There were two mice in non-littermate miR-21^{fl/fl} group and one mouse in the littermate miR-21^{fl/fl} group that had concentrations > 20 ug/mL, however the majority of mice had FITC levels < 3 ug/mL. At the tissue level, there was reduced damage and immune cell infiltration in the distal colon of miR-21^{IEC-KO} mice, but no difference between groups in proximal tissue. This effect is likely due to the fact that DSS is known to primarily act on the distal colon.²¹³ MPO activity was also similar among mouse groups, though notable pro-inflammatory markers such as Lcn2, IL-6, IL-1 β and TNF- α were reduced in distal tissue of miR-21^{IEC-KO} mice and littermate miR-21^{fl/fl} groups. Interestingly IL-6 has been linked to induce miR-21-5p levels in fibroblasts.²⁷⁴ It should be noted that IL-10, an anti-inflammatory cytokine that inhibits cell apoptosis and cytokine expression pathways, was also reduced, which may be due to the lack of immune activity within these protected groups. With notable damage and presence of immune cells penetrating the submucosae, this suggests that the immunological response was in its early stages of colitis, and major factors that lead to differences in disease severity may be more prominent at the molecular level.

In contrast to males, there were no major differences in any disease metrics among female mice groups during the duration of the trial, concluding with similar DAI scores on Day 6. This does agree with results shown previously, in which females overall (ie. non-littermate and littermate WT / miR-21^{-/-} female mice) had less severe disease during DSS-induced colitis compared to male counterparts. Symptoms were appearing steadily, and therefore prolonging DSS-treatment or increasing DSS concentration dose may lead to differences in disease severity. In tissue, although not significant, there was an observable trend of reduced tissue damage in both distal and proximal tissue of miR-21^{IEC-KO} mice, but similar neutrophil activity in both groups. In female distal tissue, there were elevated levels

of both TNF- α and IL-10. TNF- α is a cytokine that activates of the NF- κ B pathway, and has been shown to induce the expression miR-21 leading to a leaky phenotype *in vitro*.¹¹⁹ With differences observed in this model, where the population of miR-21 in epithelial cells is absent, future work could look at the differences in activating and regulating M1/M2 macrophages for a unique immune response in females during DSS-induced colitis.

It is interesting that non-littermate male mice showed severe symptoms on Day 6 that led to the stoppage of the trial, despite similarly low permeability and immune activity observed among the groups. As done in earlier chapters, further investigation looked at the expression of various intercellular junction genes for differential expression in miR-21^{IEC-KO} mice. Verification of miR-21 downregulation was apparent in miR-21^{IEC-KO} male mice even with RNA extraction from whole colon tissue but was found more similar between female mouse groups. This could be in relation to the difficulties observed in obtaining a miR-21^{fl/fl} female mouse, which in addition to prenatal and post-partum developmental issues, can have ectopic expression of Cre in off-target cells or mosaicism along the intestinal epithelia³²⁹, or even compensation for the lack of miR-21 at the intestinal epithelia via expression by other cell types. Assessing known miR-21 targets like *Pdcd4* and *Pten*, both male and female mice showed a trend for higher levels within miR-21^{IEC-KO} compared to miR-21^{fl/fl} tissue, even despite a wide variance. Induction of these miR-21 targets in whole colon tissue was a surprising find in the miR-21^{IEC-KO}, suggesting a greater contribution of epithelial-specific miR-21 to the gut barrier than previously thought.

AJ genes showed little difference in mRNA levels among all male and female groups. In fact, in male distal tissue, there was only a trend for reduced *Cdh1* and *Ctnnb1*. If the trend holds true with additional replicates, future work will look at determining whether this reduction in β -catenin impacts structural integrity of the AJ¹³⁷, or leads to less activation of the Wnt signalling pathway for oncolytic activity (ie. cell survival, proliferation, migration and EMT).¹⁴⁹ E-cadherin protein levels were measured in male colon tissue with immunoblotting and confocal imaging of immunofluorescent stains. Although there was no noticeable difference in overall E-cadherin abundance, intact E-cadherin was reduced in miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups, similar to the trials performed in earlier chapters using miR-21^{-/-} mice. Nonetheless, there was no difference in protein localization or fluorescence measured between male groups. This finding is consistent with a previous study that showed higher levels of fragmented E-cadherin in miR-21^{-/-} male mice, which may have enhanced wound healing effects at the epithelial layer.²⁶⁸ Moreover, for female mice, E-cadherin levels were also measured and were

increased in both distal and proximal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} mice. In this case, both fully intact and fragmented E-cadherin was greater in miR-21^{IEC-KO}, which would typically suggest stronger IEC connectivity for communication, permeability and resistance to cell shedding.²⁶⁴ As already mentioned, perhaps with a prolonged or more concentrated DSS-treatment, this stronger adherence between cells in the intestine could help protect miR-21^{IEC-KO} female mice from DSS-induced colitis.

With a focus on AJs, and in line with earlier chapters, components of epithelial wound healing were examined for differential expression in miR-21^{IEC-KO}. Interestingly, for both male and female mice, *Tgfb β 1* expression showed promising results with a significant upregulation in distal colon tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups. TGF- β receptor 1 is a transmembrane kinase that participates in heterotetrameric complexes for specific binding affinities to TGF- β family ligands and regulates the signalling cascade via R-Smad phosphorylation.^{280,332} Thus, increased activation of IEC mobility to the wound area could support a tighter epithelial gut barrier and protect miR-21^{IEC-KO} mice from DSS-induced colitis.

Other than AJs, TJs and desmosomes also play a crucial role in gut barrier integrity, offering stability to intestinal crypts, regulation of paracellular transport for luminal contents, and permitting interaction of immune cells with the external environment.⁹⁶ In male distal tissue, both *Ocln* and *Cldn2* were notably upregulated in miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups. For *Ocln*, these results agree with our previous trials, where induction of this prominent TJ component would typically result in a less leaky gut barrier. An increase in the pore-forming *Cldn2* could be an initial consequence to inflammation, as wounds form along the epithelial layer causing an increase in porosity. In addition, it may be possible that *Cldn2* is indirectly regulated, as Burkard *et al.* (2021)¹⁵⁷ showed that desmoglein-2, which was also differentially expressed in miR-21^{IEC-KO}, regulates claudin-2 expression by sequestering PI3K. Furthermore, there was a notable decrease in *Cldn4* levels in distal tissue of male and female miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups. *Cldn4* encodes a barrier-forming claudin that has demonstrated negative regulation of *Cldn2* pore function.³³³ As *Cldn4* was observed to be induced in the DSS recovery model, this may also be characteristic of the initial inflammatory response during induced colitis prior to resolution and recovery. Indeed, at the protein level, there was a trend for increased levels of occludin (intact and fragmented) in male and female distal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups, and in female proximal tissue. This phenotype was reversed in male proximal tissue, where miR-21^{IEC-KO} mice had a trend of reduced occludin

compared to miR-21^{fl/fl} groups. The reversed phenotype observed in male proximal tissue are similar results seen by Liu *et al.* (2018)¹²³, which showed a reduction of occludin levels in miR-21^{IEC-KO} mice. Unfortunately, sex and colon region were not disclosed in this study, and mice used were sampled under healthy conditions. Nonetheless, the sex-dependent differences observed for TJ regulation in miR-21^{IEC-KO} proves an exciting avenue for further research to characterize consistently observed cleavage sites, particularly due to the differences in disease severity observed during DSS-induced colitis.

Due to previous work on claudin-4 in earlier chapters, abundance and localization of the barrier-forming claudin was also examined in miR-21^{IEC-KO} male mice with confocal imaging of immunofluorescence. Despite similar fluorescence intensity, there was a noticeable concentration of claudin-4 around the top of distal colon crypts in miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups, which had more evenly distributed claudin-4 along the distal crypt axis. This localization may support a tighter gut barrier in regions most in contact with luminal elements, like gut microbiota and microbial toxins. Moreover, previous work by Tamagawa *et al.* (2003)³³⁴ showed that claudin-4 had greater localization to the apex of the dome region of follicle-associated epithelium (FAE) and correlated with the presence of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive apoptotic cells. Therefore, although Peyer's patches are typically found in the small intestine, an alternative reasoning for this localization to the apical crypt could be to assist colon immune cells for recognition of antigens or PAMPs due to breakage in the gut epithelial barrier. Future work will determine the association of claudin-4 to FAE in miR-21^{IEC-KO} mice and whether localization modulates the innate immune response during DSS-induced colitis. Therefore, claudin-4 continues to serve a vital purpose for gut barrier integrity regarding miR-21 regulation.

Taken together, this work highlights the importance of cell-specific gene KO models in the study of IBD, particularly for deciphering how miR-21 impacts the gut epithelial barrier during inflammation. IEC-specific ablation of miR-21 demonstrated similar disease severity in male mice when compared to full miR-21 KO mice during DSS-induced colitis, with some changes in intercellular junction functionality like E-cadherin and the induction of TGF- β receptor 1 that suggest regulation by exogenous miR-21, possibly emphasizing the significance of myeloid-specific miR-21. Overall, the miR-21^{IEC-KO} model is a valuable tool to understand miR-21-regulated IEC dynamics at the gut barrier during intestinal inflammation.

Chapter 6

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Conclusion

6. Conclusion

6.1 Final Discussion

In the study of IBD, it is clear that there is much to expand on regarding the dynamics within various relevant cell types. Research continues to elucidate more on cellular perspectives during inflammation; from GI-associated immune cell activation causing the inflammatory response by the host, to the dynamics of IECs impacted by that same inflammatory response, to external factors that play a crucial role in how the response is delivered and ultimately resolved. And yet, it has only been around 30 years since the discovery of miRNAs³³⁵, each participating in a multitude of signalling pathways and cellular processes involved in intestinal inflammation. In humans, latest estimates suggest approximately 1900 known miRNA loci that regulate genes, with potentially 2700 more novel canonical miRNAs yet to be studied.³³⁶ Of those thousands of miRNAs, one particular miRNA of interest to this project was the well-studied miR-21, a key anti-inflammatory mediator in immune cells, but also a pro-inflammatory modulator in non-hematopoietic cells.⁶⁸ This dual function provides the foundational basis for context-dependent research on IBD pathology.

Despite the functional roles miR-21 has on mediating processes that govern immune cell activity, little is known about the impact miR-21 has on the mucosa, composed of the outer and inner mucus layers, as well as the epithelial layer. Regarding the mucus layers, miR-21 has been shown to regulate mucus production via goblet cells, as well as the differentiation of goblet cells from stem cells of intestinal crypts.³³⁷ This negative association has consequences in structural integrity of the dense network comprised of highly glycosylated MUC proteins, particularly in the translocation of gut microbiota and their interaction with host cells.²⁰⁸ IECs like goblet cells are significant in creating the underlying gut epithelial barrier important for mediating host-microbe interactions and transport of luminal contents from digestion. Yet, few studies have deciphered how miR-21 regulate the specialized IEC dynamics during inflammation. Both the structural and functional role of IECs can be attributed to the regulation of intercellular junction complexes, located at cell peripheries between adjacent IECs. AJ proteins like E-cadherin help with cellular adhesion and structural stability via linkage to the actin cytoskeleton¹²⁹, while TJ proteins like occludin and claudin-4 control paracellular transport of water, ions and organic compounds.²⁷ Several studies have revealed the dysregulation of AJ and TJ

proteins during active IBD.⁹⁷ Furthermore, multiple studies have associated levels of miR-21 to levels of AJ and TJ in diseased tissue and intestinal epithelial cell lines.³⁵ Therefore, a major aim of this project was to determine the miR-21-specific impact on the gut barrier by investigating IEC AJs and TJs in the context of inflammation.

In designing our studies, we used *in vivo* murine models with miR-21 deficiency to evaluate changes of AJ or TJ expression in intestinal tissues during healthy and inflamed conditions. To induce inflammation, mice underwent DSS-treatment with the progression of colitis and recovery monitored each day, where miR-21 is known to be upregulated in DSS tissue.^{85,86} Tissue samples between WT and miR-21^{-/-} mice were examined phenotypically and intercellular junction expression profiles along with their barrier-associated pathways were compared at the RNA and protein level. Furthermore, *in vitro* systems were devised using Caco-2 cells transfected with miR-21 modulators for overexpression and inhibitory scenarios under an inflamed state. Here, TNF- α treatments were performed to induce inflammation on Caco-2 monolayers, which was shown to elevate the levels of miR-21.^{119,256} Similar to *in vivo* studies, the differences in the expression of intercellular junctions and their barrier-associated pathways at the RNA and protein level were compared between conditions of miR-21 induction or suppression. For AJs, we focused on their contribution to the wound healing process, using our *in vitro* system to assess how miR-21 regulates wound closure during scratch tests. We demonstrate for the first time that miR-21 may have a beneficial effect for the restitution phase during wound healing. For TJs, we expanded on their association with vesicular trafficking proteins, similarly using the *in vitro* system to assess how miR-21 impacts regulators of Golgi processes. We reveal a novel link between miR-21 with the expression and localization of Golgi-related proteins that contribute to Golgi tubulation in the retrograde transport pathway. Overall, this project highlights new avenues that further our collective understanding on miR-21-specific regulation of IEC dynamics during inflammation.

In Chapter 1, our initial studies established the *in vivo* model to examine the differences in AJ expression during miR-21 ablation. AJ targets of interest included E-cadherin, α -catenin, p120-catenin and β -catenin. E-cadherin is a primary anchor point for AJ proteins and, in addition with α -catenin, link the AJC to the IEC cytoskeleton.^{148,261} P120-catenin has been shown to stabilize E-cadherin while inhibiting AJ protein endocytosis and recycling.¹⁵⁰ Lastly, studies on β -catenin demonstrate interaction with E-cadherin to increase the level of epithelial cell adherence.²⁶⁴ In healthy conditions, our results showed an induction in E-cadherin and other AJ genes in both male and female

miR-21^{-/-} colon tissue. Moreover, DSS was used to induce colitis in WT and miR-21^{-/-} mice. Previous research using miR-21^{-/-} mice showed protection from DSS-induced colitis, attributed to the miR-21-dependent regulation of RhoB and Cdc42 at the intestinal barrier, as well as changes to gut microbiota.^{85,86} Here, we expanded on those previous findings and looked for potential cellular processes that could contribute to the protective phenotype observed during recovery from DSS-induced colitis via differential expression of AJ genes in miR-21^{-/-} mice. Since DSS causes destruction of the colon epithelial lining, the traditional DSS model was replaced by a DSS recovery model, allowing for re-establishment of the gut barrier and thus, stabilization of intercellular junctions after induced colitis. During recovery, we demonstrated induction in AJ genes E-cadherin, α -catenin and p120-catenin in both male and female miR-21^{-/-} distal colon tissue. However, to our surprise, when assessing levels of some AJ proteins in this same tissue, we saw a reduction compared to WT counterparts. This was surprising results and suggests miR-21-dependent post-transcriptional changes. Indeed, insights from oncology may prove useful, as studies on the role of AJ proteins in cancer development showed that AJ protein reduction increases cellular motility and proliferation.¹²⁹ In addition to the decrease in intact E-cadherin levels in distal colons of miR-21^{-/-} mice, perhaps AJs are not necessary during recovery from DSS-induced colitis, as the presence of stable AJs would result in less plasticity along intestinal epithelia when re-establishing gut barrier integrity after colitis.

There were notable differences between male and female miR-21^{-/-} molecular analyses obtained in the DSS recovery model. Not only were male mice generally more susceptible to DSS induced colitis, but female miR-21^{-/-} mice showed increased levels of β -catenin mRNA in both distal and proximal tissue compared to WT. β -catenin is a unique AJ protein that serves an alternative function to AJ stability. In its unbound form, β -catenin participates in Wnt signalling that mediates cellular processes like apoptosis or EMT through migration to the nucleus¹⁴⁹, which can be mediated by transcription factor BCL9-2.²⁷⁰ Therefore, future work on exploring sex differences in DSS-treated miR-21^{-/-} mice will look at β -catenin localization in intestinal tissue, and furthermore, using our *in vitro* system to determine whether the protein is colocalizing to AJs, or whether there is localization to another subcellular compartment during inflammation.

E-cadherin emerged as a consistent target of miR-21 regulation, known to have miR-21 seed regions in its 3'-UTR. During inflammation, dysregulation of E-cadherin has led to the deterioration of gut barrier function and the manifestation of IBD.¹³¹ In IBD

patient tissues, multiple studies have revealed a downregulation of E-cadherin mRNA and protein level^{97,135}, and polymorphisms in the *CDH1* gene have been associated with IBD.⁷⁰ Our findings suggested not only direct targeting of miR-21 to the *CDH1*-3'UTR seed region, but also an induction of E-cadherin through immunofluorescence during miR-21 ablation and that this induction is mainly cleaved E-cadherin which occurs in a miR-21-dependent manner. Studies demonstrated that fragmentation of E-cadherin resulted in improved wound healing in Caco-2 monolayers, and that this cleavage can be caused by internalized neutrophil elastase.^{268,338} It is unclear whether cleavage of E-cadherin is due to its localization in intestinal tissue, or whether cleavage is done prior to localization, along with the functional purpose of this cleavage. Future work will measure the abundance and activity of neutrophil elastase in miR-21^{-/-} mice under healthy conditions and after DSS-treatment to determine its impact on barrier integrity.

An essential process that occurs during recovery from colitis is wound healing, required for the tightening of the gut barrier in resolving the inflammatory response. E-cadherin is known to be a mediator of wound healing in IECs.²⁶⁴ In addition to E-cadherin regulation, Fan *et al.* (2022)²⁵³ demonstrated that junctional protein JAM-A was essential for wound healing in the gut by establishing focal adhesion points between adjacent IECs. We revealed that expression of JAM-A mRNA was induced in male and female miR-21^{-/-} mice during recovery from DSS-induced colitis. Moreover, another major pathway in wound healing is TGF- β signalling. TGF- β family proteins have been shown to support tissue repair by attracting monocytes and fibroblasts to the wound site, upregulating ECM deposition and inhibiting metalloproteinases, as well as promoting epithelialization after EMT.¹⁹⁶ Not only was TGF- β ligand induced in miR-21^{-/-} mice, but several TGF- β receptors were also upregulated within DSS-treated miR-21^{-/-} mice during recovery compared to WT. Interestingly, previous studies by Wang *et al.* (2012)²⁷² showed that TGF- β induced levels of miR-21 in EMT models of HaCat keratinocytes, and is associated with an anti-proliferative, pro-migratory phenotype. Furthermore, an inhibitor of TGF- β signalling, Smad7, was previously shown to be a direct target of miR-21, confirmed through reduction of Smad7 protein levels in rat renal tubular epithelial cells during the overexpression of miR-21.²⁵⁵ Therefore, since miR-21^{-/-} colon tissue showed an overall induction of TGF- β family genes, and isolated miR-21^{-/-} IECs also showed increased levels of inhibitory *Smad7*, it is likely that miR-21 acts in both ways to fine-tune TGF- β signalling. Future work will determine which specific TGF- β family proteins are true miR-

21 targets via *in vitro* assessment of luciferase activity with bioluminescent vector constructs of TGF- β family 3'-UTR seed regions, similar to what was done for E-cadherin.

Using the *in vitro* Caco-2 system under TNF- α -induced inflammation, we revealed unexpectedly that the closure of artificial wounds was improved during the overexpression of miR-21. Even more surprisingly, this improvement can be matched with a high concentration of miR-21 inhibitor as well, indicating that changes to miR-21 levels away from baseline under TNF- α -induced inflammation can support wound closure. For overexpression, the improved wound healing effect could be attributed to the specific induction of miR-21-3p during overexpression from analysed RNA lysates of test groups, and thus hindering this mature form could prevent wound healing pathways to take effect. Indeed, Wu *et al.* (2020)²⁷⁹ demonstrated that miR-21-3p agonist injected into dorsal wounds of mice had a significantly higher wound closure rate compared to control mice, associated with direct targeting of *SPRY1* mRNA. In this case, the various mature forms of miR-21, and whether it originated endogenously or engulfed from the external environment, could have specific roles in the wound healing process. For inhibition, this would lead to a more expected induction of TGF- β signalling and downregulation of competing pathways that induce cellular proliferation, such as Wnt signalling.²⁵⁶ Put together, the benefit of miR-21 modulation away from baseline levels during TNF- α -induced inflammation suggests that miR-21 has a temporal role in the full wound healing process, where its absence can enhance the initial step of epithelial cell migration, while its presence can repress an inhibitor of TGF- β signalling, as well as enhance the subsequent steps of cell proliferation and differentiation once epithelial cells reach the wound site.

In Chapter 2, we expanded on utilizing the *in vivo* miR-21 deficient model as well as the *in vitro* Caco-2 system with miR-21 modulators to investigate miR-21-specific changes in TJ gene expression. Chosen TJ targets of interest included occludin, various claudins (claudin-1, 2, 3, 4, 7, 15) and ZOs (ZO-1, 2, 3). This panel of TJ components was selected not only for their appearance in miR-21 prediction databases, but also for their relevance during IBD in maintaining gut barrier integrity, while also serving the multiple functions of the TJ itself. Occludin was the first integral TJ protein to be identified.³³⁹ One study showed that in Madin-Darby canine kidney (MDCK) cells, overexpression of occludin enhanced barrier tightness with increased TEER, while a separate study demonstrated that *Ocln*^{-/-} mice had histological phenotypes similar to chronic inflammation and poor TJ integrity in epithelial tissues.³⁴⁰ Regarding claudins, these are transmembrane proteins that have two major types: sealing claudins (ex. claudin-1, 3, 4) and pore-forming

claudins (ex. claudin-2, 7, 15). These proteins modify the amount of permeability through the gut barrier.¹²⁸ ZO-1 is a cytosolic scaffolding protein that stabilizes TJ complexes through its many protein-protein interaction motifs.¹²⁸ It has been proposed that the regulation of both occludin and ZO-1 is linked to miR-21 expression through the downregulation of several miR-21 targets such as PTEN and RhoB.^{120,123,124} Here, our novel focus was on IECs isolated from WT and miR-21^{-/-} mice to determine IEC-specific changes caused by miR-21 ablation. Interestingly, there were few differences between female mouse groups, however there was a significant upregulation of *Cldn4* and *Cldn15* in miR-21^{-/-} IECs compared to WT. Downregulation of *Cldn4* has been observed to increase barrier permeability⁹⁵, while *Cldn15* reduction can impact normal vitamin-D signalling and ion transport across the intestinal membrane.^{179,296} Thus, under healthy conditions, these claudins may play significant roles in general IEC homeostasis.

Subsequent experiments assessed the differences in TJ gene expression in miR-21^{-/-} mice during recovery from DSS-induced colitis. Past studies have shown that both occludin and ZO-1 were drastically reduced in IECs at the luminal surface during IBD⁹⁷, but little research has investigated the involvement of TJs during recovery from intestinal inflammation. We demonstrated that during recovery from DSS-induced colitis, *Cldn3* and *Cldn4* were upregulated in miR-21^{-/-} distal colon tissue compared to WT. This is in line with previous research that showed a negative association of claudin-4 with miR-21 levels in Caco-2 cells, attributed to the induction of Akt and Jnk signalling pathways that reduce TJ integrity.¹²⁰ To our knowledge, no link between claudin-3 regulation and miR-21 has been made. During colitis induction, multiple studies using *Cldn3*^{-/-} mice exhibited a hyper-permeable gut phenotype and immune cell infiltration into the lamina propria compared to WT counterparts.^{283,298} Future work will examine claudin-3 protein abundance and localization in the absence of miR-21 to expand on functional differences in IEC TJs.

In addition to TJ components, we also assessed desmosome genes *in vivo* during healthy and diseased states. For desmosomes, the two main types of components are desmocollins and desmogleins that each interact with the IEC cytoskeleton through binding to linking armadillo family proteins.¹⁵³ Traditionally, desmocollins and desmogleins serve redundant functions and only differ in their interacting partners.³⁴¹ Within the intestine, only desmocollin-2 and desmoglein-2 are expressed, and in conjunction with their appearance in our miR-21 prediction database search, were selected for investigation on gut barrier stability. To our knowledge, there is no literature linking desmosome regulation to miR-21 targeting. In healthy conditions, we saw no difference in the expression of *Dsc2*

or *Dsg2* between WT and miR-21^{-/-} mice. However, during recovery from DSS-induced colitis, miR-21^{-/-} male mice showed a significant upregulation in *Dsg2* in distal colon tissue compared to WT, but not *Dsc2*. This significance of *Dsg2* was also emphasized in *in vitro* analyses performed by Ungewiß *et al.* (2017)³⁰¹ using human desmosome-deficient cells or *DSG2* inhibitors, showing impaired barrier integrity which was restored by targeted induction of p38MAPK activity. In their study, re-expression of *DSC2* did not rescue barrier integrity nor p38MAPK activity.³⁰¹ Furthermore, findings by Gross *et al.* (2019)¹⁵⁵ revealed that *Dsg2*^{IEC-KO} mice had increased intestinal permeability in healthy conditions and during inflammation, with alterations in other desmosome and TJ components. Future work will look at desmoglein-2 protein abundance and localization to understand desmosome stability within miR-21^{-/-} intestinal tissue and through use of the *in vitro* Caco-2 system with miR-21 modulators.

One of the most consistent targets within our *in vivo* data was claudin-4. When protein levels were further assessed in miR-21^{-/-} male colon tissue of the DSS recovery model, there was a notable induction of claudin-4 compared to WT through western blotting and fluorescence microscopy. This is in line with work done by Nakata *et al.* (2017)¹²⁰ that connected claudin-4 to miR-21 regulation in an indirect manner. In their study, proteomic analyses during miR-21 modulation revealed ARF4 was significantly differentially expressed, and through knockdown assays with *ARF4* siRNA showed a negative correlation to claudin-4 levels. ARF4 is known to localize to the Golgi, and upon activation by the GEF GBF1 can induce COPI vesicle formation.³¹¹ Importantly, ARF4 has been demonstrated to negatively regulate the functioning of the Golgi in packaging protein into endosomes and transport for recycling during stress.³¹³ Our investigation into these Golgi processes revealed that although there was a decrease of *Gbfl* in male miR-21^{-/-} IEC isolate samples compared to WT under healthy conditions, there was an induced trend for *Arf4* and *Gbfl* in both male and female miR-21^{-/-} distal colon tissue during recovery from DSS-induced colitis. Interestingly, western blotting for Arf4 showed a decrease in male miR-21^{-/-} distal tissue compared to WT. To our knowledge, there is no other literature that characterizes Arf4 abundance or function within murine IECs in either healthy or diseased states. The notable mismatch between RNA and protein expression here and in some intercellular junction targets of interest in general indicate that translation of targets is being hindered, and future work will look to determine whether miR-21 is regulating Arf4 through translational repression in a direct or indirect manner. Additionally, we evaluated the abundance and localization of GBF1 using our *in vitro* Caco-2 system with miR-21

modulators. Our results showed that GBF1 colocalized with Golgi marker GLG1 in TNF- α -treated Caco-2 cells during overexpression of miR-21 and lost in the presence of a miR-21 inhibitor, suggesting miR-21 induces greater activation of ARFs like Arf4. It is possible that GBF1 translocation to the Golgi, which requires interaction with PIPs, may be facilitated through the upregulation of PIP₃ caused by miR-21 targeting of *PTEN* mRNA. Indeed, Meissner *et al.* (2018)³¹⁵ demonstrated that GBF1 preferentially bound to PIP₃ among other PIPs in the PI3K-Akt pathway. Future work will expand more on the functional consequences of the retrograde transport pathway during inflammation by assessing IEC-specific COPI gene and protein expression, evaluating the localization of ARF4, and in addition, assess the localization of GBF1 during re-expression of PTEN.

In Chapter 3, we build on the previous chapters by investigating the contribution of IEC-specific miR-21 to gut barrier integrity and the development of intestinal inflammation. We created a murine IEC conditional KO of miR-21 using traditional breeding techniques surrounding the Cre-Lox system. By attaching the Cre recombinase gene to the IEC-specific *Vill* promoter³²⁸ and following a breeding protocol with miR-21^{fl/fl} mice as described by Park *et al.* (2012)³²⁰, we showed that newly formed male miR-21^{IEC-KO} mice had a downregulation of miR-21 in whole colon tissue. However, levels of miR-21 were more similar between whole colon tissue of female miR-21^{fl/fl} and miR-21^{IEC-KO}, which could be in relation to the difficulties we observed in obtaining littermate miR-21^{fl/fl} female mice. There were littermate miR-21^{fl/fl} mice born, but post-weaning and after separation into their own cages in preparation for the trial, all five mice died of advanced autolysis in all major organs. This finding was not previously reported in the literature, and studies that have used the Cre-Lox system to create conditional miR-21 KO mice either only used males⁸² or did not specify sex of mice used in their study.^{123,326,327} Interestingly, female miR-21^{fl/fl} mice were able to be produced in the separated non-littermate miR-21^{fl/fl} breeding colony. Since there can be a dysregulation in miR-21 levels sourced through follicular fluid³³⁰ or that extracellular vesicles secreted by pregnant mothers³³¹ can result in suppression of embryo development, this could explain the absence or abnormalities observed for littermate miR-21^{fl/fl} female mice. Future work will deepen the characterization of the conditional miR-21 KO strain by assessing Cre and miR-21 expression across multiple tissues.

A novel contribution to the field by this project was the focus on miR-21 from epithelial origin and whether this sub-population contributes to pathogenesis. To continue previous work done using miR-21^{-/-} mice, the newly created miR-21^{IEC-KO} strain was put

through traditional DSS treatment to assess impact on disease severity during DSS-induced colitis. We included mice from non-littermate and littermate lineages to compare differences in mouse upbringing, as metabolites of parental origin and the overall change in gut microbiome due to genotypically distinct parents are important factors to consider in any colitis model. We revealed in male mice notable weight loss, diarrhea and stool blood differences starting on Day 5, where there was strong separation of DAI scores on Day 6 between the non-littermate miR-21^{fl/fl} group with littermate miR-21^{fl/fl} and miR-21^{IEC-KO} mice. There were also notable pro-inflammatory markers such as Lcn2, IL-6, IL-1 β and TNF- α that were reduced in distal tissue of miR-21^{IEC-KO} and littermate miR-21^{fl/fl} groups compared to non-littermate miR-21^{fl/fl} mice. Importantly, there was a decrease in Day 6 DAI and increase in colon length for miR-21^{IEC-KO} compared to littermate miR-21^{fl/fl} mice. To some extent, protection was observed for both miR-21^{IEC-KO} and littermate miR-21^{fl/fl}. These results agree with findings from Chapter 1 DSS-recovery trials, where protection was observed in non-littermate miR-21^{-/-} but was lost in littermate miR-21^{-/-} compared to WT counterparts during the DSS-treatment phase. Our results in non-littermate mice are also in line with previous studies.^{85,86} Altogether, this indicates a significant contribution of epithelial-specific miR-21 on the integrity of the gut barrier than previously thought, and perhaps more strikingly, the absence of IEC-specific miR-21 during upbringing may strengthen the gut barrier in preparation to better handle induced inflammation by DSS.

Subsequent RNA and protein analyses investigated AJ components like E-cadherin in miR-21^{IEC-KO} mice. Through western blotting, we showed reduced intact E-cadherin protein in miR-21^{IEC-KO} male mice compared to non-littermate and littermate miR-21^{fl/fl} counterparts, but similar levels of total E-cadherin. These findings are consistent with Chapter 1 studies using the DSS recovery model, and once again are in line with beneficial effects of fragmented E-cadherin shown in work done by Gordon *et al.* (2019)²⁶⁸ where E-cadherin peptide fragments enhanced wound healing effects in IEC monolayers. When assessing targets of interest in signalling pathways participating in epithelial wound healing, we observed a strong induction of *Tgfbri* in miR-21^{IEC-KO} mice. Thus, due to its importance for R-Smad phosphorylation in the TGF- β signalling cascade²⁸⁰, greater activation of IEC mobility to the wound area could support improved barrier re-establishment and protect miR-21^{IEC-KO} mice from DSS-induced colitis. Future work will continue to assess IEC-specific TGF- β receptor 1 abundance and localization during inflammation *in vivo* and *in vitro* when miR-21 can be modulated.

Finally, multiple TJ targets like occludin and claudin-4 were also evaluated in DSS-treated miR-21^{IEC-KO} mice. In male distal tissue, *Ocln* was notably induced in miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups, which agrees with our previous DSS-recovery trials where induction of this prominent TJ component would typically ameliorate a leaky phenotype. Indeed, at the protein level, there was an induced trend for occludin in distal tissue of miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups. Further, we showed a notable decrease in *Cldn4* levels in distal colon tissue of miR-21^{IEC-KO} mice compared to miR-21^{fl/fl} groups. Using fluorescence microscopy, despite similar fluorescence intensity, there was a notable concentration of claudin-4 around the top of distal colon crypts in miR-21^{IEC-KO} compared to miR-21^{fl/fl} groups, which had more evenly distributed claudin-4 along the distal crypt axis. This localization could aid in tightening the gut barrier in regions where interactions with gut microbiota are more prevalent. Insights from other studies on claudin-4 in the intestine suggest localization could be beneficial as well. Tamagawa *et al.* (2003)³³⁴ showed that claudin-4 had greater localization to the apex of the dome region of FAE, correlating with the presence of TUNEL-positive apoptotic cells. Therefore, although Peyer's patches are typically found in the small intestine, this localization to the apical colon crypt could assist colon immune cells in recognition of antigens or PAMPs due to breakage in the gut epithelial barrier. Future work will determine the association of claudin-4 to small intestine FAE in miR-21^{IEC-KO} mice and whether localization affects the innate immune response during DSS-induced colitis.

6.2 Future Perspectives

Use of miRNA biology, such as in the case of highly abundant miRNAs like miR-21, in the diagnosis or treatment of IBD is still in its infancy. Conventional methods of IBD diagnosis are typically invasive and harmful, such as endoscopy or testing of biopsies, while treatments include pharmaceuticals or harmful surgical procedures.³⁵ Immunosuppressants are prescribed, however this can cause potentially dangerous risk of opportunistic infections or toxic side effects.⁵ Our work in this thesis adds to a growing body of research on miR-21 as a therapeutic target, and understanding both its cell-dependent roles at the gut epithelial barrier while emphasizing the context-specific nature of miR-21 during IEC homeostasis can be effectively utilized for regulating barrier stability targets in different stages of IBD pathology.

Up until now, connections between miR-21 and gut barrier components were only associations. Now, our research has demonstrated that not only does miR-21 have additional novel associations to the regulation of barrier components like AJs and TJs, but that miR-21 may be required in some instances for the reinforcement of epithelial integrity. Not only was there an unexpected downregulation of AJ proteins in DSS-treated miR-21^{-/-} mice but using the intestinal epithelial cell line Caco-2, cells overexpressing and inhibiting miR-21 showed a significant increase in wound healing capacity compared to control cells. We theorize that this is due in part to the positive feedback loop observed in the TGF- β pathway, of which inhibiting Smad SMAD6 is repressed during miR-21 induction, as well as the increase in AJ induction from suppressing miR-21 function. Through this finding, therapeutic usage of miR-21 should be dependent on the level of damage observed in the intestines, or to what degree lesions are spread through intestinal epithelia leading to inflammation in an IBD patient.

Even fundamental cellular processes like vesicular trafficking components were impacted through modulating miR-21 levels in our models. We demonstrated that ARF4 was downregulated in miR-21^{-/-} mice and GBF1 localised to the Golgi for ARF protein activation and Golgi remodelling. We theorize that this activation of the retrograde transport pathway could be the reason for claudin-4 downregulation during intestinal inflammation, increasing gut barrier permeability while also repressing claudin-4 regulation of pore-forming claudins. With this finding, research focusing on controlling the retrograde transport pathway could be another therapeutic strategy for helping to strengthen gut barrier integrity and reduce levels of inflammation in IBD patients.

It is unanimous that the field is progressing to more contextualized experiments regarding miRNA regulation during disease, focusing on specific miRNAs like miR-21 and the impact on cellular subpopulations. We showed lower histological damage and reduction in pro-inflammatory markers within colon tissue of DSS-treated miR-21^{IEC-KO} male mice, despite no changes in colitis disease severity to control mice. This does support a higher level of significance for miR-21 regulation of the immune cell subpopulation at the gut barrier but further illustrates context-driven methodology that is currently lacking in IBD research. Using these approaches can build on previous clinical findings produced from relevant IBD patients' samples and more importantly increase the number of collaborative opportunities among research labs.

It has been suggested that miRNAs can be used as biomarkers for decades now in a plethora of diseases.³² Of course, on their own, the levels of miR-21 or any miRNA cannot be the only metric for diagnostic criteria. Nonetheless, as researchers learn more about miR-21 and other miRNAs for their mechanistic impacts at the gut barrier, a panel of IBD-relevant miRNAs can be a novel method to determine whether a patient has IBD, or further, differentiating between whether someone has CD or UC.

Therapeutics that target miRNAs like miR-21 are also completely justified, particularly if more research on miRNA gene regulation can continue to highlight mechanistic consequences.^{35,68} For less severe IBD cases, reducing the pool of functional miR-21 through ASOs can help improve epithelial barrier integrity. Still, with these results presented here, the environment at the gut barrier is a lot more complex, but knowing in which contexts miR-21 serves harmful roles versus when miR-21 serves beneficial roles will provide further guidance to therapeutic development.

This thesis showcased the value in context-driven, targeted approaches for understanding the impact miR-21 has at the gut epithelial barrier. With a major focus on IECs and the miR-21-specific cellular processes at play during IBD, more targeted therapeutics can be developed that alleviate dysfunction caused by inflammation-induced miR-21. Like most biological processes, the function for miR-21 is complex and still necessary for certain immunological roles, acting as a switch for when the inflammatory response should shift towards resolution, which could be dysfunctional in the case of IBD. Highlighting how miR-21 can affect gut permeability and fundamental IEC dynamics show how miR-21 is a great molecular tuner of intestinal inflammation.

Chapter 7

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References

7. References

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Chapter 8

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Appendices

8. Appendices

8.1 Supplementary Figures

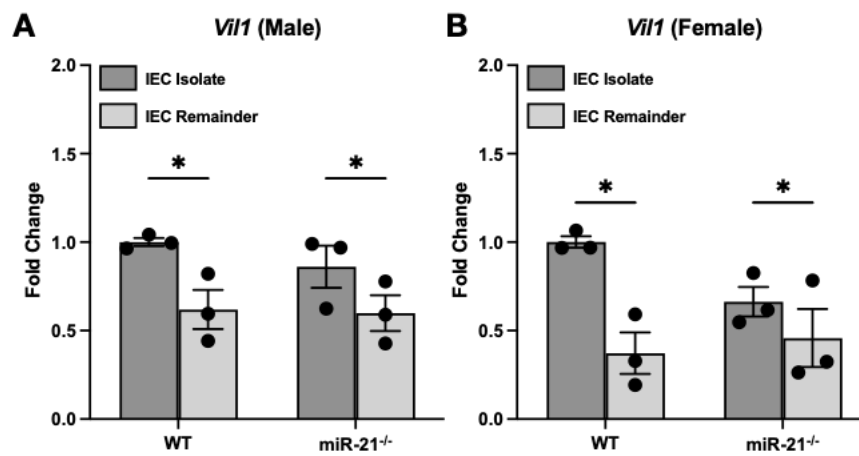


Figure 8.1.1 – Confirmation for the isolation of *Vill*⁺ IECs from colon tissue. RNA was isolated from IECs that were extracted from healthy 10-week old WT and miR-21^{-/-} male and female mice. qRT-PCR analyses of *Vill* in WT and miR-21^{-/-} (A) male and (B) female mice comparing IEC isolate and remainder fractions. Denoted are n = 3 mice per group, mean values ± SEM, *p* value calculations using two-way ANOVA with * - *p* ≤ 0.05.

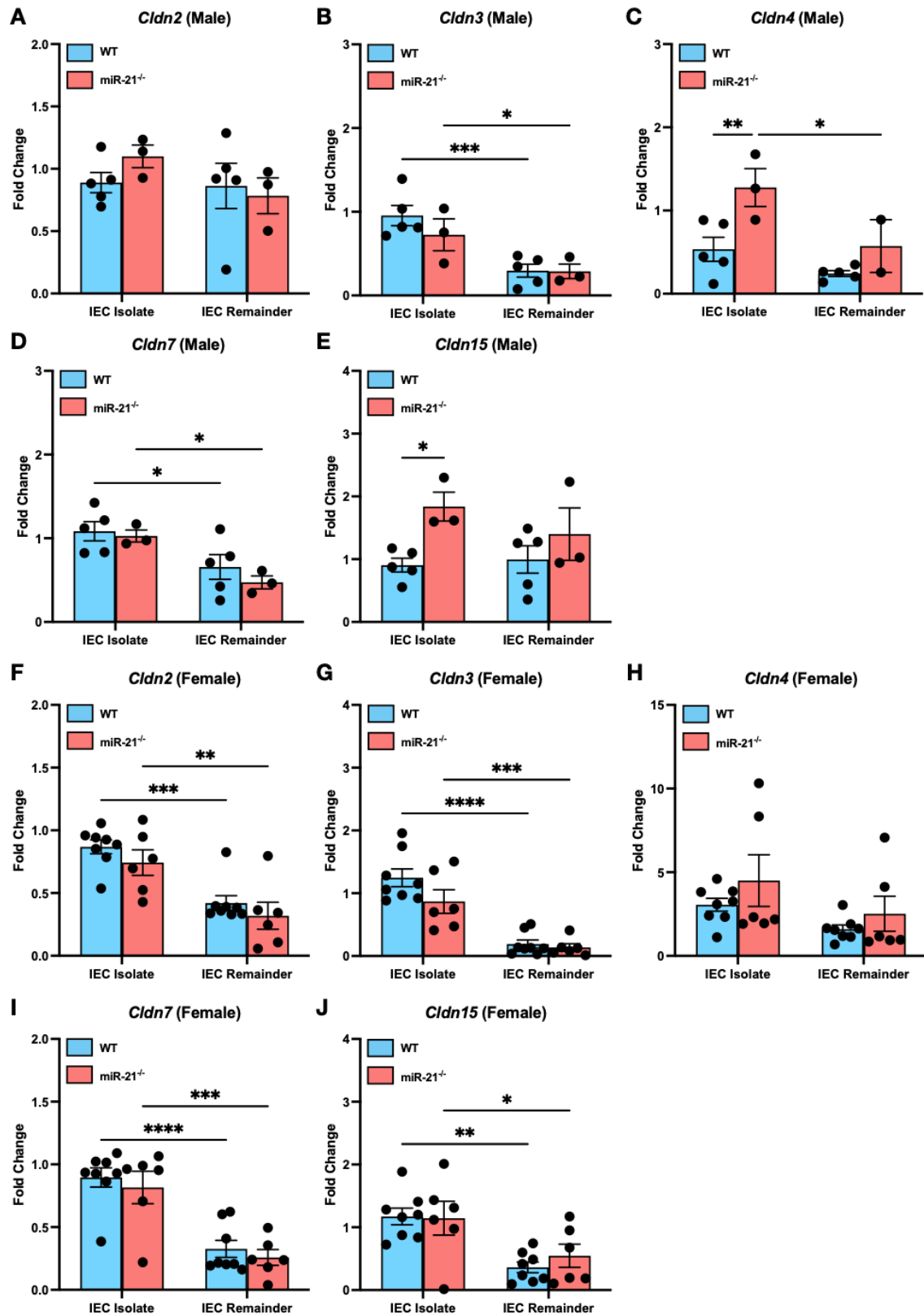


Figure 8.1.2 – *Cldn4* and *Cldn15* are significantly upregulated in IEC isolates of miR-21^{-/-} male mice compared to WT, while notable differences in gene expression between IEC isolates and remainder fractions were seen in both sexes. RNA was isolated from IECs that were extracted from healthy 10-week old WT and miR-21^{-/-} male and female mice. qRT-PCR analyses of junction genes in WT and miR-21^{-/-} (A-E) male and (F-J) female mice comparing IEC isolates and remainder fractions. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$, **** - $p \leq 0.0001$.

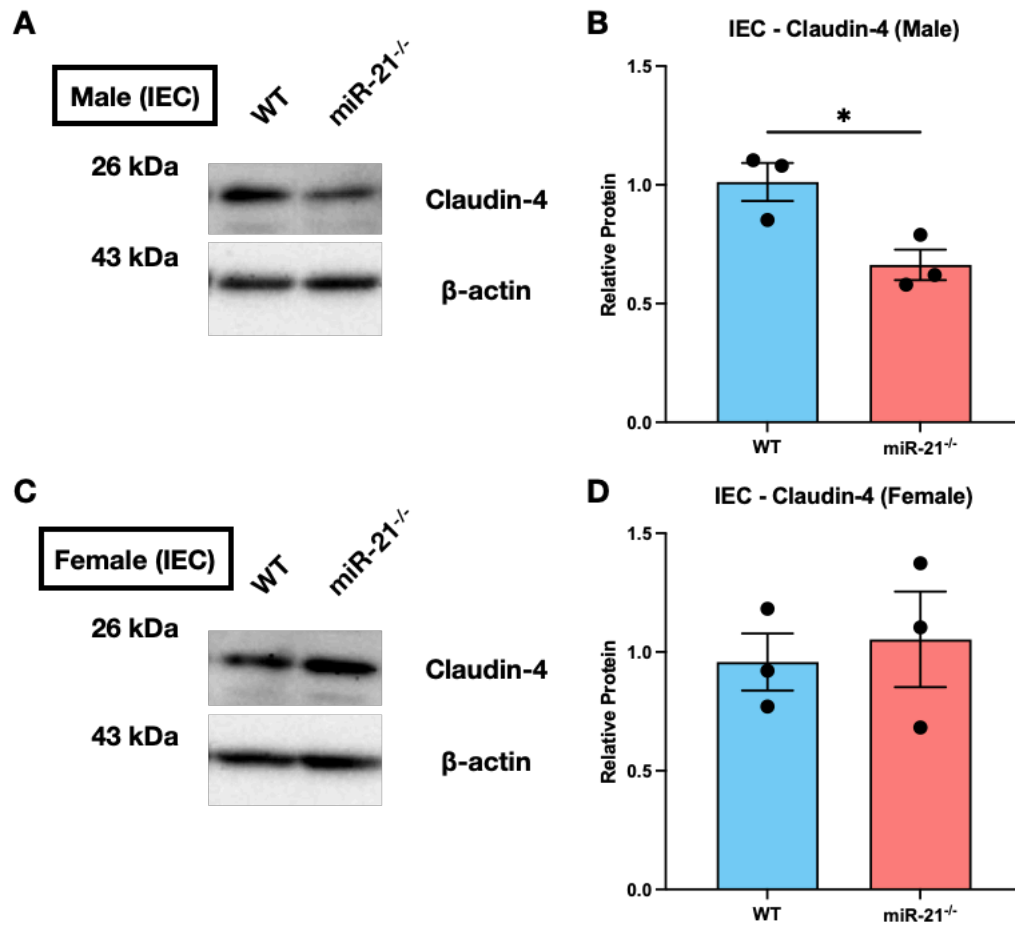


Figure 8.1.3 – Claudin-4 is significantly decreased in IEC isolates of miR-21^{-/-} male non-littermate mice under healthy conditions. Protein lysates were obtained from IECs that were extracted from healthy 10-week old WT and miR-21^{-/-} male and female mice. Immunoblots and quantification for claudin-4 of whole cell lysates from IEC isolate samples of WT and miR-21^{-/-} (A + B) male and (C + D) female mice. Denoted are n = 3 mice per group, mean values ± SEM, *p* value calculations using Welch's *t*-test with * - *p* ≤ 0.05.

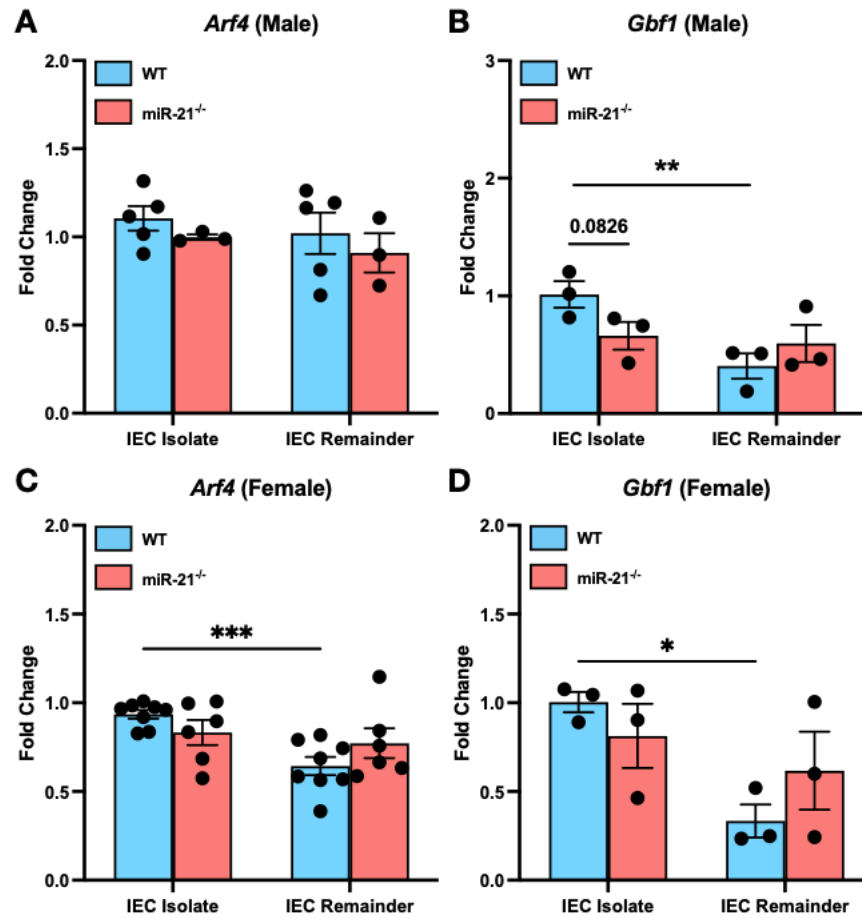


Figure 8.1.4 – *Gbfl* is reduced in IEC isolates from miR-21^{-/-} male mice under healthy conditions. RNA was isolated from IECs that were extracted from healthy 10-week old WT and miR-21^{-/-} male and female mice. qRT-PCR analyses of Golgi-related genes in WT and miR-21^{-/-} (A + B) male and (C + D) female mice comparing IEC isolates and remainder fractions. Denoted are $n \geq 3$ mice per group, mean values $\pm$ SEM, p value calculations using two-way ANOVA with * - $p \leq 0.05$, ** - $p \leq 0.01$, *** - $p \leq 0.001$.

8.2 Publications

- *Stiegeler, S., ***Mercurio, K.**, Iancu, M. A. & Corr, S. C. The impact of microRNAs during inflammatory bowel disease: Effects on the mucus layer and intercellular junctions for gut permeability. *Cells* **10**, (2021). *Equal Contribution
Note: Part of the introduction to this thesis which focuses on microRNAs, gut physiology and intercellular junctions has been published in this review paper.
- O'Callaghan, A. A., Dempsey, E., Iyer, N., Stiegeler, S., **Mercurio, K.** & Corr, S.C. Intestinal Metabolites Influence Macrophage Phagocytosis and Clearance of Bacterial Infection. *Front. Cell. Infect. Microbiol.* **11**, 1–13 (2021).

8.3 Oral Presentations

- **Mercurio K.** (2022). *The mechanistic impact of miR-21 on gut epithelial intercellular junctions and host-microbiome interactions*. Primary author of abstract, poster and flash presentation for the Microbiology Society UK's Focused Meeting, Dublin, Ireland.
- **Mercurio, K.** (2022). *The mechanistic impact of miR-21 on intercellular junctions, gut microbiota and susceptibility to intestinal inflammation*. Presentation for the School of Genetics and Microbiology Early Career Researcher Seminar Series, Dublin, Ireland.

8.4 Poster Presentations

- **Mercurio K.**, Corr S. C. (2024). *The impact of miR-21 on epithelial intercellular junctions influencing gut inflammation and barrier repair*. Primary author of abstract and poster for the European Congress of Immunology, Dublin, Ireland.
- **Mercurio K.**, Corr S. C. (2024). *Investigation epithelial-specific mechanisms of miR-21 influence on intestinal inflammation and IBD*. Primary author of abstract and poster for the International Congress of Mucosal Immunology, Copenhagen, Denmark.
- **Mercurio K.**, Corr S. C. (2022). *The mechanistic impact of miR-21 on gut epithelial intercellular junctions and host-microbiome interactions*. Primary author of abstract and poster for the School of Genetics and Microbiology Early Career Researcher Symposium, Dublin, Ireland.
- **Mercurio K.**, Corr S. C. (2022). *The mechanistic impact of miR-21 on gut epithelial intercellular junctions and host-microbiome interactions*. Primary author of abstract and poster for the APC Microbiome Symposium, Cork, Ireland.
- **Mercurio K.**, Corr S. C. (2022). *The mechanistic impact of miR-21 on intercellular junctions and susceptibility to intestinal inflammation*. Primary author of abstract and poster for the Irish Society of Immunology, Maynooth, Ireland
- **Mercurio K.**, Corr S. C. (2022). *The mechanistic impact of miR-21 on intercellular junctions and susceptibility to intestinal inflammation*. Primary author of abstract and poster for the Dublin University Microbiological Society's Focused Meeting, Dublin, Ireland.