MICRORNA-146A REGULATES NOVEL ASPECTS OF INTESTINAL HOMEOSTASIS AND IMMUNOMETABOLISM

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

MicroRNAs (miRNAs) are small, noncoding RNA regulators of gene expression that have many important functions within the immune system. While various critical immunologic functions for specific miRNAs have been uncovered, less is known about the roles of these molecules within the intestinal and adipose microenvironments. Recently, many studies have described the complex intestinal interface, which contains host immune cells and epithelial cells interacting with the microbiota in a manner that promotes symbiosis. Further, there is emerging evidence that miRNAs have evolved to fine tune host gene expression networks and signaling pathways that modulate cellular physiology in the intestinal tract. Here, I first review the present knowledge of the influence miRNAs have on both immune and epithelial cell biology in the mammalian intestines and the impact this has on the microbiota. Next, my work demonstrates the role of one specific miRNA, microRNA-146a (miR-146a), in intestinal homeostasis and disease. miR-146a has previously been shown to have anti-inflammatory function within the immune system and is required to downregulate inflammation in mammals. I find that this miRNA constrains multiple parameters of intestinal immunity and increases murine colitis severity. Further, because miR-146a regulates intestinal homeostasis and populations of the gut microbiota, I hypothesized that this molecule may also be important in regulating immunometabolism in a model of diet-induced obesity. I demonstrate that miR-146a is required to prevent obesity, diabetes, and metabolic disease

during high-fat diet. miR-146a was found to regulate multiple networks of gene expression in adipose tissue macrophages both during dietary homeostasis and metabolic disease, and these miR-146a-dependent pathways converge upon inflammation and cell metabolism. Altogether, miR-146a constrains immune responses both within the intestine and adipose tissue, and can both prevent or promote disease, depending on disease and context. This institutes the importance of studying miRNA functions within multiple tissues types and disease contexts, as novel roles for these molecules may be established in various situations.

This is dedicated to my love, Nicholas, and

to those who use science to make the world a better place.

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LIST OF ABBREVIATIONS

miR/miRNA.....microRNA
Th17.....T helper 17 cell
Treg.....T regulatory cell
Tfh.....T follicular helper cell
DSS.....dextran sodium sulfate
IPA.....Ingenuity Pathway Analysis
GSEA.....Geneset Enrichment Analysis
IBD.....inflammatory bowel disease
HFD.....high-fat diet
NC.....normal chow (standard diet)
DIO.....diet-induced obesity
NFκB.....nuclear factor kappa-light-chain-enhancer of activated B cells
Traf6.....TNF receptor-associated factor

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CHAPTER 1

INRODUCTION: MICRORNAS AND THE IMMUNE SYSTEM

Inflammation is a biological response to foreign entities, characterized by heat, pain, redness, and swelling, and requires intricate regulation from a variety of molecules. If unchecked, an inadequate inflammatory response will result in harm to tissue by the invading pathogens, which include bacteria, viruses, or other entities. On the other hand, excess and unregulated inflammation can lead to tissue damage by an overreaction of the immune system. The need for a tolerable level of inflammation is referred to as the Goldilocks principle, where the response needs to be "just right" to limit harm to the host. Low-grade, chronic inflammation, a result of unresolved immune responses, is common in developed countries and is associated with many of the top causes of mortality in humans (Hu et al., 2014). This chronic condition is characterized by continuous and unregulated production of proinflammatory cytokines, antibodies, reactive oxygen species, and other inflammatory factors (Hu et al., 2014). While chronic inflammation is frequent and occurs in various diseases, including cancer, neurodegeneration, autoimmunity, heart disease, and type 2 diabetes, the factors that trigger and regulate inflammation during these diseases are not well understood (Medzhitov, 2008; Murakami and Hirano, 2012). A wide body of research is currently invested in determining the pathways and molecules that keep inflammation adequate and controlled.

One set of molecules required to regulate inflammation are microRNAs (miRNAs). miRNAs are a class of small noncoding RNAs, which do not encode for a protein but still exhibit function within cells. MiRNAs are conserved throughout evolution, as they are found in viruses, plants, *C. elegans*, *Drosophila melanogaster*, mammals, and many others clades (Ha and Kim, 2014). These small, noncoding RNAs are one of the most abundant types of gene found within the genome (Ha and Kim, 2014).

A recent study identified more than 3,700 novel miRNAs in the human/primate genome, in addition to the ~2,800 that have been previously annotated (Londin et al., 2015), bringing the total number of miRNAs to approximately 6,500. Many of these newly predicted miRNAs are specific to organism and cell type (Londin et al., 2015). It is estimated that more than 60 percent of the human transcriptome contains conserved miRNA binding sites, and this number may be even higher with the potential of non-canonical miRNA binding sites that have not yet been predicted (Ha and Kim, 2014). This demonstrates the importance of miRNAs in regulating gene expression, as we have evolved a large number of genes that encode for these small noncoding molecules. Furthermore, protein-coding genes that require tight regulation, such as oncogenes and inflammatory genes, have a large proportion of miRNAs that target them (Calin et al., 2004; Thomson et al., 2011). For example, the gene cyclin-dependent kinase inhibitor 1A (CDKN1A), an important factor downstream of tumor suppressor pathways, has been shown to be targeted by 28 miRNAs (Wu et al., 2010). Many of the miRNAs that target this particular mRNA are dysregulated in cancers. In short, miRNAs are an extensive class of molecules that are required for proper development and function of organisms.

Genes encoding for miRNAs are located on DNA and are transcribed as any other protein-coding mRNA, by RNA polymerase II and Pol II-associated factors (Cai et al., 2004; Lee et al., 2004). The initially transcribed miRNA, known as a primary miRNA or pri-miRNA, is typically long (>1 kilobases) and forms a stem-loop structure of about 33-35 base pairs (bp), with the stem-loop containing the mature sequence (Ha and Kim, 2014). Next, the ribonuclease DROSHA and cofactor DGCR8 cleave the miRNA into a smaller hairpin structure of approximately 65 nucleotides, referred to as a pre-miRNA

(Ha and Kim, 2014; Lee et al., 2003). The pre-miRNA is then exported from the nucleus into the cytoplasm through a nuclear pore complex consisting of Exportin 5 and RAN-GTP (Ha and Kim, 2014; Lund et al., 2004; Yi et al., 2003). Once in the cytoplasm, the miRNA is further cleaved by another endonuclease, known as Dicer, into a small RNA duplex structure approximately 21-25 nucleotides in length (Ha and Kim, 2014). Next, the miRNA duplex structure is unwound and one strand of the miRNA is selectively loaded onto a protein called Argonaute (AGO). This structure, containing AGO, the mature miRNA, and other proteins, is referred to as the RNA-induced silencing complex or RISC. In the mature, RISC-bound form, the miRNA then guides this complex to target mRNAs via Watson-Crick base complementarity. At this point, the miRNA, along with AGO, exhibit function by downregulating expression of the bound target mRNA. Depending on the type of AGO protein, cellular context, and extent of mRNA-miRNA base-pairing, mRNA targets are either cleaved and degraded or ribosomal translation of the mRNA is inhibited (Pratt and MacRae, 2009). Altogether, microRNAs require many steps in order to exhibit their functionality, which is to repress gene expression of target mRNAs.

MiRNA processing itself is tightly regulated, in which each step must be executed correctly in order for miRNAs to function properly. Loss of the molecules required for biogenesis of miRNAs, including DROSHA, DGCR8, and Dicer in model organisms results in lethality (Bernstein et al., 2003; Chong et al., 2010; Kanellopoulou et al., 2005; Wang et al., 2007), demonstrating the requirement of miRNAs for proper development. Conditional deletions of DROSHA, DGCR8, or Dicer, including by using floxed mice for these genes, can establish the essential role of all miRNAs within a particular cell or

tissue type. For example, conditional deletion of Dicer from hematopoietic cells using the Tie2-Cre driver results in defective development of invariant natural killer T (iNKT) cells (Zhou et al., 2009), indicating the importance of this miRNA-processing molecule in immune development. In general, expression of miRNA biogenesis enzymes is tightly regulated at each step within cells by transcription factors and regulatory proteins (Ha and Kim, 2014). Further, miRNA biogenesis is also regulated at the level of the RNA sequence and secondary structure, which can affect stability, turnover, and function (Ha and Kim, 2014; Ruegger and Grosshans, 2012).

MiRNAs do not act as on/off switches of target genes, but rather as rheostats to enforce positive and negative feedback and feedforward loops and to reduce transcriptional noise (Berezikov, 2011; Mehta and Baltimore, 2016). While a small number of these RNAs can completely downregulate target mRNAs, a majority of miRNAs only reduce expression of target genes 1.5- to 4-fold (Baek et al., 2008; Selbach et al., 2008). miRNAs can set threshold levels of mRNAs by reducing their expression to a mean level, but can also buffer and reduce variance of expression of an mRNA (Berezikov, 2011; Mehta and Baltimore, 2016); both possibilities are not mutually exclusive. Because of the now accepted concept that miRNAs act as fine tuners or "micromanagers" of gene expression and not on/off switches, testing the necessity of miRNA genes in model organisms can be difficult, as complete knockouts of miRNA genes often result in modest phenotypes (Berezikov, 2011; Lai, 2015). Testing the role of miRNAs at steady state conditions may not prove lack of functionality of these genes if no phenotype is observed; it is important to examine miRNA function in various challenges, cell types, and contexts. Altogether, miRNAs may be seemingly

inconsequential at first glance with their mild repression of mRNAs, but their evolutionary conservation and necessity in preventing disease, including cancers and autoimmune disease, shows the vast importance of these molecules in biology.

In order for miRNAs to exhibit their function, they must be expressed at the same time within the same cell as their target genes (Berezikov, 2011). This proposes another level of complexity, as these small RNAs may only target particular mRNAs in specific contexts and microenvironments. Thus, while thousands of papers have been published investigating microRNA functions, we do not yet know the full extent of their roles in all cell types, biological processes, and diseases. As determined during the studies presented in this dissertation (as well as many others), miRNAs have context-specific roles and they may both promote and inhibit disease. One may assume that since a microRNA targets a certain pathway in one context that it is not necessary to test this in multiple disease and stimulation states. However, miRNA research is still valid and important, especially as these molecules begin to be utilized in the clinic, since their complete role in targeting every mRNA in every cell type is not known. Large-scale screens, including CRISPR deletions of miRNA libraries (Wallace et al., 2016) and HITS-CLIP of Argonaute and profiling mRNA-miRNA interactions within RISC (Thomson et al., 2011), are helpful starting points for determining context-specific roles of miRNAs, and these studies are ongoing.

One biological function that miRNAs are essential for is in regulation of the immune system. Extensive research on the specific roles of miRNAs in immune function has been ongoing, with the most prominent of these genes being miR-155, miR-146a, miR-17~92, miR-181a, and miR-21 (O'Connell et al., 2010). These miRNAs and others

have been shown to be required for proper development and function in various cell types, including granulocytes, monocytes, macrophages, dendritic cells, T cells, B cells, and others. Further, immune-regulating miRNAs have been associated with infection and autoimmune disease in humans, including miR-122 for viral infections, and miR-155 and miR-146a for rheumatoid arthritis (Li and Rana, 2014; O'Connell et al., 2010; Pauley et al., 2009). The roles of these small, noncoding RNAs in the immune system have been reviewed extensively (Chen et al., 2013; Lu and Liston, 2009; Mehta and Baltimore, 2016; O'Connell et al., 2010; Xiao and Rajewsky, 2009). One up-and-coming area of research for immune-regulating miRNAs is within the intestinal immune system. The gut is a rather complex microenvironment, since it is heavily exposed to microbes, food antigens, and other foreign agents, so its immune regulation is also intricate. In Chapter 2, "MicroRNAs and the regulation of intestinal homeostasis", I review the current literature on the importance of miRNAs in the intestinal tract and discuss functional roles of specific miRNAs required for gut homeostasis and to prevent diseases, including inflammatory bowel disease. In general, while a large body of research has been put forward within the past ten years regarding the roles of miRNAs in immune system function, there are still many unknowns, especially in complex or lesser understood systems.

MicroRNA-146a, or miR-146a, is a prominent miRNA that is required for proper immune system function, and its importance within novel contexts will be presented herein. This microRNA was first described as a miRNA that regulates the immune response in 2006, when it was found to be highly upregulated in human monocytes following stimulation with LPS and other immune stimulators (Taganov et al., 2006).

miR-146a is induced by NFκB, and acts in a negative feedback loop to regulate inflammation, primarily by targeting mRNAs within the NFκB pathway, including Traf6 and Irak1 (Boldin et al., 2011; Taganov et al., 2006; Yang et al., 2012; Zhao et al., 2013). This miRNA is related to multiple diseases, including many cancers (Boldin et al., 2011; Huffaker et al., 2012; Starczynowski et al., 2011), arthritis (Lochhead et al., 2014), viral infection (Li et al., 2015), autoimmunity (Hu et al., 2014; Lu et al., 2010), and chronic inflammation (Hu et al., 2014). Loss of miR-146a in mice results in low-grade chronic inflammation, which eventually leads to a myeloproliferative disease and decreased survival (Boldin et al., 2011; Zhao et al., 2011; Zhao et al., 2013), demonstrating a requirement for this miRNA in hematopoietic development. One of the prominent roles that miR-146a plays is within T cells, where loss of this miRNA results in loss of tolerance by T regulatory cells and increased T helper 1 responses (Lu et al., 2010). Further, miR-146a is also required to regulate germinal center responses of T follicular helper and germinal center B cells (Pratama et al., 2015). In general, it is apparent that miR-146a has important roles within the hematopoietic lineage, but its cellular- and context-specific functions are not fully elucidated. In this dissertation, I explore how miR-146a influences inflammation and immune function within two unique microenvironments, the intestinal tract and adipose tissue. The work I present further demonstrates the role of this miRNA in regulating inflammatory responses but does so in novel, context-specific ways.

For my thesis project, I hypothesized that miR-146a plays an important role in regulating intestinal immune function, as well as in host metabolism. Initially, I predicted that loss of miR-146a would result in increased inflammation and therefore worsened

disease within the gut. Instead, in Chapter 3, "microRNA 146a constrains multiple parameters of intestinal immunity and increases susceptibility to DSS colitis", I demonstrate that while a loss of miR-146a does result in increased expression of immune genes, the type of genes expressed at higher levels, including intestinal barrier genes, results in protection from a model of colitis. Thus, miR-146a downregulates inflammation within the gut, but this downregulation results in worsened susceptibility to intestinal disease. This particular project further demonstrated the importance of continually testing the role of microRNAs in various tissue and disease types, as their functions may not exhibit the same outcomes in each case.

Furthermore, I hypothesized that miR-146a is necessary for preventing metabolic disease via its downregulation of inflammatory pathways. These studies are presented in Chapter 4, "Anti-inflammatory microRNA 146a protects mice from diet-induced metabolic disease", where I induced metabolic disease in mice by feeding them a high-fat diet (HFD). While my experiments demonstrated the requirement of miR-146a in downregulating inflammation during diet-induced obesity (DIO), I also uncovered novel pathways and targets that miR-146a is important in regulating outside of TLR/MyD88/NFκB signaling during metabolic disease. This study established a role for this miRNA in macrophages within the adipose tissue microenvironment, in which these innate immune cells are exposed to unique signals from adipocytes (fat cells) and local metabolites.

In general, these key findings advance a greater understanding of the role of miR-146a outside of what was previously known, and with future investigation, support the use of this miRNA as a therapeutic target for intestinal and metabolic disease. Because

miR-146a is dysregulated in human diseases of the intestine and metabolism (Runtsch et al., 2015), this work contributes to a future in utilizing this miRNA and its related pathways in treating human disease, including IBD, obesity, diabetes, and others. In the final chapter, I discuss the relationship between the presented studies on miR-146a in the gut and metabolic disease. Finally, I present future experiments and how the work performed in my graduate studies can be applied to new areas of research.

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CHAPTER 2

MICRORNAS AND THE REGULATION OF INTESTINAL HOMEOSTASIS

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MicroRNAs and the regulation of intestinal homeostasis

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The mammalian intestinal tract is a unique site in which a large portion of our immune system and the 10^{14} commensal organisms that make up the microbiota reside in intimate contact with each other. Despite the potential for inflammatory immune responses, this complex interface contains host immune cells and epithelial cells interacting with the microbiota in a manner that promotes symbiosis. Due to the complexity of the cell types and microorganisms involved, this process requires elaborate regulatory mechanisms to ensure mutualism and prevent disease. While many studies have described critical roles for protein regulators of intestinal homeostasis, recent reports indicate that non-coding RNAs are also major contributors to optimal host-commensal interactions. In particular, there is emerging evidence that microRNAs (miRNAs) have evolved to fine tune host gene expression networks and signaling pathways that modulate cellular physiology in the intestinal tract. Here, we review our present knowledge of the influence miRNAs have on both immune and epithelial cell biology in the mammalian intestines and the impact this has on the microbiota. We also discuss a need for further studies to decipher the functions of specific miRNAs within the gut to better understand cellular mechanisms that promote intestinal homeostasis and to identify potential molecular targets underlying diseases such as inflammatory bowel disease and colorectal cancer.

Keywords: microRNAs, intestine, microbiota, immune system, homeostasis, host-commensal

INTRODUCTION

A major goal of the biomedical research community is to understand mechanisms that regulate the gut immune system in a manner that maintains homeostasis, despite the presence of trillions of bacteria and other microorganisms that reside on and within the host. Gut homeostasis is defined by a proper balance of pro-inflammatory responses against harmful and/or invading microbes while tolerating non-invasive and beneficial microbes (Hooper and Macpherson, 2010). During this state, healthy proportions of commensals, tolerant immune cells, and pro-inflammatory host cells interact closely with one another. However, if this balance is disrupted, either by changes to the composition of the gut microbiota or by alterations to the host response, diseases can emerge (Round and Mazmanian, 2009).

In order to maintain homeostasis, the host intestinal mucosa contains distinct regions consisting of various cell types necessary to respond to antigens in an appropriate manner (Hooper and Macpherson, 2010; **Figure 1**). These regions include: the outer and inner mucus layers, in which invading microbes become trapped to prevent spread of infection (Johansson et al., 2011); the epithelial layer, in which physical and chemical barriers are formed to prevent dissemination of bacteria into underlying tissues (Goto and Ivanov, 2013; Peterson and Artis, 2014); the lamina propria (LP), a leukocyte-rich region that lies underneath the epithelium, housing cells that respond to microbial signals (Duerkop et al., 2009); and other immune cell-containing gut-associated lymphoid tissues (GALT) such as Peyer's Patches and mesenteric lymph nodes. Immune cell-rich gut tissues contain a diverse set of cell types, both hematopoietic and non-hematopoietic-derived, that play a role in maintaining healthy interactions with the resident microbiota. Some of these cell types are unique to the intestines and many have distinct functions within the GI tract. Several of these gut cells have been demonstrated to be required for providing both defense against pathogens and tolerance to commensals in order to prevent disease.

The gut microbiota itself heavily influences proper development and function of the intestinal immune system (Round and Mazmanian, 2009; Hooper et al., 2012; Ivanov and Honda, 2012). Indeed, germ-free mice display developmental defects within GALT and intestinal epithelial cells (IECs). One mechanism by which the microbiota can influence intestinal immune cells is via surface and cytoplasmic receptors called pattern recognition receptors (PRRs) that recognize conserved microbial motifs on both pathogens and commensals. PRR detection of microbial products initiates a variety of immune responses and developmental pathways. One class of PRRs is the toll-like receptors (TLRs), which signal primarily through the adaptor protein MyD88 to activate master transcription factors such as NF-κB. In some cases, gut microbial recognition by the host leads to proinflammatory responses resulting in increased gut inflammation (Ivanov et al., 2009). In other situations, activation of cell surface receptors by commensal products induces tolerogenic responses (Round et al., 2011; Smith et al., 2013). Additionally, recognition of commensal and food molecules by PRRs is essential for the maintenance of intestinal homeostasis (Rakoff-Nahoum et al., 2004; Abreu, 2010). This demonstrates the importance of host-commensal interactions in proper immune function and overall host physiology. While PRRs are but one mechanism thought to influence homeostasis, many of the molecular events

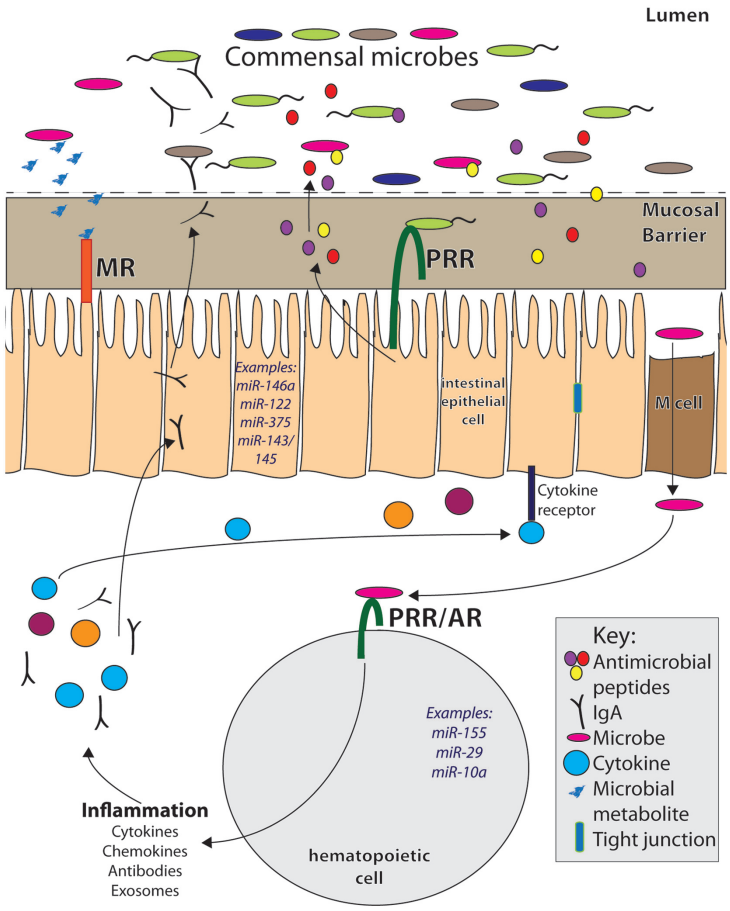

FIGURE 1 | MicroRNAs play important roles within the complex intestinal immune system. They can be expressed within hematopoietic cells in response to inflammatory signals from pattern recognition receptors (PRR) and antigen receptors (AR). In this way, miRNAs can regulate immune responses, including secretion of cytokines, chemokines, and antibodies, all of which affect intestinal homeostasis. Within intestinal epithelial cells (IECs) and other non-hematopoietic intestinal cells, miRNAs are expressed in and regulate pathways involved in secretion of antimicrobial peptides, cell renewal, and barrier permeability, among others. They also may mediate host responses to microbial metabolites, which signal to host cells via metabolite receptors (MR). Many immune signals that induce miRNAs and immune responses within the gut come from the microbiota. Altered commensal populations may mediate different miRNA responses and functions within the intestinal tract.

that mediate crosstalk between host cells and microbiota remain enigmatic.

MicroRNAs (miRNAs) have recently emerged as important mediators of immune development and responses. miRNAs are short (21–25 nucleotide), non-coding RNA molecules that are most commonly transcribed by RNA polymerase II and processed by proteins such as Drosha and Dicer (Winter et al., 2009). In their mature, RNA-induced silencing complex (RISC)-bound form, miRNAs bind to and downregulate expression of target mRNAs by degradation and/or blocking of translation. miRNAs have been demonstrated to regulate immune responses by modulating gene expression of immune-related genes. In this way, they modulate the balance between effective inflammatory responses to foreign entities and proper resolution of inflammation to

prevent tissue damage. More than 100 miRNAs are expressed within leukocytes (O'Connell et al., 2010), and many of these miRNAs have multiple targets within immune-related pathways, consistent with an important role in the immune system (Baltimore et al., 2008; Lindsay, 2008; O'Connell et al., 2010, 2012; Chen et al., 2013). Some of the most well-studied miRNAs in the immune system include miR-155, miR-146a, miRs-17∼92, and miR-181a (Baltimore et al., 2008). Many of these miRNAs regulate key signaling pathways such as Jak/Stat, TLR/MyD88, NF-κB, and Akt, which are essential for immune responses (O'Connell et al., 2012). Some miRNAs, such as miR-155, target negative regulators of the immune response to promote inflammation. Conversely, others, like miR-146a, target positive regulators of immunity to promote tolerance and resolution of responses. Despite the large amount of work done over the past decade, not all targets and functions of miRNAs in the immune system, including well-studied miRNAs, have been worked out. Furthermore, even less is known about miRNAs within the intestinal immune system.

microRNAs modulate expression of genes involved in microbial recognition and downstream immune activity, and thus may play an important role within the intestinal immune system during its interactions with the gut microbiota. Their expression in various intestinal cell types, including hematopoietic-derived leukocytes, IECs, and other specialized gut cell types points to a role in mediating homeostasis with commensal microbes and overall intestinal health (**Figure 1**). These non-coding RNAs function as rheostats to the immune response as opposed to binary switches as they act to adjust the magnitude of gene expression. miRNAs also work in feedback loops, ensuring that the immune system does not produce inappropriately strong responses while also promoting protective immunity when needed. In this way, miRNAs themselves can be considered mediators of homeostasis within the immune system as they act to buffer inflammation. Immune processes within the GI tract are coordinated differently when compared to other sites in the body as a result of regulatory mechanisms required to handle the constant exposure to microbes. Intestinal homeostasis is sensitive to even small disruptions, and therefore miRNAs make excellent candidate molecules for fine-tuning responses at this sensitive site. Multiple studies are beginning to demonstrate the importance of miRNAs within the intestine and identifying how the microbiota influences miRNA expression and function within the GI tract; some of this work will be reviewed here (**Table 1**).

miRNAs IN HEMATOPOIETIC-DERIVED GUT IMMUNE CELLS

The function of miRNAs within hematopoietic-derived immune cells has been well-studied, and it is now clear that miRNAs are deeply integrated into the molecular networks that govern mammalian immune responses (Lindsay, 2008; O'Connell et al., 2010, 2012; Chen et al., 2013). However, relatively few studies have examined the functions of miRNAs in leukocytes within the GI tract, where unique subsets of immune cells are known to reside. Recent investigations indicate that miRNAs also play important roles in hematopoietic cells within the intestines. For example, miR-155 was found to be required for protection during mucosal infection by the intestinal pathogen *Citrobacter rodentium* (Clare et al., 2013). miR-155-deficient mice displayed a higher pathogen burden within the gut, a delayed ability to clear the bacteria, and a worsened colitis phenotype as a result of infection. Interestingly, this phenotype was attributed to a defective intestinal humoral response due to a loss of miR-155 in B cells. This study demonstrates that miR-155 regulates gut B cells in a manner similar to its functions in B cells found in other microenvironments (Thai et al., 2007; Vigorito et al., 2007); however, a

Table 1 | Selected summary of studied miRNAs and their roles in the intestinal immune system, reviewed in this paper.

miRNA	Intestinal role or effect	Compartment and/or cell types involved	miRNA target(s)	Reference
miR-155	Induced by TGFβ, decreased IL-2 and IFNγ expression in lamina propria	LP T cells	InducibleT cell kinase (ITK)	Das et al. (2013)
miR-29	Decreased IL-23/Th17 gut responses, protected from colitis	Dendritic cells	IL-12p40	Brain et al. (2013)
miR-10a	Maintained Treg lineage and prevented plasticity to other T cell subsets	Peyer's patch T cells	Bcl6	Takahashi et al. (2012)
miR-146a	Reduced inflammation during intestinal ischemia reperfusion injury	Intestinal epithelial cells	IRAKI	Chassin et al. (2012)
miR-122	Increased intestinal permeability when induced with TNFa	Intestinal epithelial cells	Occludin	Ye et al. (2011)
miR-124	Associated with protection from pediatric UC	Human colonocytes	Stat3	Koukos et al. (2013)
miR-21	Overexpressed in intestinal disease, knockout mice protected from colitis	Whole murine colon	Unknown	Shi et al. (2013)
miR-143/ mIR-145	Intestinal epithelial regeneration following tissue injury (DSS)	Mesenchymal cells	Igfbp5	Chivukula et al. (2014)

different study displayed a unique role for miR-155 in the gut. Upon exposure to TGFβ, miR-155 was found to be significantly upregulated in LP T cells, contrasting with only a modest induction of miR-155 when peripheral T cells were treated with TGFβ (Das et al., 2013). Additionally, while miR-155 is known to promote inflammatory responses in many contexts, Das et al. (2013) observed immune-inhibitory effects of miR-155 in LP T cells, as evidenced by their intrinsic downregulation of both IL-2 and IFNγ expression upon induction of miR-155 by TGFβ. Importantly, gain and loss of function approaches revealed that miR-155 is indeed a repressor of these cytokines in LP T cells and identified a prominent target of miR-155 in LP T cells as inducible T-cell kinase (ITK), which normally promotes development and effector function of T cells. This work provides evidence that miR-155 may function uniquely within intestinal LP T cells, in contrast to its well-studied roles in T cells found at other anatomical locations.

A unique role for miR-29 was recently demonstrated in dendritic cells of the intestine. In this study, the intracellular pattern recognition receptor NOD2 induced expression of miR-29 in dendritic cells (Brain et al., 2013). miR-29 was found to directly target IL-12p40, which led to the downregulation of IL-23 and Th17 responses in the gut. Consequently, mice lacking miR-29 displayed worsened microbial-dependent colitis compared to WT controls. These data characterized a gut-specific role for miR-29, which had previously been shown to target genes involved in proliferation, differentiation, and fighting bacterial infections (Ma et al., 2011). These findings provide additional evidence that miRNA functions can be unique within the intestinal microenvironment compared to their functions in peripheral sites, and this may also involve their expression and function in distinct cell types.

miR-10a, a miRNA largely involved in embryonic development (Lund, 2010), has also been shown to play a distinct role within in the gut. In Peyer's Patches, which are located throughout the small intestine, miR-10a was highly expressed within regulatory T cells (Tregs; Takahashi et al., 2012). Expression of this miRNA within inducible Tregs in the Peyer's Patch constrained their plasticity and prevented conversion into T follicular helper (Tfh) cells. Takahashi et al. (2012) demonstrated that miR-10a maintained the Treg lineage within the GALT by targeting and downregulating the Tfh master regulator gene, Bcl6. The biological significance of miR-10a function within Peyer's Patch Treg cells appears to be the maintainance of tolerance, and mutations that impair this function may predispose individuals to intestinal disease. Future work will determine if the role of miR-10a in preventing Treg plasticity is unique to gut-expressed Tregs or broadly applicable to Tregs found at extra-intestinal sites.

Altogether, relatively few studies have examined gut-specific roles of well-known hematopoietic miRNAs, potentially due to assumption that their known functions in peripheral sites will carry over into the GI tract. While this may be true in some cases, it is possible that expression levels, targets, and the overall functions of miRNAs are distinct in the intestine versus extra-intestinal sites in the body. Furthermore, miRNAs that appear to play little or no roles in the systemic immune system may prove to have exclusive functions in unique populations of leukocytes within the gut. Because hematopoietic-derived immune cells interact heavily with the microbiota within the intestinal environment, understanding the roles of miRNAs within gut leukocytes will be key to understanding molecular pathways in which the host and microbiota interact, and this may differ from how a given miRNA functions in extra-intestinal sites.

miRNAs IN NON-HEMATOPOIETIC-DERIVED GUT CELLS

Intestinal epithelial cells (IECs) are now recognized as non-hematopoietic cells that exhibit immunological functions, including the ability to directly recognize microbial products via PRRs (Vaishnava et al., 2008) and to secrete mucus (goblet cells) and antimicrobial peptides (paneth cells) in response to these microbes. As miRNAs regulate many immunological and developmental pathways, they may have significant functional relevance within IECs.

Generally, disruption of miRNA processing pathways in the intestinal epithelium results in susceptibility to intestinal infection. This was demonstrated through the specific deletion of *Dicer1* within IECs in mice (Dicer1$^{loxP/loxP}$; Villin-Cre, referred to as Dicer1ΔIEC mice; McKenna et al., 2010; Biton et al., 2011). As Dicer is required for the processing of miRNAs into their mature form, its deletion abolishes miRNA function. These studies found that the deletion of Dicer from IECs results in mice with impaired growth, metabolism, and water retention. Additionally, Dicer1ΔIEC mice displayed a reduction in goblet cells, an increase in infiltrating inflammatory immune cells, and disorganized intestinal architecture. These mice also had increased intestinal permeability, decreased mucus production, and decreased Th2 cytokines and factors (Biton et al., 2011). Consequently, Dicer1ΔIEC mice were more susceptible to *Tricuris muris* infection and displayed IBD symptoms and inappropriate Th1 responses during infection. Finally, Biton et al. (2011) revealed that miR-375 was a critical IEC-expressed miRNA that was required for proper IEC differentiation and that it mediated communication with T cells to induce Th2 responses when appropriate.

One miRNA that appears to have similar targets in IECs as compared to other hematopoietic cell types is miR-146a. miR-146a directly targets essential TLR downstream signaling genes including TNF receptor associated factor 6 (TRAF6) and interleukin1 receptor-associated kinase 1 (IRAK1) to downregulate inflammation (Boldin et al., 2011; Zhao et al., 2011, 2013). In a mouse model of intestinal ischemia/reperfusion (I/R) injury, Chassin et al. (2012) showed that IEC expression of IRAK1 led to increased inflammation and tissue damage. When miR-146a was induced in IECs *in vitro*, expression of inflammatory chemokines and the effects of hypoxia were reduced. *In vivo*, miR-146a was directly injected into mice or its expression induced by DIM, and mice showed reduced disease and inflammation during I/R injury due to miR-146a targeting and downregulating expression of IRAK1 in IECs. Utilizing conditional knockout mice to examine the roles of IRAK1 and miR-146a specifically in IECs during this intestinal injury model, as well as in other intestinal disease models, would bring about further understanding of the cellular basis of miR-146a function in the gut.

microRNAs within non-hematopoietic cells may also a play a role in the development of and protection from intestinal cancers. The epithelium requires proper signaling mechanisms for normal development, differentiation, renewal, and repair of the intestinal tissue (Chivukula et al., 2014). Many of these signaling pathways require regulation by miRNAs, and colorectal cancer (CRC) can arise if dysregulated. One study found that miR-143 and miR-145, which are abnormally expressed during human CRC, are required for proper regeneration of IECs (Chivukula et al., 2014). When miRs 143 and 145 were deleted, mice were unable to undergo proper intestinal renewal and wound healing. The authors found that these miRNAs were expressed specifically within the intestinal mesenchyme and that deletion of miRs 143 and 145 from mesenchymal tissue resulted in a similar phenotype. Consistent with this, these miRNAs were found to target IGFBP5, an inhibitor of IGF signaling, which is required for regeneration of IECs. Thus, miRNAs expressed specifically within non-hematopoietic-derived mesenchymal cells are required to maintain intestinal regeneration and their dysregulation could result in improper growth and proliferation, potentially giving rise to intestinal cancers.

Another example, in which known miRNA functions are relevant in IECs, is the interplay of the RNA-binding protein LIN28 and the Let7 family of miRNAs. Let7 miRNA processing has been shown to be inhibited by LIN28 in models of cellular reprogramming, growth, and oncogenesis (Viswanathan and Daley, 2010). One group examined this interaction in the context of the intestine and found that overexpression of LIN28B in IECs led to abnormal intestinal architecture, crypt expansion, Paneth cell loss, and formation of intestinal tumors. (Madison et al., 2013). A similar phenotype was seen in miR-Let-7c2 and Let-7b deficient mice, indicating a disease-promoting role of LIN28B in targeting and degrading these miRNAs. Expression of Let-7 in the intestine reversed the hyperplasia and Paneth cell loss seen in the LIN28B overexpressing mice. This study indicates that Let-7 miRNAs are important for preventing overgrowth of IECs downstream of the oncogenic RNA-binding protein LIN28B. Expression or modulation of this miRNA family could either prevent or cause CRCs.

The ability of IECs to form tight junctions is important in creating an effective barrier between the host and commensal microbiota. Defective intestinal tight junction barriers are seen in IBD and other intestinal diseases and result in increased inflammation (Ye et al., 2011). Recently, miRNAs have been shown to play a role in regulating intestinal epithelial tight junction permeability. For example, Dicer1ΔIEC mice showed decreases in cells expressing the epithelial tight junction protein, claudin, and a weakened barrier (McKenna et al., 2010). In another study, stimulating IECs with TNFα *in vitro* led to increased expression of miR-122 (Ye et al., 2011). miR-122 directly targets occludin mRNA, which encodes a protein that forms intestinal tight junctions. Thus, expression of miR-122 increased intestinal epithelial permeability. Mice overexpressing miR-122 displayed decreased intestinal barrier function as a result of downregulated occludin expression. In this way, miR-122 may be a valid target in diseases involved in intestinal barrier and permeability, such as IBD.

Altogether, miRNAs play pivotal roles in IECs and other non-hematopoietic-derived intestinal cells, which can contribute to the protection of the host tissue from pathogenic invaders and disease. As common immunological pathways are essential within IECs, miRNAs that regulate these pathways also have relevance within these non-hematopoietic cells. For example, conditional deletion of the TLR adaptor protein MyD88 within IECs resulted in loss of barrier function and defective immunity from commensal bacteria (Vaishnava et al., 2011; Frantz et al., 2012). Thus, miRNAs that have known targets in immune signaling pathways downstream of MyD88 can also play important regulatory roles within IECs. Generally, based on the above studies and others, the expression and function of miRNAs within non-hematopoietic cells of the intestinal immune system play a large role in maintaining intestinal homeostasis.

GUT MICROBIOTA AND miRNAs

In discussing the functions and features of the mammalian intestine, one cannot ignore the large contribution to host physiology by the gut microbiota. It is estimated that approximately one hundred trillion commensal organisms reside within the human GI tract (Phillips, 2009; Hooper and Macpherson, 2010). These microbes are extremely diverse in their taxonomy, communities, and functions. The gut microbiota communicates directly with the host via the production of metabolites, peptides, and other signaling molecules. MiRNA expression by the host may play a significant role in determining how microbiota-produced signals are received by the host, and miRNAs may balance the fine line between maintaining an effective barrier and preventing inappropriate inflammation in response to the microbiota.

It is known that the microbiota modulates expression of host genes, as expression profiles of WT germ-free (GF) mice are markedly different from WT specific pathogen free (SPF) mice (El Aidy et al., 2013). New studies are beginning to examine whether this communication between host and microbiota involves miRNAs. In one such study, GF mice were colonized with microbiota from SPF mice and their miRNA profile was examined via microarray (Dalmasso et al., 2011). Nine miRNAs were differentially expressed in the ileum and colon of SPF-colonized compared with uncolonized GF mice. This differential expression of miRNAs within the mouse gut was predicted to alter the expression of hundreds of miRNA target genes. For example, miR-665, which was downregulated in SPF-colonized mice compared with GF mice, targeted the Abcc3 gene (an ATP-binding cassette transporter) in the colon. In a similar study, the differences in miRNA expression within the cecum between GF and SPF mice were examined (Singh et al., 2012). Sixteen miRNAs were expressed differentially between GF and SPF mice. Upon analyzing networks of genes involved in intestinal barrier function that may be regulated by these miRNAs, the authors propose that the gut microbiota modulates host miRNA genes, which then target and regulate the intestinal barrier.

In another investigation, the role of the microbiota in regulating host miRNA expression was examined in the context of *Listeria monocytogenes* infection (Archambaud et al., 2013). GF mice had a greater *Listeria* burden when compared to infected

SPF mice in multiple tissues. The authors hypothesized that these higher bacterial counts were due to the lack of microbiota, which prime host immune responses via changes in gene expression. They performed gene expression analysis of protein-coding and miRNA genes in SPF and GF infected and uninfected mice and observed that five miRNAs were downregulated in SPF mice during infection but not in GF mice. These included miR-378 and miR-200c. The downregulation of these miRNAs due to the presence of microbiota led to increased expression of several protein-coding genes that were predicted targets of these miRNAs. Using the set of differentially expressed target mRNA and their corresponding miRNAs, the authors revealed a miRNA-mRNA network in which microbiota-mediated miRNA expression primed the intestinal immune system to strengthen the barrier and combat *Listeria* infection. Additionally, the authors defined the ten miRNAs, including miR-143 and miR-215, that are most highly expressed within the ileum of SPF and GF mice before and during infection. These miRNAs may be considered ileal "signature" miRNAs.

While the above studies highlight potential roles for specific miRNAs in host-microbiota interactions, further investigation is necessary to reconcile differences between the studies and to find consensus miRNAs that may be more significantly involved in crosstalk between host gut tissue and the microbes that reside in this locale. So far, studies have examined miRNA expression profiles within varied intestinal sections; the microenvironments of these sections are largely different when compared to one another. Furthermore, these tissues contain a heterogeneous population of cells, which may not reveal miRNAs involved in host–microbial interactions within specific intestinal cell types. Utilizing cell sorting and RNA-sequencing to analyze gene expression may identify miRNAs that are differentially expressed within specific and/or rare gut cell types upon exposure to microbes. Additionally, further experimentation is necessary to understand cellular mechanisms and biological importance of the candidate miRNAs uncovered in these studies.

Some work has also been done to begin defining specific mechanisms by which miRNAs are induced by products of the microbiota. In one such investigation, expression of a subset of miRNAs was downregulated in human colon cells treated with butyrate, a beneficial short-chain fatty acid (SCFA) derived from commensal bacteria (Hu et al., 2011). These miRNAs, particularly those of the miR-106b family, were conversely expressed at high levels in colons of human colon cancer patients. Butyrate blocked expression of the miR-106b family to allow for increased expression of p21 in a miRNA-dependent manner. p21 is a cell cycle arrest protein important in preventing various cancers. Importantly, miR-106b reversed the anti-proliferative effects of butyrate via direct targeting of p21. Thus, a product of the microbiota regulates gene expression of host miRNAs to prevent colonic disease and cancer. The cellular mechanism by which butyrate directly modulates miRNA gene expression has yet to be investigated.

Another study demonstrated that miR-10a is highly expressed in the intestines and can be modulated by the gut microbiota through TLR signaling on dendritic cells, which suppresses expression of miR-10a (Xue et al., 2011). In experiments characterizing relevant targets and pathways, miR-10a directly targeted and downregulated expression of IL-12/23p40, an important cytokine for innate inflammatory responses in the GI tract. Mice with colitis had decreased expression of miR-10a and thus high levels of IL-12/23p40. In this instance, the microbiota signals to the host to downregulate expression of miR-10a, which targets a portion of host innate immunity. Another miRNA, miR-107, targeted a component of the IL-23 receptor, IL-23p19 (Xue et al., 2014). Like miR-10a, the expression of miR-107 was downregulated by the microbiota, as GF mice showed high levels of miR-107, which was decreased following microbial colonization. Mice with colitis displayed low levels of miR-107 and high levels of IL-23p19, indicating the importance of this miRNA in downregulating inflammation in response to commensals. Proper crosstalk between the gut microbiota and miR-107 in intestinal immune cells, which targets IL-23p19, can play a role in maintaining homeostasis.

Overall, signals from the microbiota have the ability to alter expression of miRNAs. In turn, miRNAs can target immune-related mRNAs that have the ability to impact responses to microbes and thus shape commensal communities. Thus, crosstalk between microbiota and miRNAs is required for shaping intestinal immune responses and maintaining homeostasis.

miRNAs AND HUMAN INTESTINAL DISEASE

Many human diseases are related to dysregulation of the intestinal immune system and of the microbiota, including allergies, various autoimmune diseases, and cancers (Round and Mazmanian, 2009; Kamada et al., 2013; Sears and Garrett, 2014). One of the most prominent and well-studied class of intestinal disease is inflammatory bowel disease (IBD). IBD is caused by chronic and inappropriate inflammation within the gastrointestinal tract, and this affects approximately 1.4 million Americans. Furthermore, CRC risk is significantly increased in patients that have IBD (Neurath, 2014). Together, this has placed a considerable burden on the healthcare system (Abraham and Cho, 2009). The most common types of IBD are Crohn's Disease (CD) and ulcerative colitis (UC), and each of these display different causes and pathologies. Although genetic and environmental factors are known to play a role in the etiology of IBD subtypes, the specific factors that directly cause these diseases are not fully understood. Furthermore, CD, UC, and other intestinal diseases appear to display various pathologies, outcomes, and treatment options depending on the individual (Dalal and Kwon, 2010), emphasizing the need for more diagnostic and therapeutic reagents in the clinic.

Recently, miRNA expression profiles have been shown to change in people suffering from IBD. Patients with UC and CD have distinct miRNA profiles that are unique to their disease stage when compared with healthy controls. This is true for both intestinal tissue biopsies and peripheral blood (Dalal and Kwon, 2010; Wu et al., 2011; Pekow and Kwon, 2012; Iborra et al., 2013; Lin et al., 2014). Among the miRNAs found to have altered expression during UC and CD were miR-16 (Wu et al., 2010), miR-146a (Lin et al., 2014), miR-31 (Lin et al., 2014), miR-340* (Wu et al., 2011), and miR-199a-5p (Wu et al., 2011). Some of these miRNA expression profile studies during IBD have been

summarized in two recent reviews (Dalal and Kwon, 2010; Pekow and Kwon, 2012). It is important to note that each study has sampled a different group of patients, disease types and states, as well as tissue types. This likely explains why there does not appear to be a common group of miRNAs that are associated with IBD across all studies. Future work must be done to reconcile the differences in miRNA profiles in each study and to clarify which miRNAs are common or unique to distinct disease types. These miRNA profiles might be used to accurately diagnose IBD types and predict patient prognoses. Additionally, the concept of utilizing blood miRNAs as biomarkers for intestinal disease is promising because collection would be non-invasive. Even so, further experimentation needs to be done to understand the functional significance of altered expression of specific miRNAs in various human tissues and cell types during disease.

The roles of some miRNAs and their targets during IBD and intestinal disease are beginning to be elucidated. For example, during active pediatric UC, levels of phosphorylated Stat3 were increased in colonocytes, and correlated with low expression of miR-124 in diseased patients (Koukos et al., 2013). Furthermore, miR-124 directly bound to and downregulated expression of Stat3. High levels of Stat3 and corresponding low levels of miR-124 were only seen in pediatric patients with active UC and not in adults, pediatric CD, pediatric inactive UC, nor healthy patients. This was also observed in mouse models of colitis. Dysregulation of the Stat3 pathway occurs in diseased patients due to hypermethylation of miR-124. This study implicates miR-124 and the Stat3 pathway as highly relevant potential therapeutic targets for treatment of pediatric UC. Even so, whether downregulation of miR-124 via hypermethylation is a cause or effect of IBD in children has not yet been elucidated. In another study, Brain et al. (2013) showed that human CD patients with polymorphisms in the NOD2 gene were unable to upregulate expression of miR-29. In this way, miR-29 could not repress downstream cytokine signaling pathways that were attributed to worsened disease. In general, these findings support the validity of miRNAs and their experimentally verified targets as important players in human IBD.

Single nucleotide polymorphisms (SNPs) in miRNA genes may also play a role in susceptibility to intestinal diseases (Pekow and Kwon, 2012; Gazouli et al., 2013). SNPs within miRNAs could alter their expression, processing, and functional targeting. Thus, the presence of certain miRNA SNP variants in humans can be associated with and may even be functionally relevant (Jin and Lee, 2013). The rs2910164 SNP within the miR-146a gene on chromosome 5 has been shown to decrease mature miR-146a levels and subsequently increase its target genes (Jazdzewski et al., 2008; Shao et al., 2014). This miRNA SNP has been implicated in numerous human diseases, including various cancers (He et al., 2012), papillary thyroid carcinoma (Jazdzewski et al., 2008), and sepsis (Shao et al., 2014). Even so, a general consensus within studies and meta-analyses regarding disease associations with rs2910164 has not been reached, as group size, methods of statistical analysis, and the ethnic groups sampled can affect the conclusions made in each study. In relation to the GI tract, this miR-146a SNP was associated with CD (Gazouli et al., 2013), while no significant association was observed with UC patients (Okubo et al., 2011; Gazouli et al., 2013). Patients with this SNP variant were also found to have increased risk of intestinal metaplasia and dysplasia during *H. pylori* infection (Song et al., 2013). Furthermore, this polymorphism in miR-146a predicted susceptibility to CRC and disease-specific survival outcome (Chae et al., 2013; Ma et al., 2013). MiR-146a downregulates inflammation by targeting components of the NFκB pathway (Boldin et al., 2011; Zhao et al., 2011, 2013), and these studies suggest that a change in one nucleotide within this miRNA could alter the intestinal inflammatory state via dysregulation of NFκB and other related pathways. However, the functional consequences of this polymorphism remain incompletely understood. Taken together, these findings provide evidence that the altered function of immune-related miRNA genes can influence the susceptbility to, and outcome during, intestinal diseases.

As IBD patients carry an increased risk of developing CRC, miRNAs also have potential as biomarkers, prognostic tools, and therapeutic targets during cancers of the GI tract. While multiple factors may influence CRC, miRNAs have been shown to play important roles in the molecular pathways that can give rise to intestinal cancers. These include pathways involved in inflammation, chromatin formation, stem cell signaling, apoptosis, and others (Liu and Chen, 2010; Chivukula et al., 2014). Expression profiling has indicated that miR-31, miR-21, and miR-191 are upregulated (Liu and Chen, 2010), while miR-143, miR-145, and miR-451 are downregulated in CRC tumors (Liu and Chen, 2010; Chivukula et al., 2014). Another study, using an RNA-sequencing approach, found that miR-10a-5p, miR-21-5p, miR-22-3p, miR-143-3p, and miR-192-5p are among the most abundantly expressed miRNAs in a CRC cohort (Schee et al., 2013). A pathway analysis revealed that these altered RNAs could affect many cellular signaling pathways including Wnt, MAPK, and TGFβ, all of which are linked to oncogenesis. Animal studies utilizing genetic manipulation of miRNAs within the intestines are currently being carried out to understand cellular mechanisms of CRC-related miRNAs that have been found in human studies, and a recent report has found critical roles for miRNAs 143 and 145 in pathways that can lead to CRC (Chivukula et al., 2014). Although much work remains, it has become clear that miRNAs within cells of the intestines are relevant during human IBD, CRC, and other intestinal diseases that stem from perturbations in intestinal homeostasis. This underscores the importance of miRNAs in maintaining intestinal homeostasis in humans.

FUTURE DIRECTIONS AND CONCLUDING REMARKS

In general, these investigations suggest that miRNAs are likely indispensable regulators of host-commensal interactions that are required for proper intestinal homeostasis. Because miRNAs fine-tune targets by downregulating their expression 1.2–4-fold (O'Connell et al., 2012), they prevent imbalances that eventually lead to loss of homeostasis and disease. miRNAs may also prove to be effective drug targets in terms of treating intestinal diseases. Extensive future studies are required to further define specific mechanisms of miRNA function in intestinal immunity.

This will unveil how miRNAs influence the balance between maintaining beneficial resident microbes and eliminating those that are harmful within the gut.

Tying together emerging concepts in the miRNA field with those in the GI tract and microbiota fields will bring about a comprehensive understanding of cellular processes that control intestinal homeostasis. One of these novel concepts is the transfer of miRNAs between cells via exosomes (Stoorvogel, 2012). In the future, it will be important to determine if this mode of exosomal miRNA transfer occurs and has biological relevance within the intestines to modify gut homeostasis and commensal populations. Another emerging concept regarding the microbiota involves the important role of gut commensals in contributing to extra-intestinal effects. For example, recent studies have determined that the gut microbiota can impact the biology of extra-intestinal sites, such as the brain, heart, and liver and as a consequence can influence diseases including MS, autism, cardiovascular disease, and obesity (Wang et al., 2011; Collins et al., 2012; Hsiao et al., 2013; Kamada et al., 2013; Zhao, 2013); miRNAs may play important roles in the communication process between host and microbiota at extra-intestinal sites. miRNAs may also function in cells as they respond to gut microbial metabolites and other products. Commensal-derived metabolites, such as short-chain fatty acids (SCFAs) and bile acids, play important roles in development and function of immune cells within the gut and can contribute to immunological and metabolic phenotypes within the host (Brestoff and Artis, 2013).

miRNAs and host-commensal interactions have been independently linked to human development, health, and disease in the past. Now, as we begin to explore how these systems are integrated, a greater understanding of how host-commensal interactions are regulated will undoubtedly emerge. This will provide novel therapeutic insights that will help combat the wide range of diseases that are related to the gut.

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CHAPTER 3

MICRORNA-146A CONSTRAINS MULTIPLE PARAMETERS OF INTESTINAL IMMUNITY AND INCREASES SUSCEPTIBILITY TO DSS COLITIS

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MicroRNA-146a constrains multiple parameters of intestinal immunity and increases susceptibility to DSS colitis

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ABSTRACT

Host-microbial interactions within the mammalian intestines must be properly regulated in order to promote host health and limit disease. Because the microbiota provide constant immunological signals to intestinal tissues, a variety of regulatory mechanisms have evolved to ensure proper immune responses to maintain homeostasis. However, many of the genes that comprise these regulatory pathways, including immune-modulating microRNAs (miRNAs), have not yet been identified or studied in the context of intestinal homeostasis. Here, we investigated the role of microRNA-146a (miR-146a) in regulating intestinal immunity and barrier function and found that this miRNA is expressed in a variety of gut tissues in adult mice. By comparing intestinal gene expression in WT and miR-146a-/- mice, we demonstrate that miR-146a represses a subset of gut barrier and inflammatory genes all within a network of immune-related signaling pathways. We also found that miR-146a restricts the expansion of intestinal T cell populations, including Th17, Tregs, and Tfh cells. GC B cells, Tfh ICOS expression, and the production of luminal IgA were also reduced by miR-146a in the gut. Consistent with an enhanced intestinal barrier, we found that miR-146a-/- mice are resistant to DSS-induced colitis, a model of Ulcerative Colitis (UC), and this correlated with elevated colonic miR-146a expression in human UC patients. Taken together, our data describe a role for miR-146a in constraining intestinal barrier function, a process that alters gut homeostasis and enhances at least some forms of intestinal disease in mice.

INTRODUCTION

The mammalian intestine contains a variety of cell types that coordinate complex processes in order to maintain a barrier between host tissues and resident microbes within the gastrointestinal (GI) tract. An appropriate balance of pro-inflammatory responses to support the barrier, along with mechanisms to ensure the proper degree of immunotolerance, must be achieved to receive the benefits provided by the microbiota while continuing to protect the host from microbial invasion. In order to maintain homeostasis, immunological processes within the gut require proper modulation, as dysregulation has been shown to cause infection, dysbiosis, cancer, and/or autoimmunity [1-3]. Among these unwanted outcomes is inflammatory bowel disease (IBD), which manifests itself as Crohn's Disease (CD) and Ulcerative Colitis (UC) in human patients. IBD affects millions of people around

the world, producing a substantial burden on healthcare systems [4]. Identifying risk factors, providing an accurate prognosis, and effectively treating this condition all remain challenging due the complexity of the environmental, genetic, and cellular factors involved in the development of IBD. However, these may be improved as we continue to identify and characterize regulatory mechanisms at play within the gut.

A variety of cell types are involved in promoting intestinal homeostasis, and are made up of both hematopoietic-derived leukocytes and cells of the non-hematopoietic lineage, including intestinal epithelial cells (IECs). Among the immune cells involved, specific T cell lineages play a large role in influencing gut responses. Regulatory T cells (Tregs) are required to maintain tolerance and downregulate gut immune responses [1, 5]; T follicular helper (Tfh) cells interact with B cells in the intestine to help produce antigen-specific antibodies, including IgA, against intestinal microbes to maintain barrier [6-8]; and Th1 and Th17 cells produce specific cytokines to coordinate responses against invading microbes [9, 10]. Th1 cells have been associated with Crohn's Disease in humans, while Th17 cells are implicated both in intestinal tissue damage and in intestinal tissue repair and homeostasis in other contexts [9-12]. The non-hematopoietic derived IECs maintain homeostasis by producing mucus, antimicrobial peptides, and other factors that promote tissue repair, tight junctions, and bacterial targeting [13, 14]. Both T lymphocytes and IECs express cell surface receptors, such as TLRs, that are able to recognize microbial cues and initiate appropriate responses [13, 15]. Downstream from TLRs, signaling through MyD88 and ultimately NF-κB is critical for the proper function of cells within the gut, as disruption of this pathway results in intestinal disease [16-19]. Thus, the proper control of TLR/ NF-κB signaling is essential to overall gut health.

MicroRNAs (miRNAs) are short, non-protein coding RNAs that have been shown to play significant roles in regulating cellular processes within the immune system. miRNAs function to repress their target mRNA genes by binding the 3'-UTR of targets in a mature, RNA-induced silencing complex (RISC)-bound form. Target genes are downregulated approximately 1.5 to 4 fold by miRNAs [20], in such a way that these noncoding RNAs modulate cellular processes in order to maintain equilibrium and/ or stability [20-23]. This includes miRNAs within the intestine [24], such as miR-143/145, which have recently been shown to promote IEC regeneration during stress [25]. Among important immune system-related miRNAs is microRNA-146a (miR-146a), which is largely expressed within leukocytes and is induced by pattern recognition receptors and cytokine receptors that activate NFκB [26, 27]. Once induced, miR-146a acts as a negative feedback regulator by targeting TNF receptor-associated factor 6 (Traf6) and IL-1 receptor associated kinase 1 (Irak1), both of which link MyD88 to NFκB signaling [23, 28-30]. miR-146a has also been shown to target Signal Transducer and Activator of Transcription 1 (Stat1) in T cells [31]. Recently, Inducible T-cell costimulator (ICOS), as well as other mRNAs involved in Tfh cell and germinal center biology, were shown to be targets of miR-146a [32]. Through these mechanisms, miR-146a is able to prevent excessive inflammation in response to microbial cues.

Although miR-146a plays a protective anti-inflammatory role within systemic compartments, such as the bone marrow, spleen, lymph nodes and joints [23, 27, 29, 33], its relevance within the adult GI tract remains unclear. This miRNA has been implicated in regulating pathways of intestinal disease [30, 34, 35], although studies with miR-146a-/- mice have not yet been performed in this context. Intestinal immunity and barrier function involve unique cell types and processes that are not found or do not occur in other tissues and are in place to regulate the constant exposure to microbial signals that come from resident intestinal commensal organisms. Thus, we investigated whether miR-146a also plays a host-protective role in this context. We report that miR-146a is expressed in the intestines under the steady state and functions to downregulate a subset of genes and immune cells involved in intestinal barrier function. miR-146a-/- mice displayed an altered microbiota and, surprisingly, were more protected from DSS-induced colitis compared to their WT counterparts. *In vivo* bone marrow reconstitutions demonstrated a contribution by hematopoietic-expressed miR-146a in mediating colitis severity. Consistent with a negative role in intestinal disease, miR-146a was elevated in a cohort of patients with IBD compared to healthy controls. Altogether, through its negative regulation of barrier function, miR-146a limits intestinal health during certain types of stress responses.

RESULTS

miR-146a is expressed within the intestines

We characterized the expression profile of miR-146a within gut tissues to begin to identify the cell types in which it may be functioning within the GI tract. miR-146a expression has been well-characterized within hematopoietic cells of the blood, spleen, and bone marrow [36], but its expression within intestinal tissues is not well defined. We found that mature miR-146a is expressed in both the distal colon (C) and in the small intestine (SI) (specifically in the ileum) (Figure 1a). As a control, miR-146a was not detected in intestinal tissues from miR-146a-/- mice. Furthermore, miR-146a expression was compared between small intestinal tissues and adjacent Peyer's Patches (PP), and its levels were similar in both

compartments (Figure 1b), indicating that miR-146a levels are not increased in the lymphocyte-rich Peyer's Patch. Because miR-146a has been shown to be induced by TLR/NFκB signaling [26], which can be activated by the intestinal microbiota, we examined mature miR-146a expression in the intestines of germ-free (GF) versus specific pathogen-free (SPF) mice. Equivalent expression within the small intestines and colon was observed when comparing GF and SPF mice, indicating that the presence of the microbiota has little impact on miR-146a levels in the gut (Figure 1c). To determine expression levels of miR-146a in hematopoietic versus nonhematopoietic cells of the intestines, we performed FACS-sorting of $CD45^+$ and $CD45^-$ cells from mouse colons and small intestines. miR-146a was expressed within $CD45^+$ cells of the small intestine and colon, while much lower expression was observed in $CD45^-$ cells from these tissues (Supplementary Figure 1a and 1b). Altogether, miR-146a is expressed in a variety of intestinal tissues, primarily within cells of the hematopoietic lineage, and this occurs in a microbiota-independent manner.

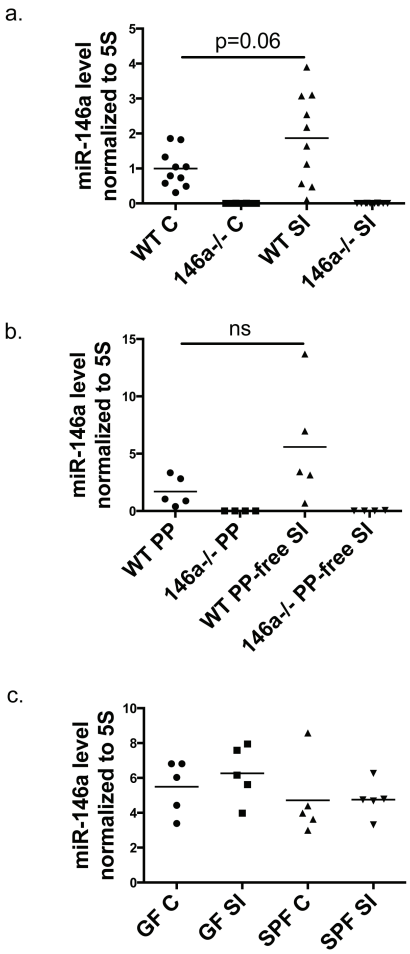

Figure 1: miR-146a is expressed within the intestinal tract. Mature miR-146a RNA expression was measured in whole colonic (C) and ileal (SI) tissue of WT C57BL/6 and miR-146a-/- mice via qRT-PCR **a.** RNA was extracted from whole WT and miR-146a-/- Peyer's Patches (PP) and an adjacent piece of small intestinal tissue lacking a PP, followed by qRT-PCR for mature miR-146a expression **b.** Mature miR-146a expression levels were measured in colons (C) and small intestines (SI) of germ-free (GF) and specific pathogen free (SPF) C57BL/6 mice using qRT-PCR **c.** All miR-146a levels were normalized to 5S rRNA; $n = 10$ (a), $n = 5$ (b), $n = 5$ (c).

miR-146a represses barrier gene expression in the intestines

To begin determining the functional role of miR-146a within the intestines, RNA was collected from the distal portions of the colon and ileum of the small intestines of WT and miR-146a-/- mice, and RNA-seq was performed to examine gene expression changes in an unbiased manner. A majority of the significant alterations in gene expression occurred within the small intestine (Supplementary Figure 2a), while substantially fewer differences were seen within the colon (Supplementary Figure 2b). In the small intestine, 289 genes were upregulated and 77 genes were downregulated greater than two fold (FDR > 10) within the small intestines of miR-146a-/- mice compared with equivalent tissues from WT mice (Supplementary Figure 2a). Among the top upregulated genes in miR-146a-/- small intestines were members of the C-type lectin antimicrobial peptide family Reg3: Reg3α, Reg3β, and Reg3γ (Figure 2a), which are expressed by IECs and function to kill gram-positive bacteria [37-39]. Reg3 proteins have been shown to play an essential role in intestinal barrier function and protection from colitis [40], indicating that miR-146a-/- mice have enhanced gut barrier function. Another highly upregulated gene in the miR-146a-/- small intestine was serum amyloid a 1 (*Saa1*), an acute phase, inflammation-promoting gene [41] that has antibacterial effects and is required for protection from colitis [42]. miR-146a-/- small intestines also had higher expression of a number of genes that produce intestinal mucus, including Muc3, Muc4, and Muc13[43]. Other important intestinal barrier genes, including interferon response genes RNaseL, Oasl1, Nos2 and Ifit1 [44-46], as well as intestinal cell adhesion molecules Ceacam1, Ceacam20, Ceacam18, and Epcam, were expressed at higher levels in miR-146a-/-

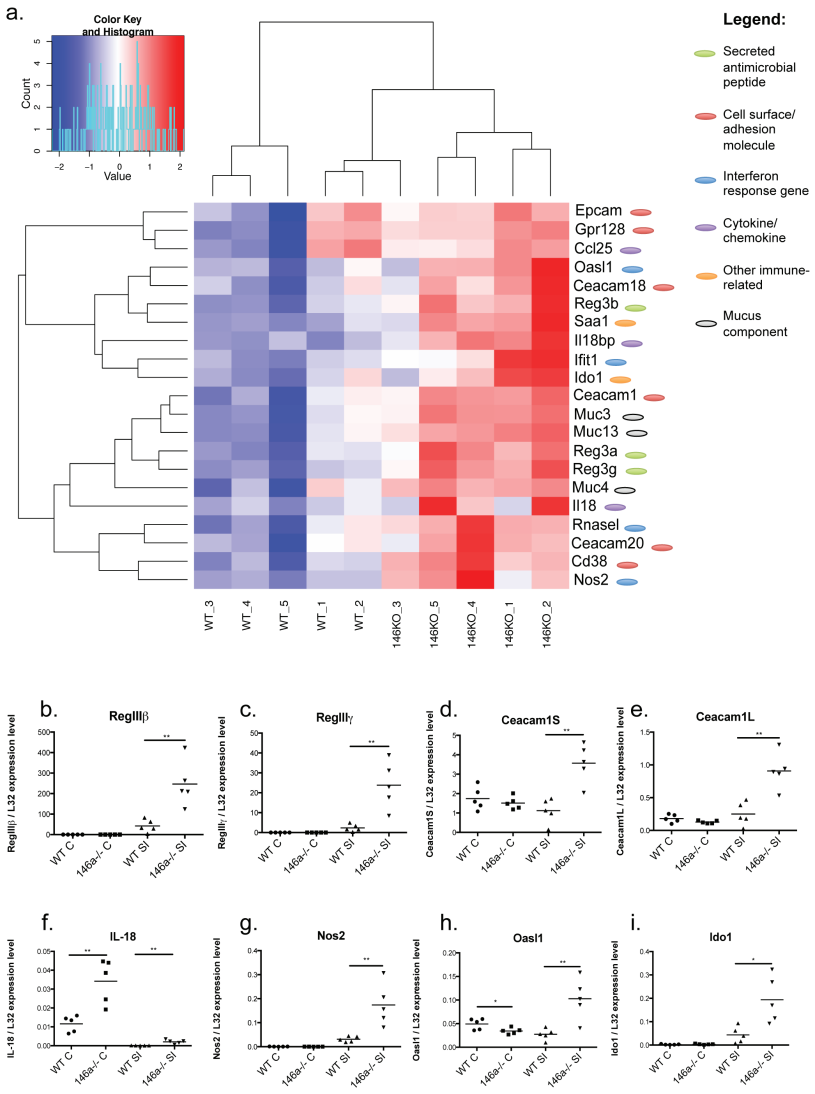

Figure 2: miR-146a regulates expression of genes important for intestinal barrier and homeostasis. RNA-seq was performed on a section of ileal tissue of WT C57BL/6 and miR-146a-/- mice. Heat map indicates fold change in gene expression of selected intestinal barrier genes comparing miR-146a-/- mice with WT, $p < 0.05$, Phred-transformed FDR > 10. Genes are categorized by their known functions within the intestine and/or immune system (antimicrobial peptide, cell surface/adhesion, interferon response, cytokine/ chemokine, immune-related, and mucus component) **a.** Select genes upregulated in miR-146a-/- small intestines and colons, including Reg3β **b.**, Reg3γ **c.**, Ceacam1S **d.**, Ceacam1L **e.**, IL-18 **f.**, Nos2 **g.**, Oasl1 **h.**, and Ido1 **i.**; were confirmed via qRT-PCR normalized to L32. $n = 5$ (a), $n = 5$ (b-i).

small intestines compared to WT [47-49]. Futhermore, a subset of genes that make up epithelial cell junctions and enhance the intestinal barrier, including claudins, occludin, and e-Cadherin [50], were modestly upregulated in mice lacking miR-146a (Supplementary Figure 3b). Also of note, the cytokine IL-18 and regulator protein IL18bp, in addition to the enzyme Ido1, were at higher levels in the absence of miR-146a. Increased IL-18 [51, 52] and Ido1 [53] levels have been shown to play a protective role in mice during experimental colitis. Several of these gene expression changes within the small intestine were confirmed via qRT-PCR including Reg3β, Reg3γ, Ceacam1 (short and long isoforms), IL-18, Nos2, Oasl1, and Ido1 (Figure 2b-i). Also of interest, IL-18 expression was found to be significantly upregulated in the colons of miR-146a-/- versus WT mice (Figure 2f). Using Targetscan and miRTarBase, 18 predicted and confirmed miR-146a targets were found to be derepressed within whole ileal tissues of miR-146a-/- mice compared with WT controls (Supplementary Figure 3a). Derepression of the confirmed miR-146a target NUMB correlated with decreased expression of downstream Shh signaling genes (Supplementary Figure 3c), which can play a role in IBD pathology [35]. Altogether, these data demonstrate that miR-146a functions to repress genes involved in intestinal barrier function and immunoprotective inflammation.

In order to identify signaling pathways that miR-146a may be directly regulating within the small intestine, we performed a pathway analysis of the RNA-seq data using the IPA software program from Ingenuity. Among the top upstream regulators predicted to be activated in the absence of miR-146a were the cytokines IL-18 and IL-22; the IL-1R, IL-18R and TLR adaptor protein MyD88; the transcription factors NFκB (RelA), Stat1, and Stat3; and type I and II interferons IFNβ and IFNγ (Supplementary Figure 4a). This predicted enhancement in signaling by MyD88 and NF-κB, as well as enhanced interferon and Stat1 signaling, is consistent with previous data demonstrating that miR-146a can directly repress Traf6 and Irak1 (both downstream from MyD88 and upstream of NFκB) [26,27,29] as well as Stat1 (IFN pathway) [31]. Further, Ingenuity predicted that miR-146a-/- mice should be resistant to inflammatory bowel disease (IBD) and colitis (Supplementary Figure 4b). Based upon these results and on previous findings that MyD88 [16, 18, 38, 54] and downstream signals promote intestinal barrier function, our data suggest that miR-146a-/- mice have enhanced barrier function within their intestinal tract through a mechanism involving enhanced MyD88 signaling.

miR-146a constrains Th17 and Treg populations within the lamina propria

In addition to the barrier gene products described above, leukocytes are also critical regulators of intestinal barrier function and participate in the regulation of these genes. Thus, we examined immune cell populations of the small intestinal and colonic lamina propria (LP), comparing WT to miR-146a-/- mice. Few differences were observed in LP myeloid ($CD11b^+$) and B cell ($B220^+$) proportions and numbers when comparing WT and miR-146a-/- mice (Supplementary Figure 5b, 5c, 5e, and 5f). However, miR-146a-/- mice had an expansion of $CD4^+$ T cells within the small intestinal and colonic LP (Supplementary Figure 5a and 5d). Upon examination of specific $CD4^+$ helper T cell populations, we observed an increase in Th17 and Treg cells in miR-146a-/- mice, both in small intestinal (Figure 3a and 3b) and colonic (Figure 3c and 3d) LP. Altogether, these results demonstrate that miR-146a plays a role in restricting $CD4^+$ helper T cell populations within the intestinal lamina propria, primarily of the Th17 and Treg cell subsets. These cell subsets have previously reported functions in intestinal barrier and tolerance [5, 11, 12], including the regulation of several barrier genes, indicating that miR-146a is playing an immunomodulatory role to shape the intestinal immune landscape.

miR-146a constrains germinal center reactions in the Peyer's Patch

In addition to the LP, T lymphocytes of gut-associated lymphoid tissues such as Peyer's Patches (PP) are also critical regulators of intestinal barrier function. In particular, T follicular helper (Tfh) cells and their interactions with germinal center (GC) B cells within the intestinal PP are essential to produce IgA and maintain intestinal homeostasis [55]. Because miR-146a plays a role in Tfh and GC B cell accumulation in extra-intestinal tissues [32,56] and is expressed within the PP (Figure 1b) we examined lymphocyte populations within this gut-associated lymphoid tissue. miR-146a-/- mice displayed an expansion in both the percentage and total number of $PD\text{-}1^+$ $CXCR5^+$ Tfh cells within the PP compared with WT mice (Figure 4a). Tfh cells interact with GC B cells within the PP to promote antigen-specific IgA responses [57]. Accordingly, the observed expansion of Tfh cells corresponded with an increase in $GL7^+$ FAS^+ GC B cells in the PP (Figure 4b). Inducible costimulator (ICOS) expression on Tfh cells is essential for their differentiation and function during germinal center responses [58] and is a direct target of miR-146a [32]. Within the PP, we observed a significant increase in ICOS expression (MFI) on total $CD4^+$ T cells and on Tfh cells upon loss of miR-146a (Figure 4c), consistent with elevated Tfh, GC B

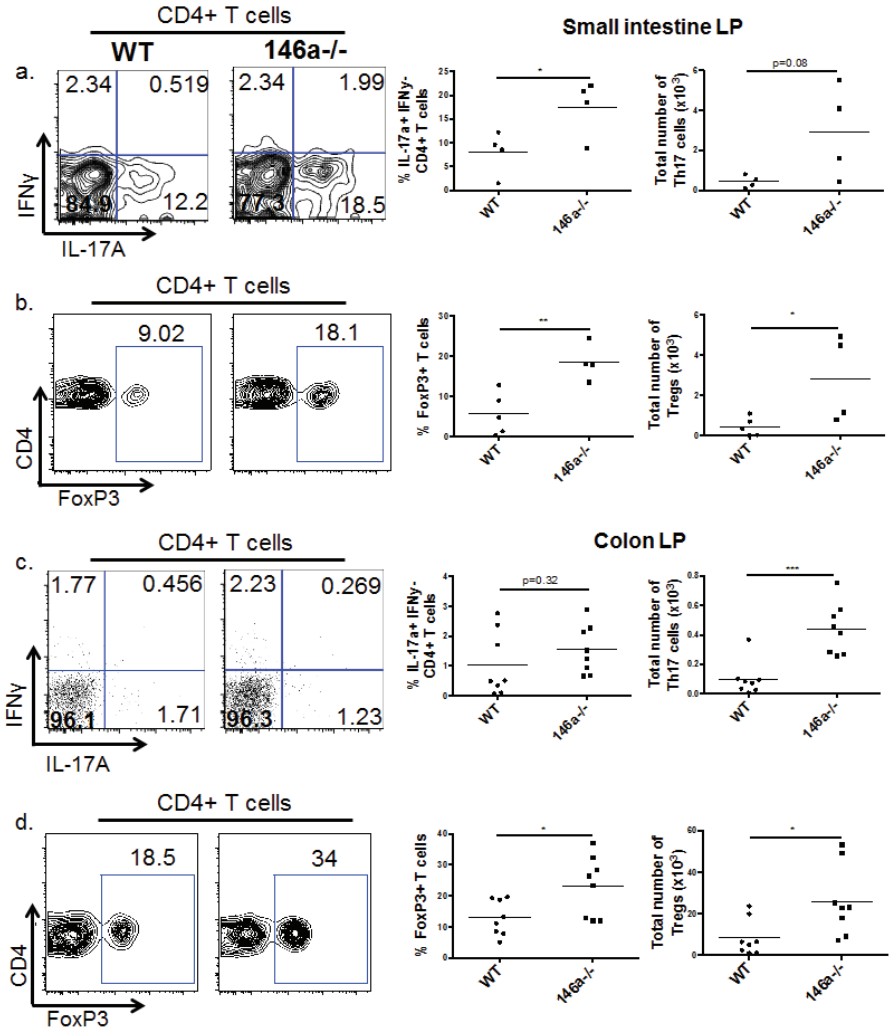

Figure 3: miR-146a constrains Th17 and Treg populations in the lamina propria. Lymphocytes were isolated from the small intestinal lamina propria, and flow cytometry was utilized to examine immune cell populations within this tissue. Displayed are representative flow plots (Wt on the left and miR-146a-/- on the right), percentages, and total numbers of IL-17A$^+$ IFNγ$^-$ (Th17) CD4$^+$ CD3e$^+$ T cells within the small intestine LP. All populations were first gated on lymphocytes using the FSC/SSC gate, then on CD3e$^+$ CD4$^+$ cells, followed by the IL-17A and IFNγ gating shown **a.** Representative flow plots, percentages, and total numbers of FoxP3$^+$ (Treg) CD4$^+$ CD3e$^+$ T cells within the small intestine LP. All populations were first gated on lymphocytes using the FSC/SSC gate, then on CD3e$^+$ CD4$^+$ cells, followed by the FoxP3$^+$ gating shown **b.** Displayed are representative flow plots, percentages, and total numbers of IL-17A$^+$ IFNγ$^-$ (Th17) CD4$^+$ CD3e$^+$ T cells within the colonic LP. All populations were first gated on lymphocytes using the FSC/SSC gate, then on CD3e$^+$ CD4$^+$ cells, followed by the IL-17A and IFNγ gating shown **c.** Representative flow plots, percentages, and total numbers of FoxP3$^+$ (Treg) CD4$^+$ CD3e$^+$ T cells within the colonic LP. All populations were first gated on lymphocytes using the FSC/SSC gate, then on CD3e$^+$ CD4$^+$ cells, followed by the FoxP3$^+$ gating shown **d.** $n = 5$ (WT) and 4 (miR-146a-/-) (a, b), $n = 8$ (c, d).

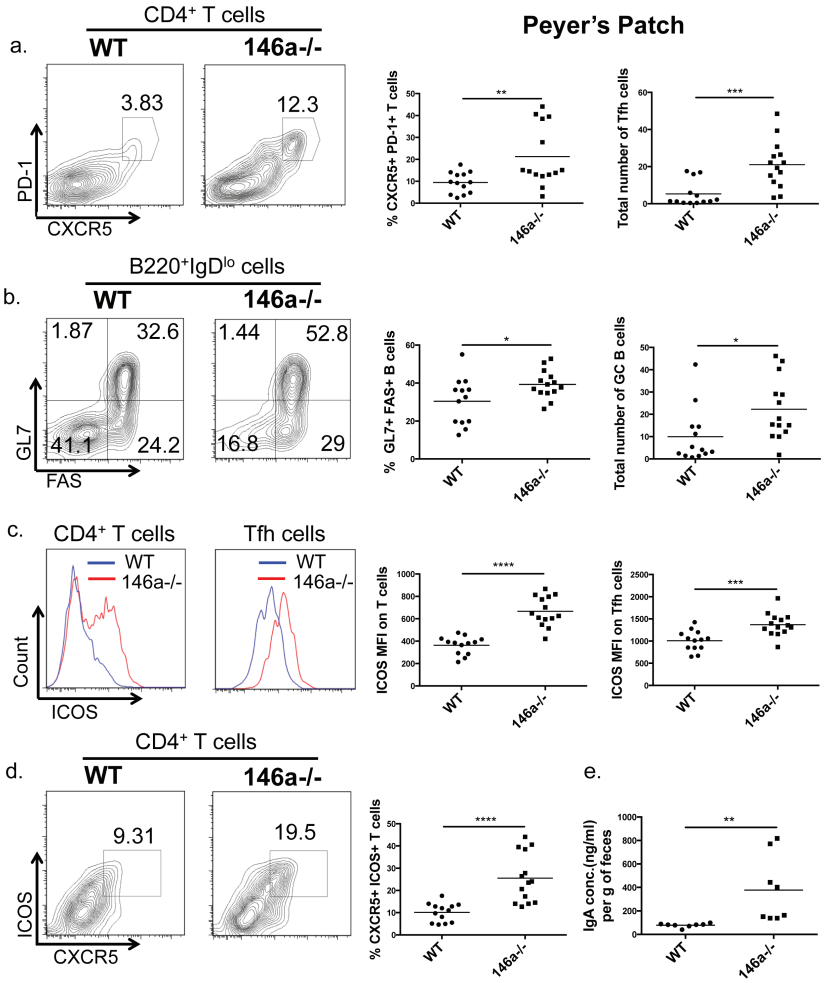

Figure 4: miR-146a constrains Tfh, germinal center (GC) B cell, and IgA levels in the intestine. Peyer's Patches were collected from WT C57BL/6 and miR-146a-/- mice, and flow cytometry was utilized to examine T and B cell populations within these GALT structures. Displayed are representative flow plots, percentages, and total numbers of $CXCR5^+$ $PD\text{-}1^+$ (Tfh) $CD4^+$ $CD3e^+$ T cells within the PP. All populations were first gated on lymphocytes using the FSC/SSC gate, then on $CD3e^+$ $CD4^+$ cells, followed by the PD-1 and CXCR5 gating shown **a.** Representative flow plots, percentages, and total numbers of $GL7^+$ FAS^+ (GC B) $B220^+$ IgD^{lo} B cells within the PP are shown. All populations were first gated on lymphocytes using the FSC/SSC gate, then on $B220^+$ IgD^{lo} cells, followed by the GL7 and FAS gating shown **b.** Representative flow plots and quantification of mean fluorescence intensity (MFI) of ICOS on $CD3e^+$ $CD4^+$ T cells (left histogram) and Tfh cells (right histogram), measured via flow cytometry with a fluorescent antibody against ICOS. All populations were first gated on lymphocytes using the FSC/SSC gate, then on $CD3e^+$ $CD4^+$ cells, followed by $PD\text{-}1^+$ and $CXCR5^+$ for Tfh cells **c.** Shown are representative flow plots and percentages of $CXCR5^+$ $ICOS^+$ $CD4^+$ $CD3e^+$ T cells within the PP. All populations were first gated on lymphocytes using the FSC/SSC gate, then on $CD3e^+$ $CD4^+$ cells, followed by the ICOS and CXCR5 gating shown **d.** Feces pellets were collected from WT and miR-146a-/- mice, weighed, and homogenized; total IgA was measured in fecal homogenates via ELISA **e.** $n = 13$ (WT) and 14 (miR-146a-/-) (a-d), $n = 8$ **e.**

cells, and a previously reported role of miR-146a in Tfh function [32]. Furthermore, the percentage of $CXCR5^+$ Tfh cells expressing ICOS was increased in miR-146a-/- mice compared with WT (Figure 4d). Consistent with an enhanced germinal center reaction, total fecal IgA levels were also elevated in miR-146a-/- compared with WT mice (Figure 4e). Tfh cells, GC B cells, and IgA have all been shown to play an important role in promoting intestinal barrier and in shaping microbial populations within the gut [55, 59-61]. Altogether, these results indicate that miR-146a-/- mice have increased Tfh and GC B cells within the intestine, correlating with elevated T cell ICOS expression and IgA levels.

miR-146a-/- mice are resistant to DSS-induced colitis

Altered intestinal inflammatory responses and barrier function have been linked to diseases within the intestinal tract. Because our RNA-sequencing (Supplementary Figure 4a and 4b) and immunological data suggested that miR-146a-/- mice might be resistant to inflammatory bowel disease (IBD) and colitis, we utilized the dextran sodium sulfate (DSS) murine colitis model to examine barrier function and disease susceptibility within the intestines. This model of intestinal inflammation, which affects both the colon and small intestine, models human Ulcerative Colitis [62-64]. After 8 days of DSS treatment, WT mice lost significantly more weight than miR-146a-/- mice (Figure 5a), and miR-146a-/- colons were longer in both DSS-treated and untreated mice (Figure 5b). The net change in colon length following DSS administration was less in miR-146a-/- mice compared to controls (Supplementary Figure 6a). Upon examination of H&E stained colonic sections by a blinded pathologist, WT colons had a complete loss of crypts and intestinal architecture, and a large infiltration of leukocytes (Figure 5e). DSS-treated miR-146a-/- colons appeared diseased, but some crypts were still in tact and inflammation and tissue damage were reduced (Figure 5f). Both WT and miR-146a-/- colons that were untreated appeared normal (Figure 5c and 5d). Altogether, WT mice showed higher histological colitis scores compared with mice lacking miR-146a (Figure 5g). Decreased expression of TNFα and IL-6 mRNAs within colons of miR-146a-/- DSS-treated animals also indicated reduced disease severity (Supplementary Figure 6b and 6c).

In order to understand the contribution of miR-146a within hematopoietic versus non-hematopoietic cells to DSS colitis susceptibility, we performed bone marrow reconstitutions, where WT or miR-146a-/- $CD45.2^+$ bone marrow was transferred to lethally irradiated WT mice carrying the CD45.1 marker. Following hematopoietic cell reconstitution, DSS was administered to bone marrow recipient animals. WT mice that received miR-146a-/- bone marrow were again more protected from DSS colitis compared with WT bone marrow recipients, as shown by weight loss differences (Figure 5h). Flow cytometric analysis of the spleen revealed that recipient bone marrow ($CD45.2^+$) made up a majority of the hematopoietic cells, indicating that the reconstitution was effective (Figure 5i). These data demonstrate that the observed colitis phenotype is mediated by miR-146a within the hematopoietic compartment. Altogether, our data reveal that miR-146a decreases intestinal barrier function resulting in elevated DSS colitis severity.

Upon examination of the intestinal landscape, one cannot ignore the crosstalk between host tissues and the 10^{14} commensal organisms that reside in this locale [65]. Owing to the clear differences in intestinal barrier and immunological parameters between WT and miR-146a-/- mice, we hypothesized that alterations to the microbiota must be taking place. To directly test this, we performed Illumina sequencing of 16s rDNA extracted from fecal pellets of both genotypes to determine if there were differences in microbial populations. Both unweighted and weighted, normalized Unifrac distances demonstrated significant differences between total microbial communities in WT versus miR-146a-/- mice (Supplementary Figure 7a). Larger and more significant Unifrac distances were observed using the unweighted measure, which is more dependent upon differences in rare bacterial taxa. Alpha diversity, as measured by multiple indices, was not significantly different between WT and miR-146a-/- mice, although a larger distribution in alpha diversity was observed in the knockout group (Supplementary Figure 7b). Upon comparison of microbial taxa that varied between WT and miR-146a-/- mice, statistically significant shifts at the phyla level were not observed (Supplementary Figure 7c). Altogether, these data indicate that host miR-146a plays some role in shaping the gut microbiota by regulating the magnitude of host-barrier function, albeit commensal community shifts were minor and within rare taxonomic groups.

Because miR-146a-/- mice had some alterations in gut microbiota composition compared to WT controls, we tested the direct contribution of these small community shifts to disease susceptibility. WT or miR-146a-/- mouse gut microbiota was transferred to broad-spectrum antibiotic pre-treated WT recipient mice via oral gavage, and DSS colitis was induced following transfer. We observed no difference in DSS disease severity when comparing recipients having received WT or miR-146a-/- microbiota. This provides evidence that the microbiota does not directly facilitate the phenotypic differences in colitis symptoms (Supplementary Figure 7d). Altogether, our data reveal that miR-146a decreases intestinal barrier function resulting in elevated DSS colitis severity, and this involves hematopoietic cells and appears to be microbiota-independent.

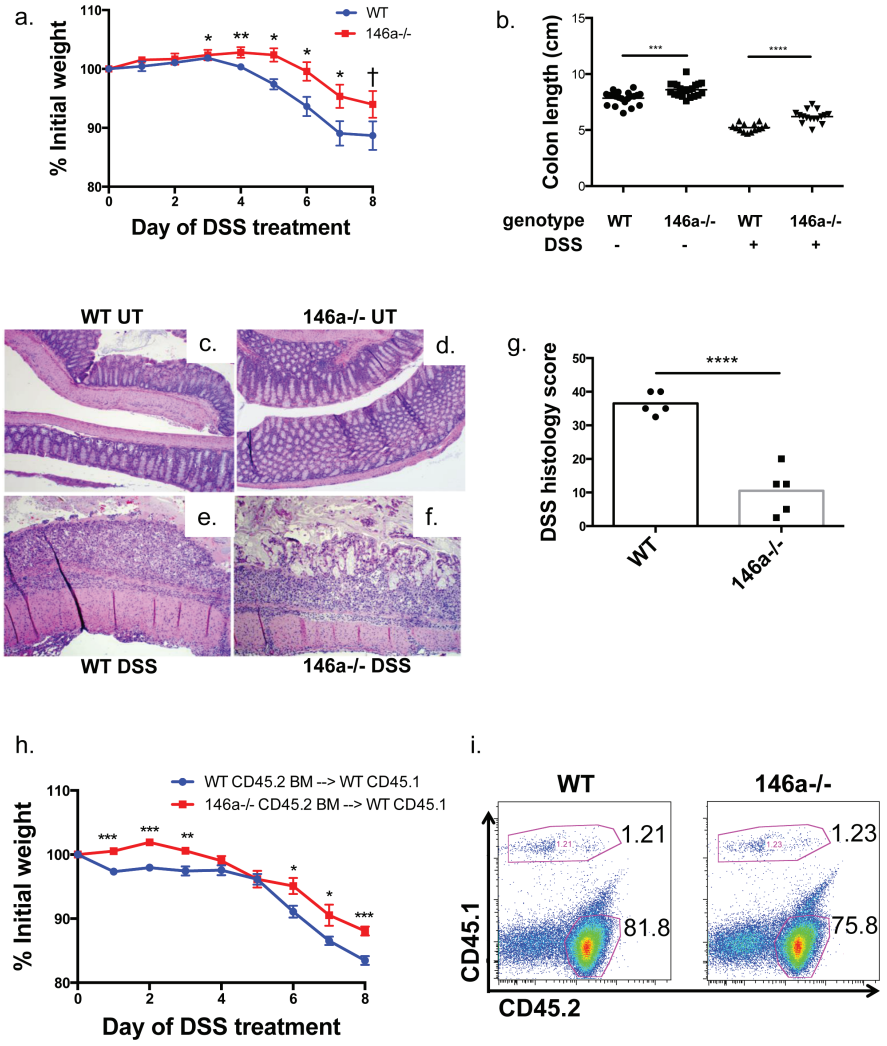

Figure 5: miR-146a-/- mice are protected from DSS-induced colitis. WT C57BL/6 and miR-146a-/- mice were treated with 3.5% dextran sodium sulfate (DSS) for eight days. Weight change of WT and miR-146a-/- mice during DSS colitis, as measured by percent initial weight **a.** Colon lengths of WT and miR-146a-/- mice before (left columns) and after DSS treatment (right columns) **b.** H&E staining of untreated (UT) and DSS-treated WT and miR-146a-/- colonic sections, shown at 10x magnification **c.-f.** Histology scores of H&E stained DSS-treated colon tissues, based on grade 3 and 4 mucosa loss, as scored by a blinded pathologist **g.** Weight change during DSS colitis following bone marrow transfer of WT or miR-146a-/- CD45.2 bone marrow into lethally irradiated WT CD45.1 recipients **h.** Representative flow cytometry plots showing percentages of donor (WT or miR-146a-/- CD45.2$^+$) and recipient (WT CD45.1$^+$) splenocytes following bone marrow reconstitution **i.** $n = 16$ (a-b); $n = 5$ (c-g); $n = 15$ (h-i).

miR-146a is overexpressed in patients with ulcerative colitis

Because we found a role for miR-146a in regulating colitis in mice, ulcerative colitis (UC) patients were examined to determine if miR-146a levels correlate with intestinal disease in humans. We obtained ulcer-free colon biopsies from non-diseased individuals (normal) and UC patients. Mature miR-146a RNA levels were measured, and we found that miR-146a expression was significantly elevated in UC patients compared to healthy controls (Figure 6). These data further demonstrate correlation of miR-146a expression in human UC and CD, in which previous studies have found associations of miR-146a expression and polymorphisms with IBD type and risk [24,66-68]. Our results indicate that higher miR-146a levels correlate with UC, and further investigation may distinguish the severity of disease and/or development of colorectal cancer (CRC) as a result of UC.

DISCUSSION

Our study provides the first genetic evidence that miR-146a, a microRNA that is important in a variety of immunological contexts, is also relevant within intestinal tissues. In previous studies, miR-146a was shown to be an anti-inflammatory miRNA by repressing genes that promote pro-inflammatory signaling. In this way, loss of miR-146a results in worsened disease susceptibility and severity in most model systems [23, 27, 29, 33, 69]. Our study is unique in that loss of miR-146a resulted in a more beneficial host outcome following DSS-induced colitis. This appears to be the result of miR-146a down-regulating expression of genes and cell types that fortify the intestinal barrier.

Previous work has shown that miR-146a down regulates pro-inflammatory pathways, including signaling downstream of the TLR/IL1R adaptor protein MyD88 in addition to IFN activated Stat1, in a variety of peripheral tissues[27, 31, 56]. Pathway analysis of our gene expression data set indicates that miR-146a represses these same pathways in the gut. MyD88 signaling is actually beneficial in the intestines, as MyD88-/- mice develop worsened DSS colitis [16-18, 54]. One mechanism used by TLR/MyD88 signaling to protect the gut is induction of antimicrobial peptides such as RegIIIγ [40], mucins [70], IL-18 [52], and other molecules that are produced by intestinal epithelial cells. Consistent with miR-146a repressing this pathway, miR-146a-/- mice are more protected from DSS colitis compared to control mice, and all of these TLR-MyD88 induced, host protective genes are elevated in the small intestines of miR-146a-/- mice. In addition to TLR/MyD88 signaling, we also see predicted de-repression of the IFN activated factor Stat1, a pathway that has also been shown to protect gut tissues. Taken together, miR-146a-/- mice appear to be protected from

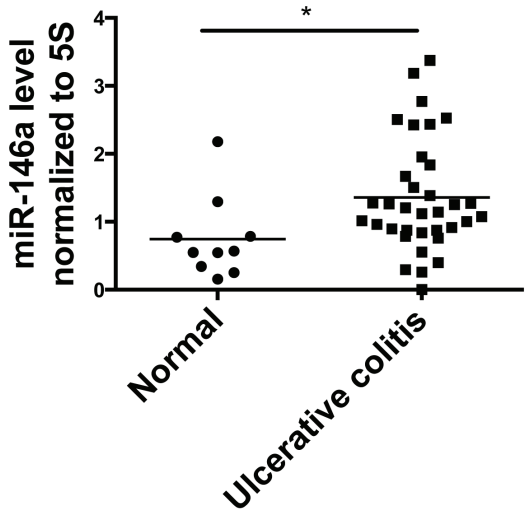

Figure 6: miR-146a expression is elevated in colon tissue from human Ulcerative Colitis (UC) patients. Colonic biopsies were collected from healthy individuals (Normal) and Ulcerative Colitis (UC) patients. qRT-PCR was used to measure levels of mature miR-146a RNA within the collected tissue, and expression was normalized to 5S rRNA. $n = 10$ (normal), $n = 35$ (UC).

DSS colitis through a mechanism involving enhanced TLR/MyD88 and IFN/Stat1 signaling. However, the additional miR-146a targets identified in our RNA-Seq experiment, as well as ICOS function in Tfh cells, also appear to be involved.

We also observed enhanced GC responses in the PPs of miR-146a-/- mice, including increases in Tfh and GC B cells and elevated IgA levels in the intestinal lumen. This correlated with increased ICOS expression on T cells, presumably through direct miR-146a targeting of ICOS mRNA and other Tfh-related genes [32]. Interestingly, we have recently reported that T cell specific MyD88 signaling is critical for Tfh cell formation and downstream formation of GC B cells and IgA production [55]. Further, this pathway impacted the host microbiota through selective IgA binding to commensal populations, and this was beneficial to host intestinal health. It has been shown that miR-146a negatively regulates downstream TLR signaling pathways (e.g. TRAF6) in $CD4^+$ T cells, and our data suggest that miR-146a acts as a negative regulator of T cell intrinsic TLR signaling that triggers increased GC responses, luminal IgA and intestinal health. Therefore, this appears to be an additional mechanism underlying the miR-146a-/- mouse gut phenotype.

Intestinal miR-146a expression is enriched in cells of the hematopoietic lineage, although this does not rule out the possibility that the observed low-level expression of this miRNA in $CD45^-$ cells may also be functional. Even so, we observed protection from colitis in WT mice upon bone marrow reconstitution with miR-146a-/- cells, indicating that it plays a required role in the hematopoietic compartment. Future work with miR-146a conditional knockout mice will allow us to determine the relative contribution of distinct cell types to the miR-146a intestinal phenotype, including an assessment of T cell and IEC intrinsic roles for this miRNA.

We have found that WT and miR-146a-/- mice have different gut microbial populations, demonstrating that miR-146a plays some role in shaping commensal microbiota. Although the biological relevance of the altered populations is currently unclear, and does not appear to impact the DSS phenotype, these altered microbial populations may be playing important roles in other intestinal conditions such as Crohn's Disease and CRC. miR-146a reshaping of the microbiota might also be relevant in extra-intestinal disorders [71], such as obesity [72] and diabetes [73, 74], multiple sclerosis [75], and infections [76-78], as both miR-146a and the microbiota have been implicated in these situations.

While miR-146a-/- mice are protected from DSS colitis, a model of intestinal barrier function, the functional role of miR-146a during CRC is unknown. In fact, miR-146a is thought of as a tumor suppressor in many cancer settings [27]. In addition, miR-146a has also been shown to play a role in protection from ischemia/reperfusion injury [30] and may contribute to early microbial colonization in the gut [34]. Thus, the benefits of miR-146a function within the gut may outweigh its adverse effects during colitis. This might explain why miR-146a has evolved to play a negative regulatory role during at least some forms of colitis.

Our study shows that miR-146a has clinical relevance within the intestine, as UC patients on average show elevated levels of this miRNA within the colon tissue. Thus, targeting miR-146a therapeutically might prove to be an effective treatment of this condition. However, one must keep in mind that using miR-146a as a therapy to treat extra-intestinal disorders may exacerbate intestinal disease. Future studies need to be done to examine the interplay of miR-146a expression within different contexts in human patients to determine how to design targeted delivery of miR-146a therapeutics. It is also possible that inhibition of miR-146a may be beneficial for some human patients with intestinal disease, but again, proper targeting to gut cells will be important

MATERIALS AND METHODS

Animals

All WT and miR-146a-/- mice are on a C57BL/6 background and were bred and housed in a specific pathogen-free mouse facility at the University of Utah, USA. WT Germ-free mice were housed in the germ-free mouse facility at the University of Utah, USA. All mice were 6-8 weeks old at time of experimentation. Experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of University of Utah, USA.

CD45 cell sorting

WT and miR-146a-/- colons and small intestines were obtained, mesenterium was removed, and the tissues were rinsed in PBS. Intestines were cut longitudinally, cleaned of mucus and feces, and cut into fragments. Fragments were then placed in a cell dissociation solution made of calcium-free HBSS with 5mM EDTA and 10uM HEPES (colon) or 1 mM DTT (small intestine) and incubated for 45 minutes at 37°C. Following cell dissociation, samples were strained into 100uM filters and flow-through was collected and placed on ice. Intestinal fragments were then placed in a digestion mix containing calcium-free HBSS, 5% FBS, 1 U/mL Dispase (Roche), 0.5 mg/mL Collagenase D (Roche), and 25 ug/mL DNase I (Worthington) and incubated at 37°C for 45 minutes. Tissue was strained through a 40uM strainer and flowthrough was collected. Cell suspensions from the cell dissociation and digestion steps were combined and resuspended in flow cytometry buffer (HBSS, 10mM

Table 1: Primer sequences

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Reg3b	CTGCCTTAGACCGTGCTTTC	ATAGGGCAACTTCACCTCAC
Reg3g	TTCCTGTCCTCCATGATCAAA	CATCCACCTCTGTTGGGTTCA
Ceacam1S	CTGGCATCGTGATTGGAGTT	CAGAAGGAGCCAGATCCG
Ceacam1L	GCGAGATCTCACAGAGCACA	GCTGGGAATTGAAGTTCAGG
Il18	GCCTCAAACCTTCCAAATCA	TGGATCCATTTCCTCAAAGG
Nos2	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGGTTCG
Oasl1	CCAGGAAGAAGCCAAGCACCATC	AGGTTACTGAGCCCAAGGTCCATC
Ido1	GGCTTTGCTCTACCACATCCAC	TAGCCACAAGGACCCAGGG
L32	AAGCGAAACTGGCGGAAAC	TAACCGATGTTGGGCATCAG

HEPES, 2mM EDTA, 0.5% w/v FBS). Cells were stained with CD45-FITC antibody (Biolegend) and 7-AAD viability staining solution (Biolegend). Fluorescence-activated cell sorting was performed using the FACS Aria (BD Biosciences) gating on singlet, live (7-AAD$^-$) cells and sorting CD45$^+$ from CD45$^-$ cells. Qiazol (Qiagen) was added to WT and miR-146a-/- small intestine and colon samples containing > 10,000 CD45$^+$ or CD45$^-$ sorted cells. RNA was extracted from sorted cells using the miRNeasy kit (Qiagen) and miR-146a expression was quantified via qPCR as described above.

Gene expression analysis

1 cm pieces of distal colon and ileum of the small intestine (SI) were removed, gently cleaned to remove mucus and feces, and rinsed in PBS. The colon and SI pieces were then placed in Qiazol (Qiagen). Tissues were homogenized and RNA was extracted using the RNeasy or miRNeasy spin column kit (Qiagen) and quality was measured. Samples were prepared and sequenced at the Microarray and Genomic Analysis Core Facility at University of Utah, USA. Libraries were prepared using RiboZero treatment and sequencing was done with Illumina TruSeq Stranded RNA-seq in 50 single-end cycles. Sequences were aligned and annotated with help of the Bioinformatics Core Facility at University of Utah, USA. Pathways were examined by uploading analyzed data into the Ingenuity IPA software program and performing a core analysis on genes upregulated or downregulated by 2-fold or more, (with Phred-transformed FDR > 10).

Quantitative real-time PCR (qRT-PCR) was performed using RNA prepared in the manner described above, which underwent randomly primed cDNA synthesis using qScript cDNA SuperMix (Quanta Biosciences). qRT-PCR was performed using SYBR green detection with GoTaq qPCR master mix (Promega) or LightCycler 480 SYBR Green I Master (Roche). All primers were purchased from Integrated DNA Technologies (IDT) and signals were normalized to L32. Primers sequences are listed in Table 1.

Mature miR-146a was quantified using the miRCURY LNA Universal RT microRNA PCR cDNA synthesis and SYBR Green Master Mix kits (Exiqon). microRNA levels were normalized to 5S rRNA (primer from Exiqon) and the LNA miR-146a primer was purchased from Exiqon.

Lamina propria and Peyer's patch isolation/flow cytometric analysis

WT and miR-146a-/- colons and small intestines were obtained; the entire colon was cut from the anus to the cecum and the first 20 cm of the small intestine beginning with the ileum was used. Mesenterium, connective tissue, and Peyer's Patches were removed; the tissues were rinsed in PBS. Intestines were cut longitudinally and mucus and feces were removed. Tissues were then cut into fragments and were then placed in a cell dissociation solution made of calcium-free HBSS (Corning) with 5mM EDTA and 10mM HEPES (colon) or HBSS with 5mM

EDTA and 1 mM DTT (small intestine) and incubated for 45 minutes at 37°C. Following cell dissociation, samples were strained into 100uM filters and flow-through was discarded. Intestinal fragments were then placed in a digestion mix containing calcium-free HBSS with 5% w/v FBS, 1 U/mL Dispase (Roche), 0.5 mg/mL Collagenase D (Roche), and 25 ug/mL DNase I (Worthington) and incubated at 37°C for 45 minutes. Tissue was strained through a 40uM strainer and flowthrough containing LP cells was collected. Cells were subjected to a 40%-80% Percoll gradient spin, then washed and resuspended in FACS buffer (HBSS, 10mM HEPES, 2mM EDTA, 0.5% w/v FBS). Cells were restimulated with PMA (Sigma), ionomycin (Sigma), and Golgi plug (BD) for 4 hours, were surface stained with fluorophore-conjugated antibodies B220-FITC (Biolegend), CD11b-APC (Biolegend), CD3e-PerCP Cy5.5 (Biolegend), and CD4-FITC (eBioscience) before they were fixed and permeablized overnight. Cells were washed and intracellulary stained with FoxP3-APC (eBioscience), IL-10-PE (eBioscience), IFNγ-PE (eBioscience), and IL-17a-APC (eBioscience) before washing and running in a flow cytometer (LSR Fortessa, BD Biosciences).

Peyer's Patches were removed from the small intestines of WT and miR-146a-/- mice. They were gently passed through a 40 μM filter in PBS in order to obtain white blood cells. Cell suspensions were then washed and resuspended in flow buffer (HBSS, 10mM HEPES, 2mM EDTA, 0.5% w/v FBS). Germinal center B cells were identified by staining with antibodies against GL7 (ebioscience), FAS (BD Pharmagen), IgD (Biolegend) and B220 (Biolegend). The Tfh cells were identified by staining with antibodies against CD3ε (eBioscience), CD4 (eBioscience), CXCR5 (ebioscience), PD-1 (Biolegend), and/or ICOS (Biolegend), and gating based on isotype or unstained controls.

Microbiota analysis

WT and miR-146a-/- mice were sacrificed; their colons were collected and cut open longitudinally. Feces was removed and flash-frozen in liquid nitrogen. Microbial DNA was extracted from feces using the PowerSoil DNA Isolation kit (MoBio). From the extracted DNA, 16S rDNA V4 region PCR amplicons were sequenced on the MiSeq platform (Illumina) using the 2x250bp protocol yielding pair-end reads with a mean merged length of ~247 bps [79]. Following sequencing, raw BCL files were retrieved from the MiSeq platform and called into fastqs by Casava v1.8.3 (Illumina). The read pairs were merged using USEARCH v7.0.1090 with the fastq_mergepairs parameters '-fastq_minovlen 20 -fastq_truncqual 5 -fastq_maxdiffs 5 -fastq_maxmergelen 350 -minhsp 8', then filtered with fastq_filter to exclude sequences with more than 0.5 expected errors over the length of the merged read. Bowtie2 v2.2.1 was used to identify and remove reads mapping to the PhiX genome. Sequences were next demultiplexed based on exact barcode matches and then clustered into 97% identity operational taxonomic units (OTUs) using the UPARSE pipeline [80]. Phylogenetic annotation of OTUs was achieved by mapping the UPARSE OTUs to the SILVA v111 database with a minimum identity of 97% [81]. The resulting OTU table representing 2,301,977 sequences in 582 OTUs was rarefied to an even depth of 21,707 reads per sample prior to calculation of alpha-diversity, beta-diversity, taxonomic summaries, and related analyses in QIIME and the phyloseq R package [82-87]. Significance of categorical variables were determined using the non-parametric Kruskal-Wallis test. Correlation between two continuous variables was determined with linear regression models, where p-values indicate the probability that the slope of the regression line is zero. Principal coordinate plots employed the Monte Carlo permutation test to estimate p-values. All p-values were adjusted for multiple comparisons with the FDR algorithm.

Colitis

Dextran sodium sulfate (DSS) colitis was induced by dissolving DSS (MP Biomedicals, 36,000-50,000 mw) in autoclaved water at a 3.5% w/v concentration. DSS water was placed in cages of WT and miR-146a-/- mice, where they drank DSS water *ad libitum* for 8 days; water was changed to fresh DSS water on day 4. Mice were weighed daily and percent weight loss was tracked over time. Mice were sacrificed and analyzed for disease severity on day 8, where colon length was measured, and colons were fixed in formalin for histology. For qRT-PCR of DSS-treated colon segments, colon tissue was subjected to a LiCl purification following RNA isolation to remove residual DSS [88].

Microbiota transfers

Donor microbiota was obtained by scraping mucus and feces from colons of WT and miR-146a-/- mice. Scrapings were placed in sterile PBS, centrifuged at 400G to remove debris, then resuspended and centrifuged at 8000G to obtain bacteria. Samples were resuspended in sterile PBS and flash frozen for gavage. Recipient mice were treated with an antibiotic cocktail of Gentamycin (Goldbio), Ampicillin (Cellgro), Neomycin Sulfate (Fisher), and Erythromycin (Fisher); each at 0.5g/L in drinking water for 7 days. After 7 days, drinking water was changed to regular water and 100ul of donor WT or miR-146a-/- bacteria was orally gavaged into recipient mice every day for 7 days. Following gavage, mice rested for 7 days to allow for microbiota stabilization. Finally, DSS colitis was administered in recipient mice using the methods described above.

miR-146a expression in human patients

Colon biopsies were obtained from normal (healthy) or Ulcerative Colitis patients. Frozen biopsy samples were processed in RNAlater-ICE transition solution overnight at -20°C and homogenized using Qiagen TissueRupter. Total RNA was extracted from the resultant tissue suspension using TRIzol LS reagent as per the manufacturer's instructions. qPCR was performed to assay expression levels of mature miR-146a using the methods described above.

Statistics

A Student's t-test was utilized to determine significant p-values when comparing two groups, unless otherwise noted. †≤0.12; * p ≤0.05; ** p≤0.01; *** p≤0.001; **** p≤0.0001. For p-values during DSS colitis, individual t-tests were performed at each time point. Outliers from experiments were removed using the Graphpad Prism 6 "identify outliers" statistical function. See "microbiota analysis" and "gene expression analysis" sections for explanation of statistics in those experiments.

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CONFLICTS OF INTEREST

We declare that there is no conflict of interest at this time.

Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget.

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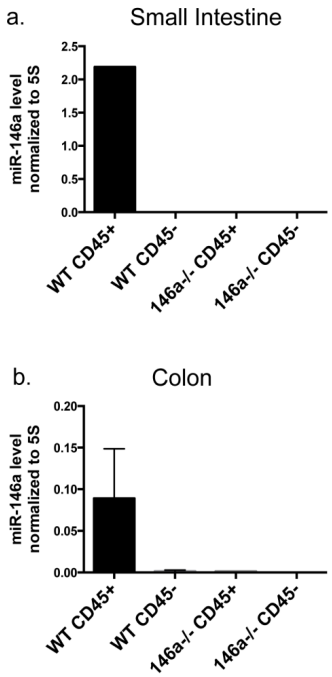
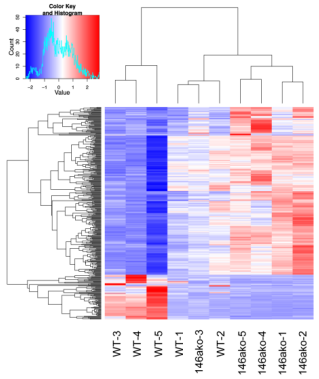
MicroRNA-146a constrains multiple parameters of intestinal immunity and increases susceptibility to DSS colitis

Supplementary Material



Supplementary Figure 1. miR-146a is enriched in CD45$^+$ cells of the colon and small intestine. Cells were dissociated from WT and miR-146a-/- small intestines and colons, stained for viability and CD45, and CD45$^+$ and CD45$^-$ cells were sorted via FACS. RNA was extracted from sorted cells, and qRT-PCR was run to measure mature miR-146a expression. miR-146a expression in CD45$^+$ and CD45$^-$ cells of the small intestine **(a)** and colon **(b)**. n=3 (a and b)

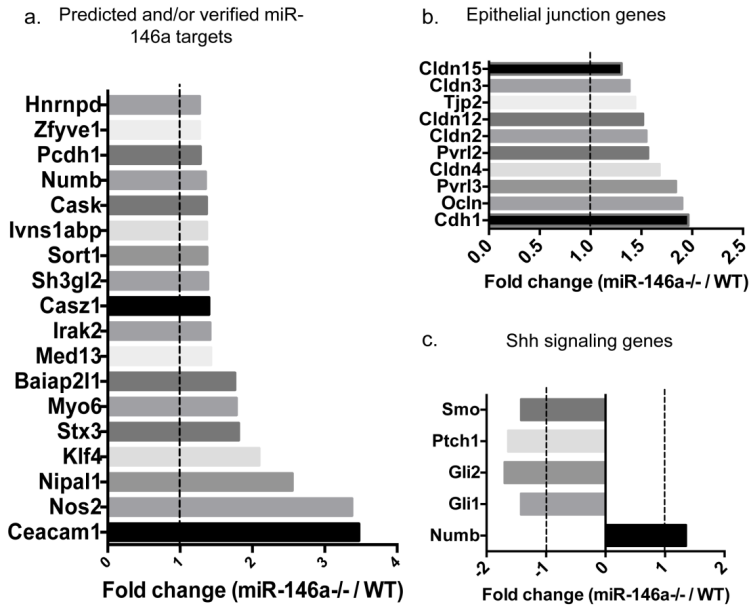
a. Significant gene expression changes in small intestine (>2-fold change, FDR>10)



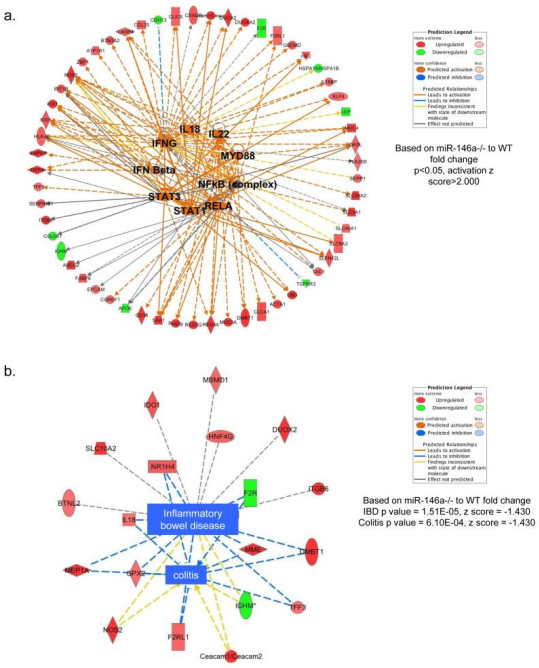
b. Significant gene expression changes in colon – miR-146a-/- over WT (FDR>10)

Gene name	Fold Change
Selenbp2	5.749662668
Tpm3-rs7	3.189507706
Edn1	2.377290752
Klhl24	2.061948947
Pbsn	-2.114540161
Ighv6-6	-2.168731855
Fam115e	-2.168880239
Neat1	-2.171490443
5830417I10Rik	-2.22453276
Prune2	-2.225547451
Tpm3	-2.292656429
Tgm4	-2.420075924
Sprr2a1	-2.546951018
Dab1	-2.57060169
Slc26a2	-2.67142886
9530053A07Rik	-2.871575472
Gm23935	-3.845753444
NA	-6.825406786

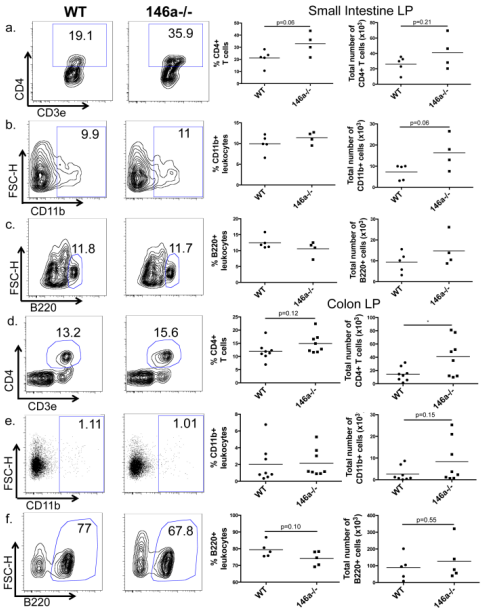
Supplementary Figure 2. miR-146a impacts global gene expression within the small intestine and regulates few colonic genes. RNA-seq was performed on ileal tissue from WT and miR-146a-/- mice. Heat map indicates a fold change in expression of genes with a >2-fold change when comparing miR-146a-/- mice with WT, $p<0.05$, Phred-transformed FDR>10 **(a)**. RNA-seq was performed on colonic tissue from WT and miR-146a-/- mice. Table indicates fold change in expression of genes with >2-fold change, comparing miR-146a-/- mice with WT, Phred-transformed FDR>10 **(b)**.



Supplementary Figure 3. miR-146a regulates the expression of epithelial junctions and predicted targets within the small intestine. Expression levels of selected predicted and/or experimentally verified mmu-miR-146a targets (according to Targetscan and miRTaRBase), comparing miR-146a-/- with WT small intestine. Targets genes shown had >1.3-fold expression change and Phred-transformed FDR>5 **(a)**. Expression levels of select intestinal epithelial junction genes in the small intestine when comparing miR-146a-/- to WT **(b)**. Expression levels of miR-146a target NUMB and downstream SHH signaling genes in the small intestine when comparing miR-146a-/- to WT **(c)**.

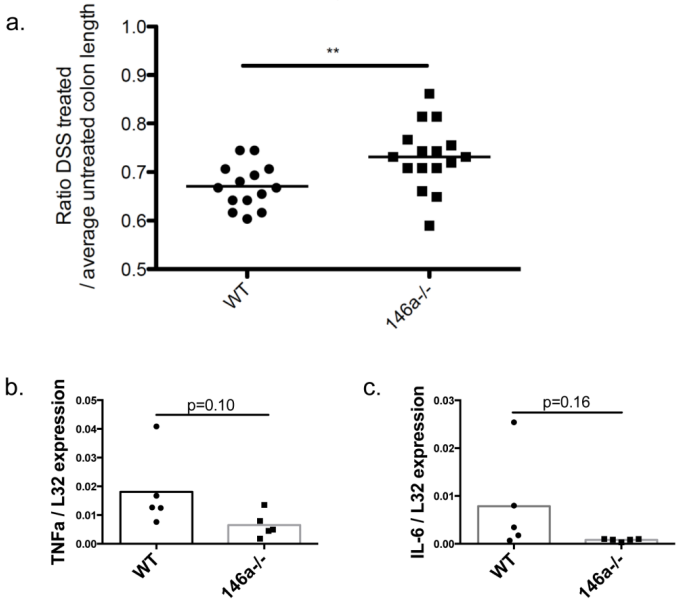


Supplementary Figure 4. IPA predictions indicate that miR-146a downregulates proinflammatory pathways in the small intestines and will enhance IBD. Ingenuity Pathway Analysis (IPA) showing predicted activation of proinflammatory pathways MyD88, NFkB, Stat1, Stat3, IFNb, IFNg, IL-22, and IL-18 within miR-146a-/- small intestines mice compared to WT; based on genes that are upregulated in RNA-seq data of ileal tissues (found in outside wheel of red and green shapes) **(a)**. Ingenuity Pathway Analysis (IPA) showing predicted inhibition of inflammatory bowel disease and colitis in miR-146a-/- mice, based on genes that are upregulated as measured by RNA-seq of the ileum **(b)**.

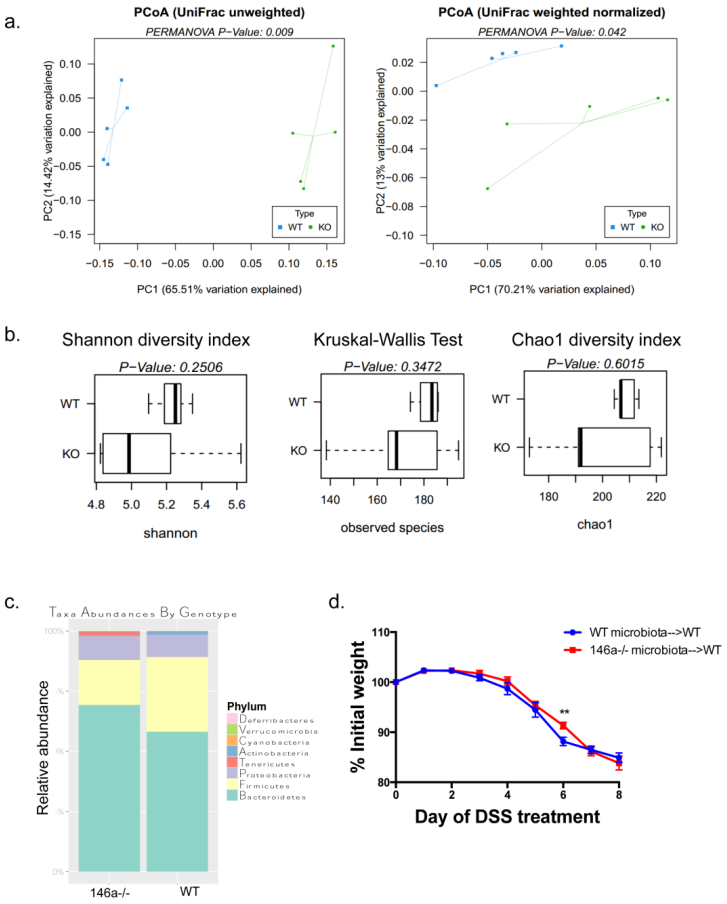


Supplementary Figure 5. miR-146a-/- mice have altered CD4$^+$ T cell populations within the intestinal lamina propria. Lymphocytes were isolated from the small intestinal lamina propria, and flow cytometry was utilized to examine immune cell populations within this tissue. Displayed are representative flow plots, percentages, and total numbers of CD4$^+$ CD3e$^+$ T cells within the small intestinal LP. All populations were first gated on lymphocytes using the FSC/SSC gate, followed by the CD4 and CD3e gating shown **(a)**. Representative flow plots, percentages, and total numbers of CD11b$^+$ cells within the small intestinal LP. All populations were first gated on lymphocytes using the FSC/SSC gate, followed by the CD11b gating shown **(b)**. Representative flow plots, percentages, and total numbers of B220$^+$ cells within the small intestinal LP. All populations were first gated on lymphocytes using the FSC/SSC gate, followed by the

B220 gating shown **(c)**. Displayed are representative flow plots, percentages, and total numbers of $CD4^+$ $CD3e^+$ T cells within the colonic LP. All populations were first gated on lymphocytes using the FSC/SSC gate, followed by the CD4 and CD3e gating shown **(d)**. Representative flow plots, percentages, and total numbers of $CD11b^+$ cells within the colonic LP. All populations were first gated on lymphocytes using the FSC/SSC gate, followed by the CD11b gating shown **(e)**. Representative flow plots, percentages, and total numbers of $B220^+$ cells within the colonic LP. All populations were first gated on lymphocytes using the FSC/SSC gate, followed by the B220 gating shown **(f)**. n=5 (WT) and 4 (146a-/-) (a-c), n=8 (d-f).



Supplementary Figure 6. DSS treated miR-146a-/- have decreased colon shortening and expression of inflammatory genes. Colon shortening in WT and miR-146a-/- mice following 3.5% DSS treatment, as calculated by DSS-treated colon length divided by average untreated colon length for each respective genotype **(a)**. Expression of TNFα **(b)** and IL-6 **(c)** in WT and miR-146a-/- DSS-treated colons, as measured by qRT-PCR. n=13 (WT) and 15 (146a-/-) (a), n=5 (b and c).



Supplementary Figure 7. miR-146a affects beta diversity of fecal microbiota, but not alpha diversity or phyla ratios. Feces was collected from WT and miR-146a-/- mice; total bacterial 16s rDNA was extracted and next-generation sequencing was performed to measure microbiota populations. Principle coordinate analysis plots of fecal microbiota comparing WT communities (blue) with miR-146a-/- (KO) communities (green),

measured via 16s rDNA sequencing; using unweighted Unifrac distance and the weighted, normalized Unifrac distance measure **(a)**. Alpha diversity measures of WT and miR-146a-/- (KO) fecal microbiota communities, using the Shannon diversity, Kruskal-Wallis, and Chao1 diversity indices **(b)**. Relative abundances of 8 major phyla within the kingdom Bacteria, comparing miR-146a-/- and WT fecal communities. No statistical differences in relative abundance were found at the phyla level. WT and miR-146a-/- mice were housed separately within a specific pathogen-free (SPF) mouse facility; n=5 **(c)**. WT or miR-146a-/- intestinal microbiota were transferred to antibiotic-treated WT mice via oral gavage, and recipient mice were administered 3.5% DSS. Weight change of WT and miR-146a-/- microbiota recipient mice during DSS colitis, as measured by percent initial weight **(d)**. n=19 (WT) and 18 (146a-/-); 3 independent experiments.

CHAPTER 4

ANTI-INFLAMMATORY MICRORNA-146A PROTECTS MICE FROM DIET-INDUCED METABOLIC DISEASE

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Abstract

Inflammation in adipose and liver tissues has clearly been associated with metabolic disorders that include obesity and insulin resistance. However, regulatory mechanisms that influence inflammation in these tissues are still being identified, and critical to our capacity to study and treat metabolic diseases that currently plague the Western world. Here, we investigated the role of microRNA-146a (miR-146a) during diet-induced obesity, as this microRNA has been shown to be reduced in patients with type 2 diabetes and to regulate inflammation in other contexts. Results reveal that miR-146a-/- mice on a high fat diet have increased weight gain due to enhanced adiposity, as well as elevated blood glucose levels and insulin resistance compared to WT controls on

the same diet. Furthermore, adipocyte hypertrophy and liver steatosis were also exaggerated in miR-146a-/- versus control mice on a high fat diet. Upon closer examination of adipose and liver tissues, we observed an increase in inflammatory genes, as well as increased NFκB activation, in the absence of miR-146a. High fat diet-fed miR-146a-/- mice also had crown-like structures, indicating excessive adipose inflammation and accumulation of macrophages. RNA-sequencing of sorted adipose tissue macrophages from WT and miR-146a-/- mice revealed a role for miR-146a in limiting inflammatory and metabolic pathways. Finally, we found that the NFκB activating protein Traf6 is a miR-146a mRNA target in adipose tissue and macrophages. Further, we demonstrate that in addition to NFκB and inflammation, miR-146a regulates AKT activation to alter the metabolic state of macrophages. Altogether, this study demonstrates that miR-146a represses metabolic tissue inflammation and metabolic disease in response to a high fat diet, and exhibits how the combination of diet and genetics can influence obesity and diabetes phenotypes.

Introduction

Obesity and the resulting chronic inflammation and metabolic disorders are prominent throughout the world, placing a large burden on the healthcare system. Dietary, genetic, environmental, psychological, socioeconomic, as well as other factors, play a role in the widespread obesity epidemic that has arisen in the past 20-30 years (Johnson et al., 2012). Recently, diabetes, heart disease, cancer, infections, and other previously correlated diseases have been directly linked to obesity through immunological pathways (Kanneganti and Dixit, 2012). During excessive caloric intake,

adipocyte stress results in secretion of adipokines that recruit and activate pro-inflammatory immune cells within adipose tissue (Rutkowski et al., 2015). This local inflammation, perpetuated by cytokine production by adipocytes and adipose tissue macrophages (ATMs), T cells, and other leukocytes, leads to further recruitment of pro-inflammatory immune cells to adipose tissue and other metabolically stressed tissues, including liver and muscle (Lumeng and Saltiel, 2011). Thus, obesity triggers chronic inflammation within the adipose tissue in addition to systemic compartments. This chronic inflammation is characterized by increased production of pro-inflammatory cytokines and adipokines such as TNFα, Leptin, IL-6, and IL-1β by many cell types including M1 macrophages and Th1 T cells (Gregor and Hotamisligil, 2011; Huh et al., 2014). While the mechanistic details of this process are still being uncovered, it is clear that macrophages play a large role in the pathogenesis of adipose and systemic inflammation during obesity.

Macrophages were initially characterized as players in obesity when they were found to present at increased numbers within adipose tissue of obese mice and humans (Weisberg et al., 2003; Xu et al., 2003). Later work found these cells to be preferentially polarized to the "classically activated" M1 state within obese adipose tissue versus the "alternatively activated" M2 macrophages, which are more prominent within lean adipose tissue (Lumeng et al., 2007). These macrophage populations play roles in maintenance and turnover of adipocytes, as well as metabolic roles, such as lipid buffering, in order to maintain homeostasis (Boutens and Stienstra, 2016). During obesity, macrophages are recruited to adipose tissue (Kamei et al., 2006; Oh et al., 2012; Yu et al., 2006) and are polarized to a more pro-inflammatory state, secreting high

amounts of cytokines like TNFα, IL-1β, and reactive oxygen species (ROS) (Boutens and Stienstra, 2016). At the metabolic level, M1 macrophages are characterized by a Warburg switch into glycolytic metabolism over oxidative phosphorylation and fatty acid synthesis, which is driven by HIF1α and other metabolic intermediates (Kelly and O'Neill, 2015; O'Neill et al., 2016). Additionally, adipose tissue macrophages (ATMs) are metabolically distinct from those found during infection, including in lipid metabolism pathways, and this metabolic activation may drive disease during obesity and diabetes (Kratz et al., 2014; Xu et al., 2013). These pro-inflammatory, metabolically activated ATMs from obese tissue have been shown to promote insulin resistance (Chawla et al., 2011; Xu et al., 2003), chronic inflammation (Boutens and Stienstra, 2016; Xu et al., 2003), and metabolic dysregulation (Chawla et al., 2011; Kratz et al., 2014; Xu et al., 2013), further perpetuating obesity and its comorbidities (Johnson et al., 2012). Even so, the pathways and molecules that play roles in ATMs during obesity and metabolic disease have not been fully elucidated.

Some regulators of metabolic inflammation, including within macrophages, are microRNAs (miRNAs). MiRNAs are ~22 nucleotide-long noncoding RNAs that downregulate gene expression by mRNA degradation and/or ribosomal inhibition upon binding to their mRNA targets via base complementarity. The roles for miRNAs in inflammation and immunity have been widely studied, and it is now accepted that many particular miRNAs are essential for proper immune system function (Lee et al., 2014; O'Connell and Baltimore, 2012; O'Connell et al., 2010). One such miRNA is microRNA 146a (miR-146a), which is an anti-inflammatory miRNA that dampens the immune response through targeting mRNAs that activate NFκB and inflammation, including

Traf6, Irak1, and Stat1 (Boldin et al., 2011; Lu et al., 2010; Taganov et al., 2006). miR-146a plays a role in many immune phenotypes and diseases, including hematopoiesis (Boldin et al., 2011; Starczynowski et al., 2011; Zhao et al., 2013; Zhao and Starczynowski, 2014), cancer (Boldin et al., 2011; Huffaker et al., 2012; Zhao and Starczynowski, 2014), Lyme Disease arthritis (Lochhead et al., 2014), autoimmunity (Hu et al., 2014; Lu et al., 2010), immunization (Pratama et al., 2015), intestinal homeostasis (Runtsch et al., 2015), and many others. This miRNA has been shown to be important within the macrophage response (Alexander et al., 2015; Boldin et al., 2011; Lochhead et al., 2014; Zhao et al., 2013) and polarization (Huang et al., 2016; Li et al., 2015), as loss of miR-146 leads to hyper-responsive and more pro-inflammatory macrophages (Zhao et al., 2013). Even so, the cell-intrinsic roles of miR-146a are not well understood. Because miR-146a is important for downregulating the inflammatory response, including in macrophages, and because increased immune activation is associated with obesity and metabolic disease, we hypothesized that miR-146a is important for dampening inflammation during diet-induced obesity and preventing metabolic disease. This is further corroborated by data in which this miRNA is associated with diabetes (Balasubramanyam et al., 2011; Baldeon et al., 2014; Bhatt et al., 2016; Feng et al., 2011; Yang et al., 2015) and is suggested to regulate metabolic processes (Payne, 2013).

Here, we show that loss of miR-146a resulted in worsened obesity, fatty liver disease, and diabetes during diet-induced obesity (DIO). MiR-146a-/- mice had increased inflammation, including NFκB activation, in metabolic tissues both before and after onset of metabolic disease. miR-146a was expressed within adipose tissue and regulated accumulation of crown-like structures during high fat diet. Upon RNA-sequencing

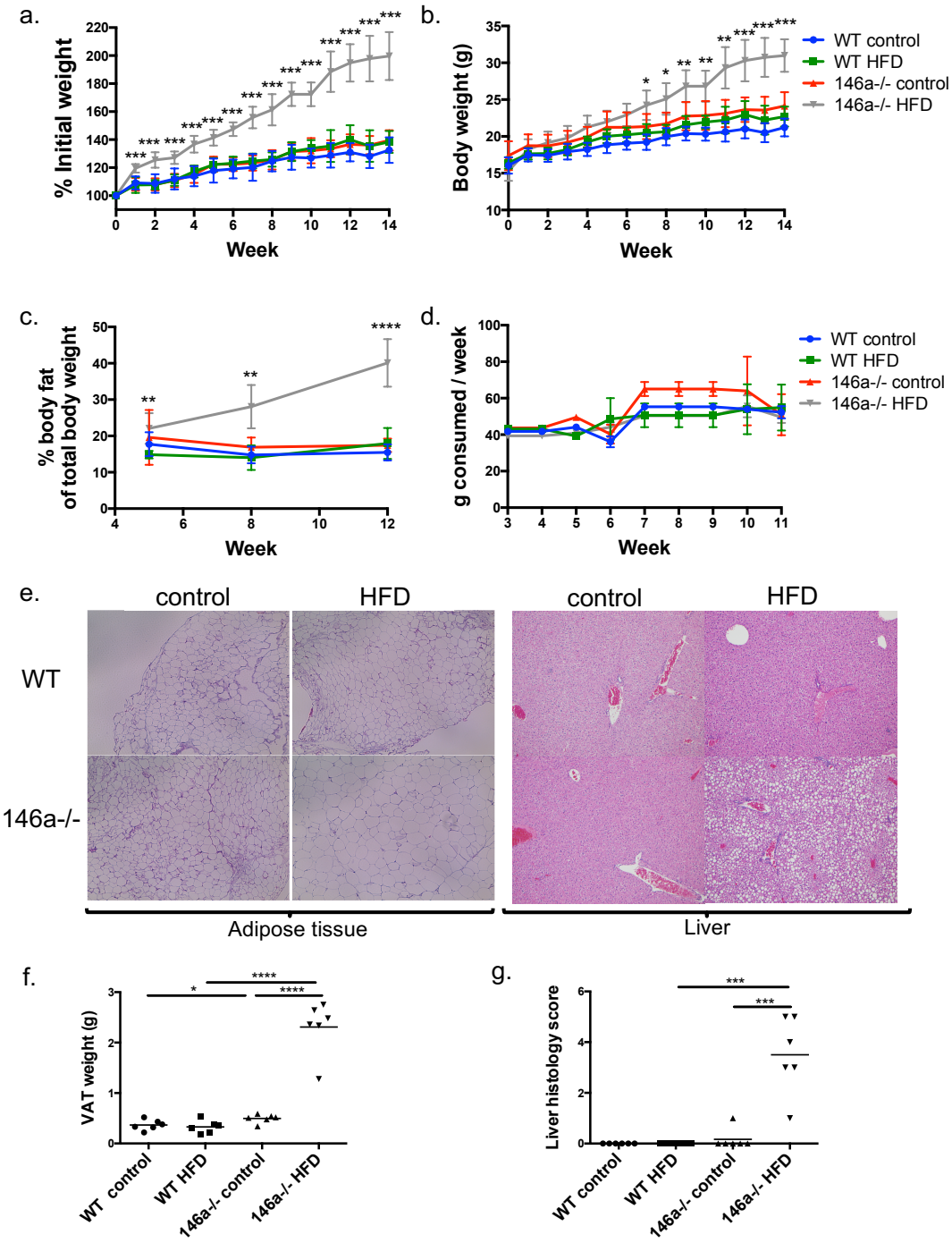
analysis of sorted white adipose tissue macrophages, we found that miR-146a regulates multiple inflammatory and metabolic pathways both before and during obesity, including NFκB and PI3K/mTOR/AKT. Furthermore, loss of miR-146a resulted in upregulation of lipid metabolism genes in adipose tissue macrophages during high fat diet. We found *Traf6* to be a target of miR-146a during DIO, and show that miR-146a-/- macrophages were hyper-responsive and upregulated inflammatory gene expression upon TLR activation. Futher, obese adipose tissue and macrophages had increased activation of AKT upon loss of miR-146a. This creates a novel link of miR-146a to AKT and metabolic signaling downstream via Traf6. Altogether, these data demonstrate important functions for miR-146a in immunometabolic pathways within macrophages during obesity and metabolic disease and that this miRNA is required to prevent metabolic disease.

Results

miR-146a is required to prevent obesity during a high fat diet

In order to study the role of miR-146a in the development of obesity and metabolic disease, we fed female B6 WT or miR-146a-/- mice a control diet with 10% kcal from fat or a high fat diet (HFD) with 45% kcal from fat. Over time, WT mice on a HFD or control diet, and miR-146a-/- control diet mice, all gained some weight albeit the groups were not statistically different from one another (Figure 4.1a and b). In contrast, miR-146a-/- mice fed a HFD gained significantly more body weight compared to the other groups, both as a measure of percent initial weight (Figure 4.1a, 4.S1a) and weight in grams (Figure 4.1b). This was seen with both male and female miR-146a-/- mice on a

Figure 4.1. miR-146a is required for protection from weight and fat gain, adipocyte hypertrophy, and liver steatosis during diet-induced obesity. C57BL/6 WT and miR-146a-/- mice were placed on a control diet of 10% kcal/fat (control) or a high-fat diet of 45% kcal/fat (HFD). Percent weight gain of WT and miR-146a-/- mice over time of diet, based on initial weight in grams **(a)**. Body weight in grams of WT and miR-146a-/- mice over time of diet **(b)**. Body composition of mice was measured using TD-NMR. Shown is percent body fat of WT and miR-146a-/- at timepoints 5, 8, and 12 weeks during diet treatment **(c)**. Amount of chow consumed by one mouse each week over time of diet **(d)**. (Left) H&E staining of representative sections of visceral adipose tissue at week 14 of diet treatment. (Right) H&E staining of representative sections of liver tissue at week 14 of diet treatment **(e)**. Weight of reproductive visceral adipose tissue (VAT) at 14 weeks of diet **(f)**. Histology scores of H&E stained liver tissues, measuring steatosis and inflammatory foci within livers at 14 weeks of diet **(g)**.


a.
% Initial weight
Week
b.
Body weight (g)
Week
WT control
WT HFD
146a-/- control
146a-/- HFD
c.
% body fat of total body weight
Week
d.
g consumed / week
Week
WT control
WT HFD
146a-/- control
146a-/- HFD
e.
control
HFD
control
HFD
WT
146a-/-
Adipose tissue
Liver
f.
VAT weight (g)
WT control
WT HFD
146a-/- control
146a-/- HFD
g.
Liver histology score
WT control
WT HFD
146a-/- control
146a-/- HFD

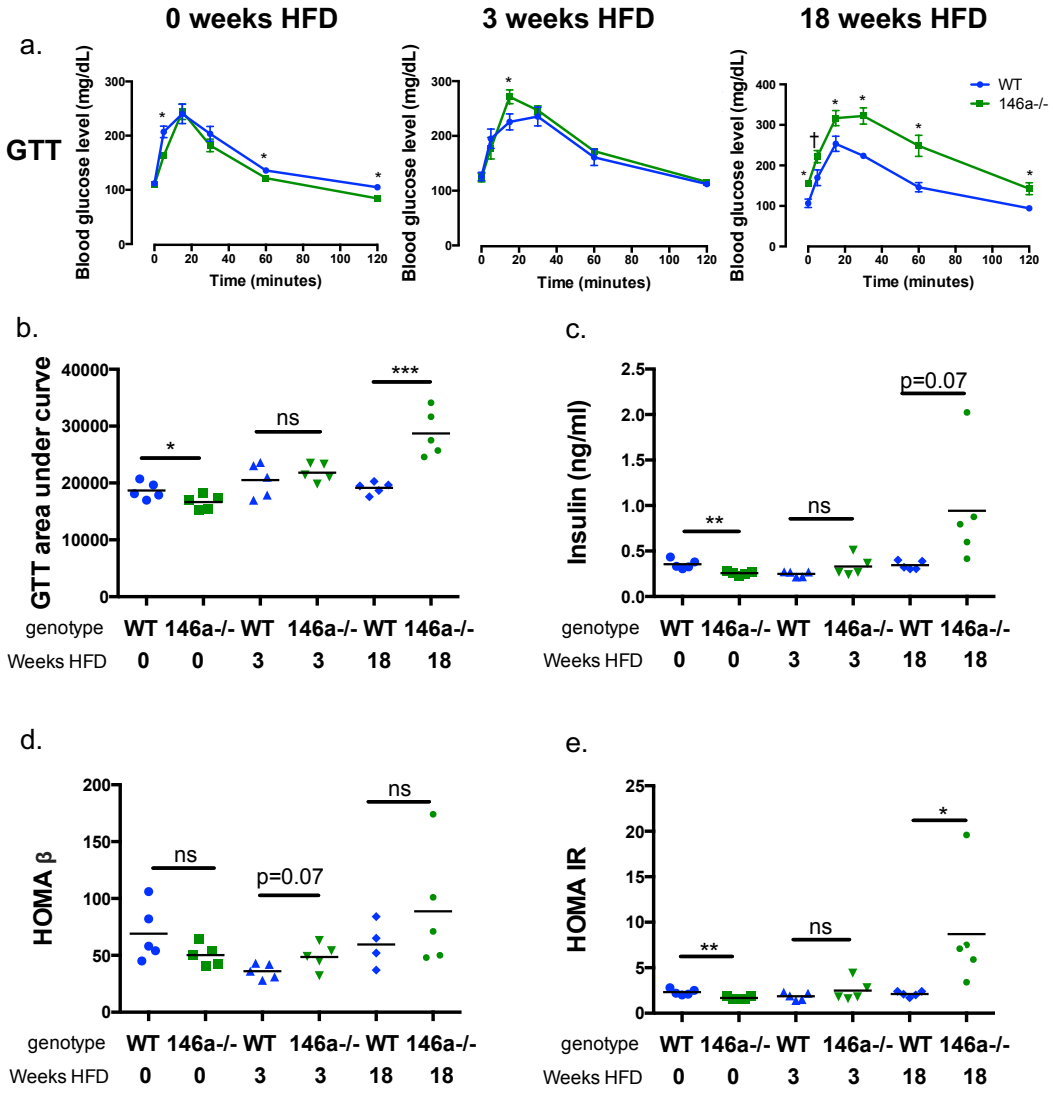
high fat diet (4.S1b) and also in miR-146a-/- mice purchased from Jackson Labs compared to WT Jackson C57BL/6 controls (Figure 4.S1c and d). Upon performing an NMR body composition analysis, we found that miR-146a-/- mice on a HFD gained significantly more body fat than the other groups, with a body composition of ~40% body fat by week 12 after beginning the diet compared to ~20% for the other groups (Figure 4.1c). The significant weight and fat gain by miR-146a-/- HFD mice was not caused by an increase in food consumption, as the groups consumed a similar amount of food over the course of the diet (Figure 4.1d). This was corroborated by a metabolic chamber measurement of food and drink consumption over 5 days, where minimal differences in food consumption were observed until 18 weeks on HFD, in which miR-146a-/- mice consumed less food than WT (Figure 4.S2d). Altered heat production, decreased movement, respiratory exchange ratio, VO_2, and VCO_2 were measured in miR-146a-/- HFD mice, indicating metabolic disease in these animals, while few changes in these metabolic parameters were observed at earlier timepoints of HFD treatment (Figure 4.S2a-S2c). Upon histological examination of the adipose tissue following the diet regimens, WT control, WT HFD, and miR-146a control mice all showed normal adipose organization and architecture (Figure 4.1e, left). However, miR-146a-/- mice fed a HFD displayed hypertrophied adipocytes, indicating dysfunctional adipocytes and metabolic disease (Rutkowski et al., 2015) (Figure 4.1e, left). Visceral adipose tissue was removed from these mice, and miR-146a-/- HFD fat pads were significantly larger in weight compared with the other three groups (Figure 4.1f). Further, histology of livers from miR-146a-/- HFD mice showed steatosis, while fatty liver was not observed in the WT groups and miR-146a-/- mice on a control diet (Figure 4.1e, right). Liver histology

scores, measuring portal inflammation and steatosis, were quantified according to a previously published scoring system (Dixon et al., 2004). miR-146a-/- HFD mice had significantly increased liver histology scores compared with WT control, WT HFD, and miR-146a-/- control groups (Figure 4.1g), indicating miR-146a-/- HFD mice have worsened metabolic disease within the liver. Further indicative of obesity and metabolic dysregulation, miR-146a-/- mice fed HFD for 17 weeks had elevated Leptin serum protein levels, while miR-146a-/- mice at 2 weeks HFD did not (Figure 4.S1e). These data indicate that the combination of a miR-146a deficiency and HFD are both required for the exacerbated weight gain and metabolic disease to occur in this model of diet-induced obesity.

miR-146a protects mice from hyperglycemia during a high fat diet

Obesity is closely associated with glucose dysregulation, and recent reports have found decreased miR-146a levels in patients with type 2 diabetes (Balasubramanyam et al., 2011; Baldeon et al., 2014; Feng et al., 2011). Thus, we next examined glucose homeostasis in mice lacking miR-146a during DIO. miR-146a-/- mice fed HFD showed higher blood glucose levels during fasting and glucose tolerance tests (GTT), compared with the WT control, WT HFD, and miR-146a-/- control diet groups (Figure 4.S3a and S3b). GTTs were performed on fasted WT and miR-146a-/- mice at 0 weeks HFD, 3 weeks HFD, or 18 weeks HFD in order to determine if diabetic phenotypes precede or succeed the enhanced obesity in miR-146a-/- mice. Results indicate that miR-146a-/- mice showed little difference in fasting blood glucose and during GTT at timepoints 0 and 3 weeks HFD compared with WT (Figure 4.2a and 2b). However, after 18 weeks on

Figure 4.2. miR-146a is required for protection from diabetic phenotypes during diet-induced obesity. Blood glucose levels of WT (blue) and miR-146a-/- (green) mice given 45% HFD for 0 weeks, 3 weeks, or 18 weeks, following injection of glucose at 0 minutes and measured over time for 120 minutes **(a)**. Area under curve of glucose tolerance tests (GTT) for WT and miR-146a-/- mice given HFD for 0, 3, or 18 weeks **(b)**. Serum levels of insulin in WT and miR-146a-/- mice given HFD for 0, 3, or 18 weeks, measured via ELISA **(c)**. Homeostatic assessment (HOMA) beta levels, measuring beta cell function of WT and miR-146a-/- mice given HFD for 0, 3, or 18 weeks **(d)**. Homeostatic assessment (HOMA) IR levels, measuring insulin resistance of WT and miR-146a-/- mice given HFD for 0, 3, or 18 weeks **(e)**.


a.
GTT
0 weeks HFD
3 weeks HFD
18 weeks HFD
Blood glucose level (mg/dL)
Time (minutes)
WT
146a-/-
b.
GTT area under curve
c.
Insulin (ng/ml)
p=0.07
d.
HOMA β
e.
HOMA IR
ns
genotype WT 146a-/- WT 146a-/- WT 146a-/-
Weeks HFD 0 0 3 3 18 18

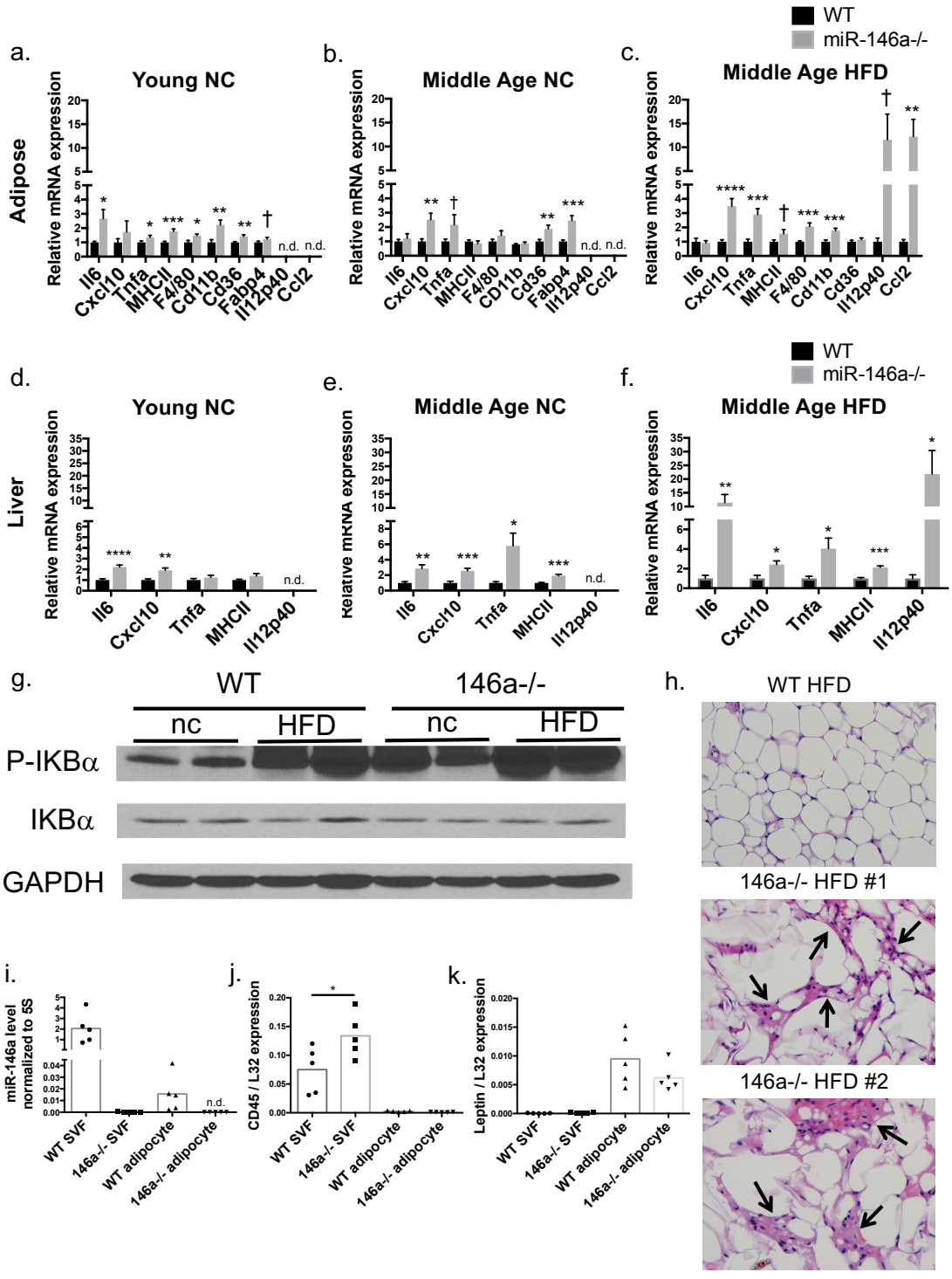
a HFD, miR-146a-/- mice had significantly higher blood glucose levels than WT mice at resting, and during GTT, with an increase in the GTT area under curve (Figure 4.2a and 2b). These data indicate that miR-146a-/- mice are not glucose intolerant on a low-fat normal chow diet or during early stages of DIO, but develop an inability to properly regulate glucose after adiposity has begun.

miR-146a-/- and WT mice also had comparable fasting serum insulin levels at 0 weeks and 3 weeks HFD, yet miR-146a-/- mice had notably higher levels of serum insulin at 18 weeks HFD (Figure 4.2c), revealing that miR-146a mice can still make insulin. Increased insulin levels also indicate insulin resistance, as hyperinsulinema is a common symptom in type 2 diabetes patients (Shanik et al., 2008). HOMA-β and HOMA-IR, which are based on fasting blood glucose and insulin levels and measure pancreatic beta cell function and insulin resistance, respectively, were calculated. No differences in HOMA-β were measured between WT and miR-146a-/- mice (Figure 4.2d), and pancreatic architecture of miR-146a-/- mice appeared normal upon H&E staining (Figure 4.S3c), both consistent with miR-146a-/- mice being able to produce insulin. On the other hand, HOMA-IR was increased in the miR-146a-/- HFD group at 18 weeks, demonstrating that miR-146a-/- mice develop insulin resistance over time following DIO (Figure 4.2e). Altogether, these data show that miR-146a is required in mice to prevent development of a type 2 diabetes phenotype during DIO.

miR-146a constrains inflammation in metabolic tissues during a normal and high fat diet

miR-146a has previously been shown to minimize inflammation in several contexts; therefore, we hypothesized that it may be regulating inflammatory gene expression within metabolic tissues, including adipose tissue and liver, which could influence adiposity and insulin resistance. To determine whether gene expression changes were due to high fat diet, inflammatory gene expression was measured via qPCR in whole visceral white adipose tissue (WAT) and liver tissue from WT and miR-146a-/- mice on normal chow and high fat diet at different ages. 8-week-old (young) miR-146a-/- mice fed a standard diet (normal chow or NC) had increased WAT expression of Il6, Tnfa, MHCII, and Cd36 (Figure 4.3a) and increased expression of IL6 and Cxcl10 in the liver (Figure 4.3d) compared to WT. Similar changes were also observed in 5-month-old mice, where these inflammatory and fatty acid transporter genes were higher in miR-146a-/- adipose (Figure 4.3b) and Il6, Cxcl10, Tnfa, and MHCII were increased in miR-146a-/- liver (Figure 4.3e). In 5-month-old mice fed high fat diet, we observed increases in a variety of inflammatory genes, including MHCII, Cxcl10, Il12p40, Ccl2, Tnfa, F4/80, and Cd11b in WAT (Figure 4.3c), and Il6, Cxcl10, Tnfa, Ifng, and Il12p40 in liver tissue (Figure 4.3f) from miR-146a-/- compared with WT mice. Additionally, miR-146a-/- mice had increased NFκB activation, as measured by phosphorylated IKBα, compared to WT controls, and this activation was further increased in mice fed a HFD (Figure 4.3g). As a control, Total IKBα and GAPDH levels remained constant. These data indicate that the increased inflammation in miR-146a-/- mouse tissues occurs in the absence of a high fat diet, and is likely a preexisting condition that sensitizes these mice

Figure 4.3. miR-146a constrains inflammation and NFkB activation before and during HFD and is expressed in adipose tissue. qRT-PCR expression levels of various mRNAs (shown on x axis) measured in whole visceral adipose tissue of 8-week-old WT (black) or miR-146a-/- (grey) mice fed a standard diet (NC). Expression levels were measured relative to housekeeping gene L32 and normalized by setting WT expression to 1 **(a)**. qRT-PCR expression levels of various mRNAs (shown on x axis) measured in whole visceral adipose tissue of 20-week-old WT (black) or miR-146a-/- (grey) mice fed a standard diet (NC). Expression levels were measured relative to housekeeping gene L32 and normalized by setting WT expression to 1 **(b)**. qRT-PCR expression levels of various mRNAs (shown on x axis) measured in whole visceral adipose tissue of 20-week-old WT (black) or miR-146a-/- (grey) mice fed a 45% high fat diet (HFD). Expression levels were measured relative to housekeeping gene L32 and normalized by setting WT expression to 1 **(c)**. qRT-PCR expression levels of various mRNAs (shown on x axis) measured in liver of 8-week-old WT (black) or miR-146a-/- (grey) mice fed a standard diet (NC). Expression levels were measured relative to housekeeping gene L32 and normalized by setting WT expression to 1 **(d)**. qRT-PCR expression levels of various mRNAs (shown on x axis) measured in liver of 20-week-old WT (black) or miR-146a-/- (grey) mice fed a standard diet (NC). Expression levels were measured relative to housekeeping gene L32 and normalized by setting WT expression to 1 **(e)**. qRT-PCR expression levels of various mRNAs (shown on x axis) measured in liver of 20-week-old WT (black) or miR-146a-/- (grey) mice fed a high fat diet (HFD). Expression levels were measured relative to housekeeping gene L32 and normalized by setting WT expression to 1 **(f)**. Western blot for phosphorylated IKBa, total IKBa, and GAPDH in lysates of whole visceral white adipose tissue collected from 20-week-old WT or miR-146a-/- mice fed a standard diet (normal chow, NC) or high fat diet (HFD) **(g)**. H&E staining of visceral adipose tissue of WT and miR-146a-/- mice fed high fat diet (HFD) for 18 weeks. Black arrows indicate areas of crown-like structures and/or inflammation. Two representative examples of miR-146a-/- are shown, with one representative WT mouse **(h)**. The stromal vascular fraction (SVF) and adipocytes were fractionated from whole visceral adipose tissue of WT and miR-146a-/- mice fed a standard diet. Expression of mature miR-146a was measured using qRT-PCR, relative to 5S rRNA **(i)**. The stromal vascular fraction (SVF) and adipocytes were fractionated from whole visceral adipose tissue of WT and miR-146a-/- mice fed a standard diet. Expression of *Ptprc* (CD45) was measured using qRT-PCR **(j)**. The stromal vascular fraction (SVF) and adipocytes were fractionated from whole visceral adipose tissue of WT and miR-146a-/- mice fed a standard diet. Expression of *Leptin* was measured using qRT-PCR **(k)**.


a.
b.
c.
WT
miR-146a-/-
Adipose
Young NC
Middle Age NC
Middle Age HFD
Relative mRNA expression
Il6
Cxcl10
Tnfa
MHCII
F4/80
Cd11b
Cd36
Fabp4
Il12p40
Ccl2
n.d.
d.
e.
f.
Liver
g.
WT
146a-/-
nc
HFD
P-IKBα
IKBα
GAPDH
h.
WT HFD
146a-/- HFD #1
146a-/- HFD #2
i.
miR-146a level normalized to 5S
j.
CD45 / L32 expression
k.
Leptin / L32 expression
WT SVF
146a-/- SVF
WT adipocyte
146a-/- adipocyte

to obesity and glucose dysregulation in response to elevated amounts of fat in their diet. Even so, DIO further activates inflammation in miR-146a-/- mice, as the fold change increase in gene expression compared to WT was further boosted during HFD. Upon conducting a blinded histological examination of H&E stained WT and miR-146a-/- visceral adipose tissues following HFD for 18 weeks, we observed more crown-like structures within miR-146a-/- compared to WT mice (Figure 4.3h). This indicates an infiltration of immune cells to adipose tissue in miR-146a-/- mice during DIO. Altogether, miR-146a constrains inflammation and adipose tissue macrophages upon aging and diet-induced obesity.

Because miR-146a has known roles within immune cells, and because immune cells present in adipose tissue cause inflammation that drives metabolic disease, we hypothesized that this miRNA is expressed in immune cells to promote exacerbated metabolic disease. In order to assess the local expression of miR-146a within adipose tissue, the leukocyte-containing stromal vascular fraction (SVF) and adipocytes of WT and miR-146a-/- visceral adipose tissue (VAT) were separated, and qPCR was performed on RNA purified from these fractions. Mature miR-146a was highly expressed in the SVF and at lower levels within the adipocyte fraction of WT mice (Figure 4.3i). As a control, mature miR-146a was not detected in miR-146a-/- SVF and adipocyte fractions. To ensure proper separation of the SVF and adipocyte fractions, we assayed *Ptprc* (CD45) expression, a white blood cell marker that was specifically detected in the SVF (Figure 4.3j), and *Leptin*, which was only expressed in the adipocyte fraction (Figure 4.3k). These results indicate that miR-146a is expressed within the stromal vascular fraction of visceral adipose tissue and at lower levels within adipocytes,

suggesting that it has a regulatory function within adipose tissue immune cells.

Exaggerated obesity and hyperglycemia in miR-146a-/- mice is not dependent on miR-155

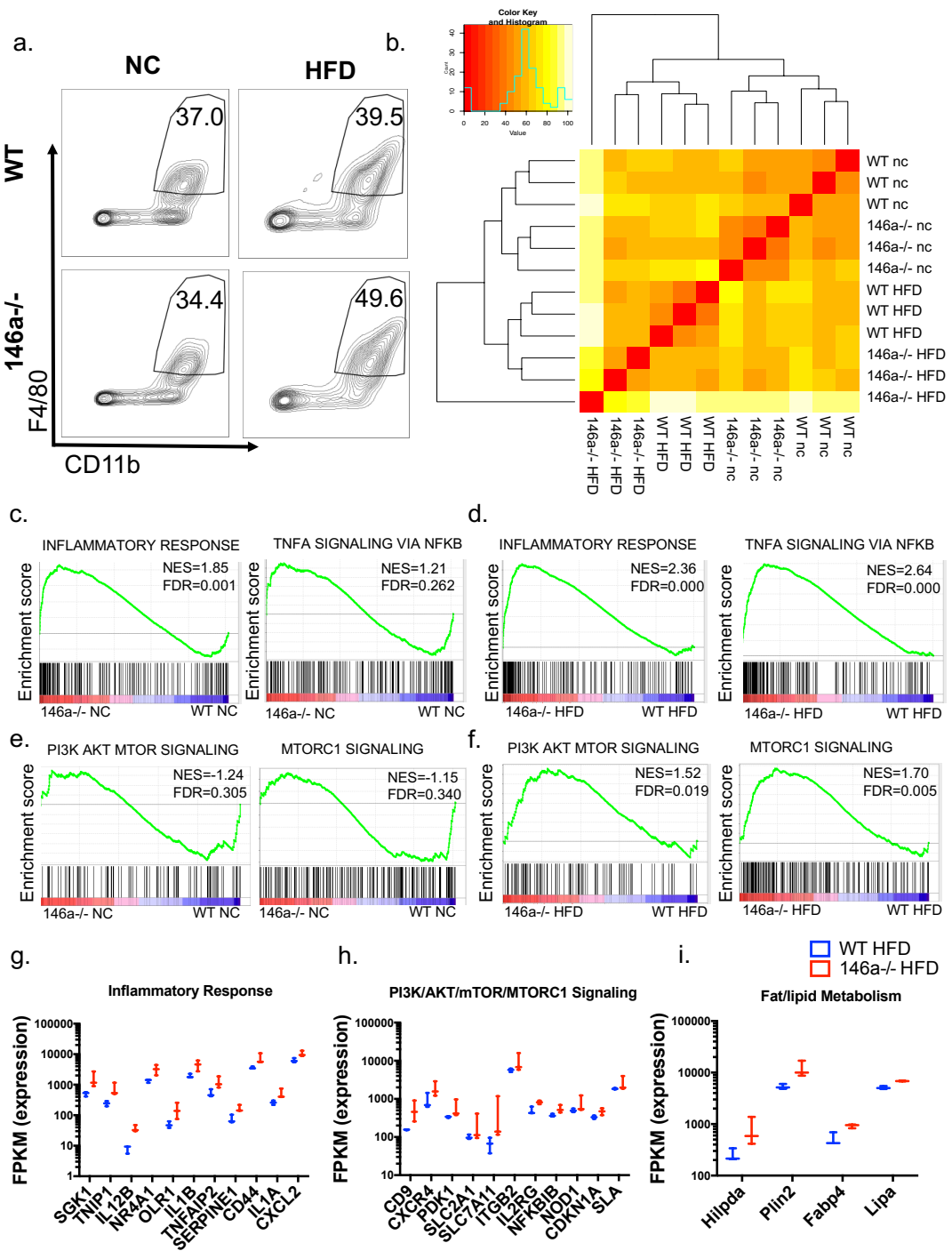
We have previously shown that some of the age-dependent chronic inflammatory phenotypes exhibited by loss of miR-146a are dependent upon miR-155, an inflammation-promoting miRNA (Hu et al., 2014; Huffaker et al., 2012). Thus, we wanted to test whether or not the metabolic disease and inflammatory phenotypes in miR-146a-/- mice during DIO were also dependent on miR-155 expression. To test this, we fed a HFD to WT, miR-155-/-, miR-146a-/-, and miR-155-/- miR-146a-/- (double knockout or DKO) mice for a period of 12 weeks and measured weight gain over time. miR-155-/- mice had a similar increase in weight over time in response to a high fat diet compared with WT mice, while miR-146a-/- and DKO mice were both significantly larger than both WT and miR-155-/- (Figure 4.S4a and S4b). Fasting blood glucose levels also followed this pattern, with DKO mice having levels similar to miR-146a-/- mice, and increased when compared to WT and miR-155-/- mice (Figure 4.S4c). Further, miR-146a-/- and DKO mice also had larger visceral adipose tissue fat pads (Figure 4.S4d), a greater percent body fat (Figure 4.S4e), and reduced lean body composition (Figure 4.S4f) compared with WT and miR-155-/- mice. These results demonstrate that miR-155 does not promote obesity and metabolic disease in the miR-146a-/- DIO model. This also suggests that our miR-146a-/- mouse obesity and metabolic disease phenotype may be T cell-independent, as our previous models showed that miR-155 plays a T cell-intrinsic role during age-dependent chronic inflammation or antitumor immunity upon loss of

miR-146a (Hu et al., 2014; Huffaker et al., 2012).

Enhanced inflammatory gene expression and mTor signaling by adipose tissue macrophages from miR-146a-/- mice on a high fat diet

Macrophages are dominant in adipose tissue during obesity (Weisberg et al., 2003; Xu et al., 2003) and promote much of the inflammation that causes metabolic disease and other comorbidities (Boutens and Stienstra, 2016). Since these cells and macrophage hallmark genes were accumulated in adipose tissue of miR-146a-/- mice fed high fat diet (Figure 4.3a-c, 3h), we hypothesized that miR-146a in macrophages was contributing to obesity and metabolic disease. To identify the genes and pathways that miR-146a might be regulating in macrophages *in vivo*, we isolated adipose tissue macrophages from adult (5-month-old) WT and miR-146a-/- mice fed a standard diet (NC) or given a 45% HFD for 15 weeks. As expected, the miR-146a-/- mice treated with HFD gained significantly more weight and fat than their WT counterparts (data not shown). Adipose tissue was removed from all four groups (WT normal chow (NC) or HFD, miR-146a-/- NC or HFD), digested, and $CD45^+$ $CD11b^+$ $F4/80^+$ cells were sorted using fluorescence activated cell sorting (FACS). A comparable proportion of macrophages were sorted from NC animals, while a trending increase in percentage of macrophages was measured in miR-146a-/- HFD compared to WT (Figure 4.4a, Figure 4.S5a). The total number of macrophages sorted was slightly increased in 146a-/- NC animals compared to WT NC but not different in HFD (Figure 4.S5b). RNA was extracted from sorted macrophages, and RNA-seq was performed on these cells. Gene expression by these cells clustered with their respective genotypes and diet treatments,

Figure 4.4. miR-146a regulates gene expression in adipose tissue macrophages during standard and high fat diet**.** Representative fluorescence activated cell sorting (FACS) plots of $CD11b^+$ $F4/80^+$ cells sorted from live, singlet, $CD45^+$ cells collected from the stromal vascular fraction of visceral adipose tissue from 20-week-old WT and miR-146a-/- mice fed a standard diet (NC) or high fat diet (HFD). Each sample contained cells from 3 mice combined in order to obtain enough RNA, and 3 samples were collected for each genotype, for a total of 9 mice per genotype and treatment **(a)**. Heat map showing total gene expression of sorted adipose tissue macrophages from WT and miR-146a-/- mice fed standard diet (nc) or high fat diet (HFD), as measured by RNA-seq. Red color indicates 0% dissimilarity to respective compared sample, while yellow indicates more dissimilarity in total RNA expression **(b)**. (Left) Geneset enrichment analysis plot showing enrichment of inflammatory response geneset in miR-146a-/- mice fed a standard diet (NC) as compared with WT. Black bars indicate enrichment of individual genes in the set in miR-146a-/- (red) or WT (blue), while green line indicates enrichment score of genes within that set. NES=normalized enrichment score; FDR=false discovery rate, where FDR<0.25 is statistically significant. (Right) Geneset enrichment analysis plot showing no statistical enrichment of the TNFa signaling via NFkB geneset in miR-146a-/- mice fed NC as compared with WT, as FDR>0.25 **(c)**. (Left) Geneset enrichment analysis plot showing enrichment of inflammatory response geneset in miR-146a-/- mice fed a high fat diet (HFD) as compared with WT. Black bars indicate enrichment of individual genes in the set in miR-146a-/- (red) or WT (blue), while green line indicates enrichment score of genes within that set. NES=normalized enrichment score; FDR=false discovery rate, where FDR<0.25 is statistically significant. (Right) Geneset enrichment analysis plot showing enrichment of the TNFa signaling via NFkB geneset in miR-146a-/- mice fed HFD as compared with WT **(d)**. (Left) Geneset enrichment analysis plot showing no statistical enrichment of PI3K/AKT/mTOR signaling in miR-146a-/- mice fed a standard diet (NC) as compared with WT. Black bars indicate enrichment of individual genes in the set in miR-146a-/- (red) or WT (blue), while green line indicates enrichment score of genes within that set. NES=normalized enrichment score; FDR=false discovery rate, where FDR<0.25 is statistically significant. (Right) Geneset enrichment analysis plot showing no statistical enrichment of mTORC1 signaling in miR-146a-/- mice fed a standard diet (NC) as compared with WT **(e)**. (Left) Geneset enrichment analysis plot showing enrichment of PI3K/AKT/mTOR signaling in miR-146a-/- mice fed a high fat diet (HFD) as compared with WT. Black bars indicate enrichment of individual genes in the set in miR-146a-/- (red) or WT (blue), while green line indicates enrichment score of genes within that set. NES=normalized enrichment score; FDR=false discovery rate, where FDR<0.25 is statistically significant. (Right) Geneset enrichment analysis plot showing enrichment of mTORC1 signaling in miR-146a-/- mice fed a high fat diet (HFD) as compared with WT **(f)**. FPKM values of the top hits within the inflammatory response GSEA geneset upregulated in miR-146a-/- adipose tissue macrophages compared to WT, as determined by RNA-seq **(g)**. FPKM values of the top hits within the PI3K/AKT/mTOR/mTORC1 signaling GSEA genesets upregulated in miR-146a-/- adipose tissue macrophages compared to WT, as determined by RNA-seq **(h)**. FPKM values of fat and lipid metabolism genes upregulated in miR-146a-/- adipose tissue macrophages compared to WT, as determined by RNA-seq **(i)**.


a.
NC
HFD
WT
146a-/-
37.0
39.5
34.4
49.6
F4/80
CD11b
b.
Color Key and Histogram
WT nc
WT nc
WT nc
146a-/- nc
146a-/- nc
146a-/- nc
WT HFD
WT HFD
WT HFD
146a-/- HFD
146a-/- HFD
146a-/- HFD
c.
INFLAMMATORY RESPONSE
NES=1.85
FDR=0.001
TNFA SIGNALING VIA NFKB
NES=1.21
FDR=0.262
Enrichment score
146a-/- NC
WT NC
d.
INFLAMMATORY RESPONSE
NES=2.36
FDR=0.000
TNFA SIGNALING VIA NFKB
NES=2.64
FDR=0.000
146a-/- HFD
WT HFD
e.
PI3K AKT MTOR SIGNALING
NES=-1.24
FDR=0.305
MTORC1 SIGNALING
NES=-1.15
FDR=0.340
f.
PI3K AKT MTOR SIGNALING
NES=1.52
FDR=0.019
MTORC1 SIGNALING
NES=1.70
FDR=0.005
g.
Inflammatory Response
FPKM (expression)
SGK1
TNIP1
IL12B
NR4A1
OLR1
IL1B
TNFAIP2
SERPINE1
CD44
IL1A
CXCL2
h.
PI3K/AKT/mTOR/MTORC1 Signaling
CD9
CXCR4
PDK1
SLC2A1
SLC7A11
ITGB2
IL2RG
NFKBIB
NOD1
CDKN1A
SLA
i.
WT HFD
146a-/- HFD
Fat/lipid Metabolism
Hilpda
Plin2
Fabp4
Lipa

indicating that genes are regulated by both miR-146a and type of diet (Figure 4.4b). Average FPKM (reads) of mRNAs show that gene expression patterns of adipose tissue macrophages are unique to both diet type and genotype, and few genes overlapped between genotypes and diets (data not shown).

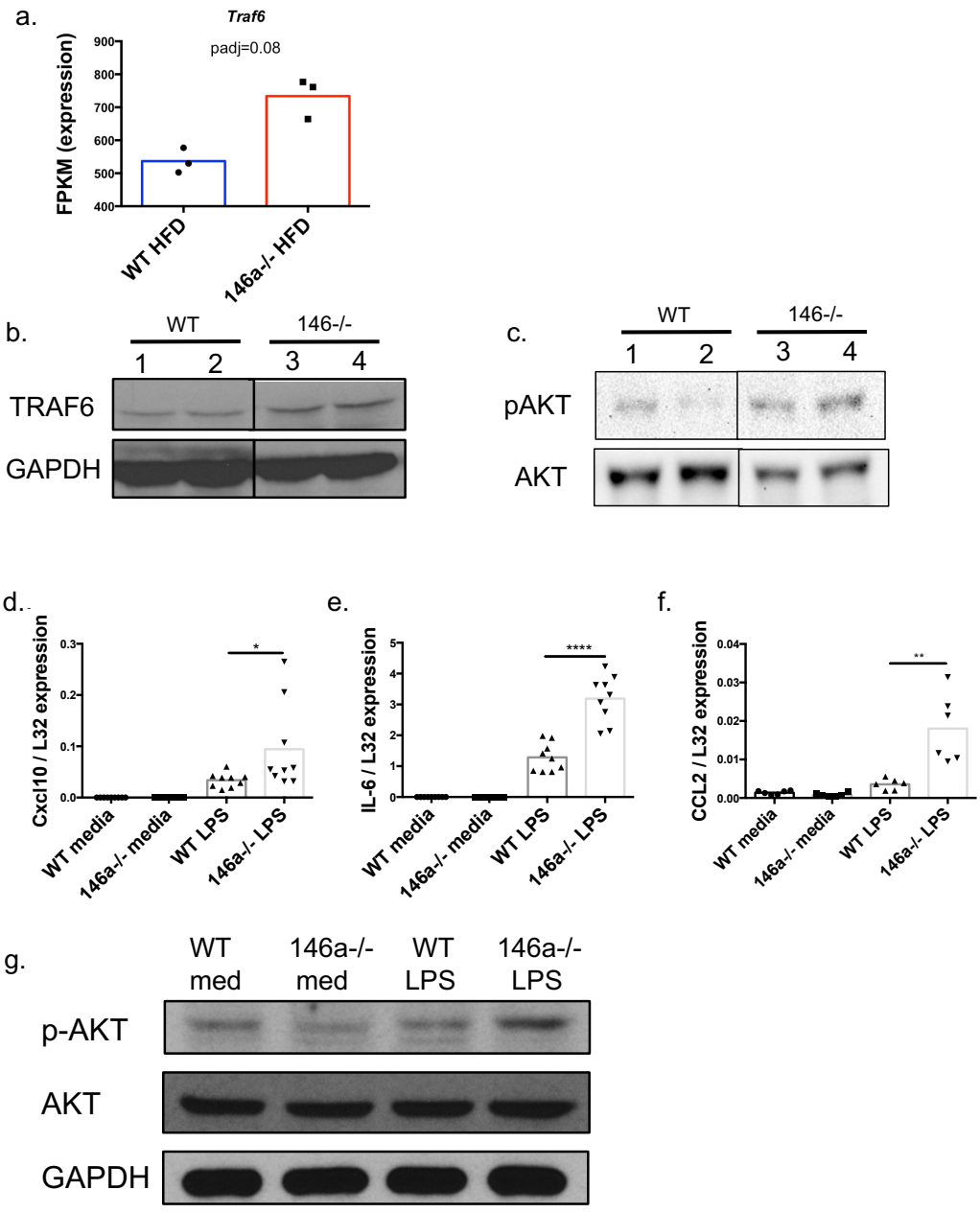
In order to examine adipose tissue macrophage genes that are regulated by miR-146a during NC and HFD, we performed both an Ingenuity Pathway Analysis and a Geneset Enrichment Analysis. Among the significant hits for gene sets enriched in miR-146a-/- macrophages were inflammatory pathways, including interferon gamma and interferon alpha response for normal chow, glycolysis and hypoxia for HFD, and IL6/Jak/Stat3 signaling and complement for both diet treatments (Figure 4.S5c and 5d). Of note, the top two pathways enriched in miR-146a-/- HFD macrophages were 'TNFa signaling via NFkB' and 'Inflammatory Response' (Figure 4.4d, Figure 4.S5c), which were also significantly enhanced in miR-146a-/- NC cells (Figure 4.4c, Figure 4.S5d). While PI3K/AKT/mTOR and mTORC1 signaling were not significantly altered in NC cells (Figure 4.4e), these genesets were enriched specifically in miR-146a-/- HFD macrophages (Figure 4.4f, Figure 4.S5c). This indicates that miR-146a regulates inflammatory pathways in adipose tissue macrophages both from lean (NC) and obese (HFD) adipose tissue. Furthermore, this miRNA regulates a unique subset of macrophage genes during HFD but not on a standard diet. Inflammatory response genes that were upregulated in miR-146a-/-HFD adipose tissue macrophages included *Il12b*, *Il1b*, *Il1a*, and *Cxcl2* (Figure 4.4g). Genes from the enriched PI3K/AKT/mTOR/mTORC1 geneset included *Txnrd1*, *Pdk1*, *Asns*, and *Cdkn1a* and *b* (Figure 4.4h). Notably, we independently observed that another set of genes involved in fat metabolism was

upregulated in miR-146a-/- macrophages during HFD (Figure 4.4i). These genes, which included *Hilpda*, *Plin2*, *Fapb4*, and *Lipa*, play roles in uptake and metabolism of lipids, and have previously been reported to be uniquely expressed in adipose tissue macrophages during diet-induced obesity (Xu et al., 2013). Altogether, miR-146a regulates gene expression of adipose tissue macrophages, particularly of the inflammatory response, both during a standard diet and DIO. These data also indicate that miR-146a regulates a unique subset of metabolic pathways within adipose tissue macrophages during DIO.

miR-146a targets Traf6 and downregulates AKT and inflammation in macrophages

To further understand the mechanism by which miR-146a is regulating the inflammatory response in adipose tissue macrophages, we analyzed predicted miR-146a targets within our RNA-seq dataset. Among the relevant predicted targets from Targetscan, we observed increased Traf6 mRNA expression in miR-146a-/- HFD macrophages compared with WT (Figure 4.5a). Traf6 is a *bona fide* miR-146a target in many other inflammatory contexts (Boldin et al., 2011; Yang et al., 2012). Thus, we measured protein levels of Traf6 within the adipose tissue of adult high fat diet-fed WT and miR-146a-/- mice. Traf6 levels were increased in HFD-treated miR-146a-/- adipose tissue, indicating that this miRNA is regulating Traf6 within this context (Figure 4.5b). Futhermore, because the RNA-seq of ATMs showed enhanced PI3K/AKT/mTOR signaling upon loss of miR-146a (Figure 4.4f, 4.4h), we measured activation in these pathways in obese adipose tissue. miR-146a-/- white adipose tissue had increased

Figure 4.5. miR-146a targets Traf6 and downregulates inflammation in macrophages upon stimulation. FPKM values of Targetscan predicted miR-146a-5p target *Traf6* anticorrelated with miR-146a expression adipose tissue macrophages during HFD, where expression was significantly increased in miR-146a-/- adipose tissue macrophages compared to WT, as determined by RNA-seq **(a)**. Western blot for Traf6 and GAPDH in lysates of whole visceral white adipose tissue collected from 20-week-old WT or miR-146a-/- mice fed a high fat diet (HFD) **(b)**. Western blot for phospho-AKT and AKT in lysates of whole visceral white adipose tissue collected from 20-week-old WT or miR-146a-/- mice fed a high fat diet (HFD) **(c)**. Expression of *Cxcl10* in WT and miR-146a-/- bone marrow-derived macrophages double-stimulated with media control or LPS **(d)**. Expression of *Il6* in WT and miR-146a-/- bone marrow-derived macrophages double-stimulated with media control or LPS **(e)**. Expression of *Ccl2* in WT and miR-146a-/- bone marrow-derived macrophages double-stimulated with media control or LPS **(f)**. Western blot for phosphorylated AKT, total AKT, and GAPDH in lysates from WT and miR-146a-/- bone marrow-derived macrophages double-stimulated with media control or LPS **(g)**.


a.
Traf6
padj=0.08
FPKM (expression)
900
800
700
600
500
400
WT HFD
146a-/- HFD
b.
WT
146-/-
1
2
3
4
TRAF6
GAPDH
c.
WT
146-/-
1
2
3
4
pAKT
AKT
d.
Cxcl10 / L32 expression
0.3
0.2
0.1
0.0
*
WT media
146a-/- media
WT LPS
146a-/- LPS
e.
IL-6 / L32 expression
5
4
3
2
1
0
****
WT media
146a-/- media
WT LPS
146a-/- LPS
f.
CCL2 / L32 expression
0.04
0.03
0.02
0.01
0.00
**
WT media
146a-/- media
WT LPS
146a-/- LPS
g.
WT med
146a-/- med
WT LPS
146a-/- LPS
p-AKT
AKT
GAPDH

phosphorylation of AKT at serine 473, indicating enhanced AKT activation (Figure 4.5c). Traf6 has previously been shown to be involved in the activation of AKT (Yang et al., 2009). Further, since Traf6 also activates the production of cytokines and chemokines through Toll-like receptors and NFκB, we measured the requirement of miR-146a in this process in macrophages. Cultured miR-146a-/- bone marrow-derived macrophages were hyper-responsive to LPS stimulation compared with WT, as evidenced by significant increases in *Cxcl10*, *Il6*, and *Ccl2* mRNA expression, demonstrating that miR-146a regulates inflammatory gene expression *in vitro* (Figure 4.5d, 4.5e, and 4.5f). These cells also expressed increased levels of phosphorylated AKT compared with WT (Figure 4.5g), validating the results from GSEA and demonstrating that miR-146a regulates AKT activation in macrophages. Altogether, these data suggest that miR-146a is working through Traf6 within adipose tissue to suppress the inflammatory and metabolic response in macrophages.

Discussion

We have demonstrated that miR-146a is required for protection from obesity and metabolic disease during a high fat diet (HFD). We observed significant weight gain, body fat accumulation with corresponding loss of lean mass, adipocyte hypertrophy, and fatty liver disease (steatosis) in miR-146a-/- HFD animals. These mice also became diabetic, as characterized by glucose intolerance and insulin resistance, but only at later timepoints of diet-induced obesity, indicating that loss of miR-146a alone does not cause diabetes. Importantly, miR-146a-/- fed a standard diet did not become obese, diabetic, or show other metabolic phenotypes compared with WT; the HFD was also required in

order to trigger metabolic disease. We measured increased inflammatory gene expression and NFκB activation in adipose tissue from miR-146a-/- mice at a young age, and with and without HFD. Many of these genes were signature genes of proinflammatory macrophages, including Cxcl10, F4/80, and MHCII. Intriguingly, expression of fatty acid transporter genes Cd36 and Fabp4 was increased upon loss of miR-146a even without HFD, suggesting that lipid metabolism pathways may also be altered within adipose tissue of these animals.

Inflammation alone, within this particular context of chronic inflammation triggered by lack of miR-146a, cannot trigger metabolic disease. This was evidenced by the data showing middle aged knockout mice fed normal chow had increased inflammation in adipose tissue and liver but did not show worsened obesity and metabolic disease compared to WT mice fed a standard diet. The high fat diet must also be providing additional signals that lead to adiposity, adipocyte hypertrophy, steatosis, and diabetes when miR-146a is not expressed. The likely culprit of this trigger may be pro-inflammatory adipokines that are expressed by adipocytes during caloric excess and a need for increased fat storage. Thus, miR-146a is required in immune cells to downregulate inflammation that may be initially signaled from adipocytes. We found that this miRNA is highly expressed within the stromal vascular fraction of adipose tissue, which is rich in leukocytes, and at much lower levels in purified adipocytes. This indicates a role for miR-146a within immune cells, although its cell-intrinsic function within macrophages during DIO has not yet been established. Even so, it is entirely possible that this miRNA may play an important cell-intrinsic role within adipocytes, and this possibility must be tested in the future by generating conditional knockout mice

lacking miR-146a within adipocytes.

miR-146a regulates gene expression in adipose tissue macrophages, as it is significantly altered in these cells, both from mice fed a normal chow (NC) and HFD. Because the gene expression pattern was unique to each group (WT vs. miR-146a-/-; NC vs. HFD), this indicates that miR-146a is regulating macrophage genes that are uniquely important during lean and obese states. As expected, miR-146a is regulating the inflammatory response in adipose tissue macrophages, further corroborating that this miRNA is required to downregulate inflammation during disease. While miR-146a is downregulated in type 2 diabetes patients (Balasubramanyam et al., 2011; Baldeon et al., 2014), it will be important to test the expression levels of this miRNA in obese versus lean patients. Potentially, an individual with decreased expression of miR-146a may be at greater risk of developing obesity, type 2 diabetes, and other metabolic diseases if fed a diet rich in fat. miR-146a may become an important miRNA to study in this regard, as it may be an important therapeutic target to treat metabolic disease. As there has been a recent and renewed call to start treating obesity and diabetes with anti-inflammatory drugs (Donath, 2014; Sears and Ricordi, 2011), miR-146a is feasible as a target, since we have now demonstrated its role in this context.

In general, miR-146a is an important switch on the convergence of inflammation and metabolism, particularly in macrophages. Further work must be done to characterize the metabolic state of these cells in the context of miR-146a expression, and it may play an important metabolic role in other immune cells. Because miR-146a-/- macrophages had higher expression of fat and lipid metabolism genes during DIO, it is possible that this miRNA is regulating lipid uptake and processing within macrophages, which could

alter inflammation status. Further, the glycolysis geneset was significantly enriched in macrophages lacking miR-146a during HFD compared to WT; this may point to this miRNA regulating the Warburg switch that occurs in macrophages during inflammation (Kelly and O'Neill, 2015; O'Neill et al., 2016). In general, miR-146a may play a large role in metabolic repurposing of immune cells, and further work must be done to investigate this. Overall, miR-146a is essential to prevent metabolic disease during diet-induced obesity, and may be valid therapeutically to treat obesity, diabetes, as well as other forms of metabolic inflammation.

Materials and Methods

Animals

All WT and miR-146a-/- mice are on the C57BL/6 background and were bred and housed in a specific pathogen-free mouse facility at the University of Utah, USA. WT and miR-146a-/- mice were also purchased from Jackson Labs in order to ensure phenotypes were repeatable in mice exposed to different housing. Experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of University of Utah, USA.

Diet induced obesity and metabolic analysis

Mice were placed on control diet (10% kcal fat; Research Diets D12450Bi) or high fat diet (HFD) (45% kcal fat; Research Diets D12451i) from 4-6 weeks of age for 12 to 18 weeks. Mice were weighed weekly, and food consumption was tracked by weighing food weekly. Body composition, including body fat, lean mass, and fluid, was measured

via TD-NMR using the Bruker Minispec Body Composition Analyzer at the University of Utah. Heat production, movement, VO_2 max, VCO_2 max, respiratory exchange ratio (RER), and food and drink consumption were measured using CLAMS metabolic cages; this work was performed by the Metabolic Phenotyping Core, a part of the Health Sciences Cores at the University of Utah. Serum Leptin levels were measured by collecting serum from mice treated with HFD for 2 or 17 weeks and quantifying via MAGPIX, using the mouse metabolic hormone panel (Millipore). This work was also performed in the Metabolic Phenotyping Core Facility at the University of Utah. Following sacrifice, adipose, liver, and pancreas tissues were removed and tissues were formalin fixed and H&E stained for histological analysis. Liver histology score was based on a previously published scoring system from Dixon et al. Hepatology (2004). All histological scoring was performed blindly by a trained pathologist, Dr. Mary Bronner, at the University of Utah.

Glucose and insulin analysis

For glucose tolerance tests (GTT), mice were fasted for 6 hours prior to experimentation. Glucose was injected into mice at a concentration of 1g/kg, and blood glucose levels were measured at timepoints 0 (prior to injection), 5, 15, 30, 60, and 120 minutes following injection using a Bayer Contour glucometer. Blood for this experiment was taken from tail nicks. For insulin ELISA, serum was collected from 6-hour fasted mice, and insulin was measured using a mouse insulin ELISA kit (Crystal Chem). The homeostasis model of β-cell function (HOMA-B), the β-cell function index, was calculated using the formula: HOMA-B = [insulin (microunits per milliliter)20]/[glucose

(millimoles per liter) − 3.5] and homeostasis model assessment insulin resistance index (HOMA-IR), the insulin resistance index, was calculated using the formula: HOMA-IR = [glucose (millimoles per liter)insulin (microunits per milliliter)/22.5]. Both HOMA-B and HOMA-IR were calculated using fasting values (Matthews et al. Diabetologia 1985).

Adipose and liver gene expression and protein analysis

Approximately 0.1 grams of whole adipose tissue was collected from reproductive visceral white adipose tissue or liver from mice. Young normal chow (NC) mice were ~8 weeks in age, middle-aged NC mice were ~20 weeks in age, and middle-aged HFD mice were ~20 weeks in age fed HFD since ~6 weeks in age. RNA was extracted from these tissues using miRNeasy kits (Qiagen), cDNA was made using qScript cDNA Synthesis Kit (Quanta), and GoTaq Master Mix (Promega) and LC480 (Roche) measured expression of various genes. Primer set sequences are as follows: For Western blots, levels of phosphorylated IKBα (Cell Signaling), IKBα (Cell Signaling), Traf6 (Abcam), phosphorylated AKT (Cell Signaling), AKT (cell signaling), and GAPDH were measured from whole white adipose tissue lysates of WT or miR-146a-/- mice.

Adipocyte and stromal vascular fraction separation

Visceral reproductive white adipose fat pads were removed from mice, minced into pieces, and placed in a digestion buffer containing HBSS, Collagenase D (Roche), and Dispase (Worthington) for 1 hour at 37 degrees. The homogenates were then placed on ice for 30 minutes, and samples were spun to separate adipocytes from the SVF. Adipocytes, which float on the top, were removed and considered the adipocyte fraction.

Supernatant was then removed to obtain the pelleted stromal vascular fraction (SVF). Red blood cell lysis buffer was added to the SVF pellet and pellet was spun and washed. RNA was collected from the adipocyte and SVF fractions via Qiazol/miRNeasy Kit (Qiagen), and miR-146a levels were measured using miRCURY LNA RT PCR (Exiqon) and the miR-146a primer provided by Exiqon.

RNA-seq of adipose tissue macrophages

WT and miR-146a-/- mice were treated with a standard chow (Teklad 2920, Envigo) or HFD from 6 weeks of age (Research Diets D12451i) and were sacrificed at 20 weeks old. Visceral adipose tissue was removed from mice, minced into pieces, and placed in a digestion buffer containing HBSS, Collagenase D, and Dispase for 1 hour at 37 degrees. The homogenates were then placed on ice for 30 minutes, and samples were spun to separate adipocytes from the SVF. Adipocytes, which float on the top, were removed and discarded. Red blood cell lysis buffer was added to the SVF pellet, which was then spun and washed. SVF pellets from three different mice were combined in order to obtain enough cells for sorting and RNA collection. Cells were stained with antibodies to CD45, CD11b, and F4/80, and live, singlet, $CD45^+$ $CD11b^+$ $F4/80^+$ cells were sorted using a FACS Aria (BD). RNA was then collected via Qiazol/RNeasy Kit (Qiagen). Library preparation used was Illumina TruSeq Stranded RNA Kit with Ribo-Zero Gold and RNA-seq was performed using Illumina HiSeq 50 cycle single-read sequencing version 4 at the University of Utah High Throughput Genomics Core Facility. Sequence alignment was performed through the University of Utah Bioinformatics Core Facility (Dr. Timothy Mosbruger), and Geneset Enrichment Analysis (GSEA) and Ingenuity

Pathway Analysis software were used to examine types of genes that were up or downregulated in miR-146a-/- macrophages. Predicted miR-146a targets were obtained via Targetscan for mmu-miR-146a-5p.

Bone marrow-derived macrophage stimulations

Bone marrow was isolated from WT or miR-146a-/- mice, red blood cell-lysed, and plated in DMEM complete media with mouse MCSF. At 4 days of culture, cells were spiked with additional media containing MCSF. At day 7, cells were stimulated with LPS (Sigma) for 24 hours. At the 24-hour timepoint, media was removed, and cells were stimulated with a second hit of fresh media containing LPS for an additional 2-6 hours. Protein lysate or RNA was collected using Qiazol/miRNeasy kit (Qiagen) and Western or qRT-PCR was performed on these cells.

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Figure 4.S1. miR-146a is required to prevent weight gain and Leptin accumulation during high fat diet. Each line shows an individual experimental repeat where young female WT and miR-146a-/- mice were placed on high fat diet and weight gain was measured over time, as a percent of initial weight measured week 0. Comparison of weight gain in male WT and miR-146a-/- mice placed on HFD for 11 weeks with female WT and miR-146a-/- mice placed on HFD for 11 weeks, as measured by percent initial weight **(a)**. Weight gain, as measured by percent initial weight, of young, 6-week-old C57BL6/J and miR-146a-/- mice purchased from Jackson Laboratories and placed on HFD for 13 weeks **(b)**. Weight gain, as measured by weight in grams, of young, 6-week-old C57BL6/J and miR-146a-/- mice purchased from Jackson Laboratories and placed on HFD for 13 weeks **(c)**. Levels of Leptin protein in serum of 6-hour fasted WT and miR-146a-/- mice that were placed on HFD for 2 weeks or 17 weeks **(d)**.

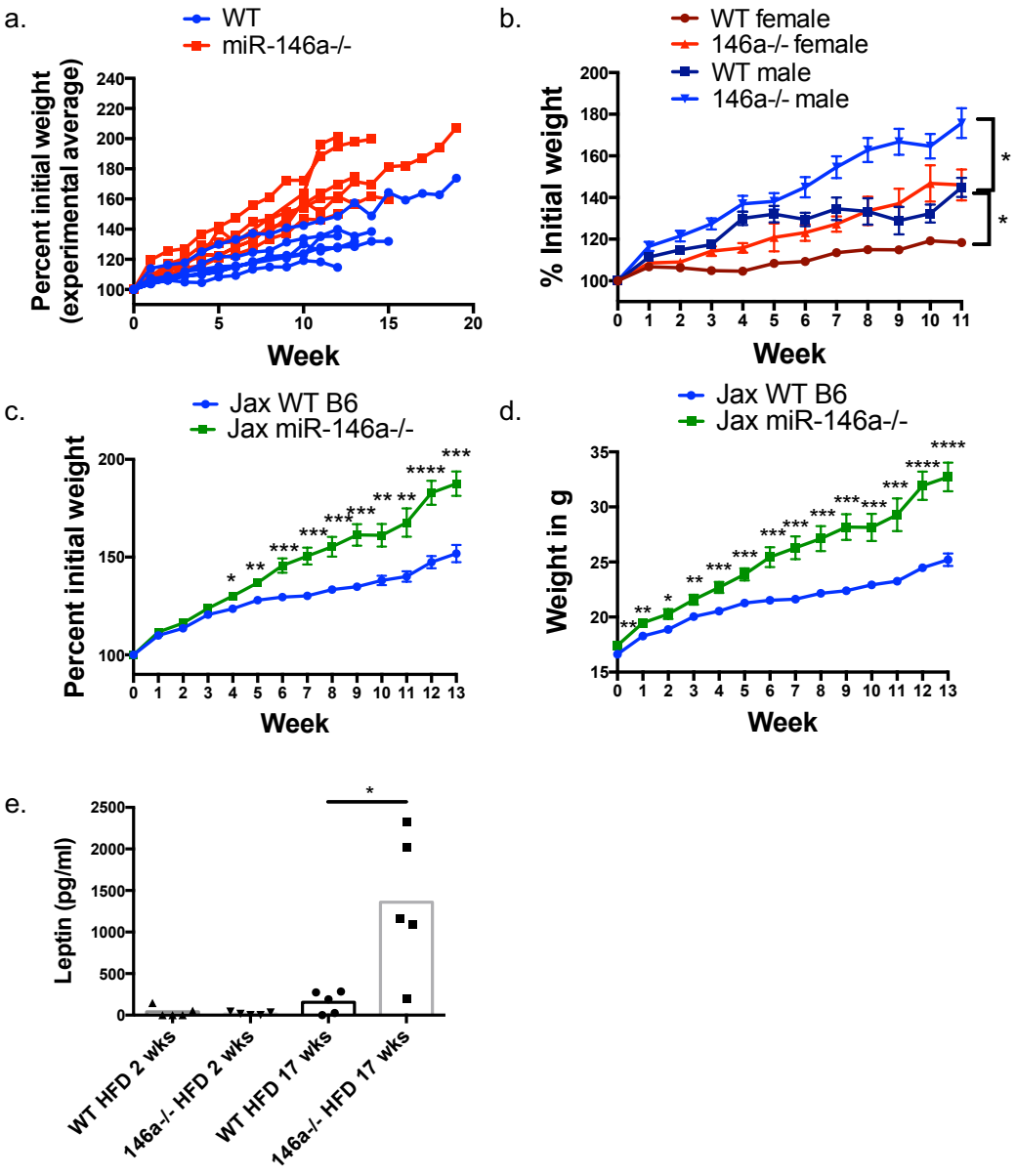

a.
WT
miR-146a-/-
Percent initial weight (experimental average)
Week
b.
WT female
146a-/- female
WT male
146a-/- male
% Initial weight
Week
c.
Jax WT B6
Jax miR-146a-/-
Percent initial weight
Week
d.
Jax WT B6
Jax miR-146a-/-
Weight in g
Week
e.
Leptin (pg/ml)
WT HFD 2 wks
146a-/- HFD 2 wks
WT HFD 17 wks
146a-/- HFD 17 wks

Figure 4.S2. miR-146a-/- show altered metabolic phenotypes upon diet-induced obesity. WT (blue) and miR-146a-/- (green) mice were placed on HFD for 0 weeks, 3 weeks, or 18 weeks and metabolic parameters were measured in metabolic chambers. Heat production by mice in kcal/hour, at day and night **(a)**. XY Movement of mice, measured at day and night **(b)**. Respiratory exchange ratio of mice, calculated by VO_2 and VCO_2 max levels, measured at day and night **(c)**. Amount of drinking water consumed in ml, measured at day and night **(d)**. Amount of drinking food consumed in ml, measured at day and night **(e)**.

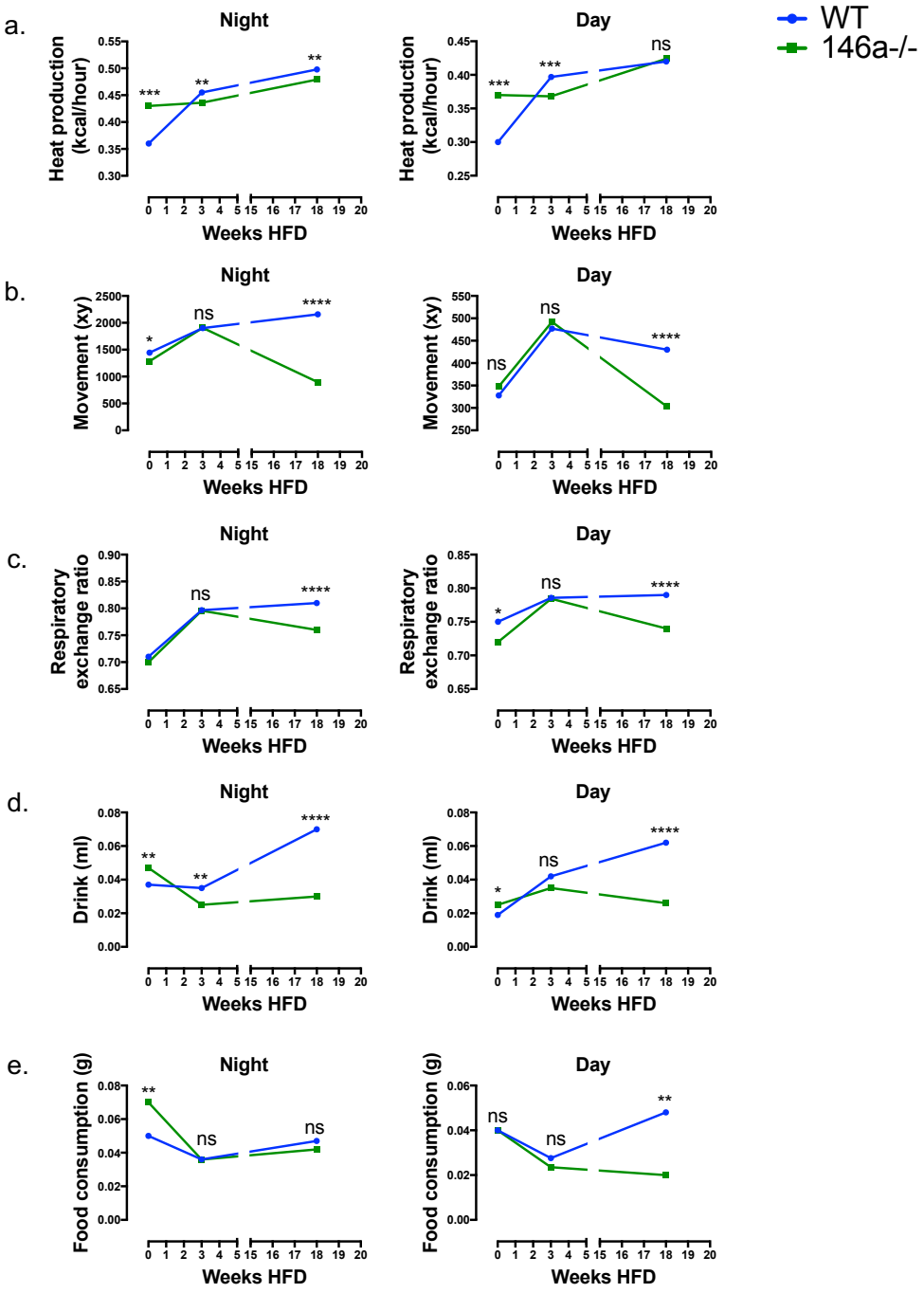
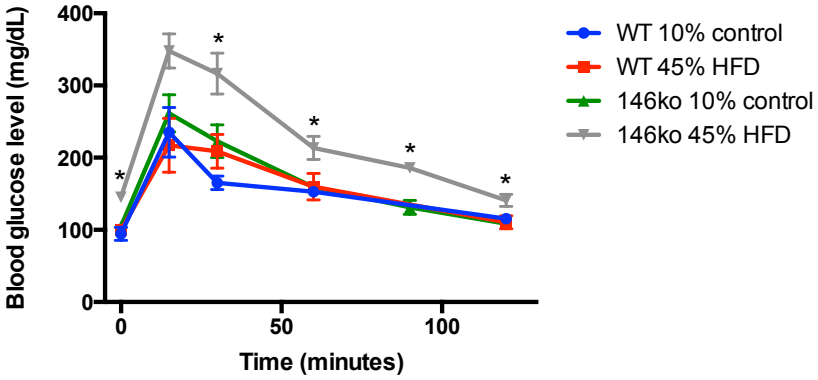
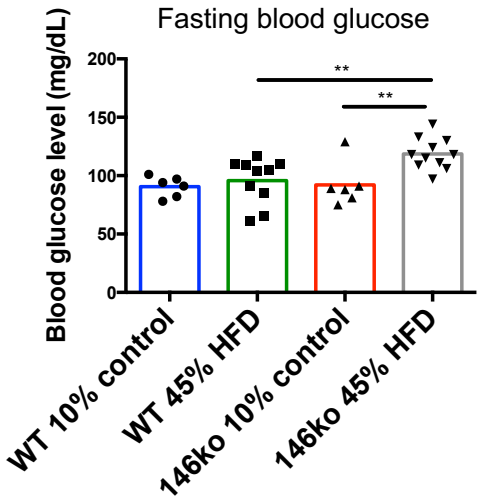

a.
b.
c.
d.
e.
Night
Day
WT
146a-/-
Heat production (kcal/hour)
Movement (xy)
Respiratory exchange ratio
Drink (ml)
Food consumption (g)
Weeks HFD
ns

Figure 4.S3. miR-146a protects from high blood glucose levels during diet-induced obesity but does not alter pancreatic architecture. C57BL/6 WT and miR-146a-/- mice were placed on a control diet of 10% kcal/fat (control) or a high fat diet of 45% kcal/fat (HFD). Blood glucose levels following injection of glucose at 0 minutes and measured over time for 120 minutes **(a)**. Blood glucose of 6-hour fasted WT and miR-146a-/- mice fed a control diet or high fat diet (HFD) **(b)**. H&E staining of representative sections of pancreas at week 14 of diet treatment **(c)**.

a.


Blood glucose level (mg/dL)
400
300
200
100
0
0
50
100
Time (minutes)
*
*
*
*
*
WT 10% control
WT 45% HFD
146ko 10% control
146ko 45% HFD

b.


Fasting blood glucose
Blood glucose level (mg/dL)
200
150
100
50
0
**
**
WT 10% control
WT 45% HFD
146ko 10% control
146ko 45% HFD

c.

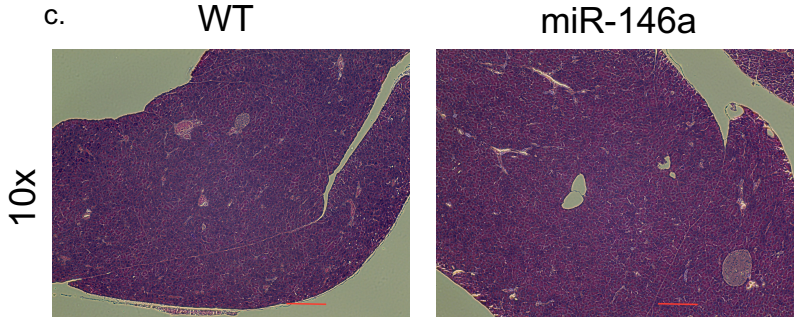

WT
miR-146a
10x

Figure 4.S4. Weight gain in miR-146a-/- mice during DIO is not dependent upon miR-155. WT, miR-155-/-, miR-146a-/-, and miR-155-/- miR-146a-/- (DKO) mice were placed on 45% kcal/fat HFD for 12 weeks. Percent weight gain over time of diet, based on initial weight **(a)**. Body weight in grams of WT, miR-155-/-, miR-146a-/-, and DKO mice over time of diet **(b)**. Blood glucose levels of WT, miR-155-/-, miR-146a-/-, and DKO mice following a 6-hour fast, at 15 weeks HFD **(c)**. Weight of reproductive, visceral fat pads harvested from WT, miR-155-/-, miR-146a-/-, and DKO mice following 15 weeks HFD **(d)**. Body composition of mice was measured using TD-NMR. Shown is percent body fat of WT, miR-155-/-, miR-146a-/-, and DKO HFD-treated mice at week 14 during diet treatment **(e)**. Percent lean of total body weight of WT, miR-155-/-, miR-146a-/-, and DKO HFD-treated mice at week 14 during diet treatment **(f)**.

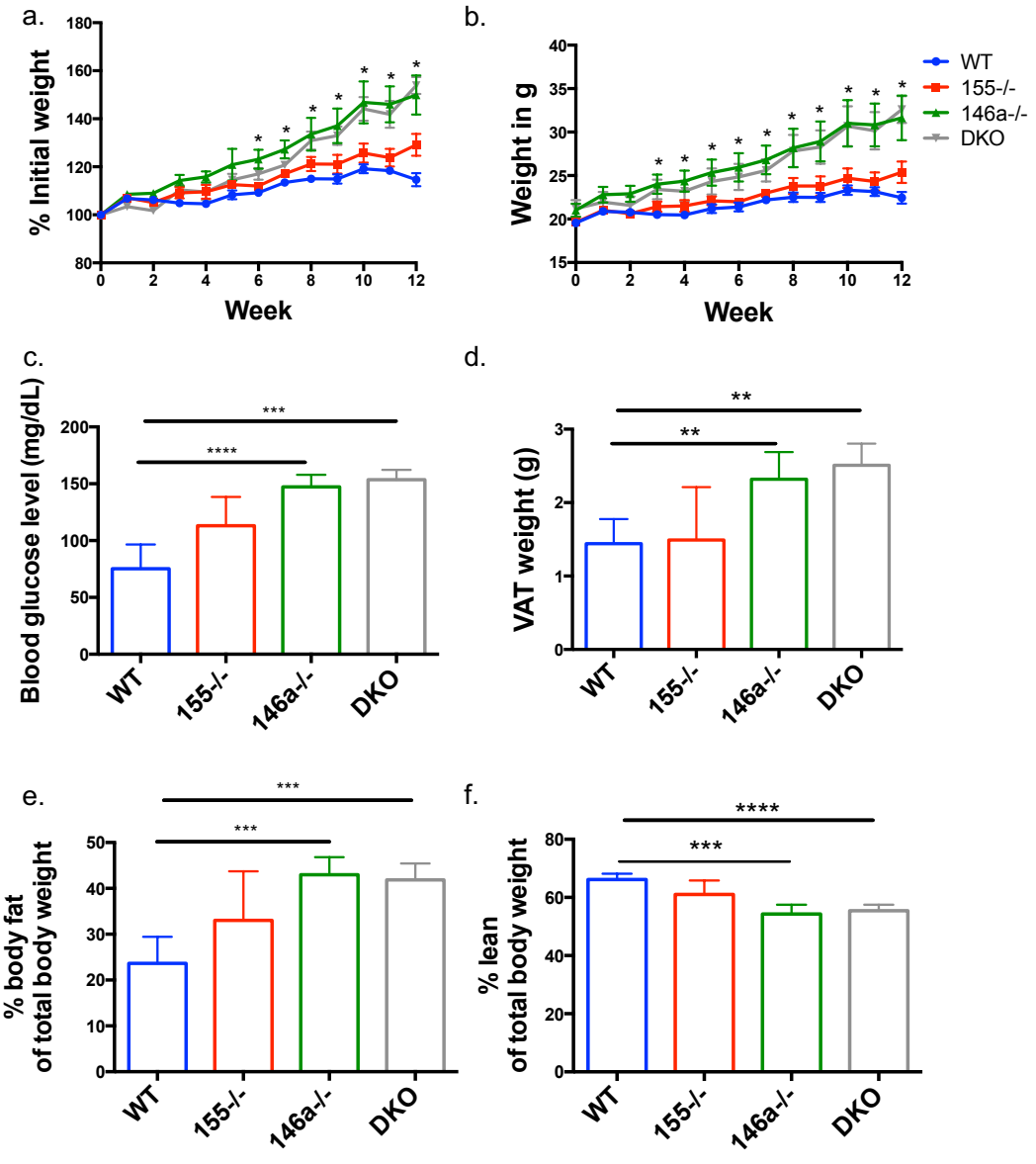
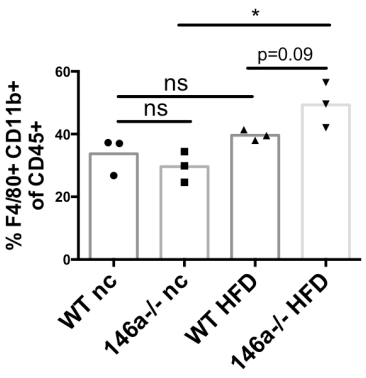
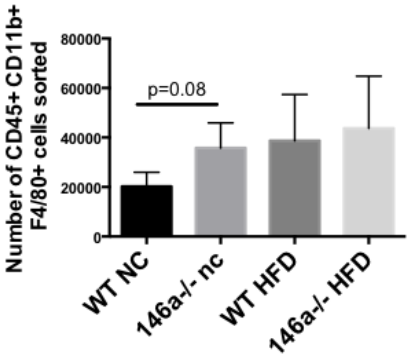

a.
% Initial weight
Week
b.
Weight in g
Week
WT
155-/-
146a-/-
DKO
c.
Blood glucose level (mg/dL)
WT
155-/-
146a-/-
DKO
d.
VAT weight (g)
WT
155-/-
146a-/-
DKO
e.
% body fat of total body weight
WT
155-/-
146a-/-
DKO
f.
% lean of total body weight
WT
155-/-
146a-/-
DKO

Figure 4.S5. RNA-seq of adipose tissue macrophages upon loss of miR-146a. Percentages of $CD11b^+$ $F4/80^+$ cells sorted from live, singlet, $CD45^+$ cells collected from the stromal vascular fraction of visceral adipose tissue from 20-week-old WT and miR-146a-/- mice fed a standard diet (NC) or high fat diet (HFD). Each sample contained cells from 3 mice combined in order to obtain enough RNA, and 3 samples were collected for each genotype, for a total of 9 mice per genotype and treatment **(a)**. Total numbers of $CD11b^+$ $F4/80^+$ cells sorted from live, singlet, $CD45^+$ cells collected from the stromal vascular fraction of visceral adipose tissue from 20-week-old WT and miR-146a-/- mice fed a standard diet (NC) or high fat diet (HFD). Each sample contained cells from 3 mice combined in order to obtain enough RNA, and 3 samples were collected for each genotype, for a total of 9 mice per genotype and treatment **(b)**. Significant genesets upregulated in macrophages from miR-146a-/- high fat diet (HFD) mice, as compared with WT, according to Geneset Enrichment Analysis (GSEA). NES=normalized enrichment score; FDR=false discovery rate. FDR q-values below 0.25 are considered statistically significant **(c)**. Significant genesets upregulated in macrophages from miR-146a-/- normal chow (nc) mice, as compared with WT, according to Geneset Enrichment Analysis (GSEA). NES=normalized enrichment score; FDR=false discovery rate. FDR q-values below 0.25 are considered statistically significant **(d)**.

a.



b.



c. Genesets increased in miR-146a-/- HFD

Geneset (Hallmark genes)	NES	FDR q-val
TNFA_SIGNALING_VIA_NFKB	2.6386719	0
INFLAMMATORY_RESPONSE	2.3580816	0
IL6_JAK_STAT3_SIGNALING	2.1659997	0
COMPLEMENT	2.1218636	0
IL2_STAT5_SIGNALING	2.0260806	2.62E-04
ALLOGRAFT_REJECTION	1.9283742	4.30E-04
REACTIVE_OXIGEN_SPECIES_PATHWAY	1.8080266	0.001608506
MTORC1_SIGNALING	1.6976037	0.005041469
G2M_CHECKPOINT	1.635474	0.008235074
HEME_METABOLISM	1.6285411	0.007957065
HYPOXIA	1.6138178	0.008822143
MITOTIC_SPINDLE	1.5576462	0.014513454
TGF_BETA_SIGNALING	1.5518762	0.014680671
APOPTOSIS	1.5311283	0.017364034
PI3K_AKT_MTOR_SIGNALING	1.5193748	0.018843114
SPERMATOGENESIS	1.497906	0.022776036
P53_PATHWAY	1.4809158	0.02465755
ESTROGEN_RESPONSE_LATE	1.4652648	0.02672713
UNFOLDED_PROTEIN_RESPONSE	1.4550822	0.028324483
E2F_TARGETS	1.4020414	0.046144336
KRAS_SIGNALING_UP	1.3982054	0.04564533
WNT_BETA_CATENIN_SIGNALING	1.3954952	0.044916987
ESTROGEN_RESPONSE_EARLY	1.3518641	0.063512504
ANGIOGENESIS	1.3478694	0.062811695
NOTCH_SIGNALING	1.3408898	0.064391315
KRAS_SIGNALING_DN	1.2805339	0.103409514
UV_RESPONSE_UP	1.2800627	0.09988134
ADIPOGENESIS	1.2154886	0.15921585
GLYCOLYSIS	1.1679655	0.21559803

d. Genesets increased in miR-146a-/- nc

Geneset (Hallmark genes)	NES	FDR q-val
INTERFERON_GAMMA_RESPONSE	2.323837	0
INTERFERON_ALPHA_RESPONSE	2.2484753	0
ALLOGRAFT_REJECTION	2.1879375	0
COMPLEMENT	2.0502415	0
INFLAMMATORY_RESPONSE	1.8536447	5.65E-04
KRAS_SIGNALING_UP	1.7598661	0.00294924
IL2_STAT5_SIGNALING	1.5682718	0.027151238
COAGULATION	1.5324342	0.034844965
IL6_JAK_STAT3_SIGNALING	1.4479833	0.07185547
ESTROGEN_RESPONSE_EARLY	1.3687286	0.12711988
KRAS_SIGNALING_DN	1.3124099	0.18215932
APICAL_SURFACE	1.3071978	0.17438523
APICAL_JUNCTION	1.264015	0.22563073
MITOTIC_SPINDLE	1.2435552	0.24216191
P53_PATHWAY	1.2314073	0.24486062

CHAPTER 5

CONCLUDING REMARKS AND FUTURE DIRECTIONS

MicroRNA 146a is one of the most prominently studied microRNAs and has correlations with many human diseases (Li et al., 2010; Rusca and Monticelli, 2011). Importantly, it is now clear that this miRNA plays a role in intestinal diseases and cancers, as well as during metabolic disease. The work presented in this dissertation demonstrates that miR-146a regulates inflammation within the gut, adipose tissue, and liver microenvironments and is required to maintain homeostasis in these tissues at steady state and during immunologic challenge. These data corroborate the findings that miR-146a is associated with inflammatory bowel disease, type 2 diabetes, and other related diseases (Balasubramanyam et al., 2011; Baldeon et al., 2014; Lin et al., 2014; Nunez Lopez et al., 2016; Rong et al., 2013; Runtsch et al., 2015; Schaefer et al., 2015). My work indicates large gene expression networks that are regulated, indirectly or directly, by miR-146a. Interestingly, some of these general gene sets and pathways are overlapping, with many of these being immune-related. Other non-inflammation gene networks that overlapped included metabolic disease and lipid metabolism (data not shown). This suggests two things: first, that miR-146a directly regulates metabolic processes; secondly, that the inflammatory pathways regulated by miR-146a are tightly involved with metabolism. Lipid metabolism was a top pathway of genes upregulated in the small intestine (Ingenuity; data not shown) and in adipose tissue macrophages during obesity (Figure 4.4); thus, this novel role of miR-146a must be investigated further. Of course, the immune system is tightly linked to many other biological processes including metabolism and membrane trafficking, and these are now emerging fields of study. My findings add to another layer of regulation to consider in these new fields in addition to protein-coding genes—post-transcriptional regulation by microRNAs. Through the recent

studies I reviewed in Chapter 2, it is clear that microRNAs regulate intestinal homeostasis, but further functional studies in this complex microenvironment are essential.

Because miR-146a typically prevents disease through its anti-inflammatory function, I was initially surprised at the results presented in Chapter 3, where I observed that loss of this miRNA during a model of colitis actually resulted in less severe disease. Indeed, these mice showed enhanced immune responses in the intestine, but within this context, miR-146a is actually repressing genes that can promote gut barrier function. This was shown at the levels of gene expression (Figure 3.2), immune cell composition (Figures 3.3 and 3.4), and colitis susceptibility (Figure 3.5). This suggests that if this microRNA is utilized in the clinic as a treatment, one should consider the possibility of compromising barrier function in the gut and note this as a possible risk.

One of the main future directions needed to pursue in this area is in regards to the role miR-146a plays in intestinal cancers, including colorectal cancer. Correlative data involving miR-146a expression in gut malignancies exists, but functional studies about the direct involvement of this miRNA in intestinal cancer have not yet been published. It is possible that the same genes that enhance barrier and immune function upon loss of miR-146a may also promote intestinal cell proliferation and cancer. One important future experiment would be to cross miR-146a-/- mice with APC^{min} mice. APC^{min} mice are a genetic model of colorectal cancer and spontaneously develop tumors in their intestines. Perhaps loss of miR-146a in this model, which will promote NFκB activation (Figure 3.S4) and other potentially pro-proliferative pathways, may lead to worsened tumor growth. An alternative hypothesis is that the types of genes and immune cells that are in

excess in miR-146a-/- mice are helpful in combating intestinal tumors or creating an anti-tumor environment. Another remaining unanswered question related to this study is the cell-intrinsic role of miR-146a in the intestine. Many of the changes that occurred in the gut upon loss of miR-146a were with T cell populations, including increases in Th17, Treg, and Tfh cells in the colon and small intestine. The function of cell-intrinsic miR-146a in T cells has not yet been elucidated, especially during colitis and intestinal homeostasis. Further, loss of miR-146a resulted in upregulation of antimicrobial peptides, mucus components, and epithelial junction genes, many of which are expressed solely in intestinal epithelial cells (IECs). Thus, it is possible that miR-146a has function in these non-hematopoietic cells, which would open up a previously undiscovered area for studies of this miRNA. In addition to homeostasis, intestinal development is a complex field that is not fully understood. Based on the phenotypes observed and the gene expression pathways altered in miR-146a-/- mice, I predict that miR-146a may also play a role in intestinal stem and progenitor cells. For example, intestines from miR-146a-/- mice show decreased Sonic hedgehog signaling pathway gene expression (Figure 3.S3), which is highly important for proper intestinal development (Akiyoshi et al., 2006; Lees et al., 2005; Merchant, 2012). Examination of the expression and function of miR-146a in intestinal stem and progenitor cell populations would tie in this miRNA with cancer and would further implicate miR-146a as a target for therapy during intestinal cancers.

In Chapter 4, I demonstrated that miR-146a is required to protect from metabolic disease during a metabolic challenge, diet-induced obesity. When this microRNA was deleted from mice fed a high fat diet, these animals developed obesity, type 2 diabetes, fatty liver disease, as well as other phenotypes. These conditions are all interrelated and

commonly present themselves concurrently in humans, solidifying this study as valid for potential in translational studies to human therapy. One cell type that promotes these metabolic diseases in this model is the hematopoietic-derived macrophage, and miR-146a was highly expressed in hematopoietic cells of the adipose tissue. More evident, RNA-sequencing of adipose tissue macrophages on normal chow and high fat diet exposed a requirement for miR-146a in these cells present in metabolic tissues. Even so, the role of this miRNA in adipocytes has not yet been elucidated. Previous work indicates that miR-146a has function in adipocytes, at least *in vitro* (Roos et al., 2016; Wu et al., 2016). Further, crosstalk commonly occurs between macrophages and adipocytes (Sorisky et al., 2013; Xie et al., 2010), with similar pathways playing opposing roles in one cell versus another. Thus, it is possible that miR-146a in macrophages, adipocytes, and perhaps other cell types plays either/or combined and antagonistic roles during metabolic disease.

One of the most important things that I have learned from these studies is that macrophages in the adipose tissue microenvironment can behave very differently from macrophages in other tissues: for example--macrophages in the skin responding to an infection. Thus, miR-146a regulating the M1/M2 dichotomy may not apply in the adipose tissue. Instead, this small, noncoding RNA seems to affect genes that are important for adipose tissue inflammation and lipid metabolism. Thus, I hypothesize that miR-146a regulates lipid metabolism pathways that affect lipid buffering by macrophages during obesity. This hypothesis could also carry over into studies regarding miR-146a during heart disease (Bronze-da-Rocha, 2014; Li et al., 2015), in which lipid buffering by macrophages is also important (Boutens and Stienstra, 2016). Answers to these questions about regulation of metabolic pathways and immune cell function in metabolic tissues by

miR-146a will provide novel insight into its function outside of the well-studied roles in NFκB and inflammation.

It is important to note that processes occurring in the intestine are interconnected with those that occur in the adipose tissue and liver. Upon eating a meal, intestinal cells are the first to interact with the compounds introduced, and eventually, these are processed by the liver and may be delivered to adipose tissue. It is now apparent that the gut microbiota also plays a direct role in susceptibility to metabolic disease, including obesity (Rosenbaum et al., 2015; Sonnenburg and Backhed, 2016), further relating these processes. Because loss of miR-146a resulted in altered intestinal homeostasis and a varied makeup of microbial communities within the gut, it is possible that these changes also play a role during metabolic disease. Gut microbes utilize and produce metabolites, which can directly signal to host cells and tissues (Rooks and Garrett, 2016). I previously performed a study measuring metabolites within the feces and blood of young and old WT and miR-146a-/- mice fed a standard and high fat diet. Indeed, differences in specific metabolites were observed (data not shown), but future work addressing the functionality of these changes must be performed. Furthermore, while results in pilot experiments suggest that the microbiota does not play an observable role in metabolic disease upon loss of miR-146a (data not shown), more experimentation is required, especially with germ-free mice. These studies would solidify the link between intestinal homeostasis and metabolism, in which miR-146a likely has function. Altogether, I have clearly demonstrated this microRNA has a functional role in the gut and during immunometabolism, and future work will provide additional insight into the molecular mechanisms of miR-146a.

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