

The emerging role of miRNAs in inflammatory bowel disease: a review

Christopher G. Chapman and Joel Pekow

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Abstract: Inflammatory bowel disease (IBD), comprised of ulcerative colitis and Crohn's disease, is believed to develop as a result of a deregulated inflammatory response to environmental factors in genetically susceptible individuals. Despite advances in understanding the genetic risks of IBD, associated single nucleotide polymorphisms have low penetrance, monozygotic twin studies suggest a low concordance rate, and increasing worldwide IBD incidence leave gaps in our understanding of IBD heritability and highlight the importance of environmental influences. Operating at the interface between environment and heritable molecular and cellular phenotypes, microRNAs (miRNAs) are a class of endogenous, small noncoding RNAs that regulate gene expression. Studies to date have identified unique miRNA expression profile signatures in IBD and preliminary functional analyses associate these deregulated miRNAs to canonical pathways associated with IBD pathogenesis. In this review, we summarize and discuss the miRNA expression signatures associated with IBD in tissue and peripheral blood, highlight miRNAs with potential future clinical applications as diagnostic and therapeutic targets, and provide an outlook on how to develop miRNA based therapies.

Keywords: epigenetics, inflammatory bowel disease, microRNA

Introduction

Inflammatory bowel disease (IBD), comprised of the chronic inflammatory conditions ulcerative colitis (UC) and Crohn's disease (CD), is believed to result from a dysregulated, continuous inflammatory response to enteric microbes in genetically susceptible individuals. Despite the prevailing hypothesis of a genetic basis to IBD, associated single nucleotide polymorphisms (SNPs) have low penetrance, monozygotic twin studies suggest a low concordance rate (UC 10–15% and 30–35% in CD) [Spehlmann *et al.* 2008], and increasing worldwide IBD incidence [Molodecky and Kaplan, 2010] leave gaps in our understanding of IBD heritability and, simultaneously, highlight the importance of the environment in modifying the development and progression of IBD. Current and future treatments are designed primarily to target the inflammatory cascade as a mechanism to abate continuous disease activity. In order to develop novel therapeutic and diagnostic strategies, further understanding of underlying molecular

pathophysiological mechanisms of host disease activation is required.

In this era of genome wide association studies (GWAS), epigenetics has only recently emerged as a mechanistic layer for the complexities of gene expression regulation. Results from studies to date indicate that GWAS account for 23% and 16% of the heritability in CD and UC, respectively [Khor *et al.* 2011]. The missing heritability is likely derived from genetic, epigenetic and non-genetic (including environmental) components. Epigenetics is the study of modifications in regulation of gene expression that occur without change to DNA sequence and operates at the interface between environment and heritable molecular and cellular phenotypes. MicroRNAs (miRNAs) are single stranded, evolutionary conserved, 18–24 nucleotide long unique class of noncoding RNAs that exert epigenetic post-transcriptional effects on gene regulation. miRNAs bind to complementary 3' untranslated regions (UTRs) of targeted protein-encoding messenger

Correspondence to:
Joel Pekow, MD
Section of
Gastroenterology,
Hepatology and Nutrition,
University of Chicago, 900
East 57th Street, MB # 9,
Chicago, IL 60637, USA
[jpekow@medicine.bsd.
uchicago.edu](mailto:jpekow@medicine.bsd.uchicago.edu)
Christopher G. Chapman, MD
University of Chicago,
Chicago, IL, USA

RNAs (mRNAs), resulting in decreased stability and repression of translation. Investigations into the biologic function of miRNAs have discovered an emerging common theme of adapting to physiologic and pathophysiologic environmental stresses and restoring or altering gene expression in fully differentiated tissues [Leung and Sharp, 2010; Mendell and Olsen, 2012]

Recent studies have identified distinct tissue and peripheral blood miRNA expression profiles in IBD. Analyses to validate key miRNA regulated pathways in cell based and various animal models of experimentally induced IBD have only recently begun to elucidate the functional importance of miRNAs in IBD pathogenesis, but already provide clear evidence of GWAS susceptibility gene overlap. As more evidence and knowledge of miRNA function and dysregulation in IBD is accrued, the opportunity for novel miRNA based biomarkers and therapeutics is rapidly approaching.

The present review aims to summarize the current literature on miRNAs in IBD and to explore the opportunities and limitations in utilizing miRNAs as biomarkers and therapeutic targets in patients with IBD.

miRNA general overview

Since the first discovery of miRNA in 1993 [Lee *et al.* 1993; Wightman *et al.* 1993], the identification of protein-coding targeting miRNA homologs across many vertebrate species has confirmed an evolutionary conserved mechanism of post-transcriptional gene regulation [Pasquinelli *et al.* 2000]. To date, there are now over 2500 known mature human miRNA transcripts [miRbase release 20].

miRNAs are transcribed from intronic, intergenic or exonic DNA into a hairpin-stem and loop primary transcript-miRNA (pri-miRNAs). After enzymatic maturation and transport into the cytoplasm, a single strand is loaded into the RNA-induced silencing complex (RISC) containing an Argonaute protein, the catalytically active RNase, forming a mature miRNA complex capable of silencing mRNA *via* 3'-UTR binding. The mature miRNA:RISC silences target gene mRNA by binding the miRNA 'seed sequence', 6–8 nucleotides with extensive, but not necessarily completely complementary sequences, to the 3'-UTR of the mRNA. The binding results in mRNA

translation repression or degradation thereby controlling protein synthesis.

The imperfect binding required for miRNA:mRNA targeting allows for a single miRNA to target hundreds of genes and a single mRNA may have multiple 3'-UTR binding sites allowing targeting by multiple miRNAs. Given this depth of complexity, it has been estimated that miRNAs regulate up to 60% of human protein coding genes [Beitzinger and Meister, 2010; Eiring *et al.* 2010]. While the canonical mechanism of action is translational repression, further adding to the intricacy is the recent evidence of miRNA mediated gene activation [Majid *et al.* 2010]. This has been demonstrated in the regulation of the IBD-relevant cytokine, tumor necrosis factor (TNF)- α , whereby miR-369-3p transitions to activator from repressor of TNF α translation when cells in culture are growth arrested [Vasudevan *et al.* 2007].

In addition to the biogenesis of miRNA, regulation of miRNA expression may be dependent on chromosomal aberration (malignancy), alterations in miRNA processing machinery, SNPs (within a target mRNA or within the miRNA) and transcription factor binding, as well as other epigenetic mechanisms of gene expression regulation including DNA cytosine modifications. This is exemplified by epigenetic regulation of miR-124 in pediatric patients with UC, where DNA hypermethylation of the CpG island located in its promoter region results in miRNA downregulation [Koukos *et al.* 2013].

miRNA profiling in IBD

miRNA in mucosal tissues: UC

In 2008, Wu and colleagues completed the first miRNA profiling study to examine miRNA expression in IBD. They examined sigmoid colon biopsies from patients with active UC, inactive UC, chronic active CD, irritable bowel syndrome and microscopic colitis compared with healthy control subjects. They identified 11 miRNAs with significantly different expression in active UC patients *versus* controls including 8 with increased (miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195 and let-7f) and 3 with decreased (miR-192, miR-375, and miR-422b) expression [Wu *et al.* 2008].

Over the past four years, further research in active UC *versus* healthy controls has identified additional

dysregulated miRNAs, including confirmation of upregulation of miR-21 [Takagi *et al.* 2010; Yang *et al.* 2013]. Further reported miRNA upregulation includes miR-155 [Takagi *et al.* 2010], miR-31 [Fasseu *et al.* 2010; Lin *et al.* 2014], miR-126 [Fasseu *et al.* 2010; Feng *et al.* 2012], miR-7, miR-135b, miR-223, miR-29a, miR-29b, miR-127-3p, miR-324-3p [Fasseu *et al.* 2010], miR-150 [Bian *et al.* 2011], miR-20b and miR-125b-1 [Coskun *et al.* 2013]. Downregulated miRNA in active UC *versus* healthy controls include miR-188-5p, 215, 320a, 346 [Fasseu *et al.* 2010], miR-200b [Chen *et al.* 2013b], let-7, miR-125, miR-101, miR-26, and in pediatric UC only, miR-124 [Koukos *et al.* 2013].

miRNA in mucosal tissues: CD

Assessment of miRNA expression in CD has predominantly focused on Crohn's colitis. However, one report examined 5 patients with active colonic CD and 6 patients with chronically active terminal ileal CD compared with 13 healthy controls [Wu *et al.* 2010]. In the patients with chronically active terminal ileal CD relative to healthy controls, miR-16, miR-21, miR-223 and miR-594 were increased. Similarly examining miRNA expression in Crohn's colitis relative to healthy controls, they identified overexpression of miR-23b, 106a and 191, while expression of miR-19b and miR-629 were decreased. There was no overlap between the miRNA expression patterns of patients with CD of the colon as compared with the ileum.

Fasseu and colleagues, examining 8 patients with Crohn's colitis compared with 10 healthy controls, identified 23 miRNAs significantly upregulated in active CD tissues (miRs-9, -21, -22, -26a, -29a, -29c, -30b, -31, -34c-5p, -106a, -126, -126*, -127-3p, -130a, -133b, -146a, -146-3p, -150, -155, 181c, -196a, -324-3p, -375). Five of these miRNAs were specific for active CD (miRs-9, 126, -130a, -181c and -375), while the remaining 18 were also upregulated in quiescent CD tissue [Fasseu *et al.* 2010].

Recently, miRs-196 [Brest *et al.* 2011], -106b [Lu *et al.* 2014] and -31 [Huang *et al.* 2014; Lin *et al.* 2014] have also been reported to be upregulated in active colonic CD. Analyzing 15 sigmoid colon CD patients *versus* healthy controls, Huang and colleagues reported miR-141 to be downregulated in colonic CD and further utilized *in vivo* mouse models to confirm miR-141 regulated

colonic leukocyte trafficking by targeting CXCL12 β during intestinal inflammation [Huang *et al.* 2014]. Nguyen and colleagues similarly examined eight active colonic CD patients and found only miR-7 to be downregulated relative to six healthy controls [Nguyen *et al.* 2010].

miRNA in peripheral blood: UC

In addition to tissue expression of miRNAs, several recent analyses have examined miRNA expression in the peripheral blood from patients with IBD. Initially the expression of miRNAs in plasma and serum were thought to reflect the extrusion of miRNAs from relevant remote tissues or organs [Mitchell *et al.* 2008]. Preliminary studies in IBD, however, have been conflicting, as initial studies found dissimilar miRNA expression profiles compared with tissues, leading to the suggestion that peripheral blood miRNAs may reflect circulating leukocytes rather than expression in remote tissues. However, more recent investigations have been able to demonstrate several previously reported dysregulated tissue miRNAs with increased expression in peripheral blood (miRs-16, -21 and -155) [Paraskevi *et al.* 2012; Yang *et al.* 2013].

A comparison of whole peripheral blood samples from 13 active UC patients with 13 healthy control subjects was performed using microarray technology and quantitative real time PCR (qRT-PCR) [Wu *et al.* 2011]. Nine UC-associated peripheral blood miRNAs were found to be differentially expressed by qRT-PCR (increased: miRs-28-5p, -151-5p, -103-2*, -199a-5p, -340*, -362-3p, -532-3p, miRplus-E127; decreased: miR-505*). A 7.2 fold decreased expression of miR-505* was noted in active UC patients' blood. Conversely, miR-103-2* and miR-362-3p demonstrated the greatest increase in expression in active UC patients with a 3.1 and 5.2 fold increase, respectively. When active UC patients were divided into pan colitis and distal colitis subgroups, there was no significant difference in expression of these miRNAs. Of the dysregulated miRNAs reported in the study of active UC compared with normal controls, miRs-28-5p, -151-5p, -199a-5p, -340* and miRplus-E1271 were increased in the peripheral blood of patients with active UC but not in inactive UC. In contrast, miRs-103-2*, -362-3p and -532-3p were increased, and miR-505* was decreased in the blood of both inactive and active UC patients.

Given the cell specific nature of miRNA expression and gene regulation, Duttagupta and colleagues completed a microarray analysis of miRNA expression levels from different hematological fractions as noninvasive predictors for the diagnosis of UC [Duttagupta *et al.* 2012]. They examined microvesicle, peripheral blood mononuclear cells (PBMC) and platelet fractions from a cohort of 20 normal and 20 affected individuals diagnosed with UC, and found a distinct diagnostic signature of 31 miRNAs with increased expression that derive specifically from the platelet fraction; however the PBMC and microvesicle fractions were not predictive.

miRNA in peripheral blood: CD

Further testing the hypothesis that peripheral blood miRNAs can distinguish IBD subtypes, Wu and colleagues also examined 14 active CD *versus* 13 healthy controls with microarray and qRT-PCR, and confirmed seven miRNAs with significant changes in expression (increased: miRs-199a-5p, -362-3p, -340* and -532-3p; decreased: miR-149*, miR-plus-F1065) [Wu *et al.* 2011]. Of these miRNAs, miR-362-3p demonstrated the most significant difference in expression with a 4.7 fold increase seen in the peripheral blood of active CD patients. When the active CD patients were subgrouped into Crohn's ileitis and Crohn's colitis patients, there was no significant difference in expression of these miRNAs.

miRs-199a-5p, -362-3p, -532-3p and miRplus-E1271 were increased in the peripheral blood of patients with active CD but not in the blood of patients with inactive CD compared with healthy controls. In contrast, the peripheral blood of both active and inactive CD patients exhibited increased expression of miR-340*. Similarly, miRplus-F1065 was decreased only in the blood of active CD patients while miR-149* was decreased in the blood of both active and inactive CD patients.

In addition to studying UC, Paraskevi and colleagues analyzed whole peripheral blood from 128 active CD patients compared with 162 healthy controls using the same subset of published miRNAs known to be relevant in IBD. They found 11 miRNAs were upregulated in peripheral blood CD (miRs-16, -23a, miR-29a, miR-106a, miR-107, miR-126, miR-191, miR-199a-5p, miR-200c, miR-362-3p and miR-532-3p) [Paraskevi *et al.* 2012].

Using serum instead of whole peripheral blood, Zahm and colleagues examined 46 predominantly active, ileocolonic pediatric CD patients compared with 32 healthy controls using a low density microarray approach with qRT-PCR confirmation. The authors identified 11 miRNAs with significantly increased expression (miRs-16, -484, -30e, -106a, 195, -20a, -21, -140, let-7b, -192 and -93) [Zahm *et al.* 2011]. There were no significantly decreased miRNAs in this analysis. Similarly using serum, Iborra and colleagues examined 18 CD patients (9 active, 9 inactive) with colonic involvement and 33 healthy controls, and identified 6 miRNAs expressed differentially exclusively for CD (increased: miRs -27a*, -140-3p, -140-5p, -16, -195; decreased: miR-877) [Iborra *et al.* 2013]. Comparable with UC, these studies demonstrated some overlap between CD tissues expression and peripheral blood of CD patients (miR-16, -21). The most often cited dysregulated miRNAs in IBD isolated from tissues and peripheral blood are summarized in Table 1.

miRNA in indeterminate colitis

In clinical practice, 10% of patients with IBD of the colon cannot be classified with a diagnosis of CD or UC based on usual standard clinical testing including colonoscopy, imaging, laboratory tests and histology [Tremaine, 2011]. This distinction often has significant prognostic and therapeutic implications, especially in those failing medical therapy and considering surgery. Serologic markers when used in combination, including perinuclear antineutrophil cytoplasmic antibodies (pANCA) and antimicrobial antibodies [anti *Saccharomyces cerevisiae* antibody (ASCA), anti-OmpC, anti-I2 and anti-CBir1] have relative low sensitivity in distinguishing colonic CD and UC [Prideaux *et al.* 2012]. As such, there is great interest in examining if miRNA profiles can discriminate between CD and UC.

The previously mentioned analysis by Fasseu and colleagues identified 15 miRNAs with significantly distinct alteration of expression between noninflamed UC and CD colonic tissue. Initially, 6 were published (miRs-150, -196b, -199a-3p, -199b-5p, -223, -320a) [Fasseu *et al.* 2010]; the same group published an additional 10 in a subsequent patent application (miRs-142-3p, -142-5p, -146a, -146b-5p, -181d, -182, -199a-5p, -203, -299-5p, -328) [US Patent Application: US20130143764A1, 6 June 2013]. Using this miRNA profile, they were able to correctly

Table 1. Most frequently reported dysregulated microRNA in inflammatory bowel disease tissue and peripheral blood.

miRNA	Expression	Disease cohort	Studies	Samples	Methodology	Reference
Ulcerative colitis: tissue						
miR-21	Upregulated	Active UC <i>versus</i> control	4	Sigmoid and colon biopsies	miRNA microarray and qRT-PCR, <i>in situ</i> hybridization	Wu 2008; Takagi 2010; Yang 2013; Feng 2012
miR-126	Upregulated	Active UC <i>versus</i> control	3	Sigmoid and colon biopsies	miRNA microarray and qRT-PCR	Feng 2008; Fasseu 2010; Feng 2012
miR-31	Upregulated	Active UC <i>versus</i> control	2	Colon tissue/ biopsies	Genome-wide small RNA sequencing and qRT-PCR	Lin 2013; Fasseu 2010
miR-29a	Upregulated	Active UC <i>versus</i> control	2	Sigmoid and colon biopsies	miRNA microarray and qRT-PCR	Wu 2008; Fasseu 2010
Ulcerative colitis: peripheral blood						
miR-151-5p	Upregulated	Active UC <i>versus</i> control	2	Peripheral blood	miRNA microarray and qRT-PCR	Paraskevi 2012; Wu 2011
miR-199a-5p	Upregulated	Active UC <i>versus</i> control	2	Peripheral Blood	miRNA microarray and qRT-PCR	Bian 2011; Wu 2011
Crohn's disease: tissue						
miR-31	Upregulated	Active CD <i>versus</i> control	3	Sigmoid and colonic biopsies	Small RNA sequencing, miRNA microarray and qRT-PCR	Lin 2013; Fasseu 2010; Huang 2013
miR-21	Upregulated	Active CD <i>versus</i> control	2	Ileal and colonic biopsies	miRNA microarray and qRT-PCR	Fasseu 2010; Wu 2010
miR-106a	Upregulated	Active CD <i>versus</i> control	2	Sigmoid and colonic biopsies	miRNA microarray and qRT-PCR	Fasseu 2010; Wu 2010
Crohn's disease: peripheral blood						
miR-16	Upregulated	Active CD <i>versus</i> control	3	Serum, peripheral blood	miRNA microarray, LDA qRT-PCR, qRT-PCR	Paraskevi 2012; Zahm 2011; Iborra 2013
miR-106a	Upregulated	Active CD <i>versus</i> control	2	Serum, peripheral blood	LDA qRT-PCR, qRT-PCR	Paraskevi 2012; Zahm 2011
miR-195	Upregulated	Active CD <i>versus</i> control	2	Serum	miRNA microarray and qRT-PCR	Zahm 2011; Iborra 2013
miR-199a-5p	Upregulated	Active CD <i>versus</i> control	2	Peripheral blood	miRNA microarray and qRT-PCR	Paraskevi 2012; Wu 2011
miR-532-3p	Upregulated	Active CD <i>versus</i> control	2	Peripheral blood	miRNA microarray and qRT-PCR	Paraskevi 2012; Wu 2011
miR-362-3p	Upregulated	Active CD <i>versus</i> control	2	Peripheral blood	miRNA microarray and qRT-PCR	Paraskevi 2012; Wu 2011

CD, Crohn's disease; LDA qRT-PCR, low density array quantitative real time polymerase chain reaction; miRNA, micro RNA; qRT-PCR, quantitative real time polymerase chain reaction; UC, ulcerative colitis.

identify the IBD diagnosis in 15 of 16 patients [Lin *et al.* 2014].

Peripheral blood miRNA assessment has also been demonstrated to successfully distinguish active CD and UC. Utilizing 14 active CD patients and 13 active UC patients, Wu and colleagues identified 10 miRNAs that were significantly increased and 1 miRNA that was significantly decreased in the blood of active UC patients compared with active CD patients (increased: miR-28-5p, -103-2*, 149*, -151-5p, -340*, -532-3p,

-plus-E1153, -3180-3p, -plus-E1035 and -plus-F1159; decreased miR-505*) [Wu *et al.* 2011].

miRNAs as therapy in IBD: potential IBD targets for miRNA therapy

In the broadest of terms, direct miRNA based therapeutics comprise two core strategies: miRNA antagonism and mimicry. Physiologic miRNA overexpression resulting in pathologically decreased target gene expression can be inhibited by using miRNA antagonists, while miRNA underexpression

resulting in increased target function can be restored by using miRNA mimics.

With the discovery of miRNA dysregulation in IBD, followed by the preliminary data confirming functionality in canonical IBD pathogenic pathways, there has been significant interest and opportunity in developing miRNA based therapeutics. Recent preclinical *ex vivo* cellular culture methods and *in vivo* animal models, and most recently human clinical trials, have already indicated that strategically designed synthetic oligonucleotide based inhibition and mimic replacement can be effective drugs in cardiac dysfunction [Thum *et al.* 2008], lymphoma [Babar *et al.* 2012], and hepatitis C [Janssen *et al.* 2013]. While endogenous miRNAs are stable in peripheral blood [Chen *et al.* 2008] due to packaging in exosomes or microvesicles, targeted therapeutic *in vivo* delivery of miRNAs will need to be stable in serum, avoid rapid enzymatic degradation and renal clearance, reduce off-target effects, and overcome the large negative charge and high molecular weight to allow for increased cellular uptake [Pecot *et al.* 2011]. Thus, a significant determinant to the successful development of miRNA based therapy will be dependent on design and delivery methods. The miRNAs confirmed to have altered expression in IBD patients with pathophysiologic mechanism are highlighted as having the highest potential for future therapy and are summarized in Table 2.

CD miRNA in autophagy

GWAS have identified genes regulating autophagy, immunity-related GTPase family M (IRGM) [Parkes *et al.* 2007] and autophagy-related 16-like 1 [*S. cerevisiae*] (ATG16L1) [Hampe *et al.* 2007], as susceptibility genes in CD. Additionally, CD associated SNPs in nucleotide-binding oligomerization domain-containing protein 2 (NOD2) [Ogura *et al.* 2001] have also been associated with autophagy by physically interacting with ATG16L1 in regulating autophagy and bacterial clearance by intestinal epithelial cells [Homer *et al.* 2010]. While there have been over a dozen miRNAs that directly regulate autophagic signaling and activity in certain cancer cell lines, their role in regulating autophagy and bacterial clearance by intestinal epithelial cells as part of the molecular pathways of CD pathogenesis is only recently now being elucidated.

ATG16L1

miR-106a was one of the first reported miRNAs to be differentially expressed in patients with IBD. Due to sharing of a similar seed sequence, miR-106a belongs to the broadly conserved miR-17 family. Both miR-106a and -106b have been reported to be oncogenes with correlation to cell cycle proliferation in cancer [Ivanovska *et al.* 2008]. Furthermore, the miR-17 family has been identified to target multiple autophagy genes including ATG16L1. Using a human cell line *ex vivo* cell culture system, Zhai and colleagues first identified that miR-106b *via* binding to the 3'-UTR reduced ATG16L1 expression thereby decreasing autophagic activity [Zhai *et al.* 2013]. This work was followed by that of Lu and colleagues who confirmed that miR-106b (and miR-93) targets ATG16L1 in colon cell lines and corroborated, in human colonic tissue, increased expression of miR-106b correlating to downregulated ATG16L1 in patients with active CD [Lu *et al.* 2014]. To accomplish this, the investigators not only utilized miR-106b and miR-93 mimics, but also purchased synthetic anti-mRNA oligonucleotides (AMOs) against miR-106b and miR-93.

Furthermore, using the same human cell line, Zhai and colleagues subsequently identified miR-142-3p as a miRNA target of ATG16L1, with transient overexpression resulting in decreased ATG16L1 mRNA, protein levels and decreased autophagic activity [Zhai *et al.* 2014]. Recently, using both an *ex vivo* cell culture model and confirming with an *in vivo* mouse enterocyte model, Nguyen and colleagues reported that adherent-invasive *Escherichia coli* (AIEC), a bacterium found to be highly prevalent in ileal mucosa of CD patients, leads to activation of the nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) pathway, inducing strong expression of miR-30c and miR-130a, which in turns suppresses ATG5 and ATG16L1 [Nguyen *et al.* 2014]. Upregulation of these miRNAs were confirmed *via* CD ileal biopsy specimens supporting both their experimental findings and the theory that defects in autophagy-mediated handling by CD-associated bacteria is in part regulated by miRNA. These findings indicate the importance of miRNA regulation of autophagy in CD pathogenesis and may be a target for future therapy.

IRGM

IRGM encodes an autophagic protein involved in the innate immunity against intracellular

Table 2. MicroRNA: potential targets for therapy correlated to tissue expression data.

miRNA	Pathway	Target	Tissue Expression data (Reference)	Effects (Reference)	Therapy
miR-106b	Autophagy	ATG16L1	Upregulated in human colonic tissue of CD patients (Lu, 2014)	Decreases autophagic activity (Zhai, 2013)	Antagonism
miR-142-3p	Autophagy	ATG16L1	Upregulated in CD human patients (Fasseau – patent)	Decreases autophagic activity (Zhai, 2014)	Antagonism
miR-30c and miR-130a	Autophagy	ATG5 and ATG16L1	Upregulated in human ileal CD patients (Singh, 2006)	Decreases autophagic activity, increased expression driven by AIEC (Singh, 2006)	Antagonism
miR-196	Autophagy	IRGM	Upregulated in human CD (Brest, 2011)	Downregulates IRGM protective variant (c313C) (Brest, 2011)	Antagonism
miRNA family 29a, 29b, and 29c	Cytokine production	IL-23 subunit, IL-12p40	CD patients with NOD2 SNP variant unable to upregulate miR-29 (Bae, 2013)	Inability to downregulate production of IL-12p40 (IL-23) (Bae, 2013)	Mimicry
miR-29b	Anti-fibrotic		Down regulated in human CD strictured tissue		Mimicry
miR-21	Pro-fibrotic, barrier permeability		Upregulated in ileal CD tissue (Fasseu, 2010; Wu, 2010)	Increases barrier permeability (Yang, 2013)	Antagonism
miR-192	Cytokine production	Macrophage inflammatory peptide (MIP)-2 α	Downregulated in UC tissue (Wu, 2008)	Decreases TNF- α induced MIP-2- α expression (Wu, 2008). *Has also been shown to be pro-fibrotic	Mimicry in UC*
miR-126	Immune system activation	I κ B α	Upregulated in active UC (Wu, 2008; Fasseu, 2010; Feng, 2012)	Increases inflammatory response <i>via</i> NF- κ B signaling (Feng, 2012)	Antagonism
miR-155	Immune system activation	SOCS-1	Upregulated in active UC and CD tissues (Takagi, 2010; Fasseu, 2010)	Loss of negative feedback regulation on LPS-induced macrophage activation, JAK/STAT signaling, and antigen presentation by dendritic cells (Sonkoly, 2010)	Antagonism
miR-106a	Cytokine production	IL-10	Upregulated in active CD tissue and peripheral blood (Fasseu, 2010; Wu, 2010; Paraskevi, 2012; Zahm, 2011)	Decreases IL-10 production in non-colon cell lines (Sharma, 2009)	Antagonism

ATG16L1, autophagy-related 16-like 1; CD, Crohn's disease; IL, interleukin; IRGM, Immunity-related GTPase family M; JAK, Janus kinase; LPS, lipopolysaccharide; NF- κ B, nuclear factor kappa light chain enhancer of activated B cells; NOD2, nucleotide-binding oligomerization domain-containing protein 2; SNP, single nucleotide polymorphism; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; UC, ulcerative colitis.

pathogens including AIEC [Singh *et al.* 2006; McCarroll *et al.* 2008; Lapaquette *et al.* 2010]. SNPs in or near the *IRGM* gene have been strongly associated with an increased risk of developing CD in Caucasian populations of European descent. Brest and colleagues reported that a known *IRGM* polymorphism (c313C>T) is located within the 3'-UTR seed sequence, the

site necessary for RISC to complex with miRNA-mRNA. miR-196, which is highly expressed in intestinal epithelia of patients with CD, downregulates the *IRGM* protective variant (c313C), but not the risk associated variant/SNP (c313T) in a tissue specific manner, highlighting the importance of target mRNA polymorphisms in miRNA mediated regulation [Brest *et al.* 2011].

NOD2

In silico analysis of NOD2 3'-UTR reveals 11 putative miRNA binding sites that include 2 putative miR-192 binding sites, implicating miRNAs in the regulation of NOD2 expression and inflammatory responses [Chuang *et al.* 2014]. Using a HT-29 *ex vivo* colon epithelial cell culture system, Chen and colleagues identified miR-122 targeting of NOD2, inhibiting innate immune system activation *via* the NF- κ B pathway [Chen *et al.* 2013a]. This was followed by Chuang and colleagues who demonstrated that four additional miRNAs (miR-192, miR-495, miR-512 and miR-671) attenuate innate immune responses *via* suppression of NOD2 signaling using a HCT-116 colonic epithelial cell model [Chuang *et al.* 2014]. While it is becoming clear that multiple miRNAs are involved in the regulation of NOD2 expression, the proportional effects of the miRNAs on downstream immune responses did not correlate solely with NOD2 expression levels and is suggestive of miRNAs having multiple targets in downstream cytokine signaling pathways.

NOD2 appears to not only be regulated by miRNAs but also mediates its downstream effects *via* miRNAs. Brain and colleagues demonstrated that NOD2 induces expression of the miRNA family 29a, 29b and 29c in dendritic cells, and with NOD2 activation, miR-29 downregulates IL-23 production by targeting of its subunit, IL-12p40 [Brain *et al.* 2013]. Further, dendritic cells from Crohn's patients homozygous or compound heterozygous for NOD2 SNPs were associated with failure to induce miR-29 upon NOD2 activation and increased release of interleukin (IL)-12p40 after infection with AIEC. Decreased function of this immunoregulatory pathway in small bowel intestinal dendritic cells may be responsible for producing excess IL-23, commonly observed in the small intestinal mucosa in Crohn's patients. Given the efficacy of ustekinumab, a monoclonal antibody against IL-12 and IL-23, in inducing and maintaining remission in patients with moderate-to-severe CD resistant to anti-TNF treatment [Sandborn *et al.* 2012], one may be able extrapolate the efficacy of miR-29 mimicry as another mechanism to reduce IL-23 production. With regard to potential off-target effects, miR-29a and -29c have been reported to be tumor suppressors across multiple malignancies [Bae *et al.* 2014].

miRNAs related intestinal fibrosis: miR-29, miR-200b, miR-21, miR-192

Multiple studies in non-IBD populations have implicated miRNAs in organ fibrosis which has

recently been extensively reviewed [Vettori *et al.* 2012]. Some of the miRNAs established to be relevant in IBD include three 'antifibrotic' miRNAs (-29, -200b, -141) and two 'profibrotic' miRNAs (-21 and -192). Other miRNAs not reviewed here but potentially having a role in IBD fibrosis and warranting further investigation include miRNAs -142-3p, 155 and 199a/b.

Antifibrotic. miR-29 may play an important role in the development of CD intestinal fibrosis in addition to IL-23 production. miR-29 regulates expression of collagen genes and is significantly downregulated in models of liver fibrosis [Roderburg *et al.* 2011] as well as in patients with systemic sclerosis [Maurer *et al.* 2010]. Preliminary research investigating miRNA profiling in CD strictures *versus* nonstrictured tissue has since highlighted miR-29b to be down regulated in strictured tissue. Stimulation with transforming growth factor (TGF)- β , a profibrotic cytokine, significantly downregulates miR-29b expression by CD myofibroblasts, and induced expression of miR-29b resulted in a decrease of collagen III mRNA and protein levels [Biancheri, 2013].

Similarly, miR-200b, also associated with liver fibrosis [Murakami *et al.* 2011] and therapeutically demonstrated to reduce renal tubule fibrosis [Oba *et al.* 2010], was demonstrated to be downregulated by TGF- β and the re-introduction of miR-200b resulted in decreased epithelial to mesenchymal transition (EMT) [Chen *et al.* 2013b]. In addition to regulating colonic leukocytic trafficking by targeting CXCL1 β during murine colitis and human CD, miR-141, a member of the miR-200 family, is also involved in EMT-related fibrosis.

Profibrotic. miR-21 has been demonstrated to be elevated in ileal CD tissue and is also reported to be involved in EMT and fibrosis. The most direct evidence of its role in organ fibrosis comes from investigations in cardiac and pulmonary fibrosis [Liu *et al.* 2010]. Thum and colleagues reported miR-21 overexpression in fibroblasts from failing hearts relative to normal myocardium with miR-21 targeting crucial pathways for fibroblast survival and activation [Thum *et al.* 2008]. Furthermore, injecting an AMO for miR-21 (antagomiR-21) in mice resulted in inhibition of interstitial fibrosis and attenuation of cardiac dysfunction. Similarly, use of an intraperitoneal AMO against miR-21 in a murine model of renal fibrosis resulted in decreased macrophage

infiltration and collagen gene expression [Zarjou *et al.* 2011].

TGF β also upregulates miR-192 expression. This increase in expression results in the reduction of its target, Smad-interacting protein 1 (SIP1), causing increased collagen gene expression [Kato *et al.* 2007]. Despite this shared pathway in fibrosis, miR-192 has not yet been reported to be dysregulated in CD.

miRNAs associated epithelial barrier and immune function in IBD pathogenesis: miR-192, miR-21, miR-126, miR-155, miR-106a
miR-192. Functional analyses in UC, begun by Wu and colleagues in 2008, found miR-192 to be the most highly expressed miRNA of the UC-associated miRNAs in human colon tissues; in UC tissue with active inflammation there was a 47.1% decrease compared with healthy controls. Macrophage inflammatory peptide (MIP)-2 alpha, a CXC chemokine also expressed by epithelial cells and previously implicated in IBD and murine colitis, was found to have a binding site for miR-192 and discordant expression with the miRNA in UC tissues. In a cell culture model, a miR-192 mimic was able to inhibit TNF α induced MIP-2a expression [Wu *et al.* 2008].

Given the decreased expression in active UC, the therapeutic role of miR-192 in IBD may be the result of mimicry. The intravenous administration of an antagomir to silence miR-192 demonstrated a reduction of miRNA-192 in the liver and kidney [Krutzfeldt *et al.* 2005], highlighting the importance of tissue specific delivery methods to prevent unintended off-target effects. Future consideration of miR-192 as therapy requires consideration of the profibrotic component of miR-192 overexpression, which as result, has been a target for inhibition using AMO therapy in renal fibrosis. Further, miR-192 functions as a tumor suppressor as evidenced by ability to induce cell cycle arrest and its reduced expression in primary cancers [Georges *et al.* 2008].

miR-21. Due to the crucial role miR-21 plays in a multitude of biological functions and diseases including development, multiple cancers, cardiovascular diseases and inflammation, miR-21 was one of the first reported [Lagos-Quintana *et al.* 2001] and most widely studied miRNAs. Previous studies in IBD have demonstrated that miR-21 is increased by 354.6% in active UC tissues

compared with controls and was also increased in patients with ileal CD [Wu *et al.* 2008, 2010].

In addition to fibrosis, miR-21 has also been demonstrated to have a role in epithelial barrier function as well as adaptive immune response. Using an *ex vivo* colon epithelial cell model, Yang and colleagues demonstrated that miR-21 mimics resulted in loss of tight junction proteins and increased barrier permeability [Yang *et al.* 2013]. In a dextran sodium sulfate (DSS) induced model of colitis, miR-21 knockout mice had decreased CD3 and CD68 positive cells, intestinal permeability, and apoptosis of epithelial cells relative to wildtype mice [Shi *et al.* 2013]. miR-21 also has a significant role in T-cell function, with highest expression in effector T cells, followed by memory T cells, and lowest in naive T cells [Wu *et al.* 2007]. One potential target for miR-21 is IL-12p35, a subunit of IL-12. IL-12 is a key cytokine released from macrophages and dendritic cells involved in adaptive immune responses. However, the clinical importance of the interaction between miR-21 and IL-12p35 remains unclear in active IBD as IL-12 is typically elevated in IBD tissue and there is therapeutic benefit of anti-IL-12 monoclonal antibodies [Sandborn *et al.* 2012].

miR-126. miR-126 was demonstrated to be upregulated in tissues from patients with active UC [Wu *et al.* 2008]. Feng and colleagues confirmed this finding in a separate cohort of UC patients but also, using an *ex vivo* colon cell model, identified a target of miR-126 to the 3'-UTR of I κ B α , a protein which inhibits activation of the NF- κ B signaling pathway [Feng *et al.* 2012]. With regard to potential off-target effects, miR-126 decreases apoptosis in acute myeloid leukemia by downregulating a tumor suppressor [Li *et al.* 2008], but in solid tumors has been demonstrated to act as a tumor suppressor itself in gastric [Feng *et al.* 2010] and nonsmall cell lung [Crawford *et al.* 2008] cancers.

miR-155. miR-155 is one of the most studied miRNAs with *in vitro* and *in vivo* reports indicating its central regulatory role in innate and adaptive immunity. In response to inflammatory mediators such as bacterial lipopolysaccharide, interferon- β and toll-like receptor ligands, miR-155 expression is induced in antigen presenting cells such as macrophages and plasmacytoid dendritic cells. Upon activation with antigen, B and T cells rapidly induce expression of miR-155 [Plank

et al. 2013]. miR-155 targets suppressor of cytokine signaling (SOCS)-1, which is a negative regulator of lipopolysaccharide-induced macrophage activation, Janus kinase/a signal transducer and activator of transcription (JAK/STAT) signal pathway activation, as well as antigen presentation by dendritic cells [Evel-Kabler *et al.* 2006]. Increased expression of miR-155 has also been described in other inflammatory disorders including rheumatoid arthritis [Stanczyk *et al.* 2008], multiple sclerosis [Murugaiyan *et al.* 2011] and atopic dermatitis [Sunkoly *et al.* 2010]. In addition to its effects on the immune system, miR-155 has been reported to be an oncogene in multiple malignancies [Soriano *et al.* 2013]. AMOs against miR-155 using a locked nucleic acid (LNA) anti-miR-155 lead to *in vitro* and *in vivo* repression of granulocyte-colony stimulating factor, a regulator of granulopoiesis produced by activated macrophages during acute inflammatory responses [Worm *et al.* 2009].

miR-106a. IL-10 is an anti-inflammatory, immunoregulatory cytokine with promoter polymorphisms linked to IBD and is secreted by multiple cell types including T-cell subsets, B cells and antigen presenting cells such as macrophages and dendritic cells. IL-10 knockout mice are a well-established murine model of CD as they develop a chronic ileocolitis. Although IL-10 injection in several animal models of colitis has demonstrated therapeutic efficacy, IL-10 based therapy in humans have only had modest benefit [Marlow *et al.* 2013]. miR-106a, as a member of the miR-17 family, targets IL-10, and has been demonstrated to regulate IL-10 expression in noncolon cell lines [Sharma *et al.* 2009].

miRNAs as therapy in IBD: design and delivery of miRNA agents

miRNA antagonism (Figure 1a-c)

Anti-microRNA oligonucleotides. AMOs are synthetic oligonucleotides with reverse complementary sequences to mature miRNAs that can tightly bind to their miRNA targets with high specificity as well as optimal stability and pharmacokinetics necessary to block miRNA function. Since naked RNA is rapidly degraded in the blood stream secondary to significant nuclease enzymatic degradation [Czuderna *et al.* 2003], AMOs are chemically modified to confer nuclease stability, increase binding affinity, as well as facilitate entrance into the cell and miRNA-induced silencing complex

[Lapierre *et al.* 2011]. These oligonucleotide chemical modifications may include nucleotide modifications to the phosphate backbone and/or sugar or use of nonribose backbones [Lennox and Behlke, 2011]. Commonly investigated sugar modifications include antisense 2'-O-methyl (2'-OMe) and 2'-O-methoxyethyl (2'-MOE) oligoribonucleotides, and 2',4'-methylene bridge (LNA) allowing for increased binding affinity and reduced degradation. Further modifications including phosphorothioate (PS) modification *via* the substitution of a sulfur atom to the phosphate backbone further prevents nuclease mediated degradation but at the cost of reduced binding affinity [Lennox and Behlke, 2011]. Although not involved in miRNA targeting, the efficacy of synthetic chemically modified oligonucleotides was demonstrated with Mipomersen, a subcutaneously delivered, 20 nucleotide 2'-MOE-PS oligonucleotide that hybridizes directly with apolipoprotein B-100 mRNA in the liver, resulting in mRNA degradation [Stein *et al.* 2012]. Mipomersen was approved in January 2013 by the US Food and Drug Administration (FDA) for the treatment of homozygous familial hypercholesterolemia.

LNA modified AMOs have very high binding affinity, which provides the benefit of allowing for shorter oligonucleotide lengths, maintenance of seed sequence, preservation of specificity and improved efficacy. Pure LNA modified oligonucleotides can result in self-dimerization decreasing efficacy, though this can be reduced if DNA or other nucleotides are inserted between LNA nucleotides. Such an AMO, a 15 nucleotide LNA/DNA-ASO (Miravirsen) optimized to target and inhibit miR-122, was developed by Santaris Pharmaceuticals as treatment for hepatitis C. In a phase IIa dose-finding clinical trial, subcutaneous administration in 36 treatment naïve hepatitis C patients demonstrated therapeutic effect with minimal short-term side effects [Janssen *et al.* 2013].

miRNA sponges. The broadly conserved miR-17 family, which includes miRs-17, -20a, -20b, -93, -106a and -106b, has been identified to target multiple autophagy genes and thus has a predicted role in the pathogenesis of IBD. Although a single endogenous miRNA can target multiple pathways [Li *et al.* 2007], chemically modified antisense AMOs presumably have a more limited target depth. When miRNAs are grouped as a family, due to shared seed sequences, elimination

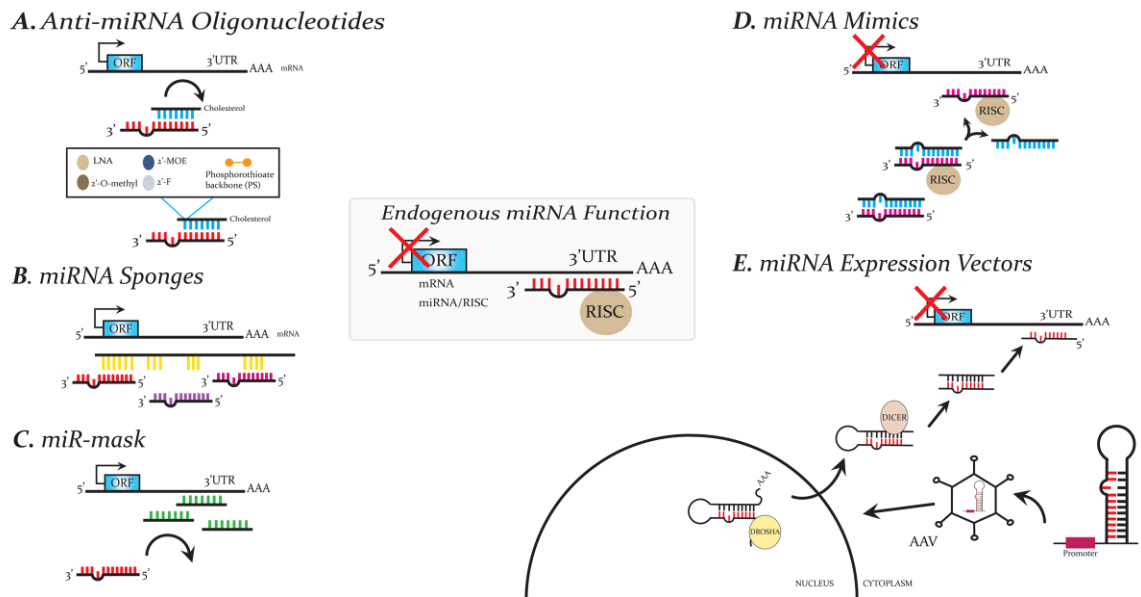


Figure 1. Methods of microRNA (miRNA) therapeutics. Endogenous mature miRNAs (center) loaded into the RNA-induced silencing complex (RISC) form a mature miRNA complex capable of silencing mRNA via 3'-UTR binding. The binding results in mRNA translation repression or degradation thereby controlling protein synthesis. (A) Anti-mRNA oligonucleotides are synthetic oligonucleotides with reverse complementary sequences to mature miRNAs that can tightly bind to their miRNA targets with high specificity to block miRNA function. These oligonucleotides utilize chemical modifications including 2'-O-methyl (2'-OME) and 2'-O-methoxyethyl (2'-MOE) oligoribonucleotides, and 2',4'-methylene bridge (LNA), and phosphorothioate (PS) modification to confer nuclease stability and/or increase binding affinity. (B) miRNA sponges involve plasmid constructs with strong promoters, containing multiple tandem binding mRNA target sites to the miRNAs of interest allowing simultaneous competitive inhibition of multiple miRNAs. (C) miRNA-masks are inhibitory miRNA antisense oligodeoxyribonucleotides that bind to the 3'-UTR of the target mRNA blocking the access of miRNAs. (D) miRNA mimics are double strand RNA in which one strand is identical to the endogenous mature miRNA of interest and paired with the complete or partially complementary 'passenger strand' which is required for loading into the RISC. (E) miRNA expression gene vectors, such as DNA plasmids or viral vectors including adeno-associated viral (AAV) vectors, allow for constitutive expression of miRNA constructs.

LNA, locked nucleic acid; ORF, open reading frame; UTR, untranslated region.

of a single miRNA may result in other miRNAs from the family producing a compensatory effect. miRNA sponges involve plasmid constructs with strong promoters, containing multiple tandem binding mRNA target sites to the miRNAs of interest allowing simultaneously competitive inhibition of multiple miRNAs [Lu *et al.* 2009]. Further investigation is needed for *in vivo* applications, but due to concern for serious undesirable side effects, initial applications of the technique have not definitively determined if expression will achieve sufficient levels to sequester miRNAs [Liu *et al.* 2013].

miR mask. miRNA masking is an inhibitory microRNA antisense oligodeoxyribonucleotide (ASO) utilizing a single-stranded 2'-OMe modified oligoribonucleotide (or other chemically

modified oligonucleotide) that instead of binding to a target miRNA like an AMO, binds to the 3'-UTR of the target mRNA by a fully complementary mechanism. As a result, the miRNA mask blocks the access of miRNAs to the binding 3'-UTR site, effectively de-repressing the target mRNA, in a gene specific manner [Choi *et al.* 2007]. Although studies have demonstrated their effectiveness *in vitro*, there is no data using *in vivo* models with this methodology.

miRNA mimicry/replacement therapy (Figure 1 d, e)

In UC, miR-192, miR-375, miR-422b, miR-188-5p, 215, 320a, 346 [Fasseu *et al.* 2010], miR-143, miR-145 [Pekow *et al.* 2012], let-7, miR-125, miR-101, miR-26, and in pediatric UC only,

miR-124 [Koukos *et al.* 2013] have all been reported to be downregulated compared with normal, healthy controls. In Crohn's colitis, miR-19b, miR-629 [Wu *et al.* 2010] and miR-7 [Nguyen *et al.* 2010] were decreased. In theory, replenishing these cellular miRNAs using miRNA mimics containing the same nucleotide sequence as the endogenous miRNAs may provide therapeutic restitution of physiological pathways lost in IBD and has the potential to have a greater therapeutic effect than drugs with a single protein or enzyme target since miRNAs target multiple genes/pathways.

The two main methods for restoring miRNA activity include the delivery of synthetic oligonucleotide miRNA mimics and miRNA expression gene vectors, such as DNA plasmids [Sibley *et al.* 2010] or viral vectors. miRNA mimics are double stranded RNA in which one strand, 'the guide strand', is identical to the endogenous mature miRNA of interest and paired with the complete or partially complementary 'passenger strand'. Similar to AMOs, to avoid nuclease digestion and aid with cellular uptake, chemical modifications such as 2'-OMe and cholesterol conjugation can be made. The double stranded structure is required for loading the guide strand into the RISC [Behlke, 2008; Garzon *et al.* 2010; Henry *et al.* 2010]. In addition to Miravirsen, the only other miRNA therapy to reach clinical trial investigation to date is MRX34 (Mirna Therapeutics, Inc.). Delivered intravenously, MRX34 is a liposome-formulated miR-34a mimic designed to restore the function of tumor suppressor miR-34a and was initiated in a multicenter Phase I study to evaluate the safety in patients with unresectable primary liver cancer or those with liver metastases from other cancers in May 2013 [ClinicalTrials.gov identifier: NCT01829971]

Delivery of miRNA agents

AMOs with chemical modifications: Delivery of nucleic acid based therapeutics is challenging due to the high molecular weight, anionic charge and enzymatic degradation. However, the promise of manipulating post-translational gene expression has invigorated research into overcoming these obstacles. AMOs with additional chemical modifications such as conjugation with cholesterol were developed to aid with *in vivo* delivery. The use of high doses of 'antagomirs', AMOs with 2'-OMe and PS phosphate backbone modifications with 3'-end cholesterol, has successfully demonstrated

target tissue delivery and survival of naked AMOs with intravenous administration without a delivery vessel such as nanoparticle, exosome or microvesicle [Krutzfeldt *et al.* 2005, 2007].

Viral vectors. Several viral vectors, including adenoviral, lentiviral, retroviral and adeno-associated viral (AAV), have been investigated as potential methods of delivery for miRNA agents. Of these, recombinant AAV-based vector systems has emerged as most promising as they do not usually integrate into the host genome, are nonpathogenic in humans, allow for sustained high levels of expression with high transduction efficiency, and *via* natural and manipulated capsid structures offer tissue specificity. AAV vectors have been demonstrated to successfully achieve antitumoral activity in animal models of hepatocellular carcinoma (HCC) *via* replacement of miR-26a [Kota *et al.* 2009] and in treatment of human patients with hemophilia B [Nathwani *et al.* 2011]. Similar methods have been used *in vivo* to inhibit miRNAs as direct injection of AAV2-expressed miRNA sponges into the eyes of mice resulted in retinal inhibition of miR-96, miR-182 and miR-183 [Krol *et al.* 2010]. Therapeutic use of AAV vectors have been used in human patients in clinical trials, and while generally well tolerated, a concern with this delivery system remains toxicity secondary to the immunogenicity of the AAV capsid [Mingozzi *et al.* 2007].

In IBD, viral vector therapies have been used in experimental models of colitis. Lindsay and colleagues demonstrated intravenous and rectal infusion of an adenoviral vector containing IL-10 in 2,4,6-trinitrobenzene sulfonic acid (TNBS) colitis [Lindsay *et al.* 2002] and IL-10 knockout (IL-10^{-/-}) mice [Lindsay *et al.* 2003] resulted in reduced colitis. Also using an IL-10 knockout mouse, Polyak and colleagues subsequently demonstrated amelioration of colitis following pseudotype 10 AAV-mediated restorative delivery of IL-10 *via* superior mesenteric artery (SMA) injection. Further work by Polyak and colleagues tested the tropism and transduction efficiency of AAV serotypes 1–10 *via* small bowel lavage, enema and SMA injection [Polyak *et al.* 2012]. Only SMA injection resulted in significant intestinal transduction in which case AAV serotypes 4, 7, 8, 9 and 10 demonstrated optimal small intestinal and colonic transduction. The extent of tissue distribution needs to be further clarified as transduction was also identified in other gastrointestinal locations such as the stomach and liver;

other studies have also demonstrated AAV 7, 8 and 9 transduction in nonintestinal tissues. Interestingly, AAV10 has been demonstrated in monkeys to maintain higher levels in the ileum and lymphoid tissues, spleen, and lymph nodes [Mori *et al.* 2008].

Nanoparticles. Although cholesterol conjugation allows for increased AMO cellular entry, this mechanism is dependent on the presence of the transmembrane protein SID1 [Wolfrum *et al.* 2007]. With possible implications for IBD therapy, leukocytes are known to have low expression SID1, making AMO targeting of leukocytes *via* cholesterol conjugation alone potentially less effective [Zhang, 2009]. There are several nanoparticle delivery systems (<1 μm diameter) currently being investigated for oligonucleotides delivery including lipid-based systems, polyethyl-amine (PEI) based systems, dendrimers and polylactide-coglycolide (PLGA) particles, as well as other mechanisms including natural polymers (chitosan, protamine, atelocollagen), exosome, and inorganic materials such as functionalized gold and silica. The differences between these methods have recently been reviewed in detail [Zhang *et al.* 2013]. Often utilizing biodegradable matrices, these nanoparticles offer advantages by being more cost-effective, less immunogenic and less toxic with higher loading densities and more favorable pharmacokinetics than virus based therapy. Despite being less efficient than viral vectors, nanoparticles offer the advantage of being able to alter surface ligands, allowing for targeting of cell surface specific receptors to augment cellular uptake in target tissues. Although not yet in use for miRNAs, this mechanism allowed Peer and colleagues to create a liposome-based, $\beta 7$ integrin-targeted, nanoparticle system to deliver cyclin D1 (CyD1) small interfering RNA (siRNA) for targeting specific leukocyte subsets involved in intestinal inflammation [Peer *et al.* 2008]. These nanoparticles were equipped with a targeting capacity by covalently attaching a monoclonal antibody against the integrin $\beta 7$ to hyaluronan that was attached to the outer surface of the liposome. Using a DSS colitis model, they found that delivery of CyD1 siRNA entrapped in this nanoparticle resulted in suppression of TNF α and IL-12 as well as drastic reduction in intestinal tissue damage. Similarly studying siRNA, Zhang and colleagues found naked siRNAs unable to significantly penetrate mucosal tissues [Zhang *et al.* 2006]. As a result, they designed anti-TNF α siRNA with liposomes as carrier vehicles and

delivered it topically *via* rectal enema. In a DSS colitis model, the authors demonstrated significantly decreased TNF α mRNA expression and reduced histologic inflammation with this delivery system.

Cell-to-cell delivery. In an approach that also holds potential for intestinal disorders, Xiang and colleagues were able to engineer nonpathogenic *Escherichia coli* to produce a short hairpin RNA (shRNA) targeting a mammalian gene *in vitro* and *in vivo* (transkingdom RNA interference). Upon oral or intravenous administration, *E. coli* encoding shRNA induced significant gene silencing in the intestinal epithelium and, in human colon cancer xenografts in mice, successfully demonstrating feasibility of transkingdom RNA interference [Xiang *et al.* 2006]. In addition to holding potential therapeutic implications, it raises the significant question if natural transkingdom interactions play a role in dysbiosis *via* miRNA interactions [Masotti, 2012]. An alternative cell based delivery model takes advantage of the fact primary B lymphocytes contain the RNase III enzymes (Drosha and Dicer) necessary for miRNA synthesis. Almanza and colleagues reported primary B lymphocytes could be successfully genetically programmed with nonviral plasmid DNA for the biogenesis and delivery of AMOs *in vivo* to CD8+ T lymphocytes [Almanza *et al.* 2013].

Off-target effect/side effects

miRNAs have emerged as regulators of post-transcriptional gene expression involved in multiple cellular processes and pathways including apoptosis and cellular differentiation, and as a result, are predicted to regulate the expression of 60% of all human protein coding genes [Friedman *et al.* 2009]. Each miRNA can target numerous mRNAs [Bushati and Cohen, 2007; Bartel, 2009] and individual mRNAs can be targeted by multiple miRNAs creating complex biological regulation [Krek *et al.* 2005; Hon and Zhang, 2007; Dombkowski *et al.* 2011]. Furthermore, predicted miRNA/mRNA targets are not necessarily restricted to a particular functional category or biological pathway. These factors raise the concern for off-target effects of therapies directed at miRNAs, potentially leading to unintended side effects [Bushati and Cohen, 2007; Bartel, 2009].

In the landmark investigation of Miravirsin, the miR-122 LNA AMO, investigators demonstrated

therapeutic efficacy with dose-dependent hepatitis C viral load reduction with no significant toxic effects, aside from mild injection site reactions and a transient slight increase in serum liver enzymes [Janssen *et al.* 2013]. However, this therapy was only short term, and the fact that miR-122 is a tumor suppressor for HCC and that miR-122 knockout mice have a high risk of fatty liver, fibrosis and HCC warrants close attention [Hsu *et al.* 2012; Tsai *et al.* 2012].

Conclusion

It has only been 6 years since the initial report of miRNA dysregulation in IBD. Since that time, investigations have described miRNA expression profiling in both tissue and peripheral blood in CD and UC, and are currently focusing on delineating the functional pathways in which miRNAs exert post-transcriptional gene expression regulation. This line of investigation has established miRNAs as regulators of canonical IBD pathways, most notably autophagy and intracellular bacterial processing. Despite these successes, there remain significant hurdles to realizing miRNAs in IBD diagnostics and therapy.

miRNAs have the benefit of being obtained with relatively minimally invasive procedures, are stable in circulation and tissues, and can be rapidly quantified by real-time PCR or microarrays. As a result, the potential utility of miRNAs as biomarkers holds significant promise and is closer to clinical realization than miRNA based therapeutics. However, answers to fundamental questions regarding tenets of biomarker assessment are still needed. The studies in IBD to date have been limited by small sample size without control of patient heterogeneity, including subphenotypes, disease extent or duration, or active medication use. From a practical consideration, there is no standard protocol for evaluating miRNAs with studies investigating peripheral blood miRNA expression profiles from whole blood, cell-free and isolated cell fractions. Intra- and inter-patient variability also needs to be addressed as the miRNA content in peripheral blood can be derived from multiple sources including distant tissues, such as cancers or sites of inflammation, and by circulating blood cells – with reported perturbations in blood cell counts and hemolysis altering plasma miRNA biomarker levels by up to 50 fold [Pritchard *et al.* 2012].

With regard to therapeutics, a significant limitation in the investigations to date has been the

inability to account for the cell type specificity of miRNA expression. The determination of physiologic relevance can only be validated in functional studies in cell types that are actually demonstrating variance in miRNA expression and, to date, there have been no mucosal tissue IBD studies to investigate the cell type specific expression of miRNAs. The importance of this point has recently been demonstrated in the case of miR-150, a highly specific miRNA for leukocytes [Kent *et al.* 2014]. In mucosal tissue of patients with UC, CD and murine models of experimental colitis, miR-150 has been reported to be upregulated. However, this upregulation may represent a colitis associated increase in mucosal leukocyte infiltration as opposed to holding a pathophysiologic relevance. Investigations into miRNA function in IBD will continue to be challenging given the high rate of inflammatory cell infiltration, epithelial/mesenchymal changes related to fibrosis, and turnover of epithelial cells.

A further limitation to the investigations to date has been the utilization of immortalized colon cancer cell lines for the majority of functional studies. These models may not be able to adequately recapitulate the environment of normal cells, and recent reports have demonstrated disparate miRNA expression profiles between normal and malignant cell lines despite being from the same organ [Kent *et al.* 2014].

These limitations highlight the infancy of investigation of miRNAs in IBD. Despite these hurdles, the need for better understanding of the pathophysiology of IBD for advancement in disease monitoring and therapy, as well as the emerging pathophysiologic mechanistic data overlapping with GWAS implicated risk genes, and the recent successful phase IIa studies of miRNA therapy in humans, all support the continued vigorous study of miRNAs as biomarkers and potential therapeutic targets in IBD.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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