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MIR106B and MIR93 Prevent Removal of Bacteria from Epithelial Cells by Disrupting ATG16L1-Mediated Autophagy

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Abstract

BACKGROUND & AIMS—Variants in genes that regulate autophagy have been associated with Crohn's disease (CD). Defects in autophagy-mediated removal of pathogenic microbes could contribute to pathogenesis of CD. We investigated the role of the micro-RNAs (miRs) MIR106B and MIR93 in induction of autophagy and bacterial clearance in human cell lines, and the correlation between MIR106B and autophagy-related gene 16L1 (ATG16L1) expression in tissues from patients with CD.

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Author Contributions: C. L. conceived, designed and performed the experiments, analyzed the results, and wrote the draft of the manuscript (Figures 1, 4, 5, and Supplementary Tables 1 and 2). J. C. designed and performed the experiments, analyzed the results, and wrote the manuscript (Figures 2, 3, 4, 6, 7, Supplementary Figures 1–6, 10, and 11). H.X. performed the experiments and analyzed the results (Figure 6, Supplementary Figures 8, 9 and 11). X. Z., Q. H., Y. L. L., G. J., Y. S., and B. X. analyzed the results and modified the manuscript. R. X. Z. and Y. W. performed the experiments and analyzed the results (Figure 7A and Supplementary Figure 7). K. D. W. participated in the analysis of intracellular AIEC with confocal microscope. R. C. and J. L. designed the experiments, analyzed the results, and edited the manuscript. Z. X. X. conceived and designed the experiments, analyzed the results, and wrote the manuscript. All the authors discussed the results and commented on the manuscript.

METHODS—We studied the ability of MIR106B and MIR93 to regulate ATG transcripts in human cancer cell lines (HCT116, SW480, HeLa, and U2OS) using luciferase report assays and bioinformatics analyses; MIR106B and MIR93 mimics and antagonists were transfected into cells to modify levels of miRs. Cells were infected with LF82, a CD-associated adherent-invasive strain of *Escherichia coli*, and monitored by confocal microscopy and for colony-forming units. Colon tissues from 41 healthy individuals (controls), 22 with active CD, 16 with inactive CD, and 7 with chronic inflammation were assessed for levels of MIR106B and ATG16L1 by *in situ* hybridization and immunohistochemistry.

RESULTS—Silencing Dicer 1, an essential processor of miRs, increased levels of ATG protein and formation of autophagosomes in cells, indicating that miRs regulate autophagy. Luciferase reporter assays indicated that MIR106B and MIR93 targeted *ATG16L1* mRNA. MIR106B and MIR93 reduced levels of ATG16L1 and autophagy; these increased following expression of ectopic ATG16L1. In contrast, MIR106B and MIR93 antagonists increased formation of autophagosomes. Levels of MIR106B were increased in intestinal epithelia from patients with active CD, whereas levels of ATG16L1 were reduced, compared with controls. Levels of CMYC were also increased in intestinal epithelia of patients with active CD, compared with controls. These alterations could impair removal of CD-associated bacteria by autophagy.

CONCLUSIONS—In human cell lines, MIR106B and MIR93 reduce levels of ATG16L1 and autophagy, and prevent autophagy-dependent eradication of intracellular bacteria. This process also appears to be altered in colon tissues from patients with active CD.

Keywords

inflammatory bowel disease; microRNA; cell biology; infection

Introduction

Autophagy is a cellular self-digestion process characterized by sequestration of bulk cytoplasm and organelles in autophagic vesicles and delivery of them to the lysosome, where the materials sequestered in the vesicles are degraded and recycled to general nutrient stores to maintain essential cellular functions under stress conditions, such as nutrient starvation.^{1,2} Specialized autophagy mediates the degradation, processing and removal of a variety of intracellular substrates, playing a role in a myriad of cell autonomous processes. Notably, a number of studies have implicated autophagy in the removal of invading pathogenic microbes (namely xenophagy) as an innate line of defense against viral and bacterial infections.^{3,4} Within murine intestinal epithelium, autophagy was shown to be important for the biology of Paneth cells, which produce secretory granules containing antimicrobial peptides.⁵ In addition, autophagy also functions to facilitate the immune system of the host organism to mount adaptive immune responses against pathogen invasion by modulating antigen presentation.⁶ Thus autophagy may play a multifaceted role in host defense against intracellular pathogens.

Autophagy related gene (ATG) 16L1 was originally identified as a component of a multiprotein complex containing ATG5-ATG12 showing sequence homolog with yeast Apg16L.⁷ During autophagy, the ATG16L1 complex acts as an E3-like enzyme to mediate ATG8 (also known as light chain 3, LC3) lipidation, which is essential for autophagosome formation.⁸ Genome-wide association studies (GWAS) have identified ATG16L1 polymorphism (T300A) being a risk variant for Crohn's disease (CD), an inflammatory bowel disorder (IBD) affecting 50–200 per 100,000 people in the western world.^{9–11} Further studies showed that ATG16L1 T300A is less capable of mediating antibacterial autophagy in a *Salmonella Typhimurium* infection model.¹²

While significant progress has been made in identifying genetic loci linked to CD and ulcerative colitis, recent meta-analysis of GWAS data in IBD has shown that 163 of the common genetic loci discovered so far account for only a minority of the genetic attributes of the disease.¹³ Single gene mutations appear to have limited effects on the development of CD.¹⁴ Thus, additional mechanisms or synthesized action of multiple genes may contribute to the dysfunction of autophagy and the development of CD.

miRNAs are a family of non-coding short RNAs, which suppress gene expression by selectively base-pairing to the complementary mRNAs.^{15,16} miRNAs mediate post-transcriptional silencing of target genes through specifically targeting homologous mRNAs for cleavage or inhibition of protein synthesis, regulating a wide spectrum of cellular processes.^{15,16} Brest et al reported that the miR-196 family of miRNAs down-regulate the CD protective variant (c.313C) but not the risk-associated allele (c.313T) of immunity-related GTPase family M protein 1 (IRGM1) gene in CD patients.¹⁷ The subsequent loss of IRGM1 expression compromises the control of intracellular replication of CD-associated adherent invasive *Escherichia coli* (AIEC) by autophagy.¹⁷ These results emphasize the role of miRNA-mediated gene silencing in the development of CD. In this report, we provide evidence showing that MIR106B and MIR93 suppress autophagy-mediated removal of bacteria in epithelial cells by targeting ATG16L1.

Materials and Methods

Cell Lines

Human cell lines HCT116, SW480 (colon cancer), HeLa (cervical cancer), and U2OS (osteosarcoma) were maintained in the lab. HCT116/GFP-LC3, SW480/GFP-LC3, and U2OS/GFP-LC3 were parental cell lines stably transfected with GFP-LC3 and established in the lab.¹⁸ HCT116 cells were grown in McCoy's 5A medium with 10% fetal bovine serum (FBS). Other cells were cultured in Dulbecco modified Eagle medium (DMEM) with L-glutamine and 10% FBS. HeLa/mCherry-LC3 cell line was established by stably transfecting pmCherry-LC3.

Bacterial Strain and Invasion Assay

CD-associated AIEC strain LF82 was used for invasion assay.¹⁹ Details are described in Supplementary Materials and Methods.

Transfection of siRNA, miRNA Precursors and Antagonists, DNA Constructs and Luciferase Reporter Assay

Detailed information is described in Supplementary Materials and Methods.

Fluorescence Microscopy

Fluorescence signals were analyzed with an Olympus IX51 fluorescence microscope or a Zeiss LSM 710 confocal microscope. Details are described in Supplementary Materials and Methods.

In situ Hybridization and Immunohistochemistry (IHC)

Protocol for the application of human tissues was approved by IRB in the University of Alabama at Birmingham (Protocol No. N120831003). Colon tissues from 41 healthy individuals, 22 active CD, 16 inactive CD, and 7 chronic inflammation of colon were provided by the University Hospital and purchased from US Biomax, Inc. (Rockville, MD, #BC05002). Protocols for *in situ* hybridization and IHC have been previously reported^{20,21} and detailed in Supplementary Materials and Methods.

Statistics

All data presented are representative of 3 or more experiments, each with similar results. Quantitative data are shown as mean $\pm$ SD. Statistical significance was determined using Student's *t* test if not specially indicated (* $P < .05$ and ** $P < .01$).

Results

Silencing of Dicer Enhances the Expression of ATGs and Autophagosome Formation

To determine whether abrogation of miRNA biogenesis is sufficient to induce ATG expression and autophagy, small interfering RNA (siRNA)-mediated knockdown of Dicer was performed.²² We transfected Dicer 1 siRNA into U2OS/GFP-LC3 cells, a cell line we previously established for monitoring autophagy.¹⁸ Upon serum starvation, GFP-LC3 in control cells redistributed from a diffuse cytoplasmic location (Figure 1*Ai*) to discrete vesicular structures (Figure 1*Aii–iii*), an indication of autophagy.¹⁸ The GFP-LC3 puncta were induced as early as 4 h after serum-starvation and the number of cells with punctate GFP-LC3 increased in a time-dependent manner (Figure 1*Ai–iii*, 1*B*). In contrast, silencing of Dicer 1 with siRNA induced autophagosome formation even in FBS-replete conditions (Figure 1*Aiv*). The autophagosome/autolysosome number further increased upon the serum starvation (Figure 1*Av, vi*, 1*B*). siRNA-mediated ATG9 depletion led to a decrease in GFP-LC3 puncta in cells with or without FBS (Figure 1*Avii–ix* and 1*B*), suggesting that the observed GFP-LC3 puncta represent autophagosomes rather than an artifact of transfection. The autophagosome formation was also confirmed biochemically by immunoblot analysis of LC3 (Figure 1*C*), as LC3-II was increased upon Dicer 1 RNAi (Figure 1*C*). Dicer 1 depletion is likely to have a widespread effect on cellular protein expression given its pleiotropism. Thus, to determine whether the autophagy machinery might be targeted by potential miRNAs, we assayed key autophagy-related gene products. Indeed, expression of ATG1, ATG9A, and ATG16L1 was up-regulated following Dicer 1 depletion (Figure 1*C*). Together, our data indicate that miRNAs may be implicated in the regulation of autophagy gene expression and the subsequent autophagosome/autolysosome formation.

MIR106B and MIR93 Inhibit Autophagy under Physiological and Stress Conditions

The involvement of miRNA biogenesis in regulating ATGs is consistent with our previous findings that miRNAs including MIR-17–92 and MIR-106B–25 clusters were down-regulated during human monocytes differentiation into DCs, during which autophagy was induced.²³ The majority of these miRNAs are predicted to target ATGs, in particular ATG16L1, according to public algorithms for miRNA prediction, e.g. Target Scan, PITA, and miRanda (Supplementary Table 2). To validate the role of miRNAs in autophagy regulation, we first assessed MIR106B and MIR93 levels in cells transfected with Dicer 1 siRNA. We focused on MIR106B and MIR93 since the bioinformatics prediction revealed that this family of miRNAs possesses the highest number of matched target sites in ATGs (Supplementary Table 2). Indeed, siRNA of Dicer 1 efficiently reduced MIR106B and MIR93 expression (Supplementary Figure 1). We then transfected MIR106B and MIR93 mimics to HCT116/GFP-LC3 and SW480/GFP-LC3 cells followed by treatment with rapamycin to induce autophagy.¹⁸ Cells transfected with control miRNA showed a diffused GFP-LC3 signal with basal levels of autophagosome/autolysosome (2–4 puncta/cell) (Figure 2*A* and *B* and Supplementary Figure 2). Rapamycin treatment led to a substantial increase in autophagy with an average of 11–20 puncta being present in each cell (Figure 2*A* and *B* and Supplementary Figure 2). In contrast, transfection of either MIR106B or MIR93 dramatically reduced autophagosome/autolysosome formation (2–8 puncta/cell) in either the presence or absence of rapamycin (Figure 2*A* and *B* and Supplementary Figure 2). Consistent with the fluorescence analysis of GFP-LC3, immunoblots showed that MIR106B and MIR93 transfection reduced LC3 lipidation (LC3-II isoform) (Figure 2*C* and

Supplementary Figure 2). Furthermore, upon MIR106B and MIR93 transduction, ATG16L1 protein level was markedly reduced although the mRNA level of ATG16L1 was not significantly changed (Figure 2C–F). In contrast, ATG5–ATG12 and ATG7 displayed only marginal alterations (Figure 2C). The action of the miRNA was validated by the down-regulation of p21 (Figure 2C and Supplementary Figure 2), a well-characterized target of MIR106B and MIR93.²⁴

To assess autophagic flux, we treated the cells with bafilomycin A1 (Baf A1), an agent that prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes.² Baf A1 treatment markedly enhanced the accumulation of autophagosomes in cells transduced with control miRNA (Supplementary Figures 3 and 4). In stark contrast, cells stably expressing MIR106B or transiently transduced with MIR106B and MIR93 mimics only slightly responded to Baf A1 and showed substantially reduced amounts of punctate LC3 (Supplementary Figures 3 and 4), suggesting that reduction in the steady-state levels of autophagy indicators caused by MIR106B and MIR93 was not due to an accelerated autophagy flux (substrate degradation), but most probably resulted from a suppressed autophagy activity. Thus, we have demonstrated that MIR106B and MIR93 are active regulators of autophagy, which likely act to target ATG16L1 to inhibit autophagy activity.

Depletion of Endogenous MIR106B and MIR93 Induces Autophagy

To further validate the role of MIR106B and MIR93 in regulating autophagy, we examined the effects of MIR106B and MIR93 inhibitors on autophagy in HCT116/GFP-LC3 and SW480/GFP-LC3 cells. Transduction of a scramble miRNA inhibitor exhibited little effect on the extent of autophagy with only isolated LC3 puncta (Figure 3A and B and Supplementary Figure 5), whereas transduction of MIR106B and MIR93 inhibitors led to a substantial decrease in endogenous MIR106B and MIR93 and an increase in punctate LC3 (Figure 3A, B, and D, and Supplementary Figure 5). Treatment of the cells with rapamycin further enhanced the presence of autophagy structures (Figure 3A and B and Supplementary Figure 5). As opposed to the miRNA mimics, MIR106B and MIR93 inhibitors increased the expression of p21 and LC3-II (Figure 3C and Supplementary Figure 5). Consistently, ATG16L1 expression was increased in inhibitor-treated cells while ATG16L1 mRNA levels were not altered (Figure 3C and E). Unexpectedly, autophagy induced by MIR106B and MIR93 inhibitors were not associated with loss of p62 (Figure 3C and Supplementary Figure 5), a selective autophagy substrate commonly observed to be degraded during active autophagy.^{1,2,25} Instead, p62 levels were modestly increased in MIR106B and MIR93 inhibitors treated cells (Figure 3C and Supplementary Figure 5), which is consistent with previous report showing that p62 is a direct target of MIR106 cluster.²⁵ Thus, p62 levels under the treatments of MIR106B and MIR93 mimics or inhibitors may reflect a balance between autophagy-mediated degradation and the direct action of the miRNAs (Figures 2C and 3C). Hence, we have demonstrated the role of endogenous MIR106B and MIR93 in autophagy induction.

MIR106B and MIR93 Target ATG16L1

Both MIR106B and MIR93 mimic and inhibitor assays suggest that ATG16L1 is a target of these miRNAs (Figures 2C and 3C and Supplementary Figures 2–5). To determine whether ATG16L1 is directly regulated by MIR106B and MIR93, a fragment of the 3'-untranslated region (3'-UTR) of ATG16L1, which contains two binding sites for MIR106B cluster (Figure 4A), was sub-cloned into the pmir-GLO dual luciferase reporter vector at the 3' end of the coding sequence of firefly luciferase to generate pmir-GLO-ATG16L1-3'-UTR-WT (wild-type) and mutant construct lacking the miRNA binding sites, pmir-GLO-ATG16L1-3'-UTR-MUT. Significantly lower luciferase activity was detected in cells

transfected with pmir-GLO-ATG16L1-3'-UTR-WT as compared with cells transfected with pmir-GLO-ATG16L1-3'-UTR-MUT (Figure 4B), suggesting that repression of the luciferase activity by the miRNAs depends on these specific recognition sequences in the 3'-UTR of the ATG16L1 transcript. To further validate the ATG16L1 targeting activity of the miRNAs, we transfected MIR106B and miR93 mimics together with either pmir-GLO-ATG16L1-3'-UTR-WT or pmir-GLO-ATG16L1-3'-UTR-MUT. Ectopic MIR106B and MIR93 mimics resulted in attenuation of the luciferase activity derived from pmir-GLO-ATG16L1-3'-UTR-WT but not the mutant 3'-UTR, whereas negative control nucleotides had no effect (Figure 4C). Together, our data demonstrated that ATG16L1 is a direct target of the MIR106B and MIR93.

Over-expression of ATG16L1 Rescues MIR106B and MIR93- Induced Autophagy Suppression

ATG16L1 forms complexes with ATG5-ATG12 conjugates to promote elongation and closure of autophagosome via an E3-ligase-like role in LC3 lipidation and localization to the autophagosome membrane.²⁶ Given our data suggesting that MIR106B and MIR93 reduced autophagosome formation by targeting 3'-UTR of ATG16L1 (Figures 2C, 3C, 4B and C), we expected that over-expression of ATG16L1 would rescue the defects in autophagy caused by MIR106B and MIR93. To assess this postulation, we made FLAG-ATG16L1 expression constructs with 3'-UTR-WT or 3'-UTR-MUT linked to the stop code of ATG16L1 and established ATG16L1 expression cell lines in HCT116/GFP-LC3 cells (Figure 4D). The cells were then transfected with MIR106B and MIR93 mimics. MIR106B and MIR93 mimics markedly down-regulated the expression of ATG16L1 construct with 3'-UTR-WT, but not construct with 3'-UTR-MUT (Figure 4D). Consistently, ATG16L1 construct with 3'-UTR-MUT, but not with 3'-UTR-WT, rescued MIR106B and MIR93-induced autophagy suppression and triggered punctate GFP-LC3 redistribution although fewer autophagosomes were present as compared to control miRNA transduced cells (Figure 4E). The incomplete rescue of autophagy formation suggests additional targets of MIR106B and MIR93 in autophagy regulation.²⁷ Together, these data support the conclusion that ATG16L1 is an important mediator for MIR106B and MIR93 induced autophagy inhibition.

MIR106B Impairs the Removal of CD-associated AIEC in Epithelial Cells

It was reported that dysregulation of ATG16L1 and the resultant autophagy deficiency play an important role in the development of CD.^{10,12,28} To determine whether MIR106B and MIR93-mediated ATG16L1 depletion would affect acute removal of CD-associated intracellular pathogens, we employed HeLa/mCherry-LC3 as host cells. CD-associated AIEC strain LF82, which was originally isolated from chronic ileal lesion of a CD patient and was able to invade and proliferate in epithelial cells,^{9,19} was used as an infecting pathogen. The bacteria were transformed with pFPV25.1/GFP-mut3 to express green fluorescent protein (GFP). In response to AIEC LF82 infection, autophagy was induced in HeLa/mCherry-LC3 cells (Figure 5A) and endogenous MIR106B levels were marginally reduced (Supplementary Figure 6). The cells were transfected with a MIR106B mimic followed by AIEC LF82/pFPV25.1/GFP-mut3 infection. Intracellular bacterial infection prompted autophagy formation in control negative oligonucleotides (N1) transfected cells (Figure 5B and Supplementary Figure 7) and the pathogens partially co-localized with autophagosome/autolysosome (Figure 5C), a process that may mediate bacterial clearance. In contrast, MIR106B mimic transfection reduced AIEC LF82-induced autophagy (Figure 5B and Supplementary Figure 7), with fewer bacteria being associated with autophagosome/autolysosome (Figure 5C). We determined intracellular bacteria numbers by counting colony-forming units (CFU) from bacterial cultures of the cell lysates. A significantly higher number of intracellular AIEC LF82 bacteria were identified in cells transfected with

MIR106B mimic (Figure 5D and E). Taken together, our data suggest that MIR106B can act to disrupt autophagy-mediated elimination of pathogenic bacteria in infected human cells.

MIR106B Is Overexpressed in Intestinal Mucosa with Lower ATG16L1 Expression in CD Individuals

To investigate the pathological correlation between MIR106B and ATG16L1 in CD, we analyzed MIR106B and ATG16L1 expression using *in situ* hybridization and IHC respectively in 86 colon tissue samples consisting of healthy, active CD, inactive CD, and chronic inflammation of colon. We first validated the *in situ* hybridization detection of MIR106B using U2OS cells stably expressing MIR106B (Supplementary Figure 8). Healthy colon tissues showed low levels of MIR106B with isolated small focuses of expression in intestinal epithelia (Figure 6A and C and Supplementary Figures 9 and 11). In contrast, around 80% of the colon tissues from individuals with active CD exhibited higher levels of MIR106B (Figure 6A and C and Supplementary Figures 9 and 11). Intestinal epithelia in the actively inflamed mucosae exhibited the highest levels of the MIR106B (Figure 6A and Supplementary Figure 11). Mucosae from individuals with inactive CD displayed a mild increase in MIR106B expression with a predominantly epithelial distribution (Supplementary Figures 9 and 11). Interestingly, comparing MIR106B levels in active, inactive (quiescent), and normal (uninvolved) mucosae within the same patients revealed that MIR106B was consistently elevated in inflamed focuses, but only weakly expressed in normal and quiescent sites (Supplementary Figures 9 and 11), suggesting that MIR106B expression is causally linked to the onset of CD and may serve as an indicator for active CD.

Next, we examined ATG16L1 expression in the colon tissue samples. In healthy mucosa, ATG16L1 was expressed in epithelia and lamina propria with the expression being stronger in the epithelial compartment (Figure 6B and Supplementary Figure 10). In contrast, ATG16L1 expression was strikingly weak in the intestinal epithelia from individuals with active CD (Figure 6B and C and Supplementary Figure 10). Consistent with the uneven levels of MIR106B expression (Figure 6A and Supplementary Figure 9), ATG16L1 was undetectable in some of the CD intestinal epithelial cells (Figure 6B and Supplementary Figures 10 and 11). ATG16L1 in inactive areas of diseased mucosae showed an appreciable weak expression as compared with normal tissues, but was stronger than that of active lesions (Supplementary Figure 10). As opposite to the expression pattern of MIR106B in serial tissue sections of same individuals with CD (Supplementary Figure 8), ATG16L1 staining exhibited a decrease trend in the order of normal, quiescent, and inflamed mucosae (Supplementary Figures 10 and 11). Together, our findings show that MIR106B expression in CD correlates with down-regulated ATG16L1 in human intestinal epithelia and active inflammation.

c-Myc, a MIR106B and MIR93 Transcriptional Regulator, Is Highly Expressed in Intestinal Mucosa of CD Individuals

c-Myc was reported to be highly expressed in CD, in particular active CD individuals.^{29–31} Recent reports also showed that c-Myc regulates the expression of miRNAs including MIR106B and MIR93 through a transcriptional mechanism.^{15,16,27} Thus, we interrogated the role of c-Myc in regulating MIR106B and MIR93 levels. We assessed c-Myc in colon tissues from healthy and CD individuals with IHC. We found that c-Myc levels were markedly higher in intestinal epithelia from individuals with active CD than those from healthy individuals (Figure 7A and B). There was an intense staining of c-Myc in the nuclei of epithelial cells from CD samples, whereas the signal in cells from healthy individuals was relatively weak and more cytoplasmic (Figure 7A). c-Myc expression is positively correlated with MIR106B levels (Figure 7C). Further, MIR106B and MIR93 levels were considerably increased in cells transfected with c-Myc as compared with those in cells transfected with

vector alone (Figure 7D and E). Accordingly, c-Myc overexpression reduced the levels of p21 and ATG16L1 in the cells (Figure 7D). Thus, our data support a model that increased c-Myc in CD individuals enhances MIR106B and MIR93 expression, which reduces autophagosome formation and intracellular bacterial removal by targeting ATG16L1 (Figure 7F).

Discussion

Autophagy has been shown to be essential for the clearance of intracellular microbes in mammalian cells.^{1–4,32} However, the underlying mechanism controlling autophagy in these cells remains unknown. We previously reported that the miR-106 cluster were among the miRNAs down-regulated during human monocyte differentiation into dendritic cells.²³ We also noted that ATG16L1 and ATG9 levels were substantially increased and that autophagy was markedly induced during the dendritic cell differentiation (Lu & Xu, unpublished data). In the current study, we further characterized that MIR106B and MIR93 decreased LC3 lipidation and reduced the number of autophagy puncta in human epithelial cells (Figure 2, Supplementary Figures 2 and 3). More importantly, expression of ATG16L1 with a mutated 3'-UTR rescued MIR106B and MIR93-mediated autophagy suppression (Figure 4D and E), supporting the notion that MIR106B and MIR93 inhibit autophagy by targeting ATG16L1. In a most recent report, Kwon's group demonstrated that MIR106A and MIR106B regulate ATGs and fine tune autophagy.³³ Together, current evidence presented in this study and by other groups^{27,33} clearly reveals that MIR106B cluster plays an important role in regulating autophagy.

Mounting evidence suggests the involvement of autophagy defects in the etiology of CD.^{3,4,32,34} ATG16L1 and two additional genes, NOD2 and IRGM1, whose aberrations are frequently associated with CD, contribute to the removal of the bacteria by regulating autophagy in intestinal epithelial cells and peripheral dendritic cells.^{17,28,35} Dysregulation or mutations of these genes disrupt autophagy and delay the removal of intracellular bacteria, leading to the chronic inflammatory pathology in affected individuals.^{5,17,28,35} MIR106A was previously found to be up-regulated in patients with CD.³⁶ Several groups further demonstrated that MIR106A family miRNAs play a role in the onset and/or relapse of inflammation from mucosal tissues in IBD patients.^{36,37} We found that MIR106B was up-regulated in colon mucosa of individuals with CD, which correlated with reduced ATG16L1 and active inflammation (Figure 6 and Supplementary Figures 9–11). Further, we established that MIR106B disrupted the removal of CD-associated LF82 strain bacteria in epithelial cells. Thus, MIR106B serves as a likely pathogenic factor of CD via targeting ATG16L1 and ATG16L1-mediated autophagy. In addition to ATG16L1, MIR106B and MIR93 appear to also target p62 directly independent of autophagy (Figures 2C and 3C), which is intriguing because p62 has been proposed to be a selective autophagy substrate and a mediator of cargo recognition in autophagy.^{1,2,25} The contribution of microRNA-mediated down-regulation of p62 to CD is currently unclear; future studies are needed to determine whether reduced p62 levels may further interfere with autophagy-mediated pathogen clearance.

Regulation of ATG16L1 and the risk variant ATG16L1 T300A in particular in CD has not been fully understood. The Xavier and Netea groups demonstrated that ATG16L1 expression responded to intracellular bacterial infection or exposure to bacterial components.^{10,38} T300A risk variant reduced ATG16L1 stability and inflammatory responses, such as the production of IL-1 β ,³⁸ whereas Hampe et al. failed to find marked alteration of ATG16L1 at mRNA and protein levels in a pilot analysis of samples from normal and CD individuals.³⁹ The differences among these findings may derive from patient samples or unclear status of the patients (active or inactive).^{38,39} Interestingly, our data

showed that instead of the entire epithelial tissue, MIR106B was expressed at a strikingly high level in a small population of CD epithelial cells, which may account for complete loss of ATG16L1 staining in some of the cells. The significance of such mosaic pattern of MIR106B and ATG16L1 expression is currently unclear. However, it is conceivable that persistent presence of pathogenic bacteria in even a fraction of cells could potentially contribute to the chronic and episodic nature of the disease. Further studies are needed to validate the expression of the MIR106B cluster in larger numbers of CD intestinal tissue samples, especially in the context of disease remission and relapse.

MIR106B and MIR93 cluster is located within intron 13 of the *minichromosome maintenance protein 7 (MCM7)* gene, which is transcriptionally regulated by c-Myc.^{27,40} Thus, MIR106B and MIR93 cluster is frequently amplified with the host gene MCM7 under the promotion of c-Myc.^{40,41} Consistently, we found that ectopic expression of c-Myc markedly increased MIR106B and MIR93 expression and decreased the levels of ATG16L1 and p21 (Figure 7D and E). Interestingly, c-Myc was found to be highly expressed in active CD in this study (Figure 7A and B) and in previous reports.^{29–31} Thus, it seems rational to propose that increased c-Myc in CD enhances MIR106B and MIR93 expression, which reduces autophagosome formation by targeting ATG16L1, thereby suppressing intracellular bacterial removal (Figure 7F).

CD is poorly understood and refractory to clinical managements; therefore, there is an immediate need to identify both genetic and non-genetic risk factors of the disease.^{11,32} Based on our data, we speculate that individuals with MIR106B and MIR93 mediated loss of ATG16L1 expression will manifest an altered antibacterial activity of intestinal epithelial cells and an abnormal presence of CD-associated intracellular bacteria with substantial impacts on the outcome of intestinal inflammation. Our findings may eventually contribute to the improvement of CD therapeutics through the use of MIR106B and MIR93 inhibitors and/or autophagy activators.

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Abbreviations used in this paper

3'-UTR	3'-untranslated region
AIEC	adherent-invasive <i>Escherichia coli</i>
ATG16L1	autophagy related gene 16L1
Baf A1	bafilomycin A1
CARD15	caspase recruitment domain gene
CD	Crohn's disease
CFU	colony forming units
GFP	green fluorescent protein

GWAS	genome-wide association studies
IBD	inflammatory bowel disorder
IEC	intestinal epithelial cell
IHC	immunohistochemistry
IRGM1	immunity-related GTPase family M protein 1; 9
MCM7	minichromosome maintenance protein 7
MDP	muramyl dipeptide
miRNA (miR)	microRNA
MOI	multiplicity of infection
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
qPCR	quantitative reverse-transcription polymerase chain reaction
siRNA	small interfering RNA

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Figure 1.

Autophagy flux is elevated after silence of Dicer 1. (A) Elevated autophagy flux by silencing Dicer 1. U2OS/GFP-LC3 cells were transfected with siRNAs against Dicer 1, ATG9A, or negative control for 2 days. Cells were then cultured in serum free medium for 4 h or 6 h. White arrows show the punctate GFP-LC3 fluorescence (autolysosomes/autophagosomes). (B) Average numbers of GFP-LC3 puncta per cell in (A). (C) Expression of ATGs after silence of Dicer 1 by Western blot detection. GAPDH served as a loading control.

Figure 2.

MIR106B and MIR93 down-regulate ATG16L1 and inhibit autophagy. HCT116 cells were transfected with MIR106B and MIR93 mimics or negative control (N1) for 48 h. The cells were then treated with 10 nM rapamycin for additional 6 h. (A) GFP-LC3 fluorescence in the cells. White arrows indicate the punctate GFP-LC3. Scale bar: 20 μ m. (B) Average numbers of GFP-LC3 puncta per cell in (A). (C) Detection of ATGs in MIR106B and MIR93 mimic-treated cells by Western blot. GAPDH served as a loading control. (D) Quantitation of MIR106B and MIR93 in cells transfected with MIR106B and MIR93

mimics by qPCR. (E) ATG16L1 mRNA levels in cells transfected with MIR106B and MIR93 mimics.

Figure 3.

Depletion of endogenous MIR106B and MIR93 induces autophagy. HCT116/GFP-LC3 cells were transfected with MIR106B and MIR93 inhibitors or scramble oligonucleotides (Ctrl) for 48 h. Cells were then treated with 10 nM rapamycin for additional 6 h. (A) GFP-LC3 fluorescence in the cells. White arrows indicate the punctate GFP-LC3 fluorescence. Scale bar: 20 μ m. (B) Average numbers of GFP-LC3 puncta per cell in (A). (C) Western blot detection of ATGs in MIR106B and MIR93 inhibitor-treated HCT116/GFP-LC3 cells. GAPDH served as a loading control. (D) Determination of endogenous MIR106B and

MIR93 in cells transfected with MIR106B and MIR93 inhibitors by qPCR. (E) ATG16L1 mRNA levels in cells transfected with MIR106B and MIR93 inhibitors.

Figure 4. MIR106B and MIR93 target ATG16L1. (A) Sequences of mature MIR106 family members and MIR106B sequence aligned with human, chimpanzee, mouse, rat ATG16L1 3'-UTRs. Mutation of MIR106B binding site sequence is shown below. (B, C) Luciferase reporter assays in HCT116 cells. pmir-GLO-ATG16L1-3'-UTR-WT and its mutant pmir-GLO-ATG16L1-3'-UTR-MUT were used to determine targeting of ATG16L1 3'-UTR by endogenous miRNAs (B) or by transfected MIR106B and MIR93 mimics (C). (D, E) HCT116/GFP-LC3 cells stably transfected with FLAG-ATG16L1 with WT or mutant 3'-UTR were transfected with MIR106B and MIR93 mimics or N1 for 48 h. (D) Western blot analysis of FLAG-ATG16L1. (E) After transfection with MIR106B and MIR93 for 48 h,

cells were treated with 10 nM rapamycin for additional 6 h. The numbers of GFP-LC3 puncta per cell were calculated.

Figure 5.

MIRNA-106B impairs the removal of intracellular bacteria in epithelial cells. (A) AIEC strain LF82 was used to infect HeLa/mCherry-LC3 cells (MOI = 10) for the indicated time. The mCherry-LC3 fluorescence was captured with Zeiss LSM 710 Confocal Microscope following the treatment. (B, C) Confocal microscopy examination of autophagy flux (B) and LC3 (red)-associated LF82 bacteria (green) (C) in MIR106B overexpressed cells. (D, E) MIR106B reduces the removal of intracellular LF82 bacteria in HeLa and U2OS cells. Cells in 6-well plate were transduced with MIR106B mimic or control (N1) for 48 h and then infected with LF82 (MOI=10) for 2 h. After the infection, cells were either washed and

lysed or cultured in regular medium for additional 2 or 8 h before lysis for bacterial colonel formation assay. Total colony numbers per well from two groups were presented.

Figure 6.

In situ hybridization of MIR106B and IHC of ATG16L1 in intestinal mucosa. (A) Representative *in situ* hybridization of the colon tissues from healthy controls (n = 41) or active CDs (n = 22) labeled for MIR106B. Scale bar: 20 μ m. (B) Representative IHC staining of ATG16L1 in serial human colon tissues as detected in (A). Scale bar: 20 μ m. (C) Quantitation of MIR106B and ATG16L1 expression in human colon disease spectrum tissues. Masked reading was performed by 3 different investigators with same criteria to evaluate the staining. Low: overall negative or weak staining; High: overall moderate or

strong staining. The Pearson's chisquare test was used to analyze the distribution difference of MIR106B or ATG16L1 between healthy and active CD tissues ($P < .05$).

Figure 7.

High expression of c-Myc in CD correlates to elevated MIR106B. (A) Assessment of c-Myc in colon tissues from healthy (n=41) and CD (n=22) individuals by IHC. Scale bars are 50 μ m for upper panel and 20 μ m for lower panel. (B) H-scores of c-Myc in (A) (see Supplementary Materials and Methods for an explanation of H scores). (C) Correlation between c-Myc and MIR106B in colon tissues from healthy (n=41) and active CD (n=22) individuals. The Pearson's chi-square test was used to analyze the significance of the correlation ($P < .05$). (D, E) Overexpression of c-Myc increases MIR106B and MIR93 and reduces ATG16L1. HCT116 cells were transfected with pCMV-c-Myc or empty vector for

48 h. Whole cell lysates (protein) or total RNA were isolated for immunoblot (*D*) or qPCR analysis (*E*). (*F*) A schematic model for elevated MIR106B and MIR93 in CD.