Published in final edited form as:

Inflamm Bowel Dis. 2013 October ; 19(11): 2295–2301. doi:10.1097/MIB.0b013e31829e71cf.

miR-106b fine tunes ATG16L1 expression and autophagic activity in intestinal epithelial HCT116 cells

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Abstract

Background—miRNAs regulate gene expression at the post-transcriptional level. ATG16L1, an essential component for autophagy and a risk gene for Crohn's disease, contains two binding sites in the 3'UTR for miR-17 family, including miRs-20a, -93, -106a, and -106b. The purpose of this study was to assess the effects of these miRNAs on ATG16L1 expression and autophagic activity in HCT116 cells.

Methods—The functional binding sites in the ATG16L1 3'UTR were evaluated by transfection of pMIR-GLO vectors bearing the WT or mutant 3'UTR into cells for luciferase reporter assay. The miRNA regulation of ATG16L1 expression was determined by qRT-PCR and Western blot. The miRNA regulation of autophagic activity was evaluated by examining LC3II formation using Western blot and confocal imaging.

Results—Both miR-106a and miR-106b mimics inhibited starvation-induced autophagy. miR-106b mimic reduced ATG16L1 protein expression. Luciferase reporter assays showed that mutating the binding sequence at the position 1036-1042 abrogated miR-106b regulation of ATG16L1 3'UTR luciferase activity. In addition, miR-106a and miR-106b overexpression inhibited the expression of several other autophagy genes, including ATG12.

Conclusions—miR-106b targets ATG16L1 and modulates autophagy, partially through the binding site at the 3' end of ATG16L1 3'UTR. miR-106a regulates autophagy, possibly irrelevant to ATG16L1 regulation. Both miR-106a and miR-106b regulate multiple autophagy genes so that may play an integral role in fine-tuning autophagy.

Keywords

ATG16L1; autophagy; Crohn's disease; microRNA; miR-106b

MicroRNAs (miRNAs) are small RNA molecules that regulate over 30% of human protein-coding genes by binding to the 3'UTR of mRNAs that results in mRNA cleavage and/or translational arrest.^{1, 2} A single miRNA can regulate hundreds of genes simultaneously and an mRNA may be subject to regulation by multiple miRNAs coordinately.¹ Based on the

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miRBase database, over 1000 mature human miRNAs have been identified so far.³ Therefore, miRNAs constitute a complex regulatory network in managing almost each physiological and pathological process. The dysregulated miRNAs have implicated in human diseases, including inflammatory bowel disease (IBD).⁴ We have previously reported that several miRNAs, including miR-106a, are differentially expressed in tissues of IBD patients.⁵⁻⁷ We are interested in assessing whether these dysregulated miRNAs are involved in the IBD pathogenesis.

miR-106a belongs to the miR-17 family, which includes miRs-17, -20a, -20b, -93, and -106b. These miRNAs share the same seed region and therefore may target common mRNAs.⁸ An *in silico* analysis reveals that the members of miR-17 family target over 12 autophagy genes (Table, Supplemental Digital Content 1, <http://links.lww.com/IBD/A217>) such as ULK1, BECN1, ATG12, ATG5, and ATG16L1. These genes act sequentially in the autophagy process, with most of them involved in the autophagosome formation, a central event in autophagy;⁹ in particular, ATG12, ATG5, and ATG16L1 constitute an important protein conjugation system required for the autophagosome formation.¹⁰ In addition, miR-17 family members are predicted to target some signaling molecules that orchestrate autophagy activation, including PTEN, STAT3, and MAPK1. Therefore, miR-17 family miRNAs may play an integral role in regulating autophagic function by targeting diverse autophagy genes and autophagy regulators at once.

Among the IBD-associated miRNAs, miR-106a especially attracted our attention because it is predicted to target ATG16L1, a genetic susceptibility gene for Crohn's disease (CD). Human data support a strong association of ATG16L1 T300A polymorphism with CD.¹¹⁻¹³ Animal studies have demonstrated that ATG16L1 defect or low protein expression increases inflammatory response and produces CD-like pathology.¹⁴⁻¹⁶ Cumulated evidence suggests that autophagy in the intestinal epithelium behaves as a defensive strategy for clearance of microbes,¹⁷⁻¹⁹ but autophagy impairment results in intestinal epithelial dysfunction and IBD pathogenesis. In this study, we assessed the regulatory effects of miR-106a and other members of miR-17 family on ATG16L1 expression and autophagic activity in human intestinal epithelial HCT116 cells.

MATERIALS AND METHODS

Cell Culture

HCT116 cells were previously obtained from American Type Culture Collection and grown in McCoy 5A medium (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO₂ incubator. We chose this intestinal epithelial cell line because it expresses miR-17 family miRNAs, is less sensitive to transfection procedure-produced cell damage, and undergoes spontaneous mutations very slowly over time.

3'UTR Construct

ATG16L1 3'UTR is 1324 nucleotides (nt) in length, containing two putative binding sites for miR-17 family miRNAs with the seed regions at 449-455 and 1036-1043. The pMIR-GLO

reporter vectors (Promega, Madison, WI) containing the full-length ATG16L1 3'UTR or a truncated 3'UTR fragment were created previously (Zhai Z, Wu F, Chuang AY, et al. Submitted). The truncated 3'UTR was 697 nt in length, starting at the 5' end of the full-length 3'UTR. Mutant full-length and truncated 3'UTRs were generated by GenScript (Piscataway, NJ). For the truncated 3'UTR, the sequence at 449-454 (-CACTTT-) was replaced by -GTGAAA-; for the full-length 3'UTR, the sequences at 449-454 (-CACTTT) and 1036-1042 (-GCACTTT-) were replaced by -GTGAAA- and -CGTGAAA-, respectively.

Transfection

All miRNA mimics and mimic negative control (miR-NC) were from Sigma (St. Louis, MO). Transfections with miRNA mimics, pMIR-GLO ATG16L1 3'UTR vectors or corresponding negative control were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in OPTI-MEM1 reduced serum medium (Gibco, Grand Island, NY) for 5 h, then cell culture medium was added. At indicated time points, cells were further incubated in Earle's balanced salt solution (EBSS, Sigma) for autophagy induction or directly harvested for luciferase activity and gene expression assays.

Luciferase Reporter Assay

The firefly and renilla luciferase activities were analyzed using the commercial Dual-Luciferase reporter assay system (Promega) on a microplate luminometer, and the firefly luciferase activity was normalized to the renilla luciferase activity.

Real-time RT-PCR for mRNA

Total RNA was isolated using TRIzol reagent (Invitrogen) and 500 ng RNA was used for cDNA synthesis using the SuperScript VILO cDNA synthesis kit (Invitrogen). The relative amount of a test mRNA to the housekeeping gene GAPDH was determined using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). The thermal profile for qPCR amplifications on the LightCycler 480 real-time PCR system (Roche Diagnostics, Indianapolis, IN) was 95°C for 2 min followed by 45 cycles of 95°C for 10s, 56°C for 20s, and 60°C for 30s. Primers used are listed in the Table, Supplemental Digital Content 2, <http://links.lww.com/IBD/A218>.

Real-time RT-PCR for Mature miRNAs

cDNA was synthesized from total RNA using the NCode VILO miRNA cDNA synthesis kit (Invitrogen) followed by qPCR using the NCode EXPRESS SYBR GreenER miRNA qRT-PCR kit (Invitrogen). The thermal profile was 95°C for 5 min followed by 40 cycles of 95°C for 15s, 56°C for 1 min, and 60°C for 20s. The expression of miRNAs was calculated relative to the small nuclear RNA, RNU6B. The forward primers for miRs-20a, -20b*, -31*, -93, -106a, -106b, -130b, let-7i, and RNU6B were 5'-TAAAGTGCTTATAGTGCAGGTAG-3', 5'-ACTGTAGTATGGGCACTTCCAG-3', 5'-TGCTATGCCAACATATTGCCAT-3', 5'-AAAGTGCTGTTCGTGCAGGTAG-3', 5'-AAAAGTGCTTACAGTGCAGGTAGC-3', 5'-TAAAGTGCTGACAGTGCAGAT-3', 5'-CAGTGCAATGATGAAAGGGCAT-3', 5'-TGAGGTAGTAGTTTGTGCTGT-3', and 5'-

CGCAAGGATGACACGCAAATTCG-3', respectively. A universal reverse primer provided in the qRT-PCR kit was used.

Western Blot

The immunoblot was incubated with mixed primary antibodies (1:1,000 dilution each) and then Alexa Fluor 680 (Invitrogen) and IRDye 800CW (LI-COR Biosciences, Lincoln, NE) conjugated secondary antibodies (1:10,000 dilution each). The immunocomplexes were visualized on an Odyssey infrared imaging system (LI-COR Biosciences) and band intensities quantified using the ImageJ64. The primary antibodies included rabbit anti-ATG16L1, LC3B, ULK1, ATG12, ATG5, and SQSTM1/p62, and mouse anti-BECN1 (Cell Signaling, Danvers, MA) as well as goat anti- β -actin and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA).

Confocal Imaging

Confocal imaging was carried out as previously described (Zhai Z, Wu F, Chuang AY, et al. Submitted) to evaluate autophagy activation using increasing LC3 puncta or LC3II formation as an indicator.

Statistical Analysis

GraphPad Prism 4.0 was used for the statistical analysis. Differences between the treatment groups were analyzed by one-way ANOVA with Bonferroni's or Dunnett's post-tests. A value $P < 0.05$ was considered statistically significant.

RESULTS

Mature miR-106 Expression in Nutrient Deprived Cells

To test miRNA regulation of ATG16L1, we first determined the expression levels of four miR-17 family members and four other miRNAs in HCT116 cells with or without starvation for 4 h. miRs-20b*, -31*, -130b, and let-7i are predicted by *in silico* bioinformatic tools including MicroCosm to target the ATG16L1 3'UTR at the positions 482-488, 93-98, 1034-1040, and 281-287, respectively. Significant increases in the expression of miRs-20b*, -93, -106b, 130b, and let-7i were observed under starvation conditions (Fig. 1A). Of note, we previously observed a modest but significant increase in the ATG16L1 gene expression shortly after starvation treatment, peaking at about 1 h followed by a gradual decrease to the baseline level at about 6 h (Zhai Z, Wu F, Chuang AY, et al. Submitted). Interestingly, we observed a similar, continual increase in miR-106b expression during the 6-h starvation period (Fig. 1B), suggesting that the increasing miR-106b expression may help control the magnitude of ATG16L1 expression.

miR-106 Regulates ATG16L1 Expression and Autophagy

To further assess the potential role of miRNAs in regulating ATG16L1, we initiated a series of miRNA mimic transfection studies. First, we assessed the effect of miRNA mimics on the ATG16L1 expression in HCT116 cells. Up to concentrations of 50 nM, none of the miRNA mimics tested were able to regulate ATG16L1 mRNA expression (Fig. 2A). However, we

observed that transfections of miRs-106b, -130b, and let-7i mimics resulted in reduced ATG16L1 protein expression (Fig. 2B). Our results indicated that these three miRNAs regulate ATG16L1 expression at the level of protein rather than mRNA expression. To determine whether the miRNA influence on ATG16L1 protein expression results in a specific functional alteration in the HCT116 cells, we further assessed the transfection effect of these miRNA mimics on the cellular autophagic activity. Our results support previous findings that starvation induces the conversion of LC3I to LC3II (Fig. 2C).²⁰ Transfections of several mimics inhibited the conversion between the two LC3B forms, including miRs-20a, -106a, -106b, and -130b, and the transfections of miR-106a and miR-106b mimics resulted in the greatest inhibition of LC3II conversion. The results suggest that miR-106b has the capacity to down-regulate the autophagic activity, possibly in part via the regulation of ATG16L1 protein expression, but the miR-106a regulation of autophagic activity may be via other mechanisms.

miR-106 Clusters Regulate ATG16L1 Expression and Autophagy

The miR-106a and miR-106b genes are located on chromosomes and are grouped with other miRNAs into clusters. The miR-106a~363 cluster is located on the X chromosome and the miR-106b~25 cluster is located on chromosome 7.⁸ In this study, we tested the co-transfection effects of these two cluster miRNA mimics. HCT116 cells were transfected with miR-106a~363 cluster mimics (miRs-18b, -19b, -20b, and -106a, 50 nM each) or miR-106b~25 cluster mimics (miRs-106b, -93, and -25, 50 nM each). Figure 3A and B show miR-106b~25 cluster mimics reduced ATG16L1 mRNA and protein expression. In contrast, transfections of the miR-106a~363 cluster mimics had no effect on ATG16L1 mRNA and protein expression. Consistent with our results from transfecting the individual miR-106a and miR-106b mimics, the transfection of the miR-106a~363 and miR-106b~25 cluster mimics reduced the baseline and starvation-induced autophagy as indicated by Western blot analysis for LC3II conversion (Fig. 4A). In support of the Western blot analysis findings, immunofluorescence microscopy for LC3 demonstrated increased LC3 puncta by starvation, which were inhibited by transfections of the miR-106a~363 and miR-106b~25 cluster mimics (Fig. 4B and 4C).

miR-106b Targets ATG16L1 3'UTR at the Seed Region 1036-1043

In accordance with our earlier observations (Zhai Z, Wu F, Chuang AY, et al. Submitted), we demonstrated that by transfecting pMIR luciferase reporter vectors bearing the ATG16L1 full-length or truncated 3'UTRs into HCT116 cells, the full-length 3'UTR and the truncated 3'UTR reduced luciferase activities by approximately 72.6% and 57%, respectively (Fig. 5A). To assess the underlying mechanism of miR-106b in the modulation of ATG16L1 expression and autophagy, we co-transfected individual miRNA mimics with the ATG16L1 full-length 3'UTR vector. We found that among the tested mimics, miR-106b and miR-93 mimics significantly reduced the luciferase activity by an additional 18% and 13.5%, respectively (Fig. 5B). Since miR-106b and miR-93 are members of miR-106b~25 cluster, we evaluated whether the miR-106b~25 cluster miRNAs can interact with the predicted binding sequences in the ATG16L1 3'UTR. We obtained a mutated full-length 3'UTR vector containing mutations at two putative seed regions 449-454 and 1036-1042 for miR-17 family miRNAs. We co-transfected the cells with the WT or mutated full-length 3'UTR vectors and

miR-106b~25 cluster mimics or miR-NC. We found that miR-106~25 cluster mimics inhibited the luciferase activity in the WT full length 3'UTR vector by 27.2%, however, the inhibitory effect was abrogated in the mutated full-length 3'UTR vector (Fig. 5C). Moreover, we got a truncated 3'UTR with mutations at position 449-454 and co-transfected the cells with the WT or mutated truncated 3'UTR vectors and miR-106b cluster mimics, we found that the cluster mimics had no influences on the luciferase activity in either WT or mutated truncated 3'UTR vectors (Fig. 5D). These data suggest that miR-106b~25 cluster miRNAs interact with the seed region at 1036-1043, but not the one at 449-455. To understand the role of individual miRNAs in the miR-106b~25 cluster, we co-transfected the cells with the WT or mutated full-length 3'UTR vectors and each individual miR-106b~25 cluster mimics. Our results were consistent with the results in Figure 5B; in particular, transfection of either the miR-106b or the miR-93 mimic inhibited the luciferase activity in the WT full-length 3'UTR vector by an additional 17.8% and 14%, respectively. In contrast, miR-25 has no regulatory effect (Fig. 5E). Since the combinatory effect of miR-106b and miR-93 mimics in Figure 5C was better than that of individual miRNA mimics, our results suggest that these two miRNAs have additive regulatory effects.

miR-106 Influences the Expression of Other Autophagy-related Genes

Our results indicate that the miR-106a~363 and the miR-106b~25 clusters may influence autophagy via different autophagy gene targets. This is further indicated by the differential effects of miR-106a and miR-106b on ATG16L1 protein expression. The inhibition of autophagy by miR-106a and miR-106b may result from the regulation of multiple autophagy genes and/or autophagy regulators. Table, Supplemental Digital Content 1, <http://links.lww.com/IBD/A217>, lists several predicted target genes for miR-106. Figure 6A-D show that transfection of miR-106a and miR-106b mimics significantly inhibited ATG12 gene expression, marginally reduced BECN1 gene expression ($P=0.07$ each), but had no effect on the ULK1 and ATG5 gene expression. Western blot analysis demonstrated that both miR-106a and miR-106b mimics reduced the amounts of free ATG12 protein, but not the ATG5-bound ATG12 (Fig. 6E). We also observed that miR-106a mimic reduced BECN1 protein expression and miR-106b reduced ULK1 protein expression. However, both miR-106a and miR-106b had no effect on ATG5 protein expression and even showed an increasing effect on SQSTM1/p62 protein expression (Fig. 6E). The increasing p62 may be related to the inhibition of autophagy by miR-106. Figure, Supplemental Digital Content 3, <http://links.lww.com/IBD/A219> shows the effects of miR-106a and miR-106b overexpression on the mRNA expression levels of other putative target genes in HCT116 cells, including RB1CC1, ATG2A, ATG2B, ATG4B, ATG10, and ATG14 as well as MAPK1. We found both miR-106a and miR-106b mimics reduced the mRNA expression of RB1CC1, ATG2B, and MAPK1, but increased ATG4B gene expression. In addition, miR-106b inhibited ATG14 mRNA expression. Our results suggest that miR-106 has a widespread modulating effect throughout the autophagy pathway.

DISCUSSION

Autophagy is a cellular degradation mechanism that participates in cell survival, apoptosis, immunity and inflammation.¹⁰ It is thought to balance the beneficial and harmful effects of

immunity and inflammation, and play a critical role in intestinal inflammation, IBD, and colorectal cancer.²¹ miRNA dysregulation may represent a new mechanism in the IBD pathogenesis.⁴ miRNAs usually do not switch on/off the expression of target genes, but fine-tune the magnitude of gene expression. However, dysregulated miRNAs will alter the target gene expression profile and as a result produce the related disease phenotypes. Since BECN1 was reported in 2009 as a miR-30a target in several cancer cell lines,²² over 12 autophagy genes have been identified as miRNA targets in various cancer cell lines,²³ including ATG16L1 as a target of miR-142-3p in intestinal epithelial cells (Zhai Z, Wu F, Chuang AY, et al. Submitted). All these findings suggest that miRNA-mediated changes in the autophagy gene expression may result in autophagy dysfunction and intestinal pathology.

The broadly conserved miR-17 family have been found to be significantly elevated in a variety of cancers and to promote tumor proliferation.^{8, 24} miR-17 family members are also involved in inflammation. It has been found that miR-106a downregulates the anti-inflammatory cytokine IL-10 production.^{25, 26} Interestingly, deletion of miR-106b~25 cluster alone or in combination with miR-106a~363 cluster does not produce obvious phenotypes.²⁷ In a chemically-induced acute colitis model, several members of miR-17 family were found to be significantly up-regulated, including mmu-miRs-106a, -106b, -20a, -20b, and -93.²⁸ In mouse hematopoietic cells, miRNAs in the miR-17 family have been identified to target SQSTM1/p62.²⁹ In mouse myoblast cell line C2C12 cells, miR-20a and miR-106b were found as regulators of ULK1.³⁰ Moreover, In HCT116 cells, miR-106b has been identified to target p21, a cell cycle regulator, through the protein translational inhibition mechanism.^{31, 32} p21 is thought to play an important in IBD cancers.³³ We found that in HCT116 cells miR-106a and miR-106b had no influences on p62 protein expression, but miR-106b may somewhat modulate ULK1 protein expression.

In this study, we demonstrated that miR-106b up-regulation inhibits ATG16L1 expression and autophagy in HCT116 cells. Luciferase reporter assays indicate that this miRNA directly targets ATG16L1 3'UTR via the predicted binding site at position 1036-1043. Further observations suggest that miR-93 can also bind to the 3'UTR sequence. Even though the miR-93 regulation of ATG16L1 3'UTR may be not enough to affect ATG16L1 expression, it has a combinatory effect with miR-106b.

ATG16L1 3'UTR contains conserved binding sites for hundreds of miRNAs, some of them being IBD-associated. Generally, the binding sites at either end of a miRNA 3'UTR are more likely to be functional than those in the middle.³⁴ Our data suggest that the 5' end of ATG16L1 3'UTR is more functional. Luciferase reporter assay demonstrated that the first half of 3'UTR at the 5' end (697 nt of total 1324 nt) contributes around 80% of the full length of 3'UTR regulatory function and the second half of 3'UTR at 3' end (627 nt of total 1324 nt) contributes the left 20%. Therefore, though miR-106b is able to regulate ATG16L1 expression by binding to the binding site at the 3' end, it may just play a minor or limited role in controlling ATG16L1 expression and function. Nevertheless, the regulation of ATG16L1 by miR-106b will undoubtedly affect the autophagy pathway.

Noted that miR-17 family miRNAs regulate autophagy, possibly via multiple mechanisms. Of them, miR-106a seems to regulate autophagy function that is not a result of ATG16L1

regulation. In addition to targeting the putative genes that are involved in various steps in the autophagy process from initiation to promotion and completion, miR-17 family miRNAs may directly affect certain signaling molecules that could positively or negatively regulate the autophagy pathway, further indicating that miRNA regulation of autophagy is complex. In the present study, we mainly focused on the influence of miR-17 family miRNAs on ATG16L1 expression. However, our results indicate that miR-106a and miR-106b may regulate other autophagy genes as well, such as BECN1 and ATG12, suggesting that miR-106b and other miR-17 family members may regulate multiple autophagy-related genes and that play an integral role in fine-tuning autophagy.

Taken together, we have collected evidence supporting miR-106b regulation of autophagy, partially through a direct interaction with the ATG16L1 3'UTR. miR-106a also modulates autophagy, but it functions likely via other autophagy genes and/or autophagy regulators. Due to the nature of multiple target genes for the members of miR-17 family, their regulation of autophagy and underlying mechanisms need to be thoroughly assessed. Though the regulatory effects of miR-106a and miR-106b on the autophagy pathway may be modest, further studies are warranted to investigate their significance in the intestinal inflammation and IBD pathogenesis.

Acknowledgments

Supported by the National Institute of Health (RO3 DK086549), NIH T32 Gastrointestinal training grant (DK07074), and the NIH DDRCC core grant (P30 DK42086).

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FIGURE 1.

Mature miRNA expression in nutrient deprived HCT116 cells. (A) HCT116 cells were incubated in EBSS for 4 h, and then the expression levels of eight miRNAs that are predicted to target the ATG16L1 3'UTR were determined by qRT-PCR and normalized by RNU6B. The data are expressed as the mean $\pm$ SEM (n=3-6). (B) qRT-PCR analysis of miR-106a and miR-106b expression in the cells starved for 0-6 h. The data are expressed as the mean $\pm$ SEM (n=3). * P <0.05 and ** P <0.01 vs the corresponding non-starved control.

FIGURE 2.

miR-106 regulation of ATG16L1 expression and autophagic activity. (A) HCT116 cells were transfected with miRNA mimics or miR-NC (50 nM), and 24 h later, the ATG16L1 mRNA expression levels were analyzed by qRT-PCR and normalized by GAPDH. The data are expressed as the mean $\pm$ SEM (n=3). (B) At 24 h after transfection with 20 nM miRNA mimics, the ATG16L1 protein expression was assayed by Western blot and the band densities were quantitated and normalized to that of the corresponding loading control. (C) At 48 h post-transfection of 40 nM mimics, cells were starved for another 4 h and then autophagy was monitored based on the LC3 turnover by Western blot. The band densities were quantitated and the ratios of LC3II/LC3I calculated.

FIGURE 3.

miR-106 cluster regulation of ATG16L1 expression. HCT116 cells were transfected with miR-NC (200 nM), miR-106a~363 cluster mimics (miRs-18b, Supported by the -19b, -20b, and -106a, 50 nM each) or miR-106b~25 cluster mimics (miRs-106b, -93, and -25, 50 nM each). At 24 or 48 h post-transfection, cells were directly collected or incubated in EBSS for another 4 h. (A) qRT-PCR analysis of ATG16L1 mRNA expression. The data are expressed as the mean $\pm$ SEM (n=3). ** P <0.01 vs miR-NC. (B) Western blot analysis of ATG16L1

protein expression. The band densities were quantitated and the ratios of ATG16L1/GAPDH calculated.

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FIGURE 4.

miR-106 cluster regulation of autophagic activity. As in Fig. 3, HCT116 cells were transfected with miR-NC, miR-106a~363 cluster mimics or miR-106b~25 cluster mimics. At 24 or 48 h post-transfection, cells were directly collected or incubated in EBSS for another 4 h. (A) Autophagy was assessed based on the LC3 turnover by Western blot. The band densities were quantitated and the ratios of LC3II/LC3I calculated. (B) LC3II puncta were visualized by confocal imaging of cells immunostained for nucleus and LC3 using DAPI (blue) and Alexa Fluor 488 secondary antibody conjugates (green), respectively. Representative imaging are shown. (C) LC3II puncta per cell were quantitated by randomly counting 15-20 cells for each treatment group and the data are expressed as the mean $\pm$

SEM. ** $P < 0.01$ vs the baseline control transfected with miR-NC. ## $P < 0.01$ vs the starved cells transfected with miR-NC.

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FIGURE 5.

miR-106b targets ATG16L1 3'UTR. (A) HCT116 cells were transfected with the vectors containing the full-length 3'UTR, truncated 3'UTR or an empty pMIR-GLO vector (200 ng/ml). Firefly luciferase activity was measured 48 h later and normalized to renilla luciferase activity. (B) The cells were co-transfected with the ATG16L1 full-length 3'UTR vector (200 ng/ml) and a miRNA mimic (20 nM), and luciferase reporter assays were carried out 24 h after transfection. (C) The cells were co-transfected with the WT or mutated full-length 3'UTR vector (200 ng/ml) and miR-106b~25 cluster (miRs-106b, -93, and -25, 40 nM each) mimics or miR-NC (120 nM). At 48 h post-transfection, dual luciferase activities were determined. (D) The cells were co-transfected with the WT or mutated truncated 3'UTR vector (200 ng/ml) and miR-106b~25 cluster (miRs-106b, -93, and -25, 40 nM each) mimics or miR-NC (120 nM). At 24 h post-transfection, dual luciferase activities were determined. (E) The cells were co-transfected with the WT or mutated full-length 3'UTR vector and a miRNA mimic (40 nM), and 24 h later, luciferase activities were determined. The data are expressed as the mean $\pm$ SEM (n=3-8). * P <0.05 and ** P <0.01 vs the corresponding control.

FIGURE 6.

miR-106 regulation of ULK1, BECN1, ATG12, ATG5, and SQSTM1/p62 expression. HCT116 cells were transfected with a miRNA mimic (40 nM). At 48 h post-transfection, (A-D) qRT-PCR analysis of indicated gene mRNA expression. The data are expressed as the mean $\pm$ SEM (n=3-6). ** P <0.01 vs miR-NC. (E) Western blot analysis of indicated gene protein expression. The band densities were quantitated by ImageJ64 software and the ratios of ATG16L1/ β -actin calculated and normalized. The data represent the mean of three experiments with different cell preparations.