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BASIC RESEARCH PAPER

Elevated *Mirc1/Mir17-92* cluster expression negatively regulates autophagy and CFTR (cystic fibrosis transmembrane conductance regulator) function in CF macrophages

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ABSTRACT

Cystic fibrosis (CF) is a fatal, genetic disorder that critically affects the lungs and is directly caused by mutations in the *CF transmembrane conductance regulator (CFTR)* gene, resulting in defective CFTR function. Macroautophagy/autophagy is a highly regulated biological process that provides energy during periods of stress and starvation. Autophagy clears pathogens and dysfunctional protein aggregates within macrophages. However, this process is impaired in CF patients and CF mice, as their macrophages exhibit limited autophagy activity. The study of microRNAs (*Mirs*), and other noncoding RNAs, continues to offer new therapeutic targets. The objective of this study was to elucidate the role of *Mirs* in dysregulated autophagy-related genes in CF macrophages, and then target them to restore this host-defense function and improve CFTR channel function. We identified the *Mirc1/Mir17-92* cluster as a potential negative regulator of autophagy as CF macrophages exhibit decreased autophagy protein expression and increased cluster expression when compared to wild-type (WT) counterparts. The absence or reduced expression of the cluster increases autophagy protein expression, suggesting the canonical inverse relationship between *Mirc1/Mir17-92* and autophagy gene expression. An *in silico* study for targets of *Mirs* that comprise the cluster suggested that the majority of the *Mirs* target autophagy mRNAs. Those targets were validated by luciferase assays. Notably, the ability of macrophages expressing mutant F508del CFTR to transport halide through their membranes is compromised and can be restored by downregulation of these inherently elevated *Mirs*, via restoration of autophagy. In vivo, downregulation of *Mir17* and *Mir20a* partially restored autophagy expression and hence improved the clearance of *Burkholderia cenocepacia*. Thus, these data advance our understanding of mechanisms underlying the pathobiology of CF and provide a new therapeutic platform for restoring CFTR function and autophagy in patients with CF.

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Introduction

Production of functional proteins requires multiple steps, including gene transcription and posttranslational processing. MicroRNAs (*Mirs*) can regulate individual stages of these processes.[1] *Mirs* are an evolutionarily, conserved class of small (~21–24 nucleotides) noncoding RNAs that play key roles in the transcriptional and posttranscriptional regulation of gene expression.[1] Specific *Mirs* have been identified to regulate *Cftr (cystic fibrosis transmembrane conductance regulator)* gene expression, but their actions in the context of both autophagy and CF have not been investigated. Autophagy is a highly regulated biological process that provides energy during periods of stress and starvation. This process is impaired in CF patients and CF mice, as their macrophages exhibit limited autophagy activity.

CF is an autosomal recessive disease caused by mutations in the Cftr gene that result in defective CFTR function in many organs; however, the majority of CF-associated morbidity and mortality arises from pulmonary infection and inflammation.[2] The Cftr gene encodes a transmembrane chloride channel that is regulated by ATP hydrolysis and expressed in various cell types including epithelial cells and macrophages.[3] In epithelial cells, the CFTR channel conducts anions and plays a critical role in regulating the volume and composition of airway surface liquid,[4] a thin layer of aqueous fluid and mucus covering the airway surface whose properties include facilitating mucociliary clearance, bacterial killing, and epithelial cell homeostasis.[5] The function of the CFTR channel in macrophages remains unclear although our recent work demonstrates that defective CFTR function is accompanied by an impaired innate

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immune response to specific infections.[6-11] In CF patients with the most common mutation, F508del, the mutant form of the protein fails to traffic properly to the plasma membrane. This leads to a critical lack of fluid exchange across the membrane. Should the F508del-CFTR traffic to the cell membrane in response to therapy, the mutant protein then regains partial channel function in epithelial cells.[12,13] Heterozygote humans and mice do not suffer pathological symptoms despite the fact that their cells exhibit only 50% functional activity of the CFTR channel.[14] Thus, small improvement of CFTR channel function in F508del homozygotes is accompanied by a significant improvement in ensuing symptoms as reported by several clinical trials.[15,16]

Recent studies have implicated reduced autophagy activity in a number of physiological and pathophysiological processes such as aging, cancer, neurodegenerative diseases, innate immunity, and CF.[10,11,17-19] Autophagy functions to yield energy and nutrients during stress or starvation of the cell.[20] In addition, autophagy can also restrict specific pathogens within macrophages and improve clearance of misfolded protein aggregates that cannot be managed by proteasomes.[21] The process of autophagy involves formation of double-membrane compartments (phagophores) that engulf nonfunctional organelles and cytoplasm. These phagophores then mature into autophagosomes that fuse with lysosomes to form autolysosomes, within which the autophagic cargo is degraded and recycled for protein and ATP synthesis via degradative enzymes from the lysosome.[22,23] Autophagosome formation is mediated by a series of autophagy-promoting molecules including Atg5, Atg12, Atg16, Atg7, Atg8 (mammalian MAP1LC3/LC3 and GABARAP) and Vps30/Atg6 (BECN1/Beclin1).[22] Thus, the absence or reduction in expression of one factor can markedly impair the autophagy process.

Burkholderia (*B.*) *cenocepacia* is notorious for infecting CF patients and is resistant to the majority of antibiotics. In healthy macrophages, *B. cenocepacia* is cleared by autophagy. However, macrophages from CF humans and mice fail to control *B. cenocepacia* due to impaired autophagy activity.[10,11,24] CF mice allow *B. cenocepacia* to establish infection in their lungs, which triggers an intense, and often lethal, inflammatory response.[10,11,24] Therefore, clearance of *B. cenocepacia* from the lungs of CF mice reflects the amount of autophagy activity.

Reports have demonstrated the extent to which *Mirs* regulate CFTR expression and function[16,25,26] yet their role in the context of autophagy has yet to be investigated. Thus, considering the strong implications of *Mirs*, both in autophagy and CF, and given the lack of current evidence linking these 2 processes, we investigated specific *Mirs* that regulate autophagy-related genes whose expression is altered in CF patients.

In this report, we used *in silico* approaches to identify the importance of the *Mirc1/Mir17-92* cluster which generates a single polycistronic transcript that yields 6 mature *Mirs*: *Mir17, Mir18a, Mir19a, Mir20a, Mir19b*, and *Mir92*.[27] The polycistronic *Mirc1/Mir17-92* cluster was initially linked to tumorigenesis as published by our group and others.[27-34] The role of the *Mirc1/Mir17-92* cluster in CF has not been investigated. We demonstrate that members of the *Mirc1/Mir17-92* cluster target multiple essential autophagy factors. In addition, we find that several specific *Mirs* comprising the *Mir17HG/Mir17-92* cluster are overexpressed in CF human and murine macrophages with corresponding reduced expression of their predicted autophagy-targeted genes. *Mirs* comprising the *Mir17HG/Mir17-92* cluster exhibit a trend toward upregulation in CF cells, especially *Mir17* and *Mir20a*, which are significantly increased in murine macrophages. Importantly, *Mir17* is also significantly upregulated in macrophages derived from CF patients. Luciferase assays validated that both *Mir17* and *Mir20a* target *Atg7* and *Atg16l1*. Notably, reducing the inherently elevated expression of *Mir17* and *Mir20a* improves ATG7 and ATG16L1 expression both in vitro and in vivo. In addition, reducing *Mir17* and *Mir20a* expression improves CFTR function by restoring autophagy expression. In this regard, targeting *Mir17* was more efficient. Accordingly, *B. cenocepacia* clearance is improved in CF mice after intra-tracheal treatment with antagomirs to *Mir17* and *Mir20a*. Containment of *B. cenocepacia* upon administration of these specific antagomirs is accompanied by improved expression of targeted autophagy proteins. Our study advances our understanding of the mechanism underlying defective autophagy in CF and provides a novel therapeutic approach for restoring CFTR function and autophagy.

Results

Expression of autophagy proteins is reduced in primary CF (F508del) macrophages

CF macrophages exert weak autophagic activity[10,11,18] yet the underlying mechanisms behind this reduction remain unknown. To investigate the potential mechanism for compromised autophagy activity in CF cells, we examined the expression of autophagy-related proteins in wild-type (WT) C57/BL6J and CF murine macrophages by western blot using specific antibodies. Notably, the expression of members of the ATG12–ATG5 protein complex and ATG7 was decreased in CF macrophages when compared to WT cells (Fig. 1A). However, ATG12 protein and mRNA levels were comparable in WT and F508del macrophages (Figs. 1A and S1A).[10] To evaluate if the low expression level of autophagy family member proteins reduces the basal autophagy activity in resting WT and CF macrophages, the number of macrophages exhibiting more than 5 LC3 labeled-autophagosomes (puncta) was quantified using confocal microscopy. LC3 is a cytosolic autophagy protein that is recruited to phagophores; the subsequent autophagosomes appear as donut-shaped structures called puncta. Significantly fewer resting CF macrophages had more than 5 puncta when compared to their WT counterparts (Fig. 1B and C). In addition, to determine autophagy activity in response to stimulation, macrophages were starved and those expressing more than 5 puncta were quantified. CF macrophages failed to increase their puncta content in response to starvation in contrast to WT macrophages (Fig. 1B and C). Therefore, CF macrophages fail to mount an autophagic flux response upon starvation. Together, these data provide evidence that CF

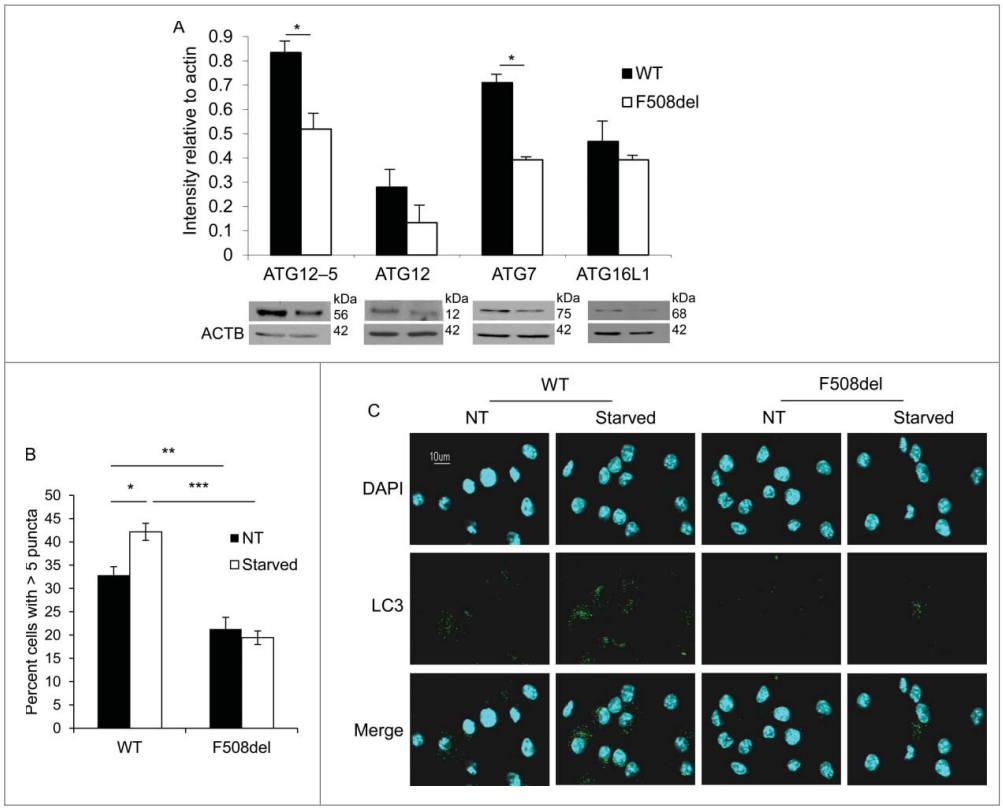

Figure 1. Primary F508del (CF) murine macrophages exhibit weak autophagic flux during resting and starvation conditions. (A) Western blot for basal level autophagy proteins in WT (black bars) and F508del primary mouse macrophages (white bars). Densitometry analyses of western blot bands were normalized to their respective loading control bands using ImageJ software to control for loading. Panels shown represent 3 independent experiments displaying similar results. (B) Scoring of the percentage of macrophages harboring more than 5 puncta in WT and F508del macrophages before (NT, black) and after 2 h of starvation (starved, white). Data are representative of scoring the means ± SEM of 900 WT macrophages and 700 F508del macrophages. (C) Confocal microscopy representing autophagy activity of resting and starved WT and F508del macrophages. Blue (DAPI) stain indicates nuclei and green stain indicates LC3 puncta (autophagosome formation). Asterisks (*) indicates $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.

macrophages exhibit lower expression of essential ATG proteins and are characterized by weak autophagic activity.

Expression of the Mirc1/Mir17-92 *cluster is elevated in primary CF macrophages resulting in the reduction of several autophagy molecules*

Given the lack of current evidence regarding autophagy regulation in CF, we investigated predicted *Mirs* that may play a role in modulating the autophagy process and whether their expression is altered in CF cells. Using 2 online web servers that predict targets of specific *Mirs*, TargetScan and MirBase, we determined that *Mir101* and the *Mirc1/Mir17-92* cluster are predicted to target autophagy-related mRNAs. *Mir101* was reported to target *STMN1, RAB5A*, and *ATG4D*, each of which is an important autophagic regulator.[35] The *Mirc1/Mir17-92* cluster is predicted to target *Atg4, Atg5, Becn1, Atg7, Atg12, Atg16l1* and *Lc3* (Table S1). To determine whether the expression of *Mir101* and the *Mirc1/Mir17-92* cluster are altered in CF macrophages, we performed quantitative real-time PCR (qRT-PCR) on RNA lysates from murine WT and CF macrophages. Expression of *Mir101* was not statistically different between WT and CF macrophages (Fig. S1B), whereas the *Mirc1/Mir17-92* cluster expression was significantly elevated in CF macrophages (Fig. 2A). While members of the cluster exhibit a trend toward upregulation in murine CF macrophages, *Mir17* and *Mir20a* were significantly increased (Fig. 2A). To determine whether these findings are consistent with human CF pathology, expression of *Mirs* comprising the cluster was assessed in macrophages derived from blood monocytes of CF patients. Several members of the cluster were upregulated, but only *Mir17* was significantly upregulated in both human and mouse samples (Fig. 2B). These data suggest that the inherently elevated expression level of *Mir17HG/Mir17-92* cluster members in CF macrophages contributes at least in part to the reduced expression of several autophagy-related genes that potentially impair autophagy activity in CF macrophages.

To determine the extent to which the elevated *Mirc1/Mir17-92* cluster contributed to the reduced expression of autophagy molecules, their expression was examined in the absence of cluster expression. Macrophages lacking the *Mirc1/Mir17-92* cluster were obtained from *Mirc1/Mir17-92*$^{-/-}$ floxed mice.[36] Since a complete knockout of the cluster is embryonic lethal, these mice were obtained by breeding a Lyz2/LysM-cre mouse with a *Mirc1/Mir17-92*$^{-/-}$ floxed mouse, thus, knocking out the *Mirc1/Mir17-92* cluster specifically in the myeloid lineage.[27] The protein lysates of WT and *Mirc1/Mir17-92*$^{-/-}$ macrophages were analyzed by western blot using specific autophagy antibodies. ATG12–ATG5, ATG7, and ATG16L1 protein levels were substantially elevated in the absence of the *Mirc1/Mir17-92* cluster, but not ATG12, (Fig. 2C) as were their corresponding mRNA expression (Fig. 2D). To determine if the elevated

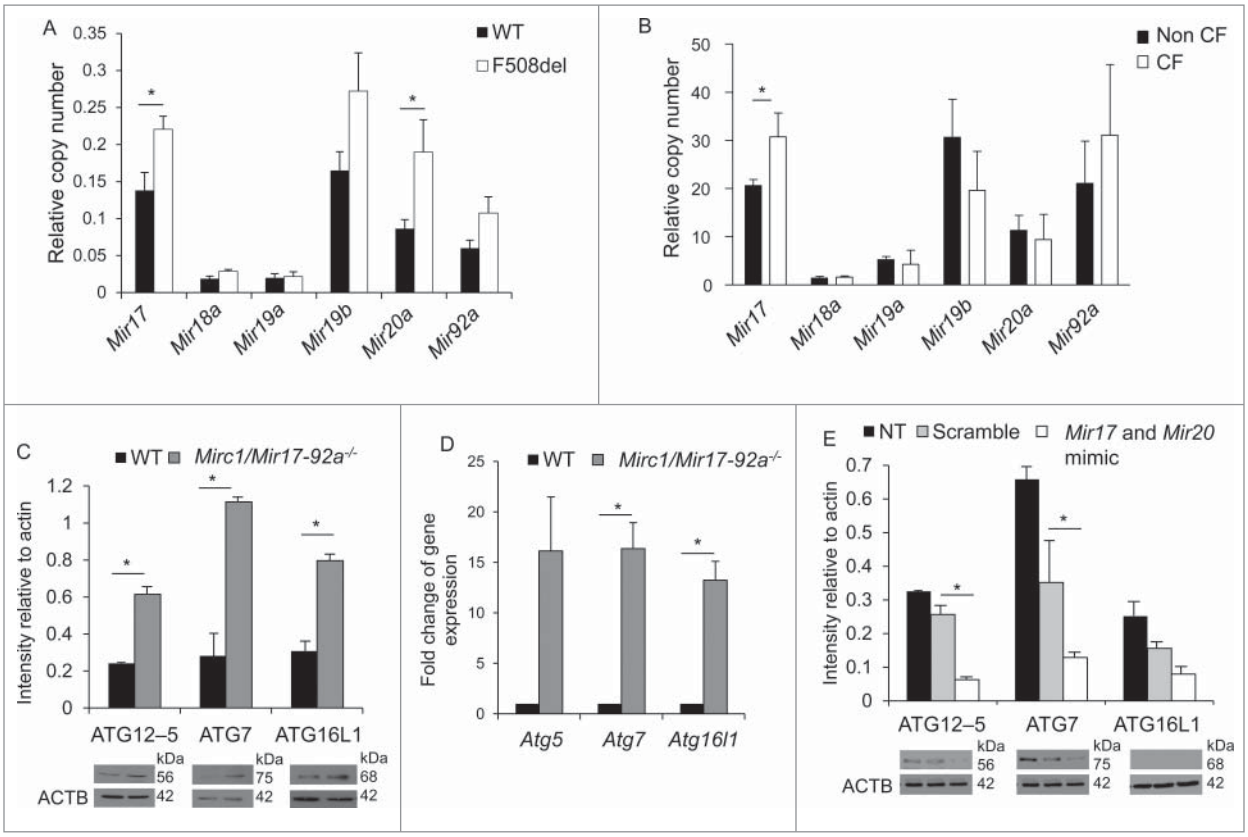

Figure 2. *Mirc1/Mir17-92* cluster expression is elevated in primary homozygous F508del macrophages and targets several autophagy molecules. (A) Quantitative real-time PCR (qPCR) representing the expression of *Mirc1/Mir17-92* cluster members in resting WT (black bars) and F508del (white bars) murine macrophages. Student 2-tailed *t* test was used to determine significance at $p \leq 0.5$. WT macrophage cluster expression compared to F508del macrophage cluster expression was also significant as calculated by 2-way ANOVA $p \leq 0.05$. WT macrophages $n = 5$ and F508del macrophages $n = 4$ for *Mirc1/Mir17-92* analysis. Graphs are representative of compiling means ± SD. (B) qPCR representing the expression of *Mir17HG/Mir17-92* cluster in human blood monocyte-derived macrophages from 6 non-CF (black bars) and 6 CF (white bars) patients. Data shown represent the means ± SD. Student 2-tailed *t* test was used to assess significance. (C) Western blot for autophagy proteins in WT (black bars) and *Mirc1/Mir17-92*$^{-/-}$ macrophages (gray bars). Densitometric analyses of bands were normalized to their respective actin loading control bands using imageJ software. Each blot is representative of 3 independent experiments displaying similar results. (D) qPCR results for autophagy-regulating genes in WT (black bars) and *Mirc1/Mir17-92*−/− macrophages (gray bars). Data are presented as fold change compared to WT normalized to 1 and are presented as the means ± SD, $n = 3$. Student 2-tailed *t* test was used to determine significance at $p \leq 0.05$. (E) Western blot for autophagy proteins in *Mirc1/Mir17-92*$^{-/-}$ macrophages untreated (NT, black bars), transfected with scramble control nucleotides (gray bars), or *Mir17* and *Mir20a* mimics (white bars). Protein levels were normalized to their respective actin levels and quantified by ImageJ software, $n = 3$. * indicates $p \leq 0.05$ for differences between transfected scrambled controls and mimics.

levels of ATG proteins in *Mir17-92*$^{-/-}$ macrophages are directly associated with low levels of the cluster, we transfected *Mir17-92*$^{-/-}$ macrophages with *Mir17* and *Mir20a* mimics to restore their expression and assessed the autophagy protein expression profile. Overexpression of *Mir17* and *Mir20a* led to a reduction in ATG12–ATG5 and ATG7 expression when compared to their scrambled controls (Fig. 2E). Collectively, these data provide strong evidence that the *Mir17-92* cluster modulates the expression of essential autophagy-related genes in macrophages.

Mir17 *and* Mir20a *target the 3′-untranslated region of* Atg7 *and* Atg16l1

We have shown that several microRNAs comprising the cluster are elevated in human and murine CF macrophages; however, *Mir17* and *Mir20a* exhibit the most significantly increased expression in mice, and *Mir17* is elevated in human CF macrophages (Fig. 2A and B). Therefore, we focused on further characterizing *Mir17* and *Mir20a* in CF mice. Targeting only 2 *Mirs* is a more feasible therapeutic approach to take in the future, as it would potentially reduce off-target effects. To confirm the binding of *Mir17* and *Mir20a* to the 3′ untranslated (3′-UTR) regions of *Atg7* and *Atg16l1*, NIH3T3 cells were transiently transfected with a luciferase reporter construct containing the full length 3′-UTR of *Atg7, Atg16l1* or an empty vector luciferase reporter. Transfection with *Mir17* or *Mir20a* mimics reduced luciferase activity from cells transfected with either *Atg7*-3′-UTR-luc or *Atg16l1*-3′-UTR-luc constructs (Fig. 3A and B). Notably, mutations in the 3′-UTR of *Atg7* (M-*Atg7*) or *Atg16l1* (M-*Atg16l1*) eliminated the *Mir17* and *Mir20a* mimic-mediated reduction of luciferase activity (Fig. 3A and B). To further verify the relationship between *Mir17* and *Mir20a* and the expression of their respective targets *Atg7* and *Atg16l1*, we inhibited the expression of these *Mirs* in CF macrophages using specific antagomirs. CF macrophages transfected with either antagomir17 or antagomir20a elicited a nonsignificant increase in ATG7 and ATG16L1 protein expression (Fig. S1C). However when transfected together, antagomir17 and antagomir20a significantly increased the

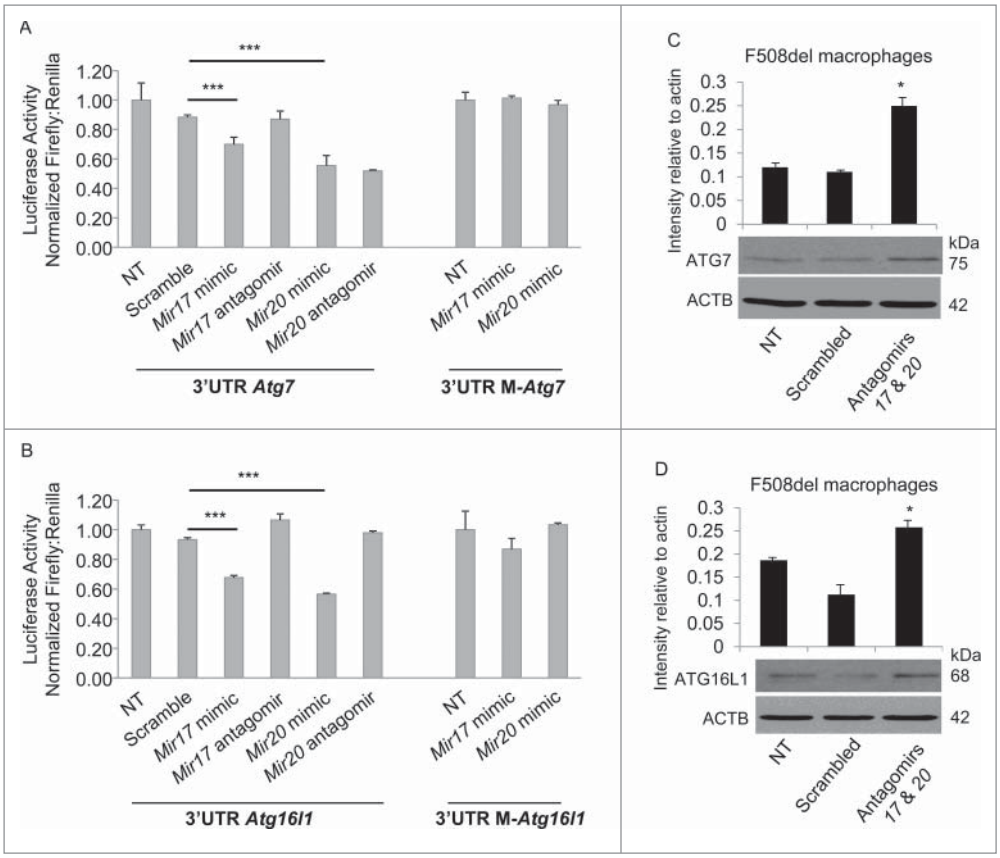

Figure 3. *Mir17* and *Mir20a* target the 3′UTRs of *Atg7* and *Atg16l1*. (A and B) NIH3T3 cells transfected with luciferase reporter constructs containing the 3′UTR of murine *Atg7* (A) or *Atg16l1* (B), or their mutated 3′UTR (M-*Atg7*, M-*Atg16l1*), were not treated (NT) or treated with scramble control nucleotides (A and B), or *Mir17* or *Mir20a* mimics or their corresponding antagomirs. Antagomir17 and antagomir20a were transfected to reduce corresponding endogenous miRNA levels. Luciferase expression was normalized to the level of Renilla measured. Data shown are representative of the average luciferase production conducted in quadruplicate ± SD. n = 3 and asterisks indicate significant differences by one-way ANOVA. (C and D) Western blot for (C) ATG7 or (D) ATG16L1 in F508del macrophages transfected with antagomir17 and antagomir20a. The blot is representative of 3 independent experiments displaying similar results and protein bands were normalized to their respective ACTB bands and quantified by ImageJ software. * indicates $p \leq 0.05$; and ***, $p \leq 0.001$.

expression of ATG7 and ATG16L1 when compared to scramble-transfected cells (Fig. 3C and D). Therefore, transfection with specific antagomir*17* and antagomir20a improved the expression of ATG7 and ATG16L1, whereas transfection with scramble antagomirs did not (Fig. 3A and B). Taken together, these data demonstrate that *Mir17* and *Mir20a* target the *Atg7* and *Atg16l1* genes and modulate their expression in CF macrophages.

Pulmonary delivery of antagomir17 and antagomir20a improves the expression of targeted autophagy genes and autophagy activity in CF mice

To determine whether reducing the elevated expression of *Mir17* and *Mir20a* in vivo improves the expression of targeted autophagy genes, we delivered antagomirs against *Mir17* and *Mir20a* or scrambled control intratracheally, once a day for 3 d, to mice. The lungs were harvested, homogenized and analyzed for the expression of *Mir17* and *Mir20a* by qRT-PCR. We found that this regimen effectively lowered the expression of elevated *Mir17* and *Mir20a* in the lungs of CF mice and did not alter the expression of other members of the cluster (Fig. 4A). To determine the extent to which decreasing the expression of *Mir17* and *Mir20a* rescues the expression of targeted autophagy genes in vivo, lung homogenates were analyzed for the mRNA levels of autophagy genes *Atg5*, *Atg7* and *Atg16l1* by qRT-PCR and western blot. Delivery of *Mir17* and *Mir20a* antagomirs to the lungs of CF mice resulted in a statistically significant increase of mRNA levels of *Atg7* and protein levels of ATG7 and ATG16L1 (Fig. 4B and C).

B. cenocepacia is primarily cleared by autophagy in the lungs of healthy mice and represents an often fatal infection in CF patients who suffer from reduced autophagy activity. Stimulation of autophagy by rapamycin in live mice and their macrophages contains the infection.[10] Thus, we examined bacterial loads in the lungs of antagomir-treated CF mice to determine the functional consequences of targeted reduction of *Mir17* and *Mir20a*. Notably, CF mice treated with *Mir17* and *Mir20a* antagomirs improved clearance of *B. cenocepacia* infection compared to their counterparts treated with scrambled control antagomirs (Fig. 5A). Both groups harbored similar bacterial loads at 4 h (data not shown).[15] Improved autophagy activity as indicated by improved bacterial clearance, was accompanied by significant reductions in the expression of *Mir17* and *Mir20a* in lung tissues of mice treated with the corresponding antagomirs even in the presence of *B. cenocepacia* (Fig. 5B). Therefore, targeting *Mir17* and *Mir20a* in vivo improves autophagy activity in live CF mice.

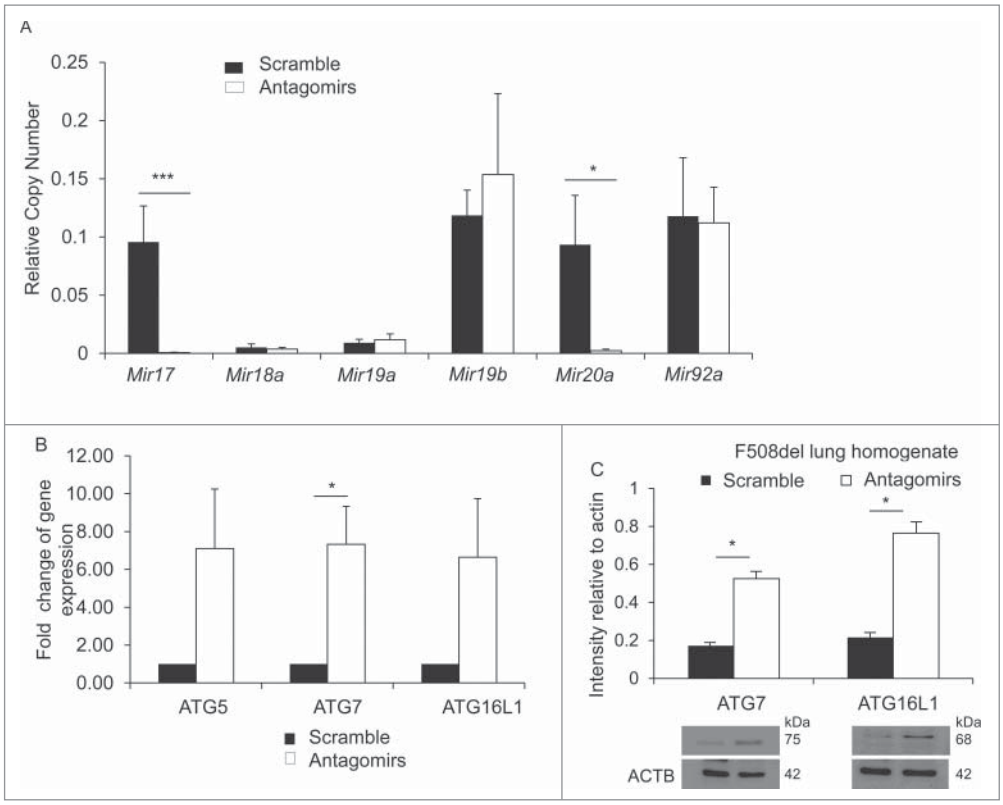

Figure 4. Decreasing the expression of *Mir17* and *Mir20a* in live F508del mice improves the expression of autophagy molecules. (A) Expression of members of the *Mirc1/Mir17-92* cluster in F508del mice intratracheally treated with scrambled control (black bars) or antagomirs to *Mir17* and *Mir20a* (white bars) as measured by qPCR. Data are presented as the mean ± SD and are representative of n = 6. Student 2-tailed *t* test was used to determine significance for *Mir17* and *Mir20a*. Significance assessed at $p \leq 0.05$ (B) Expression of autophagy-regulating genes in F508del murine lung homogenate after intra-tracheal treatment with scramble (black bars) or antagomir17 and antagomir20a (white bars) assessed by qPCR. Data shown are representative of the average expression analyzed in duplicate and presented as fold-change compared to WT normalized to one ± SD (n = 3). Student 2-tailed *t* test was performed and asterisks indicate significant differences at $p \leq 0.05$. (C) Western blot for autophagy proteins, ATG7 and ATG16L1, in F508del lung homogenates post-intra-tracheal administration of scrambled control (black bars) or antagomir17 and anatgoMir20a (white bars). The blots shown are representative of 3 independent experiments displaying similar results and protein production bands were normalized to their respective ACTB bands and quantified by ImageJ software. * indicates $p \leq 0.05$; and ***, $p \leq 0.001$.

CFTR function is impaired in primary F508del macrophages and is improved by reducing the levels of Mir17 *and* Mir20a *via improved autophagy*

As demonstrated above, correcting the inherently elevated levels of *Mir17* and *Mir20a* in CF macrophages in vitro and in vivo improved autophagy activity; however, the impact of these *Mirs* on CFTR function remains unknown. To determine the effect of reducing *Mir17* and *Mir20a* on the function of the F508del-CFTR channel, primary CF macrophages were transfected with antagomirs against *Mir17* and *Mir20a* or a scramble control. After 48 h, CFTR function was assessed using the fluorescent and halide-sensitive compound 6-methoxy-N-(3-sulfopropyl)-quinolinium (SPQ) after modification of a protocol used to measure CFTR function in airway epithelial cells.[37,38] CFTR was activated using a "cocktail" containing forskolin, cpt-cAMP and 3-isobutyl-1-methylxanthine/IBMX to increase cAMP, since CFTR is a cAMP-activated channel. Results demonstrated that resting CF macrophages exhibited significantly reduced CFTR activity compared to WT cells. Forty-eight h after transfection of combined antagomirs against *Mir17* and *Mir20a*, an increase in fluorescence was detected in CF macrophages (Fig. 6). These results indicate that reducing the expression of inherently elevated *Mir17* and *Mir20a* in CF macrophages improves F508del-CFTR function. Furthermore, to determine if this improvement in CFTR function was mediated by increased autophagy activity in antagomir-treated cells, CF macrophages were transfected with antagomir17 and antagomir20a in the presence or absence of the autophagy inhibitor 3-methyladenine (3-MA). The function of CFTR was determined by the SPQ assay as described above. Notably, inhibition of autophagy activity by 3-MA abolished the beneficial effect on CFTR function in CF macrophages achieved via decreasing *Mir17* and *Mir20a* expression (Fig. 6). Collectively, these findings provide evidence that restoration of CFTR function upon reduction of *Mir17* and *Mir20a* is mediated by autophagy. Together, our data provide evidence that correcting elevated levels of *Mir17* and *Mir20a* in CF improves autophagy and CFTR function.

Discussion

CF symptoms have been attributed to defective CFTR function in epithelial cells where impairment of CFTR function reduces chloride secretion, thereby impairing mucociliary clearance due to dehydration.[15] Our data support the notion that the pathobiology of CF is multi-factorial, including functional impairment of autophagy in macrophages, as indicated by increased

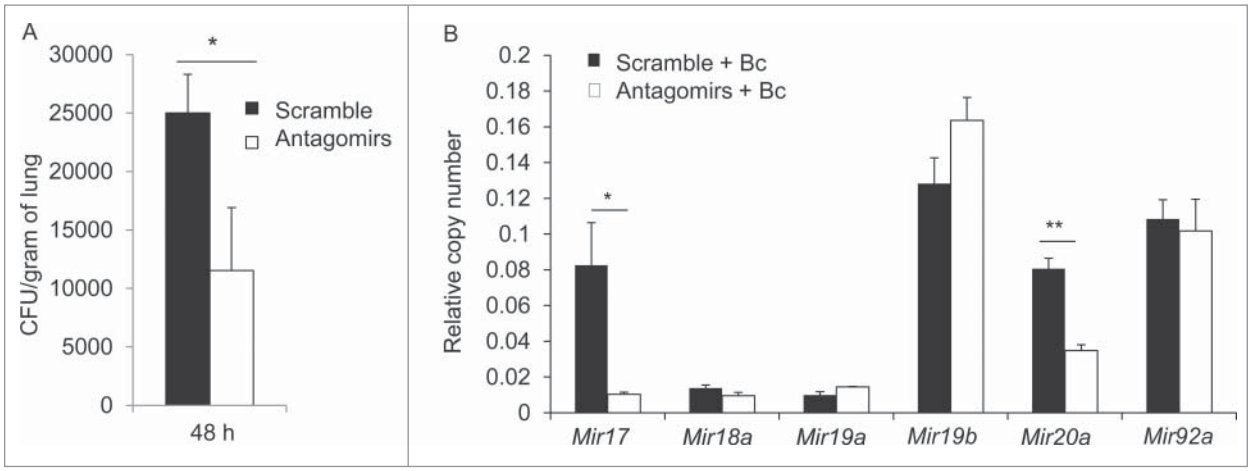

Figure 5. Targeting *Mir17* and *Mir20a* in F508del mice increases autophagy-mediated clearance of *Burkholderia cenocepacia*. F508del mice were intratracheally treated with scrambled control (black bars) or antagomir17 and antagomir20a (white bars), then infected with *B. cenocepacia*. (A) After 48 h of infection, lungs were homogenized and plated for colony forming units (CFUs). Data shown represent the average CFU/gram of lung ± SD, n = 4 per condition. Students 2-tailed *t* test was used to determine significance (*) at $p \leq 0.05$. (B) qPCR of individual *Mirs* within the *Mirc1/Mir17-92* cluster in lung homogenates. Data represent the mean expression ± SD, n = 4 per condition. Student 2-tailed *t* test was used to determine significance. * indicates $p \leq 0.05$; **, $p \leq 0.01$.

susceptibility to specific bacterial infections and inflammation.[39] Macrophages expressing mutant F508del-CFTR on both alleles inherently exert reduced autophagy activity, which is associated with exacerbated inflammatory responses.[10,11,19,40] In this regard, autophagy controls bacterial infection and IL-1β production in macrophages.[41] The link between the CFTR mutation and the autophagy impairment has established macrophages as major players in CF pathology.[10,11,42] Emerging studies also investigate neutrophil function in CF.[43,44] Thus, CF pathobiology encompasses several immune cells, in addition to epithelial cells, recognizing that CF is a newly identified immune deficiency disorder.

All previous work in CF macrophages has focused on various phenomena such as impairment of bacterial clearance or exacerbated IL-1β production.[24,25] Here, we examine CFTR function in macrophages using a modified SPQ assay initially used for epithelial cells. This is the first report to directly demonstrate that halide transport is impaired in F508del macrophages. Therefore, it is plausible to conclude that impairment of CFTR function in macrophages is responsible for innate immune deficiency in CF immune cells.

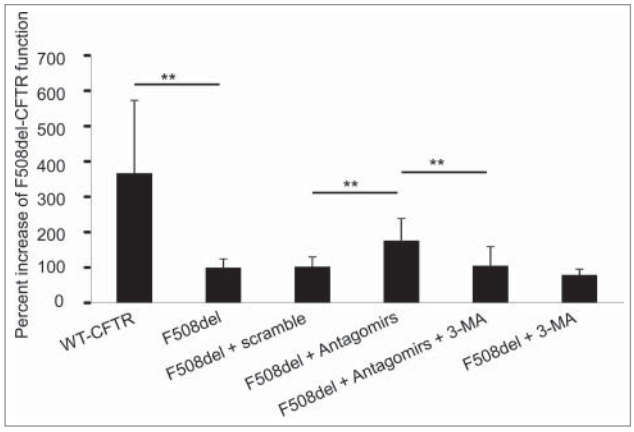

Figure 6. CFTR function is reduced in F508del murine macrophages and partially restored by decreasing *Mir17* and *Mir20a* expression levels via autophagy. F508del macrophages were untransfected, transfected with scramble control or antagomir17 and −20a (Antagomirs) for 48 h, and 3-methyladenine (3-MA) for 6 h to inhibit autophagic flux. Cells were loaded with SPQ dye, quenched using an iodide buffer, and fluorescence output was measured. Data are presented as means of percent increase of F508del-CFTR ± SD from 5 independent experiments. Student 2-tailed *t* test was used to determine significance of antagomir transfection, and 3-MA inhibition. ** indicates $p \leq 0.01$.

Defective autophagy plays a critical role in the pathology of several pulmonary diseases including chronic obstructive pulmonary disease, pulmonary hypertension, idiopathic lung fibrosis, and CF.[22,45] We have previously demonstrated in a CF mouse model, that treatment with rapamycin, an autophagy-inducing drug, improves autophagy activity and reduces the lung inflammatory response both in vitro and in vivo.[10,11,24,46,47] Rapamycin treatment also improves CFTR function in airway epithelial cells.[19,40,48] The use of rapamycin reinforces the concept that restoration of autophagy activity is beneficial for CF patients. However, rapamycin elicits severe side-effects and thus cannot be used for the young CF population. Therefore, alternative approaches to improve autophagy in CF are needed.[39,49]

Despite the rapidly growing literature describing defective autophagy in CF, the underlying mechanisms remain to be fully elucidated. To characterize the impaired autophagy activity and inherently reduced expression of autophagy proteins observed in CF macrophages, we examined the expression of several *Mirs* predicted to target autophagy. *Mir101* and the *Mirc1/Mir17-92* cluster were identified. However, only the *Mirc1/Mir17-92* cluster was overexpressed in both human and mouse CF macrophages. Within this cluster, only *Mir17* was significantly elevated in both human and mouse samples. Downregulation of inherently elevated *Mir17* and *Mir20a* in murine macrophages results in improved CFTR channel function. Other *Mirs* within the cluster were unregulated only to a small extent. Thus, it appears that correcting *Mir17* and *Mir20a* is sufficient to restore significant autophagy activity in mice. Since only *Mir17* is significantly elevated in human macrophages, it is possible that reducing its expression alone will be sufficient to improve CFTR functions and bacterial clearance in CF patients.

We observe that both *Mir17* and *Mir20a* mimics were able to downregulate *Atg7* and *Atg16l1*; however, the *Mir20a*

antagomir failed to elevate *Atg7* expression, suggesting that *Mir17* has more specificity toward *Atg7* than *Mir20a*. Notably, the seed sequences of *Mir17* and *Mir20a* are the same but have different duplex structures causing different silencing efficacies for their targets. Online computational prediction data showed that the free energy release between *Mir17* and *Atg7* or *Atg16l1* is higher than between *Mir20a* and *Atg7* or *Atg16l1* suggesting that *Mir17* binds to the 3′UTR of *Atg7* or *Atg16l1* more stably and complementary than *Mir20a*. Thus, we propose that targeting *Mir17* can be used in conjunction with CFTR potentiators that are demonstrating partial efficacy in clinical trials. Our findings are corroborated by the recent clinical trials demonstrating the restoration of defective autophagy in CF airways can be achieved by proteostasis regulators, such as cystamine and its reduced form, cysteamine.[50] As suggested above, these agents also rescue and stabilize F508del-CFTR at the plasma membrane.[48,51]

Improved autophagy activity may be a result of the release of sequestered essential autophagy proteins, such as ATG7, from SQSTM1/p62 collections within F508del aggregates.[10,18,19,39-41] The disassembly of these aggregates would allow for the autophagy molecules to traffic and localize to the phagophore membrane, and also permit folded F508del-CFTR protein to reach the plasma membrane and facilitate chloride transport. This mechanism was suggested by the Luciani group and ours.[10,19,39]

Another potential regulatory element of autophagy is the existence of 2 paralogs of the cluster, *Mir106a-363* and *Mir106b-25*. Each paralog shares high sequence similarity with one another and intersects the predicted targets. The role of autophagy regulation by the paralogs is unknown, although their expression is dispensable early in life, whereas expression of the *Mirc1/Mir17-92* cluster is required for normal development.[52]

In addition to the *Mirc1/Mir17-92a* cluster, other *Mirs* also play a role in the function of the CFTR protein.[25,26,53] *Mir138* was identified to regulate CFTR expression through its interaction with the transcriptional regulatory protein SIN3A.[25] *Mir384, Mir494* and *Mir1246* are involved in the post-transcriptional regulation of CFTR channel synthesis.[25] Individuals carrying the F508del mutation exhibit increased expression of *Mir145, Mir223*, and *Mir494* in bronchial epithelium that correlates with decreased CFTR expression.[16] Therefore, these findings suggest that overexpression of a variety of *Mirs* cooperate to disrupt several functions in the CF cell.

We demonstrate that inherently elevated *Mirc1/Mir17-92* expression contributes to decreased autophagy in CF cells and that defective CFTR protein function is improved when autophagy activity is enhanced by reducing the expression of *Mir17* and *Mir20a*. Several reports demonstrate that partial restoration of CFTR function is sufficient for improving CF pathobiology. More than 80% of CF individuals carry at least one allele with the F508del-CFTR mutation,[54] which leads to misfolding of the CFTR protein preventing its proper trafficking to the plasma membrane.[3,55] These heterozygote individuals express 50% of the normal amount of CFTR protein and secrete 50% of the airway surface fluid and chloride ions compared to healthy non-CF individuals.[56,57] However, heterozygote humans and mice do not exhibit pathological symptoms.[58-60] Furthermore, it is important to note that increasing the expression of F508del-CFTR in only 10% of CF epithelial cells is sufficient to improve the level of CFTR-mediated chloride ion transport.[61] Similarly, expression of CFTR in 25% of airway cells is sufficient to restore normal mucus transport due to proper fluid homeostasis.[15] Therefore, the positive effect we found on autophagy in response to antagomir17 and antagomir20 is promising.

Importantly, cancer has emerged as an increasingly significant problem affecting the CF community as the average life expectancy for CF patients now exceeds 40 y.[62,63] The fact that elevated expression of the *Mirc1/Mir17-92* cluster is associated with several types of malignancies, and our findings that this cluster is elevated in CF patients, raises growing concerns about cancer predispositions in the CF population.[29,64,65] Current strides in research aim to prolong and improve the life of CF patients, however CF patients are prone to different types of cancers later in life.[62,65,66] Elevated expression of the *Mirc1/Mir17-92* cluster in CF may contribute to this predisposition. It has also been shown that the *Mirc1/Mir17-92* cluster is highly expressed in intestinal tissue in CF patients.[26] Should high levels of the *Mirc1/Mir17-92* cluster correlate with increased susceptibility to cancer in CF, it would be even more critical to find approaches to control expression of the *Mirc1/Mir17-92* cluster throughout the patient's life in an effort to improve bacterial clearance and chronic inflammation early in life, and prevent cancer at later stages in life. However, it will be important to carefully titrate the expression of *Mir17* and *Mir20a* to normal physiological levels, rather than abolish them, given the importance of these *Mirs* in regulating diverse biological pathways.

Despite advancements in CF research, this disease remains without a cure. Our data build a foundation for the development of novel therapeutics to improve autophagy and CFTR protein function in CF patients by targeting *Mir17*. This approach can be applied to any cell type in CF should the elevated expression of the cluster be a global phenomenon rather than a macrophage-specific one. This approach may also be used in other disease conditions characterized by weak autophagy accompanied by upregulation of the *Mirc1/Mir17-92* cluster or its members.

Materials and methods

Mice and bone-marrow-derived macrophages (BMDMs)

All animal experiments were performed according to protocols approved by the Animal Care and Use Committee (IACUC) of the Ohio State University College of Medicine. Wild-type (WT) C57BL/6 mice were obtained from Jackson Laboratories. F508del mice were obtained from Case Western Reserve University. *Mirc1/Mir17-92*$^{-/-}$ floxed mice were generously donated from the lab of Dr. Clay Marsh at The Dorothy M. Davis Heart and Lung Research Institute. All mice were housed in the OSU vivarium. BMDMs were isolated as previously described.[10,11]

Immunoblotting

Macrophages were lysed in lysis buffer solution (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 142 mM KCl, 1% Ige-Pal

[Sigma Aldrich, I-3021]) supplemented with a protease inhibitor cocktail (Roche Applied Science, 11 836 170 001). Thirty micrograms of protein were separated by sodium dodecyl sulfate-12% PAGE and transferred to polyvinylidene difluroide membranes (Bio-Rad Laboratories, 162-0117). Membranes were probed for ATG12–ATG5, ATG12, ATG16L1, ATG7 and ACTB/actin (Sigma-Aldrich, A0856, A8731, SAB2501677, A2856 and Abcam ab8226, respectively). Protein bands were detected with secondary antibodies conjugated to horseradish peroxidase; Rabbit: GE Healthcare, NA934; mouse: ThermoFisher Scientific, A28177; goat: Santa Cruz Biotechnology, sc-2020 followed by enhanced chemiluminescence reagents (Amersham/GE Health Care-Life Sciences, RPN 2106)

Confocal microscopy

Immunofluorescence microscopy experiments were performed as previously described.[10,11] Antibodies used to stain autophagosomes were rabbit anti-LC3 (Abgent, AP1805a) followed by fluorescent secondary antibodies (Molecular Probes, A11008). Nuclei were stained with the nucleic acid dye 4′,6′-diamino-2-phenylindole (DAPI). Samples were analyzed with a FluoView FV10i confocal microscope.

Quantitative real-time PCR (qRT-PCR) for expression of Mirs

Total RNA was isolated from cells that were lysed in Trizol (Invitrogen Life Technologies, 15596-026). Chloroform (Fisher Scientific, 268320010), isopropanol (Fisher Scientific, BP2618-212), and glycogen (Fisher Scientific, 10814010) were used to isolate total macrophage RNA and its concentration was measured by Nanodrop. Expression of mature *Mir17, Mir18a, Mir19a, Mir19b, Mir20a, Mir92a, Mir101*, and *snoRNA202* as an endogenous control, were analyzed by first converting the RNA to cDNA by priming with specific primers (Applied Biosystems, Assay ID 2308, 2422, 395, 396, 580, 431, 002253, 001232, respectively) using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, 4366596). PCR was conducted according to the manufacturer's guidelines. For qRT-PCR, cDNA was primed with specific TaqMan primers listed above and assayed using TaqMan Universal PCR MasterMix (Applied Biosystems, 4304437) and Applied Biosystems ABI 7900HT real-time PCR system. Expression was calculated as relative copy numbers. Ct values of each *Mir* were subtracted from the average Ct of the internal control, *snoRNA Snord68/MBII-202* (mouse) or *SNORD48/RNU48* (human), and the resulting ΔCt was used in the equation: relative copy numbers $= (2^{-\Delta\Delta Ct})$

Quantitative real-time PCR for expression of autophagy genes

Total RNA was isolated from cells lysed in Trizol. *Atg5, Atg7*, and *Atg16l1* mRNA expression was assessed using SYBR Green PCR Master Mix (Life Technologies, 4309155). Briefly, Ct values of each target gene were subtracted from the average Ct of the housekeeping gene, *Gapdh*, and the resulting ΔCt was used.

Downregulation of elevated Mir17 and Mir20a in vitro and in vivo

Antagomir17 and antagomir20a (Applied Biosystems, MH12412 and AM10057, respectively) were diluted to 100 nM in phosphate-buffered saline (Gibco, 14190-144) and transfected into macrophages using the Lipofectamine LTX and PLUS reagents (Life Technologies, 15338100) for 48 h according to the manufacturer's instructions. For in vivo studies, 2-mo-old F508del mice were anesthetized via isofluorane (Ohio State University Veterinary Hospital) and intratracheally received antagomir17 and antagomir20 or scramble control (GE Dharmacon, IH-310561-08-0020, IH-310514-07-0020, or IN-001005-01-20, respectively) at 25 μg per mouse in 24-h intervals for 72 h.[67,68] The dose was derived from our previous publications.[69] The antagomirs were reconstituted in siRNA buffer (GE Dharmacon, B2000UB100) and diluted in 1X phosphate-buffered saline prior to administration. After 3 treatments, the mice were sacrificed and lungs were isolated. One lobe of the lung was homogenized in Trizol for RNA isolation. The other lobe was homogenized in lysis buffer with protease inhibitor, as described previously, for protein analysis. Homogenization was accomplished using the Qiagen Tissue Lyser (Qiagen, 85600) and accompanying 5-mm stainless steel beads (Qiagen, 69989). Another set of mice was treated as described above then infected intratracheally with *B. cenocepacia* and colony-forming units (CFUs) were quantified from homogenized lungs as previously described.[10]

Luciferase reporter assays

The NIH3T3 cell line was co-transfected with a plasmid containing either the *Mir* 3′UTR target clone for murine *Atg7, Atg16l1* or control vector (GeneCopoeia, MmiT035820-MT01, MmiT036321-MT01 or CmiT000001, respectively) and *Mir* mimics to *Mir17* and *Mir20a* (Applied Biosystems, 4464066 or AM17101, respectively) using Lipofectamine 2000 according to the manufacturer's protocol. Antagomir17 and antagomir20a were also transfected separately to downregulate endogenous *Mir17* or *Mir20a* expression within the NIH3T3 cells. Firefly and Renilla Luciferase activities were measured consecutively by using the Dual-Luciferase Reporter Assay system (Promega, E1910) 24 h after transfection. The seed regions (5′ nucleotides 2 through 8) of mature *Mir17* and *Mir20a/b* are predicted to bind with perfect complementarity to the sequence 5′-GCACUUU-3′, which is present in the *Atg7* and *Atg16l1* 3′UTR. Site-directed mutagenesis was used to introduce 3 point mutations within these 7 nucleotides of the *Atg7* and *Atg16l1* 3′UTR luciferase reporters to further test *Mir17* and *Mir20a/b* binding specificity. Primers for introducing point mutations were designed using the QuikChange Primer Design online tool to be compatible with the QuikChange Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies, 210514). A list of mutagenesis primers can be found in Table S2.

CFTR function

CFTR channels can transport chloride as well as iodide. Therefore, CFTR function was assessed by measuring iodide efflux

using the fluorescent and halide-sensitive dye 6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ) as previously described by our group.[70] Macrophages were briefly plated in a 96-well plate. Cells were loaded with SPQ (Molecular Probes, M-440) using hypotonic shock and were incubated with 10 mM SPQ in Opti-MEM:water (1:1) for 15 min at 37°C. Cells were then washed and incubated twice for 10 min with fluorescence quenching NaI buffer (130 mM NaI, 5 mM KNO_3, 2.5 mM $Ca[NO_3]_2$, 2.5 mM $Mg[NO_3]_2$, 10 mM D-glucose, 10 mM *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic] acid/HEPES, pH 7.4). Subsequently, cells were switched to a dequenching isotonic $NaNO_3$ buffer (identical to NaI buffer except that 130 mM NaI was replaced with 130 mM $NaNO_3$) in the presence of 20 μM forskolin (Abcam, ab120058) and 100 μM 8-(4-chlorophenylthio)-c-AMP (Sigma-Aldrich, c3912) to activate CFTR. Nonspecific increase in fluorescence was measured by incubating the cells with the activation cocktail and the specific CFTR inhibitor GlyH101 (10 μM; Calbiochem, 219671. Fluorescence was measured using the plate reader VICTOR X3 (Perkin Elmer) with excitation wavelength at 350 nm and DAPI emission filter.

Statistical analysis

All experiments were performed at least 3 independent times unless stated otherwise and yielded similar results. Comparisons of groups for statistical difference were done using 2-tailed Student *t* test or ANOVA when described. P-value ≤ 0.05 was considered significant. * indicates $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.

Abbreviations

3-MA	3-methyladenine
BMDMs	bone marrow-derived macrophages
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFUs	colony-forming units
*Mir*s	microRNAs
qRT-PCR	quantitative real-time PCR
SPQ	6-methoxy-N-(3-sulfopropyl)-quinolinium
UTR	untranslated region
WT	wild type

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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