Elevated miR-155 promotes inflammation in Cystic Fibrosis by driving hyper-expression of Interleukin-8

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Cystic Fibrosis (CF) is characterized by a massive pro-inflammatory phenotype in the lung arising from profound expression of inflammatory genes, including interleukin-8 (IL-8). We have previously reported that IL-8 mRNA is stabilized in CF lung epithelial cells, resulting in concomitant hyper-expression of IL-8 protein. However, the mechanistic link between mutations in CFTR, and acquisition of the pro-inflammatory phenotype in the CF airway, has remained elusive. We have hypothesized that specific microRNAs might mediate this linkage. To identify the potential link, we have screened a miRNA library for differential expression in [ΔF508]-CFTR and [wildtype]CFTR lung epithelial cell lines. Of 22 differentially and significantly expressed miRNAs, we find that expression of miR-155 is more than 5-fold elevated in CF IB3-1 lung epithelial cells in culture, compared to control IB3-1/S9 cells. Clinically, miR-155 is also highly expressed in CF lung epithelial cells, and circulating CF neutrophils biopsied from CF patients. We report here that high levels of miR-155 specifically reduce levels of SHIP1, thereby promoting PI3K/Akt activation. However, over-expressing SHIP1 or inhibition of PI3K in CF cells, suppresses IL-8 expression. Finally, we find that phospho-Akt levels are elevated in CF lung epithelial cells, and are specifically lowered by either antagomir-155, or elevated expression of SHIP1. We therefore suggest that elevated miR-155 contributes to the pro-inflammatory expression of IL-8 in CF lung epithelial cells, by lowering SHIP1 expression, and thereby activating the PI3K/Akt signaling pathway. These data suggest that miR-155 may play an important role in the activation of IL-8-dependent inflammation in CF.

Introduction

Cystic fibrosis (CF)², is the most common autosomal recessive disease in the U.S. and Europe, is caused by mutations in the cystic fibrosis trans-membrane conductance regulator (CFTR) gene (1-4). CFTR mutations, of which the most common is [ΔF508]CFTR, cause a massive pro-inflammatory phenotype in the lung, which manifests in the airway by high levels of IL-8 and other pro-inflammatory cytokines and chemokines. (5-7). IL-8 is the most potent known chemotactic agent for neutrophils (8), and is constitutively secreted from CF lung epithelial cells (9). The enhanced secretion of IL-8 seems to be an intrinsic property of the CF epithelium, since fetal CF lung epithelium also spontaneously secretes high IL-8 levels...
into the airway as early as the 15th week of gestation (10,11). However, the mechanism by which a mutation in CFTR causes up-regulated levels of IL-8 expression remains poorly understood.

The expression of pro-inflammatory genes such as IL-8 is known to be regulated by post-transcriptional mechanisms. For example, the stability of mRNAs encoding many inflammatory genes, including IL-8, is regulated by interactions between AU-rich elements (AREs) in the 3'-untranslated region (3'-UTR), and specific ARE binding proteins. In the case of CF lung epithelial cells in culture, which constitutively secrete high levels of IL-8, we have recently reported that high levels of IL-8 mRNA are sustained by mutation-dependent reduction in the ARE binding protein tristetraprolin (TTP) (12). We also find a similarly low level of TTP in primary CF lung cells, which had been obtained acutely by brush biopsy of CF patients. Finally, we find experimentally that elevation in TTP directly reduces the stability of IL-8 mRNA, and, concomitantly, reduces the level of secreted IL-8. Thus hypersecretion of IL-8 from the CF lung epithelium seems to involve a loss of post-transcriptional regulation by TTP. However, it also became clear that low levels of TTP might not be the only mechanism underpinning CF lung inflammation. For example, as mentioned above, the CF airway is characterized not only by high levels of IL-8, but also by high levels of TNFα, IL-6, and many other potent pro-inflammatory analytes (5-7). Furthermore, many of the CF-specific pro-inflammatory processes have been associated with up-regulation of both TNFα/NFκB signaling (13,14), and TGFβ-1 signaling (15). Thus the CFTR mutation appears to target a pro-inflammatory regulatory mechanism with simultaneous deleterious effects on many pro-inflammatory genes.

MicroRNAs (miRNAs) may provide such a pleiotropic mechanism. miRNAs mediate mRNA instability by action in the 3'-UTRs of target genes (16-19). There are nearly 1000 unique miRNAs in the human genome, each of which individually targets ca. 200 different mRNAs (20-25). Furthermore, it has been reported that, in response to certain kinds of stress, miRNAs can switch from a normally translational repressor mode to that of a translational activator (26,27). Taken together, it appears that a relatively few miRNAs can regulate as much as 20-30% of the human genome. Recently, specific miRNAs have been reported to be associated with diabetes (28,29), cancer (30-33), heart disease (34,35), cell cycle (36), and development (17). Importantly, functional suppression of miRNAs can be achieved, both in vitro and in vivo, by antagonirs, which are chemically engineered oligonucleotides that are anti-sense to miRNAs (37). However, the possibility of an association between specific miRNAs and the pathophysiology of cystic fibrosis has not yet been described. Thus, if aberrant elevation of a CF-specific miRNA could be identified, then it might lead to a candidate therapeutic agent.

We report here the results of a hypothesis-based discovery study for miRNAs associated with the pro-inflammatory phenotype for cystic fibrosis. We specifically hypothesized that uniquely expressed miRNAs might aid in identifying the mechanism by which mutant CFTR induces the characteristic pro-inflammatory phenotype in the CF lung. To test this hypothesis, we have performed a comprehensive and systematic analysis of all miRNAs in CF IB3-1 lung epithelial cells that are differentially affected by the presence of natural abundance [ΔF508]CFTR. Among 22 miRNAs that are aberrantly expressed in CF cells, we have identified miR-155 as the most abundantly elevated species, both in vitro and in vivo. Furthermore, we find that a reduction in miR-155, mediated by either antisense or antagonir constructs, results in suppression of IL-8 mRNA, and concomitantly reduced
expression of IL-8 protein. The mechanism of miR-155 action in CF cells is to inhibit translation of SHIP1, an inositol phosphate phosphatase. Loss of SHIP1 results in elevated signaling of the PI3K/Akt pathway, with downstream effects on the IL-8 system. We suggest that miR-155 may play an important role in the regulation of inflammation in CF lung epithelial cells.

**Experimental Procedures**

**Reagents**- LHC-8 media, Trypsin-EDTA (0.05%) and Lipofectamine transfection Reagent were purchased from Invitrogen (New York, NY). Bronchial Epithelial Growth Media (BEGM) and the normal human bronchial epithelial cells (NHBE) were purchased from Lonza. miRVana kit and RiboPure kit for isolation of total RNA from CF cells was obtained from Ambion Inc. (Austin, TX). Taqman Low Density V1 arrays, miRNA primer pools, and pMIR-Report Luciferase vector were purchased from Applied Biosystems (Foster City, CA). Wortmannin was purchased from EMD Chemicals (Gibbstown, NJ), and CFTRinh-172 was obtained from Sigma Chemical (St. Louis, MO).

**Cell culture**- IB3-1 CF lung epithelial cells and the control CFTR-repaired IB3-1/S9 cells were maintained in LHC-8 serum free medium in humidified 5% CO₂ as previously described (13).

**RNA isolation**- Total RNA was isolated from the IB3-1 and IB3-1/S9 cells using miRVana isolation kit (Ambion). The primary bronchial epithelial cells were obtained from lung brush biopsies of CF patients as described earlier (12) and the blood was collected from CF patients and controls under a USUHS Institutional Review Board approved protocol. The cells were stored in RNA later or Trizol and total RNA was isolated using the miRVana kit.

**Real-Time Quantification of miRNAs by Stem-Loop RT-PCR**- Multiplex Reverse Transcription was performed with TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). Following reverse transcription, each RT reaction was diluted and mixed with TaqMan Gene expression Master Mix (2X). 100 µl of the RT reaction-specific PCR reaction mix was loaded into the corresponding fill ports of the TaqMan Low Density Human MicroRNA Panel v1.0 (Early Access). The CF brush biopsy samples were similarly analyzed using the Taqman v2.0 Low Density Arrays. Individual microRNA assays were performed using specific TaqMan MicroRNA assays Kit (ABI).

**miRNA expression arrays**- miRNA expression arrays were probed essentially as described (Ambion). Five micrograms of total RNA from IB3-1 or IB3-1/S9 cells were end-labeled with 30 µCi of γ³²P dATP (3000 Ci/mmmole) by T4 polynucleotide kinase and purified using the QIAgen Nucleotide Removal Kit. Membranes were first prehybridized in MicroHyb Hybridization Buffer (ResGen) at 37°C for at least 30 min followed by an overnight hybridization in the same solution containing RNA probe. Following hybridization membranes were washed twice with 2 × SSC/0.5% SDS at 37°C. The second wash was performed in 1 × SSC/0.5% SDS at 37°C. Membranes were exposed to a phosphor storage screen, scanned using Phosphor Imager, and hybridization signals were quantified using Image Quant software (Molecular Dynamics).

**Statistical Data Analyses**- Real Time PCR data was analyzed using the R and the Bioconductor package, as well as with STATMINER (statistical analyses package from ABI). Data were filtered for Ct values <35 and the data were normalized to the endogenous control gene RNU44. If an assay measurement was not detected in both experimental subgroups, the assay was not included in the pairwise statistical analysis. Furthermore, if an assay measurement was not detected in more than 50% of samples in an
experimental sub-group, it was deemed undetected for that subgroup. For all detectable assays, an un-paired student t-Test was performed on the delta Ct values. Adjusted p-values (False Discovery Rates) were calculated using the Benjamini Hochberg procedure. Fold changes were calculated using the comparative Ct method. Hierarchical clustering based upon Euclidean distances was performed on differentially expressed samples with p-values <0.05.

**Illumina mRNA expression processing and analysis** - Beadarray data were obtained from whole-genome expression HumanRef-8 v2.0 as well as human HT-12 BeadChips using the iScan System and BeadScan software (Illumina, San Diego, CA). Non-background, non-normalized array data was generated using BeadStudio 3.2.7 software. Preprocessing of array data by model-based or offset background correction and robust spline normalization was performed using MATLAB or the Lumi 1.8.3 package from Bioconductor 2.3 on the R 2.8 programming language platform. The processed Illumina array data is MIAME compliant and has been submitted to the NCBI Gene Expression Omnibus (GEO) database. Processed array data was analyzed using Ingenuity Pathways Analysis (IPA). MicroRNA and mRNA relationship analysis was generated using TargetScan Release 5.1 (Whitehead Institute) for miRNA biological target prediction and Ingenuity Pathways Analysis (IPA).

**RESULTS**

**CF lung epithelial cells express mutation-specific miRNAs.** As shown in Table 1, we have used two independent methods to identify CF-specific microRNAs in CF lung epithelial cells. Using the new technology, quantitative Taqman® qPCR miRNA array platform, we find that of 365 miRNAs tested, only 22 significantly distinguish between the natural abundance IB3-1 CF cell, and the [wildtype]CFTR-repaired daughter cell, IB3-1/S9 (see Table 1, left panel). For this analysis we analyzed three independent cultures of both IB3-1 and IB3-1/S9 cells, and identified any miRNAs for which the fold difference was at least ca. 50%, and the P value for the difference was <0.05. Of the 22 differentially expressed miRNAs, 18 were elevated in the CF cells, and four were reduced. The data in Table 1, ordered by fold change, indicate that the miRNAs with the highest differential expression (> 4-fold) are miR-155 and let-7c. We also validated the miR-155 and miR-let7c data with independent Taqman® miRNA assays (see Figure 1B). Of the remaining 16 elevated miRNAs, all were changed by 1.3 to 3.4-fold. Of the four down-regulated miRNAs, only miR-615 has a > 2-fold reduction, while miR-660, miR-194 and miR-192 exhibit reductions only in the 1.2 to 1.6-fold range.

However, these differently expressed miRNAs all appear to contribute to a composite CF microRNA signature. For example, Figure 1A shows that when all 22 microRNAs are compared using a hierarchical cluster algorithm, the dendrogram clearly distinguishes between three independent experiments with CF IB3-1 and three independent experiments with CFTR-repaired IB3-1/S9 daughter cells. Thus, in spite of quantitative differences in expression, all of the significantly aberrant miRNAs, from the most aberrant miR-155 to the least, appear to make a concerted contribution to the CF phenotype in the CF IB3-1 lung epithelial cell system.

To further validate the Taqman® data, we also tested the CF lung epithelial cell samples using a conventional, semi-quantitative Ambion Bioarray® platform (see Table 1, right panel). The data with this different type of system consistently replicated 12 of the 18 miRNAs predicted by the Taqman® platform. The common directions of change are indicated by up-or down arrows. Horizontal double headed arrows indicate saturated
expression. The validated miRNAs included miR-155 and many of the other miRs with predicted high-fold differences. Several of the low-fold changes are also confirmed. However, consistent saturation problems were encountered with two of the high-fold different miRs, miR-let7c, and miR-21, and were not pursued further. Inasmuch as we had independently validated the miR-let7c by PCR (Figure 1B), we did not pursue this technical saturation problem further. Either of the microRNAs with ≤ 2-fold difference from control, 6 were not detectable at all on the microarray platform (marked “ND”). Based on low levels in either platform, we also chose to not pursue this technical sensitivity problem any further. We therefore conclude that both the quantitative Taqman® and the semi-quantitative Ambion Bioarray® platforms agree with similar identifications for most of the miRNAs with high fold-differences between CF IB3-1 and [wildtype]CFTR-repaired IB3-1/S9 cells, including miR-155.

Ex-vivo clinical CF cells also exhibit elevated miR-155 levels. To test the extent to which microRNA data from the IB3-1 cell system might parallel microRNA expression in cells from CF patients, we used the Taqman® qPCR miRNA array platform to measure microRNA expression in bronchial brushings and neutrophils obtained from CF patients homozygous for ΔF508 mutation. As a control for the CF lung epithelial cells obtained by brush biopsy, we measured miR-155 in primary non-CF normal human bronchial epithelial cells (NHBE). As a control for CF neutrophils, we used neutrophils from normal individuals. Of the 22 differentially expressed microRNAs found to be differentially expressed in the CF IB3-1 cell system, only massively elevated miR-155 expression was noted. We therefore validated this finding using independent Taqman® miRNA assays. As shown in Figure 1C, we find that miR-155 is significantly elevated in CF bronchial brushings (CFBB vs NHBE, 10.8-fold elevated), and in CF neutrophils (CD66+ CF vs control neutrophils, 2.4-fold elevated). We conclude that these differences therefore appear to closely parallel the differences in the CF lung epithelial IB3-1 cell system (IB3-1 vs IB3-1/S9, 8.6-fold elevated). Additionally, the inhibition of wt-CFTR function with CFTR-172 inhibitor in CFBE41o-wtCFTR cells, a human bronchial epithelial cell line, has been shown to produce a similar effect on inflammatory signaling as is observed in CF cells (38). As depicted in Figure 1D, treatment of the IB3-1/S9 CFTR-repaired controls cells with CFTR-172 inhibitor induces an 8-fold up-regulation of miR-155. These data indicate that elevation in miR-155 expression is closely tied to failure of CFTR channel activity, either by chemical inhibition or by mutation. The data thus consistently suggest that further specific interest in miR-155 is warranted.

Aberrantly expressed miRNAs have predicted effects on mRNA expression in CF cells. To further understand the mechanism by which miR-155, and the other CF-specific microRNAs, might affect the CF phenotype, we used the ILLUMINA® bead chip system to analyze levels of ca. 24,000 transcripts, in both CF IB3-1 and [wildtype]CFTR-repaired IB3-1/S9 cells. Table 2 is ordered by starting with top-ranked miR-155, and then proceeding in the order of declining values of fold-elevation, followed by fold-reduction in CF cells. For each of the miRNAs, the changes are listed for the top six specific mRNAs, chosen from among the predicted target mRNAs for the respective miRNA. In each box, mRNAs that are targeted by more than one of the CF-specific microRNAs are bolded.

For example, in the case of miR-155, the top six reduced mRNAs are shown. Two mRNAs, ELL2 (Elongation Factor, RNA Polymerase II, 2; 1.98-fold reduced) and
PIK3R1 (Phosphoinositol 3 kinase regulatory subunit 1 [p85α, GRB1]; 1.63-fold reduced), are in bold, meaning that they are predicted to also be targets of other miRs in this dataset. In this instance, they are also targeted by miR-576-5p and miR-21, respectively. The remaining top four miR-155-dependent mRNAs include HMGB3 (High Mobility Box 3; 1.94-fold reduced), INADL (Channel-Interacting PDZ Domain Protein, 1.75-fold reduced), MIDN (Midbrain Nucleolar Protein, 1.66-fold reduced), and INPP5D (phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 [SHIP1], 1.56-fold reduced).

Each of the sub-tables in Table 2 can be analyzed in the same way. However, since we wish to establish a basis for experimental priority among these 22 microRNAs, we are faced with a complex problem. The hierarchical cluster analysis in Figure 1A suggests that all 22 miRNAs may cooperate to affect the CF phenotype. However, as suggested by Table 2, the bolded target mRNAs suggest that confluence of effects could be due multiple microRNAs affecting expression of more than one mRNA. Although this could be a basis for cooperation, it does not help with the priority problem. Alternatively, one mRNA, which is affected by a specific miRNA, could have an indirect, downstream effect on an entirely separate mRNA, and thus its mRNA targets. Finally, there might be multiple effects of the CFTR mutation on multiple miRNAs. To bypass these complexities for the moment, we have decided to focus on miR-155. The practical reasons are (i) that miR-155 exhibits the highest level of CF-specific difference; (ii) that miR-155 is the only known microRNA in the human genome to target INPP5D/SHIP1, a well known effector for regulation of inflammation; and (iii) ex-vivo CF lung epithelial cells and CF neutrophils also express high levels of miR-155.

**Antagomir-155 reduces miR-155 and IL-8 expression in CF cells.** Since miR-155 expression has been consistently associated in the literature with suppression of inflammation and cancer (39-43), we hypothesized that down-regulating miR-155 might also suppress the IL-8-associated pro-inflammatory phenotype in CF cells. To reduce the levels of miR-155 in CF cells, we decided to deploy an antagonim against miR-155. Antagomirs are metabolically stable antisense constructs against specific microRNAs, which are further modified with cholesterol or other moiety to permit efficient entry into cells. We therefore synthesized antagomir-155, and a scrambled control, and tested each for potency and efficiency as a suppressor of both miR-155 and IL-8 expression in our CF cell system. As shown in Figure 2A, incubation of IB3-1 CF cells with antagomir-155 (2.5 μM) effectively down-regulates miR-155 expression by ca. 85%. Concomitantly, we observed a corresponding decrease in IL-8 mRNA levels of ca. 70% (Figure 2A), as well as a ca 11-fold decrease in IL-8 protein levels (see Figure 2B). The scrambled antagonim control has no effect on miR-155 expression (see Figure 2A). These data thus firmly establish the specificity of antagomir-155 as a negative effector for IL-8 expression in CF lung epithelial cells, both ex-vivo and in cell culture.

**Inflammation-related mRNAs are targeted by antagomir-155 in CF cells.** We next used the ILLUMINA bead chip HT-12 array system to identify those pro-inflammatory or anti-inflammatory mRNAs, in addition to IL-8, which were significantly targeted by antagomir-155 in CF lung epithelial cells. We find 34 mRNAs in this category whose expression is significantly affected by treatment with antagomir-155. Using a conventional hub-and-spoke connectivity analysis, Figure 2C uses a color code to depict when the change is elevated (red), or reduced (green). The spokes identify...
literature-based relationships among the individual mRNAs. As expected, the major down-regulated hubs included IL-8 (7 spokes), but also IL-6 (5 spokes), and ITGB2 (integrin beta 2, 7 spokes). Major up-regulated hubs included IL-10 (8 spokes) and RAC1 (7 spokes). However, all of the hubs are connected, either directly or indirectly, and many of the low spoke number singlets and doublets have important inflammatory functions. We conclude that the collective and concurrent changes in expression of the cytokine and chemokine genes in response to antagomiR-155 treatment can contribute to attenuation of the inflammatory phenotype in CF cells. Importantly, the reduced mRNAs (green coded), such as IL-8 and IL-6, are only indirectly connected to the antagomir-155, since a direct effect on the mRNAs would have caused the signals to increase, not decrease. The problem to be solved is therefore what that indirect connection might be in the CF cell.

**miR-155 targets SHIP1 in CF cells to promote mRNA stability of the IL-8.** Although INPP5D/SHIP1 is not a classical inflammation-associated gene, and is not included as such by the IPA database in Figure 2C, it is predicted to be targeted only by miR-155 (44). Furthermore, low levels of SHIP1 have recently been directly linked to pro-inflammatory processes involving granulocyte/monocyte populations, through regulation by elevated miR-155 (44,45). We therefore hypothesized that high levels of miR-155 in CF cells might also directly affect the CF pro-inflammatory phenotype by suppressing SHIP1 expression. We further anticipated that antagomir-155 might reverse the process and the phenotype. Using conventional qPCR, we find that that SHIP1 mRNA is expressed at a relatively high level in control IB3-1/S9 cells (ca. 3.5 fold) compared to CF IB3-1 cells (see Figure 3A). As further shown in Figure 3A, we find that suppression of miR-155 in CF cells with Antagomir-155 also up-regulates SHIP1 mRNA expression by ca. 3-fold. Consistently, we had observed similar suppression of SHIP1 mRNA expression in IB3-1 cells, compared to IB3-1/S9 control cells, when measured by the Illumina bead chip array platform (see Table 2). In this case, we had observed a ca. 1.6 fold reduction in INPP5D/SHIP1 in CF IB3-1 cells, when miR-155 levels were elevated by ca. 5 fold (see Table 1). Thus both conventional qPCR and Illumina-based methods suggest that SHIP1 expression is systematically and significantly reduced in CF cells, but can be elevate to near control levels when CF cells are treated with antagomir-155.

To gain more direct evidence that SHIP1 might be the target of miR-155 in CF cells, we developed a luciferase reporter assay in which the pMIR-Report vector was constructed with [wildtype] or [mutant] SHIP1 3′-UTR sequences. These reporter plasmids were then transfected into CF IB3-1 cells. As an alternative to treatment with the antagomir-155 construct, we further transfected the CF cells with a conventional anti-miR-155. A demonstration that anti-miR 155 knocks down levels of miR-155 in a dose-dependent manner in CF IB3-1 cells is shown in Supplemental Figure S2. As shown in Figure 3B, we find that [wildtype] SHIP1 3′-UTR diminishes luciferase expression, while luciferase expression is returned towards control levels by a partially inactivating mutation engineered into the SHIP1 3′-UTR. However, when these cells are additionally transfected with anti-miR-155, to knock down miR-155 expression, the luciferase reporter activity is only slightly elevated over control, and is independent of whether or not [wildtype] or [mutant] SHIP1 3′-UTR sequences in the luciferase reporter are also present.

In previous work we have shown that IL-8 mRNA is greatly stabilized in CF cells, both in vitro and in vivo, and that IL-8 protein
expression rates were also elevated as a consequence (12,46). We therefore hypothesized that if SHIP1 levels were exogenously elevated in CF cells, then the half-life of IL-8 mRNA would be reduced, and the expression levels of IL-8 protein would also be reduced. To test this hypothesis, we over-expressed SHIP1 in CF IB3-1 cells, and used qPCR to measure the levels of IL-8 mRNA following actinomycin D treatment. As shown in Figure 3C, following 16 hours post-transfection with an expression plasmid for SHIP1, the half-life of IL-8 in the CF IB3-1 cells was reduced nearly 3-fold when SHIP1 was over-expressed. Corresponding to the enhanced degradation of IL-8 mRNA, we also concomitantly observed a significant reduction in IL-8 protein secretion into the incubation medium at the 16 hour post-transfection time point (see Figure 3D). These data clearly show a close relationship between miR-155 dependent low SHIP1 levels in the CF cell, and the pro-inflammatory CF phenotype manifest by stabilized IL-8 mRNA and elevated IL-8 protein expression.

To further test the potentially countervailing effects of miR-155 and SHIP1, we compared the influence of antagonir-155 and siRNA-mediated knockdown of SHIP1 in CF cells. As shown in Figure 4A (effect on IL-8 mRNA), and Figure 4B (effect on SHIP1 mRNA), knockdown of SHIP1 in CF cells reduces the already low levels of SHIP1 (see Figure 4B) by a further 40%. As anticipated, there is no measurable effect on the already high levels of IL-8 mRNA (see Figure 4A). As also anticipated, the treatment of the CF cells with antagonir-155 doubles the level of SHIP1 mRNA (see Figure 4B), while reducing IL-8 mRNA levels by ca. 70% (see Figure 4A). However, when antagonir-155 and siSHIP1 are simultaneously added to the CF cells, SHIP1 mRNA levels are significantly reduced towards SHIP1 knockdown levels, while IL-8 levels are slightly, but significantly elevated towards SHIP1 knockdown levels. Thus miR-155 and SHIP1 have mutually countervailing effects on SHIP1 and IL-8 mRNA levels in CF lung epithelial cells. The next critical question is how the information from the miR-155/SHIP1 system is translated to effects on IL-8 mRNA stability in CF cells.

**SHIP1 destabilizes IL-8 mRNA by suppressing PI3K/Akt signaling.** The default function of SHIP1, an inositol 5 phosphatase, is to interfere with PI3K signaling to Akt. The enzymatic function of PI3K is to convert PIP2 to PIP3, and the PIP3 product functions to activate Akt (protein kinase B). However, SHIP1 hydrolyzes PIP3 back to PIP2, thereby blocking PI3K signaling to Akt. Therefore, to test whether this PI3K activity per se might be relevant to how low levels of SHIP might lead to stabilization of IL-8 mRNA in CF cells, we treated CF IB3-1 cells with the PI3K inhibitor Wortmann, and tested for effects on IL-8 mRNA stability. As shown in Figure 5A, 300 nM Wortmann causes a reduction in IL-8 mRNA stability of ca. 50% (~2-fold change in half-life). Thus either raising the levels of SHIP1 to hydrolyze the PI3K product PIP3 to PIP2 (Figure 3C), or blocking primary production of PIP3 itself, as evidenced by blocking PI3K with Wortmann (Figure 5A), have the same destabilizing consequences for IL-8 mRNA in CF cells.

This result suggest that SHIP1-dependent failure of Akt activation by the PI3K product of PI3K activity may be a crucial signaling intermediate in stabilizing IL-8 mRNA in CF lung epithelial cells. To test this hypothesis, we examined the influence of antagonir-155, and SHIP1 over expression, on the activation state of Akt. Figure 5B shows that the parental CF IB3-1 cell has high resting levels of phospho-Akt, compared to levels in the [wildtype]CFTR-repaired IB3-1/S9 cells. This is consistent with the low levels of SHIP1, which we have shown are typical of CF cells. However, to further test whether Akt
activation in CF cells was specifically, and reciprocally, dependent upon miR-155 and SHIP1, we treated IB3-1 cells with either antagomir-155 or the SHIP1 expression plasmid. As shown in Figure 5B, antagomir-155, by lowering miR-155 expression, reduces activation of Akt to virtually undetectable levels. On the other hand, expression of SHIP1 in the CF cells raises phospho-Akt levels to approximately 50% of the levels seen in the [wildtype]CFTR-repaired IB3-1/S9 cells. We conclude that the mechanism by which miR-155 induces stabilization of IL-8 mRNA depends directly on suppression of Akt signaling by SHIP1.

**miR-155 stabilizes IL-8 mRNA by activation of MAPKinases.** In CF cells, both in vitro and in vivo, we have previously identified intrinsically reduced levels of the ARE-interacting protein tristetraprolin (TTP) (12), and increased levels of MAPK signaling (46), as being independently responsible for IL-8 mRNA stabilization. Nonetheless, in these previous studies, the CF-specific drivers for either of these specific activities had yet to be identified. As shown in Figure 6A, the baseline transcriptome for CF IB3-1 versus repaired IB3-1/S9 cells shows that in the CF cells there are intrinsic elevations (color-coded in red) in mRNAs for PI3K/ p85 and PDK1. Elevated mRNAs are also found along the downstream signaling pathway towards mTOR, including TSC2, Rheb and mTOR itself. The downstream PI3K/AKT effector c-Raf is reduced in expression, but MEK1/2 and ERK1/2 are, as anticipated, significantly elevated. However, as shown in Figure 6B, when these cells are treated with an anti-miR-155, the mRNA for PI3K/p85 is reduced in CF cells relative to control, as is mTOR, and the entire signaling pathway comprising c-RAF, MEK1/2, and ERK1/2. Thus elevated levels of miR-155 differentially control downstream levels and activation of MAPK activities. These data therefore suggest that the mechanism by which elevated miR-155 causes stabilization of IL-8 in CF lung epithelial cells is through loss of SHIP1, activation of PI3K/Akt signaling, and downstream activation of MAPK signaling.

**DISCUSSION**

In this paper we show that CF lung epithelial cells, both in vitro and in vivo, hyperexpress miR-155. The downstream consequence of elevated miR-155 includes activation of PI3K/Akt signaling through specific reduction in SHIP1. Furthermore, we show here that in CF cells, intrinsic activation of PI3K signaling occurs through intrinsic miR-155-dependent down-regulation of SHIP1. This leads to activation of downstream MAPKs, with consequent stabilization of IL-8 mRNA, and hyper-expression of IL-8 protein (46). SHIP1, an inositol 5-phosphatase, controls PI3K/Akt signaling by hydrolyzing the PIP3 product of PI3K enzymatic activity to PIP2. Thus the low levels of SHIP1 found in CF cells results in elevated levels of PIP3, and consequent elevated signaling of the PI3K/Akt pathway. We have validated this conclusion by raising or lowering miR-155 using antagomir-155, or using anti-miR-155. We also use expression plasmids and siRNA for SHIP1 to verify that raising or lowering levels of miR-155 is mirrored by reciprocal effects of SHIP1. Thus, as summarized in Figure 7, the effects of raising miR-155, lowering SHIP1, and activating PI3K/Akt is to activate MAPKinases, thereby stabilizing IL-8 mRNA and increasing the levels of IL-8 protein expression. These data thus lend further support to the concept that the pro-inflammatory phenotype of the CF airway is due to increased stability of the IL-8 mRNA and subsequent increased expression of IL-8 protein (12,46).

**SHIP1 and PI3K/Akt signaling** The only predicted and validated microRNA to target SHIP1 is miR-155 (44), and the connection of
SHIP1 to activation of PI3K/AKT signaling has been validated using both pharmacologic and molecular methods. For example, in both hematologic and other cell types, repression of endogenous SHIP1, caused by over expression of miR-155, leads to an increase in Akt signaling (47). This increase in Akt activity is secondary to the loss of SHIP1, and thus failure to hydrolyze PIP3, the active product of PI3K enzyme activity (48,49). Consistently, metabolically stabilized analogues of PIP3 (PtdIns(3,4,5)P3), utilizing methylene phosphonates or phosphorothioates in place of phosphate, have been shown to inhibit SHIP1, thereby ensuring long-lived PI3K/Akt activation (50). A different type of chemical inhibitor for SHIP1, 3-alpha-aminocholestane, has a similar biological effect on blood cell production and numbers of immune regulatory cells in the circulation (51). The opposite experiment, with small molecule agonists of SHIP1, results in inhibition of PI3K/AKT signaling (52). It has also been suggested that SHIP1 can interact directly with the PI3K complex by means of an adaptor protein, ITAM-containing receptor (53).

At the level of intact animals, knockout mice for SHIP1 have been created and studied. It has been reported, for example, that in SHIP1(-/-) knockout mice, there is an increased number of neutrophils and macrophages, due to enhanced progenitor survival. Consistently, it has been demonstrated in many systems that PI3K activity is necessary for survival of different types of progenitor cells (54,55). The SHIP1(-/-) mice also exhibit osteoporosis and significant neutrophil infiltration into the lungs (48). It is possibly not just by coincidence alone that both osteoporosis and neutrophil lung infiltrates are characteristic of the CF phenotype in humans.

miR-155-dependent activation of PI3K/Akt signaling and downstream activation of MAPKinases. In CF cells, both \textit{in vitro} and \textit{in vivo}, we have previously shown that increased levels of MAPK signaling contribute to IL-8 mRNA stabilization (46). Nonetheless, in these previous studies, the CF-specific driver for MAPK activation had yet to be identified. In the case of IL-8 mRNA stabilization by MAPK signaling, the experiments showed destabilization of IL-8 mRNA and reduced expression of IL-8 protein by both pharmacological and molecular inhibitors of ERK1/2 (\textit{viz.}, U0126; or Mnk-1), p38 (\textit{viz.}, SB203580; or p38-AGF), the mediator of p38 action, MK2 (\textit{viz.}, MK2-KR), or JNK-1/2 (\textit{viz.}, SP600125; or JNK-APF and MEK7). Thus the chemical biology of MAPK-dependent stabilization of IL-8 mRNA is well understood at the pharmacological and molecular levels. However, the puzzle had remained as to how mutations in CFTR might cause MAPK activities to be intrinsically elevated in CF cells, both \textit{in vitro} and \textit{in vivo}.

The new data in Figure 6 suggest that elevated miR-155 contributes to the mechanism of intrinsic MAPK activation and dependent IL-8 mRNA stabilization in CF cells. The data in this paper clearly show that elevated levels of miR-155 cause decreased levels of SHIP1, with enhanced PI3K/Akt signaling as a principal consequence. Mechanistically, activation of PI3K converts PIP2 to PIP3, which binds to and activates PDK1 (PI3K Dependent Protein Kinase 1). With SHIP1 levels low in CF cells, there is more PIP3 available. Activated PDK1 then binds to and phosphorylates Akt (Protein Kinase B). This activated complex has multiple signaling possibilities including interaction with mTOR, and activation of cRAF and Rheb. cRAF then activates MEK1/2, ERK1/2, and other MAPK elements. The data in Figure 6 clearly substantiate this hypothesis in terms of reciprocal miR-155 changes in mRNA expression for the individual signaling proteins. However, exactly how activated MAPKs stabilize IL-8 mRNA remains a subject for future study.
Intrinsic elevation of miR-155 in cystic fibrosis cells. The specific basis for the pro-inflammatory phenotype in the CF airway remains an enigma, and may be the central pathophysiologic problem confronting our understanding of this disease. Most disease-inducing mutations in the CFTR gene, including [ΔF508]CFTR, are associated with intrinsic activation of the TNFα/NFκB signaling pathway (13,14), leading to production of IL-8, and other pro-inflammatory mediators. However, how the mutations in CFTR induce TNFα/NFκB signaling remains a mystery. Nonetheless, studies in diffuse large B cell lymphomas (DLBCL) have shown that TNFα causes elevated levels of miR-155 (43). Therefore, it is possible that the same mechanism by which mutant CFTR is responsible for activation of TNFα/NFκB signaling may also be responsible for activating miR-155 in CF lung epithelial cells (see Figure 7).

Potential translational impact of antagonimr-155 for Cystic Fibrosis. Our study clearly indicates that antagonizing miR-155 is a potent method of suppressing IL-8 and other pro-inflammatory genes in CF cells, and therefore might have potential as a candidate CF therapeutic. As indicated in Figure 2C, antagonimr-155, while lowering the level of IL-8 mRNA, has the opposite effect on the anti-inflammatory cytokine IL-10. Low levels of IL-10 have recently been suggested as a mechanism for up-regulation of miR-155 (57), possibly via effects on toll-like receptors (58). Such an approach has recent precedents. Intravenous administration of antagonimirs against miR-122, miR-92a, miR-196 and miR-221/222 were among the first examples shown to suppress functions of endogenous miRzs in vivo. In the case of antagomir-122, increases in a set of genes associated with cholesterol biosynthesis were demonstrated in mouse liver (37). An antagonimr to miR-92a ("antagomir-92a") has been shown to aid in angiogenesis and functional recovery of ischemic tissues in intact mice (56). Administration of antagomir-196 into intact chicken eggs has been shown to regulate Hox gene expression and vertebral development in the enclosed developing chick (57). Finally, antagomir -221/-222 has been shown to regulate the progression of human melanoma Me665/1 cells in athymic nude mice (58). What is most striking is the fact that in all cases cited, the antagonimirs were not only functional, but also apparently non-toxic. We therefore conclude that the potential translational value of antagomir-155 for cystic fibrosis therapeutics needs to be fully investigated.

FOOTNOTES
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The abbreviations used are: CF, Cystic Fibrosis; miRNA, microRNA; IL-8, Interleukin-8; IL-6, Interleukin-6; TNF-α, Tumor necrosis factor-α; TTP, Tristetraprolin; ARE, Adenine uridine rich element; UTR, Untranslated region; MAPK, mitogen-activated protein kinase; IPA, Ingenuity Pathway Analysis; PtdIns, Phosphatidylinositol; PIP3, PtdIns (3,4,5)P3; PIP2, PtdIns (3,4,)P2.

REFERENCES

1. Pollard, H. B. (2000) Anat Rec 259, FMIII-IX
2. Frizzell, R. A. (1999) Physiol Rev 79, S1-2
3. Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R., and Smith, A. E. (1990) Cell 63, 827-834
4. Kopito, R. R. (1999) Physiol Rev 79, S167-173
5. Dean, T. P., Dai, Y., Shute, J. K., Church, M. K., and Warner, J. O. (1993) Pediatr Res 34, 159-161
6. Richman-Eisenstat, J. B., Jorens, P. G., Hebert, C. A., Ueki, I., and Nadel, J. A. (1993) Am J Physiol 264, L141-418
7. Armstrong, D. S., Grimwood, K., Carlin, J. B., Carzino, R., Gutierrez, J. P., Hull, J., Olinsky, A., Phelan, E. M., Robertson, C. F., and Phelan, P. D. (1997) Am J Respir Crit Care Med 156, 1197-1204
8. Roebuck, K. A. (1999) J Interferon Cytokine Res 19, 429-438
9. Bonfield, T. L., Panuska, J. R., Konstan, M. W., Hilliard, K. A., Hilliard, J. B., Ghnaim, H., and Berger, M. (1995) Am J Respir Crit Care Med 152, 2111-2118
10. Khan, T. Z., Wagener, J. S., Bost, T., Martinez, J., Accurso, F. J., and Riches, D. W. (1995) Am J Respir Crit Care Med 151, 1075-1082
11. Tirouvanziam, R., de Bentzmann, S., Hubeau, C., Hinnrasky, J., Jacquot, J., Peault, B., and Puchelle, E. (2000) Am J Respir Cell Mol Biol 23, 121-127
12. Balakathiresan, N. S., Bhattacharyya, S., Gutti, U., Long, R. P., Jozwik, C., Huang, W., Srivastava, M., Pollard, H. B., and Biswas, R. (2009) Am J Physiol Lung Cell Mol Physiol 296, L1012-1018
13. Eidelman, O., Srivastava, M., Zhang, J., Leighton, X., Murtie, J., Jozwik, C., Jacobson, K., Weinstein, D. L., Metcalf, E. L., and Pollard, H. B. (2001) Mol Med 7, 523-534
14. Srivastava, M., Eidelman, O., Zhang, J., Paweletz, C., Caohuy, H., Yang, Q., Jacobson, K. A., Heldman, E., Huang, W., Jozwik, C., Pollard, B. S., and Pollard, H. B. (2004) Proc Natl Acad Sci USA 101, 7693-7698
15. Wojnarowski, C., Frischer, T., Hofbauer, E., Grabner, C., Mosgoeller, W., Eichler, I., and Ziesche, R. (1999) Eur Respir J 14, 1136-1144
16. Ambros, V. (2001) Cell 107, 823-826
17. Ambros, V. (2003) Cell 113, 673-676
18. Ambros, V. (2004) Nature 431, 350-355
19. von Roretz, C., and Gallouzi, I. E. (2008) J Cell Biol 181, 189-194
20. Kim, V. N. (2005) Nat Rev Mol Cell Biol 6, 376-385
21. Kim, V. N. (2005) Mol Cells 19, 1-15
22. Bagga, S., and Pasquinelli, A. E. (2006) Genet Eng (N Y) 27, 1-20
23. John, B., Enright, A. J., Aravin, A., Tuschl, T., Sander, C., and Marks, D. S. (2004) PLoS Biol 2, e363
24. John, B., Sander, C., and Marks, D. S. (2006) *Methods Mol Biol* **342**, 101-113
25. Krek, A., Grun, D., Poy, M. N., Wolf, R., Rosenberg, L., Epstein, E. J., MacMenamin, P., da Piedade, I., Gunsalus, K. C., Stoffel, M., and Rajewsky, N. (2005) *Nat Genet* **37**, 495-500
26. Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I., and Filipowicz, W. (2006) *Cold Spring Harb Symp Quant Biol* **71**, 513-521
27. Vasudevan, S., Tong, Y., and Steitz, J. A. (2007) *Science* **318**, 1931-1934
28. Poy, M. N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., Macdonald, P. E., Pfeffer, S., Tuschi, T., Rajewsky, N., Rorsman, P., and Stoffel, M. (2004) *Nature* **432**, 226-230
29. Poy, M. N., Spranger, M., and Stoffel, M. (2007) *Diabetes Obes Metab* **9 Suppl 2**, 67-73
30. Blenkiron, C., Goldstein, L. D., Thorne, N. P., Spiteri, I., Chin, S. F., Dunning, M. J., Barbosa-Morais, N. L., Teschendorff, A. E., Green, A. R., Ellis, I. O., Tavare, S., Caldas, C., and Miska, E. A. (2007) *Genome Biol* **8**, R214
31. Blenkiron, C., and Miska, E. A. (2007) *Hum Mol Genet* **16 Spec No 1**, R106-113
32. Hwang, H. W., and Mendell, J. T. (2006) *Br J Cancer* **94**, 776-780
33. Jovanovic, M., and Hengartner, M. O. (2006) *Oncogene* **25**, 6176-6187
34. van Rooij, E., and Olson, E. N. (2007) *Physiol Genomics* **31**, 365-366
35. Ikeda, S., Kong, S. W., Lu, J., Bisping, E., Zhang, H., Allen, P. D., Golub, T. R., Pieske, B., and Pu, W. T. (2007) *Physiol Genomics* **31**, 367-373
36. Rane, S., Sayed, D., and Abdellatif, M. (2007) *Cell Cycle* **6**, 1850-1855
37. Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschi, T., Manoharan, M., and Stoffel, M. (2005) *Nature* **438**, 685-689
38. Vij, N., Mazur, S., and Zeitlin, P. L. (2009) *PLoS ONE* **4**, e4664
39. Bolisetty, M. T., Dy, G., Tam, W., and Beemon, K. L. (2009) *J Virol* **83**, 12009-12017
40. Stahl, H. F., Fauti, T., Ullrich, N., Bopp, T., Kubach, J., Rust, W., Labhart, P., Alexiadis, V., Becker, C., Hafner, M., Weith, A., Lenter, M. C., Jonuleit, H., Schmitt, E., and Mennerich, D. (2009) *PLoS ONE* **4**, e7158
41. Tili, E., Croce, C. M., and Michaille, J. J. (2009) *Int Rev Immunol* **28**, 264-284
42. Banerjee, A., Schambach, F., Dejong, C. S., Hammond, S. M., and Reiner, S. L. (2009) *Eur J Immunol*
43. Pedersen, I. M., Otero, D., Kao, E., Miletic, A. V., Hother, C., Ralfkiaer, E., Rickert, R. C., Gronbaek, K., and David, M. (2009) *EMBO Mol Med* **1**, 288-295
44. O'Connell, R. M., Chaudhuri, A. A., Rao, D. S., and Baltimore, D. (2009) *Proc Natl Acad Sci U S A* **106**, 7113-7118
45. Cremer, T. J., Ravneberg, D. H., Clay, C. D., Piper-Hunter, M. G., Marsh, C. B., Elton, T. S., Gunn, J. S., Amer, A., Kanneganti, T. D., Schlesinger, L. S., Butchar, J. P., and Trindadapani, S. (2009) *PLoS ONE* **4**, e8508
46. Bhattacharyya, S., Gutti, U., Mercado, J., Moore, C., Pollard, H. B., and Biswas, R. *Am J Physiol Lung Cell Mol Physiol* **300**, L81-87
47. Yamanaka, Y., Tagawa, H., Takahashi, N., Watanabe, A., Guo, Y. M., Iwamoto, K., Yamashita, J., Saiioh, H., Kameoka, Y., Shimizu, N., Ichinohasama, R., and Sawada, K. (2009) *Blood* **114**, 3265-3275
48. Rauh, M. J., Sly, L. M., Kalesnikoff, J., Hughes, M. R., Cao, L. P., Lam, V., and Krystal, G. (2004) *Biochem Soc Trans* **32**, 785-788
49. Ooms, L. M., Horan, K. A., Rahman, P., Seaton, G., Gurung, R., Kethesparan, D. S., and Mitchell, C. A. (2009) *Biochem J* **419**, 29-49
50. Zhang, H., He, J., Kutateladze, T. G., Sakai, T., Sasaki, T., Markadieu, N., Erneux, C., and Prestwich, G. D. *ChemBioChem* **11**, 388-395
51. Brooks, R., Fuhler, G. M., Iyer, S., Smith, M. J., Park, M. Y., Paraiso, K. H., Engelman, R. W., and Kerr, W. G. *J Immunol* **184**, 3582-3589
52. Ong, C. J., Ming-Lum, A., Nodwell, M., Ghanipour, A., Yang, L., Williams, D. E., Kim, J., Demirjian, L., Qasimi, P., Ruschmann, J., Cao, L. P., Ma, K., Chung, S. W., Duronio, V., Andersen, R. J., Krystal, G., and Mui, A. L. (2007) *Blood* **110**, 1942-1949
53. Peng, Q., Malhotra, S., Torchia, J. A., Kerr, W. G., Coggeshall, K. M., and Humphrey, M. B. *Sci Signal* **3**, ra38
54. Ebner, S., Dunbar, M., and McKinnon, R. D. (2000) *J Neurosci Res* **62**, 336-345
55. Dubrovska, A., Kim, S., Salamone, R. J., Walker, J. R., Maira, S. M., Garcia-Echeverria, C., Schultz, P. G., and Reddy, V. A. (2009) *Proc Natl Acad Sci U S A* **106**, 268-273
56. Bonauer, A., Carmona, G., Iwasaki, M., Mione, M., Koyanagi, M., Fischer, A., Burchfield, J., Fox, H., Doebele, C., Ohtani, K., Chavakis, E., Potente, M., Tjwa, M., Urbich, C., Zeiher, A. M., and Dimmeler, S. (2009) *Science* **324**, 1710-1713
57. McGlinn, E., Yekta, S., Mansfield, J. H., Soutschek, J., Bartel, D. P., and Tabin, C. J. (2009) *Proc Natl Acad Sci U S A* **106**, 18610-18615
58. Felicetti, F., Errico, M. C., Segnalini, P., Mattia, G., and Care, A. (2008) *Expert Rev Anticancer Ther* **8**, 1759-1765
FIGURE LEGENDS

**Fig. 1.** miRNA expression profile in CF cells. (A) The miRNA expression profile in IB3-1 CF cells compared to that in control CFTR-repaired IB3-1/S9 cells shows significant differences (P value ≤ 0.05, n = 3) in the expression of 22 miRNAs (listed on the right-side). (B) Individual miRNA assays were performed for the two miRNA, miR-155 and let-7c, which exhibit maximum up-regulation, to validate the expression profiling data. (C) The miR-155 up-regulation was further determined by individual Taqman assay specific for miR-155 in IB3-1 CF cells vs IB3-1/S9 control cells, CFBB vs NHBE and CD66+ CF vs Controls. (D) Treatment of the IB3-1/S9 CFTR-repaired control cells with CFTRinh-172 for 1h with the indicated doses induces miR-155 expression in the [WT]CFTR cells (p<0.05).

**Fig. 2.** Effect of antagomir-155 on IL-8 expression in CF lung epithelial cells. Treatment of IB3-1 CF cells with antagomir-155 (2.5 μM) or with mock treatment for 24 h leads to (A) down-regulation of miR-155, and IL-8 mRNA levels and (B) IL-8 protein levels. The mRNA levels were analyzed by qRT-PCR and IL-8 protein levels were analyzed by ELISA. Data (p<0.01, denoted by asterisk) reflect average of three or more independent experiments and error bars on graphs represent SEM. (C) The corresponding mRNA levels were determined with Illumina HT-12 arrays and subsequent IPA analyses of the data indicate the diminution of the inflammatory phenotype. While both pro-inflammatory genes, IL-8 and IL-6, are down-regulated in antagomir-155-treated cells, the anti-inflammatory gene IL-10 is up-regulated.

**Fig. 3.** SHIP1 regulates the expression of IL-8 gene in CF cells. SHIP1 expression was examined as a potential mediator of the miR-155 dependent pro-inflammatory state in CF. (A) SHIP1 expression, examined by qPCR, is significantly diminished in IB3-1 CF cells compared to control IB3-1/S9 cells (~3.5 fold). This relates to an inverse miR-155 expression profile in these cells. Additionally, suppression of miR-155 in CF cells with antagomir-155 also up-regulated SHIP1 expression as expected. (B) Luciferase reporter assays were performed in IB3-1 CF cells transfected with pMIR-Report vectors, either controls or those containing SHIP1 3′-UTR target sequences of miR-155 (both wt and mt), in the presence or absence of anti-miR-155. The data reflect averages of at least three independent experiments (* indicates p<0.05 and ** indicates p>0.05). (C) IB3-1 cells were transiently transfected with expression plasmid encoding SHIP1 (0.5 μg/2 x 10⁶ cells). RNA was isolated from IB3-1/SHIP1 and IB3-1 cells after actinomycin D treatment for the indicated time intervals and the remaining mRNA was analyzed by quantitative real time PCR. Increased expression of SHIP1 promotes rapid degradation of IL-8 mRNA (* indicates p<0.001). (D) The level of IL-8 protein secreted by IB3-1/SHIP1 cells were analyzed by ELISA 16 hours after transfection, also exhibit a diminution in IL-8 protein (* indicates p<0.01).

**Fig. 4.** miR-155 and SHIP1 regulate IL-8 expression in CF cells. IB3-1 cells were incubated with siSHIP1(30nM), or antagomiR-155, alone or with both for 24 h. The isolated RNA was analyzed by qRT-PCR for (A) IL-8 mRNA or (B) SHIP1 mRNA expression. The data reflect averages of at least three independent experiments (* indicates p<0.05).

**Fig. 5.** PI3K/Akt activity regulates IL-8 mRNA stability in CF cells. (A) IB3-1 cells treated with the pharmacological inhibitor wortmannin (300 nM) have significantly potentiated
rapid decay of IL-8 mRNA (* indicates p<0.001). (B) Both phospho-Akt as well as total Akt protein levels were analyzed by Western blot in IB3-1/S9 CFTR-repaired control cells as well as in the IB3-1 cells mock-treated or treated with antagomiR-155 or transfected with SHIP1 expression vector. GAPDH protein levels were also analyzed as endogenous control. The relative quantitation of pAkt/total Akt is also depicted in the bar graph below.

Fig. 6. miR-155 stabilizes IL-8 mRNA by activation of MAPKs. The mRNA expression profile in IB3-1 Cf cells, IB3-1/S9 control cells and IB3-1 cells treated with anti-miR-155 was analyzed by Illumina bead arrays. Subsequently, Ingenuity Pathway analyses (IPA) of the data was performed: (A) IB3-1 cells compared to IB3-1/S9 cells and (B) IB3-1 cells treated with anti-miR-155 compared to IB3-1 cells.

Fig. 7. Regulation of IL-8 gene expression in CF cells. A model appropriately summarizes the pro-inflammatory status of CF cells due to increased expression of miR-155. The aberrant up-regulation of miR-155 leads to increased IL-8 levels via down-regulation of the expression of intermediate target gene of miR-155, SHIP1 (INPP5D).

TABLE LEGENDS

Table 1. miRNA expression levels in IB3-1 CF cells relative to control cells (p<0.05).
The miRNAs which exhibit altered expression, analyzed by Taqman qPCR miRNA arrays, are listed with respective fold changes in expression in the IB3-1 CF cells compared to IB3-1/S9 control cells: the 18 miRNAs up-regulated (↑) and the four that are down-regulated (↓) in CF cells are indicated. Additionally, the miRNA expression profile obtained by miRNA microarrays (Bioarray, Ambion) is also depicted. The miRNAs which indicate similar expression profile by these two independent methods are indicated in bold.

Table 2. TargetScan-predicted miRNA target genes detected on Illumina arrays. The mRNA levels corresponding to the 20 miRNA expressions for CF IB3-1 and CFTR-repaired IB3-1/S9 cells, using ILLUMINA bead chip HumanRef-8 v2.0 arrays. These arrays can analyze ~24,000 transcripts per sample. The target mRNAs are listed: fold increases in IB3-1/S9 control cells compared to IB3-1CF cells for the miRNAs up-regulated (grey) and fold increases in the IB3-1cells compared to IB3-1/S9 for the miRNAs down-regulated (white) in CF cells. The miRNAs, miR- 423-3p and miR-615-3p were not present in the TargetScan database and were excluded from this analysis. Genes targeted by multiple miRNAs are indicated in bold.
| miRNA          | Chromosomal location | Fold change | p-value | CF vs IB3-1 | IB3-1/S9 | CF vs IB3-1/S9 |
|---------------|----------------------|-------------|---------|-------------|----------|----------------|
| hsa-miR-155   | 21q21.3              | 5.12↑       | 0.024328| 504.5       | <1       | ▲              |
| hsa-let-7c    | 21q21.1              | 4.41↑       | 0.037099| 13682.25    | 14904.75 | <—>           |
| hsa-miR-576-5p| 4q25                 | 3.43↑       | 0.029524| NP          | NP       | —              |
| hsa-miR-23b   | 9q22.32              | 3.23↑       | 0.017488| 20921       | 8693.5   | ▲              |
| hsa-miR-126   | 9q34.3               | 3.12↑       | 0.008423| 504.5       | <1       | ▲              |
| hsa-miR-27b   | 9q22.32              | 2.88↑       | 0.03101 | 5062.75     | 3266.5   | ▲              |
| hsa-miR-100   | 11q24.1              | 2.88↑       | 0.003527| 10665.25    | 3388     | ▲              |
| hsa-miR-99a   | 21q21.1              | 2.59↑       | 0.016107| 1080        | 129.5    | ▲              |
| hsa-miR-424   | Xq26.3               | 2.42↑       | 0.018096| ND          | ND       | —              |
| hsa-miR-125b  | 11q24.1              | 2.34↑       | 0.03134 | 8599.5      | 2455.5   | ▲              |
| hsa-miR-31    | 9p21.3               | 2.03↑       | 0.005089| 48058.25    | 25164    | ▲              |
| hsa-miR-491-5p| 9p21.3               | 2.03↑       | 0.024976| ND          | ND       | —              |
| hsa-miR-21    | 17q23.1              | 2.00↑       | 0.00751 | 19468.75    | 21233.25 | <—>           |
| hsa-miR-10a   | 17q21.32             | 1.95↑       | 0.004423| ND          | ND       | —              |
| hsa-miR-125a-5p| 19q13.33            | 1.81↑       | 0.034058| 6543.5      | 2396.75  | ▲              |
| hsa-miR-423-3p| 17q11.2             | 1.74↑       | 0.013898| 2066        | 641.5    | ▲              |
| hsa-miR-301   | 17q22                | 1.70↑       | 0.010314| ND          | ND       | —              |
| hsa-miR-328   | 16                   | 1.34↑       | 0.010555| ND          | ND       | —              |
| hsa-miR-660   | Xp11.23              | 2.07↓       | 0.02013 | NP          | NP       | —              |
| hsa-miR-192   | 11q13.1              | 1.57↓       | 0.04724 | ND          | ND       | —              |
| hsa-miR-194   | 1q41                 | 1.41↓       | 0.04156 | <1          | 46.75    | ↓              |
| hsa-miR-615-3p| 12                   | 1.28↓       | 0.020786| NP          | NP       | —              |

NP: Not present;  ND: Not Detectable;  <—>: Saturated expression
| miR-155 | let-7c | miR-576-5p | miR-23b | miR-126 |
|-------|-------|------------|--------|--------|
| Symbol | Fold  | Symbol | Fold  | Symbol | Fold  | Symbol | Fold  |
| ELL2   | 1.98  | UNC5A   | 4.79  | ELL2   | 1.98  | CEBPA  | 2.33  |
| HMGB3  | 1.94  | ARID3B  | 2.73  | CASZ1  | 1.91  | SNX5   | 2.18  |
| INADL  | 1.75  | DLC1    | 2.46  | RNF165 | 1.74  | STK35  | 2.16  |
| MIDN   | 1.66  | CPA4    | 2.20  | CCDC64 | 1.70  | FO9B   | 2.15  |
| PIK3R1 | 1.63  | GAB2    | 2.12  | ZNF536 | 1.68  | PCDHA4 | 2.13  |
| INPP5D | 1.56  | FAM125B | 2.01  | KIAA0406 | 1.64 | FAM125B | 2.01  |

| miR-27b | miR-99a/100 | miR-424 | miR-125a/b | miR-31 |
|--------|------------|---------|------------|-------|
| Symbol | Fold  | Symbol | Fold  | Symbol | Fold  | Symbol | Fold  |
| RIMS4  | 3.36  | PPFIA3 | 1.36  | COL12A1 | 2.71  | STMN3  | 3.09  |
| SNAP25 | 2.68  | ADCY1  | 1.35  | CDK6    | 2.55  | ARID3B | 2.73  |
| CDK6   | 2.55  | IGIF1R | 1.23  | TXNIP   | 2.26  | BRSK2  | 2.56  |
| FO9B   | 2.15  | EIF2C2 | 1.20  | PCDHA4  | 2.13  | ARID3A | 2.55  |
| ELL2   | 1.98  | PPP3CA | 1.15  | FKBPA1  | 2.12  | GAB2   | 2.12  |
| ZFP36  | 1.47  | CCDC21 | 1.14  | ELL2    | 1.98  | DPP9   | 2.07  |

| miR-491-5p | miR-21 | miR-10 | miR-301a | miR-328 |
|------------|--------|--------|---------|--------|
| Symbol     | Fold  | Symbol | Fold  | Symbol | Fold  | Symbol | Fold  |
| GIPC1      | 1.56  | CDK6   | 2.55  | CDK6   | 2.55  | NPTX1  | 13.77 |
| KCNK5      | 1.56  | NTF3   | 2.46  | TPM4   | 2.04  | SNAP25 | 2.68  |
| MGAT4B     | 1.41  | ZCCHC3 | 1.74  | LPHN1  | 1.96  | RTN1   | 2.60  |
| KIAA0664   | 1.40  | CNTFR  | 1.67  | RNF165 | 1.74  | RUNX3  | 2.46  |
| PBX2       | 1.39  | PIK3R1 | 1.63  | KIAA0247 | 1.64 | DLC1   | 2.46  |
| CIDEC      | 1.35  | CIDEC  | 1.35  | FLRT2  | 1.62  | TXNIP  | 2.26  |

| miR-194 | miR-192 | miR-660 |
|--------|--------|--------|
| Symbol | Fold  | Symbol | Fold  | Symbol | Fold  |
| CNTNAP2 | 6.55  | PPM2C  | 2.84  | DIAPH2 | 1.87  |
| ZFHX4   | 4.09  | ALCAM  | 2.25  | APPL2  | 1.85  |
| LPHN2   | 3.69  | DIAPH2 | 1.87  | ESRRG  | 1.61  |
| DACH1   | 3.05  | PUNC   | 1.51  | EIF2C1 | 1.56  |
| HS3ST2  | 2.78  | ARFIP2 | 1.44  | G3BP2  | 1.53  |
| THBS1   | 2.70  | ATF1   | 1.42  | IL-8   | 4.22  |
Figure 1

A. Cluster analysis with pVal 0.05 Type Euclidean

B. Relative miRNA (IB3-1 vs IB3-1/S9)

C. Relative miR-155

D. Relative miR-155 with CFTRinh-172 (nM)

miR-576, miR-155, miR-328, miR-125a, miR-301, miR-424, miR-126, miR-31, miR-21, let-7c, miR-491, miR-10a, miR-125b, miR-27b, miR-99a, miR-100, miR-23b, miR-423, miR-194, miR-192, miR-660, miR-615

CF Control
Figure 2

A

![Graph showing % Remaining RNA for miR-155 and IL-8 mRNA under different conditions.](image)

B

![Graph showing IL-8 Protein Secreted (pg/ml) under different conditions.](image)

C

![Pie chart illustrating hub and spoke model with gene interactions.](image)

| Hub       | Spoke |
|-----------|-------|
| IL-10     | 8     |
| IL-8      | 7     |
| ITBG2     | 7     |
| RAC1      | 7     |
| IL-6      | 5     |
| ARHGDIAl  | 5     |
Figure 3
Figure 4

(A) % Remaining IL-8 mRNA

(B) % Remaining SHIP1 mRNA
Figure 5

A

% Remaining IL-8 mRNA

IB3-1

IB3-1+Wm
(300nM)

Time after Act D (h)

0 1 2 3

100 10 0

B

IB3-1/59
IB3-1
AntagomirR155
IB3-1+ShP1

pAKT

AKT

GAPDH

% Remaining IL-8 mRNA

Relative pAKT/AKT

0 25 50 75 100

1 2 3
Figure 6
Figure 6
Figure 7

WT-CFTR → ΔF508-CFTR

NF-κB → miR-155 → PI3K

SHIP1 → Wortmannin

Akt → pAkt

code: [mutant]CFTR state

NF-κB → IL-8

mRNA stability
Elevated miR-155 promotes inflammation in cystic fibrosis by driving hyper-expression of interleukin-8
Sharmistha Bhattacharyya, Nagaraja S. Balakathiresan, Clifton Dalgard, Usha Gutti, David Armistead, Cathy Jozwik, Meera Srivastava, Harvey B. Pollard and Roopa Biswas

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