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miR-17 overexpression in cystic fibrosis airway epithelial cells decreases IL-8 production

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Take home message: Overexpression of miR-17 in cystic fibrosis airway epithelial cells decreases interleukin-8 protein production.

Abstract

Interleukin-8 levels are higher than normal in the cystic fibrosis (CF) airways causing neutrophil infiltration and non-resolving inflammation. Overexpression of microRNAs that target IL-8 expression in airway epithelial cells may represent a therapeutic strategy for CF.

IL-8 protein and mRNA were measured in CF and non-CF bronchoalveolar lavage fluid and bronchial brushings (n=20 per group). miRNAs decreased in the CF lung and predicted to target IL-8 mRNA were quantified in βENaC-transgenic, *Cftr-/-* and wild type mice, primary CF and non-CF bronchial epithelial cells and a range of CF versus non-CF airway epithelial cell lines or cells stimulated with lipopolysaccharide, *Pseudomonas*-conditioned medium or CF bronchoalveolar lavage fluid. The effect of miRNA overexpression on IL-8 protein production was measured.

miR-17 regulates IL-8 and its expression was decreased in adult CF bronchial brushings, βENaC-transgenic mice and bronchial epithelial cells chronically stimulated with *Pseudomonas*-conditioned medium. Overexpression of miR-17 inhibited basal and agonist-induced IL-8 protein production in F508del-CFTR homozygous CFTE29o$^-$ tracheal, CFBE41o$^-$ and/or IB3 bronchial epithelial cells.

These results implicate defective CFTR, inflammation, neutrophilia and mucus overproduction in regulation of miR-17. Modulating miR-17 expression in CF bronchial epithelial cells may be a novel anti-inflammatory strategy for CF and other chronic inflammatory airway diseases.

Word count: 198

Introduction

Cystic fibrosis (CF) is a multisystem disorder, however its pulmonary manifestations are largely responsible for the associated high morbidity and mortality [1, 2]. A striking feature of CF lung disease is the high level of infiltrating neutrophils. The large numbers of neutrophils that accumulate within the CF lung secrete proteases that overwhelm the normal anti-protease and anti-microbial defences, and together with neutrophil-derived oxidants cause derangement of the respiratory epithelial surface and promote pro-inflammatory gene expression [3]. Thus neutrophils and their products play a major role in pulmonary inflammation in CF. Importantly neutrophil proteases, such as neutrophil elastase, and other neutrophil- or microbial-derived proinflammatory factors present in the CF lung, can exacerbate neutrophil-dominated inflammation by inducing expression of interleukin-8 (IL-8) [4]. IL-8 is abundantly present in the CF lung and is a potent neutrophil chemokine. Thus inhibiting IL-8 expression represents an anti-inflammatory strategy to control excessive neutrophil-dominated lung inflammation in CF.

IL-8 is expressed by bronchial epithelial cells and macrophages in the lung. CF is largely an airway disease, and the vast surface area of the potentially inflamed CF bronchial epithelium represents a major source of IL-8. Recent studies suggested that strategies designed to interfere with IL-8 gene transcription using transcription factor decoys may have therapeutic potential for CF [5, 6]. siRNA-mediated inhibition of IL-8 expression in polarised airway epithelial cells is another strategy [7]; however interfering with IL-8's post-transcriptional regulation is also possible.

MicroRNAs (miRNAs) are short endogenous non-coding single-stranded RNAs that function as post-transcriptional negative regulatory molecules. They modulate target gene expression via target mRNA degradation and/or translational repression and are implicated as key regulators in almost all biological processes [8, 9]. miRNAs bind to miRNA recognition

elements (MREs) largely located in the 3'UTR of target mRNAs. Therapeutic modulation of miRNA levels is possible by the use of over-expression with synthetic miRNA mimics or antisense inhibition by anti-miRs [10]. In the CF lung where IL-8 expression is persistently elevated, we speculate that miRNAs regulating IL-8 expression may be reciprocally decreased. If so an overexpression approach using miRNA mimics may have therapeutic potential.

The first *in vivo* CF miRNA profiling studies were carried out using CF versus non-CF bronchial brushings [11]; however the role of miRNA in modulating IL-8 was not addressed at that point. Hu *et al*. have demonstrated that IL-8 is susceptible to modulation by miR-520b in breast cancer cells [12], whilst Yu *et al.* have shown that the miR-17/20 cluster regulates the IL-8 3'UTR [13]. Other studies have reported that miR-203 and miR-93/106b regulate IL-8 expression [14, 15]. In CF IB3 cells, CF bronchial brushings and CF neutrophils Bhattacharyya *et al*. reported how increased levels of miR-155 indirectly elevate IL-8 [16]. More recently Fabbri *et al.* showed that miR-93 regulates IL-8 production in CF airway epithelial cell lines in response to heat-killed *Pseudomonas aeruginosa* [17].

Here we quantify IL-8 mRNA and protein expression levels in clinical samples from the lungs of people with and without CF. We investigate the contribution of the altered miRNA expression profile in CF bronchial brushings to the higher than normal IL-8 levels and explore reasons for the reduced levels of miR-17, a validated regulator of IL-8. We compare the findings to candidate miRNA expression in βENaC-transgenic mice with CF-like lung disease and *Cftr-/-* mice, and explore the potential to decrease basal and agonist-induced IL-8 expression by CF bronchial epithelial cells using miRNA mimics.

Experimental procedures

In silico analysis.

Bioinformatic analysis was performed using the miRNA target prediction databases TargetScan 6.2, MicroRNA.org, PITA, and Microcosm to search for miRNAs predicted to target the 3'UTR sequence of human IL-8 or the 3'UTR of murine KC/CXCL1.

Bronchoalveolar lavage fluid and bronchial brush sampling, miRNA profiling, primary epithelial cell culture and qRT-PCR.

Following written informed consent under a protocol approved by Beaumont Hospital ethics review board (i) bronchoalveolar lavage (BAL) fluid was recovered from CF (n = 12, 27±1.4 yr, M/F 4:8) and non-CF control individuals (n = 12, 6±4.8 yr, M/F 3:9), as previously described [18] and (ii) bronchial brushings were sampled and RNA isolated as previously described [11] from CF (n = 8, 28.2± 4.6 yr, M/F 4:4) and non-CF control individuals (n = 8, 48.5±6.02 yr, M/F 4:4). CF individuals were confirmed by sweat testing and/or genotyping and are listed in Supplementary Table 1. Non-CF control individuals were undergoing exploratory bronchoscopy in the investigation into idiopathic cough. Five of the adult CF and non-CF bronchial brush samples were previously used for miRNA expression profiling [11], the remainder were used for subsequent miRNA validation studies and qRT-PCR.

Following written informed consent under a protocol approved by the ethics review board at Our Lady's Children's Hospital Crumlin, primary epithelial cultures were established from 7 CF (3±0.61 yr, M/F 5:2, 5 ΔF508/ΔF508, ΔF508/C.1766+1G>A and ΔF508/unknown) and 2 non-CF (0.54±0.45 yr, M/F 2:0) bronchial brushings collected as part of the Study of Host Immunity and Early Lung disease in CF (SHIELD CF) as previously

described [19]. In addition mRNA expression profiling data was used from 3 ΔF508/ΔF508 and 3 non-CF bronchial brushings previously reported in [20].

Interleukin-8 ELISA

IL-8 protein concentrations in BAL fluid and cell supernatants were determined by sandwich ELISA (R&D Systems).

Measurement of mRNA expression levels by qRT-PCR.

Total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen). mRNA expression was measured using LightCycler® 480 SYBR Green I Master Mix (Roche) on the Roche LC480 Lightcycler. Expression of IL-8 mRNA relative to β-actin was determined using the $2^{-\Delta\Delta Ct}$ method. Primers used were as follows: IL-8 Forward 5'-TTTTGCCAAGGAGTGCTAAAGA-3', Reverse 5'-AACCCTCTGCACCCAGTTTTC-3' and β-actin Forward 5'-GGACTTCGAGCAAGAGATGG-3' Reverse 5'-AGGAAGGAAGGCTGGAAGAG-3'. qRT-PCR was also performed for STAT3, TIMP2 and p21 using the following primers: STAT3 Forward 5'-GGACATCAGCGGTAAGACCC-3', Reverse 5'-GCTCTCTGGCCGACAATACT-3', TIMP2 Forward 5'-CTCATTGCAGGAAAGGCCGA-3' Reverse 5'-GGAGGAGATGTAGCACGGGA-3' and p21 Forward 5'-GTGGACCTGTCACTGTCTTGT-3' Reverse 5'-GGTAGAAATCTGTCATGCTGGTCT-3'. All qRT-PCR experiments were performed in triplicate and included no-template controls.

Measurement of human miRNA levels

cDNA was generated from total RNA using Taqman MicroRNA Reverse Transcription kits (Applied Biosystems). miRNA expression was measured on the Roche

6

LC480 Lightcycler with TaqMan assays (Applied Biosystems). Expression of miRNAs relative to U6 snRNA was determined using the $2^{-\Delta\Delta Ct}$ method. qRT-PCR experiments in cell lines were performed in triplicate, a minimum of three times and included no-template controls.

Mouse studies.

Experimental animals were housed in a specific pathogen-free animal facility at the University of Heidelberg and had free access to chow and water. The βENaC-transgenic (βENaC-Tg) mouse was originally generated on a mixed genetic background (C3H/He x C57BL/6) and was backcrossed to the C57BL/6 background as described previously [21]. Transgene positive mice were identified by PCR of genomic DNA [22]. For transcript analyses, we collected whole lung homogenates from 2-week-old mice and trachea and main stem bronchi from 6-week-old mice as previously described [23], i.e. at ages when βENaC-Tg mice were reported to exhibit CF-like lung disease [24]. Gut corrected *Cftr* knockout (*Cftr-/-*) mice overexpressing human CFTR in the intestine under control of the fatty acid binding protein promoter were kindly provided by Dr. Jeffrey A. Whitsett, backcrossed to the C57BL/6 background and genotyped as previously described [21, 25]. Transcript analyses in *Cftr-/-* mice were performed in bronchi of 30 week old mice, i.e. at an age when *Cftr-/-* mice were reported to exhibit multiorgan pathology [26]. Total RNA was isolated using TRIzol reagent (Invitrogen). Real-time qRT-PCR for mmu-miR-17 and mmu-let-7b normalised to U6 snRNA was performed with Taqman assays (Applied Biosystems). Bronchoalveolar lavage (BAL) was performed in 6-week-old βENaC-Tg mice and 30-week-old *Cftr-/-* mice, and differential cell counts were determined from cytospin preparations as previously described [24]. In all experiments, age matched wild-type (WT) littermates served as controls.

Luciferase reporter assays

HEK293 cells from the European Collection of Cell Cultures ($2x10^5$ in triplicate) were transiently co-transfected for 24 h with an IL-8 3'UTR firefly luciferase reporter vector containing the full length 3'UTR (250 ng) (kindly provided by R. Pestell, Thomas Jefferson University [13]), a constitutive *Renilla* luciferase vector (100 ng) and 30 nM synthetic Pre-miR miRNA Precursors (PM) or AntimiR miRNA Inhibitors (AM) (Applied Biosystems) as indicated or with a scrambled control. Transfections were performed using Genejuice (Novagen,) for plasmid DNA and Ribojuice (Novagen) for miRNA in OptiMEM reduced serum media (Life Technologies) as recommended. AntimiRs do not always degrade their target miRNA and we measured variable antimiR-specific target miRNA knockdown of 20-80% using transfection conditions that were optimised previously to ensure uniform transfection across all wells [27]. Lysates were prepared 36 h post transfection and luciferase activities measured using the Luciferase assay system (Promega) and coelenterazine (Marker Gene Technologies). Relative luciferase activity was calculated.

16HBE14o-/CFBE41o⁻, 9HTEo-/CFTE29o⁻ and S9/IB3 epithelial cell studies

The human non-CF 16HBE14o- and 9HTEo- and F508del homozygous CFBE41o⁻ and CFTE29o⁻ bronchial and tracheal epithelial cell lines were obtained as a gift from D. Gruenert (California Pacific Medical Centre Research Institute, San Francisco, CA) [28, 29]. IB3 cells are a CF bronchial epithelial cell line (F508del/W1282X), S9s are their isogenic non-CF counterpart and both were obtained from Pamela Zeitlin, Johns Hopkins Children's Centre, MD, USA [30]. 16HBE14o-/CFBE41o⁻ and 9HTEo-/CFTE29o⁻ cells were grown in MEM+GlutaMax (Gibco) with 10% fetal calf serum (FCS, Gibco) and 1% penicillin-streptomycin (Pen-Strep, Gibco). 9HTEo-/CFTE29o⁻ cells were supplemented with 1X non-essential amino acids (Lonza). IB3 and S9 cells were grown in LHC-8 medium (Gibco) with

5% FCS and 1% Pen-Strep. In some experiments S9 cells were grown up to a density of 5×10^5 cells/cm^2 and chronically stimulated (5 days) [31] with 1% *Pseudomonas*-conditioned medium (PCM) [27] in LHC-8 medium (Gibco) with 1% FCS and 1% Pen-Strep. Medium was changed every day.

Cells were seeded at 1×10^5, 0.5×10^5 and 0.8×10^5 cells/cm^2 for 16HBE14o-/CFBE41o$^-$, 9HTEo-/CFTE29o$^-$ and S9/IB3 respectively and were transfected with 30 nM scrambled control or PMs using Ribojuice transfection reagent (Novagen). In initial experiments, cells were co-transfected with a fluorescently labelled non-targeting miRIDIAN miRNA mimic (Dharmacon) to monitor and optimise transfection efficiency; >80-90% of cells were transfected as monitored by epifluorescence and miRNA expression was increased following PM transfection [24]. Mature miRNA expression was measured in transfected cells to ensure efficient transfection. Twenty-four or 48 hours post transfection cells were left unstimulated or stimulated with *Pseudomonas aeruginosa* lipopolysaccharide (LPS) (10 µg/ml, Sigma-Aldrich, Kilkenny, Ireland), 1% PCM or 10 µl/ml CF BAL fluid for a further 24 h. Supernatants were retained for IL-8 protein analysis.

Statistical analysis.

All analyses were performed using GraphPad PRISM 4.0 (San Diego CA). Results are expressed as the mean ± SEM and were compared by student *t*-test or ANOVA as indicated. For ANOVA, the post-hoc Dunnett test was used for pair-wise comparison Differences were considered significant at $p \leq 0.05$.

Results

Levels of IL-8 are increased in CF versus non-CF bronchoalveolar lavage fluid and bronchial brushings.

Many studies have reported higher than normal levels of IL-8 in the CF lung. In order to confirm this observation in our patient cohort, levels of IL-8 protein were measured in BAL fluid collected from people with and without CF. Figure 1A shows significantly higher levels of IL-8 in CF versus non-CF BAL fluid samples.

Next IL-8 mRNA expression was measured in a selection of CF and non-CF bronchial brushings. Figure 1B shows that expression of IL-8 mRNA, relative to β–actin mRNA, is significantly increased in CF *in vivo*.

miRNAs predicted to target the IL-8 3'UTR in CF bronchial epithelium

A selection of miRNA target prediction databases was interrogated to identify miRNAs with a possible role in regulation of IL-8. Figure 2A shows the number of miRNAs predicted to target the IL-8 3'UTR by TargetScan 6.2, MicroRNA.org and PITA/Microcosm. These lists were cross-compared with miRNAs known to be decreased *in vivo* in CF bronchial brushings [11]. Figure 2B shows details of miRNAs selected for further study on the basis of (i) the relative expression level (significant or not) of the miRNA in CF versus non-CF bronchial epithelium, (ii) the number of predicted MREs in the IL-8 3'UTR, and (iii) the miRSVR score. The selected miRNAs were: let-7b, miR-17 and miR-203 which are all significantly decreased *in vivo* in CF bronchial brushings, and miR-19b and miR-125b which are predicted to target IL-8 but the expression of which is unaltered between CF and non-CF brushings. Of the selected miRNAs levels of let-7b and miR-17 were independently validated by miRNA assay as being lower than normal in the remaining original CF versus non–CF bronchial brush samples used in the profiling (miR-17:1±0.42 vs. 0.38±0.21, let-7b: 1±0.85

10

vs. 0.57±0.21 for control versus CF bronchial brushings, respectively albeit not significantly).

Figure 2C depicts the full length human IL-8 3'UTR with predicted binding locations for the

selected miRNAs of interest.

miR-17 is the most effective regulator of an IL-8 3'UTR luciferase reporter

In order to determine which of the chosen miRNAs regulate expression of IL-8,

HEK293 cells were transiently transfected with an IL-8 3'UTR luciferase reporter vector. Co-

transfection with PMs showed that PM-let-7b and PM-17 significantly decreased luciferase

gene expression compared to a scrambled control (Figure 3A). PM-203, PM-19b and PM-

125b did not decrease luciferase expression so these miRNAs were not explored further.

Luciferase assays were repeated using AMs to knockdown the expression of let-7b

and miR-17. Under identical transfection conditions AM-17, but not AM-let-7b, significantly

increased luciferase expression (Figure 3B). miR-221 is not predicted to target IL-8 and AM-

221 did not decrease luciferase activity. These data indicated that miR-17 is the most

effective regulator of IL-8 from the miRNAs decreased *in vivo* in CF versus non-CF

bronchial brushings.

Expression of miR-17 target genes in CF versus non CF bronchial brushings.

In addition to IL-8, other targets of miR-17 include STAT3, TIMP2, p21, fibronectin

and FoxA1 [32-36]. mRNA array data from CF and non-CF bronchial brushings was

analysed to determine whether the expression of these genes is increased in CF [20].

Supplementary Figure 1 shows that whilst there was no increase in expression of fibronectin

or FoxA1, there was a trend towards increased levels of STAT3, TIMP2, p21 and IL-8

transcripts in the CF samples, however due to the small sample size and/or the possibility that

miR-17 may affect only the protein rather than both the mRNA and protein levels of these targets, differences were not statistically significant.

Expression of mmu-miR-17 in airway tissues from βENaC-transgenic mice and Cftr-/- mice.

miR-17 and let-7b are conserved in mammals. In order to determine whether expression of these miRNAs is altered in mice with CF-like lung disease, the levels of mmu-miR-17 and mmu-let-7b were measured in whole lung homogenates and native airway tissues (trachea and main stem bronchi) from 2-week-old and 6-week-old βENaC-Tg mice and wild-type littermates and normalised to U6 snRNA. Similar to human CF bronchial brushings, the expression of mmu-miR-17 was significantly decreased in the airway tissues of the βENaC-Tg mice at 2 and 6 weeks (Figure 4A). Mmu-let-7b expression tended to be decreased in βENaC-Tg compared to wild-type mice, however this difference did not reach statistical significance.

We also quantified miR-17 and let-7b levels in *Cftr-/-* mice compared to WT littermates. There was no significant difference in let-7b expression. Surprisingly miR-17 levels were higher in *Cftr-/-* versus WT mice (Figure 4B). In contrast to βENaC-Tg mice that develop a robust spontaneous neutrophilic inflammation, we did not detect increased neutrophil counts in the lungs of *Cftr-/-* mice (Supplementary Figure 2). Further *Cftr-/-* mice, unlike βENaC-Tg mice, do not develop goblet cell metaplasia and airway mucus obstruction [24, 37]. These results suggest that factors associated with chronic airways disease may contribute to reduced miR-17 levels in CF-like lung disease in vivo, and may be responsible for the observed differences in miR-17 levels in βENaC-Tg versus *Cftr-/-* mice.

Expression of miR-17 in CF and non-CF cell lines, primary cells and under chronic inflammatory conditions.

miR-17 levels were decreased *in vivo* in CF bronchial brushings and in βENaC-Tg mice with spontaneous airway neutrophilia and mucus obstruction. Next we quantified its levels in a selection of CF and non-CF airway epithelial cells lines and primary bronchial cells from children with and without CF. We did not observe a consistent difference in miR-17 levels across all of the CF versus non-CF cell lines (Figure 5); miR-17 levels were slightly or significantly decreased in IB3 and CFBE cells versus their non-CF counterparts, respectively, whereas miR-17 was significantly increased in CFTE versus HTE cells. The CFTE effect may be cell line- or tracheal-specific. In contrast to the adult bronchial brushings, we did not observe a significant decrease in miR-17 levels in primary CF cells from children although miR-17 levels showed a trend towards reduction in age-matched non-CF primary cells. Whilst this may be an age-specific effect, it is also possible that the effect may be due to a lack of specific microbial and host-derived pro-inflammatory factors in the pediatric CF lung samples, and/or removal of these stimuli in the process of establishing the primary cell cultures. To test this we examined miR-17 expression in S9 cells that underwent chronic (5-day) stimulation with *Pseudomonas*-conditioned medium. Figure 5 shows that miR-17 expression is significantly decreased under these conditions.

Pre-miR-17 inhibits basal and LPS-induced IL-8 protein production in CF bronchial and tracheal epithelial cells

In order to test whether miR-17 overexpression can inhibit basal or stimulus-induced IL-8 expression in human CF airway epithelial cells, CFBE41o⁻ and CFTE29o⁻ cells were transfected with PM-17 and treated, or not, with 10 µg/mL LPS to induce IL-8 expression. mRNA levels of IL-8 and other miR-17 gene targets were quantified in the scrambled control

versus the PM-17-transfected cells (Supplementary Figure 1B); only IL-8 was decreased in LPS-stimulated CFTEs transfected with PM-17. Figure 6 shows that compared to PM-let-7b which had no significant effect on basal or LPS-induced IL-8 protein production, overexpression of miR-17 significantly decreased basal (62.5% and 46.5%) and LPS-induced IL-8 protein production (44.6% and 43.7%) in the CFBE41o$^-$ and CFTE29o$^-$ cell lines, respectively. In the tracheal but not bronchial cells PM-let-7b significantly increased basal IL-8 protein production; this may be a cell line- or tracheal-specific effect.

Pre-miR-17 inhibits agonist-induced IL-8 protein production in IB3 CF bronchial epithelial cells

Finally the effect of PM-17 on IL-8 protein production induced by LPS and other pro-inflammatory agonists representative of the CF lung was evaluated in the CF airway epithelial line, IB3 as this cell line was previously used for CF miRNA studies by Bhattacharyya *et al*. [16]. Similar to treatment with LPS, stimulation of IB3 cells with PCM or CF BAL fluid for 24 h led to significant increases in IL-8 protein production (Figure 7). Although PM-17 had no effect on basal IL-8 protein production in these cells, it significantly inhibited agonist-induced IL-8 protein production in all cases.

Discussion

miR-17 post-transcriptionally regulates IL-8 expression. Here we show that overexpression of miR-17 can interfere with basal and CF lung-specific agonist-induced IL-8 protein production from CF airway epithelial cells *in vitro*, suggesting that a pre-miR-17-based medicine could have potential to treat the inflammatory manifestations of CF lung disease.

miRNA profiling identified miRNAs that are altered *in vivo* in CF bronchial brushings [11]. Of the miRNAs that were significantly decreased in the CF samples a number were predicted to target the IL-8 3'UTR. Taking into account their miR-SVR scores, three miRNAs were chosen for this study; let-7b, miR-17 and miR-203. IL-8 is a validated target of these and other miRNAs [12-15]. Notwithstanding that miR-203 levels were lower than normal in CF bronchial brushings and that there are four putative miR-203 MREs in the IL-8 3'UTR, PM-203 was ineffective in the luciferase reporter gene studies, therefore it was not explored further. Instead a comparison between miR-17 and let-7b was performed by knocking them down and determining if this could enhance expression of the IL-8 3'UTR luciferase reporter gene. Based on these studies miR-17 was found to be more effective than let-7b at regulating IL-8. This observation was substantiated in the CF airway epithelial cell studies whereby PM-17, but not PM-let-7b, significantly impaired basal and agonist-induced IL-8 protein production.

miR-17 is encoded on the miR-17~92 gene cluster on chromosome 13. This cluster has a role in tissue development and miR-17 is known to be important in lung epithelial bud morphogenesis [38] and proliferation of lung epithelial progenitor cells [39, 40]. Largely overexpressed in lung cancers [41], there are few reports of under expression of miR-17 in the lung, with the recent exception of pulmonary fibrosis and asthma [42, 43]. Fabbri *et al.* have identified miR-93 as a miRNA that regulates IL-8 expression and that may be

implicated in CF pathology [17]. Interestingly miR-93 and miR-17 are also part of the same miRNA gene family – the miR-17 microRNA precursor family – but unlike miR-17, miR-93 is encoded on chromosome 7. We previously reported that miR-93* is increased rather than decreased in CF versus non-CF bronchial brushings [11] however there is no data to suggest that miR-93* regulates IL-8 expression. In addition to IL-8, other targets of miR-17 include STAT3, TIMP2, p21, fibronectin and FoxA1 [32-36]. We examined expression of these mRNAs in bronchial brushings and CF airway epithelial cells transfected with PM-17 however there were no major changes in the levels of these transcripts, except for IL-8, in the prospectively collected CF bronchial brushings or LPS-stimulated CFTE cells transfected with PM-17.

In the lungs of βENaC-Tg mice expression of miR-17 was decreased compared to their WT counterparts. In contrast, miR-17 was increased rather than decreased in *Cftr-/-* versus WT mice. In the context of CF, altered miRNA expression in the airways could occur in response to increased chemokine production, airway neutrophilia or mucus obstruction, amongst others. To the best of our knowledge, in *Cftr-/-* mice the hyper-inflammatory response with robust lung neutrophilia and increased chemokine production is only observed when mice are challenged with *Pseudomonas* or LPS. In the absence of such challenges, these mice have not been reported to develop spontaneous CF-like neutrophilic airways disease with increased CXCL1 and CXCL2 levels and mucus plugging, but have been shown to develop alveolar abnormalities with parenchymal interstitial thickening and recruitment of macrophages in the alveoli that was most pronounced in older mice on the C57BL6 background [26, 44]. For this reason, we used aged (30 week old) *Cftr-/-* mice. Consistent with previous reports of alveolar infiltration with macrophages we found that BAL macrophages were elevated in the *Cftr-/-* mice versus wild-type controls (Supplementary Fig 2B). However, we did not detect spontaneous neutrophilia in the *Cftr-/-* mice. While a subtle

increase in neutrophils was reported [45] this was not observed in other mice with loss of *Cftr*, albeit on a different genetic background [46]. In contrast, βENaC-Tg mice develop robust spontaneous neutrophilic inflammation in addition to CF-like airways mucus obstruction [22, 24, 37]. Further *Cftr-/-* mice, unlike βENaC-Tg mice, do not develop goblet cell metaplasia and airway mucus obstruction [24, 37], however the CF mouse model used in the current study is on a gut-corrected background, which may change the overall inflammatory phenotype. Mucus obstruction and the lack of spontaneous airway neutrophilia and inflammation are key differences between the two models that may be responsible for the observed differences in miR-17 levels. Interestingly inflammation in the mouse lung is controlled differently than in humans. Mice do not express IL-8, instead they express a number of functional homologues of IL-8 with KC/CXCL1 considered to be the closest. Murine CXCL2, CXCL5 and CCL2 also share overlapping functions with IL-8. None of these transcripts or KC/CXCL1 is predicted to be regulated by mmu-miR-17 or mmu-let-7b.

We were initially surprised that miR-17 levels were not decreased in CF versus non-CF primary airway epithelial cells but given that these cells were of pediatric origin they are less likely to have been exposed to as many chronic inflammatory stimuli as the cells in the adult CF bronchial brushings. Further removal of any such stimuli in the process of establishing the primary cell cultures, or an age-related effect, may also account for our observations. In order to test the pro-inflammatory theory we subjected airway epithelial cells to chronic stimulation with *Pseudomonas*-conditioned medium. These conditions significantly decreased miR-17 expression and go some way towards explaining the mechanism by which miR-17 is decreased in the adult CF lung. Taken together the data from the basal and agonist-stimulated cell lines, pediatric primary bronchial epithelial cells, adult bronchial brushings, and βENaC-Tg and *Cftr-/-* mice indicate that defective CFTR, the pro-inflammatory milieu of the CF lung, neutrophilia, goblet cell metaplasia and mucus

17

obstruction are all factors that could contribute to altered miR-17 expression. The data do not demonstrate that decreased miR-17 expression *in vivo* in the CF lung is solely responsible for increased IL-8 expression.

CF lung therapeutics are targeted at liquefying mucus, decreasing inflammation, killing infectious microbes and restoring CFTR-mediated chloride ion conductance. A number of anti-inflammatory approaches indirectly target IL-8 production, for example corticosteroids, NSAIDs and possibly macrolides. In addition to IL-8, leukotriene B4 (LTB4) and other less abundant chemotactic peptides are also present in the CF lung. Strategies targeting LTB4 in CF were unsuccessful in the past [47, 48]. That strategy not only inhibited neutrophil chemotaxis but was also cytotoxic against neutrophils, thereby killing whatever neutrophils managed to migrate to the lung in response to other chemokines. The goal in controlling the excessive neutrophil infiltration into the CF lung is to restore chemotaxis to normal rather than sub-normal levels. In this regard we aim to inhibit abnormal expression of IL-8 whilst leaving intact the normal processes which are necessary and sufficient for physiological neutrophil infiltration into the lung.

miR-medicines are attractive therapeutic candidates [10]. Unlike DNA-based approaches, which require nuclear delivery, miRNAs only need to be delivered to the cytoplasm, and may be more benign to cells in terms of eliciting innate immune responses. Nonetheless challenges exist with respect to their delivery and efficacy. We have demonstrated that it is possible to deliver functional PMs into CF bronchial epithelial cells using polyethyleneimine nanoparticles [49]. A similar strategy could be used to deliver miRNA mimics that target IL-8. Examining off target effects of any such approach will be necessary in order to determine the specificity of the inhibition observed. Thus nanomedicines designed to increase levels of under expressed endogenous miRNAs represent a new alternative to the existing therapeutic strategies that treat the pulmonary manifestations

of cystic fibrosis. Our results highlight miR-17 as a potential candidate for more in depth drug development studies for CF and potentially other chronic neutrophilic lung diseases.

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Figure Legends.

Figure 1. IL-8 levels in CF and non-CF bronchoalveolar lavage (BAL) fluid and bronchial brushings. (A) IL-8 protein levels (pg/ml) were measured in BAL fluid from 12 CF and 12 non-CF subjects measured by ELISA. Data are represented as mean ± SEM and were compared by *t* test (***p <0.0001). (B) Relative expression of IL-8 mRNA in bronchial brushings, (CF; n=8, non-CF; n=8) measured by qRT-PCR. Data are represented as mean ± SEM and were compared by *t* test (**p <0.01).

Figure 2. miRNAs predicted to target IL-8 in CF bronchial epithelium. (A) *In silico* analysis of miRNAs predicted to regulate IL-8. The miRNA target prediction databases TargetScan version 6.2, MicroRNA.org, PITA and Microcosm Targets were used to identify predicted miRNA sites in the full length 1252 nucleotides human IL-8 mRNA (NM_000584). (B) miRNAs selected for further study. Number of MREs in IL-8 3'UTR, miR-SVR score, relative expression in CF versus non-CF bronchial brushings and significance [11]. (C) Binding sites of selected miRNAs in the IL-8 3'UTR predicted by Targetscan 6.2 and microRNA.org.

Figure 3. miR-17 regulates IL-8. Relative luciferase activity in HEK293 cells (1×10^5 in triplicate) transiently transfected with pGL3-IL-8-3'UTR and a constitutive *Renilla* luciferase reporter plasmid pRLSV40 and co-transfected with (A) synthetic premiR (PM) or (B) antimiR (AM) for miRNAs as indicated. Firefly luciferase activity was normalized to the *Renilla* luciferase activity. Data are represented as mean ± SEM and were compared by *t* test (**p <0.01, *p <0.05, n=3)

Figure 4. mmu-miR-17 expression in βENaC-Tg and *Cftr-/-* vs. wild-type mice**.** Relative expression of murine miR-17 and let-7b was determined by qRT-PCR using individual TaqMan murine miRNA assays and normalised to U6 snRNA in whole lung homogenates of (A) 2-week-old (n=5 each) and trachea and bronchi of 6-week-old (n=6 each) wild type and βENaC-Tg mice or (B) bronchi of 30-week-old (n=6 each) wild type and *Cftr-/-* mice Data are represented as mean ± SEM and were compared by *t* test (*p <0.05).

Figure 5. miR-17 in cell lines, primary cells and under chronic inflammatory conditions. Relative expression of miR-17 was determined by qRT-PCR by TaqMan miRNA assays and normalised to U6 snRNA in IB3 (n=6), CFTE29o⁻ (n=3) and CFBE41o⁻ (n=3) epithelial cell lines, CF pediatric primary epithelial cultures (n=7), and S9 cells stimulated for 5 days with 1% *Pseudomonas*-conditioned medium (n=5). Data are represented as fold-change ± SEM compared to S9 (n=8), 9HTEo- (n=3), 16HBE14o- (n=3), non-CF primary epithelial cells (n=2), or untreated S9 cells (n=5), respectively (i.e. dotted line at 1-fold). Data were compared by *t* test Data were compared by *t* test (*p <0.05, **p <0.01).

Figure 6. Effects of miRNA over-expression on IL-8 protein production in CFBE41o- and CFTE29o- cells stimulated with LPS. IL-8 (pg/ml) was measured by ELISA in the supernatant of cells (1 x 10⁵/ml) transfected with a synthetic Pre-miR (PM) for let-7b and miR-17, or a scrambled control and stimulated with LPS (10μg) for 24 h. Data are represented as mean ± SEM and were compared to untreated (***p <0.0001) or scrambled control (*p <0.05, **p <0.005, ***p <0.0001) by *t* test.

Figure 7. Effects of miRNA over-expression on IL-8 protein production in IB3 cells stimulated with LPS, *Pseudomonas*-conditioned medium or CF BAL fluid. IL-8 (pg/ml) was

measured by ELISA in the supernatant of cells (1 x 10^5/ml) transfected with a synthetic pre-miR (PM) for miR-17 or a scrambled control (Scr) and stimulated with LPS (10µg), 1% *Pseudomonas*-conditioned medium or 10 µl CF BAL fluid for 24 h (n=3). Data are represented as mean ± SEM and were compared by *ANOVA* (*p <0.05, **p <0.01, ***p <0.001).