MicroRNA-145 Antagonism Reverses TGF- β Inhibition of F508del CFTR Correction in Airway Epithelia

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Abstract

Rationale: MicroRNAs (miRNAs) destabilize mRNA transcripts and inhibit protein translation. miR-145 is of particular interest in cystic fibrosis (CF) as it has a direct binding site in the 3'-untranslated region of CFTR (cystic fibrosis transmembrane conductance regulator) and is upregulated by the CF genetic modifier TGF (transforming growth factor)- β .

Objectives: To demonstrate that miR-145 mediates TGF- β inhibition of CFTR synthesis and function in airway epithelia.

Methods: Primary human CF (F508del homozygous) and non-CF airway epithelial cells were grown to terminal differentiation at the air-liquid interface on permeable supports. TGF- β (5 ng/ml), a miR-145 mimic (20 nM), and a miR-145 antagonist (20 nM) were used to manipulate CFTR function. In CF cells, lumacaftor (3 μ M) and ivacaftor (10 μ M) corrected mutant F508del CFTR. Quantification of CFTR mRNA, protein, and function was done by standard techniques.

Measurements and Main Results: miR-145 is increased fourfold in CF BAL fluid compared with non-CF ($P < 0.01$) and increased 10-fold in CF primary airway epithelial cells ($P < 0.01$). Exogenous TGF- β doubles miR-145 expression ($P < 0.05$), halves wild-type CFTR mRNA and protein levels ($P < 0.01$), and nullifies lumacaftor/ivacaftor F508del CFTR correction. miR-145 overexpression similarly decreases wild-type CFTR protein synthesis ($P < 0.01$) and function ($P < 0.05$), and eliminates F508del corrector benefit. miR-145 antagonism blocks TGF- β suppression of CFTR and enhances lumacaftor correction of F508del CFTR.

Conclusions: miR-145 mediates TGF- β inhibition of CFTR synthesis and function in airway epithelia. Specific antagonists to miR-145 interrupt TGF- β signaling to restore F508del CFTR modulation. miR-145 antagonism may offer a novel therapeutic opportunity to enhance therapeutic benefit of F508del CFTR correction in CF epithelia.

Keywords: microRNA; TGF- β ; CFTR modulation; cystic fibrosis; lumacaftor

Cystic fibrosis (CF) is the most common monogenic fatal respiratory disease, affecting 1 in 3,500 white individuals (1, 2). TGF (transforming growth factor)- β has been identified repeatedly as a genetic modifier of CF disease progression, with direct effects on CFTR (cystic fibrosis transmembrane conductance regulator) synthesis and

function (3–8). We and others have identified an association between increased TGF- β and diminished CFTR transcription, protein levels, and ion channel function, suggesting a feedback loop in which increased TGF- β diminishes CFTR activity, and loss of CFTR function increases TGF- β -dependent secretion and signaling

(9, 10). As TGF- β is a pluripotent regulator of cellular functions including cell differentiation, repair, and remodeling (11, 12), it is important to ascertain the precise mechanisms by which TGF- β modifies CFTR function and CF lung disease.

MicroRNAs (miRNAs) are small (20–22 nucleotides) noncoding RNAs that

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At a Glance Commentary

Scientific Knowledge on the Subject:

Improving F508del CFTR (cystic fibrosis transmembrane conductance regulator) correction is a critical unmet therapeutic need for patients with cystic fibrosis (CF). TGF- β (transforming growth factor)- β is a genetic modifier of CF that decreases CFTR synthesis and function. MicroRNAs (miR) are small noncoding RNA that post-transcriptionally regulate gene expression. Manipulation of miR-145 may be relevant to improving F508del CFTR correction as miR-145 is stimulated by TGF- β and has a direct binding site on CFTR.

What This Study Adds to the Field:

miR-145 mediates TGF- β inhibition of CFTR synthesis and function in lumacaftor-corrected CF airway epithelia through diminished availability of F508del CFTR mRNA transcripts and protein translation. In CF primary polarized airway epithelia, antagonists to miR-145 reverse TGF- β downregulation of CFTR and enhance lumacaftor/ivacaftor correction of F508del CFTR function.

may regulate more than 60% of protein-coding genes in human cells (13–15). miRNAs have emerged as important modifiers of lung development and pulmonary fibrosis (16, 17), modifying mRNA transcript stability and protein translation in a sequence-specific manner (18). Candidate miRNAs have been identified that regulate CFTR expression and function (15, 19). Among these, miR-145 is of significant interest to our group as it has a direct binding site on the 3'-untranslated region (3'-UTR) of the CFTR mRNA transcript (20–22) and is upregulated by TGF- β in fibrotic disease (23). In the era of CFTR modulator development (24–27), delineating mechanistic factors that partially restore or inhibit CFTR function acquires enhanced significance for optimizing strategies for CFTR modulation. Similarly, increased awareness of acquired CFTR dysfunction (28–31) in non-CF respiratory disease extends the significance of TGF- β

inhibition of CFTR levels and function. In the current study, we build on the initial reports of miRNA in CF to investigate the contribution of miRNA to TGF- β -associated CF lung disease and the impact on F508del CFTR corrector response. Specifically, we hypothesize that miR-145 mediates TGF- β downregulation of CFTR in airway epithelia, and conduct a series of experiments to test the benefit of miRNA antagonism to augment CFTR correction in the setting of TGF- β -associated disease. Some of these results previously have been reported in the form of abstracts presented to the North American Cystic

Fibrosis Conference and American Thoracic Society international meetings (32, 33).

Methods

Cell Culture

Primary polarized non-CF and CF (homozygous F508del) human airway epithelial cells were cultured by the Gregory Fleming James Cystic Fibrosis Research Center Tissue Procurement Core at the University of Alabama at Birmingham, utilizing established techniques (27, 34, 35). Briefly, cells were initially expanded in growth medium and then differentiated on

Figure 1. MicroRNA (miRNA) expression profiles in human airway epithelial cells. (A) miR-145 (miR-145) predicted binding site on 3'-untranslated region (UTR) of CFTR (cystic fibrosis transmembrane conductance regulator) mRNA that is highly conserved across species (TargetScan 7.1). (B) Luciferase reporter activity for miR-145 binding to CFTR 3'-UTR was measured in CFBE410^o cell lines. Each data point represents summary data from three independent experiments ($n = 3$). (C) Expression profiles of CFTR-specific miRNAs (miR-145, miR-101, and miR-494) in non-cystic fibrosis (CF) and CF primary airway epithelial cells. Each miRNA data point represents summary data from three biological replicates ($n = 3$ donors). (D) Increased miR-145 in F508del CFBE cell line ($n = 3$). All experiments were repeated at least three times. Quantitative PCR data were analyzed by the $\Delta\Delta C_t$ method. Data are presented as the median of normalized expression (to U6 small nuclear RNA) with interquartile range. WT = wild type. * $P < 0.05$; ** $P < 0.01$.

collagen-coated Costar Transwell filters (0.3 cm² at density) for experimentation after 6–8 weeks of culture at the air–liquid interface (29). Complementary human bronchial epithelial CFBE41o[−] cell lines stably transduced with wild-type CFTR (wild-type CFBE) and F508del CFTR (F508del CFBE) were similarly cultured. Unless specified otherwise, results were from at least three separate donors with the experiments performed in triplicate.

BAL Fluid Exosomes

BAL was performed per clinical routine (36, 37), and remnant fluid was collected and processed as previously described (38). In brief, exosomes were isolated by differential centrifugation (300 g for 10 min, then 2000 g

for 20 min, then 10,000 g for 30 min) and then pelleted at 150,000 g for 2 hours before resuspension in phosphate-buffered saline and centrifuging at 500,000 g for 15 minutes. Exosome quantification was performed with the NanoSight NS300 (Malvern Instruments), stained with Qtracker 565 (Life Technologies) and visualized with a 488-nm laser module and long-pass filter.

miRNA Isolation and qPCR Assays

miRNA was isolated from cells and human BAL-derived exosomes, using an miRNeasy mini kit according to the manufacturer's instruction (Qiagen). Isolated RNAs were processed for real-time qPCR, using QIAgility robot and Rotor-Gene Q PCR cyclers system (Qiagen) in accordance with

the manufacturer's instructions (see www.qiagen.com). TaqMan probes for *CFTR*, *TGF-β*, *PAI* (plasminogen activator inhibitor)-1, *BMP2* (bone morphogenetic protein 2), and *LTBP2* (latent TGF-β-binding protein 2) genes, and miRNAs including miR-101, miR-145-5p, miR-494, and small nucleolar RNA U6, were purchased from Applied Biosystems.

Transfection

Primary airway epithelial cells were grown on Transwell permeable supports (Corning Costar) to terminal differentiation with transepithelial resistance greater than 500 Ω on 24-well plates. Transfection utilized Lipofectamine RNAiMAX (Invitrogen) with miRNA precursors

Figure 2. TGF (transforming growth factor)-β stimulates microRNA (miR)-145 in cystic fibrosis (CF) BAL fluid and airway epithelial cells. (A) Increased miR-145 in human CF BAL-derived exosomes ($n = 7$) compared with non-CF ($n = 5$). (B and C) TGF-β transcription and signaling (plasminogen activator 1 [PAI-1] expression) are increased in human CF BAL-derived exosomes ($n = 6$) compared with non-CF ($n = 4$) samples. (D) Baseline expression of TGF-β transcription and signaling (PAI-1) is increased in CF airway epithelial cells ($n = 3$) without stimulation. (E) Addition of exogenous TGF-β (5 ng/ml) further doubles miR-145 in non-CF and CF epithelial cells compared with DMSO control ($n = 3$). All quantitative PCR experiments were repeated at least three times. Data points represent summary data from each donor. Normalized data expression is presented as median value with interquartile range. DMSO = dimethyl sulfoxide. * $P < 0.05$; ** $P < 0.01$.

(miR-145 mimic) and miRNA inhibitors (for miR-145) or miR-145 negative control (Ambion, Life Technologies) at a final concentration of 20 nM.

3'-UTR Reporter Assay

Wild-type CFBE and F508del CFBE cell lines were transfected with human CFTR 3'-UTR (GenBank accession No. NM_000492.3) reporter plasmid pEZX-MT06 (100 ng) (GeneCopoeia), together with miR-145 mimic or miR-145 negative control (25 nM) (Ambion, Life Technologies). After 24–36 hours, firefly and *Renilla* luciferase reporter activities were measured with a Luc-Pair Duo-Luciferase assay kit 2.0, according to the manufacturer's instructions (GeneCopoeia).

Immunoblots

Western blot analysis was performed and quantified as described previously (39). Human anti-CFTR antibodies were obtained from Cell Signaling and Millipore.

Ussing Chamber Measurements for CFTR Function

Epithelial cells grown to confluence were mounted in modified Ussing chambers. Short-circuit current (Isc) was obtained with an epithelial voltage clamp as described previously (34). Briefly, the mucosal bathing solution was changed to a low Cl⁻ solution containing 1.2 mM NaCl, 115 mM sodium gluconate, plus 100 μ M amiloride followed by addition of agonists (20 μ M forskolin, and/or 10 μ M ivacaftor) to the mucosal surface. CFTR inhibitor 172 (CFTR_{inh}-172) (10 μ M; Sigma-Aldrich) was added to the

bathing solution at the conclusion of each experiment to block CFTR-dependent Isc.

Regulatory Approval

All studies involving human subjects or material were reviewed and approved by the University of Alabama Institutional Review Board (Protocols X081204008, F070813009).

Statistical Analysis

Data comparisons were performed with SAS version 9.4 (SAS Institute) and GraphPad Prism version 7.1 (GraphPad Software Inc.). *In vitro* experiments with primary airway epithelia studies utilized biospecimens collected from multiple donors ($n = 4$ CF and $n = 5$ non-CF biological replicates),

Figure 3. TGF (transforming growth factor)- β inhibits CFTR (cystic fibrosis transmembrane conductance regulator) synthesis and function in airway epithelial cells. (A) TGF- β (5 ng/ml) significantly diminishes CFTR mRNA in non-cystic fibrosis (CF) and F508del CF primary polarized airway epithelial cells. (B) Immunoblots indicate similar reduction in CFTR protein (C band, ~170 kD). (C) Densitometric analysis for CFTR protein C band normalized to β -actin ($n = 3$ donors). (D and E) Representative tracing of short-circuit current in modified Ussing chamber as a measure of CFTR function in (D) non-CF and (E) CF primary polarized epithelia (forskolin/VX-770: positive stimulation, higher value reflects increased CFTR function; CFTR_{inh}-172: negative stimulation, more negative value reflects greater CFTR current response). All investigations involved at least three CF and non-CF donors, with studies repeated in triplicate. Data points indicate summary data from each donor and are presented as median with interquartile range. * $P < 0.05$. DMSO = dimethyl sulfoxide; Isc = short-circuit current reflective of CFTR-dependent ion transport; MW = protein marker molecular weight; VX-770 = ivacaftor, a CFTR potentiator.

with each biospecimen analyzed in triplicate ($n = 3$ technical replicates). Technical replicates from each donor were averaged to generate a single summary value for each biological replicate under each condition. For comparison of values across multiple donors and conditions, data distribution was analyzed for normality. Because cells from each sample donor were manipulated under parallel treatment conditions, these values from different groups were not considered independent measures. As results from the same donor were correlated, statistical methods for correlated outcomes were applied for group comparisons. If departure from normality was not observed, repeated-measures ANOVA was conducted for multiple-group comparisons and paired t test for two-group comparisons. In these comparisons across groups, data were represented as mean value (SD). If nonnormality could not be presumed, nonparametric comparisons utilizing the Friedman test and Wilcoxon signed-rank test were conducted. A P value not exceeding 0.05 was considered significant.

Results

Identification of TGF- β - and CFTR-Relevant miRNAs

We determined the expression profile of a panel of relevant CFTR-specific miRNAs in human BAL fluid and primary airway epithelial cells (AECs). Before *in vitro* assays, we computationally screened miRNAs predicted to regulate the CFTR gene (TargetScan 7.1; see Figure E1 in the online supplement), in which three candidate miRNAs (miR-101, miR-145, and miR-494) exhibited direct, well-conserved binding sites on the 3'-UTR of CFTR mRNA across species that had supportive literature reporting significance in CF and/or influence on CFTR transcript stability (20–22). Identification of these CFTR-specific miRNAs was further confirmed by two other popular prediction algorithms (miRWalk and miRanda; Table E1). miR-145 was emphasized for its direct relevance to TGF- β -associated respiratory disease and established binding site of CFTR mRNA (40–43).

To confirm whether miR-145 directly binds to the 3'-UTR of the human CFTR transcript (as shown in Figure 1A), we transfected CFBE cell lines with a commercially available CFTR 3'-UTR reporter construct together with a miR-145

mimic or a negative control precursor miRNA. When the reporter plasmid containing the CFTR 3'-UTR cloned upstream of its promoter region is bound by the specific miRNA, luminescence is suppressed. Addition of the miR-145 mimic to CFBE cells significantly decreased luciferase reporter activity by more than 50% ($P < 0.01$), demonstrating direct binding of miR-145 to the human CFTR 3'-UTR (Figure 1B).

Expression of miRNA-145 in Airway Epithelia

We first evaluated miRNA expression profiling in RNA samples isolated from human AECs to quantify relevance to human disease. The extraction method included all small RNAs including miRNAs.

For miRNA expression screening in primary epithelial cells, we examined CFTR-specific miRNAs (miR-145, miR-101, and miR-494) and identified significantly increased miR-145 (approximately 10-fold) in F508del CF primary AECs (Figure 1C). Evaluation of human bronchial epithelial cell lines similarly demonstrated a twofold increase in miR-145 expression in the F508del CFBE cell line compared with wild-type CFBE cells (Figure 1D). These expression profiles indicate the relevance of increased miR-145 expression in CF epithelia.

Expression of miR-145 in CF BAL Fluid Exosomes

To confirm the relevance of miR-145 to CF pulmonary disease, we isolated

Figure 4. MicroRNA (miR)-145 inhibits CFTR (cystic fibrosis transmembrane conductance regulator) in airway epithelial cells. (A) Reduction in CFTR mRNA after administration of miR-145 mimic (20 nM). (B) Immunoblot of CFTR protein (C band) after administration of miR-145 mimic (top). Densitometric analysis for CFTR protein band normalized to β -actin (bottom). All investigations involved at least three donors ($n = 3$). Each data point indicates a summary value from each donor. Data are represented as median with interquartile range. ** $P < 0.01$. (C) Representative tracing of change in wild-type CFTR function in a single donor after administration of miR-145 mimic. CF = cystic fibrosis; CFTRinh-172 = an inhibitor of CFTR; Ctrl = miRNA control; Isc = short-circuit current reflective of CFTR-dependent ion transport; mimic = miR-145 agonist.

exosomes from CF and non-CF BAL fluid. miRNA qPCR assays identified increased miR-145 expression (>fivefold) in CF BAL samples compared with the non-CF control group (Figure 2A). As TGF- β is a major stimulus of miR-145 in non-CF disease, we also measured TGF- β and a representative downstream signal (PAI-1) by qPCR. TGF- β mRNA expression was increased threefold in CF BAL exosomes (Figure 2B) and TGF- β signaling (PAI-1) was upregulated >10-fold compared with non-CF samples (Figure 2C).

TGF- β Stimulates miR-145 Expression in Airway Epithelial Cells

To evaluate the source of increased miR-145 in CF epithelia, we tested the idea that TGF- β stimulates miR-145 expression in AECs. Under basal conditions, CF primary AECs manifest a threefold increase in both TGF- β transcription and signaling (represented by PAI-1 expression) compared with non-CF cells (Figure 2D). Previous reports have suggested that TGF- β exposure upregulates miR-145 expression in smooth muscle and fibroblasts (23, 44, 45), but no previous studies have examined

the miR-145 response to TGF- β in airway epithelial cells. Exogenous TGF- β (5 ng/ml) added to the basolateral medium of non-CF and CF primary AECs increased miR-145 expression more than twofold (Figure 2E). These data suggest that TGF- β stimulation upregulates miR-145 transcription in airway epithelia.

TGF- β Inhibits CFTR Synthesis and Function in Airway Epithelia

To establish a signaling pathway connecting TGF- β , miR-145, and CFTR function we first determined whether

Figure 5. MicroRNA (miR)-145 antagonism reverses TGF (transforming growth factor)- β inhibition of CFTR (cystic fibrosis transmembrane conductance regulator) in airway epithelia. (A and B) Response of representative TGF- β signals BMP2 and LTBP2 to miR-145 antagonism (20 nM) and/or TGF- β (5 ng/ml) stimulation. (C) Immunoblots for CFTR protein in setting of TGF- β stimulation and/or miR-145 antagonism. (D) Densitometric analysis for CFTR protein band normalized to β -actin. (E) Representative tracing from a single donor of wild-type CFTR functional response to TGF- β stimulation and miR-145 antagonism. (F) Cumulative data from biological replicates ($n = 3$ non-CF donors) in response to forskolin. All investigations involved at least three donors, each repeated in triplicate. Data points represent a summary value from each donor. Data represented as median with interquartile range. * $P < 0.05$; ** $P < 0.01$. AM = miR-145 antagonist; BMP2 = bone morphogenetic protein 2; CFTRinh-172 = an inhibitor of CFTR; Ctrl = control; FSK = forskolin; ΔI_{sc} = change in short-circuit current reflective of alteration in CFTR-dependent ion transport; LTBP2 = latent TGF- β -binding protein 2.

TGF- β can alter CFTR expression. Exogenous TGF- β added to basolateral medium of non-CF and CF primary AEC monolayers grown on Transwell filters at the air-liquid interface for 24 hours significantly diminished CFTR transcript and protein levels (Figures 3A–3C). These reductions in CFTR mRNA and protein levels after TGF- β stimulation led to representative reduction in both wild-type and F508del CFTR channel function in airway epithelia evaluated by short-circuit current (Isc) measurement in modified Ussing chambers (Figures 3D and 3E). This functional assay measured CFTR ion channel activity in which CFTR Isc was stimulated with the cyclic AMP agonist forskolin and then potentiated (CF cells) with ivacaftor (VX-770), followed by functional inhibition with CFTR_{inh}-172.

miR-145 Downregulates CFTR in Primary Airway Epithelial Cells

We then tested the hypothesis that miR-145 mediates TGF- β inhibition of CFTR synthesis and function in airway epithelia. The CFTR gene harbors highly conserved nucleotide sequences (7–8 nucleotides long through 3'-UTR) that are specifically bound by miR-145 (Figure 1A), blocking CFTR mRNA translation or stability. Binding of miRNA-145 to the CFTR 3'-UTR has been reported previously as well as by our assays (Figure 1B) (19, 21, 22), but its impact on CFTR biological function in primary airway epithelia remains suboptimally defined. Non-CF primary polarized AECs were transfected basolaterally with a 20 nM concentration of a miR-145 mimic (double-stranded RNAs that mimic endogenous precursor of mature miR-145), or miR-145 negative control for 48 hours. Overexpression of miR-145 by mimics led to a significant decrease in CFTR mRNA (Figure 4A) and protein levels (Figure 4B) ($P < 0.05$). The functional consequence of the miR-145 mimic on ion transport is shown in Figure 4C.

Anti-miR-145 Attenuates TGF- β Signaling in Airway Epithelia

As TGF- β increased miR-145 expression in airway epithelial cells, we hypothesized that a miR-145-specific antagomir would interrupt TGF- β signaling. This miR-145 antagonist is a single-stranded RNA-based oligonucleotide designed to bind specifically to miR-145 and inhibit miRNA-induced

silencing activity. To assess this novel TGF- β -inhibitory strategy, non-CF primary polarized AECs were transfected with a miR-145 antagonist and TGF- β for 24 hours. We then examined a number of TGF- β pathway-specific genes including BMP2 and LTBP2. Inhibition of miR-145 significantly downregulated the expression of BMP2 and LTBP2 in primary AECs (Figures 5A and 5B), indicating that miR-145 antagonism has broad applicability to diminish TGF- β signaling in airway epithelia.

miR-145 Antagonism Reverses TGF- β Suppression of CFTR in Primary AECs

The above data indicate that TGF- β negatively regulates CFTR function through increased miR-145 expression in AECs and that miR-145 antagonism inhibits TGF- β signaling. We then hypothesized that antagonism to miR-145 would limit TGF- β inhibition of wild-type CFTR. To investigate this mechanism, we transfected non-CF primary AECs basolaterally with 20 nM anti-miR-145 or a scrambled control for 48 hours. In TGF- β -treated non-CF primary AECs, miR-145 antagonism significantly reversed TGF- β inhibition of CFTR protein synthesis (Figures 5C and 5D). Consistent with these biochemical data, miR-145 antagonism reversed TGF- β inhibition of wild-type CFTR function by restoring approximately 80% of forskolin-sensitive short-circuit currents (control, 22.6 [4.3] $\mu\text{A}/\text{cm}^2$; TGF- β , 10.8 [0.9] $\mu\text{A}/\text{cm}^2$; TGF- β + miR-145 antagonist, 19.7 [3.2] $\mu\text{A}/\text{cm}^2$; $P < 0.05$) (Figures 5E and 5F). These data demonstrate a significant benefit for miR-145 antagonists to reverse TGF- β inhibition of wild-type CFTR.

Investigation of miR-145 Manipulation in Lumacaftor-Corrected F508del CF Primary Airway Epithelia

miR-145 replicates TGF- β inhibition of F508del CFTR correction in CF primary airway epithelial cells. Correction of F508del with lumacaftor is antagonized by TGF- β . If miR-145 is an intermediate in the biochemical pathway employed by TGF- β to reduce CFTR expression, we predicted miR-145 should also antagonize CFTR corrector agents. To test this hypothesis, we transfected primary polarized CF AECs with a 20 nM concentration of miR-145 mimic or negative control for 48 hours.

Figure 6. TGF (transforming growth factor)- β and microRNA (miR)-145 inhibit F508del CFTR (cystic fibrosis transmembrane conductance regulator) correction in cystic fibrosis primary airway epithelial cells. (A) TGF- β inhibition of lumacaftor-corrected F508del CFTR function. Cumulative data from biological replicates ($n = 4$ cystic fibrosis [CF] donors) in response to forskolin and VX-770 (ivacaftor). (B) miR-145 inhibition of lumacaftor-corrected F508del CFTR function ($n = 3$ CF donors). Investigations involved at least three CF donors, with studies repeated in triplicate and summary data from each donor represented as median with interquartile range. $*P < 0.05$. Ctrl = control; FSK = forskolin; ΔIsc = change in short-circuit current; Lum = lumacaftor (a corrector of F508del CFTR).

A topic relevant to current CFTR therapeutic development is the impact of miR-145 on CFTR correction. F508del CFTR primary AECs were treated with 3 μM lumacaftor (VX-809) and TGF- β for 24 hours. First, we replicated previous reports of TGF- β inhibition of CFTR correction (9), with significant reductions in CFTR channel activity in CF primary AECs (Figure 6A). In lumacaftor-treated F508del CF AECs, miR-145 mimics

similarly abolished the F508del CFTR corrector benefits (control, 15.6 [4.4] $\mu\text{A}/\text{cm}^2$; lumacaftor, 32.9 [7.2]; lumacaftor + miR-145 mimic, 14.5 [3.2]; $P < 0.05$) after forskolin/ivacaftor stimulation (Figure 6B), implying that miR-145 mediates TGF- β downregulation of CFTR, likely through transcriptional instability that decreases the available substrate for lumacaftor correction.

miR-145 Antagonism Restores CFTR Correction in TGF- β -Exposed Airway CF Epithelia

Anti-miR-145 showed a substantial biochemical benefit in our non-CF primary airway epithelial cells by antagonizing TGF- β signaling and restoring CFTR function. We further investigated the benefit of miR-145 antagonism in CF primary airway epithelial cells. Cells

transfected with the specific miR-145 antagonist underwent basolateral treatment with TGF- β and lumacaftor for 24 hours. As in non-CF cells, miR-145 antagonism blocked TGF- β stimulation of BMP2 and LTBP2 to baseline levels, indicating that miR-145 antagonism attenuates TGF- β signaling in CF epithelia.

We then hypothesized that TGF- β nullification of lumacaftor CFTR

Figure 7. MicroRNA (miR)-145 antagonism reverses TGF (transforming growth factor)- β suppression of CFTR (cystic fibrosis transmembrane conductance regulator) correction in primary cystic fibrosis (CF) airway epithelial cells. (A) Immunoblots for CFTR protein (C band) after TGF- β stimulation and miR-145 antagonism. CFBE cell lines (wild-type and F508del) in the first two lanes serve as controls to demonstrate antibody detection of both CFTR C and B bands. Antagonists to miR-145 interrupt TGF- β diminution of CFTR protein to lumacaftor-corrected levels. (B) Densitometric analysis of immunoblots from primary CF airway epithelial cells ($n = 4$). (C) Representative tracing of F508del CFTR function in response to TGF- β stimulation and miR-145 antagonism. miR-145 antagonism nearly completely restores lumacaftor correction of CFTR function. (D) Cumulative data from biological replicates ($n = 4$ CF donors) in response to forskolin/ivacaftor stimulation. Summary data reflect TGF- β inhibition of lumacaftor F508del CFTR correction and subsequent restoration by miR-145 blockade. All investigations involved at least three CF donors, with studies from each donor repeated in triplicate. Summary data from each donor represented as median with interquartile range. * $P < 0.05$; ** $P < 0.01$. AECs = airway epithelial cells; AM = miR-145 antagonist; Ctrl = control; ΔIsc = change in short-circuit current; FSK = forskolin, a CFTR agonist to stimulate channel opening; inh-172 = CFTR_{inh}-172, an antagonist to induce channel closure; Lum = lumacaftor, a CFTR corrector; VX-770 = ivacaftor, a CFTR potentiator; WT = wild type.

Figure 8. MicroRNA (miR)-145 antagonism enhances F508del CFTR (cystic fibrosis transmembrane conductance regulator) correction. (A) Augmented lumacaftor response with miR-145 antagonism even in the absence of TGF- β stimulation. (B) Cumulative data from biological replicates ($n = 4$ cystic fibrosis donors) in response to forskolin/ivacaftor stimulation. Summary value from each donor represented as median and interquartile range. $*P < 0.05$. AM = miR-145 antagonist; Ctrl = control; FSK = forskolin, a CFTR agonist to stimulate channel opening; inh-172 = CFTR_{inh}-172, CFTR antagonist to induce channel closure; Isc = short-circuit current; Lum = lumacaftor, a CFTR corrector; VX-770 = ivacaftor, a CFTR potentiator.

corrector benefit could be reversed by miR-145 antagonism. CF primary AECs were treated basolaterally with lumacaftor and miR-145 antagonists for 24 hours and then with TGF- β for another 24 hours. We first identified that utilization of miR-145 antagonism restored the F508del corrector benefit in TGF- β -treated CF AECs. miR-145 inhibition significantly augmented Δ F508-CFTR protein synthesis (Figures 7A and 7B) and nearly completely restored channel function, diminishing TGF- β -inhibitory effects in CF primary AECs (control, 14.5 [4.2] μ A/cm²; lumacaftor, 30.3 [7.3] μ A/cm²; lumacaftor + TGF- β , 16.0 [7.4] μ A/cm²; lumacaftor + TGF- β + miR-145 antagonist, 26.4 [7.9] μ A/cm²; $*P < 0.05$) after forskolin/ivacaftor stimulation (Figures 7C and 7D).

As CF primary polarized cells have elevated TGF- β signaling and miR-145 expression at baseline (Figures 1C and 2A), we then examined the benefit of miR-145 antagonism in CF AECs treated with lumacaftor only (e.g., in the absence of exogenous TGF- β stimulation). Lumacaftor-corrected CF primary AECs were exposed to miR-145 antagonists or a miRNA control for 48 hours. miR-145 antagonism significantly improved lumacaftor benefit in CF AECs, augmenting F508del CFTR corrector function by more than 25% (control, 14.1 [4.6]; lumacaftor, 25.0 [10.4]; lumacaftor + miR-145 antagonist, 32.0 [10.5]; $*P < 0.05$) after

forskolin/ivacaftor stimulation (Figures 8A and 8B).

Together, these data indicate the potential benefit of miR-145 antagonists to improve CFTR corrector benefit in CF epithelia, in both the setting of increased TGF- β signaling and under basal CF conditions.

Discussion

This study is the first to identify miR-145 as a targetable mechanism linking TGF- β signaling to diminished CFTR function with direct consequences on CFTR modulator strategies. TGF- β is a well-established genetic modifier of CF lung disease (3), with more profound modifier effects in the setting of partial CFTR function or adverse environmental exposure (5). We and others (9, 10) have previously shown a relationship between TGF- β exposure and CFTR functional inhibition, and the present findings now provide a direct mechanism for this response. miR-145 has been identified previously as a significant modifier of TGF- β pathobiology in pulmonary hypertension and fibrosis with specific relevance to smooth muscle and myofibroblast phenotype (23, 43, 44, 46, 47), and now we have identified a similar role for this miRNA in CF airway epithelia. With rapidly expanding knowledge of miRNA (40, 41, 48) and the reported preclinical success utilizing miR-145

in animal models of asthma and pulmonary hypertension (46, 49, 50), manipulation of miR-145 may offer a novel avenue to augment the *in vivo* CFTR modulator response in the setting of TGF- β -associated lung pathology. Our results extend previous miRNA reports (20–22) in CF to now identify miR-145 as a targetable obstacle limiting the efficacy of F508del CFTR correction in the setting of increased TGF- β signaling.

Our results provide a mechanism through which TGF- β inhibits CFTR synthesis and function in non-CF cells and suppresses lumacaftor correction of F508del CFTR in CF epithelia (Figure 9). As an endogenous biomolecule, miR-145 regulates many gene products including CFTR. TGF- β upregulates miR-145 that binds the 3'-UTR of CFTR mRNA to decrease transcript stability, block translation, or both. In the setting of CFTR modulation, miR-145 reduces the amount of mutant F508del CFTR substrate available for correction. In acquired CFTR dysfunction in non-CF disease, miR-145 limits CFTR protein translation to reduce the number of available channels to facilitate ion transport. Antagonism to miR-145 restores F508del CFTR correction and wild-type CFTR function, suggesting a general applicability to improving CFTR modulator strategies across genotypes.

Our study focused on miR-145 to mediate TGF- β pathobiology in CF and limit CFTR correction because

Figure 9. Schematic illustrating proposed mechanisms of microRNA (miR)-145-mediated TGF-β (transforming growth factor)-β downregulation of CFTR (cystic fibrosis transmembrane conductance regulator) in airway epithelia. (A) Mechanism: Increased TGF-β in cystic fibrosis (CF) epithelia stimulates miR-145 to inhibit CFTR protein translation and reduce availability of F508del CFTR substrate for lumacaftor corrector. (B) Intervention: miR-145 antagonists inhibit miR-145 binding to CFTR, thereby increasing F508del CFTR synthesis and enhancing corrector function. Isc = short-circuit current; Lum = lumacaftor, a CFTR corrector.

this miRNA uniquely overlapped computationally predicted miRNA data sets for TGF-β and CFTR (TargetScan miRNA target prediction tool, release 7.1). Among CFTR-relevant miRNAs, miR-101, miR-145, and miR-494 are commonly reported (15, 19, 20, 22). The data set for TGF-β-relevant miRNA in lung disease is broader, but similarly includes miR-145 among many others (44, 45, 51, 52). We then evaluated human CF BAL fluid and airway epithelia for expression of these miRNAs, of which miR-145 was found to be robustly increased. Our concomitant findings of increased TGF-β and downstream signal PAI-1 suggest that increased TGF-β signaling in CF cells is one stimulus for this increased miR-145 expression. These initial computational

predictions and screening measures suggest miR-145 is available for TGF-β manipulation in lung tissue and may contribute to CFTR dysfunction in respiratory epithelia.

miR-145 regulation by TGF-β has not been studied extensively before in CF disease. Our data now provide multiple contributions to this literature. First, we show that miR-145 is significantly increased in CF primary polarized primary epithelia and BAL exosomes compared with non-CF. Even under basal conditions, the miRNA milieu is altered in primary CF cells. Our data in primary airway epithelial cells suggest that this elevation is at least partially secondary to increased TGF-β expression and signaling that we have previously identified in CF BAL fluid and

lung tissue (36, 53). The increased expression of miR-145 in CF epithelia may limit the efficacy of mutant CFTR correction, with this hypothesis confirmed by the efficacy of miR-145 antagonism to augment CFTR modulation even in the absence of exogenous TGF-β stimulation.

An additional important finding is that TGF-β stimulation further increases miR-145 expression in both CF and non-CF airway epithelia. Our findings suggest an important application and mechanism for TGF-β to contribute to acquired CFTR dysfunction in non-CF respiratory disease. These data indicate that increased TGF-β levels in CF may be an obstacle to CFTR restoration and provides a mechanistic rationale to pursue nuanced TGF-β signaling antagonists as a possible adjunct to CFTR modulator development. Beyond direct binding to the CFTR mRNA 3'-UTR, both miR-145 and TGF-β may have additional secondary effects on CFTR promoter activity or protein stability. One relevant example of this secondary regulation is miR-145 inhibition of KLF4 (Krüppel-like factor 4), which influences cellular response to injury and differentiation (23, 41, 43, 54, 55). Although we have focused specifically on miR-145 antagonism, application of alternative TGF-β inhibitory strategies may also diminish miR-145 expression and preserve CFTR restoration.

We have focused on F508del CFTR correction for our studies as this is the most common mutation, with 90% of patients with CF having at least one F508del mutation and more than 50% of patients with CF being homozygous for this mutation (1, 2, 56). Although F508del correctors have generated *in vitro* promise, the *in vivo* response has not been as robust as desired (24, 26, 57). Our previous data on increased TGF-β in CF (36, 53, 58, 59) suggest that some of the suboptimal *in vivo* results of lumacaftor may be influenced by increased TGF-β signaling in CF lungs. The present findings in both non-CF and CF primary epithelia reinforce this concern and suggest that modulator efficacy should be evaluated in the setting of CF pulmonary conditions that are known to influence CFTR stability. Although the current data emphasize downregulation of F508del correction, the mechanism of miRNA-inhibited transcript stability suggests that suppression of CFTR-directed therapeutics

may affect next-generation modulators and additional genotypes.

Our data have the overt limitation of utilizing *in vitro* conditions rather than an *in vivo* model. Although the study of CFTR modulator response in primary polarized airway epithelial cells has successfully laid the foundation for CFTR modulator development (27, 35), pertinent questions remain concerning the *in vivo* consequence of increased TGF- β stimulation on CFTR expression in airway epithelia, including a threshold definition separating pathophysiological TGF- β conditions from baseline. The dose of 5 ng/ml is commonly utilized to replicate TGF- β -stimulated conditions for *in vitro* studies (60–63) and is consistent with the dose-finding range of 0.1–10 ng/ml that we detected in our previous report (9). Our previous studies of increased TGF- β in CF BAL fluid (36) and circulating plasma (58) support this dose

administration. An important next step in this field will be to quantify the consequences of TGF- β stimulation on CFTR function *in vivo*.

In summary, our article reports a plausible, targetable miRNA mechanism linking increased TGF- β signaling to diminished F508del CFTR correction in CF airway epithelia. Beyond the important context of CFTR therapeutics, our results provide an applicable mechanism for TGF- β -derived acquired CFTR deficiency in non-CF disease. We have identified miR-145 as the most likely miRNA to connect these two pathways and completed a series of experiments to demonstrate the necessity and sufficiency of miR-145 to mediate CFTR inhibition in airway epithelia. Specifically, miR-145 alone diminishes CFTR function and limits the potency of F508del CFTR correction in non-CF and CF airway epithelia. Relatedly, miR-145

antagonism reverses TGF- β inhibition of CFTR function and enhances lumacaftor/ivacaftor CFTR modulation in TGF- β -exposed epithelia. These findings have direct relevance to advancing the *in vivo* benefit of CFTR modulators with broad application to studies of acquired CFTR dysfunction in TGF- β -associated respiratory disease. ■

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