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Original Article

Investigation of HE4 expression concerning epithelial-mesenchymal transition (EMT) in cystic fibrosis epithelial cells

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ABSTRACT

Background: Elevated levels of human epididymis protein 4 (HE4) have been observed in cystic fibrosis (CF), where its expression is directly affected by impaired CFTR through the NF-κB pathway in p.Phe508del-CFTR CFBE 41o− cells *in vitro*. Dysfunctional CFTR is also linked to epithelial-mesenchymal transition (EMT) in CF. However, no data is available whether HE4 expression changes and is associated with CF-related EMT.
Methods: The level of EMT was compared between CFBE 41o− cells with the p.Phe508del-CFTR mutation and wt-CFTR by measuring epithelial and mesenchymal markers using fluorescence microscopy and RT-qPCR. EMT was also induced by TGFβ1 in p.Phe508del-CFTR CFBE cells from 24 to 120 h to explore relationship between EMT development and the expression of HE4 and MMP9. VX-445/VX-661/VX-770 treatment restored CFTR function to observe changes in EMT phenotype. The direct effects of HE4 on EMT and MMP9 levels were examined in CFBE cells where HE4 expression was reduced through transfection with HE4-specific siRNA. Cell proliferation was assessed by Ki67 positivity.
Results: CFBE cells expressing p.Phe508del-CFTR were more prone to be mesenchymal than wt-CFTR CFBE cells and showed higher basal HE4 and MMP9 levels. In response to TGFβ1, even lower E-cadherin with higher N-cadherin and MMP9 levels were observed in p.Phe508del-CFTR CFBE cells compared to untreated cells. In contrast, HE4 expression decreased after 48 h and continued to decline up to 120 h. CFTR modulator treatment could reverse the EMT phenotype, normalizing HE4 and MMP9 levels. Finally, downregulated HE4 promoted EMT, resulting in higher MMP9 expression and reduced cell proliferation in p.Phe508del-CFTR CFBE cells.
Conclusion: EMT is accompanied by decreasing HE4 expression and upregulated MMP9 level in the airway epithelial cells of CF.

1. Introduction

Cystic fibrosis (CF) is the most common life-threatening monogenic disorder among Caucasians, affecting approximately 100,000 people worldwide. It is a multisystem disease characterized by pancreatic insufficiency, intestinal blockage, and male infertility; however, the leading cause of illness and death is progressive respiratory failure. Lung decline results from ongoing airway inflammation, recurrent infections, and tissue remodeling, leading to lung fibrosis [1,2]. CF results from mutations in the *CFTR* gene, which encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein. This apical membrane anion channel mediates chloride and bicarbonate transport in epithelial cells. The most common mutation, *p.Phe508del-CFTR*, disrupts protein folding and trafficking, while other variants impair CFTR synthesis, gating, conductance, or cause complete protein loss [3,4].

Beyond its well-known role as an ion channel and regulator of

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; MMP9, matrix metalloproteinase 9; EMT, epithelial–mesenchymal transition; HE4, human epididymis protein 4; TGFβ1, transforming growth factor beta 1; IF, immunofluorescence; MFI, mean fluorescence intensity; DMSO, dimethyl sulfoxide; DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; CFTR-KO, CFTR-knockout.

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epithelial transport, CFTR has recently been linked to various cellular functions, including epithelial differentiation, polarization, and proliferation [5]. Proper CFTR function supports tight junctions' structural and functional integrity, is vital for epithelial barrier properties, and helps efficiently repair airway epithelia after injury [6]. In contrast, airway regeneration is delayed, and epithelial cell differentiation is impaired in CF [7], with altered pro-inflammatory and matrix metalloproteinase (MMP) responses, such as increased production of interleukin-8 and MMP9 [8]. Additionally, hyperproliferation of CF airway epithelial cells has been observed, and epidemiological data show an increased risk of malignancies, especially gastrointestinal cancers [9]. These findings support a tumor-suppressive role for CFTR [10] and suggest possible links between its dysfunction, the development of epithelial–mesenchymal transition (EMT), and oncogenesis [11].

EMT is a conserved developmental cellular process in which epithelial cells undergo transcriptional reprogramming toward a mesenchymal phenotype [12]. This process, driven by transcription factors like TWIST1, involves the loss of epithelial junctional proteins, cytoskeletal reorganization, and the activation of mesenchymal markers, such as Vimentin, α-SMA, and N-cadherin [13]. Transforming growth factor beta 1 (TGFβ1) is one of the main drivers of EMT, and its total level was elevated in CF tissue compared to normal lung tissue, promoting myofibroblast differentiation and tissue fibrosis [14]. However, it remains unclear whether abnormal CFTR function and/or secondary events, such as bacterial infection and inflammation also contribute to EMT activation in CF [15,16].

Our group has studied the expression of human epididymis protein 4 (HE4) in CF through both *ex vivo* and in vitro studies [17–19]. Elevated serum HE4 levels were positively linked to the severity of pulmonary dysfunction and overall disease severity in untreated CF cohorts [17], while plasma HE4 levels inversely correlated with lung function improvement in CF patients receiving CFTR-specific therapies, such as VX-445/VX-661/VX-770, called Kaftrio® [18]. Additionally, HE4 expression was directly affected by impaired CFTR function via the NF-κB pathway in p.Phe508del-CFTR CFBE 41o− cells in vitro [19]. However, no data is available on how HE4 expression changes in CF-related EMT.

This study investigated HE4 and MMP9 expression in dysfunctional CFTR-triggered EMT in vitro. Our results clearly demonstrate for the first time that high basal HE4 expression caused by inflamed CF airway epithelial cells decreases during EMT development, which may contribute to increased MMP9.

2. Methods

2.1. Reagents

CFTR correctors *elexacaftor* (VX-445) (S8851) and *tezacaftor* (VX-661) (S7059), and CFTR potentiator *ivacaftor* (VX-770) (S1144) were purchased from Selleck Chemicals (Houston, TX, USA). TGFβ1 (Thermo Fisher Scientific Inc., Waltham, MA, USA) was ordered from Sigma-Aldrich (St. Louis, MO, USA). Except for TGFβ1 (dissolved in 40 mM acetic acid and 0.1% BSA), all reagents were dissolved in DMSO (dimethyl sulfoxide, Sigma-Aldrich).

2.2. Cell cultures

CFBE 41o− cell cultures stably expressing wt-CFTR or p.Phe508del-CFTR variant [20] were grown in Minimum Essential Medium Eagle (EMEM) with Earle's Balanced Salt Solution (EBSS) and 1% L-glutamine (Lonza, Walkersville, MD, USA), 10% fetal bovine serum (FBS, Sigma-Aldrich), and 5 μg/ml puromycin (Sigma-Aldrich) at 37 °C with 5% CO_2. CFBE cells were seeded in 6-well plates (250.000 cells per well). As in a prior study, TGFβ1 (15 ng/mL) was used for 48 h to induce EMT phenotype, confirmed by fluorescence microscopy and RT-qPCR [13]. Besides EMT marker analysis via both techniques, supernatants for HE4 protein levels were collected after treating CFBE cells with elexacaftor (3 μM), tezacaftor (5 μM), and ivacaftor (10 μM) (ETI) for 48 h in the presence of TGFβ1. These drugs were applied under conditions similar to recent in vitro studies [18–22]. Additionally, the change in HE4 expression was monitored over time during EMT development in TGFβ1-stimulated p.Phe508del-CFTR CFBE cells (25.000 cells per well) from 24 to 120 h compared to baseline (PBS) by measuring HE4 concentrations in supernatants using ELISA (see below). As controls, non-epithelial cell cultures, specifically human pulmonary mesenchymal stem cells and human lung fibroblasts of CF origin (ScienCell Research Laboratories, Carlsbad, CA, USA), were analyzed for HE4 expression.

2.3. Total RNA extraction

According to the manufacturer's recommendations, total RNA from CFBE cell culture samples was isolated using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). The purity and concentration of the isolated RNA samples were verified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The total RNA samples were stored at −80 °C before analysis.

2.4. RT-qPCR analysis

Complementary DNA (cDNA) synthesis was conducted using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fisher Scientific, Vilnius, Lithuania) following the manufacturer's instructions. An initial RNA amount of 1000 ng per reaction was used. Quantitative PCR was carried out on a LightCycler 480 qPCR instrument (Roche Diagnostics, Mannheim, Germany) with LightCycler 480 SYBR Green I Master mix (Roche Diagnostics) and gene-specific primers (10 μM, Integrated DNA Technologies, Leuven, Belgium). The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min. For normalization, we used the reference gene *RPLP0* (*36B4*). To study EMT development, E-cadherin, N-cadherin, Vimentin and TWIST1 mRNA levels were quantified in both CFBE cell lines and in p.Phe508del-CFTR CFBE cells after 48 h of TGFβ1 treatment, while HE4, IL1B, and MMP9 mRNA expression were measured in parallel. Primer sequences for cDNA amplification are listed in Suppl. Table 1.

2.5. Immunofluorescence (IF) microscopy

Detection of EMT-specific phenotype in CFBE cell cultures was visualized using immunofluorescence staining for EMT markers. For this purpose, wt-CFTR and p.Phe508del-CFTR CFBE cells were cultured on 6-well plates for 2 days and transferred onto sterile uncoated microscope slides at a density of 5 × 10^4 cells per slide. Basal positivity for EMT characteristics in both cell lines was first observed based on genotype. Simultaneously, the EMT process was further induced by TGFβ1 (15 ng/mL) for 48 h in p.Phe508del-CFTR CFBE cells, compared to control samples with vehicle (PBS, baseline). When studying the impact of CFTR modulator treatment on EMT, p.Phe508del-CFTR CFBE cells were incubated with ETI or DMSO (baseline) for 48 h in the absence or presence of TGFβ1 administration. Cells were then fixed with ice-cold methanol-acetone (50:50 v/v) for 10 min. Non-specific antibody binding sites were blocked with FBS (Sigma-Aldrich) for 15 min. For primary labeling of EMT markers, monoclonal mouse anti-human E-cadherin (610,181) and anti-N-cadherin (610,920) (250 μg/mL, BD Biosciences), as well as polyclonal rabbit anti-HE4 (AB273130) and anti-MMP9 (AB73734) (100 μg/mL, Abcam), were used for 1 hour, followed by secondary staining with FITC-labeled goat anti-mouse (A11029) and goat anti-rabbit (A11034), or rhodamine-conjugated goat anti-mouse IgG (2 mg/mL, dilution 400x, Thermo Fisher Scientific Inc.) for 1 hour. Ki67 protein staining (AB15580) (100 μg/mL, Abcam) was used to

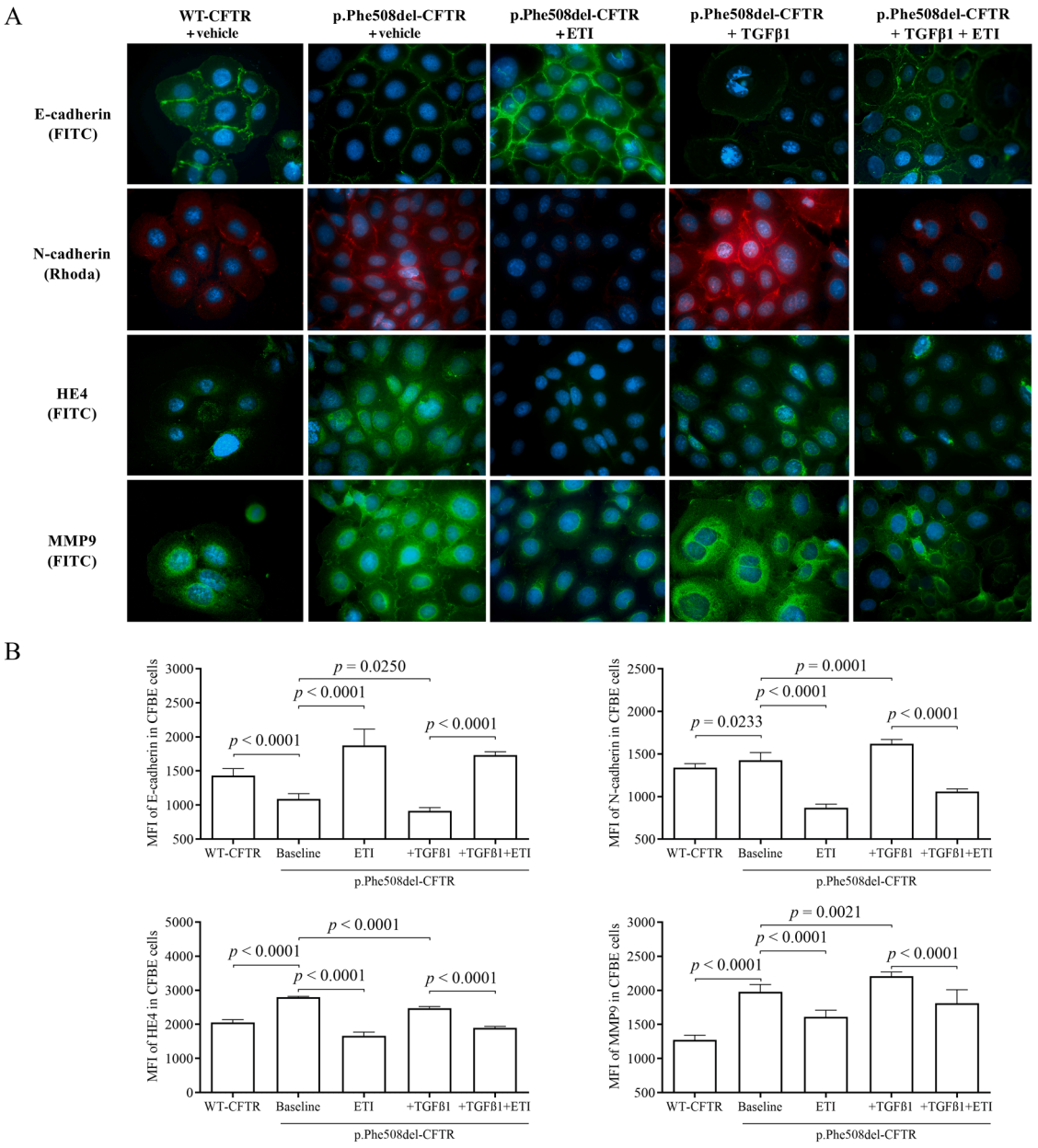


Fig. 1. Representative images of CFBE cells expressing *p.Phe508del-CFTR* vs wt-CFTR immunostained for basal and TGFβ1-induced EMT markers with HE4 and MMP9 with or without ETI. The level of these markers was analyzed by IF staining (A) and the mean fluorescence intensity (MFI) was calculated for each marker to quantify the degree of positivity (B) to compare the two cell lines with each other for evaluating the trend for EMT development. Mean ± SEM is depicted, $n = 6–8$ samples/condition. The Mann-Whitney U test was performed to compare two groups of data.

detect actively dividing cells in the growth phase of the cell cycle, with and without HE4 expression, manipulated with siRNA transfection (see below). Cell nuclei were stained with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, 283 nM), and samples were observed using a Zeiss Axio Scope A1 fluorescent microscope (HBO 100 lamp) (Carl Zeiss Microimaging GmbH, Göttingen, Germany). DAPI: excitation at G 365 nm, emission BP 445/50 nm; fluorescein: excitation BP 470/40 nm, emission BP 525/50 nm; rhodamine: excitation BP 546/12 nm, emission BP 575–640 nm. Images were analyzed with ZEN 2012 software (Carl Zeiss Microimaging GmbH). The mean fluorescence intensity (MFI) was calculated for each marker to quantify positivity levels. The specificity of immunostaining was confirmed by incubating cells with only the secondary antibody, which showed no background staining.

2.6. Downregulation of HE4 expression by siRNA transfection in CFBE cells

The p.Phe508del-CFTR CFBE cells were seeded at a density of 25.000 per well and cultured for 24 h before transfection. HE4 (*WFDC2*) expression in mutant CFBE cells was silenced using a specific siRNA (20 pmol, ID: 4392,420, Invitrogen, Carlsbad, CA, United States) for 48 h in the absence or presence of TGFβ1 pretreatment. Control samples were treated with NEG01 siRNA (Silencer Select Negative Control No.1, 20 pmol, ID: 4390,846, Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. Transfection efficiency was assessed by measuring HE4 protein expression through immunofluorescence (IF) and mRNA levels via RT-qPCR. Additionally, levels of EMT markers and MMP9 were evaluated. GAPDH mRNA expression was also measured to verify the specificity of the siRNA transfection. Furthermore, MMP9

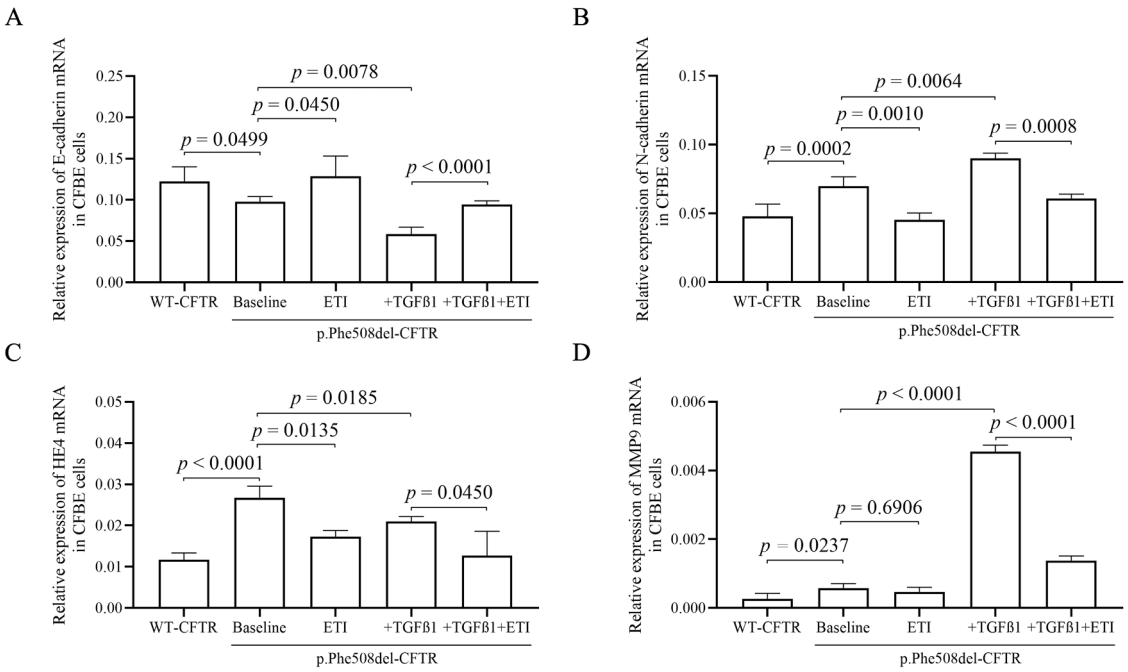


Fig. 2. Investigation of mRNA expression of EMT markers (A, B) with HE4 (C) and MMP9 (D) in CFBE cells expressing *p.Phe508del-CFTR* after treatment with TGFβ1 with or without ETI. RT-qPCR examination was applied, and the reference gene *RPLP0* (*36B4*) was used for normalization. Mean ± SEM is depicted, n = 5–6 samples/condition. The Mann-Whitney U test was performed to compare two groups of data.

protein concentrations in cell supernatants were quantified by ELISA to confirm the effect of altered HE4 expression on MMP9 levels. The proliferation rate of CFBE cells treated with HE4 siRNA was compared to cells treated with NEG01 siRNA by analyzing Ki67 positivity through IF.

2.7. Immunoassays

Chemiluminescent microparticle immunoassay (Architect-i1000SR, Abbott Diagnostics, Wiesbaden, Germany) was used to analyze protein levels of HE4 in the supernatant from CFBE cell cultures after different treatments as outlined above, as well as in non-epithelial cell lines. MMP9 levels were measured using a commercially available ELISA kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). N-cadherin protein concentrations in the supernatant of CFBE cells were determined by a commercially available ELISA kit according to the manufacturer's protocol (Wuhan Fine Biotech Co., Wuhan, China). All measurements were performed in a blinded manner by the analyst.

2.8. Cell viability assay

To assess the cell viability after TGFβ1 treatment, a Thiazolyl Blue Tetrazolium Bromide (MTT)-based cell viability assay (Sigma-Aldrich) was performed. CFBE cells (25,000 per well) were sub-cultured into PLL-precoated 24-well plates (SPL Life Sciences, Naechon-Myeon, Korea) and then exposed to TGFβ1 for 48 h. After treatment, supernatants were removed and 300 µL of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) solution in Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich) was added to each well. After incubation for 3 h (37 °C, 5% CO_2, humidified atmosphere), the MTT solution was discarded and blue formazan crystals were solubilized in 300 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich), and optical density was determined at 570 nm. Cells treated with H_2O_2 (50 µM) for 48 h were used as positive control, while samples stimulated by TNF-α (100 ng/mL) for the same period was applied as a negative control.

2.9. Ethics statement

This study received approval from the Hungarian Scientific and Research Ethics Committee (permit number: ETT-TUKEB No IV/10,417–3/2020/EKU), with consent granted on December 29, 2020, in accordance with the World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects.

2.10. Statistical analysis

The Kolmogorov-Smirnov test was used to assess the normality of the data. Data are presented as mean ± standard error of the mean (SEM). The Mann-Whitney U test was performed to compare two groups, while comparison of multiple groups was performed using Kruskal–Wallis test. $P < 0.05$ was considered statistically significant. Analyses were conducted using GraphPad Prism, version 9.0 (GraphPad Software, La Jolla, CA, USA).

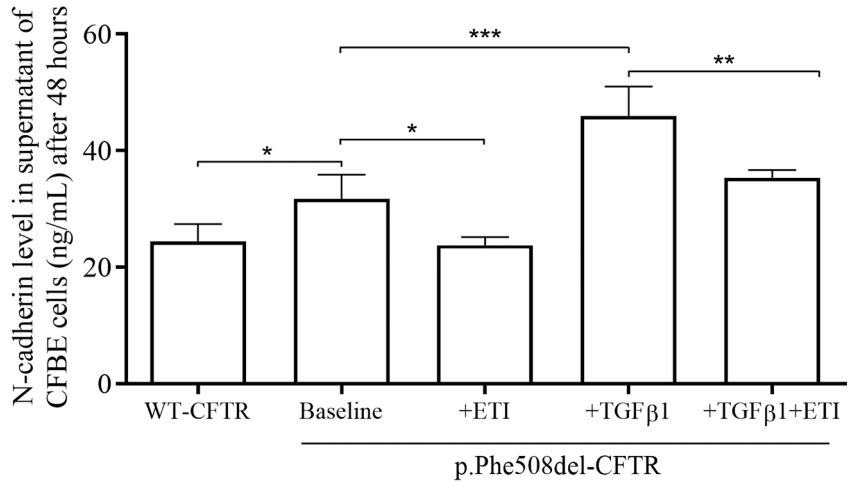
3. Results

3.1. CFBE cells expressing p.Phe508del-CFTR demonstrate EMT characteristics compared to wt-CFTR CFBE cells

Human CF airway epithelial cells, using p.Phe508del-CFTR CFBE 41o- cell cultures and control wt-CFTR CFBE 41o- cells, were first analyzed through combined immunofluorescence (IF) and transcript investigations to evaluate the native expression of EMT markers. IF analysis revealed that E-cadherin expression was decreased, while N-cadherin positivity was higher in CF versus normal epithelial cells (Fig. 1A). Additionally, if HE4 and MMP9 protein expression were detected, the baseline mean fluorescence intensity (MFI) values for both markers were increased in p.Phe508del-CFTR CFBE cells compared to wt-CFTR counterparts (Fig. 1B).

In parallel, mRNAs were quantified, and both cell lines showed similar differences in the mRNA levels of these biomarkers (Fig. 2A–D). We also measured the mRNA expression of Vimentin as an additional mesenchymal marker and IL1B, which functions as a pro-inflammatory

A



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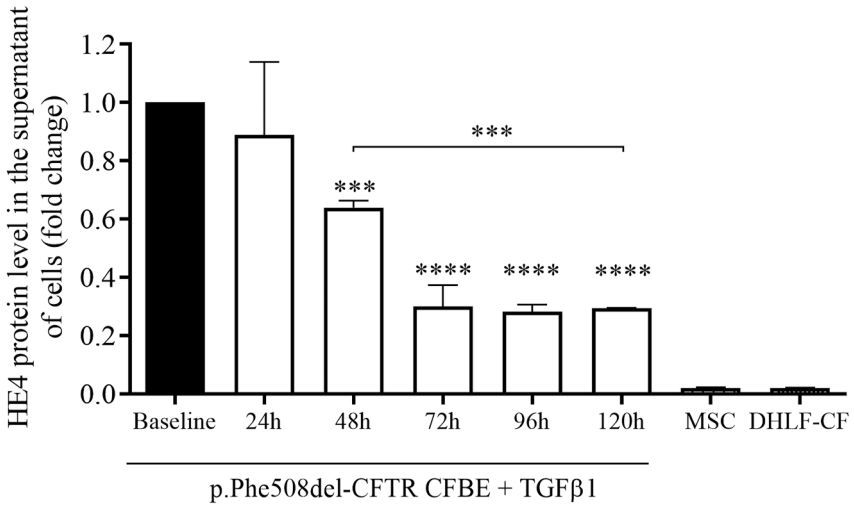

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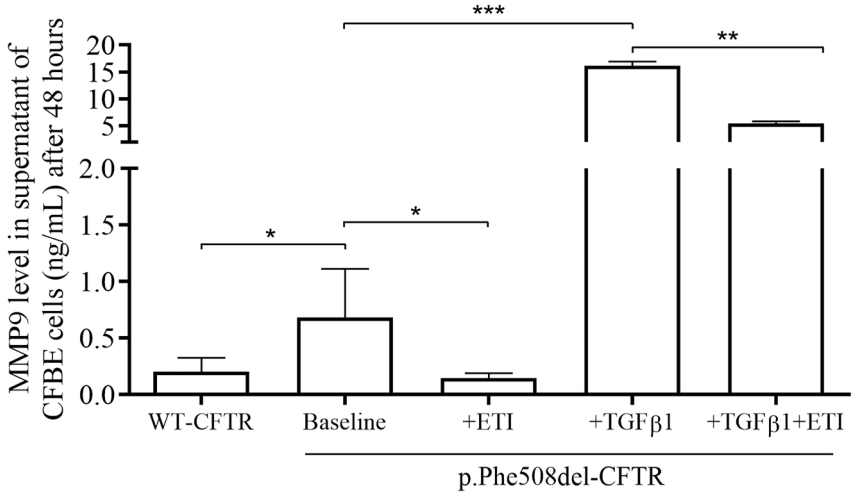

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Fig. 3. Analysis of N-cadherin (A), HE4 (B) and MMP9 (C) protein concentrations in the supernatant of CF epithelial cell cultures and non-epithelial cell lines. N-cadherin and MMP9 protein levels were measured in the supernatant of wt-CFTR, as well as untreated, ETI, TGFβ1, and TGFβ1+ETI-treated p.Phe508del-CFTR CFBE cells by ELISA (A, C). CFBE cells expressing *p.Phe508del-CFTR* were treated with TGFβ1 for 24 to 120 h to analyze the change in HE4 expression. In parallel, human pulmonary mesenchymal stem cells and CF-derived human lung fibroblasts were examined for HE4 (B). Mean ± SEM is depicted, $n = 5–6$ samples/condition. The Mann-Whitney U test was performed to compare two groups of data, while comparison of multiple groups was performed using Kruskal–Wallis test.

mediator in these samples, and both mRNA levels were significantly higher in p.Phe508del-CFTR CFBE cells compared to controls (Suppl. Figure 1A-B). Furthermore, we analyzed TWIST1 mRNA level which mediates EMT [13], and its elevated expression underlined the development of mesenchymal phenotype in basal as well as TGF-β1-treated mutant CFBE cells (Suppl. Figure 1C). These data suggest epithelial disruption and the induction of mesenchymal-specific cellular features in native CF bronchial epithelial cells.

3.2. Rescue of CFTR function results in a restored epithelial phenotype in p.Phe508del-CFTR CFBE cells

Next, p.Phe508del-CFTR CFBE cells were treated with ETI to revert EMT phenotype, and higher E-cadherin and reduced N-cadherin expression were observed (Fig. 1A). Then, we aimed to investigate whether TGFβ1 could further enhance EMT in CF airway cells. MTT assay was first performed to assess cell viability following TGF-β1 treatment at different concentrations and time points, and no cytotoxic effects were observed at 15 ng/mL of TGF-β1 for up to 120 h or at even larger concentration for 48 h (Suppl. Figure 2). This treatment resulted in even lower E-cadherin levels and increased N-cadherin and MMP9 expression at the protein level in p.Phe508del-CFTR CFBE cells compared to untreated cells, as seen in IF images. Surprisingly, HE4 expression at both protein and mRNA levels decreased in response to TGFβ1 relative to its baseline levels induced in p.Phe508del-CFTR CFBE cells (Fig. 1A–B and Fig. 2C). These findings suggest that 'provoked' EMT by TGFβ1 may be linked to reduced HE4 expression in CF.

We then aimed to demonstrate that these changes under EMT could be reversed using CFTR modulator treatment in vitro. For this purpose, p.Phe508del-CFTR CFBE cells co-incubated with TGFβ1 and ETI were analyzed for EMT biomarkers. According to IF tests, E-cadherin positivity increased and normalized, while N-cadherin returned to baseline levels or even lower. Importantly, HE4 and MMP9 expression were significantly reduced, reflecting CFTR rescue by ETI (Fig. 1A). RT-qPCR also measured these biomarkers, and N-cadherin, Vimentin, and IL1B expression returned to their baseline values (Fig. 2B and Suppl. Figure 1). Moreover, HE4 and MMP9 mRNA levels were substantially decreased in the presence of TGFβ1 and ETI together vs TGFβ1 alone (Fig. 2C–D). N-cadherin was also evaluated at protein level by ELISA and similar changes were observed in response to distinct treatments above (Fig. 3A). These results confirm that TGFβ1 is the primary trigger to induce EMT with decreasing HE4 levels, and there is a direct relationship between CFTR dysfunction and EMT development in CF.

3.3. Lack of HE4 expression among mesenchymal cellular conditions

As HE4 expression was reduced in p.Phe508del-CFTR CFBE cells due to TGFβ1 administration for already 24 h, these epithelial cells were incubated with this mediator to monitor HE4 expression under long-term TGFβ1 treatment. Importantly, HE4 expression gradually and significantly further decreased after 48 h, continuing up to 120 h in response to TGFβ1 (Fig. 3B). As controls, other cell lines of non-epithelial origin were analyzed for HE4, and no detectable protein levels were observed in either human pulmonary mesenchymal stem cells or human lung fibroblasts with CF origin (Fig. 3B).

Furthermore, protein levels of MMP9 were also measured by ELISA in wt-CFTR and p.Phe508del-CFTR CFBE cells, as well as in the latter cell line treated with or without ETI in the absence or presence of TGFβ1. Similar to the findings of IF and RT-qPCR measurements, baseline MMP9 concentrations were higher in the supernatant of p.Phe508del-CFTR CFBE cells compared to wt-CFTR CFBE cells which could be suppressed by ETI. The protein level was further increased in the mutant cells after activation with TGFβ1; however, TGFβ1-induced MMP9 expression was significantly reduced by ETI (Fig. 3C). According to these results, HE4 expression decreased due to prolonged exposure to TGFβ1 during advanced EMT in CF epithelial cells, which is linked to the development of a more severe mesenchymal phenotype, while HE4 is already absent in mesenchymal cells, such as CF fibroblasts.

3.4. Downregulated HE4 expression contributes to EMT, elevated MMP9 expression and decreased cell proliferation

HE4-specific siRNA artificially lowered HE4 expression in p.Phe508del-CFTR CFBE cells to examine the effects of TGFβ1during EMT in CF. IF and RT-qPCR analyzed cellular responses by measuring changes in EMT markers and MMP9 levels in HE4 deficiency. After 48 h, HE4 siRNA significantly reduced HE4 protein and mRNA levels in mutant CFBE cells compared to those transfected with NEG-01 siRNA, confirming transfection success (Fig. 4A and B). Notably, the control gene GAPDH mRNA level remained unchanged (Suppl. Figure 3A).

EMT was induced due to downregulation of HE4 expression, showing decreased E-cadherin and increased N-cadherin as well as MMP9 positivity detected by IF (Fig. 4A). Furthermore, we analyzed the impact of downregulated HE4 in unstimulated and TGFβ1-stimulated cells. For this purpose, HE4 siRNA and TGFβ1 were administrated together in p.Phe508del-CFTR CFBE 41o− cells vs cells with NEG-01 siRNA + TGFβ1, and these results have been compared to the data of HE4 siRNA-treated cells without TGFβ1 stimulation. Alterations in studied parameters at mRNA level measured in unstimulated cells were identical to those samples on TGFβ1 (Fig. 4B). Based on these data, we think that reduced HE4 expression in CFBE cells seems to be a key cellular event in EMT, while elevation in MMP9 protein expression is more substantial when decreased HE4 expression was associated with TGFβ1 treatment as well (Suppl. Figure 3B). Finally, the extent of cell proliferation during EMT with reduced HE4 was assessed via Ki67 positivity, which was significantly decreased in p.Phe508del-CFTR CFBE cells after HE4 siRNA treatment compared to control cells with NEG-01 siRNA (Fig. 4A), indicating that EMT status was associated with decreased cell proliferation.

4. Discussion

Recent investigations have provided strong evidence for the activation of EMT events in chronic lung diseases, such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and CF [15]. TGFβ1, a key mediator and powerful inducer of EMT, is significantly overexpressed in these disorders and in the airways of CF patients [15,23]. These conditions share several clinical and molecular features, including airway obstruction, ongoing inflammation, fibrotic remodeling, and common gene expression patterns [24]. CFTR-impaired endothelial cells display an exaggerated pro-inflammatory phenotype linked to increased reactive oxygen species, higher activation markers, defective autophagy, and mitochondrial issues [25]. Notably, EMT in CF is often partial, resulting in cells with hybrid EMT phenotypes [16,25]. This aligns with the idea of EMT as a spectrum of flexible, intermediate states rather than a complete epithelial-to-mesenchymal transition [26].

This study examined HE4 and MMP9 expression in CF-related EMT cellular conditions in vitro. We aimed to show that altered HE4 levels

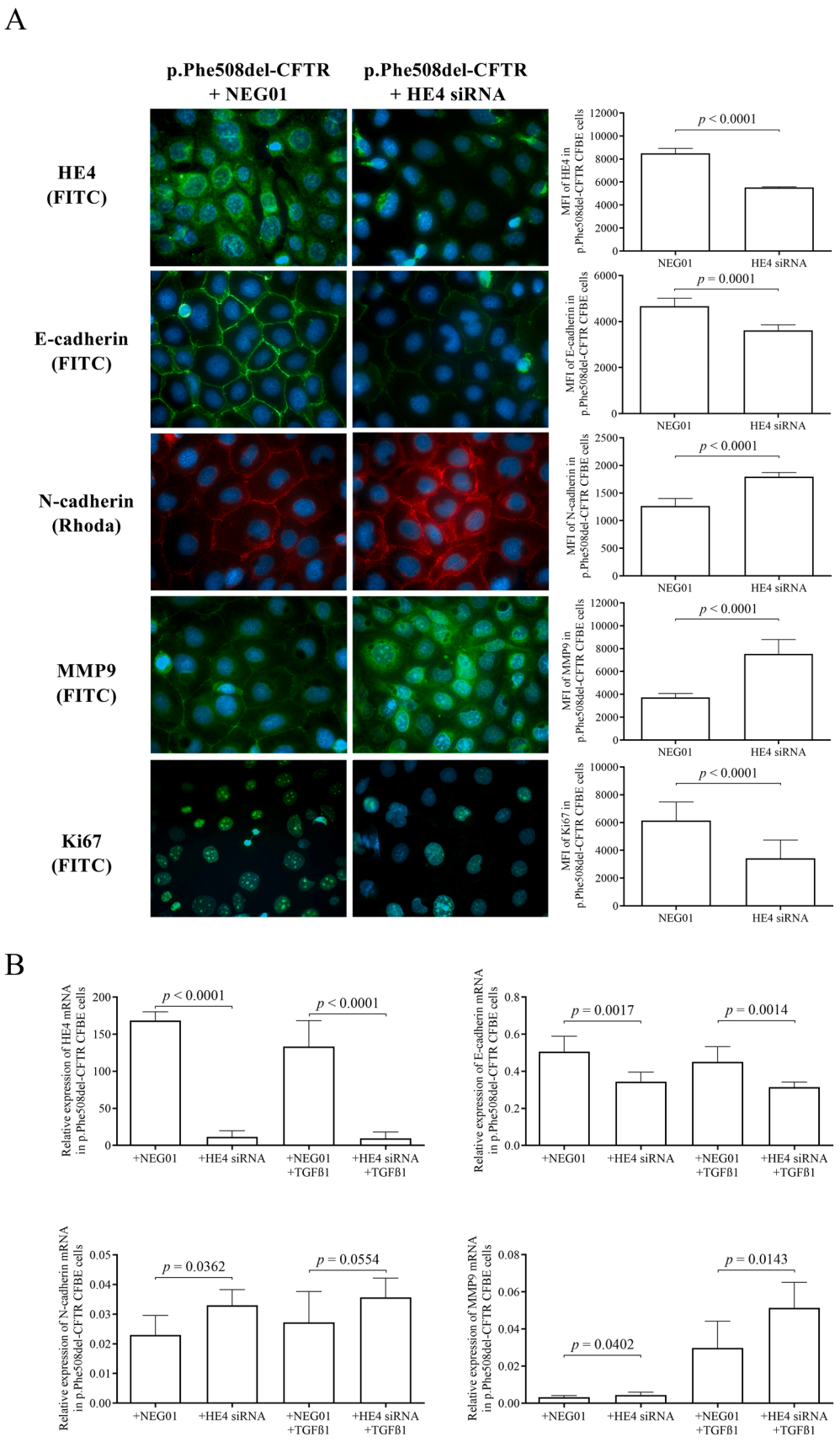

Fig. 4. Effects of downregulated HE4 expression by siRNA treatment on EMT and MMP9 expression. The level of EMT biomarkers was analyzed by IF staining (A) and RT-qPCR examination (B). The efficacy of this transfection was monitored via HE4 mRNA, and the MMP9 protein level was measured in the absence of HE4 expression (B). Mean ± SEM is depicted, $n = 4–5$ samples/condition. The Mann-Whitney U test was performed to compare two groups of data.

are linked to EMT development in CFBE cells, which contribute to increased MMP9 expression. To do this, p.Phe508del-CFTR CFBE 41o− cell cultures were compared to control wt-CFTR CFBE cells using IF,RT-qPCR and ELISA, based on native EMT biomarker expression. CF epithelial cells showed decreased E-cadherin and increased N-cadherin, Vimentin, and IL1B expression. Additionally, both HE4 and MMP9 protein levels were higher in CFBE cells carrying p.Phe508del-CFTR compared to controls. Consistent with the protein data, transcript analysis revealed more pronounced differences in mRNA levels of these markers between the two cell lines. These native EMT features align with previous findings where E-cadherin appeared weaker in CF bronchial epithelium tissue compared to controls and stained positively for N-cadherin, Vimentin, and Collagen I. Moreover, polarized p.Phe508del-CFTR CFBE 41o− cells showed increased expression of these mesenchymal markers compared to wt-CFTR cells without manipulation [13,16]. Interestingly, N-cadherin and Vimentin levels were lower in p.Gly551Asp-CFTR than in p.Phe508del-CFTR CFBE cells [16]. Overall, these findings indicate a shift toward a mesenchymal-like and pro-inflammatory phenotype in native CF bronchial epithelial cells, suggesting disruption of epithelial integrity and induction of EMT-related molecular traits under basal conditions.

To explore whether EMT could be further enhanced in CF airway epithelial cells, p.Phe508del-CFTR CFBE cells were treated with TGFβ1. As expected, TGFβ1 exposure caused a significant decrease in E-cadherin and an increase in N-cadherin and MMP9 protein levels, as shown by IF and ELISA. Interestingly, HE4 expression was notably reduced after TGFβ1 stimulation compared to its elevated baseline in CFBE cells homozygous for p.Phe508del-CFTR, suggesting that activated EMT is linked to HE4 downregulation in CF. Recently, consistent with our current findings, TGFβ1 treatment also induced EMT in CFBE cell lines by decreasing epithelial markers (e.g., E-cadherin, Claudin-1) and increasing N-cadherin levels. The mesenchymal marker Vimentin increased more markedly in p.Phe508del-CFTR than in p.Gly551Asp-CFTR CFBE cells [16]. These data imply that HE4 is usually expressed in normal epithelial cells, but its level decreases when the epithelium loses its epithelial phenotype.

Next, we explored whether pharmacological CFTR rescue could reverse these EMT-related changes. Therefore, basal p.Phe508del-CFTR CFBE cells were treated with ETI as well as being co-treated with TGFβ1 and the triple CFTR modulator combination. To monitor the restoration of CFTR function by ETI, we recently applied a whole-cell patch clamp technique under the same in vitro experimental conditions we also used in this study. ETI pretreatment resulted in successfully corrected p.Phe508del-CFTR function based on altered peak current densities [18]. Hence, the improvement of CFTR function by ETI was acknowledged in these experiments. We here showed a restoration of epithelial features, evidenced by increased E-cadherin and decreased N-cadherin expression by IF. With these immunostaining data, RT-qPCR confirmed normalization of E-cadherin, N-cadherin, Vimentin, and IL1B mRNA levels. However, HE4 and MMP9 mRNA remained significantly lower in response to ETI and in the presence of TGFβ1 and ETI compared to TGFβ1 alone. According to other studies, p.Phe508del-CFTR cells displayed a clear link between mutant CFTR correction efficacy and reduction in mesenchymal marker levels: VX-661 alone did not significantly alter EMT marker levels. Conversely, the VX-661/VX-770 combination significantly decreased Vimentin levels. When VX-661/VX-445, combined with VX-770, was added to p.Phe508del-CFTR CFBE cells, the correction was markedly more effective at substantially reducing N-cadherin and Vimentin levels. In contrast, when treated with these compounds, wt-CFTR CFBE cells showed no significant changes in epithelial or mesenchymal marker levels [13]. VX-770 and VX-809 increased CFTR expression and lowered mesenchymal markers CDH2 and TAGLN [25].

ETI treatment partially restored tight junction integrity by increasing claudin-1 levels and the E-/N-cadherin ratio in mutant CFBE cells [16]. In a rat model, the pathways involved in EMT were shown to depend on CFTR mutation, as lower expression of type 1 Collagen was observed in CFTR-knockout (CFTR-KO) rat lung tissue and primary cell cultures compared to p.Phe508del-CFTR lungs and cells, especially after TGFβ1 treatment. Additionally, Rho-associated protein kinase inhibitor Y27632 reversed changes in EMT-related genes in CFTR mutant, but not CFTR-KO cells [24]. Overall, the level of functional CFTR closely correlates with the epithelial/mesenchymal state of CFBE cells.

We then examined how HE4 is regulated over the long term under continuous stimulation. Continuous exposure to TGFβ1 caused a steady and significant decrease in HE4 levels up to 120 h. HE4 expression was also checked in non-epithelial cell lines to see if this effect is specific to certain cell types. Interestingly, no HE4 protein was detected in human pulmonary mesenchymal stem cells or in CF-derived human lung fibroblasts, confirming that HE4 is limited to epithelial cells. Wang et al. also observed a reduction in *WFDC2* (HE4) in nasal mucosal epithelial cells from people with chronic rhinosinusitis, indicating an abnormal mix of epithelial and mesenchymal cells caused by EMT during chronic inflammation. Importantly, as we found, fibroblasts did not express HE4 [27].

Additionally, MMP9 protein levels were measured in native wt-CFTR and p.Phe508del-CFTR CFBE cells, as well as in mutant cells treated with TGFβ1 with or without ETI. Consistent with the immunofluorescence and RT-qPCR data, baseline MMP9 levels were higher in p.Phe508del-CFTR CFBE cell supernatants than in wt controls which was lowered by ETI alone. MMP9 expression increased further after TGFβ1 stimulation, but this increase was significantly reduced by co-treatment with ETI. Plasma MMP9 was elevated in CF patients before CFTR-specific treatment and decreased following Trikafta® (which is ETI) [28]. Since TGFβ1 can induce MMP9 expression in other human cells, such as meningeal cells, through activation of ERK and Smad pathways [29], it is likely that high levels of TGFβ1 partly regulate the increased MMP9 expression seen in CF [8–26].

HE4 expression was artificially silenced in unstimulated and TGFβ1-stimulated p.Phe508del-CFTR CFBE cells using a HE4-specific siRNA approach. HE4 knockdown induced EMT-like changes. Additionally, MMP9 mRNA and protein expression was increased, suggesting a direct functional link between HE4 and MMP9 expression. This molecular connection was first shown by others when HE4 directly interacted with MMP9 protein in fibrotic kidney lysates, as demonstrated by immunoprecipitation with an anti-HE4 antibody followed by western blotting analysis [30].

We systematically analyzed IL1B mRNA level under EMT conditions as well in parallel to EMT characteristics, HE4 and MMP9. The reason was that CF airway epithelium exhibits chronic inflammation with elevated pro-inflammatory cytokines including IL-1β/NLRP3 activation, which is essential to CF lung pathophysiology and shapes epithelial behavior [31]. Although non-CF bronchial epithelial cells showed that IL-1β enhanced TGFβ1-induced EMT, this established connection in lung epithelial biology supports the plausibility that IL-1β might act similarly in inflamed CF epithelia as well [32]. Based on our data, EMT was associated to a higher mRNA level of IL-1β especially after exposure to TGFβ1 suggesting a link between inflammation and EMT in CF.

Finally, assessment of cell proliferation using Ki67 staining revealed a significant decrease in proliferative activity in p.Phe508del-CFTR CFBE cells after HE4 silencing compared with NEG-01 control cells, indicating that EMT induction under reduced HE4 expression is associated with decreased proliferative potential. Similarly, knocking down HE4 decreased EMT development, cell proliferation, and Ki67 expression in ovarian cancer cells in vitro [33].

This study has several limitations. We did not perform additional experiments to further characterize EMT in CFBE cells, such as a wound healing assay. The direct effect of EMT development on CFTR expression in CFBE cells was not examined when HE4 was measured. Lastly, the mechanistic connection between altered HE4 expression by TGFβ1 and the relationship between HE4 and MMP9 was not fully investigated; therefore, more research is needed to clarify the details of the regulatory

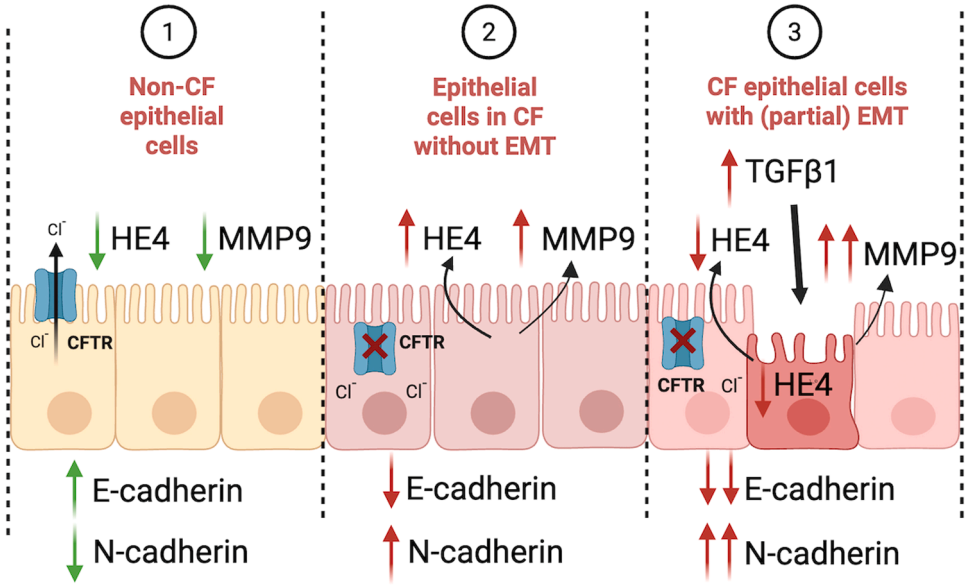

Fig. 5. Schematic representation of progressive epithelial cell changes and EMT development in CF. Functional CFTR maintains regular ion transport and epithelial integrity in healthy epithelium with high E-cadherin and low N-cadherin expression (phase 1). CFTR dysfunction and intrapulmonary inflammation in CF increase HE4 and MMP9 levels (phase 2). Upon chronic presence of TGFβ1, epithelial cells undergo (partial) EMT characterized by decreased E-cadherin, increased N-cadherin and MMP9, and reduced HE4 expression (phase 3).

mechanism.

In conclusion, TGFβ1 functions as a key inducer of EMT in CF airway epithelial cells, along with a decrease in HE4 expression and an increase in MMP9 levels (Fig. 5).

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Suppl. Figure 1. Quantifying Vimentin (A), IL1B (B) and TWIST1 (C) mRNA expression in CFBE cells expressing *p.Phe508del-CFTR* vs wt-CFTR under TGFβ1 treatment with or without ETI administration. RT-qPCR was performed to measure the levels of an additional EMT biomarker and a pro-inflammatory mediator. Mean ± SEM is depicted, n = 5–6 samples/condition. The Mann-Whitney U test was performed to compare two groups of data.

Suppl. Figure 2. Cell viability test in with TGFβ1-treated p.Phe508del-CFTR CFBE cells. In the cell viability assay, CFBE cells were treated with TGFβ1 for 48 h, followed by an MTT-based cell viability test. Results were shown as a percentage of viability of vehicle-treated (baseline) cells. As controls, cells treated with H_2O_2 (50 μM) or TNF-α (100 ng/mL) were used. Mean ± SEM is depicted, n = 5–6 samples/condition.

Suppl. Figure 3. Quantification of control gene GAPDH mRNA (A) and MMP9 protein expression in CFBE cells expressing p.Phe508del-CFTR under HE4 siRNA treatment with or without TGFβ1. RT-qPCR was performed to measure the levels of these mRNAs. Mean ± SEM is depicted, n = 4–5 samples/condition. The Mann-Whitney U test was performed to compare two groups of data.

Suppl. Table 1. Sequences of the primers for cDNA amplification for RT-qPCR analysis.

CRediT authorship contribution statement

Marianna Pócsi: Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing – review & editing. **György Jázon Balla:** Investigation, Data curation. **Ferenc Fenyvesi:** Investigation, Methodology. **Ágnes Rusznyák:** Investigation, Methodology. **Zsolt Fejes:** Investigation, Methodology. **István Balogh:** Validation. **Milan Macek Jr.:** Supervision, Writing – review & editing, Resources. **Margarida D. Amaral:** Supervision, Writing – review & editing, Resources. **Béla Nagy Jr.:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2026.03.019.

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