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Original Article

FAM13A is a modifier gene of cystic fibrosis lung phenotype regulating rhoa activity, actin cytoskeleton dynamics and epithelial-mesenchymal transition

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Abstract

Background: Cystic fibrosis (CF) lung disease severity is highly variable and dependent on several factors including genetic modifiers. Family with sequence similarity 13 member A (*FAM13A*) has been previously associated with lung function in the general population as well as in several chronic lung diseases, such as chronic obstructive pulmonary disease (COPD), we examined whether *FAM13A* is a modifier gene of CF lung phenotype. We also studied how *FAM13A* may contribute to the physiopathological mechanisms associated with CF.
Methods: We investigated the association of *FAM13A* with lung function in CF French patients (n = 1222) by SNP-wise analysis and Versatile Gene Based Association Study. We also analyzed the consequences of FAM13A knockdown in A549 cells and primary bronchial epithelial cells from CF patients.
Results: We found that *FAM13A* is associated with lung function in CF patients. Utilizing lung epithelial A549 cells and primary human bronchial epithelial cells from CF patients we observed that IL-1β and TGFβ reduced FAM13A expression. Knockdown of FAM13A was associated with increased RhoA activity, induction of F-actin stress fibers and regulation of epithelial-mesenchymal transition markers such as E-cadherin, α-smooth muscle actin and vimentin.
Conclusion: Our data show that FAM13A is a modifier gene of CF lung phenotype regulating RhoA activity, actin cytoskeleton dynamics and epithelial-mesenchymal transition.

Keywords: Cystic fibrosis; Modifier genes; FAM13A

Abbreviations: CF, Cystic fibrosis; FAM13A, Family with sequence similarity 13 member A; COPD, Chronic obstructive pulmonary diseases; CFTR, CF transmembrane conductance regulator; GAP, GTPase activating protein; VEGAS, Versatile Gene Based Association Study; EMT, epithelial to mesenchymal transition; KNoRMA, Kulich normalized mortality adjusted CF-specific lung phenotype; hAECBs, human airway epithelial cells from bronchi; MAF, minor allele frequency; FEV_1, forced expiratory volume in 1 s; siFAM13, siRNA for FAM13A; siCTRL, siRNA negative control.

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1. Introduction

Cystic fibrosis (CF) is a monogenic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. However, lung disease severity is highly variable and dependent of several factors including genetic modifiers [1,2]. The modifier genes are indeed thought to contribute to approximately 50% of the lung phenotype in CF patients harboring the common *CFTR* F508del mutation [3]. Family with sequence similarity 13 member A (*FAM13A*) has been previously shown to be associated with the lung function in the general population [4] as well as in patients with asthma, COPD, idiopathic pulmonary fibrosis and lung cancer [5–10]. The protein sequence of FAM13A contains a rho GTPase activating protein (GAP) domain. GAPs inactivate GTPases by the conversion of GTP to GDP. GTPases are known to play a role in the actin cytoskeleton and remodeling as they regulate assembly of focal adhesions and F-actin stress fibers [11]. Therefore, variants affecting the on/off switch of this GTPase feedback loop via the FAM13A-GAP domain may modify disease progression possibly by dysregulating Rho signaling and disrupting the cytoskeleton. The most common rho GTPases include RhoA, RhoB, RhoC, Rac1 and Cdc42. Perturbed rho GTPase signaling is associated with several lung diseases, including asthma, pulmonary hypertension, chronic obstructive pulmonary disease (COPD), and lung cancer [12]. In chronic lung diseases, RhoA is the most commonly associated GTPase for changes in the barrier function and actin cytoskeleton. Interestingly, RhoA has been reported to be upregulated in CF cells [13].

To date, no study has explored the role of *FAM13A* as a potential candidate for modifying the CF lung phenotype. In this study, we investigated whether *FAM13A* variants associated with CF lung phenotype. Considering the numerous publications involving *FAM13A* in lung disease progression, susceptibility and severity, and the potential role of the rhoGAP domain, we also examined the function of FAM13A in the lung and how it could participate in the CF lung physiopathology.

2. Materials and methods

2.1. Patients and lung phenotype

The French CF Gene Modifier Consortium has been recently described [14]. Characteristics of 1222 French patients included in this study are shown in Table 1. Written informed consent was obtained from adults. For patients < 18 years old consent from parents or guardians was given for participation in the study. The study was approved by the French ethical committee (CPP n°2004/15) and the information collection was approved by CNIL (n°04.404).

We transformed forced expiratory volume in 1 s (FEV_1) to the Kulich normalized mortality adjusted CF-specific lung phenotype (KNoRMA) [15], a standardized consortium lung phenotype which allows for direct comparison of the lung function of CF patients irrespective of age and gender.

Table 1
Characteristics of CF patients enrolled in *FAM13A* genotyping.

Cohort	
n	1222
Mean age (±SD)	21.0 (9.2)
Range	6.0–57.6
Male n (%)	627 (51.3)
European [a] n (%)	1211 (99.1)
F508del/F508del n (%)	716 (58.6)
Pancreatic exocrine insufficient n (%)	1222 (100.0)

[a] On the basis of Eigenstrat principal components analysis and closeness to CEU.

2.2. Reagents

Lipopolysaccharide (LPS, *Pseudomonas aeruginosa* 10), anti-FAM13A (HPA038109) and anti-β-actin antibodies were from Sigma-Aldrich (Saint-Quentin Fallavier, France) and TNF-alpha (TNF-α) and IL-1-beta (IL-1β) were from Immunotools (Friesoythe, Germany). Transforming Growth Factor-beta (TGF-β) was from PeproTech (Rocky Hill, NJ, USA). Anti-E-cadherin, anti-vimentin, anti-rabbit and anti-mouse-Horseradish peroxidase (HRP) antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-RhoA antibody and rhotekin-RBD beads are from cytoskeleton (Denver, USA). Rhosin (Rho inhibitor) was from Millipore (Billirica, MA, USA). FAM13A expression (p.FAM13A) and control plasmids (p.CTRL) were from Origene (RC216561, Rockville, MD, USA). Silencer® Select siRNA for FAM13A (siFAM13) and negative control (siCTRL) were from Ambion (Austin, TX, USA). Lipofectamine 3000 was from invitrogen (Carlsbad, CA, USA).

2.3. Cell cultures

We used two types of respiratory epithelial cells: A549 (Alveolar origin; ATCC®-CCL185, Rockville, MD, USA), and commercial primary human airway epithelial cells from bronchi (hAECBs, Epithelix, Geneva, Switzerland) (Table 2). A549 cells were cultured as previously described [16] then seeded in plates (TPP, Techno Plastic Products, Trasadingen, Switzerland) as described in the figure legends. A549 cells were stimulated for 6 h with LPS, TNF-α 10 ng/mL, or IL-1β 10 ng/mL. For TGF-β experiments, A549 cells were serum starved for 6 h and then treated with 5 ng/mL of TGF-β for 24 h (mRNA quantification) or 48 h (protein expression) as indicated in the figure legends. Primary hAECBs were isolated from the bronchi of healthy individuals or CF patients and were cultured as recommended by the manufacturer.

2.4. RT-qPCR

Total RNA was extracted using a nucleospin extract II kit (Macherey Nagel, Duren, Germany). Reverse transcription (RT) was performed using the ABI high-capacity cDNA kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using an ABI StepOnePlus™. Each reaction contained 10 μL

Table 2
Characteristics of primary cells used in the study.

Patient number	Commercial reference	Age (years)	Sex	Smoker	Pathology	CFTR mutation	Used in experiments
P1	AB037801	63	M	No	No	No	Fig. 5A
P2	AB020102	67	F	No	No	No	Fig. 5A
P3	AB0053	50	F	No	No	No	Fig. 5A
P4	AB53101	73	F	No	No	No	Fig. 5A
P5	AB60001	56	F	No	No	No	Fig. 5A
P6	AB53901	37	M	No	No	No	Figs. 2B, 5A
P7	CF22002	29	F	No	CF	F508del	Fig. 5A, Supplemantary Fig. 2A
P8	CF43702	27	M	No	CF	F508del	Fig. 5A, B(lower panel), Supplementary Fig. 2A
P9	CF44502	25	F	No	CF	F508del	Fig. 5A
P10	CF60901	21	F	No	CF	F508del	Fig. 5A, Supplementary Fig. 2A
P11	CF56701	39	F	No	CF	F508del	Fig. 5A, C, Supplementary Fig. 2A–D
P12	CF43703	27	M	No	CF	F508del	Figs. 2B, 5A, B(upper panel), C–D, Supplementary Fig. 2A
P13	CF60701	21	F	No	CF	F508del	Fig. 5C

of 2× TaqMan® Fast Universal PCR Master Mix (Applied Biosystems), 0,5 μL of FAM13A (For$^{5'}$ACCCTGTTTGAAGTAGAGTATACAG$^{3'}$/Rev$^{5'}$AGACCTCTTTTACTATGATAAGCCT$^{3'}$/probe: $^{5'}$TCTGAGATGTGAAAACCTGGCT$^{3'}$), E-Cadherin (Hs01023894_m1), Alpha SMA (Hs00426835_g1), Vimentin (Hs00958111_m), GAPDH (Hs03929097_g1) or HPRT1 (Hs02800695_m1) Taqman® probe, and cDNA as the template in a final volume of 10 μL. For relative quantification, the amount of target genes was normalized to GAPDH or HPRT1 (housekeeping genes) as indicated in figure legends relative to control cells used as the calibrator and was calculated using the $2^{-\Delta\Delta Ct}$ method [17]. Each point corresponds to the mean ± SD of N experiments as indicated in the figure legends.

2.5. Immunohistochemistry

Human lung biopsy is from a 53 years old woman with lung cancer and was obtained from Hôpital Foch, Suresnes 92150, France. It was collected in a healthy zone of the lung and processed in compliance with the current French public health legislation (articles L.1235-2 and L.1245-2, code de la santé publique, www.legifrance.gouv.fr). The institution informed the patient and made sure that they were not opposed to the use of surgical samples for research purposes. The staining procedure was realized with 5-μm thick paraffin sections. Immunolabeling for FAM13A was performed on a Bond-III® automat (Leica, Leica Biosystems, Nussloch, Germany) using anti-FAM13A antibody (1/400).

2.6. Western-blot

An equal amount of protein from each sample was size-separated on 10% SDS-polyacrylamide gel and electrotransferred to a nitrocellulose membrane using iblot system (Invitrogen). Immunodetection was performed with specific antibodies followed by secondary-HRP antibodies. Bound antibodies were detected using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Between successive probes, membranes were treated with Restore Western Blot Stripping reagent (Thermo Scientific). Molecular masses were determined using the PageRuler Plus Pre-Stained Standard (Thermoscientific). Images were recorded with a Fujifilm LAS-3000 bioimaging system (Fujifilm, Stamford, CT, USA).

2.7. Fluorescence microscopy

2.7.1. F-actin staining

A549 and hAECBs were plated in 35-mm Petri dishes (iBidi, Martinsried, Germany) and 8-well μslide dishes (iBidi) respectively. Cells were fixed with ice cold PFA 4% 10 min, rinsed three times with PBS, permeabilized with PBS-0.1% Triton X-100, blocked with PBS-5% BSA, and the stained for 30 min with Alexa-594 or Alexa-488 phalloidin (Molecular probes). DAPI (Sigma-Aldrich) was used to stain the nucleus.

2.7.2. FAM13A staining

Cells were plated in 8-well μslide dishes (iBidi) fixed with ice cold PFA 4% 10 min in growth medium supplemented with 10% FCS. The cells were permeabilized 3 × 5 min with 0.1% Triton X-100 in PBS; and then, washed with PBS and incubated overnight at 4 °C with anti-FAM13A (1/100) in PBS supplemented with 4% FCS. The following day, the cells were washed 3 × 10 min with PBS and incubated for 1 h at room temperature with anti-rabbit Alexa fluor®-488 (Molecular probes, Eugene, OR, USA). Fluorescence microscopy was achieved using a Zeiss Axiovert 200 microscope with a 63× oil objective (Zeiss, Le Pecq, France).

2.8. ELISA

Concentrations of human TGF-β were measured in cell culture supernatants according to the manufacturer's instructions (Duoset, R&D Systems, Minneapolis, MN, USA). The 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was from Cell Signaling Technology (Danvers, MA, USA).

2.9. RhoA pull-down assay

RhoA and total RhoA expressions were measured using a RhoA activation assay kit (cytoskeleton, denver, OH, USA). Briefly, A549 in 60 mm dishes were transfected with siRNA

CTRL (166 pmol), siRNA FAM13A (166 pmol) or the FAM13A expression plasmid (8 μg) for 48 h using lipofectamine 3000. Protein extracts (400 μg) were incubated 2 h with 40 μL of GST-rhotekin beads, washed, suspended in Laemli buffer (2×), boiled 3 min and subjected to SDS-PAGE. For total RhoA expression, 40 μg of total protein was used. Activated/total RhoA was detected using an anti-RhoA antibody (Cytoskeleton). The membrane of total RhoA was stripped and reprobed by anti-actin antibody. RhoA activity semi-quantification was determined by using Image J (http://imagej.nih.gov/ij/) and expressed as the ratio between RhoA/total RhoA. Total RhoA activity was determined relative to β-actin expression.

2.10. Statistical analysis

Genomic information was imputed using 1000 genomes as described previously [14]. Principal component analysis (eigenstrat) indicated no population substructure. The KNorMA values were analyzed according to number of minor alleles at each locus (additive model). We tested phenotype/genotype associations SNP-wise and gene-wise using the Versatile Gene-based Association Study (VEGAS) sum statistic (v2) [18]. All SNPS with a minor allele frequency (MAF) > 0.02 (as chosen previously [19]) within the gene and at <20 kb of the gene boundaries were included. Individual SNPs p-values were adjusted with the Bonferroni rule. Gene boundaries coordinates were obtained from NCBI (GRCh37). p-Values were computed by permutations.

For in vitro experiments, differences among groups were assessed for statistical significance using Prism 6.00 software (GraphPad Software, La Jolla, CA, USA). Mann-Whitney and Wilcoxon test were used for comparison of two groups. For >2 groups, ANOVA was used with Bonferonni or Dunnet's correction. A p value < 0.05 was taken to indicate statistical significance.

3. Results

3.1. FAM13A is associated with lung disease severity in CF patients

In the GWAS, 1283 SNPs within the *FAM13A* gene with MAF > 0.02 had been genotyped and/or imputed [14]. Overall, FAM13A was associated with lung phenotype in CF patients (VEGAS sum test, $p < 0.017$). The most associated SNP was rs7682431 (unadjusted p-value $p = 5{,}28 \times 10^{-5}$, Bonferroni adjusted p-value $p = 0.068$, Fig. 1A), the GG genotype being found in those with the most impaired lung function (Fig. 1B). The 254 SNPs showing association (unadjusted p-value $p < 0.05$) with changes in lung phenotype are reported in Supplementary Table 1. Previous variants shown to be associated with COPD [5] (rs1903003, rs7671167, rs2869967) were found to be in strong linkage disequilibrium (rs1903003/rs7682431: $D' = 0.92$; rs7671167/rs7682431: $D' = 0.909$; rs2869967/rs7682431: 0.93) and to be associated in CF with the same direction (Supplementary Table 1: rs1903003 (MAF: 0.5, $p < 0.003$, $\beta = -0.09$), rs7671167 (MAF: 0,54, $p < 0.0006$, $\beta = -0.1$), rs2869967 (MAF: 0.36, $p < 0.004$, $\beta = 0.09$)).

3.2. FAM13A is expressed in lung epithelial cells

In order to understand how FAM13A might contribute to the lung phenotype, we aimed to characterize its biological function. Firstly, we assessed FAM13A protein expression level in A549 cells. We observed that FAM13A was expressed in A549 cells with an expected molecular weight of ~117 kDa that was reduced with a siRNA against FAM13A (Fig. 2A).

Efficiency of the antibody and specificity of this band was addressed using an expression plasmid for FAM13A (Supplementary Fig. 1A). Also, the maximal reduction of FAM13A expression was observed 48 h after the siRNA transfection, resulting in 60% reduction of the transcript and 90% reduction of the protein respectively (Supplementary Fig. 1B–C). Immunofluorescence analysis showed a cytoplasmic perinuclear expression in A549 and in primary bronchial cells from control or CF patients (Fig. 2B). Additionally, using sections from human lungs, we confirmed that FAM13A staining was positive in bronchial cells (Fig. 2C).

3.3. FAM13A expression is reduced in the inflammatory context

We studied the regulation of FAM13A in an inflammatory context in order to mimic some of the lung physiopathological characteristics of CF. Stimulation of A549 cells with IL-1β resulted in a 50% reduction of FAM13A mRNA and protein levels (Fig. 3A). TNFα also tended to decrease FAM13A expression, but without reaching significance, and LPS had no effect. As expected from previous studies [20], we verified that IL-1β affected the epithelial characteristics of the cells by looking at the E-cadherin marker. This marker, normally expressed in epithelial cells at the cell membrane adhering junctions, plays a key role in cell polarity and links to the actin network [21]. A concomitant significant reduction in both FAM13A and E-cadherin expressions was seen with IL-1β (Fig. 3A, right panel).

3.4. FAM13A regulates the actin dynamics via RhoA

In order to assess the cellular functions of FAM13A, its expression was decreased with a targeted siRNA. We observed that the transient reduced expression of FAM13A resulted in an increased RhoA activation (Fig. 3B). In contrast, overexpression of FAM13A led to a reduced RhoA activity (Fig. 3B). We showed that FAM13A loss by siRNA (48 h) resulted in F-actin cytoskeleton changes with the induction of stress fibers, loss of cell-cell contacts, and sparsely located cells (Fig. 3C). This effect is not observed when cells are incubated with rho inhibitor rhosin. The reduction of E-cadherin at the protein level with concomitant decreased FAM13A expression by siRNA was confirmed (Fig. 3D). Altogether, these data confirmed the role of FAM13A as a rhoGAP and RhoA as a downstream target of FAM13A. In addition, FAM13A reduction promoted changes in the epithelial cytoskeleton by loss of E-cadherin.

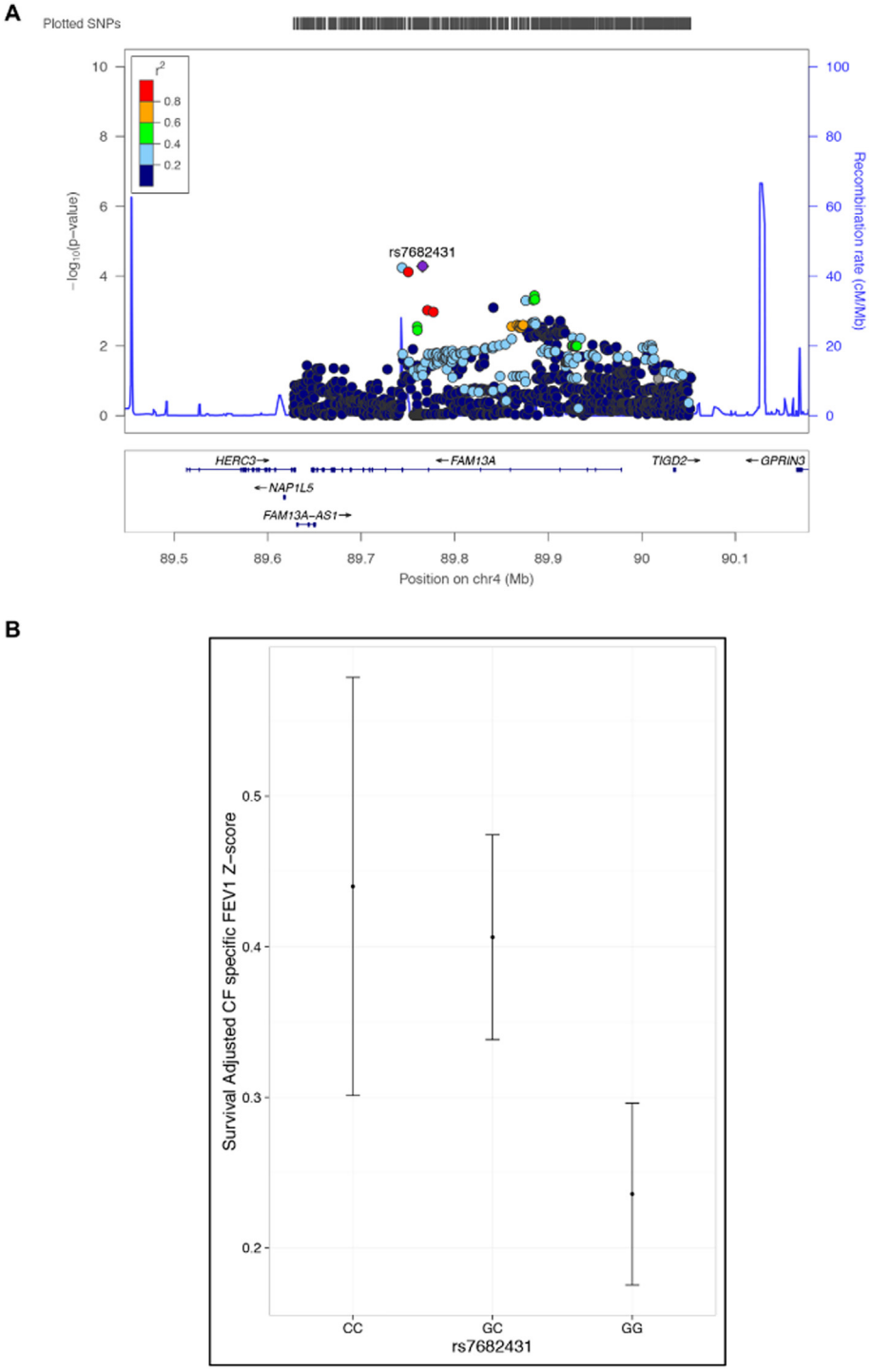

Fig. 1. *FAM13A* variants are associated with lung phenotype in CF patients. (A) LocusZoom plot of the association evidence (build GRCh37, LocusZoom viewer) in CF individuals in the *FAM13A* region with significant associated SNPs with FEV_1 in SNP-Wise analysis. Colours represent 1000 Genomes CEU linkage disequilibrium r^2 values with each SNP (p values are in column three of Supplementary Table 1). The purple diamond is the most significant SNP rs7682431. Genes within the region are shown in the lower panel, and the unbroken blue line indicates the recombination rate within the region. (B) Median survival Adjusted CF specific FEV1 Z-score on the genotype obtained for rs7682431 in the studied poupulation.

3.5. Role of FAM13A in TGFβ-induced EMT

As TGF-β is known to affect changes in Rho signaling [22] and to promote epithelial to mesenchymal transition (EMT) [23], we studied the impact of FAM13A reduction in EMT induced by TGF-β. Several markers for EMT were examined, including E-cadherin, α-smooth muscle actin (α-SMA) and vimentin. In EMT, these markers have an inverse expression

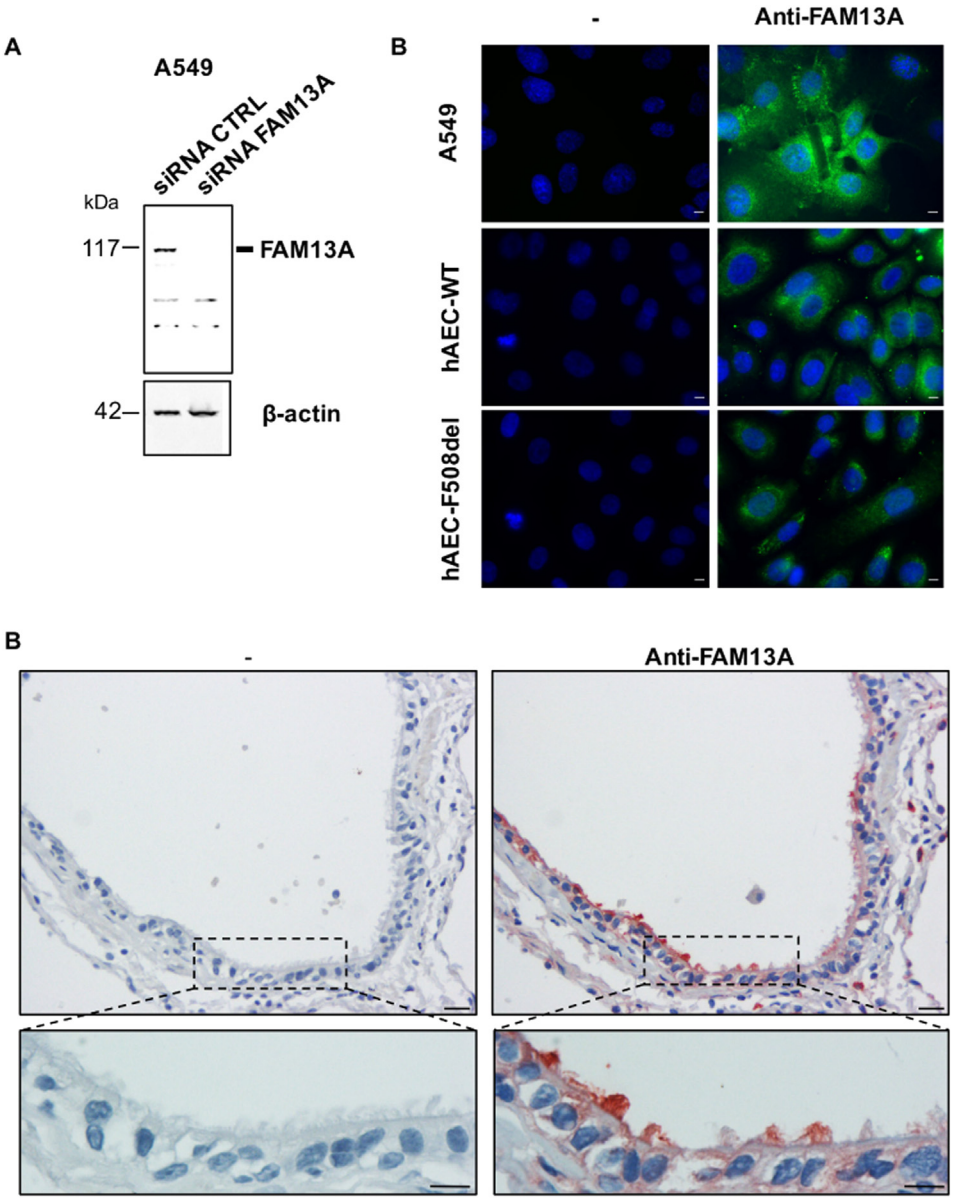

Fig. 2. Expression and localization of FAM13A in respiratory epithelium. (A) Western blot analysis of FAM13A and β-actin (loading control) expression in A549 cells seeded in 6-well plates at 3×10^5 cells and incubated 48 h with 75 pmol of a siRNA control (siRNA CTRL), or a siRNA targeting FAM13A. (B) Immunostaining of FAM13A (green) in A549 and hAECBs from WT (P7) and CF (P13) donors analyzed by fluorescence microscopy. DAPI (blue) was used to stain the nucleus. Scale bars corresponds to 10 μm. Secondary antibody was used as a control (−). (C) Immunohistology staining of FAM13A of lung tissue sections from human (upper panel: ×20, scale bars: 20 μm; lower panel: dotted rectangle, ×60, scale bars: 10 μm). Secondary antibody was used as a control (−).

profile with E-cadherin being reduced and α-SMA and vimentin increased. The qPCR analyses showed that treatments with either siRNA-FAM13A or TGF-β, resulted in 60% and 20% reduction of FAM13A at the transcriptional level respectively (Fig. 4A). This effect of TGFβ is dose and time dependant (Supplementary Fig. 1D). Treatment with siFAM13A resulted in a slight but significant decrease of expression of E-cadherin (Fig. 4A). This pattern was also observed at the protein level but without reaching statistical significance (Fig. 4B). As expected, TGF-β strongly decreased E-cadherin and induced α-SMA and vimentin. The combination of both TGF-β and siFAM13A for 24 h led to a highly significant increase in α-SMA and vimentin mRNA (Fig. 4A). This increased vimentin expression was confirmed by western blot (Fig. 4B). These effects were not due to endogenous increases in TGF-β secretion in cells with knockdown of FAM13A by siRNA, as the ELISA demonstrated no significant change in TGF-β release in siCTRL and siFAM13A treated cells (not illustrated). In addition, phalloidin staining showed that the combination of the knockdown of FAM13A with TGF-β resulted in more severe changes of the F-actin cytoskeleton than TGF-β alone (Fig. 4C).

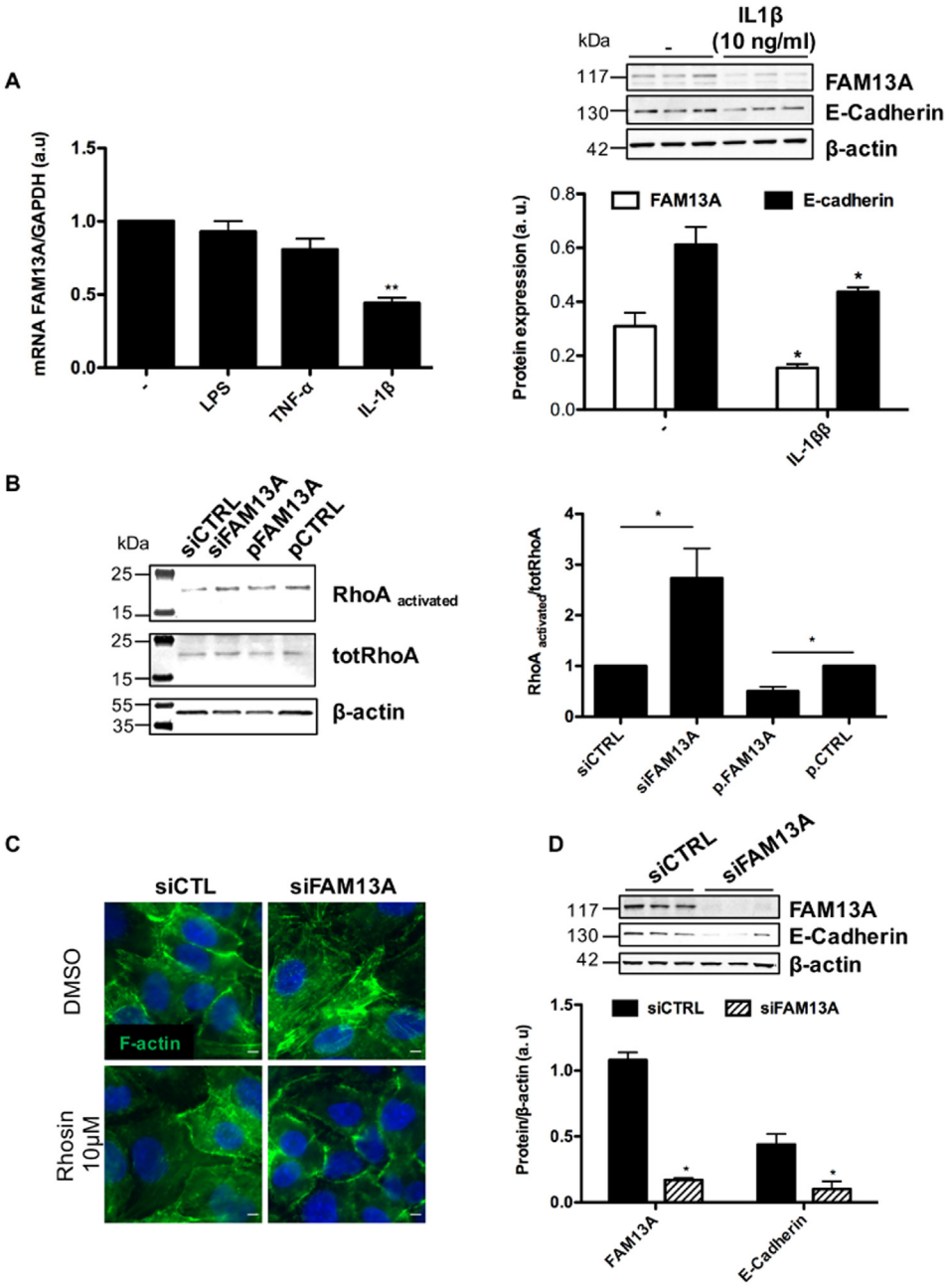

Fig. 3. FAM13A expression is downregulated by IL-1β in A549 cells and its knockdown induces F-actin stress fibers and increase RhoA activity. (A) FAM13A mRNA relative expression was assessed by qPCR in A549 cells seeded at 2×10^5 cells in 12-well plates and stimulated 6 h with LPS from *P. aeruginosa* (1 μg/mL), TNF-α (20 ng/mL), IL-1β (10 ng/mL) (Left panel). The statistical analysis consisted in ANOVA followed by Dunnett's multiple comparison test (unstimulated group (−) used as the control group) $^{**}p < 0.01$. Data are representative of the mean ± SEM of eight experiments realized in duplicate. Western blot of FAM13A, E-cadherin (E-CAD) and β-actin (loading control) in A549 stimulated with IL-1β (10 ng/mL) for 6 h. A representative experiment displayed in triplicate is shown and quantified (Right panel). The statistical analysis consisted in Mann-Whitney test (− vs. IL-1β group) $^{*}p < 0.05$. (B) Western blot of RhoA total RhoA, β-actin (loading control) after RhoA GST-Pull down in cells transfected with siCTRL, siFAM13A, pFAM13A and pCTRL (left panel). Quantification of RhoA activity expressed in arbitruary unit (a.u) (right panel). The statistical analysis consisted in Mann-Whitney test (siCTRL vs. siFAM13A group; pCTRL vs. pFAM13A group). Data are representative of the mean ± SEM four independent experiments. $^{*}p < 0.05$. (C) A549 cells transfected with siCTRL (left panel) or siFAM13A (right panel) for 48 h were further treated for 1 h with rhosin or its solvent DMSO. Cells were incubated with Alexa 488-phalloidin to observe F-actin staining. Scale bars corresponds to 10 μm. (D) Western blot of FAM13A, E-cadherin and β-actin (loading control) in A549 cells transfected with siCTRL or siFAM13A for 48 h. Quantification of protein expression is in arbitruary unit (a.u) The statistical analysis consisted in Mann-Whitney test. $^{*}p < 0.05$, $^{*}p < 0.001$.

3.6. Changes in FAM13A expression and cytoskeleton in primary bronchial epithelial cells

Using primary hAECBs, we observed that CF cells had a lower FAM13A expression level compared to WT cells (Fig. 5A). We also confirmed the inhibitory effect of IL-1β on FAM13A mRNA expression with a significant reduction of 20% in both WT and F508del cells (Fig. 5A). However, this inhibition was modest compared to the 50% decrease seen in A549 cells (Fig. 2A). The silencing of FAM13A by siRNA for

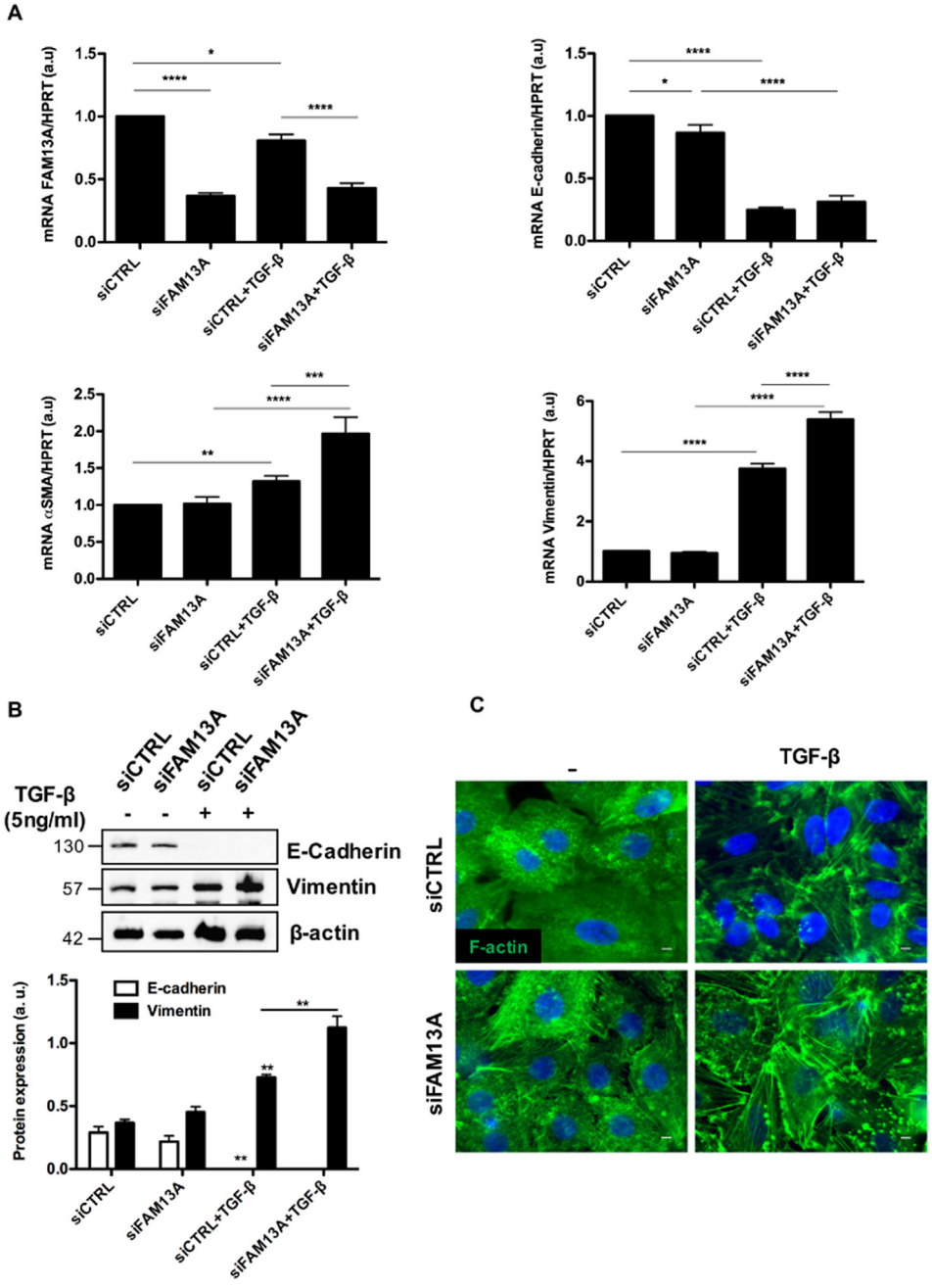

Fig. 4. FAM13A knockdown promotes E-cadherin loss and vimentin increase expressions induced by TGF-β in A549 cells. (A) FAM13A, E-cadherin, αSMA and vimentin mRNA relative quantification (HPRT used as the housekeeping gene) by qPCR of A549 seeded at 2×10^5 cells/well in 12-well plates and transfected with 30 pmol of siCTRL or siFAM13A for 24 h and then stimulated with 5 ng/mL TGF-β for 24 h. Data are representative of the mean ± SEM of four experiments realized in triplicate. The statistical analysis consisted in ANOVA followed by Bonferroni's multiple comparison test (siCTRL vs. siCTRL + TGF-β; siCTRL vs. FAM13A; siCTRL + TGF-β vs. siFAM13A + TGF-β; siFAM13A vs. siFAM13A + TGF-β). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. (B) Western-blot of E-cadherin and vimentin expression of A549 transfected with 30 pmol of siCTRL or siFAM13A for 24 h and then stimulated with 5 ng/mL TGF-β for 48 h. Quantification of E-cadherin and vimentin expression in arbitrary unit (a.u) in three independent experiments. Data are representative of the mean ± SEM of three independent experiments. The statistical analysis consisted in ANOVA followed by Bonferroni's multiple comparison test (siCTRL vs. siCTRL + TGF-β; siCTRL vs. FAM13A; siFAM13A vs. siFAM13A + TGF-β). **$p < 0.01$. (C) Alexa-488 phalloidin staining A549 transfected with siCTRL or siFAM13A for 24 h and stimulated with 5 ng/mL TGF-β for 48 h was analyzed by fluorescence microscopy.

48 h in hAECBs was efficient at the mRNA (Supplementary Fig. 2A) and protein levels as shown by immunofluorescence staining (Fig. 5B, upper panel) and was associated with changes in the F-actin cytoskeleton (Fig. 5B, lower panel). We observed that TGF-β is able to reduce FAM13A (Supplementary Fig. 2B) and regulate the expression of the EMT markers. Indeed, we detected by qPCR a reduction of E-cadherin and an increase of vimentin in a time and concentration dependent manner (Supplementary Fig. 2C and D respectively). By western blot, we observed in hAECBs from three independent CF patients,

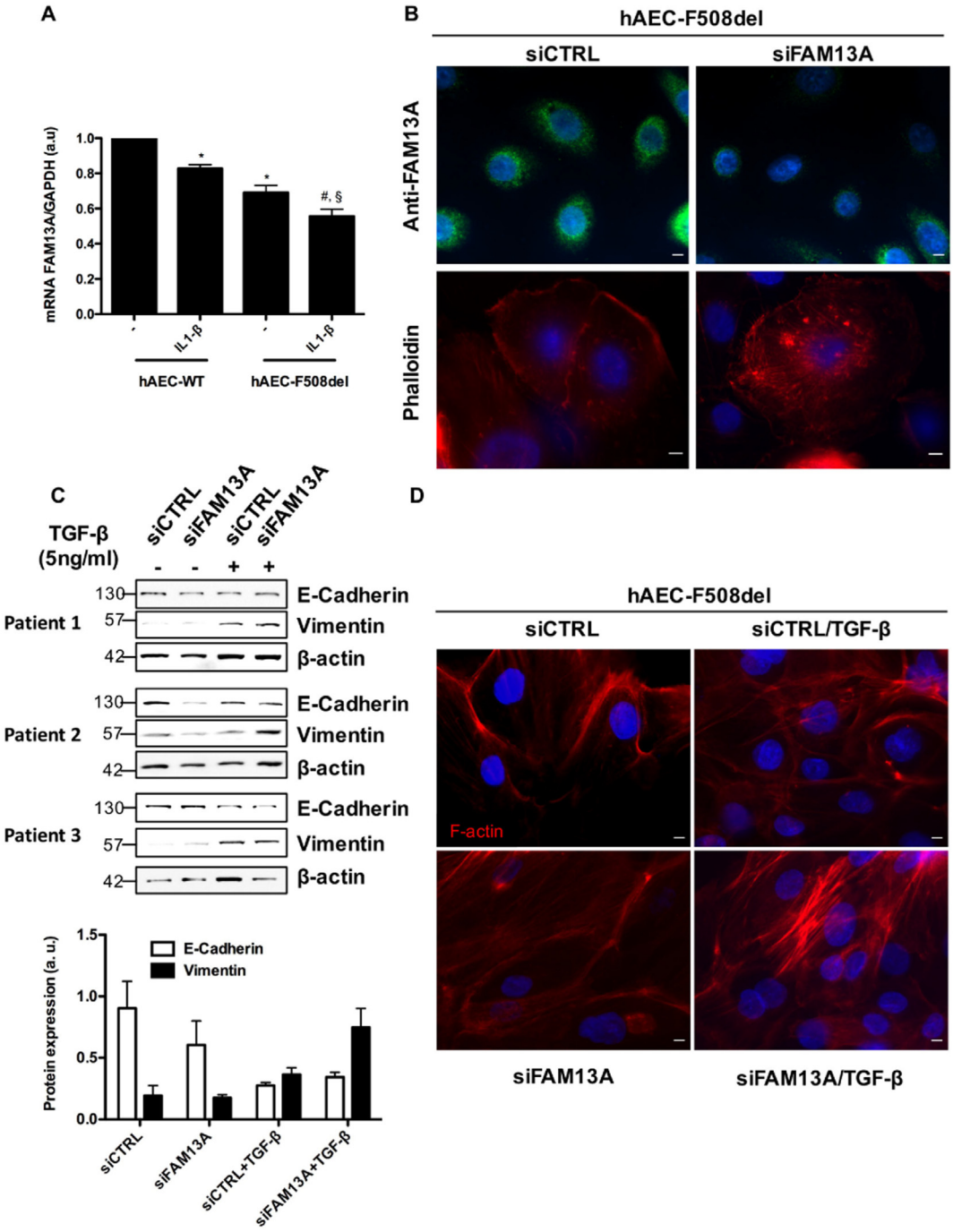

Fig. 5. FAM13A is downregulated by IL-1β in primary hAECBs from WT and CF patients and its knockdown induces F-actin stress fibers. (A) FAM13A mRNA expression by qPCR of primary hAECBs from 6 control donors (WT, P1 to P6) and 6 CF patients (P7 to P12) stimulated for 6 h with 10 ng/mL of IL-1β. Data are representative of the mean ± SEM of six independent experiments (each one including a WT and a CF patient's sample). The statistical analysis consisted in Wilcoxon test. *$p < 0.05$ (WT (−) vs. WT (IL-1), WT (−) vs. CF (−); $^{\#}p < 0.05$ (CF (−) vs. CF (IL-1)); $^{\S}p < 0.05$ (CF (IL-1) vs. WT (IL-1)). (B) Primary hAECBs from CF patients seeded at 3×10^5 cells/well in 6 well-plates were transfected with 75 pmol of siCTRL or siFAM13A for 48 h and stained for FAM13A (upper panel, P13) or F-actin (lower panel, P9). (C) Western-blot of E-CAD, vimentin and β-actin (loading control) expression of CF hAECBs (Patient 1: P12, Patient 2: P11, Patient 3: P13) transfected with 75 pmol of siCTRL or siFAM13A for 24 h and then stimulated with 5 ng/mL TGF-β for 48 h. (D) Primary hAECBs from a CF patient (P12) seeded at 5×10^4 cell/well in ibidi-8well μslide were transfected with 3 pmol of siCTRL or siFAM13A for 24 h and then stimulated for 48 h by 5 ng/mL TGF-β. Cells were stained with Alexa-594 phalloidin (red) and DAPI (Blue) was used to stain the nucleus. Scale bars corresponds to 10 μm.

that the loss of FAM13A by siRNA (72 h) was associated with a slight decreased E-Cadherin expression but had no effect on vimentin expression (Fig. 5C). However, the stimulation by TGF-β of hAECBs knockdown for FAM13A promoted the increase of vimentin expression. Consistent with the western blot, the inhibition of FAM13A by siRNA resulted in changes

in the F-actin cytoskeleton with the induction of stress fibers (Fig. 5D), which is exacerbated with TGF-β, suggestive of a more pronounced EMT phenotype. Altogether, these results suggested that as in the A549 cells, the loss of FAM13A in primary CF cells led to cytoskeletal changes and promoted EMT.

4. Discussion

In this study, we show that FAM13A is a modifier gene of CF lung phenotype. Also, in airway epithelial cells FAM13A modulation regulates RhoA activity, actin cytoskeleton dynamics and EMT. Using SNP-based analysis in a large and well-phenotyped CF cohort, we show that *FAM13A* variants associate with lung disease severity in CF patients with rs7682431 being the most significant associated SNP. *FAM13A* was originally associated with lung function in the general population [4]. Moreover, large genetic studies have also suggested the involvement of *FAM13A* in the onset and progression of other chronic lung diseases such as COPD [5,10]. Interestingly, very little is known about the role of *FAM13A* in the lung. Since we found that *FAM13A* was associated with the CF lung function phenotype, we then aimed to understand its role in lung epithelial cells especially in the inflammatory context relevant for CF. The most significant *FAM13A* variants identified across the several genetic studies, including ours, were not predicted to be damaging by classical bioinformatics analysis and were located in region for which functional tests were barely feasible. Therefore, we decided to study the function of the entire *FAM13A* gene in the inflammatory context relevant for CF.

First, we assessed the expression of FAM13A in human lung epithelial cells. FAM13A protein is detected at the molecular weight of 117 kDa and a siRNA directed against FAM13A is able to reduce its expression. We observed by immunofluorescence that endogenous FAM13A was expressed in A549 and primary hAECBs and localized in the cytoplasm with a perinuclear staining. These results were consistent with a recent study looking at exogenous FAM13A in overexpression experiments in 16HBE cells [24]. In A549 cells using the same antibody, FAM13A was also previously detected by western blot predominantly in the cytoplasm and faintly in the nucleus [25]. Immunostaining of lung sections from human detected FAM13A in bronchial cells, which was consistent with recent observations in mice [24] and human tissues [26].

CF is characterized by an excessive inflammatory response of the airways. Therefore, we evaluated the modulation of FAM13A expression by inflammatory molecules relevant in the CF physiopathology such as LPS from *Pseudomonas aeruginosa*, TNF-α and IL-1β. Indeed, these cytokines are both detected in sputum of CF patients and are known to activate rhoA and induce F-actin stress fibers [20,27]. We observed that the expression of FAM13A was indeed reduced in A549 cells stimulated by IL-1β but not modified by LPS and TNF-α. The expression of the epithelial marker E-cadherin was also downregulated by IL-1β, as previously shown in BEAS2-B cells [28], another lung epithelial cell line, which suggested some changes in cell plasticity. We confirmed this effect in primary hAECBs from both WT and CF donors. This reduction reached only 20%, but was likely relevant considering the chronic exposure of the epithelium to IL-1β in CF. Interestingly, at baseline, CF cells expressed less FAM13A which may explain the overexpression of RhoA already demonstrated in human [29] and mouse [13] CF cells in comparison to WT cells. However, this result may be biased by the age difference of the two groups of cell donors, the CF patients being younger. In a previous study comparing nasal epithelial cells from control and CF patients, FAM13A overexpression was observed in CF samples by microarray analysis (Supplementary data, fold change not available) [30]. This contrasting result may be explained by the type of cells used in this study. In fact, in CF transcriptomic profiles between nasal and bronchial cells are known to be different [31]. Clinical characteristics of the patients such as their treaments may also explain this discrepancy.

In CF, IL-1β is known to be produced in high amounts [32] and we have shown here that IL-1β induced a reduction of FAM13A expression. Thus, we evaluated the cellular consequences of a downregulation of FAM13A by loss of function experiments using a siRNA. The association of RhoGTPases with changes in the actin cytoskeleton is well described. Also, IL-1β has been shown to increase RhoA activity and induce actin stress fibers [33]. Thus, we assessed if the knockdown of FAM13A could regulate RhoA activity and how this relates to changes in the actin cytoskeleton. As expected, we observed that the knockdown of FAM13A which harbor a rhoGAP domain, increased RhoA activity whereas FAM13A overexpression decreased its activity. Interestingly, previous publications found RhoA expression to be upregulated in both CFTR knockout cells and in CFTR knockout mice [13,29]. Active RhoA plays an important role in recycling wildtype CFTR to the plasma membrane via the Na+/H+-exchanger regulatory factor isoform-1 (NHERF1). However constitutively activated RhoA does not promote recycling of F508del-CFTR to the plasma membrane, indicating that reduction of FAM13A could facilitate maintenance of F508del-CFTR in the degradative/lysosomal pathway [34]. We showed here that loss of FAM13A was markedly associated with changes in the actin cytoskeleton and changes in cell-to-cell adhesion both in A549 cells and in primary hAECBs both from CTRL and CF patients. The attenuated effect seen in hAECBs may be linked to the different origin (immortalized alveolar vs. bronchial) and type (cell line vs. primary) of cells. Interestingly, it has been shown that severe actin cytoskeleton rearrangement inhibits cAMP mediated activation of CFTR [35]. Further studies are thus needed to understand if FAM13A has a direct impact on CFTR traffic and function.

TGF-β is the most commonly associated profibrotic cytokine and its role in promoting EMT or to a myofibroblast phenotype has been well documented. In CF, TGF-β has been shown to be upregulated in the plasma of CF patients [36], and several *TGFB1* polymorphisms have been associated with an increased lung disease severity [37]. In our study, the loss of FAM13A in epithelial cells demonstrated similar changes in the actin cytoskeletal network as those seen with TGF-β in

previous publications [22]. Considering the knockdown of FAM13A by IL1-β, we examined whether TGF-β may also affect FAM13A expression. We observed that TGF-β was also able to downregulate FAM13A expression, which provided some evidence that FAM13A may play a role in the intersection of the inflammatory signaling pathways of IL-1β, RhoA and TGF-β. This result confirms a recent study involving the role of FAM13A in non-small cell lung cancer progression showing a downregulation of FAM13A by TGF-β in A549 cells [38]. Knockdown of FAM13A alone also promoted a downregulation of E-cadherin but no upregulation of αSMA and vimentin, indicative markers of loss of epithelial phenotype and the potential change to a mesenchymal phenotype via EMT. When FAM13A was silenced, TGF-β promoted this phenotype by further decreasing E-Cadherin and increasing αSMA and vimentin expressions. E-cadherin is located at cell-cell junctions and is reduced in cells where the epithelial layer integrity disintegrates; particularly with TGF-β. TGF-β driven loss of epithelial characteristics and gain of mesenchymal markers occurs through RhoA [22]. Even if a role for EMT in CF has been suggested in a transcriptomic study [39], no evidence of EMT has been shown in CF and further studies are needed. Indeed, in vivo evidence of EMT in chronic lung disease such as COPD is still controversial [40].

The full impact of FAM13A in CF lung disease is still to be resolved. Loss of epithelial integrity is associated with fibrosis [41], and reduction of FAM13A, either through a genetic polymorphism, or increased IL1-β and TGF-β with inflammation, appears to promote loss of epithelial integrity by altered RhoA signaling. In CF, loss of epithelial integrity promotes invasion and internalization of *P. aeruginosa* by airway epithelial [42]. Also, loss of RhoA-associated epithelial integrity was shown to promote the susceptibility of airway epithelial cells to *Staphylococcus aureus* [43]. Finally, actin cytoskeleton organization has been shown to impact CFTR activation [35]. In this context, another rhoGTPases, cdc42 was recently shown to impact CFTR stability [44].

Several genetic studies have found *FAM13A* associated with the lung function in different chronic lung diseases [10], as well as with airway obstruction in general [45]. FAM13A functional studies are thus needed and will help to decipher if common or different physiopathological mechanisms are involved among these different diseases. A recent study in COPD patients showed that FAM13A may influence COPD susceptibility by promoting β-catenin degradation. An increase of FAM13A expression and a concomitant decrease β-catenin downregulation was observed in comparison with healthy ex-smokers. In this study, *Fam*13A$^{-/-}$ mice were resistant to chronic cigarette smoke induced emphysema [24]. Previous publications have shown that CFTR influences the Wnt/β-catenin signaling in lung development [46] and emerging data showed that a defective CFTR/β-catenin interaction promoted intestinal inflammation in the mouse model [47]. All these results suggest that in chronic lung diseases FAM13A is at the intersection of tissue remodeling pathways that include wnt/β-catenin, EMT and TGF-β.

We showed that *FAM13A* is a modifier gene of the lung phenotype in CF patients. We identified the localization of FAM13A in both the alveolar A549 cell line, as well as in human primary bronchial cells, confirming the presence of FAM13A in lung epithelial cells. We also demonstrated the rhoGAP function of FAM13A and that knockdown of FAM13A constitutively activates RhoA. Additionally, the regulation of FAM13A by IL1-β and TGF-β was determined. These findings offer insight into the complex inflammatory milieu that promotes disease severity in CF.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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