

# Airway Surface Dehydration by Transforming Growth Factor $\beta$ (TGF- $\beta$ ) in Cystic Fibrosis Is Due to Decreased Function of a Voltage-dependent Potassium Channel and Can Be Rescued by the Drug Pirfenidone\*

Received for publication, June 9, 2015, and in revised form, September 2, 2015. Published, JBC Papers in Press, September 3, 2015, DOI 10.1074/jbc.M115.670885

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**Background:** TGF- $\beta$  is associated with worse outcome in cystic fibrosis (CF).

**Results:** TGF- $\beta$  causes mucociliary dysfunction by reducing airway surface liquid (ASL) due to decreased apical BK channel activity and down-regulation of its  $\gamma$  subunit LRRC26.

**Conclusion:** The TGF- $\beta$  inhibitor pirfenidone reverses ASL loss via BK and LRRC26 rescue.

**Significance:** Clinical trials could test the usefulness of pirfenidone in CF.

Transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ) is not only elevated in airways of cystic fibrosis (CF) patients, whose airways are characterized by abnormal ion transport and mucociliary clearance, but TGF- $\beta 1$  is also associated with worse clinical outcomes. Effective mucociliary clearance depends on adequate airway hydration, governed by ion transport. Apically expressed, large-conductance,  $\text{Ca}^{2+}$ - and voltage-dependent  $\text{K}^+$  (BK) channels play an important role in this process. In this study, TGF- $\beta 1$  decreased airway surface liquid volume, ciliary beat frequency, and BK activity in fully differentiated CF bronchial epithelial cells by reducing mRNA expression of the BK  $\gamma$  subunit leucine-rich repeat-containing protein 26 (LRRC26) and its function. Although LRRC26 knockdown itself reduced BK activity, LRRC26 overexpression partially reversed TGF- $\beta 1$ -induced BK dysfunction. TGF- $\beta 1$ -induced airway surface liquid volume hyper-absorption was reversed by the BK opener mallotoxin and the clinically useful TGF- $\beta$  signaling inhibitor pirfenidone. The latter increased BK activity via rescue of LRRC26. Therefore, we propose that TGF- $\beta 1$ -induced mucociliary dysfunction in CF airways is associated with BK inactivation related to a LRRC26 decrease and is amenable to treatment with clinically useful TGF- $\beta 1$  inhibitors.

Mucociliary clearance is an important innate defense mechanism (1, 2). The epithelium maintains an  $\sim 7$ - $\mu\text{m}$ -high periciliary layer that allows effective ciliary beating to remove mucus from the airways (3–5). Airway surface liquid (ASL)<sup>3</sup>

volume maintains this layer and hydrates mucus. Its depletion leads to mucociliary dysfunction and subsequent lung disease as exemplified by cystic fibrosis (CF).

Apical  $\text{Cl}^-$  secretion and  $\text{Na}^+$  absorption play critical roles in ASL volume homeostasis (6). Due to a genetically defective cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel, CF airway epithelia reveal reduced ASL volume that worsens under stress. Alternative pathways exist to hydrate the airway surface via calcium-activated chloride channels. However, attempts to stimulate these channels and maintain ASL volume in CF have clinically failed (7). There are several possible explanations for this outcome, including the short duration of action of the agonist used. The focus here, however, is on the driving force needed to secrete apical  $\text{Cl}^-$ .

ATP-induced apical  $\text{K}^+$  secretion, driven by apical large-conductance,  $\text{Ca}^{2+}$ -activated and voltage-dependent potassium (BK) channels, is critical for adequate ASL volume and mucociliary clearance (8, 9). Apical  $\text{K}^+$  secretion facilitates  $\text{Cl}^-$  efflux by hyperpolarizing the apical membrane and increasing the driving force for  $\text{Cl}^-$  secretion by acting as the counter ion. This creates a short circuit loop favoring airway surface hydration. In normal airway epithelial cells, inhibition of BK channels or knockdown of the BK  $\alpha$ -subunit led to periciliary fluid collapse as revealed by low ciliary beat frequency (CBF) that can be fully rescued by apical fluid addition (8, 9).

Transforming growth factor  $\beta 1$  (TGF- $\beta 1$ ) is a modifier gene in CF (10). Polymorphisms at codon 10 and 25 up-regulate TGF- $\beta 1$  synthesis (11). These TGF- $\beta 1$  genotypes have been associated with worse lung function in at least three independent large clinical studies (12). Furthermore, bronchoalveolar lavage fluid levels of TGF- $\beta 1$  were elevated in CF patients and associated with diminished lung function and recent hospitali-

\* This work was supported by a grant from the Cystic Fibrosis Foundation. The authors declare that they have no conflicts of interest with the contents of this article.

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<sup>3</sup> The abbreviations used are: ASL, airway surface liquid; CBF, ciliary beat frequency; CF, cystic fibrosis; CFBE, cystic fibrosis bronchial epithelial; CFTR,

cystic fibrosis transmembrane conductance regulator; BK channel, large-conductance,  $\text{Ca}^{2+}$ - and voltage-dependent  $\text{K}^+$  channel; LRRC26, leucine-rich repeat-containing protein 26; MTX, mallotoxin;  $I_{sc}$ , short circuit current; TER, transepithelial resistance; TEV, transepithelial voltage.

zation (13). Furthermore, increased TGF- $\beta$ 1 was found in conditioned media of CF cells (14). TGF- $\beta$ 1 also impairs therapeutic rescue of CFTR in cystic fibrosis airway epithelial (CFBE) cells (15, 16).

This study addressed the effect of TGF- $\beta$ 1 on ion transport and ASL volume regulation in CF epithelia, specifically focusing on BK channels. TGF- $\beta$ 1 reduced ASL volume and parameters of mucociliary clearance, at least in part by decreasing BK activity. This was due to the down-regulation of leucine-rich repeat-containing protein 26 (LRRC26), a subunit necessary for BK function in non-excitable tissues (17). LRRC26 overexpression, a BK opener, and the clinically used TGF- $\beta$ 1 inhibitor pirfenidone overcame the detrimental effect of TGF- $\beta$ 1. These data provide the basis of a potential novel treatment approach for patients suffering from CF.

## Experimental Procedures

**Chemicals and Solutions**—All media, buffers, and most other materials were purchased from Life Technologies and Sigma-Aldrich. Recombinant TGF- $\beta$ 1 was from R&D Systems (Minneapolis, MN), and pirfenidone was from Selleckchem (Houston, TX).

**Cell Culture**—CF lungs, homozygous for  $\Delta$ F508, were obtained from transplant recipients, approved by our Institutional Review Board. Airway epithelial cells were isolated as described previously (18, 19). CFBE cells were used fully differentiated after 4 weeks.

**Quantitative PCR and Western Blots**—mRNAs of interest were quantified by PCR using a Bio-Rad iCycler and TaqMan probes (Life Technologies), catalog number Hs02385555\_g1 for LRRC26 and catalog number Hs00266938\_m1 for KCNMA1, the pore-forming subunit of BK (8). Western blots were performed according to published methods (8). The following antibodies were used for BK: immunoprecipitation with polyclonal AP107 anti- $\alpha$  BK subunit antibody (Alomone Labs), probing with monoclonal anti- $\alpha$  BK subunit antibody (clone L6/60, NeuroMab, Antibodies Inc.). The polyclonal anti-rabbit LRRC26 antibody was from Santa Cruz Biotechnology (sc-102015 V19) and used at a dilution of 1:200.

**Ussing Chamber Electrophysiology**—Fully differentiated CFBE cells on Snapwell filters were mounted in Ussing chambers (EasyMount chamber) connected to a VCC MC6 voltage clamp unit (Physiologic Instruments, San Diego, CA). Data were acquired as described previously from intact and basolaterally permeabilized cell layers (8). Apical  $K^+$  currents were measured in the presence of apically applied 10  $\mu$ M amiloride and 30 min after basolateral permeabilization using 20  $\mu$ M amphotericin B, 10  $\mu$ M valinomycin, and 10  $\mu$ M nigericin, using a basolateral to apical  $K^+$  gradient (8).

**LRRC26 Knockdown**—Using calcium phosphate co-precipitation (Clontech Laboratories, Inc.), lentiviruses were prepared by co-transfecting HEK 293T cells with pLKO.1-puro-expressing LRRC26 shRNA (TRCN0000245794; MISSIONRNAi from Sigma-Aldrich) and packaging DNA plasmids. Virus-containing medium was collected, concentrated by polyethylene glycol (11%) precipitation, and stored at  $-80^\circ\text{C}$ . An estimation of the virus titer was performed using the p24 HIV antigen ELISA kit (PerkinElmer). Dedifferentiated cells were used for infections.

At the time of plating the cells on Transwell inserts, virus was added at a ratio of 100 ng/500,000 cells in bronchial epithelial growth medium containing Polybrene (2  $\mu$ g/ml). The infection was carried out overnight, at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . The following day, virus was removed, and bronchial epithelial growth medium was changed to air-liquid interface medium top and bottom including puromycin until cells reached confluence, when an air-liquid interface was created.

**LRRC26 Overexpression**—Full-length LRRC26 was amplified from CF cells and cloned into the pCDH-EF1-MCS-IRES-Puro vector from SBI. Viruses were made, and cells were infected and selected as above.

**Measurement of CBF**—Washing of the apical surface was suspended for the duration of the experiments. Cells were imaged on a Zeiss Axiovert 200, equipped with differential interference contrast optics. CBF was measured as described previously (29). Measurements were done within a 0.5-cm radius from the center (no influence of possible fluid menisci at the edges of the cultures). CBF was measured before and after apical fluid addition.

**ASL Volume**—ASL volumes from CFBE cells were estimated by meniscus scanning (20). Cultures were washed with PBS 24 h prior to measurements to clean the filters from mucus accumulation that may interfere with the ASL reading. Scanned menisci data were transformed to ASL volume using software generously provided by Dr. Myerburg (University of Pittsburgh).

Alternatively, the ASL of fully differentiated CFBE cells was labeled with rhodamine-dextran (Life Technologies). ASL height was measured by XZ confocal microscopy as described previously (21) using a Leica SP5 confocal microscope with a  $63\times$  glycerol immersion lens.

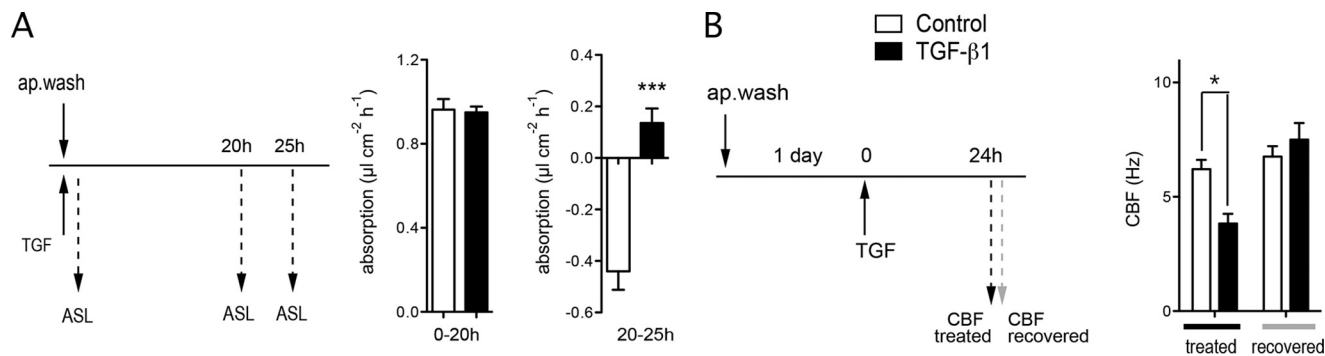
**Statistics**—GraphPad Prism (GraphPad Software, San Diego, CA) was used to analyze all results. Student's *t* test or Mann-Whitney tests were used to compare two groups as appropriate. One-way analysis of variance was used for comparisons of more than two groups followed by the appropriate post hoc tests. A  $p < 0.05$  was accepted as significant.

## Results

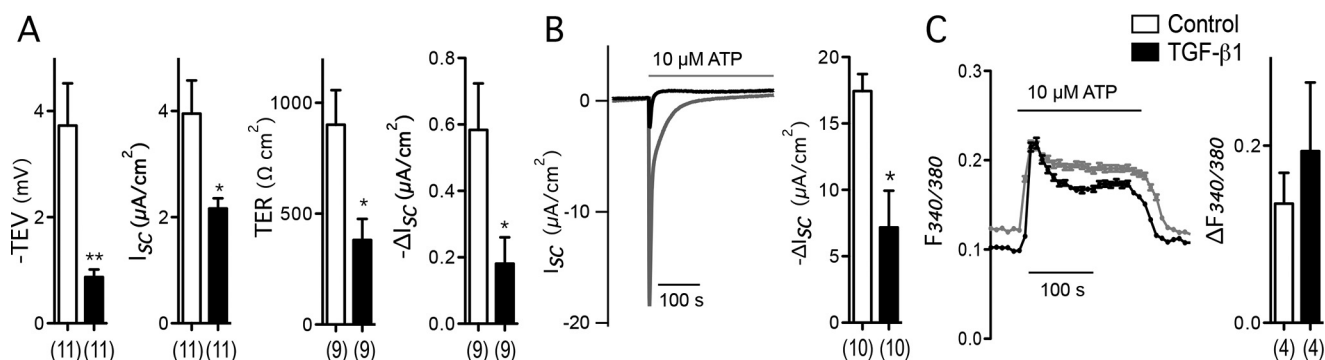
**TGF- $\beta$ 1 Decreases ASL Volume and CBF in CFBE Cells**—ASL volume was measured by meniscus scanning (20) in fully differentiated, homozygous  $\Delta$ F508 CFBE cells. Cells treated basolaterally with TGF- $\beta$ 1 (10 ng/ml) revealed fluid absorption both during the first 20 h and during the subsequent 5-h time window (Fig. 1A), whereas control cells switched to net fluid secretion in this 5-h period. TGF- $\beta$ 1 also resulted in a significant CBF decrease (Fig. 1B). ASL volume homeostasis maintains an appropriate periciliary layer height that allows normal ciliary activity. Therefore, a CBF reduction rescued by adding apical fluid is due to ASL volume loss. In fact, TGF- $\beta$ 1-induced CBF decreases were fully reversed by adding apical buffer (Fig. 1B), indicating no direct TGF- $\beta$ 1 effect on cilia.

**TGF- $\beta$ 1 Changes Transepithelial Ion Transport in CFBE Cells**—TGF- $\beta$ 1 treatment significantly decreased transepithelial voltage (*TEV*, Fig. 2A), baseline short circuit current ( $I_{sc}$ , Fig. 2A), transepithelial resistance (*TER*, Fig. 2A), and amiloride-

## TGF- $\beta$ -induced Mucociliary and BK Dysfunction in CF



**FIGURE 1. TGF- $\beta$ 1 causes excessive ASL volume absorption in CFBE cells.** *White bars, control cells. Black bars, TGF- $\beta$ 1-treated cells (10 ng/ml). Error bars: mean  $\pm$  S.E. A, ASL volume measurements by meniscus refractometry. Experimental design was as follows. Cells were apically washed (ap. wash), and 10 ng/ml TGF- $\beta$ 1 was applied basolaterally at day 0. ASL volume was measured at 0, 20, and 25 h. Volume changes were expressed as absorption (positive values) or secretion (negative values). *White bars, vehicle; black bars, TGF- $\beta$ 1-treated cells;  $\geq 3$  filters from  $\geq 3$  different donors were used in each experimental set. TGF- $\beta$ 1 causes excessive ASL absorption in CFBE lungs, by Student's *t* test. B, CBF measurements. Experimental design was as follows. Measurements were made before (treated) and after (recovered) apical buffer addition. CBF measurements of  $\geq 5$  filters from  $\geq 3$  different lungs were made. \*,  $p < 0.05$ , \*\*\*,  $p < 0.001$  by Student's *t* test.**



**FIGURE 2. TGF- $\beta$ 1 causes decreases in ATP-induced apical  $\text{K}^+$  secretion in CFBE cells.** *A, white bars, control cells; black bars, TGF- $\beta$ 1-treated cells (10 ng/ml). From left to right: transepithelial voltage (TEV) measurements in Ussing chambers with symmetrical Krebs-Henseleit buffer; baseline  $I_{sc}$ ; transepithelial resistance (TER); and amiloride-sensitive  $I_{sc}$  (10  $\mu\text{M}$ ). \*,  $p < 0.05$ , \*\*\*,  $p < 0.01$  by Student's *t* test. Numbers of filters used are indicated in parentheses below each bar. B, ATP-induced  $I_{sc}$  changes in basolaterally permeabilized CFBE cells exposed to a basolateral-apical  $\text{K}^+$  gradient and 10  $\mu\text{M}$  amiloride. C, effect of ATP on  $[\text{Ca}^{2+}]_i$ , estimated by the ratio (340 nm/380 nm) of emitted light (510 nm) from fura-2-loaded cells. Left: data plotted as mean  $\pm$  S.E. for 10 different cells. Gray line, control; black line, TGF- $\beta$ 1-treated cells. Right: summary of maximal changes.*

sensitive  $I_{sc}$  (Fig. 2A) due to epithelial sodium channel activity, an effect previously reported in alveolar epithelia (22).

**TGF- $\beta$ 1 Reduces ATP-induced Apical  $\text{K}^+$  Secretion in CFBE Cells**—Apical BK activity was measured by ATP-induced  $I_{sc}$  changes in basolaterally permeabilized CFBE cells exposed to a serosal-mucosal  $\text{K}^+$  gradient and amiloride (8). TGF- $\beta$ 1 significantly reduced ATP-stimulated BK activity (Fig. 2B).

To test whether TGF- $\beta$ 1 treatment interfered with ATP-induced  $\text{Ca}^{2+}$  signaling that opens BK, we measured the change in fura-2 fluorescence as an indicator of  $[\text{Ca}^{2+}]_i$  (Fig. 2C). TGF- $\beta$ 1 did not reduce the  $\text{Ca}^{2+}$  response to ATP, eliminating the possibility that the decreased BK activity was due to a breakdown of the upstream signaling pathway.

TGF- $\beta$ 1 treatment had no negative impact on the cellular expression of the BK channel pore-forming  $\alpha$ -subunit. TGF- $\beta$ 1 increased its mRNA and did not change protein expression significantly (Fig. 3A). Control and TGF- $\beta$ 1-treated cells responded to 5  $\mu\text{M}$  of the BK opener NS11021 (23) with a similar current change (Fig. 3B), suggesting that surface availability of the channel was not affected by TGF- $\beta$ 1.

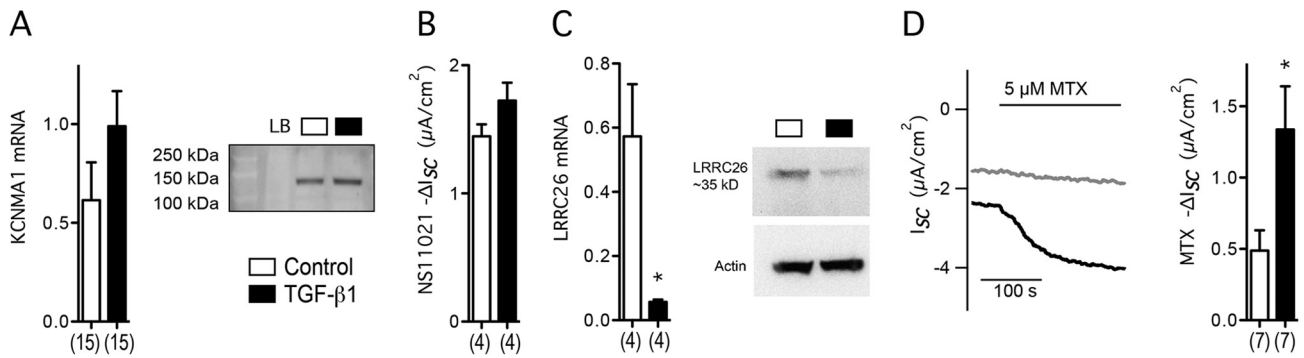
**TGF- $\beta$ 1 Changes the Functional Expression of LRRC26**—BK activity can be modified by the presence of  $\gamma$  modulatory proteins, most commonly LRRC26 (17, 24). In the presence of LRRC26, the threshold for BK channel opening at physiological

membrane potentials is left-shifted and requires a thousandfold lower  $[\text{Ca}^{2+}]_i$ , namely 100 nM instead of 100  $\mu\text{M}$  (17). This shift allows BK activity in non-excitatory cells. TGF- $\beta$ 1 decreased LRRC26 mRNA and protein expression significantly (Fig. 3C). To evaluate whether these changes translated to altered functional activity, the BK opener mallotoxin (MTX) (5  $\mu\text{M}$ ) was used. MTX potently shifts the conductance-voltage relationship of BK channels to the left (25), but only in the absence of LRRC26. Control CFBE cells did not respond to MTX, whereas TGF- $\beta$ 1-treated cells showed increased apical  $\text{K}^+$  secretion upon MTX exposure (Fig. 3D). Therefore, LRRC26 was also functionally decreased in TGF- $\beta$ 1-treated cells, leading to decreased apical activity of BK channels.

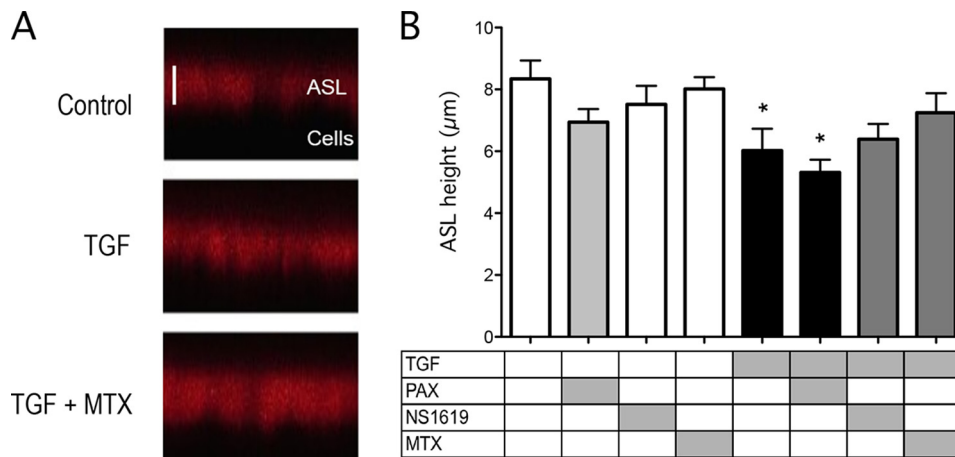
**TGF- $\beta$ 1 and Paxilline Decrease ASL Volume in CFBE Cells**—Confocal microscopy was used to confirm the effects of TGF- $\beta$ 1 and BK channel inhibitors on ASL height (Fig. 4A). Either 20 h of TGF- $\beta$ 1 alone or the combination of paxilline with TGF- $\beta$ 1 pretreatment reduced ASL height relative to control (Fig. 4, A and B). The presence of the BK opener MTX alleviated the effects of TGF- $\beta$ 1.

**LRRC26 Knockdown Decreases BK Activity**—LRRC26 was knocked down using shRNA-expressing lentiviruses. Quantitative PCR showed a significant decrease of LRRC26 expression in fully differentiated CFBE cells after they were infected in an





**FIGURE 3. TGF- $\beta$ 1 decreases ATP-induced apical  $K^+$  secretion and reduces LRRC26 expression in CFBE cells.** *A*, quantitative PCR of KCNMA1 mRNA relative to GAPDH ( $\times 1000$ ). Equal amounts of protein from pooled CFBE lysates were immunoprecipitated with polyclonal AP107 anti- $\alpha$  BK subunit antibody (Alomone Labs), electrophoresed, transferred, and probed with the monoclonal anti- $\alpha$  BK subunit antibody (clone L6/60, NeuroMab, Antibodies Inc.). TGF- $\beta$ 1 increases KCNMA1 mRNA in a non-significant manner, but does not change the  $\alpha$  BK subunit protein expression as assessed by immunoprecipitation (LB: control precipitation without antibody). *White bars*, control cells. *Black bars*, TGF- $\beta$ 1-treated cells (10 ng/ml). Numbers of filters used are indicated in parentheses below each bar. *B*, quantified current changes induced by NS11021 (5  $\mu$ M), corresponding to BK opening, indicating that BK remains at the surface upon TGF- $\beta$ 1 treatment. *C*, quantitative PCR and protein expression of LRRC26. *Left*: mRNA is shown relative to GAPDH ( $\times 1000$ ). *Right*: Western blot with pooled samples showing a decrease in LRRC26 protein expression upon TGF- $\beta$ 1 exposure. *D*, effect of 5  $\mu$ M MTX on apical  $K^+$  secretion in the presence of amiloride. Maltotxin activates BK channels only if LRRC26 is absent. *Gray line*, control; *black line*, TGF- $\beta$ 1-treated cells. \*,  $p < 0.05$  by Student's *t* test.



**FIGURE 4. TGF- $\beta$ 1 reduces ASL height.** *A* and *B*, confocal microscopy to measure ASL height using rhodamine-dextran. Cells were treated with 10 ng/ml TGF- $\beta$ 1, 5  $\mu$ M paxilline, 30  $\mu$ M NS1619, and 5  $\mu$ M MTX. *A*, XZ confocal images of ASL height. The *white bar* indicates a height of 8  $\mu$ m. ASL and cells were labeled for better orientation. *B*, analysis of ASL height (in  $\mu$ m) from  $\geq 4$  filters of  $\geq 4$  donors. MTX partially rescues TGF- $\beta$ 1-induced ASL height decreases. PAX, paxilline. \*,  $p < 0.05$  between treatments by analysis of variance followed by Dunnett's multiple comparison test.

undifferentiated state (Fig. 5A). LRRC26 protein expression was also reduced by a modest amount when assessed by Western blotting (Fig. 5B;  $\sim 0.7$ -fold control). As with TGF- $\beta$ 1, LRRC26 knockdown actually increased KCNMA1 mRNA expression (Fig. 5C). Nevertheless, knockdown cells exhibited decreased BK activity (Fig. 5D) and increased MTX sensitivity (Fig. 5E). We could not quantify ASL volume in these cells using the meniscus scanning technique because they were dry and showed mucus accumulation on their surfaces without movement (Fig. 5F), indicating ASL volume loss.

**LRRC26 Overexpression Partially Reverses TGF- $\beta$ 1-induced BK Dysfunction**—LRRC26 overexpression increased LRRC26 mRNA and protein expression as expected (Fig. 6, A and B). LRRC26 overexpression also reversed TGF- $\beta$ 1-induced BK dysfunction (Fig. 6C). As seen with TGF- $\beta$ 1 alone, the increased LRRC26 protein expression did not change the TGF- $\beta$ 1-induced increases in KCNMA1 mRNA expression, the pore-forming subunit of BK. However, this increase did not explain the rescued  $K^+$  current with LRRC26 overexpression because the current was down with TGF- $\beta$ 1 alone despite an

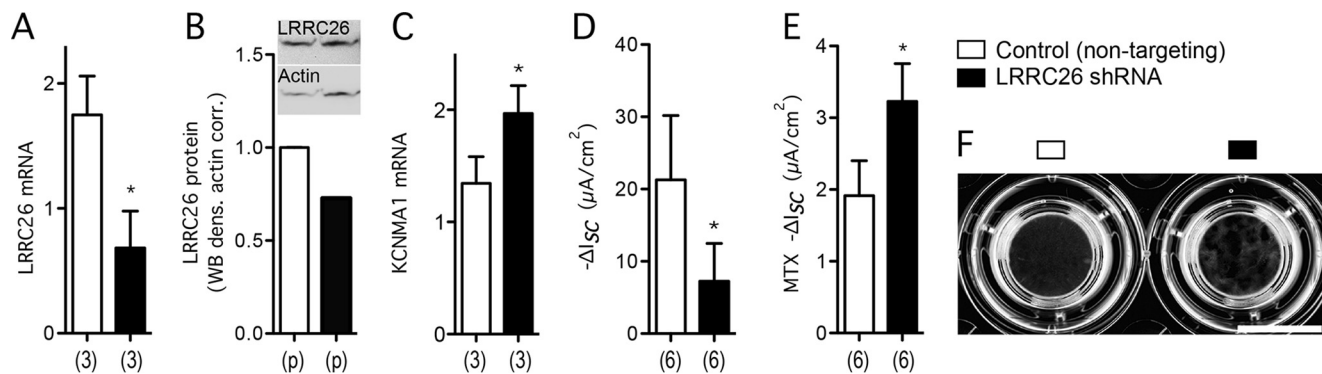
increase in KCNMA1 mRNA in the cells infected with the control plasmid.

**Pirfenidone Reverses TGF- $\beta$ 1-induced Mucociliary Dysfunction in CFBE Cells**—In normal human airway epithelial cells, the Smad3 inhibitor SIS3 (26), but not p38 MAP kinase or PIK inhibitors, partially counteracted the TGF- $\beta$ 1-induced impairments of BK activity and CBF (not shown), indicating a Smad-dependent mechanism. We therefore tested the clinically useful Smad2/3 inhibitor pirfenidone in CFBE cells. Pirfenidone (1 mg/ml) rescued TGF- $\beta$ 1-mediated BK dysfunction (Fig. 7A), LRRC26 down-regulation (Fig. 7B), and reversed MTX sensitivity (Fig. 7C). In addition, pirfenidone rescued TGF- $\beta$ 1-mediated CBF decreases (Fig. 7D) and reversed the prolonged ASL volume absorption induced by TGF- $\beta$ 1 (Fig. 7E).

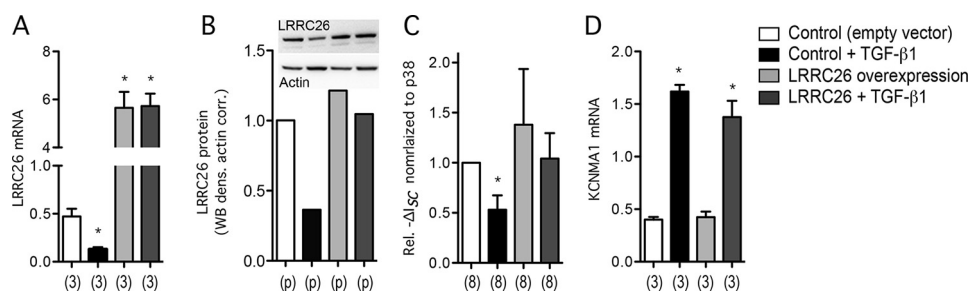
## Discussion

TGF- $\beta$ 1 is a potent physiological cytokine that is involved in differentiation and proliferation. TGF- $\beta$ 1 is increased in CF lung disease and associated with worse clinical outcome. Our results indicate that TGF- $\beta$ 1 decreased ASL volume and CBF in

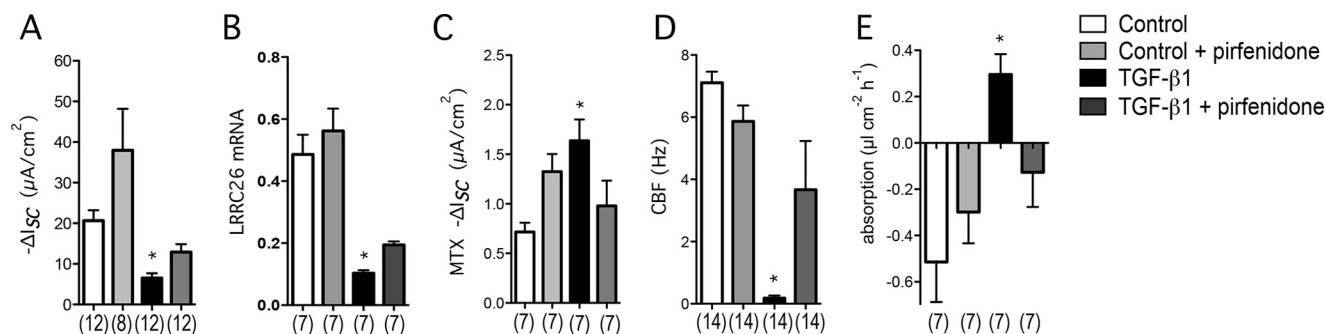
## TGF- $\beta$ -induced Mucociliary and BK Dysfunction in CF



**FIGURE 5. Partial LRRC26 knockdown still decreases BK function.** A–D, non-targeting shRNA expression (white bars) and LRRC26 shRNA expression (black bars), both via lentivirus infection. Numbers of filters used are indicated in parentheses below each bar (*p* indicates pooled samples). \*, *p* < 0.05. A–E, shRNA-mediated knockdown of LRRC26 expression results in decreased LRRC26 mRNA (A); reduced LRRC26 protein expression (pooled samples show reduction to ~0.7-fold control when corrected for actin, see inset; WB dens. actin corr., western blot densitometry corrected for actin) (B); increased KCNMA1 mRNA expression (C); BK dysfunction (D); and increased MTX responsiveness (E). F, LRRC26 shRNA dries out air-liquid interface surfaces. Shown are representative air-liquid interface cultures from non-targeting shRNA-infected cells (left) and LRRC26 shRNA-infected cells (right). The white bar is 12 mm. LRRC26 shRNA causes mucus accumulation on the cell surface and cessation of mucociliary transport.



**FIGURE 6. LRRC26 overexpression rescues TGF- $\beta$ 1-induced decreases in BK currents.** Numbers of filters used are indicated in parentheses below each bar (*p* indicates pooled samples). \*, *p* < 0.05. White bars, control; black bars, TGF- $\beta$ 1; light gray bars, LRRC26 overexpression; dark gray bars, LRRC26 overexpression plus TGF- $\beta$ 1 treatment. A, overexpression of LRRC26 increases mRNA (relative to GAPDH  $\times$  1000) and prevents reduction of mRNA for LRRC26 upon TGF- $\beta$ 1 treatment. B, quantification of the pooled samples using Western blots shows the same pattern as the mRNA (corrected for actin, see inset; WB dens. actin corr., western blot densitometry corrected for actin). C, Ussing chamber currents of permeabilized cells show a reduction in  $I_{sc}$  in control cells exposed to TGF- $\beta$ 1 and lack of a decrease when the LRRC26 protein is overexpressed and thereby maintained even in the presence of TGF- $\beta$ 1. Data are shown in relative values because of variability between samples with respect to BK currents. D, although KCNMA1 mRNA is increased upon TGF- $\beta$ 1 exposure, the BK current is decreased (see panel C and Fig. 2). Therefore, TGF- $\beta$ 1-mediated increases in KCNMA1 mRNA in LRRC26-overexpressing cells do not explain the rescue of BK currents.



**FIGURE 7. Pirfenidone partially rescues TGF- $\beta$ 1-induced changes, including BK dysfunction, LRRC26 down-regulation, CBF decreases, and ASL volume absorption.** A–E, white bars, control; light gray bars, pirfenidone; black bars, TGF- $\beta$ 1; dark gray bars, TGF- $\beta$ 1 plus pirfenidone. Pirfenidone partially rescues TGF- $\beta$ 1-mediated BK dysfunction (A) and LRRC26 mRNA reduction (B); reduces TGF- $\beta$ 1-mediated MTX responsiveness (C); reverses TGF- $\beta$ 1-mediated CBF decreases (D); and reverses the sustained ASL volume absorption (E). \*, *p* < 0.05.

CFBE cells (Fig. 8). We found that TGF- $\beta$ 1 reduced apical BK activity and that this effect was mediated by decreased expression of the  $\gamma$  modulatory protein LRRC26, which causes a shift of the voltage and  $[Ca^{2+}]_i$  needed for activation of BK (17) and thus enables BK activity in non-excitable cells. Our results confirmed that BK activity is necessary for ASL homeostasis (8) because the BK inhibitor paxilline decreased ASL volume similarly to TGF- $\beta$ 1. A BK opener was partially able to rescue ASL homeostasis in TGF- $\beta$ 1-treated CFBE

cells. However, potassium channel openers might be associated with toxicity in human beings when reaching the systemic circulation. On the other hand, it was interesting to note that a clinically useful TGF- $\beta$ 1 signaling inhibitor, pirfenidone, partially reversed the adverse effects of TGF- $\beta$ 1 on ASL volume and CBF.

Thus, targeting TGF- $\beta$  signaling might have benefits as a therapy in patients with CF. This is not only true for ASL volume but also for CFTR corrector therapies, because a BK-in-

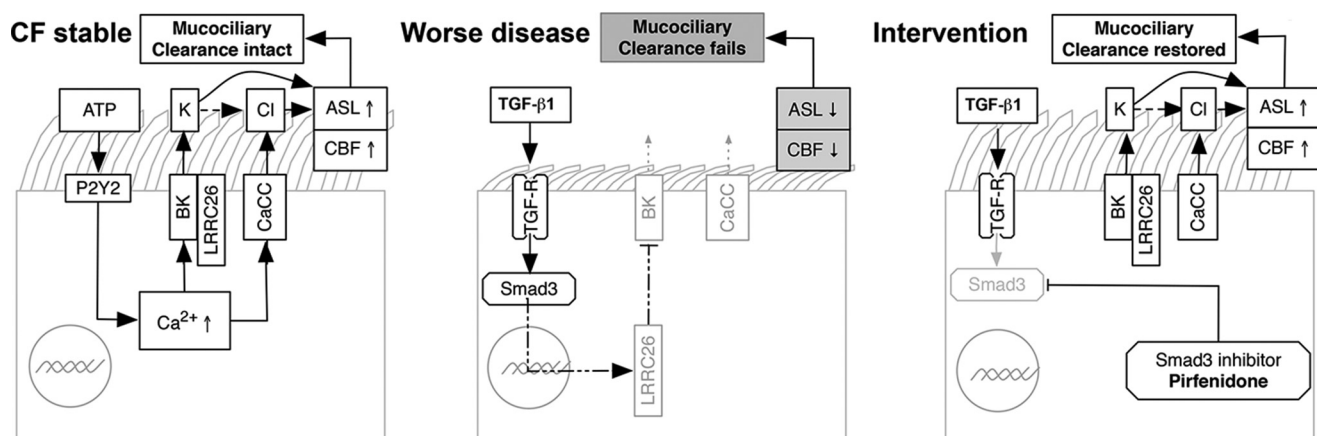


FIGURE 8. **Schematic of findings.** Under normal conditions, ATP release causes an increase in  $[Ca^{2+}]_i$  in CF airway epithelial cells that stimulates both  $K^+$  efflux through BK channels and  $Cl^-$  exit through  $Ca^{2+}$ -activated  $Cl^-$  channels (CaCC, whereby  $K^+$  efflux creates a driving force for  $Cl^-$  exit, the so-called apical loop current). This maintains ASL volume and mucociliary clearance (left). TGF- $\beta$ 1 down-regulates the BK  $\gamma$  subunit LRRC26, which renders BK less active, thereby reducing  $K^+$  efflux and causing ASL volume depletion and thus ultimately mucociliary dysfunction (middle). TGF- $\beta$  inhibitors, including the clinically useful Smad2/3 pirfenidone, can reverse TGF- $\beta$ 1-induced mucociliary dysfunction by rescuing apical  $K^+$  and  $Cl^-$  efflux and thus improve mucociliary clearance in CF (right). TGF-R, TGF receptor.

duced increase in the driving force for  $Cl^-$  secretion would be beneficial even for partially corrected CFTR.

How good are preclinical models in predicting clinical outcome? The most commonly measured clinical outcome in CF patients is a change in forced expiratory volume in the first second (FEV1). As described previously, a good correlation between preclinical data on ASL volume regulation in air-liquid interface cultures, where ASL height was brought into the normal range (*i.e.*  $\geq 7 \mu m$ ), and clinical outcome data was found using ivacaftor (10% FEV1 improvement in G551D patients) and inhaled hypertonic saline (3% improvement) (27, 28). Thus, the data presented here suggest that pirfenidone could have a significant clinical effect in CF patients. This medication is Food and Drug Administration (FDA)-approved and therefore relatively easily amenable to clinical testing.

In conclusion, we showed that BK activity is necessary for ASL homeostasis in non-stressed CFBE cells and found that TGF- $\beta$ 1 decreases BK activity by reducing functional LRRC26 expression. The use of BK openers has a significant rescuing effect, but targeting TGF- $\beta$ 1 signaling might pave the way for novel therapies for the treatment of CF and could have clinically measurable therapeutic effects via improved airway hydration.

**Author Contributions**—D. M. contributed to the overall conception of the article, partially designed, performed, and analyzed experiments shown in Figures 1, 2, 3, 5, and 6, and wrote the first draft of the paper. S. K. partially designed, performed, and analyzed experiments for Figures 3, 5, 6, and 7. N. B. partially designed, performed, and analyzed the experiments shown in Figures 1 and 7. J. S. D. cloned and tested functional LRRC26 overexpression. J. T. and R. T. designed, performed, and analyzed the experiments shown in Figure 4. M. S. conceived and supervised the work, finalized the writing of the paper, and contributed to the preparation of the figures. All authors reviewed the results, edited the paper and approved the final version of the manuscript.

**Acknowledgment**—ASL volume software was provided by Dr. Myerburg (University of Pittsburgh).

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