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The regulatory role of TG2 in Epithelial-Mesenchymal Transition in Cystic Fibrosis

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Highlights

- Increased TG2 expression in CF epithelia increases TGFβ1 levels.
- Increased TGFβ1 and TG2 leads to Epithelial Mesenchymal Transition (EMT).
- EMT leads to poor CFTR stability and increased matrix deposition
- Selective Inhibition of TG2 helps restore CFTR stability and leads to reversal of EMT.

Abstract

Cystic fibrosis (CF) is a genetic disorder caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) for which there is no overall effective treatment. Recent work indicates Tissue Transglutaminase (TG2) plays a pivotal intracellular role in proteostasis in CF epithelia and that the pan TG inhibitor cysteamine improves CFTR stability. Here we show TG2 has another role in CF pathology linked with TGFβ1 activation and signalling, induction of Epithelial-Mesenchymal Transition (EMT), CFTR stability and induction of matrix deposition. We show that increased TG2 expression in normal and CF bronchial epithelial cells increases TGFβ1 levels, promoting EMT progression, and impairs tight junctions as measured by Trans Epithelial Electric Resistance (TEER) which can be reversed by selective inhibition of TG2 with an observed increase in CFTR stability. Our data indicate that selective inhibition of TG2 provides a potential therapeutic avenue for reducing fibrosis and increasing CFTR stability in CF.

Key words

Tissue transglutaminase, Cystic fibrosis, Transforming Growth Factor β, Epithelial-Mesenchymal Transition, cystic fibrosis transmembrane conductance regulator.

1. Introduction

Cystic fibrosis (CF) is a genetic disorder caused by mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) [1]. Of the 1900 mutations now recognised to be present in CFTR the most common is F508del, all of which lead to the abnormal transport of chloride and sodium across the epithelium, resulting in multiple clinical manifestations of which lung disease characterised by chronic lung obstruction, infection and inflammation is the major cause of morbidity. CF affects not only the lungs, but also the pancreas, liver and intestines. True to its terminology fibrosis of the pancreas was noted when the disease was first recognised and uncontrolled airway remodelling is now recognised to start early in life and is linked to a poor pulmonary outcome with extensive fibrosis [2]. Transforming growth factor β1 (TGFβ1) is a key growth factors involved in fibrosis, including cystic fibrosis where increased levels are reported in lungs [3]. In addition to its pro-inflammatory properties TGFβ1 has been shown recently to inhibit the biosynthesis of CFTR and also prevents the biofunctional rescue of CFTR, especially the F508del [4]. Importantly over-expression of TGFβ1 in the CF lung has been associated with a more severe CF phenotype.

Current treatment for CF is limited and largely confined to the relief of patient symptoms. This includes use of anti-inflammatory agents and antibiotics to suppress microbial infection. Lung transplantation may be necessary as the condition progresses, but both lungs need to be, replaced to prevent infection. Recently, new therapeutic avenues have been applied to CF treatment, such as the use of CFTR correctors and potentiators to improve the cell processing and function of the mutant CFTR [5]. For example some small compounds can correct the function of F508del-CFTR in CF cells by improving its folding in the ER and stabilizing the protein during its secretion to the cell surface. Importantly in cell models, the effect of the compounds can be detected within 3 hours. However, one recent corrector that reached a clinical trial, VX-809, failed to rescue the functional defect of F508del-CFTR in nasal epithelium and did not improve lung function in patients. Recently, another family of compounds named CFTR potentiators, especially VX-770, have been shown to improve CFTR function in cell models [6]. By increasing

the open time of CFTR, VX-770 reduces Na^+ and fluid absorption and therefore prevents the dehydration of the apical surface.

In other studies the application of the pan inhibitor cysteamine has been used in cell and animal models and more recently in early phase clinical trials. When used together with epigallocatechin gallate (EGCG, a green tea flavonoid that inhibits the enzyme Casein Kinase 2), improved and sustained mutant CFTR function was reported in patient nasal epithelial cells and in sweat ducts [7]. However, cysteamine, in addition to its action as a pan but relatively poor inhibitor of transglutaminases, is also an inhibitor of cysteine proteases and has been reported to have a diverse range of other potential targets. Its ability to inhibit cysteine proteases may also be important in preventing CFTR degradation in CF and in the prevention of apoptosis but these actions remain to be proven. The other target of cysteamine Tissue Transglutaminase (TG2) is reported to play a pivotal role in proteostasis in CF epithelia demonstrating an intracellular involvement of this multifunctional enzyme in CF [7]. This work proposed that in CF the dysfunctional CFTR induces a reactive oxygen species response which in turn triggers activation of intracellular TG2 leading to protein crosslinking, culminating in blocked autophagy and protein aggregate accumulation. The work also indicated that in the CF inflammatory response, intracellular crosslinking by TG2 inhibited the anti-inflammatory peroxisome proliferator-activated receptor γ (PPARγ), leading to sustained activation of the inflammatory process. Importantly use of the pan transglutaminase inhibitor cysteamine can reverse these observations [7].

In addition to this intracellular role TG2 is also reported to have a number of extracellular functions which generally manifest themselves in a variety of human diseases. Increases in TG2 expression are normally associated with injury or stress resulting in its secretion onto the cell surface and into the extracellular matrix (ECM) through a non-conventional mechanism involving cell surface heparan sulphates [8]. In the ECM Ca^{2+} levels are high resulting in displacement of intarcellular regulator GTP, leading to the activation of TG2 where it can contribute to other important CF pathologies. For example, as referred to earlier, during childhood, CF lungs and other tissues like the pancreas, intestine and liver develop fibrosis resulting in the remodelling and dysfunction of these organs as the disease progresses for which there is presently no therapy. Extracellular TG2 is known to

be a key player in the development of tissue fibrosis via its ability to crosslink matrix proteins leading to their increased deposition and stability and via its ability to activate matrix bound TGFβ1 [9]. Recent work also suggests that TG2 mediated crosslinking of TGFβ into the matrix may prolong the activity of TGFβ [10]. Moreover, expression of TG2 can be upregulated by TGFβ1 thus, propagating the fibrotic effects of TG2 and increased levels of TGFβ1 are found in the CF lung [11].

TG2 is also reported to be involved in epithelial-mesenchymal transition (EMT) [12]. A process thought to be important in a number of fibrotic conditions [13]. Over-expressed in highly invasive cancers, TG2 mediates EMT via its ability to enhance the activation of NF-κB, TGFβ1 and TNFα [12].

In this paper we have explored the role of TG2 in CF pathology in particular its link with TGFβ1 activation and signalling, induction of EMT, CFTR stability and the induction of fibrosis. We show that the presence of TG2 in both normal and CF bronchial epithelial cells promotes EMT progression leading to increased motility and increased fibronectin (FN) expression and deposition, via a TGFβ1-stimulated pathway involving TG2 mediated matrix bound TGFβ1. We will also show that selective inhibition of TG2 reduces TGFβ1 levels and leads to reversal of this process which is accompanied by an increase in the stability and improvement in CFTR processing in CF airway bronchial epithelial cells.

2. Material and methods

2.1 Cell Culture

IB3-1 cells (a mutant tracheal epithelial cell line with a heterozygote mutation, F508del/W1282X, leading to the loss of CFTR channel function and immortalized by SV-40 transfection) and C38 cells ("add back" IB3-1 cell line which has a truncated CFTR channel with 119 residues missing but functional with elevated basal Cl- ion efflux) were obtained from Johns Hopkins University (Baltimore, USA)**.** HBEC (primary Human Bronchial Epithelial cell, isolated from surface epithelium of human bronchi) was a kind gift from Dr Lindsay Marshall (Aston University, UK). Cells were cultivated in Airway Epithelial cell Medium (AEM) containing bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, epinephrine, triiodo-L thyronine, transferrin and retinoic acid (Promocell, UK).

2.2 ALI culture

Transwell® inserts with a surface area of 0.33cm^2 and 0.4μm pore size were inserted into Transwell® companion plates. 100μg/ml solution of Collagen IV in 3% (v/v) acetic acid was added to each insert membrane and allowed to attach for 45min at room temperature. Media was then used to rinse inserts to neutralize the acidity of the solution. 3×10^4 cells / 300μl of AEM media was added to each insert and 600μl of complete media added to the well underneath each insert. The cells were cultured for 4 days. On the 3rd day, TG2 inhibitors or the TGFβ receptor inhibitor was added and cells incubated for a further 12 h, after which media was removed and the cells then exposed to air. Media in companion Transwell® plates was replaced and the inhibitor introduced. Cells were then cultured for 14 days during which basal medium with or without inhibitor was changed on alternate days. When cells cultured in ALI condition were transfected with TG2 shRNA or TG2 virus all transfections /transductions were undertaken prior to ALI culture.

2.3 Treatment of cell with Inhibitors.

All TG2 inhibitors used in this study were synthesized in the Aston laboratories. R283 is a specific TG inhibitor not selective for TG2 but shown to penetrate cells [14]. R292, R294 and R281 are peptidic water soluble inhibitors that are cell impermeable [15] and able to differentiate between TG2 and Factor XIIIa. 1-155, 1-33, 2-18 and 1-159 are peptidomimetic inhibitors that are highly selective for TG2 [16, 17]. 1-155 is cell permeable and 1-159 is cell impermeable [17]. R283 is used at 500μM, 1-155 is used at 1μM or 5μM, 1-33 is used at 50μM, 1-159 at 5 or 10μM, R294 is used at 500μM, 2-18 is used at 50μM, R281 and R292 is used at 250μM. Cells were treated for 48h with an inhibitor unless otherwise stated. At the time this work was undertaken, the inactivating antibody against TG2 that we have used previously [14] was not readily available due to its pharmaceutical development.

For inhibition of TGFβ signalling the TGFβ receptor inhibitor SB 431542 (Sigma-Aldrich, UK) was used at concentration of 10μM, which by the XTT assay was shown to be non-toxic to the cells.

2.4 Inhibition of TG2 with TG2 shRNA

5×10^5 cells were seeded into 6-well plates in 2ml of medium. The cells were incubated overnight. 10nM of TG2 shRNA (CACAAGGGCGAACCACCTGAA) was mixed with

12µl of Lipofectamine 2000 Transfection reagent and were used according to manufacturer instructions. The plates were gently swirled around, incubated for 24h at 37^{o}C, 5% CO_2 and used thereafter. The virus containing empty vectors were used as the control transduction treatment.

2.5 Overexpression of TG2

The procedure used is that reported in Wang *et al* [14]. 5×10^5 cells were sub-cultured on 6-well plates in complete media and cultured overnight. An aliquot of Lentiviral particles carrying TG2 or the TG2 mutant of interest was added to the wells and cells were incubated at 37^{o}C for 48h with two more re-infections carried out every 24h. The virus containing empty vectors were used as the control transduction treatment.

2.6 Cell migration assay

The experiment was performed using the ESSEN IncuCyte system. Graduated 96-well plates from ESSEN were pre-coated with FN and used to seed IB3 and C38 cells in the presence or absence of TG2 inhibitor R283. Once reaching 90-95% confluency, a wound was introduced on each well using Wound maker 96 instrument (ESSEN instruments). Cells were allowed to migrate at 37°C for 24 h. Cell migration was monitored and analysed by the IncuCyte software. The mean ± S.D. from 3 separate experiments was calculated and plotted using the ESSEN IncuCyte software.

2.7 Western Blotting

The cells with or without the treatments were lysed in cell lysis buffer (containing 5mM DTT, 150mM NaCl, 1mM EGTA, 1mM PMSF, 1%(V/V) protease inhibitor cocktail, 1mM Sodium orthovanadate in 50mM Tris-Cl, pH 7.4 [18]). Following pre-clearing the samples at 300×g for 5min. The proteins were separated via SDS-PAGE and the presence of the target proteins were detected via Western blotting using specific antibodies as described previously [18]. GAPDH was used as the equal loading control.

2.8 Transglutaminase activity assay

The transglutaminase activity in the cell lysates or on the cell surface was performed as described previously [8, 18]. Briefly, 96-well plates were coated with 50µl of a 5µg/ml FN overnight, 50µg protein in the whole cell lysates (WCL) was incubated on the FN coated plates in the presence of 10mM calcium, 1mM DTT, 0.1mM biotin-cadeverine (BCD) in a final volume of 100µl/well of 50mM Tris-Cl, pH 7.4. The plate was then

incubated at 37^{o}C for 2h. Following 3 washes with 50mM Tris-HCl, pH 7.4. the wells were blocked with heat inactivated 3% (w/v) Bovine Serum Albumin, BSA, in 50mM Tris-Cl, pH 7.4 (blocking buffer) for 30 min at 37^{o}C and then incubated with 100µl of peroxidise-conjugate of Extr-avidin (1:1,000 dilution) for 1h at 37^{o}C. After another 3 set of washes, transglutaminase activity was measured using SigmaFast® OPD and the reaction was stopped by 3N HCl. Absorbance was detected at 490nm using a plate reader [8].

For cell surface transglutaminase activity, 2×10^4 cells/well in serum-free medium were seeded onto FN pre-coated 96-well plates in the presence of 0.1 mM biotin-cadaverin and incubated at 37^{o}C for 2h. After removal of media the reaction was stopped by washing wells with 2mM EDTA in PBS, pH 7.4. 100µl of 0.1% (w/v) sodium deoxycholate in 2mM EDTA in PBS, pH 7.4, was then added to each well and incubated on a slow shaker for 10 min at room temperature. The transglutaminase activity was measured using Extr-avidin substrate as described above.

2.9 Immunofluorescence staining in submerged cell culture

Cells in submerged or ALI cultures with the treatments required were washed three washes with PBS, pH 7.4, the cells were fixed with 3.7 % (v/v) paraformaldehyde in PBS, pH 7.4 and permeabilized with 0.1% Triton in PBS, pH 7.4. Following blocking with 3% BSA in PBS, pH 7.4 (the blocking buffer) at 37°C for 30 min, the cells were incubated with primary antibody and fluorescence-conjugated secondary antibody at 37^{o}C for 2h, respectively. The cells were mounted in mounting medium containing 4', 6 -diamidino-2-phenylindole (DAPI). The fluorescence signals were detected using a Leica® SP5 confocal microscope.

2.10 Cell fractionation

IB3 cells were treated with TG2 inhibitors for 48h with media changed every 24h. Subcellular fraction were realized by using the "Subcellular Fractionation Kit for Cultured Cells" (Thermo Scientific, UK). Briefly, at each step, cells were lysed with a specific lysis buffer and a centrifugation undertaken according to the manufacturer's instructions to isolate each subcellular fraction. Western blotting was performed to detect the target proteins.

For the ECM fractions of the cells, IB3 cells were seeded into 60mm Petri dishes in the presence or absence of TG2 inhibitor R283 or 1-155, while addition of 1µg/ml rhTG2 was used as the control treatment. The cells were cultured for 48 h and lifted from the ECM using 2mM EDTA in PBS, pH 7.4. The remaining ECM was collected into Laemmli buffer and Western blotting was performed to detect the presence of TGFβ1 in the ECM.

2.11 Trans-Epithelial Electrical Resistance (TEER)

The TEER values were measured using Epithelial voltohmmeter (World Precision Instrument) according to the manufacturer's instructions. The final values were expressed as $\Omega \times cm^2$. The transepithelial electrical resistance values obtained in the absence of cells and the presence of collagen coating membrane were considered as background measurements, which were subtracted from the TEER values from the experimental groups. To measure TEER using electrodes media was added to the surface of cells in inserts carefully. The electrodes attached to the voltohmmeter were then inserted into each insert with the longer arm extending into the well and the shorter arm into the insert above the cells. Caution was taken not to disturb cells. The measurement was done passing AC current through the cells consequently, taking the resistance as the current passes through in ohms. This was done in triplicate with electrodes dipped intermittently in 70% (v/v) Industrial Methylated Spirit (IMS) before each use.

2.12 Total TGFβ Assay via ELISA

ELISA was performed to detect the presence of TGFβ1 as described previously [20]. Briefly, 2.5×10^5 cells were cultured on 6 well plates and allowed to settle down for 2 hours, followed by incubation with serum free medium containing supplements in the presence or absence of TG2 inhibitors. The presence of TGFβ1 was measured following the manufacture's instruction (eBiosciences). The 300µl of media was collected from cell cultures and centrufuged at 300×g for 5 minutes. The supernatant was then decanted and assayed for TGFβ1.

2.13 Reverse Transcription- Polymerase Chain Reaction (PCR)

RNA was extracted from the cells using TRIzol® followed by alcohol precipitation. Reverse-transcription was performed using the Qiagen Sensiscript® Reverse transcriptase Kit, RNA fragments were transribed into cDNA. Briefly, 50ng of RNA template for each

cell line was added to the master mix containing 10x buffer mix, 5mM of dNTP mix containing 5mM of each nucleotide, 10uM Oligo-dT primer, Sensicript Reverse Transcriptase(R) and RNAse free water to make up volume. The mix was vortexed carefully and incubated at 37°C for 60minutes. This mix containing the cDNA was then used for PCR.

2.14 Real Time- RT-PCR

Real time-PCR was performed according to the default conditions on the Stratagene® MX-3000P real-time PCR machine of which the annealing temperature was updated to accommodate the primers used for the real time-PCR. Real time-PCR was performed according to manufacturer's protocol of the Qiagen Quantitect SYBR Green® PCR kit. Primers for TGFβ1 were used in these reactions as listed below. GAPDH was used as the house keeping gene and relative levels of expression of the target genes were determined to GAPDH expression.

Table 1. Primers used in the real-time PCR study.

Primers	Forward	Reverse
TGFβ1	5'-tgcggcagtggttgagccgt-3'	5'-acctcggcggccggtagtga-3'
GAPDH	5'-tgcaccaccaacttgcttagc-3'	5'-ggcatggactgtggtcatgag-3'

2.15 Statistical analysis

Unless stated otherwise, all values are presented as the mean ± S.D. Data analyses were performed using the Student's t test. A *p* value of less than 0.05 was considered to indicate statistical significance which is indicated in the text.

3. Results

3.1 TG2 expression and activity is more elevated in IB3 cells than in C38 cells

We first ascertained the level of TG2 expressed in the CF cell, IB3 and its corrected "add-back" cell, C38, using western blot analysis (**Figure 1A**). The results show high levels of TG2 expressed in IB3 cells compared to C38 cells in whole cell lysates (**Figure 1A**) and on the cell surface (**Figure 1A**) agreeing with the work by Maiuri and colleagues [21], where high levels of TG2 were found in IB3 cells *in vitro*. The expression levels of TG2 were correlated with activity in whole cell lysates (**Figure 1B**) and with that measured at the cell surface (**Figure 1C**). IB3 cells showed high TG2

activity compared to C38 cells both in cell lysates and at the cell surface which could be significantly inhibited using the TG2 inhibitor, R283 (**Figure 1B**). Use of R283 and other TG2 inhibitors, R292 and R294 (**Figure 1C**) also led to inhibition of cell surface TG2activity. The cell lysate and cell surface activity of TG2 on C38 was shown to be significantly lower than that found in CF IB3 cells (**Figure 1B** and **C**).

3.2 EMT markers are more elevated in IB3 cells than in C38 cells

Epithelial-Mesenchymal transition (EMT) is characterized by the loss of the epithelial cell phenotype such as tight and adhesion junction proteins and the acquisition of a mesenchymal phenotype. TGFβ1 has been shown to play a key role in EMT in the lungs, kidney and pancreas [22] and this function involves nuclear restructuring via transcriptional repressors e.g. Snail, Slug and Twist. In order to show if this phenomenon was occurring in the high TG2 expressing IB3 cells, FN, N-cadherin and Slug expression was measured in both IB3 and C38 cells (**Figure 1D**). Results show increased expression of FN, N-cadherin and the transcription repressor Slug in IB3 cells compared to the C38 cells (**Figure 1D**). As a result of this mesenchymal transition, IB3 cells show an increased migratory phenotype, compared to the C38 cells (**Figure 1E** and **F**). Inhibition of TG2 activity in IB3 cells showed a significant inhibition of cell migration, while there was no significant effect found in the C38 cells treated with R283 (**Figure 1E** and **F**).

3.3 Modulation of TG2 activity regulates EMT progression

To determine the effect of TG2 knock down on EMT progression the differences in the expression of EMT markers FN, N Cadherin and Slug in IB3 cells was measured after their transfection with a Lentiviral vector containing TG2 shRNA. Cells were cultured for 48h and whole cell lysates analysed for EMT markers by western blotting (**Figure 2A**). Reduced expression of TG2 in IB3 cells correlated with a reduction in the expression of the EMT markers.

3.4 Induction of EMT in primary human bronchial epithelial cells-role of TG2

We next examined the effect of increasing TG2 expression in human primary bronchial epithelial cells (HBECs). Expression of TG2 was increased in these cells by transduction with Lentiviral particles carrying TG2 (**Figure 2B**). An increase in TG2 expression in these cells resulted in an increase in expression of EMT markers FN, N-Cadherin, Slug, and a decrease of the epithelial marker E-Cadherin (**Figure 2B**). When HBEC cells were

treated with TGFβ1, as found with the virally transduced cells showing increased TG2 expression, there was an increase in TG2 expression accompanied by an increase in the EMT markers FN, N-Cadherin and a decrease of the epithelial marker E-Cadherin (**Figure 2C**). These EMT markers could be reduced when cells were treated with TGFβ1in the presence of the TG2 inhibitor R283.

3.5 TGFβ1 activity is blocked by TG2 inhibition

Our results so far link TG2 to TGFβ1-induced EMT. Our hypothesis was that TGFβ1, through its activation by TG2, is the driver of EMT progression in CF airway bronchial epithelial cells. Therefore in this section we looked at the expression and levels of TGFβ1 in CF IB3 cells and C38 cells.

Both CF IB3 and control C38 cells express TGFβ receptors (TGFβR) I and II (**Figure 3A**) but a significantly higher TGFβ1 gene expression was found in IB3 cells compared to C38 cells (**Figure 3B**). Measurement of total TGFβ1 levels in IB3 and C38 cells indicated TGFβ1 to be significantly higher in IB3 cells compared to C38 cells which could be reduced by TG2 inhibitors R283, R292 and R294 (**Figure 3C**). The cell permeable TG2 inhibitors R283 and the less cell permeable TG2 inhibitors R292 and R294 all inhibited TGFβ1 levels present in the cell culture medium at comparable levels, suggesting that the cell surface TG2 activity is the important factor in the activation of TGFβ1.

We next looked to see if an up regulation of TG2 following treatment with TGFβ1 correlated with an increase in α-SMA in C38 cells. Our results shown in **Figure 3D** indicate that in the presence of TGFβ1, C38 cells re-oriented their structural morphology to assume a fibrous spindle shape with an increase in expression of TG2 and the myofibroblast marker, α-SMA (**Figure 3E and 3F**). This increase in TG2 expression is accompanied by an increase in FN (**Figure 3F**), and N-cadherin (**Figure 3G**) when C38 cells are stimulated with TGFβ1. When TG2 activity is reduced by the highly TG2 specific inhibitors 1-33 and 1-155 and the TG2 inhibitor R283 (**Figures 3F** and **G**), the TG2 profile and EMT profile start to reverse back to that found in C38 cells prior to TGFβ1 stimulation. Loss of the EMT markers following TG2 inhibition is accompanied by a reduction in Smad signalling both in C38 cells after treatment with TGFβ1 and in IB3 cells without TGFβ1 treatment (**Figure 3H**). This mimics the effects of TG2

expression in IB3 cells and in primary bronchial epithelial cells whereby TGFβ1 increases the expression of TG2 and TG2 in turn increases TGFβ1 levels leading to the progression of EMT (**Figure 2D**). We next looked at the effect of the TGFβ receptor inhibitor SB 431542 on EMT in IB3 cells both in isolation and in combination with the cell impermeable inhibitor R292 in order to further confirm that TGFβ1 is driving EMT and that TG2 is working via a TGFβ mechanism. **Figure 3I** shows that both the TGFβ receptor inhibitor SB 431542 [23] and R292 lead to comparable changes in the expression of TG2` and in the EMT markers FN, N-cadherin and Slug which were not substantially different overall to the changes found when the two inhibitors were combined. Moreover, **Figure 3J** demonstrates how when C38 cells are cultured in the presence of rhTGFβ1 protein, they assume a fibroblast-phenotype with a consequent loss in epithelial like cell morphology, paralleling the increase in FN deposition and expression of other EMT markers (**Figure 3F** and **G**), which can in part be reversed in the presence of the TG2 inhibitor R283.

The finding that TG2 inhibition can reduce the effect of exogenously added TGFβ1 also suggests its activity may be important in TGFβ1 signalling in addition to its role in TGFβ1 activation. Recent reports have indicated that TGFβ1 can be crosslinked into a collagen matrix by transglutaminase leading to its enhanced and prolonged signalling [10]. We therefore looked for the presence of TGFβ1 in the matrix of CF IB3 cells. **Figure 3K** shows that the matrix of IB3 cells does contain TGFβ1 in a monomeric form. Inhibition of TG2 activity using its specific inhibitors R283 and 1-155 was able to reduce the TGFβ1 monomer to around 50% of control. As the control treatment, the addition of active TG2 (1µg/ml) induced the formation of TGFβ1 polymer in the ECM (**Figure 3K**), further suggesting that TGFβ1 can act as a substrate for TG2 and that TG2 crosslinking activity is involved in the ECM deposition of TGFβ1.

3.6 The role of TG2 in ALI conditions

To confirm the results observed in submerged culture in a more physiological culture setting, IB3 and C38 cells were grown under an Air Liquid Interface (ALI) format. In IB3 cells, the protein expression of TG2 and EMT markers FN and N-Cadherin is more elevated than in C38 cells (**Figure 4A** and **B**) which agrees with the data for these cells observed in submerged culture (**Figures 1** and **2**). When IB3 cells are transfected with a

TG2 shRNA, the protein expression of TG2 and EMT markers decrease (**Figure 4C**), this is in agreement with results previously observed in submerged culture (**Figures 2A**). Moreover, when cells are incubated with the TGFβ receptor inhibitor SB 431542 or R292, either in isolation or combined, EMT markers and TG2 expression decrease in a similar manner to that found in submerged culture further, confirming that TG2 is acting via a TGFβ mechanism (**Figure 4D)**. In addition, when TG2 is knocked down using this TG2 targeted shRNA and cultured under ALI conditions the value of TEER significantly increases (**Figure 4E**).

When primary HBEC cells are transduced with TG2 containing Lentiviral particles and grown under ALI conditions the EMT marker levels increase in these cells (**Figure 4F**), which is in accordance with the results observed in submerged culture (**Figure 2C**). The TEER (**Figure 4G**) also significantly decreases when cells were transduced with TG2. This suggests, in keeping with the observed increase in EMT markers, that there is a loss in epithelial cell like morphology resulting in a loss in tight junctions with a corresponding increase in FN deposition.

3.7 Inhibition of TG2 and EMT lead to an increase in the maturation of mutant CFTR in IB3 cells

IB3 cells were treated during a 48h culture without or with a bank of different TG2 inhibitors. This time frame was chosen since we found it reduces the expression of TG2 with a parallel reduction in EMT markers. The level of the CFTR C band in the membrane fraction of the treated cells was then determined by Western Blotting (**Figure 5**). When cells are treated with Cysteamine, an increase of CFTR C-Band in the membrane fraction can be observed (**Figure 5A** and **B**). This result is in accordance with the paper by Maiuri *et al* [1]. Cysteamine is not a specific TG inhibitor, but 2-18 and 1-155 are highly specific for TG2 [24]. When these two TG2 specific inhibitors were used, an increase of CFTR C-band in the membrane fraction of IB3 cells can be observed (**Figure 5A**, **B** and **C**). When a combination of Cysteamine and 1-155 is used, a higher increase can be observed (**Figure 5B**). Importantly, inhibition of TG2 activity by its specific inhibitor 1-155 restored CFTR at the plasma membrane by around 80%, and was more efficient that Cysteamine in this function in keeping with the potency of the two inhibitors for TG2 (**Figure 5D**).

4. Discussion

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) [25]. The most common mutation is F508del, resulting in multiple organ damage amongst which lung destruction ranks first as the major cause of morbidity. The importance of TG2 in CF has been well reported, and cysteamine, a pan transglutaminase inhibitor which may have other inhibitory effects has been tested in a Phase 2 clinical trial for CF with positive outcomes [7]. A series of recent reports [1, 25] have demonstrated the intracellular involvement of TG2 in CF epithelia. It was reported that activation of TG2 in CF bronchial epithelial cells by stress related increases in intracellular Ca^{2+} lead to protein crosslinking, culminating in blocked autophagy and protein aggregate accumulation [25].

In this report, we have looked at another well-established role of TG2 related to its extracellular protein crosslinking using the well characterised CF IB3 cells and its control cells C38 (with transfected add back truncated functional CFTR). IB3 cells, like other reported CF epithelial cells tested [25], express higher levels of TG2 and higher levels of transamidase activity compared to their control cells such as C38 cells or primary human bronchial epithelial cells. This increase in TG2 expression was reported to be result of the sumoylation of TG2 resulting in its increased longevity within the cell [26]. Importantly and in keeping with the extracellular crosslinking role of TG2 an increased presence of the enzyme is found on the cell surface of IB3 cells. These cells were therefore used as the major cell model in our subsequent work together with primary bronchial epithelial cells which were used to verify our findings in these epithelial cell lines.

Increases in extracellular TG2 are now well established to play an important role in fibrosis where matrix crosslinking and activation of matrix bound TGFβ1 by TG2 contribute to fibrosis progression. This is particularly important since uncontrolled airway remodelling occurs in CF patients which is now recognized to start early in life and is linked to a poor pulmonary outcome with extensive fibrosis [27]. A similar process in CF patients also known to start in babyhood is the destruction of the pancreas which is driven by fibrosis and noted when the disease was first recognized.

This is clinically important because nearly all pancreatic insufficient CF patients develop diabetes mellitus that is disproportionately lethal to females with CF [28].

An important growth factor unequivocally linked to both TG2 and fibrosis is TGFβ which is a family of 3 members, TGFβ1-3. Working via interaction with its receptors, TGFβ1 is one of the key growth factors involved in fibrosis, including CF, with increased levels reported in CF lungs [11]. In addition to its pro-inflammatory properties TGFβ1 has been shown recently to inhibit the biosynthesis of CFTR and also prevent the bio-functional rescue of CFTR [29]. Importantly over-expression of TGFβ1 in the CF lung has been associated with a more severe CF phenotype and TGFβ1 is reported to be increased in the plasma of children with CF [30]. One major role of TGFβ1 in addition to its many other fibrotic roles is its ability to induce the mesenchymal phenotype in cells, such as epithelial cells. This process termed EMT is reported to be involved in number of different diseases: in cancer, EMT leads to the invasive phenotype in cancer cells; and during fibrosis EMT and other reported mesenchymal cell changes results in the deposition of a fibrotic matrix in organs such as lungs and kidneys [31].

Abnormal regulation of TG2 in a number of different cancer cells has been correlated with an increase in the expression of EMT markers [32-34]. The highlight of this process is the acquisition of a mesenchymal phenotype with an elevation in expression of EMT markers including FN, N-cadherin and the transcriptional marker, Slug, which facilitates invasive growth.

Given this reported link between TG2, TGFβ1 and EMT the first part of our work was aimed at studying the potential involvement of TG2/TGFβ1-related EMT in CF cells and in primary human bronchial epithelial cells.

We demonstrate that when TG2 activity is inhibited in IB3 cells either with TG2 inhibitors or specific knock down of TG2 expression this leads to a significant reduction in FN, N-cadherin, Slug and TGFβ1 expression and in TGFβ1 levels leading to a reversal of EMT.

Similarly, when TG2 is overexpressed in primary HBEC cells, an increase in expression of EMT markers can be observed at both gene and protein level. This strongly suggests that an up regulation of TG2 expression and activity can drive EMT in CF IB3 cells and in primary bronchial epithelial cells where it is not normally expressed, revealing for the

can also be first time the direct link between TG2 and EMT expression in CF bronchial epithelia. Importantly, the finding that expression of both EMT markers and TG2 in IB3 cells both in submerged an ALI culture can be reduced by the TGFβ receptor inhibitor SB 431542 [23] and the cell impermeable TG2 inhibitor R292 in a comparable manner strongly suggest that TG2 is working via a TGFβ1 mechanism. This link between TG2 and TGFβ1 is in keeping with the work of Park *et al* [35] which showed that in A549 lung cancer cells, TG2 induced EMT via TGFβ1 with an elevation of N-cadherin expression in these cells [35].

Importantly, to further prove our hypothesis, we show that inhibition of TG2 using both cell permeable and non-cell permeable TG2 inhibitors lowers the level of the TGFβ1 protein which is keeping with the extracellular activation of matrix linked TGFβ1 by TG2 [36-38]. Interestingly we also show that TG2 inhibition leads to a reduction in TGFβ1 induced Smad signalling in C38 cells after addition of exogenous TGFβ1, leading to a reversal in EMT and a reduction in the expression of TG2. This is in agreement with results from Ritter and Davies [39] where it was shown that TG2 gene expression is regulated by TGFβ1 via a TGFβ1 Response Element (TRE) on the promoter site of the TG2 gene. However these data also suggest that TG2 may have another regulatory role in TGFβ1 function related to its signalling. In keeping with the results of Niger *et al* [10] who showed that transglutaminase mediated crosslinking of TGFβ1 into collagen led to its prolonged signalling [10], we found the presence of TGFβ1 in the matrix of CF IB3 cells. Moreover in parallel with the loss of TGFβ1 induced Smad signalling in IB3 cells after TG2 inhibition there was a corresponding loss in matrix associated TGFβ1. Further evidence that TG2 is responsible for this crosslinking was shown by the finding that addition of exogenous TG2 to the IB3 cells led to polymerisation of the matrix TGFβ1 present. Although we cannot rule out other mechanisms for how TG2 is acting in TGFβ1 signalling, our data so far suggests that its crosslinking into the cell matrix may be one of them [35, 36, 38, 39].

Our data also indicate that this relationship between TG2 and TGFβ1 has the potential to lead to increases in matrix protein expression and deposition ultimately leading to the fibrosis found in the lungs and other tissues of people with CF which starts at a very early age. Although other studies have attempted to relate EMT in alveoli cells to an aberration

of TGFβ1 [40], this study has provided the interrelationship between TG2, TGFβ1 and EMT in a bronchial epithelial model of CF and for the first time show that a fibrosis-related response can be mediated via a TG2-TGFβ1-EMT driven pathway.

Importantly we can demonstrate this relationship both in CF cells grown in submerged culture and also in more physiologically related condition using ALI where we demonstrate that the presence of EMT in IB3 cells and the induction of EMT in primary bronchial epithelial cells leads to a reduction in TEER levels, which in the lung would implicate a reduction of tight junctions in the epithelial barrier. Interestingly EMT has also been shown to occur in chronic obstructive pulmonary disease [41], where patients have many comparable clinical symptoms to that of CF sufferers.

The use of CFTR potentiators and correctors has been deemed as the recent breakthrough in CF therapy [6, 42], but these have had limited success when tested in patients carrying the F508del CFTR mutation. Moreover, given reports that increased TGFβ1 levels found in CF patients can lead to lowered expression of CFTR, inhibit its translocation to the cell membrane, and in addition, block the functional correction of CFTR, we next turned our attention to looking at the effects of specifically targeted TG2 inhibitors on CFTR processing. Luciani *et al* [25] observed in CFBE41o- cells transfected with F508del CFTR that the non-selective TG inhibitor cysteamine reduced CFTR recycling and sustained the channel at the apical membrane compared to CFTR correctors, VX-325 or Corr-4a.

In this study we used a bank of TG2 inhibitors, including the cell permeable and highly potent TG2 selective peptidomimetic inhibitor 1-155, a further potent cell impermeable peptidomimetic TG2 specific inhibitor 2-18 and the cell impermeable peptidic inhibitor R281 plus cysteamine which was used as the internal positive control [16]. Our recent work demonstrated the high specificity and potency of these peptidomimetic inhibitors, which showed no toxicity when used in experimental animals [16]. All TG2 inhibitors were incubated with IB3 cells for 48h prior to testing since we demonstrated this time period lowered both TGFβ1 levels and TG2 expression (**Figure 3C** and **3H**). Following TG2 inhibition we observed that CFTR translocation to the cell membrane, an indicator of the improvement of CFTR maturity, significantly increased, in particular when cells were pre-incubated with the highly potent and TG2 selective inhibitor 1-155.

Interestingly the amount of CFTR found at the cell membrane could be enhanced even further when combined with the pan TG inhibitor cysteamine.

Our findings are therefore in keeping with those of Luciani *et al* [25] with respect to the improved translocation of the ΔF508 CFTR found when CF bronchial epithelial cells are incubated with pan TG inhibitors such as cysteamine or R283. This group demonstrated that these pan transglutaminase inhibitors stabilize and increase NBD-1:ICL interactions allowing the read through of ΔF508 CFTR copies whilst precluding their proteosomal degradation, hence, increasing the number of stable and mature CFTR copies that are trafficked to the plasma membrane. Hence we cannot rule out that our TG2 selective inhibitors are also in addition to their effects on EMT and TGFβ levels are working in part by their effects on preventing proteosomal degradation of the mutated CFTR. Importantly in this report unlike the work reported by Luciani *et al* [23], we use for the first time highly potent and TG2 selective inhibitors and confirm the importance of TG2 activity in the regulation of CFTR stability and processing. Hence in future work it would be interesting to test if these TG2 selective inhibitors can improve CFTR stability and processing in homozygous ΔF508 CFTR mouse models, particularly in the nasal epithelium where CFTR is well expressed in these mice. Unlike human CF suffers as far as we are aware there have been no reports [43] to indicate that these F508del CFTR mice have elevated levels of TGFβ in their lungs or that extensive remodeling and fibrosis occurs in the lung early in life as it does in humans. Hence any major effects of selective TG2 inhibition on these mice with respect to CFTR stability and processing if performed on young mice might be attributed to their effects on precluding proteosomal degradation of the F508del CFTR.

In summary we show that TG2 has an additional pathological role in CF related to its importance in TGFβ1 activation and signalling which sustains TG2 levels, interferes with CFTR expression and processing and in addition together with increased TG2 levels, promotes increased matrix deposition leading to fibrosis in CF patients.

5. Conclusion

This paper demonstrates that TG2 has other pathological roles in CF in addition to those reported in the literature. It demonstrates that TG2 is one of the major drivers in CF pathology, in particular in fibrosis progression via its ability to regulate TGFβ1 activation

and signalling leading to the induction of epithelial-mesenchymal transition (EMT) in CF epithelia. We demonstrate that induction of EMT in CF cells affects the epithelial barrier by impairing tight junctions as demonstrated by a reduction in TEER values, induces matrix deposition, increases cell mobility and affects CFTR stability and maturity. Inhibition of TG2 activity using either TG2-specific inhibitors or knock down of TG2 expression is able to counteract the effect of TGFβ1 and in turn reverse EMT, reduce levels of active TGFβ1 produced by CF epithelial cells and as a consequence help prevent fibrosis progression. Importantly TG2 specific inhibitors are able to help restore the function and maturity of CFTR in CF cells. This work reveals for the first time the potential of using TG2-specific non-toxic inhibitors as therapeutic agents in the treatment of CF.

6. Abbreviations

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; TGFβ1, Transforming growth factor β1; ECM, extracellular matrix; EGCG, epigallocatechin gallate; TG2, tissue transglutaminase; EMT, Epithelial-Mesenchymal transition; ALI, air liquid interface; HBEC, primary Human Bronchial Epithelial cells; LTBP1, Latent TGFβ1 binding protein 1; EMT, epithelial-mesenchymal transition; TEER; Trans Epithelial Electric Resistance; TRE, TGFβ1 Response Element; αSMA, αSmooth Muscle Actin; HBE, human bronchial epithelial; PPARγ; peroxisome proliferator-activated receptor γ; NF-κB, nuclear factor- κB; TNFα, tumour necrosis factor α; FN, fibronectin; rhTGFβ1, recombinant human TGFβ1.

7. Author contribution

M.G. was the major grant holder that funded the work. M.G. and Z.W. designed the experiments. M.G., Z.W., T.T. and S.N. wrote the manuscript. Z.W. S.N., T.T., O.A. R.B. performed the biological experiments. L.M. provided the primary HBEC cells and advised on the TEER experiments.

None of the authors of this manuscript have a financial interest related to this work.

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9. Figure legends

Figure 1. TG2 expression and activity in C38 and in IB3 cells. (A) Western blotting was performed to detect the TG2 expression in a whole lysate of IB3 and C38 cells, as well as cell surface (CS) TG2 following biotinlyation of cell surface proteins. A representative images from 3 separate experiments with the densitometry ratios show below after normalisation of protein loading with GAPDH. **(B)** TG2 activity in both IB3 and C38 cells was measured via biotin-cadaverine incorporation assay, while TG2 inhibitor R283 was used. *, $p<0.05$. **(C)** Cell surface TG2 activity in the of IB3 and C38 cells was performed as introduced in the Experimental Procedures. R283, R292 and R294 are TG2 inhibitors. n=3. *, $p<0.05$. **(D) EMT marker screening in IB3 and C38 cells.** Repesentative images showing FN, N-Cadherin and Slug proteins in IB3 and C38 cells detected by Western Blot. The densitometry ratio of the different bands is show below taken from 3 separate experiments. **(E)** and **(F)** Cell migration assay. Representative migration images of IB3 and C38 cells is shown over 12h (**E**) with or without R283 treatment and analyzed and plotted as the % relative wound density against time using an ESSEN IncuCyte system **(F)** as described in the Experimental Procedures.

Figure 2. (A) Inhibition of TG2 via shRNA or inhibitor R283 treatment blocks EMT in IB3 cells. EMT Proteins (E-Cadherin, N-Cadherin, FN, Slug) in IB3 cells when TG2 is inhibited by TG2 shRNA detected via Western blotting. **(B)** Induction of EMT in HBEC

cells via over-expressing TG2. TG2 was over-expressed using Lentiviral particles in HBEC cells, while the virus containing the empty vector was used as the control treatment as introduced in the Experimental Procedures. Mesenchymal and epithelial markers were measured via Western blotting. **(C)** Inhibition of TG2 activity via R283 treatment blocks the effect of TGFβ1-induced EMT. HBEC cells were treated with 3ng/ml rhTGFβ1 for 48h in the presence or absence of R283. GAPDH was used as the equal loading standard.

Figure 3. Effect of TG2 on TGFβ1-induced EMT. (A) The presence of TGFβ receptor I (RI) and II (RII) in IB3 and C38 cells were detected via Western blotting. **(B)** TGFβ1 expression in IB3 and C38 cells determined by RT-PCR as described in Experimental Procedures. Expression level of TGFβ1 were normalized to GAPDH expression. *, $p<0.05$. **(C)** TGFβ1 levels in IB3 and C38 cells. ELISA was performed following manufacture's instructions to detect the presence of TGFβ1. *, $p<0.05$. **(D)** Morphology changes of C38 cells induced by TGFβ1. Representative images of C38 cells treated with or without 3ng/ml TGFβ1 for 48h and the images were taken using a Nikon digital camera. **(E)** Immunofluorescence staining was performed to detect the presence of αSMA in C38 cells treated with or without TGFβ1 as introduced in Experimental Procedures. **(F-H)** C38 cells were treated with 3ng/ml TGFβ1 for 48h in the presence of various TG2 inhibitors, including R283, 1-33, 1-155 and R294. Western blotting was carried out to detect the presence of TG2, mesenchymal markers FN and N-cadherin and active phosphorylated (p) Smad2/3. GAPDH or total (t) Smad2/3 were used to ensure the equal loading and normalization of p-Smad2/3, respectively. **(I)** Effect of non-cell permeable TG2 inhibitor R292 and TGFβ receptor inhibitor SB 431542 on EMT in IB3 cells. IB3 cells were treated with R292 (250μM) and TGFβ receptor inhibitor SB 431542 (10μM) either alone or in combination for 48h. The presence of TG2, FN, N-cadherin and Slug in the cell lysates was detected via Western blotting. GAPDH was used to ensure equal loading. **(J)** Morphology of C38 cells treated with TGFβ1 in the presence or absence of R283. Representative images from C38 cells treated with 3ng/ml TGFβ1 with or without R283 as in introduced in **(F)** at 10x and 20x magnification taken using a Nikon digital camera. **(K)** Presence of TGFβ in the ECM. IB3 cells were treated with or without R283 (500μM) or 1-155 (1μM), or with the addition of 1μg/ml rhTG2 for 48h. The remaining

ECM was collected after lifting the cells with 2mM EDTA in PBS, pH 7.4, and used to detect the presence of TGFβ1 or FN via Western blotting.

Figure 4. ALI condition and TEER measurement. (A) TG2 and EMT Proteins expression in IB3 and C38 cells cultured at ALI. **(B)** Visualization by immunostaining of TG2 and EMT marker FN in IB3 and C38 cells cultured at ALI. **(C)** TG2 and EMT proteins expression in IB3 cells cultured at ALI when cells are transfected with TG2 shRNA. VC, vector control. **(D)** Presence of EMT markers in ALI in IB3 cells. IB3 cells treated with R292 (250 μM) or TGFβ receptor inhibitor SB 431542 (10μM) alone or in combination in the ALI culture was used to detect expression of the EMT markers FN, N-cadherin and Slug, as well as TG2, via Western blotting. GAPDH was used to ensure the equal loading. **(E)** The TEER values in IB3 cells cultured at ALI when cells are transfected with TG2 shRNA. *, $p<0.05$. VC, vector control. **(F)** TG2 and EMT Proteins expression in HBEC cells cultured at ALI when cells are transfected with TG2. **(G)** TEER Measurement in HBEC cultured at ALI without or with TG2 transfection. *, $p<0.05$.

Figure 5. Restoration of CFTR functions by TG2 inhibition in IB3 cells. The presence of CFTR C-band in IB3 cells were labelled via biotinlyation and detected via Western blotting. **(A)** CFTR C-Band when IB3 cells are treated with Cysteamin (250μM) or 1-155 (1μM). (**B)** CFTR C-Band when IB3 cells are treated with Cysteamine, (250μM) with 1-155 (1μM) or Cysteamine and 1-155. **(C)** CFTR C-Band (Membrane Fraction) and TG2 (Total Extract) when IB3 cells are treated with Cysteamine (250μM) or TG2 specific inhibitors 2-18 (50μM), R281 (250μM) and 1-155 (at concentrations shown). **(A)**, **(B)** and **(C)** show representative images from 3 separate experiments with the densitometry ratios show below after normalisation of protein loading with GAPDH. **(D)** The mean values ± S.D. taken from the 3 separate experiments using cysteamine at 250μM or 1-155 at 1μM as the inhibitor treatment with the DMSO control normalised to 100%. *, $p<0.05$.

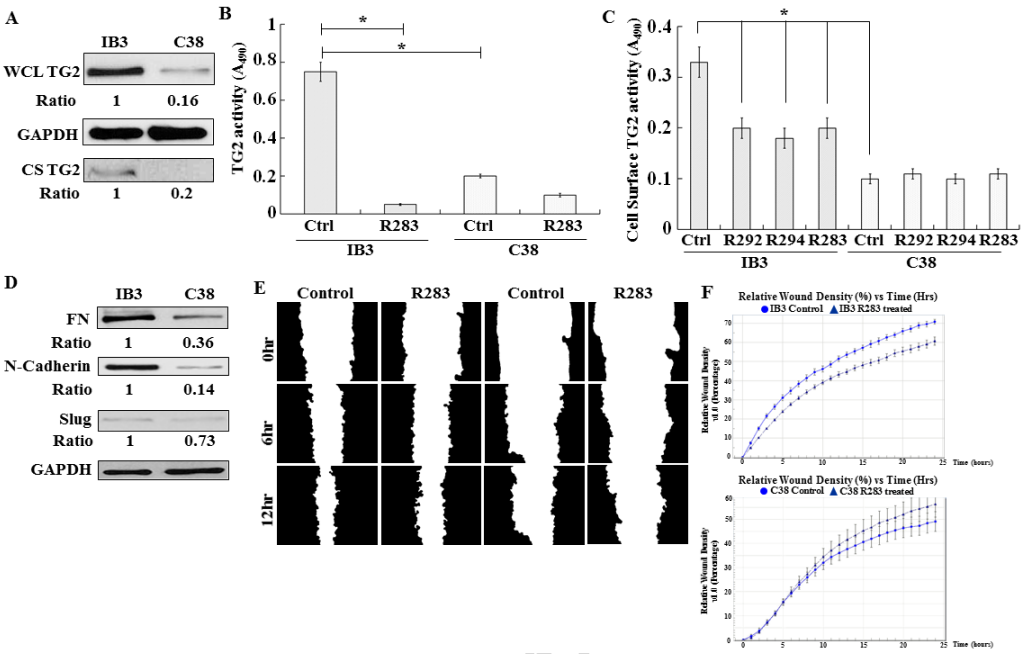


Figure 1

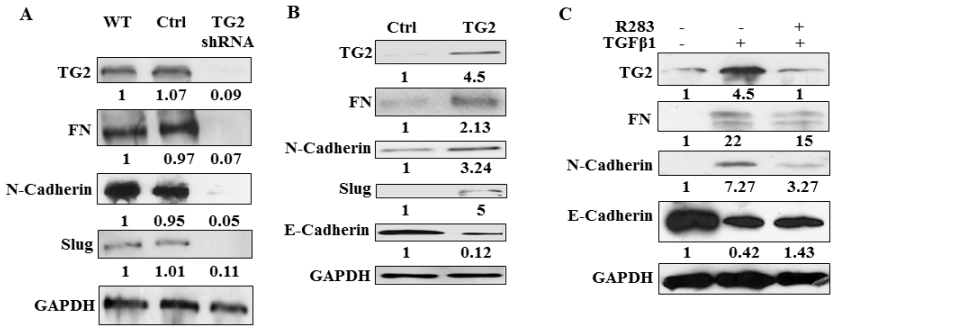


Figure 2

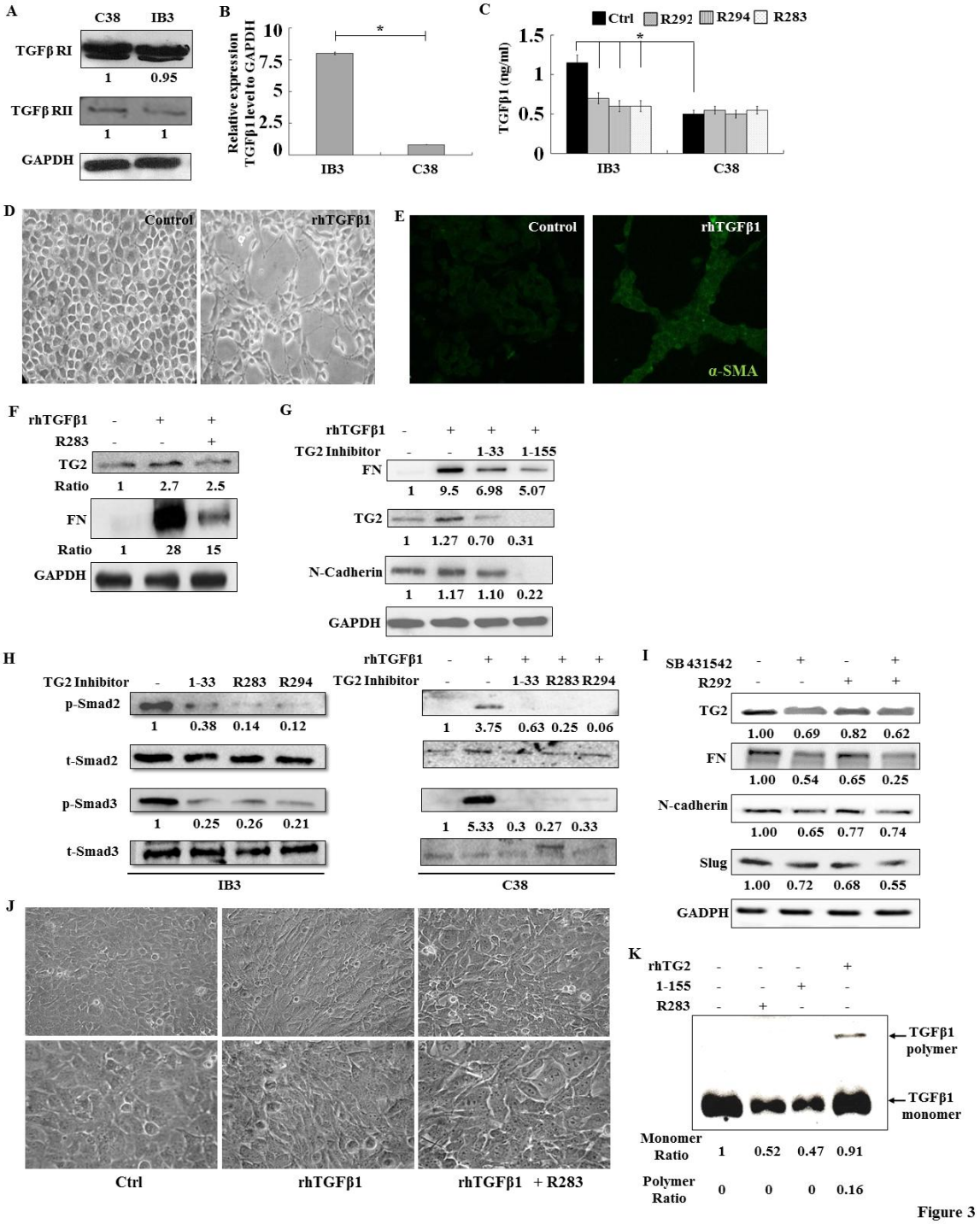


Figure 3

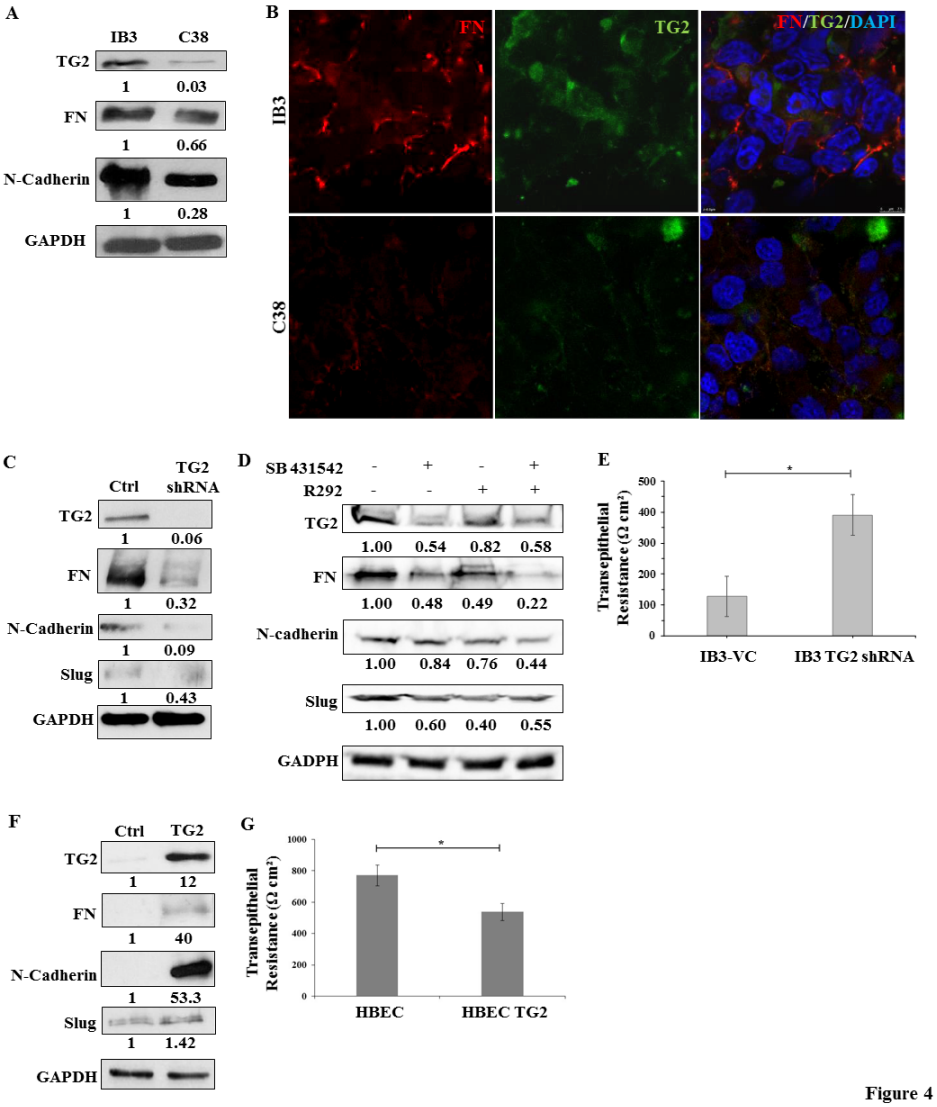


Figure 4

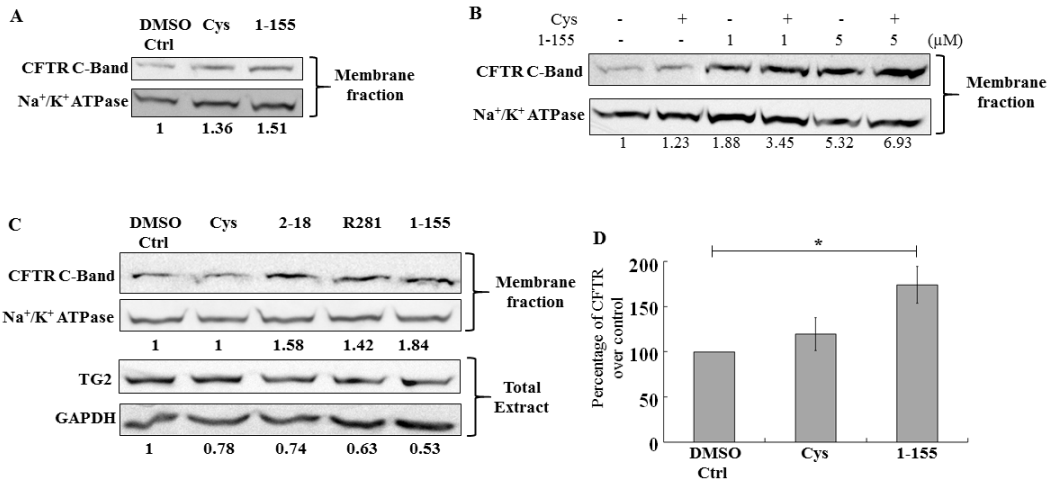
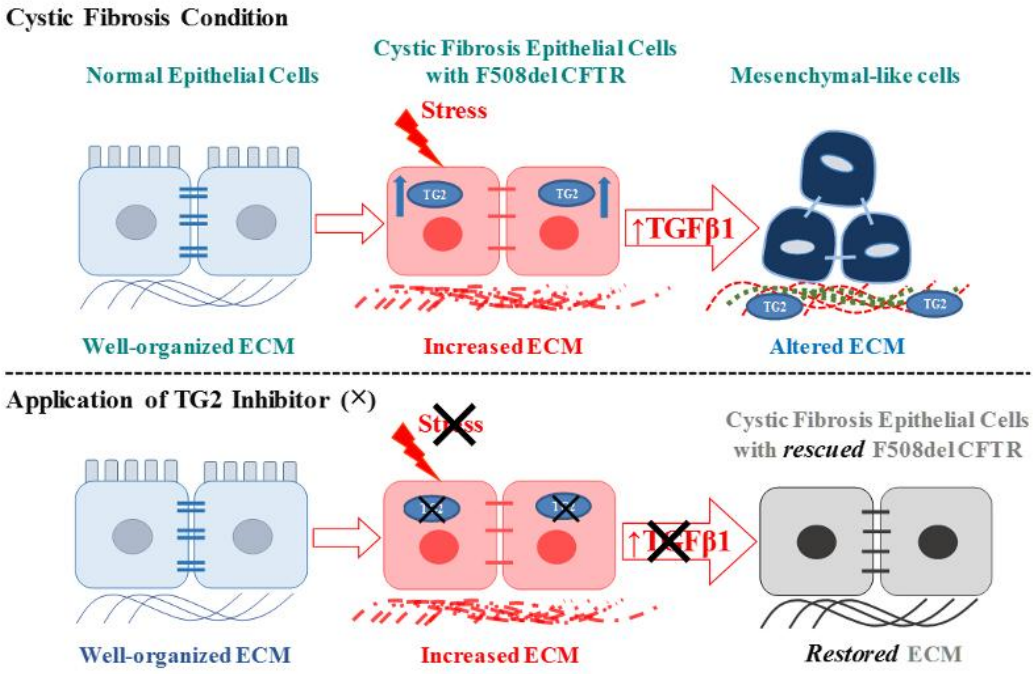


Figure 5



Graphical Abstract

Highlights

- Increased TG2 expression in CF epithelia increases TGFβ1 levels.
- Increased TGFβ1 and TG2 leads to Epithelial Mesenchymal Transition (EMT).
- EMT leads to poor CFTR stability and increased matrix deposition

- Inhibition of TG2 helps restore CFTR stability and leads to reversal of EMT.