Human lung myofibroblast TGFβ1-dependent Smad2/3 signalling is Ca\(^{2+}\)-dependent and regulated by K\(_\text{Ca}3.1\) K\(^+\) channels

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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a common and invariably lethal interstitial lung disease with poorly effective therapy. Blockade of the K\(^+\) channel K\(_\text{Ca}3.1\) reduces constitutive α-SMA and Smad2/3 nuclear translocation in IPF-derived human lung myofibroblasts (HLMFs), and inhibits several transforming growth factor beta 1 (TGFβ1)-dependent cell processes. We hypothesized that K\(_\text{Ca}3.1\)-dependent cell processes also regulate the TGFβ1-dependent Smad2/3 signalling pathway in HLMFs. HLMFs obtained from non-fibrotic controls (NFC) and IPF lungs were grown in vitro and examined for αSMA expression by immunofluorescence, RT-PCR, and flow cytometry. Two specific and distinct K\(_\text{Ca}3.1\) blockers (TRAM-34 200 nM and ICA-17043 [Senicapoc] 100 nM) were used to determine their effects on TGFβ1-dependent signalling. Expression of phosphorylated and total Smad2/3 following TGFβ1 stimulation was determined by Western blot and Smad2/3 nuclear translocation by immunofluorescence.

Results: K\(_\text{Ca}3.1\) block attenuated TGFβ1-dependent Smad2/3 phosphorylation and nuclear translocation, and this was mimicked by lowering the extracellular Ca\(^{2+}\) concentration. K\(_\text{Ca}3.1\) block also inhibited Smad2/3-dependent gene transcription (αSMA, collagen type I), inhibited K\(_\text{Ca}3.1\) mRNA expression, and attenuated TGFβ1-dependent αSMA protein expression.

Conclusions: K\(_\text{Ca}3.1\) activity regulates TGFβ1-dependent effects in NFC- and IPF-derived primary HLMFs through the regulation of the TGFβ1/Smad signalling pathway, with promotion of downstream gene transcription and protein expression. K\(_\text{Ca}3.1\) blockers may offer a novel approach to treating IPF.

Keywords: Human lung myofibroblast, Idiopathic pulmonary fibrosis, Potassium channel KCa3.1
which induces the phosphorylation of the downstream targets Smad2 and Smad3. Phosphorylated Smad2 and Smad3 then form hetero-oligomeric complexes with Smad4 and translocate to the nucleus to regulate gene expression through transcription and thus mediate the biological effects of TGFβ1 such as cell growth, differentiation and contraction [20-22]. In particular, this TGFβ1/Smad pathway contributes to myofibroblast differentiation by increasing α-smooth muscle actin expression [20,21].

Ion channels are attractive therapeutic targets in many chronic diseases. In particular, the Ca²⁺ activated K⁺ channel Kᵥ3.1 plays an important role in Ca²⁺ signalling through its ability to maintain a negative membrane potential during cellular activation, which enhances Ca²⁺ influx from the extracellular fluid [23-25]. The Kᵥ3.3.1 channel modulates the activity of several structural and inflammatory cells [26-28], but more specifically, Kᵥ3.1 blockade inhibits several TGFβ1-dependent cell processes in primary human lung myofibroblasts (HLMFs) derived from both non-fibrotic and IPF lung tissue [29]. Our previous work demonstrated that blocking Kᵥ3.1 with the selective Kᵥ3.1 blockers TRAM-34 and ICA-17043 inhibits TGFβ1-dependent HLMF wound healing, collagen secretion, contraction and Ca²⁺ influx [29]. In addition, IPF-derived HLMFs demonstrate increased constitutive αSMA expression and Smad2/3 nuclear localisation, effects which are reversed by Kᵥ3.3.1 inhibition [30].

We hypothesized that Kᵥ3.3.1 channel activity also regulates TGFβ1-dependent responses in HLMFs through the TGFβ1/Smad signalling pathway. We therefore investigated the role of the Kᵥ3.3.1 channel in TGFβ1-dependent Smad2/3 phosphorylation, Smad2/3 nuclear translocation, gene transcription and αSMA protein expression in HLMFs obtained from both healthy and IPF lung.

**Results**

**TGFβ1-dependent Smad2/3 phosphorylation is attenuated by Kᵥ3.3.1 blockade**

The ability of Kᵥ3.3.1 blockers to attenuate several TGFβ1-dependent cell processes in HLMFs [29] suggests that Kᵥ3.3.1 potentially regulates the activity of TGFβ1-dependent transcription factors. Smad2 and Smad3 are implicated as the major factors regulating HLMF differentiation and collagen secretion in response to TGFβ1 [20,21,31]. Phosphorylation is a key initial event in the activation of these Smad proteins. Using Western blot analysis, we therefore investigated the effect of TGFβ1 (10 ng/ml) on the phosphorylation of Smad2/3 and the expression of total Smad2/3 in HLMFs. Phosphorylation of Smad2/3 after TGFβ1 stimulation was examined over a time course of 5 h and peaked at 60 min (Figure 1A). No significant differences in Smad2/3 phosphorylation were seen between non-fibrotic controls (NFC) and IPF data, and data were therefore pooled for statistical analysis here and elsewhere where no disease-related differences were evident. Further experiments were therefore performed after 60-min stimulation with TGFβ1 in the presence or absence of the highly selective Kᵥ3.3.1 blockers TRAM-34 (Kd 20 nM) [24] and ICA-17043 (Kd 10 nM) [32]. TGFβ1 increased Smad2/3 phosphorylation in both NFC and IPF-derived HLMFs (P = 0.0022, paired t test). This was suppressed by both TRAM-34 and ICA-17043 (P = 0.0117 and P = 0.0144, respectively, one-way ANOVA corrected by Sidak’s multiple comparisons test) (Figure 1B, C, D).

**TGFβ1-dependent Smad2/3 nuclear translocation is disrupted by Kᵥ3.3.1 inhibition**

Once TGFβ1 has induced phosphorylation of Smad2/3, this complex interacts with Smad4, which acts as a transporter and enters the nucleus to initiate gene transcription. Inhibition of Smad2/3 phosphorylation should therefore also prevent Smad2/3 nuclear translocation. We previously demonstrated using immunofluorescence that total Smad2/3 nuclear staining was significantly greater amounts of total Smad2/3 located within the nucleus of HLMFs (P < 0.0001); furthermore, after inhibition with ICA-17043 (100 nM) significantly attenuated the nuclear translocation of Smad2/3 (P = 0.0034 and P = 0.0029, respectively, two-way ANOVA corrected by Sidak’s multiple comparison test). In contrast, the structurally related molecule, TRAM-85, which does not have channel blocking activity, did not inhibit nuclear translocation of Smad2/3 (Figure 2A, B, C, D). No significant differences were found between TGFβ1-stimulated NFC and IPF HLMF responses, and therefore, statistics were performed on pooled data. TRAM-34 (200 nM) or ICA-17043 (100 nM) significantly attenuated the nuclear translocation of Smad2/3 (P = 0.0034 and P = 0.0029, respectively, two-way ANOVA corrected by Sidak’s multiple comparison test). Thus, Kᵥ3.3.1 activity is critical for the TGFβ1-dependent phosphorylation and nuclear translocation of Smad2/3.

**TGFβ1-dependent Smad2/3 phosphorylation and nuclear translocation are Ca²⁺-dependent**

We have shown previously that Kᵥ3.3.1 channel blockade inhibits a rise in intracellular Ca²⁺ in HLMFs following
TGFβ1 stimulation [29]. This is likely due to plasma membrane depolarization which reduces Ca\(^{2+}\) entry [25,29]. If this is mechanistically important, lowering extracellular Ca\(^{2+}\) should also inhibit TGFβ1-dependent Smad2/3 phosphorylation and nuclear translocation. Myofibroblasts incubated for 1 h in Ca\(^{2+}\)-free media phosphorylated significantly less Smad2/3 in the presence of TGFβ1 (10 ng/ml) than those incubated in media containing normal external Ca\(^{2+}\), P = 0.0439 (one-way ANOVA, corrected by Sidak’s multiple comparison test) (Figure 3A, B). This was accompanied by reduced TGFβ1-dependent Smad2/3 nuclear translocation in Ca\(^{2+}\)-free media, P = 0.0114 (Figure 4A, B). This suggests that the enhancement of Ca\(^{2+}\)-influx by K\(_{Ca}\)3.1 channels is an essential requirement for the efficient TGFβ1-dependent phosphorylation and nuclear translocation of Smad2/3.

TGFβ1-dependent increases in αSMA, collagen type I and K\(_{Ca}\)3.1 mRNA are inhibited following K\(_{Ca}\)3.1 channel block

Impaired TGFβ1-dependent Smad2/3 nuclear translocation with K\(_{Ca}\)3.1 blockade predicts that TGFβ1-dependent gene transcription will also be inhibited. To assess this, we first undertook quantitative RT-PCR to examine the mRNA expression of αSMA and collagen type I in HLMFs. TGFβ1 (10 ng/ml) stimulation for 24 h
Figure 2 (See legend on next page.)
or without.

P<0.001 results are represented as median (IQR) (one-way ANOVA, (B) = 4, data pooled for statistical analysis). HLMFs

α

α

α

α

α

α

α

α

P=0.0052 and

P=0.0278, respectively

P=0.0156, Wilcoxon

<0.01 (two-way ANOVA corrected by Sidaks multiple comparison test),

<0.001, one sample

 examines the increase in nuclear translocation following TGFβ1 stimulation compared to control. TRAM-34 (200 nM) inhibited

TGFβ1-induced Smad2/3 nuclear translocation (NFC n = 4, IPF n = 4) whereas the structurally related molecule TRAM-85 without KCa3.1 blocking

properties did not inhibit nuclear translocation. NFC and IPF data were pooled for statistical analysis. (B) Representative fluorescent microscopy

images illustrate the increased expression of Smad2/3 following TGFβ1 stimulation and its movement into the nucleus, which was significantly

attenuated by TRAM-34 (200 nM). (C) ICA-17043 (100 nM) also significantly attenuated Smad2/3 nuclear translocation, which can be seen visually

in (D). (E) Quantification of Western blot analysis confirms that the total Smad2/3 in the nuclear enriched fraction is significantly increased following

TGFβ1 stimulation and attenuated by ICA-17043 (100 nM). Results are normalized to TATA Binding Protein (TBP) and representative Western blot

analysis images are shown. (F) Similarly, the total Smad2/3 was examined in the cytoplasmic-enriched fraction; however, no changes were found

following TGFβ1 stimulation or treatment with ICA-17043 (NFC n = 2 and IPF n = 3, data pooled). Results are represented as mean ± SEM ***P<0.001,

**P<0.01 (two-way ANOVA corrected by Sidaks multiple comparison test), **P<0.0001, one sample t test, ^P<0.05, paired t test.

Figure 2 Smad2/3 nuclear translocation is inhibited by KCa3.1 channel blockade. (A) The ratio of nuclear to whole cell staining of Smad2/3

demonstrates a significant increase in nuclear translocation following TGFβ1 stimulation compared to control. TRAM-34 (200 nM) inhibited

TGFβ1-induced Smad2/3 nuclear translocation (NFC n = 4, IPF n = 4) whereas the structurally related molecule TRAM-85 without KCa3.1 blocking

properties did not inhibit nuclear translocation. NFC and IPF data were pooled for statistical analysis. (B) Representative fluorescent microscopy

images illustrate the increased expression of Smad2/3 following TGFβ1 stimulation and its movement into the nucleus, which was significantly

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following TGFβ1 stimulation or treatment with ICA-17043 (NFC n = 2 and IPF n = 3, data pooled). Results are represented as mean ± SEM ***P<0.001,

**P<0.01 (two-way ANOVA corrected by Sidaks multiple comparison test), **P<0.0001, one sample t test, ^P<0.05, paired t test.

Figure 3 TGFβ1-dependent Smad2/3 phosphorylation is Ca2+ dependent. (A) A representative Western blot demonstrating Smad

2/3 phosphorylation when cells are incubated in media either containing Ca2+ or without. (B) Quantification of Western blots

showing that the increased phosphorylation of Smad2/3 after 60 min of TGFβ1 exposure (10 ng/ml) is largely dependent on the

presence of extracellular Ca2+. Phosphorylation of Smad2/3 was examined by fold change over total Smad2/3 and normalized to

β-actin (NFC n = 2, IPF n = 4, data pooled for statistical analysis). HLMFs stimulated for 1 h in Ca2+-free media phosphorylated significantly less

Smad2/3 than those incubated in media containing Ca2+. ^P<0.005, ***P<0.001 results are represented as median (IQR) (one-way ANOVA,

corrected by Sidaks multiple comparison test).

KCa3.1 block attenuates TGFβ1-induced increases in αSMA protein expression

We and others have shown previously that IPF-derived HLMFs express higher levels of αSMA than NFC HLMFs constitutively, and we showed that this is inhibited by KCa3.1 blockade. Because TGFβ1-dependent transcription of the αSMA gene is also inhibited by KCa3.1 blockers, we proceeded to investigate the effect of KCa3.1 blockers on TGFβ1-dependent αSMA protein expression in HLMFs.

Flow cytometry was used to examine αSMA expression in HLMFs following 24 h of TGFβ1-stimulation in the presence of KCa3.1 channel blockers. TGFβ1 significantly increased αSMA protein expression in both NFC- and IPF-derived myofibroblasts (P = 0.0156, Wilcoxon signed rank test). TRAM-34 (20 and 200 nM) and ICA-17043 (10 and 100 nM) both dose-dependently decreased TGFβ1-dependent αSMA expression when examined by the fold change in geometric mean fluorescent intensity, P = 0.0052 and P = 0.0278, respectively (one-way ANOVA) (Figure 6A, B, C). This indicates a
phenotypic transition of HLMFs into a more fibroblast-like phenotype, which would explain the reduced contraction, secretion and proliferative abilities demonstrated in previously published work [29].

Discussion

This study demonstrates that Ca$^{2+}$ and the Ca$^{2+}$-activated K$^+$ channel KCa3.1 play a key role in the TGFβ1-dependent activation of Smad2/3 in HLMFs. Inhibition of KCa3.1 channels therefore attenuates TGFβ1-dependent gene transcription, resulting in the inhibition of pro-fibrotic HLMF activity [30] and de-differentiation of HLMFs towards a fibroblast phenotype as indicated by a reduction in αSMA protein.

This paper provides mechanistic insight into the inhibitory effects of KCa3.1 blockers on HLMF pro-fibrotic function. Open K$^+$ channels hyperpolarize the plasma membrane and increase Ca$^{2+}$ entry following receptor-dependent cell activation [29,33]. We have demonstrated previously that blocking KCa3.1 channels reduces constitutive αSMA expression and Smad2/3 nuclear localization in NFC- and IPF-derived HLMFs, although it was not possible to detect Smad2/3 phosphorylation under these conditions [30]. We have also shown previously that KCa3.1 activity is required for a rise in intracellular Ca$^{2+}$ that occurs following exposure of HLMFs to TGFβ1 [29]. Importantly, here, we have shown that extracellular Ca$^{2+}$ and KCa3.1 activity are required for the TGFβ1-dependent phosphorylation and nuclear translocation of Smad2/3, an essential initial step in Smad2/3 activation. Thus, there is a clear mechanistic link between KCa3.1 activity and Smad2/3-dependent cell signalling. Although other signalling cascades including ras/MEK/ERK and P38 MAPK are also involved in TGFβ1 pro-fibrotic signalling, our data nevertheless suggest that the predominant regulation of the TGFβ1 signalling pathway by KCa3.1 channels occurs proximally at or above the level of Smad2/3 phosphorylation, via effects on Ca$^{2+}$ signalling.

This study both supports and extends the work of others investigating the role of KCa3.1 in renal fibrosis [34], liver fibrosis [35] and brain pathology [36]. Murine renal fibroblast proliferation and kidney fibrosis following ureteric obstruction were significantly attenuated by KCa3.1 blockers [37], while in a streptazocin mouse model of diabetes-induced renal fibrosis, KCa3.1 inhibition reduced TGFβ1, TGFBRII and phosphoSmad2/3 expression in the tissue [34,38]. Subsequent work from the same authors using human renal interstitial fibroblasts showed that TGFβ1-dependent increases in types I and IV collagen and αSMA mRNA expression were attenuated by KCa3.1 blockade, although there was only a
Figure 5 (See legend on next page.)
reduction in type IV collagen protein expression reported [34]. In contrast, we found clear evidence of reduced collagen type I mRNA expression in keeping with previous observations that KCa3.1 blockade attenuates TGFβ1-dependent HLMF collagen secretion [29] and that fibroblasts lacking the Smad3 gene have reduced induction of collagen type I gene [39]. We also found that TGFβ1-dependent αSMA gene transcription was inhibited by KCa3.1 blockers, and TGFβ1-dependent HLMF αSMA protein expression were reduced. In keeping with the work of Huang et al., we also found that TGFβ1-dependent phosphorylation of Smad2/3 was attenuated by KCa3.1 blockade [38], but in addition show that this prevents Smad2/3 nuclear translocation.

It is important that we have demonstrated the effects of KCa3.1 inhibition in parenchymal HLMFs because of the functional heterogeneity evident in fibroblasts from different tissues and between species [40-42]. Moreover, in previous studies of renal fibroblasts, the concentration of TRAM-34 (2 μM) used was relatively high [38]. We used two distinct KCa3.1 blockers at the IC50 (20 nM for TRAM-34 and 10 nM for ICA-17043) and at 10× the IC50 where >95% of channels will be blocked and found clear evidence for activity of these drugs at these

![Figure 5](see figure on previous page.)

**Figure 5** TGFβ1-dependent transcription of αSMA, collagen type I and KCa3.1 is KCa3.1 dependent. (A) TGFβ1 stimulation significantly increased αSMA and collagen I mRNA expression in HLMFs per 10^3 copies of β-Actin, which was significantly inhibited by TRAM-34 200 nM (NFC n = 4, IPF n = 4, data pooled for statistical analysis). (B) Similarly, ICA-17043 (100 nM) also significantly decreased TGFβ1-dependent increases in αSMA and collagen I mRNA expression in HLMFs (NFC n = 3, IPF n = 3, data pooled). Results are represented as mean ± SEM or median (IQR) ***P < 0.001 (one sample t test), *P < 0.05 and **P < 0.01 (paired t test or Wilcoxon signed rank test). (C, D) The fold change of TGFβ1-dependent KCa3.1 mRNA expression was significantly higher in IPF HLMFs compared to that in NFC HLMFs, *P = 0.0313 (NFC n = 4, IPF n = 4, data pooled for statistical analysis). This TGFβ1 dependent increase in KCa3.1 mRNA in IPF donors was significantly attenuated by KCa3.1 channel blockers, TRAM-34 and ICA-17043 (*P = 0.0385 and P = 0.0313, respectively, paired t test).

![Figure 6](see figure on previous page.)

**Figure 6** TGFβ1-dependent αSMA protein expression is attenuated by KCa3.1 channel block. (A) Flow cytometry measurements of αSMA expression in HLMFs show that the fold change in gMFI was significantly increased by TGFβ1 (10 ng/ml) (NFC n = 3, IPF n = 4, data pooled for statistical analysis). TRAM-34 (20 and 200nM) dose-dependently decreased TGFβ1-induced αSMA expression. (B) Similarly, ICA-17043 (10 and 100nM) significantly reduced TGFβ1-induced αSMA expression. (C) Representative fluorescent histogram showing αSMA expression in HLMFs under the above conditions. Results are represented as mean ± SEM. *P < 0.05 (paired t test); **P < 0.05, ***P < 0.01 (corrected by Dunn’s multiple comparisons test).
physiologically relevant concentrations. Importantly, the concentration of ICA-17043 used here can be achieved in vivo with oral dosing [43], although the free drug concentration will be reduced due to protein binding. Thus, pharmacological activity is likely to be achieved within human lung following oral administration.

We found previously that K\textsubscript{Ca}-3.1 inhibition reduced TGF\textbeta{}1-dependent HLMF contractility in collagen gels [29]. Our demonstration that this is associated with reduced \textalpha{}SMA gene transcription and protein expression suggests that de-differentiation towards a fibroblast phenotype with reduced contractile machinery may explain this reduced contractility, at least in part. However, attenuation of Ca\textsuperscript{2+} influx as demonstrated previously [29], with depolarization of the plasma membrane could also contribute to the effects of K\textsubscript{Ca}-3.1 blockade on HLMF contraction.

Our findings also provide a potential mechanism for the ability of K\textsubscript{Ca}-3.1 inhibition to prevent airway wall remodelling in a mouse model of asthma [44]. That study demonstrated that treatment with TRAM-34 reduced airway smooth muscle mass, peribronchial fibrosis, and sub-epithelial collagen expression. While these effects might have occurred in part through the inhibition of the associated inflammatory response, it seems likely that a direct effect on airway smooth muscle and myofibroblast activity was also relevant.

Conclusions
Myofibroblast activity is clearly a key driver of tissue fibrosis and shrinkage in IPF [45-47]. There is strong evidence that TGF\textbeta{}1-dependent signalling contributes to this fibrotic process [12,14,20,30,38,48]. However, there is also evidence that HLMFs derived from IPF lungs also exhibit greater constitutive pro-fibrotic activity, which might occur due to pre-programming by genetics, and/or re-programming by epigenetics [30,49]. Importantly, K\textsubscript{Ca}-3.1 blockade is able to inhibit both TGF\textbeta{}1-dependent and constitutive pro-fibrotic HLMF activity as shown here and in our previous study [30], respectively. This provides further support for the view that K\textsubscript{Ca}-3.1 may provide a novel and effective target for the treatment of IPF. Furthermore, the capacity for K\textsubscript{Ca}-3.1 inhibition to downregulate TGF\textbeta{}1-dependent increases in K\textsubscript{Ca}-3.1 expression suggests that inhibiting K\textsubscript{Ca}-3.1 will rapidly downregulate the TGF\textbeta{}1 axis through positive feedback (Figure 7). K\textsubscript{Ca}-3.1 knockout animals are relatively healthy, and an oral K\textsubscript{Ca}-3.1 inhibitor, ICA-17043 (Senicapoc), was well tolerated for 12 months in a phase III clinical trial of sickle cell disease [43]. There is therefore the potential for the rapid translation of K\textsubscript{Ca}-3.1-directed therapy to the clinic.

Methods

Ethics statement
All patients donating tissue gave written informed consent, and the study was approved by the National Research Ethics Service (references 07/MRE08/42 and 10/H0402/12).

Human lung myofibroblasts isolation and culture
NFC parenchymal HLMFs were derived from healthy areas of lung from patients undergoing lung resection for carcinoma at Glenfield Hospital, Leicester, UK. No morphological evidence of disease was found in the tissue samples used for HLMF isolation. IPF HLMFs were derived from patients undergoing lung biopsy for diagnostic purposes at the University of Pittsburgh Medical Center, USA, and were shown to have UIP on histological examination. HLMFs were grown from explanted lung tissue from both sources under identical conditions, using Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotic/antimycotic agents and non-essential amino acids [50,51]. The cells were cultured at 37°C in 5% CO\textsubscript{2}/95% air. The cells were studied at passages 4 to 5 for functional studies. Myofibroblasts were characterized as previously described [29]. All NFC patients gave informed written consent, and the study was approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee 2. Written informed consent was also obtained from all IPF subjects, in accordance with the responsible University of Pittsburgh Institutional Review Board.

Flow cytometry
HLMFs were grown on T25 flasks and serum starved for 24 h prior to the experiment. The HLMFs were either left unstimulated or stimulated for 24 h with TGF\textbeta{}1 (10 ng/ml), in the presence of 0.1% DMSO control, TRAM-34 (200 nM) or ICA-17043 (100 nM). The cells were detached using 0.1% trypsin/0.1% EDTA, washed then fixed and permeabilized in 4% paraformaldehyde plus 0.1% saponin (Sigma-Aldrich, St. Louis, MO, USA), antimicrobial/antimycotic agents and non-essential amino acids [50,51]. The cells were cultured at 37°C in 5% CO\textsubscript{2}/95% air. The cells were studied at passages 4 to 5 for functional studies. Myofibroblasts were characterized as previously described [29]. All NFC patients gave informed written consent, and the study was approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee 2. Written informed consent was also obtained from all IPF subjects, in accordance with the responsible University of Pittsburgh Institutional Review Board.

qRT-PCR
HLMF RNA was isolated using the RNeasy Plus Kit (Qiagen, West Sussex, UK) according to the manufacturer’s instructions. Primers were designed for ACT2A,
forward TTCAATGTCCAGCCATGTA and reverse GAAGGAATAGCCACGCTCAG, product size 222 bp from NCB1 Reference sequence NM_001141945.1 and COL1A1, forward TTCTGCAACATGGAGACTGG and reverse CGCCATACTCGAACTGGAATC, product size 151 bp from reference sequence NM_000088.3. K Ca3.1 and β-actin primers were analysed using gene-specific Quantitect Primer Assay primers (Qiagen, Hilden, Germany), Hs_KCNN4_1_SG and HS_ACTB_1_SG. All experiments were performed in duplicate. All expression data was normalized to β-actin and corrected using the reference dye ROX. Gene expression was quantified by real-time PCR using the Brilliant SYBR Green QRT-PCR 1-Step Master Mix (Stratagene, Breda, The Netherlands). PCR products were run on a 1.5% agarose gel to confirm the product amplified was the correct size, and they were sequenced to confirm the specificity of the primers. Prior to qRT-PCR, myofibroblasts were grown to confluence, serum starved for 24 h and then stimulated with TGFβ1 (10 ng/ml) in the presence of 0.1% DMSO control, TRAM-34 (200 nM), ICA-17043 (100 nM) or Ca2+-free media for 1 h. The cells were detached with 0.1% Trypsin/EDTA and washed. The protein was isolated using RIPA buffer lysis system (Santa Cruz, Heidelberg, Germany), and the total protein concentration was determined using the DC Bio-Rad protein Assay (Bio-Rad, Hemel Hempstead, UK). Of the protein, 30 μg was resolved using 10% Mini-Protean TGX precast gels (Bio-Rad, Hemel Hempstead, UK) and then transferred to an immunobilon-P polyvinylidene difluoride membrane, using Trans-blot Turbo transfer packs (Bio-Rad, Hemel Hempstead, UK) and then transferred to an immunobolon-P polyvinylidene difluoride membrane, using Trans-blot Turbo transfer packs (Bio-Rad, Hemel Hempstead, UK). The membranes were blocked with 5% milk and incubated with rabbit monoclonal anti-phospho-Smad2/Smad3 (0.231 μg/ml, Cell Signalling, Danvers, MA, USA) or rabbit monoclonal anti-smad2/smad3 (0.0087 μg/ml, Cell Signalling). Protein bands were identified by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagent (Amersham Laboratories, Buckinghamshire, UK). Immunolabelled proteins

Pro-fibrotic effects
- αSMA expression
- Excessive ECM deposition
- Contraction
- Proliferation

Figure 7 The involvement of K Ca3.1 in the TGF/Smad 2/3 signalling pathway. A diagrammatic representation of how Smad2/3 phosphorylation and subsequent nuclear translocation is reliant on an influx of extracellular Ca2+ and K Ca3.1 ion channels. TGFβ1 stimulation triggers an influx of extracellular Ca2+, which in turn opens Ca2+-activated K Ca3.1 K+ channels. K Ca3.1 opening maintains a negative membrane which in turn promotes Ca2+ entry. Phosphorylation of Smad2/3 and therefore its downstream effects such as translocation and gene transcription are heavily reliant on Ca2+. K Ca3.1 channel inhibition reduces Ca2+ entry, which in turn reduces Smad2/3 phosphorylation and nuclear translocation, and thus reduces TGFβ1-dependent gene transcription.

Western blot for SMAD proteins
Cells were grown in T75 flasks, serum starved for 24 h and stimulated with TGFβ1 (10 ng/ml) in the presence of either 0.1% DMSO control, TRAM-34 (200 nM), ICA-17043 (100 nM) or Ca2+-free media for 1 h. The cells were detached with 0.1% Trypsin/EDTA and washed. The protein was isolated using RIPA buffer lysis system (Santa Cruz, Heidelberg, Germany), and the total protein concentration was determined using the DC Bio-Rad protein Assay (Bio-Rad, Hemel Hempstead, UK). Of the protein, 30 μg was resolved using 10% Mini-Protean TGX precast gels (Bio-Rad, Hemel Hempstead, UK) and then transferred to an immunobolon-P polyvinylidene difluoride membrane, using Trans-blot Turbo transfer packs (Bio-Rad, Hemel Hempstead, UK). The membranes were blocked with 5% milk and incubated with rabbit monoclonal anti-phospho-Smad2/Smad3 (0.231 μg/ml, Cell Signalling, Danvers, MA, USA) or rabbit monoclonal anti-smad2/smad3 (0.0087 μg/ml, Cell Signalling). Protein bands were identified by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagent (Amersham Laboratories, Buckinghamshire, UK). Immunolabelled proteins
were visualized using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Buckinghamshire, UK).

SMAD nuclear translocation
HLMFs were grown on 8-well chamber slides and serum-starved for 24 h prior to the experiment. The cells were then stimulated with TGFβ1 (10 ng/ml) in the presence of either 0.1% DMSO control, TRAM-34 (200 nM), ICA-17043 (100 nM), TRAM-85 (200nM) or Ca²⁺-free media. After 1 h, the cells were fixed with methanol for 20 min on ice, blocked using 3% BSA for 1 h and immunostained using rabbit monoclonal anti-Smad2/Smad3 (0.174 μg/ml, Cell Signalling). Secondary antibody labelled with FITC (F0313, Dako) was applied, and the cells counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA). The cells were mounted with fluorescent mounting medium and cover-slipped.

Original images were captured on an epifluorescent microscope (Olympus BX50, Olympus UK Ltd., Southend-on-sea, Essex, UK); grey scale intensity was examined using Cell F imaging software (Olympus UK Ltd., Essex, UK). Matched exposures were used for isotype controls. The intensity of nuclear Smad2/3 staining was quantified by measuring the grey scale intensity of DAPI positive nuclei with a minimum of 10 random cells measured in one field for each condition.

Nuclear fraction
NFC and IPF-derived HLMFs were grown in T75 flasks, serum-starved for 24 h then stimulated with TGFβ1 (10 ng/ml) in the presence of either 0.1% DMSO control, TRAM-34 (200 nM) or ICA-17043 (100 nM). After 1 h, HLMFs were detached with 0.1% Trypsin/EDTA and washed. Nuclear and cytoplasmic extracts were isolated using the Nuclear Extract Kit (Abcam, ab113474, New territories, Hong Kong). Proteins of both the nuclear and cytoplasmic extracts were then isolated using the RIPA buffer lysis system and the concentration determined using the DC Bio-Rad protein Assay. Western blot was then performed as described above.

Statistical analysis
Experiments from an individual donor were performed either in duplicate or triplicate, and a mean value was derived for each condition. Data distribution across donors was tested for normality using the Kolmogorov-Smirnov test. For parametric data, the one-way ANOVA or repeated measures ANOVA for across-group comparisons was used followed by the appropriate multiple comparison post hoc test; otherwise, an unpaired or paired t test was used. Where appropriate, a one sample t test was used using a null hypothesis of 1. For non-parametric data, the Friedman test was used for across group comparisons followed by the appropriate multiple comparison post hoc test, or the Mann Whitney U test was used where there were two unpaired groups. GraphPad Prism for windows (version 6, GraphPad Software, San Diego, CA, USA) was used for these analyses. A value of $P < 0.05$ was taken to assume statistical significance and data are represented as mean (± SEM) or median (IQR).

Abbreviations
DMEM: Dulbecco’s modified eagle’s medium; FBS: Fetal bovine serum; HLMF: Human lung myofibroblast; IPF: Idiopathic pulmonary fibrosis; IQR: Interquartile range; NFC: Non-fibrotic control; SEM: Standard error of the mean; TGFβ1: Transforming growth factor beta 1; TGFα: Transforming growth factor beta receptor II; cSMA: α-smooth muscle actin.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
KMR performed all of the laboratory studies on human lung myofibroblasts under the supervision of PB, analysed the data and drafted the manuscript. CFB recruited and characterized patients with IPF for inclusion in the study and advised on myofibroblast cell culture; analysed and interpreted the data and revised the manuscript critically for intellectual content. HW provided TRAM-34 for the in vitro studies, analysed and interpreted the data and revised the manuscript critically for intellectual content. PB, YA and KMR conceived and designed the research, analysed the data and drafted the manuscript. All authors approved the final manuscript.

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