ORIGINAL RESEARCH

The Role of KCNMB1 and BK Channels in Myofibroblast Differentiation and Pulmonary Fibrosis

Anne M. Scruggs[1], Gintautas Grabauskas[2], and Steven K. Huang[1]

[1]Division of Pulmonary and Critical Care Medicine and [2]Division of Gastroenterology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan

ORCID ID: 0000-0002-2090-6331 (S.K.H.).

Abstract

The differentiation of fibroblasts into myofibroblasts is critical for the development of fibrotic disorders, including idiopathic pulmonary fibrosis (IPF). Previously, we demonstrated that fibroblasts from patients with IPF exhibit changes in DNA methylation across the genome that contribute to a profibrotic phenotype. One of the top differentially methylated genes identified in our previous study was *KCNMB1*, which codes for the β subunit of the large-conductance potassium (BK, also known as MaxiK or $K_{Ca}1.1$) channel. Here, we examined how the expression of *KCNMB1* differed between IPF fibroblasts and normal cells, and how BK channels affected myofibroblast differentiation. Fibroblasts from patients with IPF exhibited increased expression of *KCNMB1*, which corresponded to increased DNA methylation within the gene body. Patch-clamp experiments demonstrated that IPF fibroblasts had increased BK channel activity. Knockdown of *KCNMB1* attenuated the ability of fibroblasts to contract collagen gels, and this was associated with a loss of α-smooth muscle actin (SMA) expression. Pharmacologic activation of BK channels stimulated α-SMA expression, whereas BK channel inhibitors blocked the upregulation of α-SMA. The ability of BK channels to enhance α-SMA expression was dependent on intracellular calcium, as activation of BK channels resulted in increased levels of intracellular calcium and the effects of BK agonists were abolished when calcium was removed. Together, our findings demonstrate that epigenetic upregulation of *KCNMB1* contributes to increased BK channel activity in IPF fibroblasts, and identify a newfound role for BK channels in myofibroblast differentiation.

Keywords: fibroblast; IPF; KCNMA1; MaxiK; KCa1.1

Clinical Relevance

This study identifies for the first time that *KCNMB1* is upregulated in idiopathic pulmonary fibrosis fibroblasts and that it contributes to increased large-conductance potassium (BK) channel activity in idiopathic pulmonary fibrosis cells. Increased BK channel activity contributes to myofibroblast differentiation through increases in intracellular calcium. These findings identify a novel role for BK channels in pulmonary fibrosis and identify it as a potential target for future therapy.

Although fibrosis often develops as a result of chronic inflammation and injury, some diseases are associated with fibrosis that exceed the level of observed inflammation, and patients with these disorders often do not respond therapeutically to traditional antiinflammatory medications. Idiopathic pulmonary fibrosis (IPF) is one such disease (1) and is pathologically characterized by accumulation of fibroblastic foci, excessive extracellular matrix deposition, and progressive architectural remodeling that results in impaired gas exchange, diminished lung capacity, and eventually death (2, 3).

Fibroblasts are the major mesenchymal cells in the lung, and their activation and differentiation into myofibroblasts is critical for the development of PF (4, 5). Over the years, other investigators and we have shown that fibroblasts from the lungs of patients with IPF demonstrate differences

(Received in original form May 2, 2019; accepted in final form September 4, 2019)

Supported by grant HL127203 (to S.K.H.) from the National Heart, Lung, and Blood Institute.

Author Contributions: A.M.S. and G.G. performed the experiments and analyzed the data. S.K.H. performed the experiments, analyzed the data, and wrote the manuscript.

Correspondence and requests for reprints should be addressed to Steven K. Huang, M.D., Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical School, 6301 MSRB III, 1150 W. Medical Center Drive, Ann Arbor, MI 48109. E-mail: stehuang@umich.edu.

This article has a related editorial.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 62, Iss 2, pp 191–203, Feb 2020

Originally Published in Press as DOI: 10.1165/rcmb.2019-0163OC on September 5, 2019
Internet address: www.atsjournals.org

in gene expression, often due to epigenetic modifications, that contribute to fibrosis (6–9). Several years ago, we performed a DNA methylation array analysis that identified a number of CpG sites that were differentially methylated in fibroblasts from normal and IPF lungs (10). One of the top differentially methylated genes was *KCNMB1*.

KCNMB1 codes for the β subunit of the large-conductance (*B*ig) potassium (BK) channel (also known as MaxiK or $K_{Ca}1.1$). BK channels are known for their high conductance to potassium (11), and are gated by changes in either calcium or voltage, a feature unique compared with other potassium channels (12). The BK channel consists of the main pore-forming α subunit consisting of a single gene, *KCNMA1* (*Slo1*). The β subunits, of which there are four isoforms (*KCNMB1–4*), associate with the α subunits in a 1:1 stoichiometric ratio and serve as adapter proteins that enhance the sensitivity of the channel to changes in calcium or voltage (13–15). Although the α subunit is expressed ubiquitously in all tissues, variations in the expression of different β subunits provide an opportunity for the BK channel to exhibit pleiotropic effects among different cell types (16). The function of BK channels is well studied in smooth muscle, where increases in intracellular calcium during muscle contraction trigger the opening of the BK channels, resulting in cellular repolarization. Repolarization causes voltage-dependent calcium channels to close, thus serving as a negative-feedback mechanism that results in smooth muscle relaxation (17–19). Loss of BK channel activity, as demonstrated by *Kcnma1* and *Kcnmb1* knockout mice, results in increased vascular smooth muscle contraction, systemic hypertension, and increased bladder muscle tone (20, 21). A similar negative-feedback mechanism has been described in neurons (22).

Although the actions of BK channels are well described in neurons and cardiac and smooth muscle, much less focus has been devoted to the role of BK channels in fibroblasts, especially in the lung. BK channels have recently been shown to couple with mechanotransduction signals to promote osteoblast activation (23). BK channels also promote the migration and proliferation of synoviocytes (24), and the proliferation of dermal fibroblasts (25). The function of BK channels in lung fibroblasts, and particularly their ability to modulate myofibroblast differentiation, has not been described. Because *KCNMB1* was one of the top genes that were found to be differentially methylated between normal and IPF fibroblasts, we sought to examine how the expression of *KCNMB1* and the activity of the BK channel differ between normal and IPF cells, and how BK channels affect the expression of α-smooth muscle actin (SMA), a contractile protein whose expression distinguishes activated myofibroblasts from fibroblasts.

Here, we report that *KCNMB1* expression and BK channel function were elevated in fibroblasts from patients with IPF. As opposed to its role as a negative regulator of smooth muscle, BK channels were associated with an increase in fibroblast gel contraction and were necessary for the differentiation of fibroblasts into myofibroblasts. Increased BK channel activity resulted in an increase in intracellular calcium, which was necessary for myofibroblast differentiation. These findings identify a novel role for BK channels in the stimulation of lung fibroblasts, and provide new insights into how ion channels affect fibroblast-to-myofibroblast differentiation and contribute to the development of lung fibrosis.

Methods

Cell Culture

Normal primary human lung fibroblasts were cultured from the outgrowths of histologically normal regions of lungs that were provided for organ donation but were later deemed unsuitable for transplantation (Gift of Life). In some experiments, CCL210, a normal primary lung fibroblast line obtained from the American Type Culture Collection, was also used. IPF fibroblasts were cultured from the outgrowths of explanted lungs of patients with IPF, taken at the time of transplantation. In one experiment (Figure 1E), IPF fibroblasts were obtained from surgical lung biopsies of patients who were undergoing initial diagnosis of their disease and were believed to represent an earlier stage of lung fibrosis. All cells were isolated on standard tissue culture plates as previously described and studied between passages 3 and 6 (10). All diagnoses of IPF were confirmed by a consensus conference consisting of a multidisciplinary group of pulmonologists, pathologists, and radiologists. All tissue specimens, including those obtained after transplantation, were reviewed by a pathologist and confirmed as demonstrating a pattern of usual interstitial pneumonia. All patients consented to have fibroblasts cultured from their lungs as part of a biorepository study that was approved by the University of Michigan Institutional Review Board. Fibroblasts were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% FBS (Hyclone) and 1% penicillin/streptomycin (Invitrogen).

Electrophysiologic Studies

Whole-cell patch-clamp recordings were performed on single-cell normal and IPF fibroblasts. Cells were first plated and allowed to adhere on glass slides overnight. Cells were visualized on an inverted microscope (Nikon Ti; Mager Scientific) and recordings of current were obtained using patch pipettes with an access resistance of 3–5 MΩ and an internal solution of ∼280 mOsm consisting of 140 mM potassium glutamate, 10 mM HEPES, 10 mM EGTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM ATP, and 0.5 mM GTP, adjusted to a pH 7.3 with potassium hydroxide (KOH). The bath solution was maintained at 30 ± 0.5°C. Cells were bathed in an external solution of ∼300 mOsm consisting of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, and 10 mM mannitol, adjusted to a pH of 7.3 and maintained at 37°C. Each slide was used once per recording. The series resistances were in the range of 5–10 MΩ (typically 5 MΩ) and were compensated >80% online. Capacitance subtraction was used in all recordings. Current across the cell was measured using a current-clamp amplifier (Axopatch 200B; Molecular Devices) and recorded using DIGIDATA 1322A (Molecular Devices). The current across the cell was measured in response to voltage command pulses adjusted in 10 mV increments, from −80 to +100 mV. Cells were analyzed in vehicle control and after treatment with 100 nM of the BK channel antagonist iberiotoxin. Data were analyzed using pCLAMP9 software (Molecular Devices).

Assay of Intracellular Calcium

Levels of intracellular calcium were assayed using the FluoForte calcium assay (ENZ-51017; Enzo Life Sciences) per the manufacturer's protocol. For this purpose,

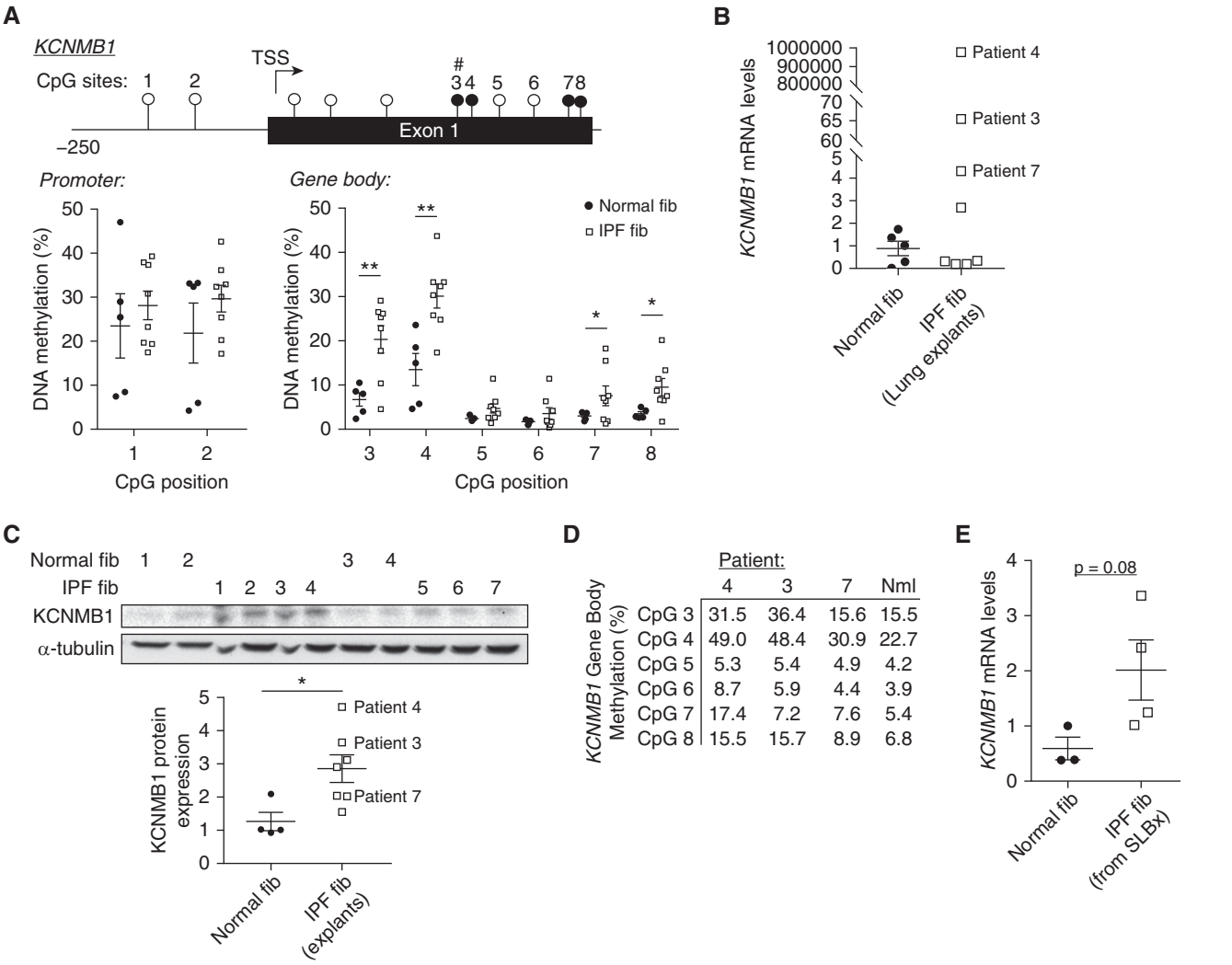

Figure 1. Lung fibroblasts from patients with idiopathic pulmonary fibrosis (IPF) exhibited DNA hypermethylation and increased expression of *KCNMB1*. (*A*) Fibroblasts (fib) cultured from the lungs of five healthy control subjects and eight patients with IPF were assessed for DNA methylation at different CpG sites along the *KCNMB1* promoter and gene body. The # sign indicates the CpG locus (position 169,748,956 on chromosome 5, Build 36.1) that was identified from a previous microarray study (10) as being hypermethylated in IPF fibroblasts. Solid circles indicate CpG sites that were found to be differentially methylated between control and IPF fibroblasts by pyrosequencing. TSS = transcription start site. (*B*) Fibroblasts cultured from healthy control lungs ($n = 5$) and lungs of patients with IPF ($n = 8$), explanted at the time of lung transplantation, were analyzed for levels of *KCNMB1* mRNA by RT-PCR. The labels on specific samples (patients 3, 4, and 7) correspond to the same fibroblast cell lines examined in *C* and *D*. (*C*) Cell lysates from normal and IPF fibroblasts were immunoblotted for KCNMB1. (*D*) The table indicates the percentage of DNA methylation of various CpG sites within the *KCNMB1* gene body in fibroblasts from patients with IPF and normal (Nml) healthy control subjects. The CpG sites listed in the table correspond to the same CpG positions indicated in *A*. (*E*) Expression of *KCNMB1* was assayed by RT-PCR in normal lung fibroblasts and fibroblasts cultured from the lungs of patients who underwent surgical lung biopsy (SLBx) for a diagnosis of IPF. $^{*}P < 0.05$ and $^{**}P < 0.01$, all analyzed by Student's *t* test.

2.5×10^4 cells were cultured in black 96-well tissue-culture–treated plates (Corning #3603; Thermo Fisher Scientific) and serum starved in DMEM overnight before addition of FluoForte dye, diluted 1:4, for 45 minutes at 37°C. Cells were then incubated for 15 minutes at room temperature before fluorescence was measured on a Functional Drug Screening System (FDSS7000; Hamamatsu Photonics) in real time with addition of reagents.

See the data supplement for additional details regarding the methods used in this work.

Results

KCNMB1 Is Hypermethylated within the Gene Body and Its Expression Is Increased in IPF Lung Fibroblasts

Our previous study using a genome-wide DNA methylation array identified *KCNMB1* as the top hypermethylated gene in fibroblasts from patients with IPF compared with fibroblasts from nonfibrotic control lungs (10). We first sought to verify these data by independently performing bisulfite sequencing of the *KCNMB1* promoter and the first exon of *KCNMB1*, which includes the CpG locus surveyed in the original microarray study. Bisulfite sequencing revealed no differences in DNA

methylation between normal and IPF fibroblasts within the *KCNMB1* proximal promoter (Figure 1A). However, within the first exon of *KCNMB1*, fibroblasts from patients with IPF exhibited significantly increased levels of DNA methylation compared with fibroblasts from healthy control lungs. This was observed at multiple CpG sites, including the CpG locus that was originally identified by the previous microarray study.

In contrast to other genes that are rich in CpG sites clustered in "CpG islands," there are no defined CpG islands and a relative paucity of CpG loci within the *KCNMB1* gene. Nonetheless, changes in the DNA methylation of just a few CpG sites in genes that demonstrate a low abundance of CpGs have been shown to powerfully regulate gene expression (26). Furthermore, DNA hypermethylation of gene bodies,

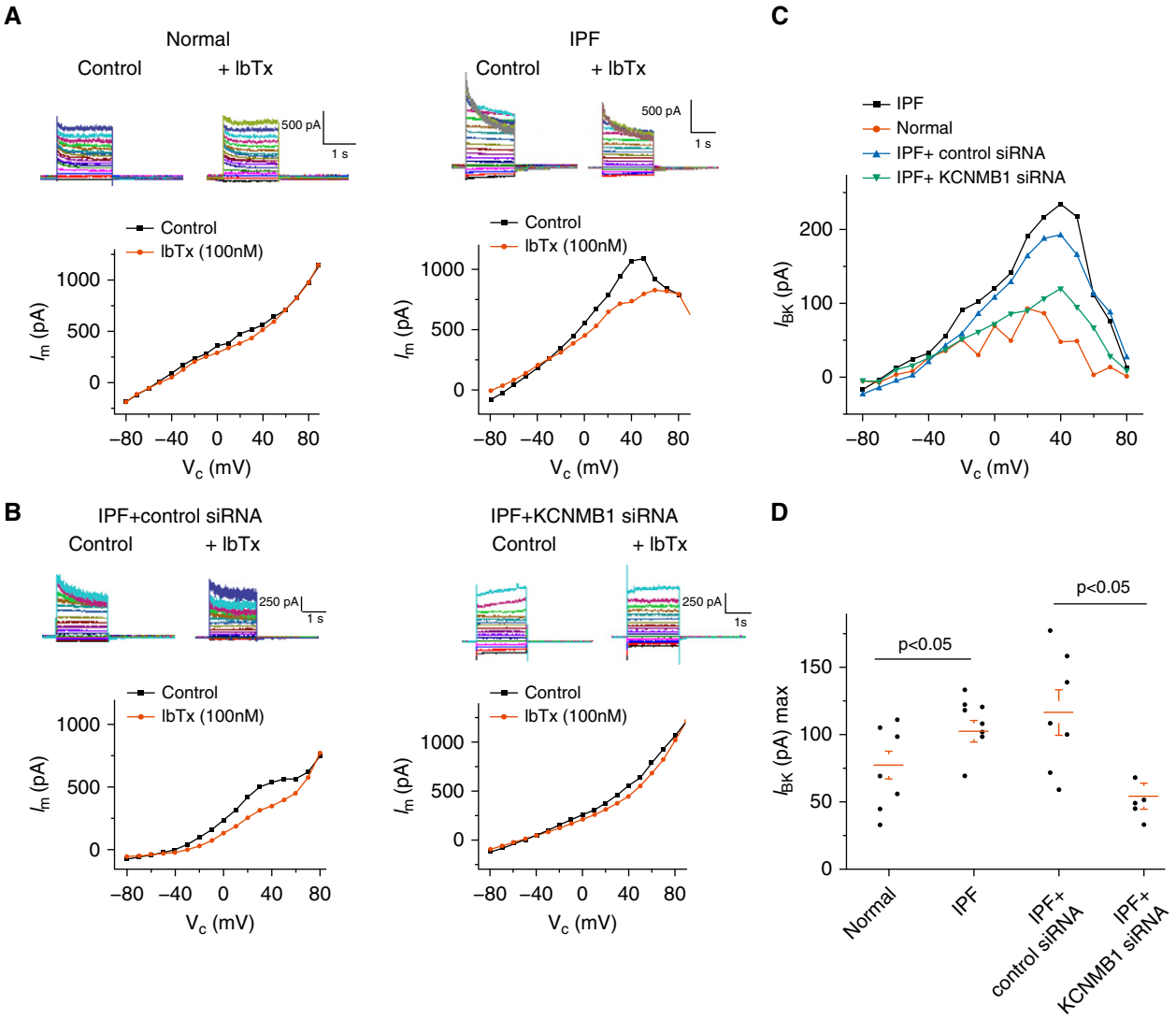

Figure 2. Fibroblasts from patients with IPF exhibited increased activity of the large-conductance potassium (BK) channel compared with normal fibroblasts. Whole-cell patch-clamp tracings were performed on nonfibrotic control fibroblasts and fibroblasts from patients with IPF that demonstrated increased KCNMB1 expression (fibroblast lines 3, 4, and 7). (*A*) Shown are representative single-cell current tracings and a graph of the current (I_m) versus voltage taken from patch clamping normal and IPF fibroblasts. Transmembrane voltage was increased in increments of 10 mV from −80 to +100 mV, and membrane current was measured by patch clamp as described in METHODS. To ascertain the current attributable to the BK channel, cells were analyzed before (blue squares) and after (red circles) treatment with 100 nM of the BK channel antagonist iberiotoxin (IbTx). (*B*) IPF fibroblasts were treated with control siRNA or siRNA against KCNMB1 for 48 hours before undergoing patch clamp. Recordings of current were measured in the presence or absence of IbTx. Shown are representative tracings and data taken from a single cell. (*C*) Current attributed to the BK channel (I_{BK}) was calculated by subtracting the current measured between cells in the presence and absence of iberiotoxin. I_{BK} was measured in a representative normal lung fibroblast (red circles), an IPF fibroblast (black squares), an IPF fibroblast treated with control siRNA (blue triangles), and an IPF fibroblast treated with KCNMB1 siRNA (green inverted triangles). (*D*) The maximum current attributable to BK channels (i.e., maximal difference in current before and after iberiotoxin treatment) was measured after patch clamping eight normal and eight IPF fibroblasts, as well as seven IPF fibroblasts treated with control siRNA, and seven IPF fibroblasts treated with KCNMB1 siRNA. Statistical analysis was performed using one-way ANOVA with Tukey's multiple-comparisons posttest. pA = picoamps.

including that of exons, is associated with an increase in gene expression (27). We thus sought to compare the expression of *KCNMB1* in fibroblasts obtained from healthy control subjects and patients with IPF. Fibroblasts from four of the eight patients with IPF demonstrated increased levels of *KCNMB1* expression, with one line exhibiting a greater than 60-fold increase, and another exhibiting a greater than 900,000-fold increase, in *KCNMB1* mRNA (Figure 1B). Patient-derived cells with high levels of *KCNMB1* mRNA also demonstrated a correspondingly high level of protein expression (Figure 1C). Because the levels of DNA methylation from IPF fibroblasts also varied, we compared the levels of *KCNMB1* methylation with its expression. Fibroblast lines that expressed increased DNA methylation correlated with those that expressed increased *KCNMB1* mRNA (Figure 1D). Fibroblasts cultured from the outgrowths of surgical lung biopsy specimens, which are often obtained at an earlier stage of disease than explants taken at the time of transplantation, demonstrated less of an increase, but still a trend, toward greater *KCNMB1* expression compared with normal cells (Figure 1E).

KCNMB1 codes for the regulatory subunit of the BK channel, which is comprised of a single gene, *KCNMA1*, that functions as the main pore-forming α subunit (13–15). Three other β subunits, *KCNMB2–4*, are also recognized to modulate BK channel activity (19). In comparison with *KCNMB1*, the expression of *KCNMA1* or any of the β subunits (*KCNMB2–4*) did not differ between IPF fibroblasts and fibroblasts from healthy control subjects (Figure E1A in the data supplement). In fact, an examination of publicly available data from a single-cell RNA-sequencing analysis by Reyfman and colleagues (28) showed that *KCNMB1* was the only β subunit of the BK channel that was appreciably expressed in lung fibroblasts (Figure E1B). *KCNMB1* expression was also observed in dendritic cells, macrophages, mast cells, and type 1 alveolar epithelial cells, whereas *KCNMB2* was expressed almost solely in ciliated cells, and *KCNMB4* was expressed predominantly in endothelial cells. Data from the single-cell RNA-sequencing analysis (28) also showed greater expression of *KCNMB1* in fibroblasts from fibrotic lungs than in those from normal control lungs (Figure E1C).

In addition to demonstrating differences in *KCNMB1* expression in cultured fibroblasts, we also sought to examine the *in situ* expression of KCNMB1 protein in lung tissue sections. In healthy control lungs, staining for KCNMB1 was observed throughout the alveolar epithelium (Figure E2A), which is consistent with findings from other investigators who have described expression of BK channels in lung epithelium (29, 30). In the lung sections from patients with IPF, KCNMB1 stained with greater intensity in areas of fibrosis, particularly among mesenchymal cells and within fibroblastic foci (Figures E2B and E2C). The hyperplastic epithelium overlying the fibroblastic foci was also noted to be highly expressed for KCNMB1. These findings demonstrate that KCNMB1 is expressed in IPF lungs and is upregulated in fibroblasts, particularly within fibroblastic foci.

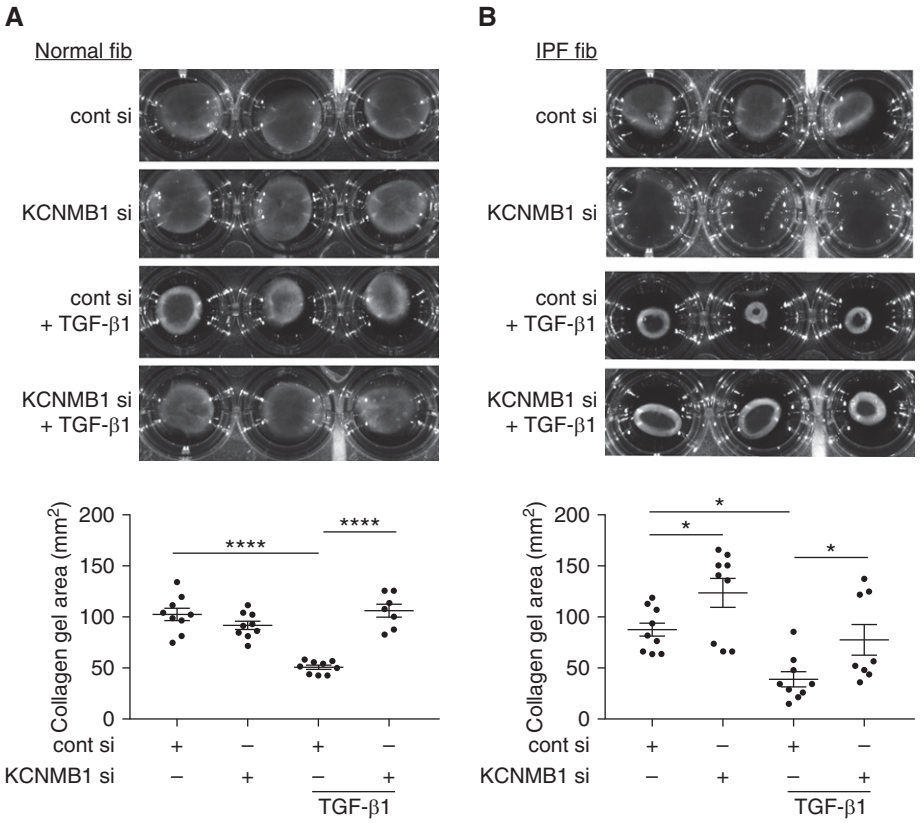

Figure 3. Silencing *KCNMB1* inhibited the capacity of normal and IPF fibroblasts to contract collagen gels. (*A* and *B*) Fibroblasts from normal healthy control subjects (*A*, $n = 3$) and patients with IPF (*B*, $n = 3$) were treated with either *KCNMB1* si or cont si for 24 hours before subsequent treatment with TGF-β1 (transforming growth factor β1; 2 ng/ml) for 24 hours. Cells were then placed in collagen gels supplemented with or without TGF-β1 and the gels were imaged after 24 hours. Gel images are from a representative experiment performed in triplicate. $^{*}P < 0.05$ and $^{****}P < 0.0001$, one-way ANOVA with Tukey's multiple-comparisons posttest. cont = control; si = siRNA.

BK Channel Activity Is Increased in IPF Fibroblasts

To examine how increased expression of *KCNMB1* affects BK channel activity, we performed single-cell patch-clamp recordings of IPF and normal fibroblasts in the presence or absence of iberiotoxin, a selective BK channel antagonist. For electrophysiology studies, we focused on the IPF fibroblast cell lines identified in Figure 1 that expressed higher levels of KCNMB1. There was no difference in resting membrane potential (voltage at $I_m = 0$) between IPF fibroblasts and fibroblasts from healthy control subjects. However, upon cellular depolarization, fibroblasts from patients with IPF exhibited higher levels of total current in response to

changes in the membrane potential (Figure 2A). Current attributed to BK channel activity, which is the difference in current before and after treatment with iberiotoxin, was greater in IPF fibroblasts than in fibroblasts from normal control subjects (Figures 2A and 2C). IPF fibroblasts treated with KCNMB1 siRNA exhibited less BK channel activity compared with those treated with control siRNA (Figures 2B and 2C). On average, IPF fibroblasts exhibited greater BK channel current than normal fibroblasts, and this was abolished when IPF fibroblasts were treated with KCNMB1 siRNA (Figure 2D).

Involvement of KCNMB1 and BK Channels in Gel Contraction and Myofibroblast Differentiation

To examine the effect of increased BK channel activity on IPF fibroblast function, we treated normal and IPF fibroblasts with control siRNA or siRNA against *KCNMB1*, and measured the ability of fibroblasts to contract collagen gels in the presence or absence of TGF-β1 (transforming growth factor β1). Expression of *KCNMB1* was effectively knocked down by siRNA (Figures E3A and E3B). Although treatment of both normal and IPF fibroblasts with TGF-β1 resulted in a robust contraction of collagen gels, this was inhibited in normal fibroblasts and IPF fibroblasts when *KCNMB1* was silenced (Figure 3). IPF fibroblasts demonstrated a greater intrinsic ability to contract collagen gels than normal fibroblasts, and this was also inhibited when *KCNMB1* was silenced (Figure 3B).

Expression of α-SMA is critical for the ability of myofibroblasts to contract wounds and is the predominant marker of myofibroblast differentiation (5). To examine the importance of BK channels in myofibroblast differentiation, we first examined the expression of *KCNMB1* in normal fibroblasts in response to TGF-β1. Although normal fibroblasts express low levels of *KCNMB1*, its expression increased in response to TGF-β1 (Figure 4A). We next treated normal fibroblasts with TGF-β1 in the presence or absence of either the BK channel agonist NS-1619 or the BK channel antagonist iberiotoxin. Expression of α-SMA increased in response to TGF-β1, and this was further enhanced in the presence of the BK channel agonist NS-1619 (Figure 4B). The BK channel antagonist iberiotoxin attenuated the ability of TGF-β1 to increase α-SMA (Figure 4C). At the doses tested, neither the agonist NS-1619 nor the antagonist iberiotoxin significantly affected cell toxicity, viability, or size (Figure E4). To further explore the effects of BK channels on myofibroblast differentiation, fibroblasts were treated with siRNA against either *KCNMA1* or *KCNMB1*. As with *KCNMB1*, levels of *KCNMA1* were effectively knocked down by siRNA (Figure E3C). Silencing of either *KCNMA1* or *KCNMB1* attenuated the ability of TGF-β1 to upregulate α-SMA (Figures 4D, 4E, and E3D). Finally, knockdown of *KCNMB1* also attenuated α-SMA expression in the presence of endothelin-1, another soluble mediator that is known to induce myofibroblast differentiation (Figures 4F and E3E). These data demonstrate that KCNMB1 and BK channels play an important role in modulating α-SMA expression and myofibroblast differentiation.

Role of Calcium in Myofibroblast Differentiation and Generation of Intracellular Calcium by BK Channels

Other investigators have demonstrated that calcium signaling is important for fibroblast activation and myofibroblast differentiation (31–33). To demonstrate the importance of calcium in TGF-β1 signaling, we first treated normal and IPF fibroblasts with TGF-β1 and measured the levels of intracellular calcium. Treatment with TGF-β1 resulted in an ∼1.4-fold increase in the levels of intracellular calcium in both normal and IPF fibroblasts (Figure 5A). To determine whether calcium is necessary for α-SMA expression, we removed calcium from cells by either culturing fibroblasts in DMEM without calcium or treating them with the calcium chelator 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-aminopolycarboxylic acid (BAPTA-AM). Levels of α-SMA expression were significantly attenuated when cells were treated with BAPTA-AM and/or additionally when cells were cultured in DMEM lacking calcium (Figure 5B).

To determine whether BK channels modulate α-SMA expression through changes in intracellular calcium, we first determined the effects of the BK channel agonist NS-1619 and the BK channel antagonist iberiotoxin on levels of intracellular calcium. As opposed to smooth muscle cells, where activation of BK channels limits calcium influx by inactivating voltage-dependent calcium channels (17–19), fibroblasts treated with NS-1619 exhibited increased levels of intracellular calcium (Figure 6A). By contrast, treatment with the BK antagonist iberiotoxin completely blocked the ability of TGF-β1 to increase intracellular calcium levels (Figure 6B). Silencing KCNMB1 with siRNA also attenuated calcium flux in response to TGF-β1 and NS-1619 (Figure 6C). To demonstrate that the increase in calcium was responsible for the promotion of α-SMA expression by BK channels, fibroblasts were cultured in DMEM lacking calcium or with BAPTA-AM before treatment with NS-1619. In contrast to what was observed when cells were cultured in normal DMEM (Figure 4B), NS-1619 was unable to upregulate α-SMA expression when fibroblasts were cultured in DMEM lacking calcium (Figure 6D). Similarly, the effects of NS-1619 were diminished when cells were treated with the calcium chelator BAPTA-AM (Figure 6E). Finally, the increase in *ACTA2* mRNA by TGF-β1, as well as the decrease in *ACTA2* when *KCNMB1* was silenced, was attenuated when cells were cultured in medium without calcium (Figure 6F). These data thus demonstrate that activation of BK channels results in an increase in intracellular calcium that appears to be crucial for the ability of BK channels to enhance α-SMA expression and myofibroblast differentiation.

Role of KCNMB1 in Mediating α-SMA in the Presence of Intermediate-Conducting Potassium and TRPV4 Agonists and Antagonists

It has been reported that intermediate-conducting potassium (IK) channels also promotes fibroblast-to-myofibroblast differentiation and the development of PF (34–37). To determine the relative importance of BK channels in the context of IK channel agonists and antagonists, normal fibroblasts were treated with *KCNMB1* siRNA in the presence or absence of TGF-β1, the IK agonist Tram-34, or the IK antagonist 1-EBIO. Neither Tram-34 nor 1-EBIO significantly affected fibroblast viability or apoptosis at the doses used (Figure E4). Silencing *KCNMB1* robustly

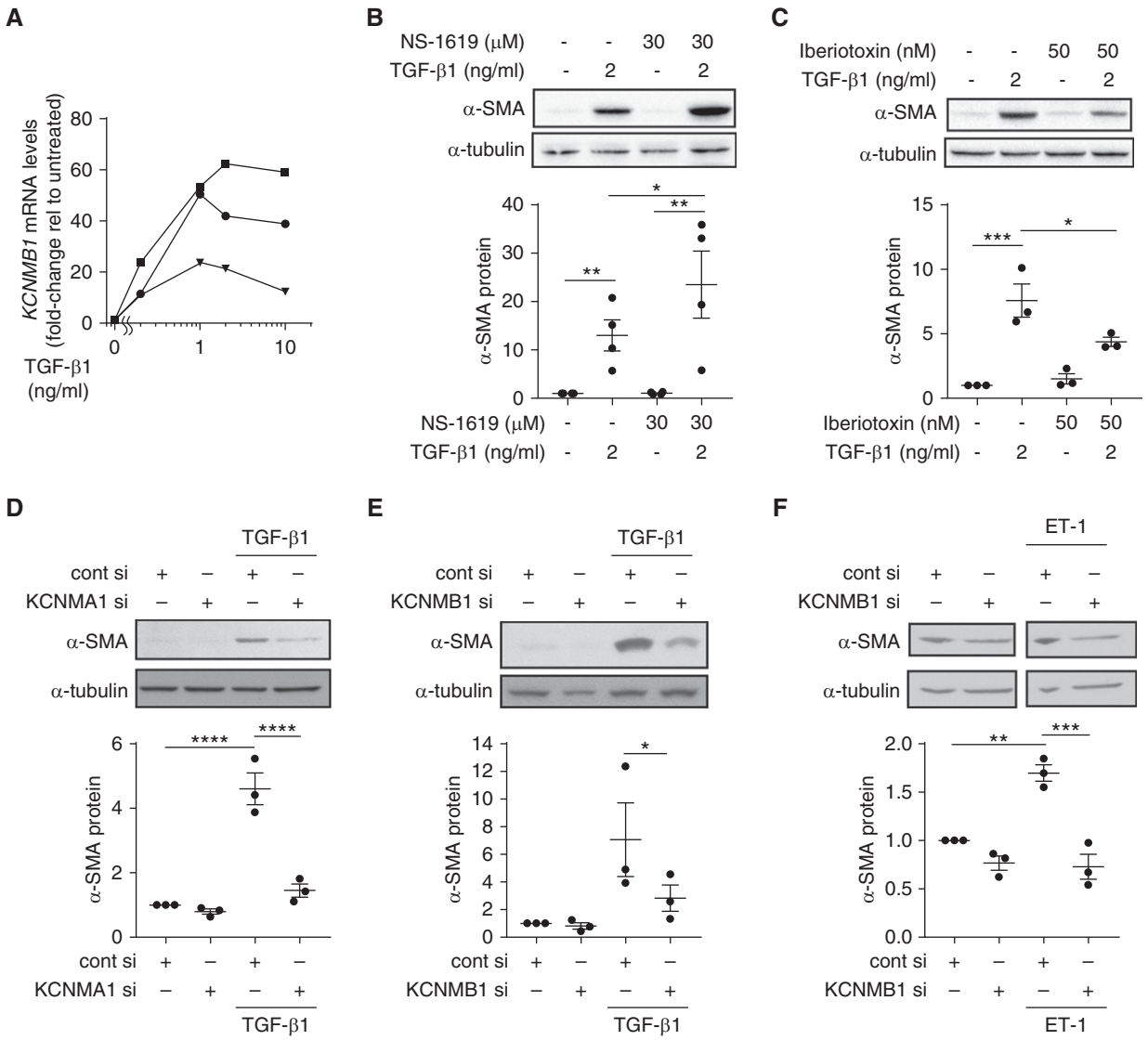

Figure 4. Activation of BK channels enhances α-smooth muscle actin (α-SMA) expression and is necessary for myofibroblast differentiation. (*A*) Normal CCL-210 lung fibroblasts were treated with increasing concentrations of TGF-β1 for 24 hours and *KCNMB1* mRNA levels were assayed by RT-PCR. Each line indicates the fold change in *KCNMB1* expression from an independent experiment ($n = 3$ total). (*B* and *C*) CCL210 fibroblasts were treated with either the BK channel agonist NS-1619 (*B*) or the BK channel antagonist iberiotoxin (*C*) with or without TGF-β1 for 24 hours, and expression of α-SMA was assayed by immunoblot. (*D* and *E*) CCL210 fibroblasts were pretreated with nontargeting control siRNA or siRNA against *KCNMA1* (*D*) or *KCNMB1* (*E*) before subsequent treatment with TGF-β1 (2 ng/ml). α-SMA protein levels were assayed by immunoblot. (*F*) CCL210 fibroblasts were pretreated with either control siRNA or siRNA against *KCNMB1* for 48 hours before subsequent treatment with 100 ng/ml of endothelin-1 (ET-1) for 24 hours. α-SMA protein levels were assayed by immunoblot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, all tested by one-way ANOVA with Tukey's multiple-comparisons posttest.

downregulated α-SMA expression in all treatment conditions. α-SMA levels were not enhanced in the presence of Tram-34, and although 1-EBIO treatment decreased α-SMA expression, levels of α-SMA were further diminished in the presence of *KCNMB1* siRNA (Figures 7A and 7B).

The TRPV4 (transient receptor potential vanilloid 4) channel has also been shown in lung fibroblasts to regulate myofibroblast differentiation (38). Although α-SMA levels were increased by the TRPV4 antagonist GSK2193874, silencing *KCNMB1* effectively inhibited this increase (Figure 7C). Silencing *KCNMB1* also inhibited the increase in α-SMA in the presence of a TRPV4 agonist, GSK1016790A (Figure 7D). These findings thus demonstrate that although various other ion channels have been implicated in activating fibroblasts and influencing fibrosis, modulating KCNMB1 expression and BK channel activity influences fibroblast function and myofibroblast differentiation to a degree that is as great as, if not greater than, that observed by activating IK or TRPV4 channels.

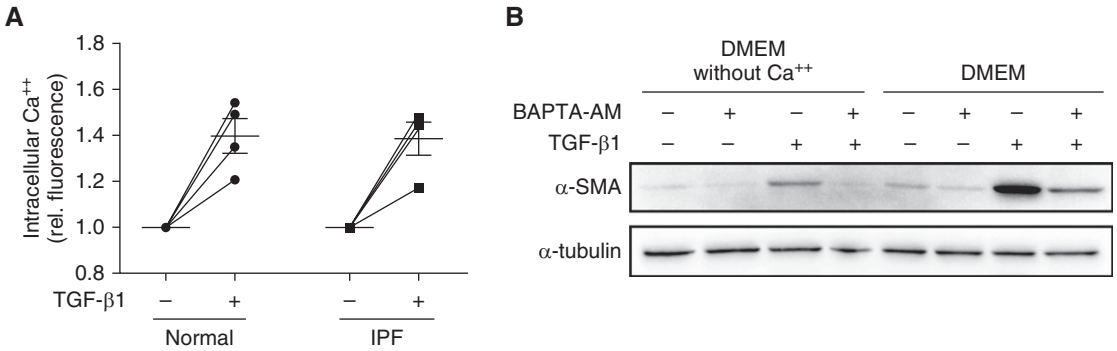

Figure 5. TGF-β1 increases calcium signaling, and calcium is required for α-SMA expression. (*A*) Fibroblasts from normal lung and the lungs of patients with IPF were treated for 30 minutes with TGF-β1 (2 ng/ml) and levels of intracellular calcium were measured by the FluoForte calcium assay. Each data point indicates values from different cell lines. (*B*) CCL210 lung fibroblasts were cultured in medium with or without calcium and treated in the presence or absence of the calcium chelator BAPTA-AM (10 μM) and TGF-β1 (2 ng/ml). Levels of α-SMA protein were assayed by immunoblot; a representative immunoblot of three independent experiments shown. BAPTA-AM = 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-aminopolycarboxylic acid; DMEM = Dulbecco's modified Eagle medium.

Discussion

We previously demonstrated that epigenetic modifications in different genes of fibroblasts from patients with IPF contribute to a profibrotic phenotype (7, 39, 40). Here, we examine how *KCNMB1*, the top differentially methylated gene identified from a previous microarray study of DNA methylation differences (10), also plays an important role in fibroblast activation. We show for the first time that *KCNMB1* is hypermethylated at the gene body, and that its expression is increased in select IPF fibroblasts. Fibroblasts from patients with IPF exhibited increased BK channel activity. Surprisingly, as opposed to their ability to promote smooth muscle relaxation as described in the literature (17, 21), BK channels promoted fibroblast activation and myofibroblast differentiation. BK channel agonists enhanced α-SMA expression, whereas antagonists inhibited α-SMA levels. α-SMA expression was also inhibited by knockdown of either *KCNMB1* or *KCNMA1*, both of which comprise the functioning BK channel. BK channel activation resulted in an increase in levels of intracellular calcium, and the increase in calcium was responsible for the ability of BK channels to upregulate α-SMA. Finally, the role of BK channels in modulating α-SMA levels was as great as, if not greater than, that of stimulation by either the IK channels or TRPV4 channels, which have been described in other studies as being important for the development of fibrosis (34–36, 38). These data thus identify *KCNMB1* as a novel epigenetically dysregulated gene that supports a profibrotic phenotype in IPF fibroblasts and may contribute to the development of PF.

Ion channels serve critical functions in the activation and homeostasis of every cell type. Traditionally, BK channels in smooth muscle (17, 21), cardiac muscle (41), and neurons (22) have received the greatest attention, but the ubiquity of BK channels and variations in β-subunit expression in different cell types suggest that its function may be diverse and cell specific. Although BK channels have been described to promote smooth muscle relaxation by repolarizing cells after initial contraction (17, 18), our studies demonstrate that in fibroblasts, BK channels promote myofibroblast differentiation and α-SMA expression. This is supported by studies in other mesenchymal cells, such as synoviocytes and dermal fibroblasts, that demonstrated that BK channels promote cell migration and proliferation (24, 25). In addition to inherent differences between fibroblasts and smooth muscle, our studies in fibroblasts focused on long-term actions of BK channels, including their ability to modulate downstream gene expression and markers of myofibroblast differentiation, rather than the short-term contractile effects emphasized in smooth muscle. Baseline expression of KCNMB1 is also higher in smooth muscle (16), whereas *KCNMB1* is induced either epigenetically in IPF fibroblasts or with TGF-β1. The inducible nature of this gene in fibroblasts indicates that its role in fibroblasts may be distinct from that in smooth muscle. Finally, whereas activation of BK channels in smooth muscle cells prevents further calcium influx by directly closing voltage-dependent calcium channels, calcium levels increased in fibroblasts after BK channel activation. The increase in calcium is a necessary step for myofibroblast differentiation, which was prevented when fibroblasts were cultured in medium lacking calcium or when cells were treated with an intracellular calcium chelator.

Activation of intracellular calcium signaling is increasingly recognized as a critical signaling event in myofibroblast differentiation and the stimulation of genes necessary for fibroblast activation (31, 32). TGF-β1 not only causes calcium to increase in fibroblasts but also contributes to oscillatory waves of calcium that promote myofibroblast differentiation (31, 33). In our experiments, we confirmed that calcium was necessary for the increase in α-SMA expression, and that BK channel activation contributes to increased intracellular calcium. How the BK channel does so is not completely clear, but one hypothesis is that BK channels physically couple with other calcium channels to exert these effects. Indeed, it has been reported that BK channels can couple with TRPV4 channels in the cells of kidney collecting ducts to promote calcium influx (42). Alternatively, BK channels may couple with ryanodine or inositol triphosphate receptors in the endoplasmic reticulum to trigger release of calcium stores (43). In future studies, we plan to identify some of the diverse mechanisms by which BK

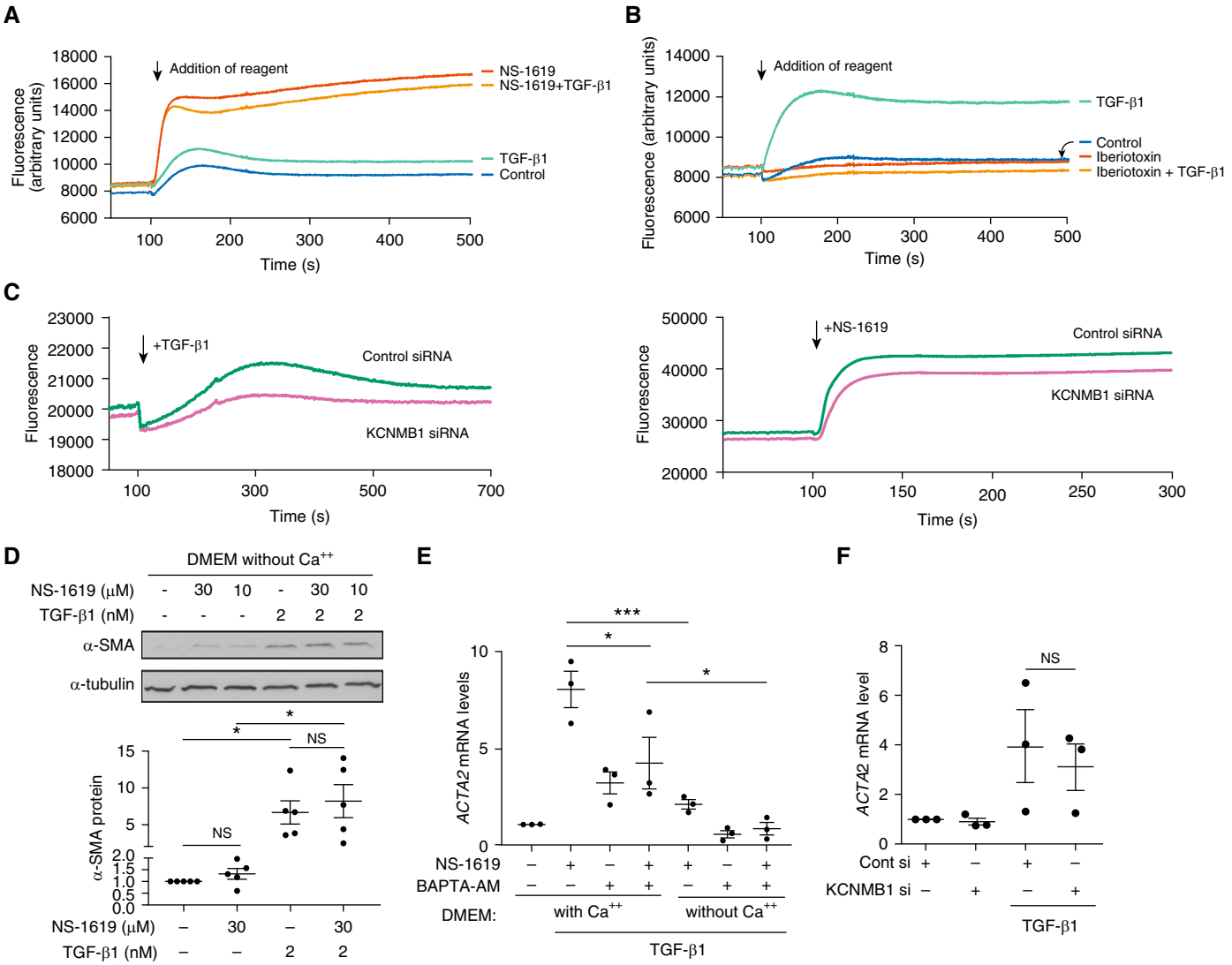

Figure 6. Activation of BK channels increases levels of intracellular calcium and is responsible for the ability of BK channels to enhance α-SMA expression. (*A* and *B*) CCL210 lung fibroblasts were treated with either the BK channel agonist NS-1619 (30 μM) (*A*) or the BK antagonist iberiotoxin (50 nM) (*B*) with and without TGF-β1 (2 ng/ml), and levels of intracellular calcium were measured over time on an FDSS7000 system using the FluoForte calcium assay. Shown are representative tracings from three independent experiments. (*C*) CCL210 fibroblasts were treated with control siRNA or KCNMB1 siRNA for 48 hours and then assayed for levels of intracellular calcium after treatment with TGF-β1 (2 ng/ml) or NS-1619 (30 μM). Shown are representative tracings from three independent experiments. (*D*) CCL210 fibroblasts were treated with varying doses of NS-1619 and TGF-β1 in DMEM lacking calcium, and levels of α-SMA protein were assayed by immunoblot. (*E*) Fibroblasts from healthy control subjects were treated with TGF-β1 (2 ng/ml) and NS-1619 (30 μM) in the presence or absence of BAPTA-AM (10 μM) or DMEM without calcium, and *ACTA2* mRNA levels were assayed by RT-PCR. (*F*) Normal lung fibroblasts were treated with either control or *KCNMB1* siRNA and then incubated in DMEM without calcium. Cells were subsequently treated with TGF-β1 (2 ng/ml) and levels of *ACTA2* were assayed by RT-PCR. $^{*}P < 0.05$ and $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple-comparisons posttest. NS = not significant.

channels promote the increase in levels of intracellular calcium, and determine how the increase in intracellular calcium affects downstream α-SMA expression.

We conclude that BK channels promote myofibroblast differentiation by increasing intracellular calcium, based on the observations that treatment with the BK channel agonist NS-1619 resulted in increased levels of intracellular calcium, and that culturing cells in calcium-free medium and/or exposed to a calcium chelator abolished the ability of NS-1619 to enhance α-SMA expression. Treatment with iberiotoxin or silencing KCNMB1 inhibited the ability of TGF-β1 to increase calcium. One limitation of these experiments, however, is that because BK channels are themselves activated by calcium, depletion of calcium from cells may itself inhibit BK channel activity and myofibroblast differentiation. Because our data support a positive-feedback mechanism whereby BK channels promote calcium flux and increased calcium activates BK channels, this interrelationship between calcium and BK channels makes it

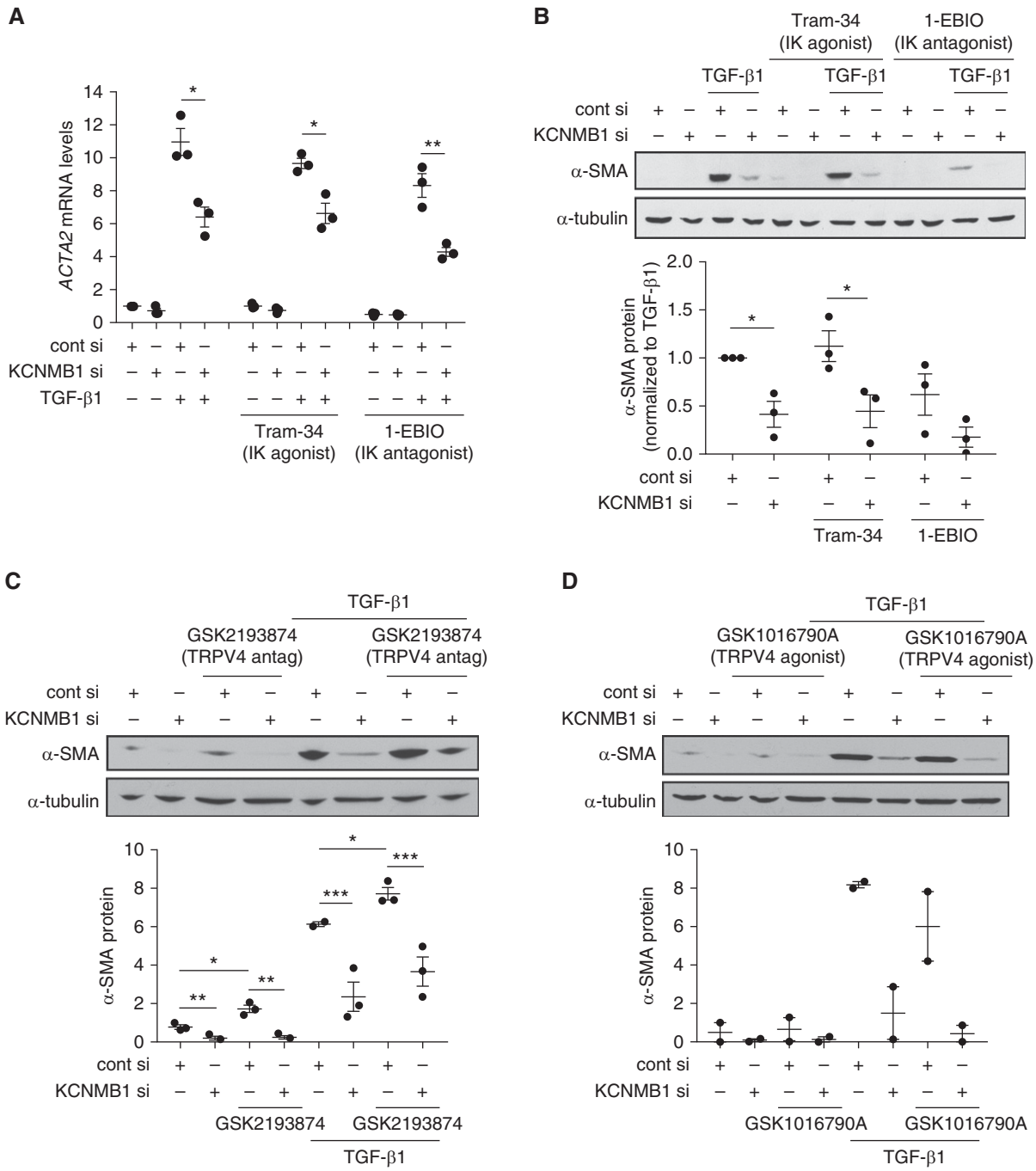

Figure 7. Combinatorial effects of BK channels, intermediate-conducting potassium (IK) channels, and TRPV4 (transient receptor potential vanilloid 4) on myofibroblast differentiation. (*A* and *B*) CCL210 lung fibroblasts were pretreated with either cont siRNA or KCNMB1 siRNA for 48 hours and then treated with TGF-β1 (2 ng/ml), the IK agonist Tram-34 (200 nM), or the IK antagonist 1-EBIO (100 μM). RNA was analyzed for levels of *ACTA2* mRNA (*A*) and protein lysates were assayed for α-SMA by immunoblot (*B*). (*C* and *D*) CCL210 fibroblasts were pretreated with either control siRNA or KCNMB1 siRNA for 48 hours and then treated with either 2 ng/ml of TGF-β1, 80 nM of the TRPV4 antagonist GSK2193874 (*C*) or 10 nM of the TRPV4 agonist GSK1016790A (*D*) for 24 hours. Cell lysates were analyzed for α-SMA expression by immunoblot. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, one-way ANOVA with Tukey's multiple-comparisons posttest for all experiments.

inherently difficult to determine what comes first, the opening of BK channels or the generation of intracellular calcium. Nonetheless, the observation that BK channels increase, rather than decrease, levels of intracellular calcium is unique to fibroblasts and has not been described in other cell types. Future studies should investigate what mediators cause BK channels to open in fibroblasts.

The suggestion that *KCNMB1* and BK channels are important for fibroblast

activation is further supported by our observations that fibroblasts from patients with IPF demonstrated greater BK channel activity by patch clamp, and that *KCNMB1* expression was upregulated by profibrotic mediators, including TGF-β1. Indeed, serum response factor and myocardin, two transcription factors that are critically important for myofibroblast differentiation, have been shown by other investigators to directly bind *KCNMB1* and promote its transcription (44). The increased activity of BK channels in IPF fibroblasts appears to be specific to increased *KCNMB1* expression, as silencing KCNMB1 in these cells abolished BK channel activity.

Although levels of *KCNMB1* expression were heterogeneous among different cell lines from patients with IPF, half of the IPF lines surveyed expressed increased levels of *KCNMB1* compared with normal fibroblasts, with two cell lines demonstrating >60-fold and >900,000-fold higher levels of *KCNMB1* mRNA, respectively. We identified increased *KCNMB1* expression in other, independently performed IPF microarray analyses, including one in fibroblasts (45) (Gene Expression Omnibus [GEO] dataset GSE87175) and one examining whole-lung tissue (46) (GEO dataset GSE32537). An analysis of single-cell RNA sequencing data also demonstrated increased *KCNMB1* expression in a fibroblast population derived from patients with IPF (28), and the heterogeneous expression suggests that there may be subpopulations that both express higher levels of *KCNMB1* and drive fibrosis. Staining for KCNMB1 in IPF tissues demonstrated varying levels of KCNMB1 expression, with increased levels of KCNMB1 found particularly within fibroblastic foci, which also supports the notion that BK channels contribute to myofibroblast generation. Further sorting and isolation of distinct fibroblast populations, which was beyond the scope of this study, would help us better understand the basis for this heterogeneity. Although we did not observe a difference in *KCNMA1* expression in our cohort of fibroblasts, other datasets comparing IPF with normal fibroblasts demonstrate differences in *KCNMA1* mRNA (GEO dataset GDS4995) (47), which further supports the importance of BK channels in fibroblast biology.

Epigenetic changes are increasingly recognized as an important mechanism in the development of IPF. Genome-wide DNA methylation changes have been described in the lung tissue of patients with IPF (48, 49) and more specifically in certain cell types of the lung, including fibroblasts (10). The epigenetic silencing of antifibrotic genes such as *THY1* (9), *PTGER2* (7), and *p14/ARF* (6) has been shown to contribute to activation of mesenchymal cells and a profibrotic phenotype. Here, we show that DNA methylation contributes to increased expression of *KCNMB1*. The increased expression was associated with DNA hypermethylation within the gene body, a phenomenon that is well described in other contexts (27). It remains to be determined whether the increase in *KCNMB1* DNA methylation by itself is sufficient to cause an increase in expression, and whether other epigenetic modifications are capable of modulating *KCNMB1* expression. All fibroblasts from either the lungs of patients with IPF or healthy control subjects were cultured in a similar manner and examined at similar passage numbers. The fact that differences in the DNA methylation of IPF and normal cells persisted in culture indicates that these epigenetic changes are stable over time and during cell division, independently of the environmental milieu. The culture conditions and the stiffness of the culture plates, which are often associated with increased levels of TGF-β1, may also influence the levels of *KCNMB1* and potentially its DNA methylation pattern.

Although certain signaling pathways are well known to modulate fibroblast differentiation, the effects of ion channels in general and the role of ion channel biology in the activation of fibroblasts have not been as well studied. Recent studies have emphasized the importance of calcium channels, such as TRPV4 (38) and transient receptor potential melastatin 7 (TRPM7) (50), in the pathogenesis of fibrosis. Potassium channels have received less attention in the context of fibroblast biology, but recent studies have shown that IK channels are also upregulated in PF and contribute to fibroblast activation through an increase in intracellular calcium (35–37). An examination of the effects of IK channel and TRPV4 channel agonists and antagonists indicates that BK channels play a greater role in modulating fibroblast activation and α-SMA expression than stimulation of either of those channels alone. Synergizing the antagonism of BK channels with IK and/or TRPV4 calcium channel antagonists may enhance the therapeutic potential of targeting each of these channels individually.

In conclusion, our finding that *KCNMB1* expression and BK channel activation are important for myofibroblast differentiation introduces a novel mechanism by which fibroblasts are activated. Understanding the mechanisms that contribute to myofibroblast differentiation may aid in the development of therapeutic targets to mitigate the morbidity and mortality of fibrotic disease. These findings provide an opportunity to gain new insights into how ion channels affect fibroblast biology, and how therapeutic targeting of BK channels may be considered in the treatment of PF and other fibrotic disorders. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

References

1. Selman M, King TE, Pardo A; American Thoracic Society; European Respiratory Society; American College of Chest Physicians. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001;134: 136–151.
2. Katzenstein AL, Myers JL. Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. *Am J Respir Crit Care Med* 1998;157:1301–1315.
3. Raghu G, Remy-Jardin M, Myers JL, Richeldi L, Ryerson CJ, Lederer DJ, *et al*.; American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, and Latin American Thoracic Society. Diagnosis of idiopathic pulmonary fibrosis: an official

ATS/ERS/JRS/ALAT clinical practice guideline. *Am J Respir Crit Care Med* 2018;198:
e44–e68.
4. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003;200:500–503.
5. Hinz B, Phan SH, Thannickal VJ, Galli A, Bochaton-Piallat ML, Gabbiani G. The myofibroblast: one function, multiple origins. *Am J Pathol* 2007;170:1807–1816.
6. Cisneros J, Hagood J, Checa M, Ortiz-Quintero B, Negreros M, Herrera I, *et al*. Hypermethylation-mediated silencing of p14(ARF) in fibroblasts from idiopathic pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L295–L303.
7. Huang SK, Fisher AS, Scruggs AM, White ES, Hogaboam CM, Richardson BC, *et al*. Hypermethylation of PTGER2 confers prostaglandin E2 resistance in fibrotic fibroblasts from humans and mice. *Am J Pathol* 2010;177:2245–2255.
8. Huang SK, Wettlaufer SH, Hogaboam CM, Flaherty KR, Martinez FJ, Myers JL, *et al*. Variable prostaglandin E2 resistance in fibroblasts from patients with usual interstitial pneumonia. *Am J Respir Crit Care Med* 2008;177:66–74.
9. Sanders YY, Pardo A, Selman M, Nuovo GJ, Tollefsbol TO, Siegal GP, *et al*. Thy-1 promoter hypermethylation: a novel epigenetic pathogenic mechanism in pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2008;39: 610–618.
10. Huang SK, Scruggs AM, McEachin RC, White ES, Peters-Golden M. Lung fibroblasts from patients with idiopathic pulmonary fibrosis exhibit genome-wide differences in DNA methylation compared to fibroblasts from nonfibrotic lung. *PLoS One* 2014;9:e107055.
11. Knaus HG, Eberhart A, Glossmann H, Munujos P, Kaczorowski GJ, Garcia ML. Pharmacology and structure of high conductance calcium-activated potassium channels. *Cell Signal* 1994;6:861–870.
12. Butler A, Tsunoda S, McCobb DP, Wei A, Salkoff L. mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. *Science* 1993;261:221–224.
13. Knaus HG, Folander K, Garcia-Calvo M, Garcia ML, Kaczorowski GJ, Smith M, *et al*. Primary sequence and immunological characterization of beta-subunit of high conductance Ca(2+)-activated K+ channel from smooth muscle. *J Biol Chem* 1994;269:17274–17278.
14. McManus OB, Helms LM, Pallanck L, Ganetzky B, Swanson R, Leonard RJ. Functional role of the beta subunit of high conductance calcium-activated potassium channels. *Neuron* 1995;14:645–650.
15. Tanaka Y, Meera P, Song M, Knaus HG, Toro L. Molecular constituents of maxi KCa channels in human coronary smooth muscle: predominant alpha + beta subunit complexes. *J Physiol* 1997;502:545–557.
16. Jiang Z, Wallner M, Meera P, Toro L. Human and rodent MaxiK channel beta-subunit genes: cloning and characterization. *Genomics* 1999; 55:57–67.
17. Petkov GV, Bonev AD, Heppner TJ, Brenner R, Aldrich RW, Nelson MT. Beta1-subunit of the Ca2+-activated K+ channel regulates contractile activity of mouse urinary bladder smooth muscle. *J Physiol* 2001;537:443–452.
18. Nakahara T, Mitani A, Kubota Y, Maruko T, Sakamoto K, Tanaka Y, *et al*. MaxiK channel-triggered negative feedback system is preserved in the urinary bladder smooth muscle from streptozotocin-induced diabetic rats. *J Smooth Muscle Res* 2004;40:97–109.
19. Tanaka Y, Koike K, Alioua A, Shigenobu K, Stefani E, Toro L. Beta1-subunit of MaxiK channel in smooth muscle: a key molecule which tunes muscle mechanical activity. *J Pharmacol Sci* 2004;94: 339–347.
20. Meredith AL, Thorneloe KS, Werner ME, Nelson MT, Aldrich RW. Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel. *J Biol Chem* 2004;279:36746–36752.
21. Sausbier M, Arntz C, Bucurenciu I, Zhao H, Zhou XB, Sausbier U, *et al*. Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice. *Circulation* 2005; 112:60–68.
22. Knaus HG, Schwarzer C, Koch RO, Eberhart A, Kaczorowski GJ, Glossmann H, *et al*. Distribution of high-conductance Ca(2+)-activated K+ channels in rat brain: targeting to axons and nerve terminals. *J Neurosci* 1996;16:955–963.
23. Rezzonico R, Cayatte C, Bourget-Ponzio I, Romey G, Belhacene N, Loubat A, *et al*. Focal adhesion kinase pp125FAK interacts with the large conductance calcium-activated hSlo potassium channel in human osteoblasts: potential role in mechanotransduction. *J Bone Miner Res* 2003;18:1863–1871.
24. Hu X, Laragione T, Sun L, Koshy S, Jones KR, Ismailov II, *et al*. KCa1.1 potassium channels regulate key proinflammatory and invasive properties of fibroblast-like synoviocytes in rheumatoid arthritis. *J Biol Chem* 2012;287:4014–4022.
25. Zironi I, Gavoçi E, Lattanzi G, Virelli A, Amorini F, Remondini D, *et al*. BK channel overexpression on plasma membrane of fibroblasts from Hutchinson-Gilford progeria syndrome. *Aging (Albany NY)* 2018;10: 3148–3160.
26. Han H, Cortez CC, Yang X, Nichols PW, Jones PA, Liang G. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Hum Mol Genet* 2011;20:4299–4310.
27. Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, *et al*. Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature* 2010;466:253–257.
28. Reyfman PA, Walter JM, Joshi N, Anekalla KR, McQuattie-Pimentel AC, Chiu S, *et al*. Single-cell transcriptomic analysis of human lung provides insights into the pathobiology of pulmonary fibrosis. *Am J Respir Crit Care Med* 2019;199:1517–1536.
29. Ridge FP, Duszyk M, French AS. A large conductance, Ca2+-activated K+ channel in a human lung epithelial cell line (A549). *Biochim Biophys Acta* 1997;1327:249–258.
30. Jovanović S, Crawford RM, Ranki HJ, Jovanović A. Large conductance Ca2+-activated K+ channels sense acute changes in oxygen tension in alveolar epithelial cells. *Am J Respir Cell Mol Biol* 2003;28:363–372.
31. Follonier L, Schaub S, Meister JJ, Hinz B. Myofibroblast communication is controlled by intercellular mechanical coupling. *J Cell Sci* 2008; 121:3305–3316.
32. Janssen LJ, Mukherjee S, Ask K. Calcium homeostasis and ionic mechanisms in pulmonary fibroblasts. *Am J Respir Cell Mol Biol* 2015;53:135–148.
33. Mukherjee S, Kolb MR, Duan F, Janssen LJ. Transforming growth factor-β evokes Ca2+ waves and enhances gene expression in human pulmonary fibroblasts. *Am J Respir Cell Mol Biol* 2012;46:757–764.
34. Grgic I, Kiss E, Kaistha BP, Busch C, Kloss M, Sautter J, *et al*. Renal fibrosis is attenuated by targeted disruption of KCa3.1 potassium channels. *Proc Natl Acad Sci USA* 2009;106:14518–14523.
35. Roach KM, Duffy SM, Coward W, Feghali-Bostwick C, Wulff H, Bradding P. The K+ channel KCa3.1 as a novel target for idiopathic pulmonary fibrosis. *PLoS One* 2013;8:e85244. [Published erratum appears in *PLoS One* 9(1).]
36. Roach KM, Feghali-Bostwick C, Wulff H, Amrani Y, Bradding P. Human lung myofibroblast TGFβ1-dependent Smad2/3 signalling is Ca(2+)-dependent and regulated by KCa3.1 K(+) channels. *Fibrogenesis Tissue Repair* 2015;8:5.
37. Roach KM, Wulff H, Feghali-Bostwick C, Amrani Y, Bradding P. Increased constitutive αSMA and Smad2/3 expression in idiopathic pulmonary fibrosis myofibroblasts is KCa3.1-dependent. *Respir Res* 2014;15:155.
38. Rahaman SO, Grove LM, Paruchuri S, Southern BD, Abraham S, Niese KA, *et al*. TRPV4 mediates myofibroblast differentiation and pulmonary fibrosis in mice. *J Clin Invest* 2014;124:5225–5238.
39. Huang SK, Scruggs AM, Donaghy J, Horowitz JC, Zaslona Z, Przybranowski S, *et al*. Histone modifications are responsible for decreased Fas expression and apoptosis resistance in fibrotic lung fibroblasts. *Cell Death Dis* 2013;4:e621.
40. Scruggs AM, Koh HB, Tripathi P, Leeper NJ, White ES, Huang SK. Loss of CDKN2B promotes fibrosis via increased fibroblast differentiation rather than proliferation. *Am J Respir Cell Mol Biol* 2018;59:200–214.
41. Xu W, Liu Y, Wang S, McDonald T, Van Eyk JE, Sidor A, *et al*. Cytoprotective role of Ca2+- activated K+ channels in the cardiac inner mitochondrial membrane. *Science* 2002;298:1029–1033.
42. Li Y, Hu H, Tian JB, Zhu MX, O'Neil RG. Dynamic coupling between TRPV4 and Ca^{2+}-activated SK1/3 and IK1 K^{+} channels plays a critical role in regulating the K^{+}-secretory BK channel in kidney collecting duct cells. *Am J Physiol Renal Physiol* 2017;312:F1081–F1089.

43. Earley S, Heppner TJ, Nelson MT, Brayden JE. TRPV4 forms a novel Ca2+ signaling complex with ryanodine receptors and BKCa channels. *Circ Res* 2005;97:1270–1279.
44. Long X, Tharp DL, Georger MA, Slivano OJ, Lee MY, Wamhoff BR, *et al*. The smooth muscle cell-restricted KCNMB1 ion channel subunit is a direct transcriptional target of serum response factor and myocardin. *J Biol Chem* 2009;284:33671–33682.
45. Rodriguez LR, Emblom-Callahan M, Chhina M, Bui S, Aljeburry B, Tran LH, *et al*. Global gene expression analysis in an *in vitro* fibroblast model of idiopathic pulmonary fibrosis reveals potential role for CXCL14/CXCR4. *Sci Rep* 2018;8:3983.
46. Yang IV, Coldren CD, Leach SM, Seibold MA, Murphy E, Lin J, *et al*. Expression of cilium-associated genes defines novel molecular subtypes of idiopathic pulmonary fibrosis. *Thorax* 2013;68:1114–1121.
47. Lindahl GE, Stock CJ, Shi-Wen X, Leoni P, Sestini P, Howat SL, *et al*. Microarray profiling reveals suppressed interferon stimulated gene program in fibroblasts from scleroderma-associated interstitial lung disease. *Respir Res* 2013;14:80.
48. Rabinovich EI, Kapetanaki MG, Steinfeld I, Gibson KF, Pandit KV, Yu G, *et al*. Global methylation patterns in idiopathic pulmonary fibrosis. *PLoS One* 2012;7:e33770.
49. Sanders YY, Ambalavanan N, Halloran B, Zhang X, Liu H, Crossman DK, *et al*. Altered DNA methylation profile in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2012;186: 525–535.
50. Du J, Xie J, Zhang Z, Tsujikawa H, Fusco D, Silverman D, *et al*. TRPM7-mediated Ca2+ signals confer fibrogenesis in human atrial fibrillation. *Circ Res* 2010;106:992–1003.