

Mechanosensitive transient receptor potential vanilloid 4 regulates *Dermatophagoides farinae*-induced airway remodeling *via* 2 distinct pathways modulating matrix synthesis and degradation

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ABSTRACT: Contributions of mechanical signals to airway remodeling during asthma are poorly understood. Transient receptor potential vanilloid 4 (TRPV4), a mechanosensitive ion channel, has been implicated in cardiac and pulmonary fibrosis; however, its role in asthma remains elusive. Employing a *Dermatophagoides farinae*-induced asthma model, we report here that TRPV4 knockout mice were protected from *D. farinae*-induced airway remodeling. Furthermore, lung fibroblasts that were isolated from TRPV4 knockout mice showed diminished differentiation potential compared with wild-type mice. Fibroblasts from asthmatic lung exhibited increased TRPV4 activity and enhanced differentiation potential compared with normal human lung fibroblasts. Of interest, TGF- β 1 treatment enhanced TRPV4 activation in a PI3K-dependent manner in normal human lung fibroblasts *in vitro*. Mechanistically, TRPV4 modulated matrix remodeling in the lung *via* 2 distinct but dependent pathways: one enhances matrix deposition by fibrotic gene activation, whereas the other slows down matrix degradation by increased plasminogen activator inhibitor 1. Of importance, both pathways are regulated by Rho/myocardin-related transcription factor-A and contribute to fibroblast differentiation and matrix remodeling in the lung. Thus, our results support a unique role for TRPV4 in *D. farinae*-induced airway remodeling and warrant further studies in humans for it to be used as a novel therapeutic target in the treatment of asthma.—Gombedza, F., Kondeti, V., Al-Azzam, N., Koppes, S., Duah, E., Patil, P., Hexter, M., Phillips, D., Thodeti, C. K., Paruchuri, S. Mechanosensitive transient receptor potential vanilloid 4 regulates *Dermatophagoides farinae*-induced airway remodeling *via* 2 distinct pathways modulating matrix synthesis and degradation. FASEB J. 31, 000–000 (2017). www.fasebj.org

KEY WORDS: TGF- β 1 • myofibroblasts • PI3K • PAI-1 • fibronectin

Allergic asthma is characterized by chronic inflammation of the airways, bronchial hyper-responsiveness, and airway remodeling (1, 2). Airway remodeling is responsible for the rapid decline in lung function that is experienced in the treatment-resistant asthmatic population compared

with nonasthmatics (3). Characteristic structural changes that occur in the asthmatic lung include thickening of the airway wall, an increase in the number of infiltrating cells, and deposition of an abnormal amount of extracellular matrix (ECM) components beneath the bronchial epithelium. These changes result in the replacement of normal lung parenchyma with scar tissue, which limits the surface of gas exchange and respiratory capacity of patients (4). Whereas airway inflammation is known to play a role in the progression of airway remodeling, the mechanisms that regulate airway remodeling remain poorly understood. Major players that contribute to subepithelial fibrosis in remodeled airways are fibroblasts and myofibroblasts, as they secrete and remodel ECM in the lung. Lung biopsy samples from several asthmatics reveal enhanced fibroblast numbers compared with normal patients, and this enhancement in the submucosal layer is correlated with the extent of thickening of the lamina reticularis (5, 6).

ABBREVIATIONS: BAL, bronchoalveolar lavage; DHLF, diseased human lung fibroblast; ECM, extracellular matrix; EGFP, enhanced green fluorescent protein; FN, fibronectin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin and eosin; KO, knockout; mLF, mouse lung fibroblast; MRTF-A, myocardin-related transcription factor-A; NHLF, normal human lung fibroblast; PAI-1, plasminogen activator inhibitor 1; PAS, periodic acid–Schiff; siRNA, small interfering RNA; α -SMA, α -smooth muscle actin; SRF, serum response factor; TRPV4, transient receptor potential vanilloid 4; WT, wild-type

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TGF- β 1 released from inflammatory cells, epithelial cells, and myofibroblasts promotes lung remodeling by inducing expression of a wide range of ECM components (7). Furthermore, enhanced expression of TGF- β 1, at both transcript and protein levels, was observed in asthmatic airways (8). In addition to soluble factors, such as TGF- β 1, increased mechanical stress has also been implicated in augmented expression of fibronectin (FN), collagen types III and V, and matrix metalloproteinase 9 *in vitro* (9), which suggests that airway remodeling may be promoted by mechanical stress that occurs during repeated episodes of airway obstruction. Supporting these findings, structural remodeling of the airways has been found in children with recurrent wheezing, regardless of their atopic status (10), and an abnormal mechanical environment has been predicted to modify the composition of ECM contributing to fibrosis in the airways (11). The ECM compartment of the airway wall is dynamic, and a shift in this balance toward enhanced accumulation of matrix results in fibrosis, which leads to distorted structure and abnormal mechanical properties.

Transient receptor potential vanilloid 4 (TRPV4) is a stretch-activated calcium channel and a mechanosensor. It is activated by both physical and chemical stimuli, such as temperature, hypotonicity, phorbol esters, endocannabinoids, and arachidonic acid and its metabolites, epoxy eicosatrienoic acids (12–15). In lungs, TRPV4 has been widely known to regulate alveolar barrier function and vascular permeability (16). TRPV4 has also been implicated in ventilator-induced lung injury (17) and has been shown to regulate inflammation and pulmonary function in chemically induced lung injury (18). Furthermore, TRPV4 gene polymorphisms have been shown to influence the development of osmotic airway hyper-responsiveness in patients with bronchial asthma (19). Recently, TRPV4 has been shown to integrate soluble and mechanical signals in the differentiation of cardiac (20) and lung (21) fibroblasts; however, neither the mechanism by which TRPV4 modulates fibroblast differentiation, nor its role in airway remodeling *in vivo* [using TRPV4 knockout (KO) mice] and the underlying molecular mechanism(s) involved has been investigated. Therefore, we attempted to understand the molecular mechanism by which TRPV4 is activated downstream of TGF- β 1 and how TRPV4 activation regulates matrix deposition and fibroblast differentiation as well as its role in the *Dermatophagoides farinae*-mediated model of asthma *in vivo* by employing TRPV4 KO mice.

We observed that *D. farinae*-mediated lung inflammation and remodeling responses were completely attenuated in TRPV4 KO mice compared with corresponding wild-type (WT) mice and, in addition, fibroblasts that were isolated from TRPV4 KO mice exhibited diminished differentiation potential compared with WT. Furthermore, fibroblasts that were isolated from patients with asthma exhibited higher TRPV4 activity and concomitant differentiation potential, as determined by α -smooth muscle actin (α -SMA) expression and enhanced matrix accumulation in response to TGF- β 1. Mechanistically, we identified that TRPV4 translocation to the membrane is enhanced in response to TGF- β 1 *via* PI3K, followed by TRPV4-dependent Rho/myocardin-related transcription

factor-A (MRTF-A) activation. TRPV4/Rho/MRTF-A, in turn, activates p38 MAPK-dependent fibrotic gene expression, which leads to enhanced collagen and FN production as well as plasminogen activator inhibitor 1 (PAI-1) activation, leading to reduced matrix degradation. Both of these events contribute to matrix accumulation and fibroblast differentiation, which contributes to airway remodeling.

MATERIALS AND METHODS

Animals

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) and TRPV4 KO mice (22) were maintained at the Comparative Medicine Unit at Northeast Ohio Medical University. All animal experiments were done in accordance with standard guidelines as approved by the Animal Care and Use Committee of Northeast Ohio Medical University.

Reagents

The following chemicals and reagents were purchased commercially: RN1734 (selective TRPV4 inhibitor) (20, 23), SB203580 (p38 inhibitor, binds to the ATP-binding pocket) (24, 25), and Y27632 dihydrochloride (Rho kinase inhibitor; all from Tocris Bioscience, Minneapolis, MN, USA) (26, 27); LY294002 (PI3K inhibitor; Cayman Chemicals, Ann Arbor, MI, USA) (28, 29); TGF- β 1 (R&D Systems, Minneapolis, MN, USA); PAI-1, phospho-Akt, phospho-p38 MAPK, p38 MAPK, PI3K, lamin, and α -tubulin Abs (Cell Signaling Technology, Danvers, MA, USA); α -SMA antibody and GSK1016790A (Sigma-Aldrich, St. Louis, MO, USA); FN Ab (Abcam, Cambridge, MA, USA); MRTF-A Ab (Santa Cruz Biotechnology Santa Cruz, CA, USA); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fitzgerald, Acton, MA, USA); all secondary Abs (Jackson ImmunoResearch, West Grove, PA, USA); Fluo-4 (Thermo Fisher Scientific, Waltham, MA, USA); nontargeting small interfering RNA (siRNA) and specific siRNA for TRPV4 (Dharmacon, Lafayette, CO, USA); specific siRNA for PI3K and p38 (Cell Signaling Technology); siLentFect lipid reagent (Bio-Rad, Hercules, CA, USA); RhoA G-LISA activation kit (Cytoskeleton, Denver, CO, USA), Diff Quik Stain Set (Siemens, Newark, DE, USA); subcellular protein fractionation kit for cultured cells (Thermo Fisher Scientific); and transcript first strand cDNA synthesis kit and light cycler 480 SYBR Green I Master Mix (Roche, Indianapolis, IN, USA).

D. farinae-induced airway remodeling

House dust mite (*D. farinae*) protein extract was purchased from Greer Laboratories (Lenoir, NC, USA). *D. farinae* extract was resuspended in PBS (Corning, NY, USA) at a concentration of 2 mg/ml. After isoflurane treatment, 6- to 8-wk-old WT and TRPV4 KO mice received *D. farinae* (25 μ g /animal) or saline intranasally $3 \times$ /wk for 5 wk. Mice were euthanized 24 h after the last intranasal instillation and the extent of lung remodeling was assessed. Bronchoalveolar lavage (BAL) fluid was collected and cellular content was analyzed as previously described (30). After BAL fluid collection, total cell number was determined by trypan blue staining. Differential cell count was analyzed using Diff Quik Stain Set. In brief, BAL fluid was cytospun (Cytospin; Thermo Fisher Scientific) onto glass slides and stained with Diff Quik Stain Set according to manufacturer protocol. A minimum of 300 cells were counted and identified as macrophages, lymphocytes, or polymorphonuclear leukocytes (eosinophils and

neutrophils) on the basis of morphologic criteria. The number of differential cells in the BAL fluid was determined from total cell counts and expressed per milliliter of BAL fluid. For histology, lungs from mice were embedded in paraffin, sectioned, and stained for hematoxylin and eosin (H&E) to monitor infiltrated cells, periodic acid–Schiff (PAS) to visualize goblet cells, or trichrome to detect collagen. PAS-positive cells (goblet cells) were identified and enumerated along the airway and expressed as goblet cells/100 μm of airway. For airway wall thickness measurements, morphometric data were obtained by image analysis of H&E-stained airways using the cellSens1.12 software calibrated for 0.01 μm (Olympus, Center Valley, PA, USA) on a computer connected to a light microscope (Olympus SC30) coupled to a digital camera. Three airways were analyzed per slide and airway wall thickness was measured at 4 predetermined sites around the airway. Airway wall thickness, the space between the airway lumen and smooth muscle layer, was measured in micrometers.

Cell culture

Validated primary normal human lung fibroblasts (NHLFs; CC-2512) and diseased [asthma; male, 27 y old, diagnosed at age 7 and on medication (Proventil, albuterol; Merck, Darmstadt, Germany)] human lung fibroblasts (DHLFs; 00194912) were obtained from Lonza (Walkersville, MD, USA). Cells from the same passage for both NHLFs and DHLFs were cultured in DMEM (Corning) that was supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA), 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine (Thermo Fisher Scientific), and maintained at 37°C in a humidified 5% CO₂ environment. Both NHLFs and DHLFs were used below passage 8 and comparable passages were used in different experiments. Primary mouse lung fibroblasts were isolated from 10-wk-old WT and TRPV4 KO mice. In brief, lungs were removed aseptically, minced into ~1 mm pieces, and incubated with agitation in 0.2% trypsin and 0.2% collagenase in DMEM that was supplemented with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine for 30 min. The resulting cell suspension was filtered with a cell strainer (40 μm ; BD Biosciences, San Jose, CA, USA) and centrifuged at 500 g for 5 min. Cell pellet was washed, resuspended, and cultured in DMEM (10% fetal bovine serum, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine) in 25 cm² tissue culture flasks and maintained at 37°C in a humidified 5% CO₂ environment.

Cell activation and treatment

NHLF and/or DHLF cells were pretreated with inhibitors RN1734 (10–50 μM), LY294002 (10–100 μM), Y27632 (5–25 μM), and SB203580 (1–20 μM) for 30 min, followed by stimulation with TGF- β 1 (2 ng/ml) for the indicated time points (48 h for α -SMA expression; 24, 48, 72, and 96 h for FN expression; 48 and 72 h for PAI-1 expression; 24 h for MRTF-A expression; 15, 30, and 60 min for phosphorylation of Akt; and 30 min for phosphorylation of p38 MAPK). Transfection of isoform-specific siRNA smart pool constructs from Dharmacon (TRPV4) or Cell Signaling Technology (PI3K α and P38 MAPK) were carried out with siLentFect transfection reagent for 48 h according to manufacturer protocol.

Calcium flux

NHLF and DHLF cells were cultured on MatTek glass-bottomed dishes to confluency (100,000 cells) and were loaded with Fluo-4/AM (4 μM) for 20 min, washed 3 times, resuspended in calcium medium (136 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.1 mM CaCl₂, 1.2 mM KH₂PO₄, 5 mM NaHCO₃, 5.5 mM glucose,

and 20 mM HEPES, pH 7.4), and subjected to an inverted Olympus IX 70 confocal microscope (FV300). Fluo-4/AM was excited at 488 nm and emission signals were collected at 517 nm. Cells were stimulated with GSK1016790A (100 nM) in the presence or absence of RN1734 (30 μM) and images were acquired every 3 s for 300 s and analyzed by using Olympus Fluoview software and Microsoft Excel. The ratio F/F₀ represents the fluorescence signal over 300 s to the (initial) fluorescence signal at 0 s (31). Fluorescent signal of at least 10 respondent cells per field was analyzed, and the average was expressed as F/F₀. Differences in the peak fluorescence signal and the initial fluorescence were calculated and expressed as $\Delta F/F_0$.

Cell lysates and Western blotting

After stimulation with the respective agonists, fibroblasts (7.5×10^4) were lysed with lysis buffer (BD Bioscience) that was supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Pierce, Rockford, IL, USA). Immunoblotting was performed as previously described (32). Western blot was incubated with ECL and protein bands were visualized by using an imager (ProteinSimple, San Jose, CA, USA) and quantified using AlphaView SA (ProteinSimple). Blots were stripped and reprobed with GAPDH or α -tubulin Ab.

Real-time quantitative PCR

Expressions of α -SMA, collagen 1A1, FN, MRTF-A, SM22, and PAI-1 transcripts in fibroblasts and FN, collagen 1A1, MUC5AC, Gob5, TGF β , TNF α , IL-5, and IL-13 transcripts in the lung tissue were determined with real-time PCR performed on Light Cycler 480 (Roche). Total RNA was isolated from fibroblasts after respective treatments with an E.Z.N.A. Total RNA Kit 1 (Omega Bio-Tek, Norcross, GA, USA). RNA from tissues was extracted by Trizol-chloroform, and DNase contamination was removed by using Ambion DNA-free DNA Removal Kit (Thermo Fisher Scientific) according to manufacturer instructions. cDNA was synthesized by using cDNA synthesis kit from Roche. Real-time PCR was performed. Levels of respective genes relative to GAPDH were analyzed and $\Delta\Delta C_t$ values were calculated and expressed as relative expression or fold change compared with control. Quality of the RNA, primers, and real time PCR reaction was validated by using proper controls, such as no RT control and water control. Real-time PCR for each sample was performed at least in triplicate and repeated in 3 different experiments.

The following primers were used: α -SMA: forward: 5'-CGGGACATCAAGGAGAACT-3', reverse: 5'-CCATCAGGCAACTCGTAACT-3'; hCOLLAGEN1A1: forward: 5'-CGATGGATTCCAGTTCGAGTATG-3', reverse: 5'-CTTGCAAGTGGT-3'; hFN: forward: 5'-ACAACACCGAGGTGACTGAGAC-3', reverse: 5'-GGACACAACGATGCTTCCTGAG-3'; hSM22: forward: 5'-TGGAGATCCCAACTGGTTTAT-3', reverse: 5'-CCCATCTGAAGGCCAATG-3'; hMRTF-A: forward: 5'-GGCTATGAGGAAGCCATGAG-3', reverse: 5'-CTCCGCTCTGAATGAGAATGT-3'; hPAI-1: forward: 5'-CATCCCCCTCTGACTACGTGG-3', reverse: 5'-CCCCATAGGGTGAGAAACCA-3'; hTRPV4: forward: 5'-TCACTCTCACCGCTACTACCA-3', reverse: 5'-CCCAGTGAAGAGCGTAATGACC-3'; hGAPDH: forward: 5'-TGCACCACCAACTGCTTAGC-3', reverse: 5'-GGCATGGACTGTGGTCATGAG-3'; mFN: forward: 5'-CACCTTCAGTAGAAGGCAGTAG-3', reverse: 5'-GTTCTCCTCCACAGCATAGATAG-3'; mCOLLAGEN1A1: forward: 5'-GAAACCCGAGGTATGCTTGA-3', reverse: 5'-GTTGGGACAGTCCAGTTCTT-3'; mMUC5AC: forward: 5'-CCATGCAGAGTCCTCAGAACAA-3', reverse: 5'-TTACTGGAAAGGCCCAAGCA-3'; mGob5: forward:

5'-ACTAAGGTGGCCTACCTCCAA-3', reverse: 5'-GGA-GGTGACAGTCAAGGTGAGA-3'; mTGF- β : forward: 5'-CTTTAGGAAGGACCTGGGTTG-3', reverse: 5'-GTGTGTCC-AGGCTCCAAATA-3'; mTNF: forward: 5'-CTATGTCTCA-GCCTCTTCTCATTC-3', reverse: 5'-GAGGCCATTGGGAA-CTTCT-3'; mIL-5: forward: 5'-AGGATGCTTCTGCACTTGAG-3', reverse: 5'-AGCTGTGTCAAGGTCTCTTTC-3'; mIL-13: forward: 5'-AACGGCAGCATGGTATGGAGT-3', reverse: 5'-TGGGTCCTGTAGATGGCATTG-3'; m GAPDH: forward: 5'-CTCCCACTCTTCCACCTTCG-3', reverse: 5'-CCACCACC-CTGTTGCTGTAG-3'.

siRNA transfection

NHLF cells were transfected with 20 nM TRPV4-specific siRNA (Dharmacon), 100 nM PI3K p110 α siRNA (Cell Signaling Technology; SignalSilence PI3 Kinase p110 α siRNA), 100 nM p38 MAPK siRNA (Cell Signaling Technology; SignalSilence p38 MAPK siRNA), or nonspecific siRNA (negative control; Dharmacon). Transfection was carried out with siLentFect transfection reagent (Bio-Rad) according to manufacturer instructions. Post-transfection, cells were treated as previously mentioned.

TRPV4 siRNA sequences are as follows: TRPV4 siGenome SmartPool: target sequence 1: GAACCCGUGUGCCAACAUG, target sequence 2: CAACCGGCCUAUCCUCUUU, target sequence 3: GACCAAAUCUGCGCAUGAA, and target sequence 4: GCACACCGCCGUACCCUUA; and non-targeting siGenome Pool#2 siRNA.

TRPV4 membrane translocation and analysis

NHLF cells were transfected with TRPV-enhanced green fluorescent protein (EGFP) (2 μ g) (22) or EGFP (Thermo Fisher Scientific) alone using siLentFect transfection reagent according to manufacturer instructions. Post-transfection, cells were pre-incubated with 50 μ M PI3K inhibitor (LY) for 30 min and stimulated with TGF- β 1 (2 ng/ml). Images were obtained at 0, 0.5, and 2 h by using EVOS fluorescence microscope ($\times$ 20 objective; Thermo Fisher Scientific). Translocation was quantified by counting the number of cells with TRPV4-EGFP at the membrane.

RhoA activation assay

RhoA activation assay was performed on NHLF cell lysates by using the G-LISA RhoA activation assay biochem kit (Cytoskeleton) according to manufacturer instructions. Rho activation was determined by absorbance and measured by using Epoch Bio-Tek microplate reader. Total Rho was determined from cell lysates by Western blotting with total Rho Ab.

MRTF-A nuclear translocation

Subcellular protein fractions were extracted from cultured NHLF cells by using the subcellular protein fractionation kit (Thermo Fisher Scientific) according to manufacturer protocol. Cytoplasmic and nuclear fractions were analyzed by using Western blotting. Purity and equal loading of nuclear fractions were assessed by lamin Ab.

Statistical analysis

Blots presented are representative of 3 experiments performed and data are expressed as means $\pm$ SEM from at least 3 experiments except where otherwise indicated. Significance was

determined using 1-way ANOVA and comparisons between the groups were determined by Tukey's multiple comparisons test (GraphPad Prism 7.01; GraphPad Software, La Jolla, CA, USA).

RESULTS

TRPV4 mediates *D. farinae*-induced airway remodeling *in vivo* and fibroblast differentiation *in vitro*

To determine the role of TRPV4 in *D. farinae*-induced inflammation and airway remodeling *in vivo*, WT and TRPV4 KO mice were subjected to intranasal inhalation of either saline or *D. farinae* antigen (25 μ g) 3 times/wk for 5 wk. Exposure to *D. farinae* resulted in the infiltration of immune cells to the lung, as determined by H&E staining, in WT mice; however, this effect was significantly attenuated in TRPV4 KO mice (Fig. 1A). This result was further confirmed by analyzing BAL fluid for the number of infiltrated cells to the lung upon *D. farinae* treatment, (Supplemental Fig. 1A). Differential cell count analysis demonstrated increased recruitment of lymphocytes and polymorphonuclear leukocytes to the lung in response to *D. farinae* challenge in WT mice compared with TRPV4 KO mice. Morphometric analysis of H&E-stained airways revealed enhanced airway wall thickening with *D. farinae* treatment in WT mice, but not TRPV4 KO mice (Fig. 1B). We also observed increased expression of TGF- β (essential for lung remodeling; Fig. 1C), IL-13 (important for goblet cell metaplasia and AHR; Supplemental Fig. 1B), IL-5 transcript (crucial for eosinophilia; Supplemental Fig. 1C), and TNF α (Supplemental Fig. 1D) transcripts only in lung tissues of WT mice in response to dust mite treatment. Furthermore, we observed an increase in the number of PAS-positive goblet cells in *D. farinae*-challenged WT mice compared with saline-treated mice. Of importance, the number of these cells was significantly lower in *D. farinae*-challenged TRPV4 KO mice (Fig. 1D, E). We next determined the role of TRPV4 on the expression of mucin gene transcripts, Gob5 and MUC5AC (Fig. 1F, G). In agreement with PAS staining, we observed marked up-regulation of MUC5AC and Gob5 transcripts with *D. farinae* challenge only in WT mice and not in TRPV4 KO mice, which suggests a vital role for TRPV4 in lung remodeling in response to *D. farinae*. As collagen deposition is also a hallmark of airway remodeling during asthma, lung sections from the above-mentioned treatments were further stained with trichrome and the extent of collagen deposition was analyzed. Again, *D. farinae*-challenged lung tissues exhibited intense trichrome staining (Fig. 1H) and increased collagen transcript in WT mice and not in TRPV4 KO mice (Fig. 1I). Of interest, WT, but not TRPV4 KO, mice treated with *D. farinae* also exhibited augmented expression of another matrix protein, FN, both at transcript (Fig. 1J) and protein levels (Fig. 1K). Taken together, these data suggest that TRPV4 KO mice were protected from *D. farinae*-induced inflammation and matrix remodeling, which indicates a role for TRPV4 in modulating lung remodeling. Total IgE levels were increased in all mouse groups treated with *D. farinae* compared with saline treated groups as reported earlier by other groups (33, 34).

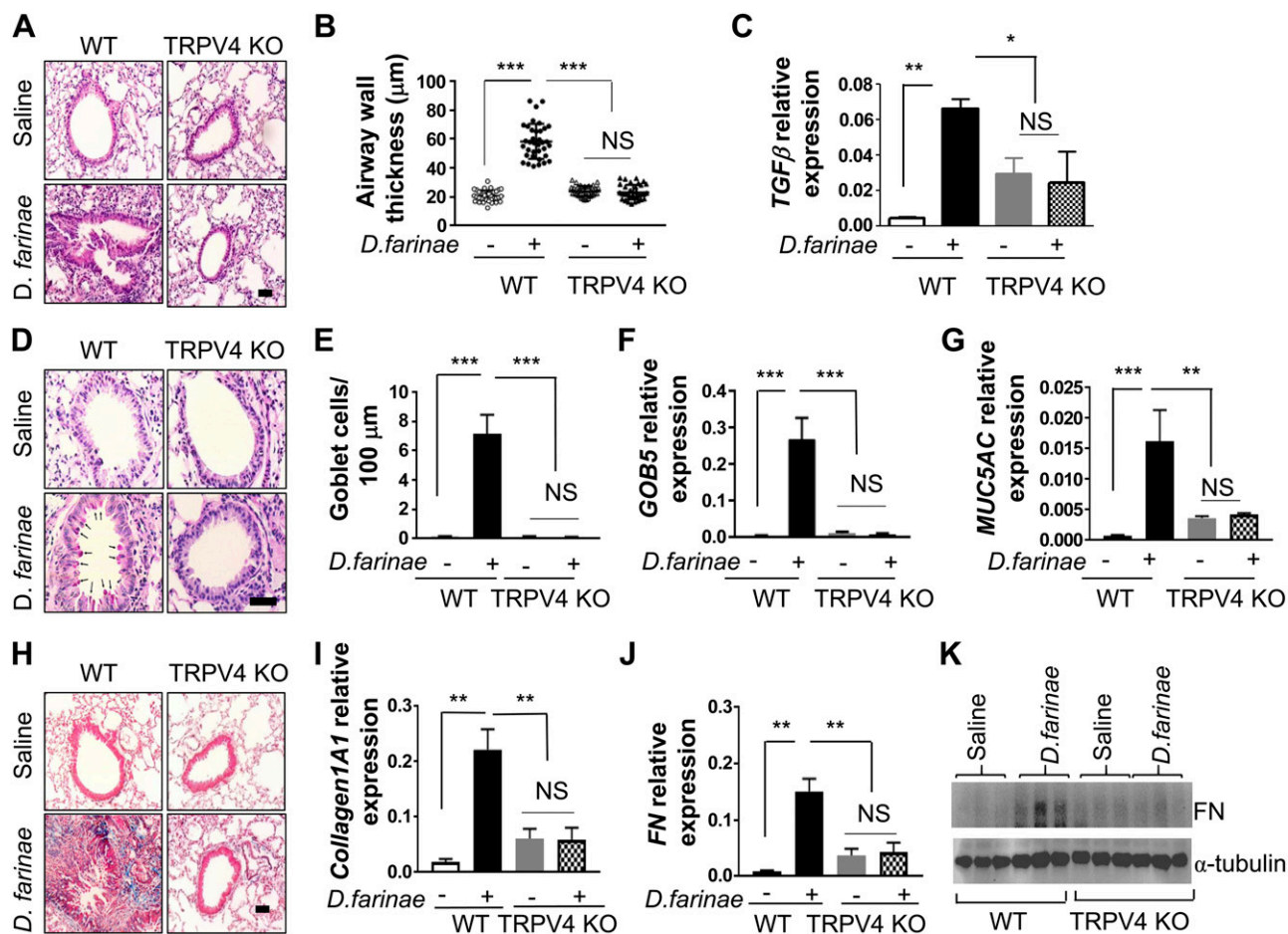


Figure 1. TRPV4 KO mice are protected from *D. farinae*-induced airway remodeling. WT and TRPV4 KO mice were treated with saline or *D. farinae* (25 μg/animal) intranasally 3 times/wk for 5 wk. Mice were euthanized 24 h after the final challenge. Paraformaldehyde-fixed lung sections (4 μm) were used for the analysis of pulmonary inflammation and remodeling. **A)** Representative photomicrographs of H&E-stained lung tissue. Original magnification, ×20. **B)** Morphometric analysis of airway thickness in H&E-stained sections, evaluated without the knowledge of the treatment groups. Three airways were analyzed per slide (per mouse) and airway wall thickness, the space between the airway lumen and smooth muscle layer was measured at 4 predetermined sites around the airway in micrometers. **C)** Expression of TGF-β1 transcripts normalized to GAPDH as determined by real-time PCR of whole lung RNA extracted from the indicated groups. **D)** Representative photomicrographs of PAS-stained lung sections showing goblet cells (arrows; original magnification, ×40). **E)** Morphometric analysis of goblet cells, evaluated as the numbers of PAS-positive goblet cells per 100 μm of airway. **F, G, I, J)** Expression of Gob5 (**F**), MUC5AC (**G**), collagen1A1 (**I**), and FN (**J**) transcripts normalized to GAPDH as determined by real-time PCR of whole lung RNA extracted from the indicated groups. **H)** Representative photomicrographs of Masson's trichrome-stained lung sections depicting collagen (original magnification, ×20). **K)** Representative SDS-PAGE immunoblot of total lung protein lysate from indicated treatments and probed for FN. The blot was stripped and reprobed with tubulin Ab. Blots were quantified by using AlphaView software. Scale bars, 100 μm. Results are means ± SEM from 3 independent experiments (**B, C, E–G, I, J**). NS, not significant. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

However, there was no difference in IgE levels between *D. farinae*-treated WT mice and TRPV4 KO mice (data not shown).

As we observed increased TGF-β1 transcript levels and enhanced remodeling changes in response to *D. farinae*, and we know that matrix remodeling is regulated primarily by differentiated fibroblasts, we asked whether preserved lung remodeling effects observed in TRPV4 KO mice were dependent on differentiation of fibroblasts. To address this, we isolated mouse lung fibroblasts (mLFs) from WT and TRPV4 KO mice and analyzed their differentiation potential (α-SMA expression) and ability to synthesize ECM protein, FN, in response to TGF-β1. TGF-β1 treatment robustly increased the expression of α-SMA

and FN in a time-dependent manner in WT mLFs; however, this response was completely attenuated in TRPV4 KO fibroblasts (**Fig. 2A**), which suggests that the absence of TRPV4 in fibroblasts may result in attenuated remodeling seen *in vivo* in response to *D. farinae*. We then asked if stimulation of fibroblasts with TGF-β1 could increase the expression and activity of TRPV4. We found that NHLFs that were exposed to TGF-β1 (2 ng/ml) had enhanced TRPV4 transcripts in a time-dependent manner up to 24 h (~5-fold) and a corresponding increase in TRPV4 protein (data not shown). To further assess TRPV4 activity in these cells, NHLFs that were treated with TGF-β1 for 24 h were loaded with Fluo-4 dye and calcium influx in response to a specific TRPV4 agonist, GSK1016790A (100 nM), was

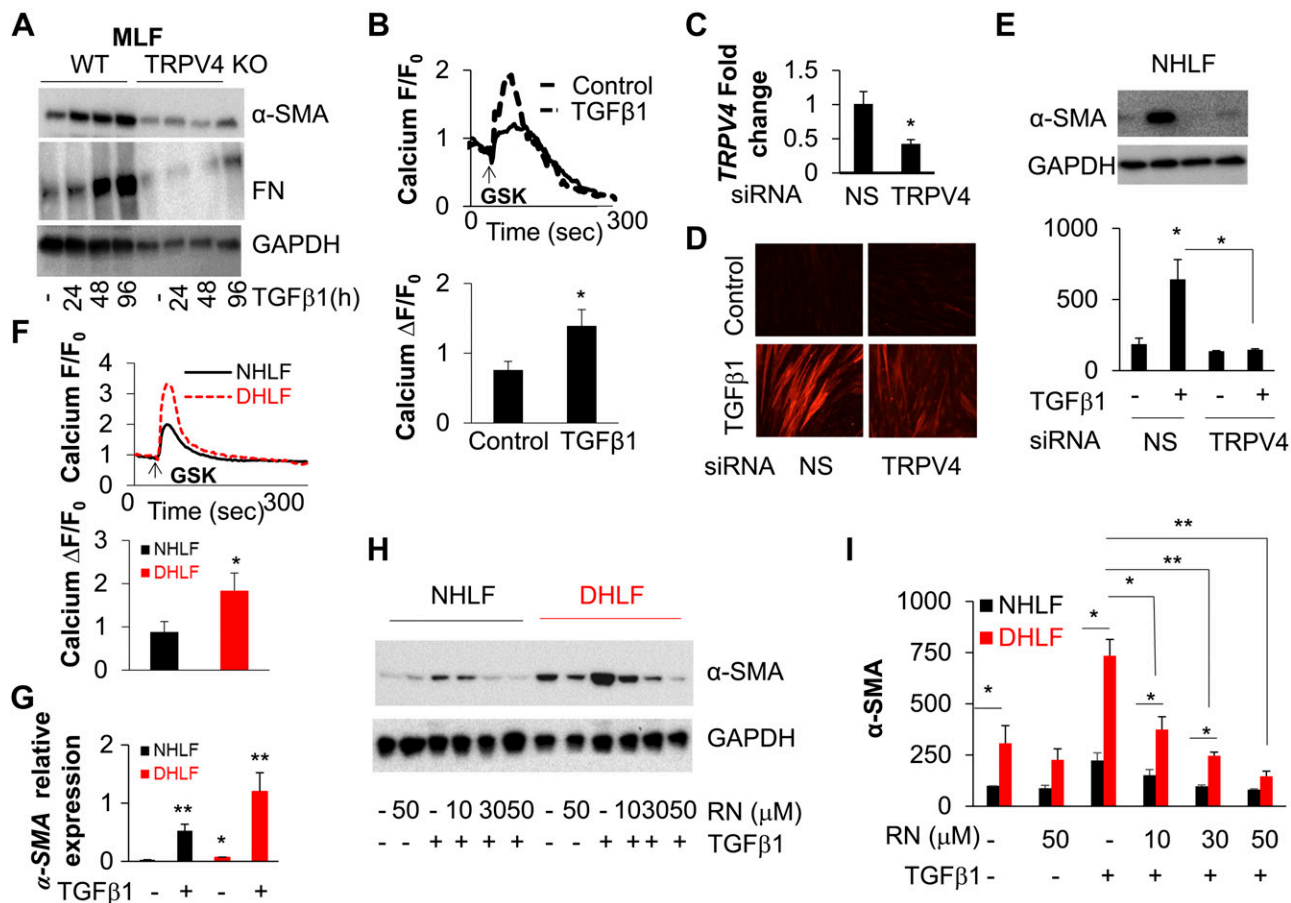


Figure 2. TRPV4 is required for TGF- β 1-mediated differentiation of NHLFs and DHLFs. **A)** mLFs isolated from WT and TRPV4 KO mice were treated with TGF- β 1 (2 ng/ml) for 24, 48, and 96 h and expression of α -SMA and FN were analyzed by Western blotting. Note that time-dependent TGF- β 1 response seen in WT fibroblasts was attenuated in TRPV4 KO fibroblasts. **B)** Effect of TGF- β 1 treatment on TRPV4-dependent calcium flux in NHLFs. NHLFs were loaded with Fluo-4 and calcium flux was measured as described in Materials and Methods in response to a specific TRPV4 activator, GSK1016790A (100 nM), and quantified. **C–E)** TRPV4 protein was knocked down in NHLFs by transfecting them with siRNA against TRPV4 (20 nM). NHLFs transfected with nonspecific (NS) siRNA were used as control. Twenty-four hours after transfection, NHLFs were treated with TGF- β 1 (2 ng/ml; 48 h). **C)** TRPV4 transcript was analyzed by real-time PCR. **D)** α -SMA localization was determined by immunofluorescence (red). **E)** α -SMA expression was analyzed by Western blotting and quantified. **F)** TRPV4-mediated calcium flux in NHLFs and DHLFs. Calcium flux, indicative of TRPV4 activity, was analyzed in NHLFs and DHLFs from same passage in response to GSK and quantified. **G–I)** Differential responses of NHLFs and DHLFs to TGF- β 1. **G)** α -SMA transcript levels both at basal and in response to TGF- β 1 (2 ng/ml; 48 h) were analyzed by real-time PCR in NHLFs and DHLFs. **H, I)** Effect of RN1734, a selective TRPV4 antagonist on TGF- β 1-induced fibroblast differentiation in NHLFs and DHLFs. Both NHLFs and DHLFs were stimulated with or without TGF- β 1 (2 ng/ml; 48 h) in the presence or absence of a TRPV4 inhibitor RN1734 (10, 30, and 50 μ M), and α -SMA protein expression was determined by Western blotting. Blots were stripped and reprobed with GAPDH Ab. Blots were quantified using AlphaView software. Results are means $\pm$ SEM from 3 independent experiments (**B, C, E–G, I**). * P < 0.05; ** P < 0.01.

measured (31, 35). TGF- β 1-treated NHLFs exhibited a robust increase in calcium flux in response to GSK1016790A compared with untreated NHLFs (Fig. 2B), which indicates that TGF- β 1 treatment can enhance the activity of TRPV4. To ascertain the role of TRPV4 in the differentiation of human lung fibroblasts, NHLFs were pretreated with increasing concentrations of TRPV4 antagonist, RN1734 (0–50 μ M), stimulated with TGF- β 1, and analyzed for α -SMA expression. To examine whether RN1734 intervention is selective, we analyzed its ability to block calcium flux in NHLFs stimulated with specific TRPV4 agonist, GSK1016790A. Pretreatment with RN1734 not only attenuated GSK1016790A-mediated calcium flux (Supplemental Fig. 2A, B), but also inhibited TGF- β 1-induced α -SMA incorporation into stress fibers

(Supplemental Fig. 2C) and expression (Fig. 2H, I) significantly. Finally, we knocked down TRPV4 expression in NHLFs by using specific TRPV4 siRNAs and analyzed differentiation potential of NHLFs in response to TGF- β 1. TRPV4 siRNA-transfected cells showed $\sim$ 60% reduction in TRPV4 expression compared with nonspecific (NS) siRNA treatment (Fig. 2C) and a corresponding reduction in α -SMA incorporation into stress fibers (Fig. 2D) and α -SMA protein expression (Fig. 2E). Taken together, these results demonstrate that TRPV4 channels are required for TGF- β 1-induced NHLF differentiation as reported earlier (20, 21). Furthermore, GSK1016790A modestly enhanced α -SMA expression in NHLF (Supplemental Fig. 2D) compared with TGF- β 1. We believe that fibroblast differentiation requires signals from both

mechanical stiffness and soluble factor (TGF- β), and TRPV4 integrates these 2 signals as has been suggested previously (20).

DHLFs express enhanced TRPV4 activity and fibroblast differentiation in response to TGF- β 1

To further evaluate the role of TRPV4 in asthma, we examined TRPV4 activity and TGF- β 1-induced differentiation potential in fibroblasts obtained from normal human lungs (NHLFs) and patients suffering from asthma exacerbation (DHLFs; validated from Lonza). First, we examined the activity of TRPV4 in NHLFs and DHLFs by loading cells with Fluo-4 dye and measuring calcium influx in response to GSK1016790A (31, 35). As shown in Fig. 2F, GSK1016790A induced a robust increase in calcium influx in DHLFs compared with NHLFs, which indicated enhanced TRPV4 activity in DHLFs. Furthermore, TGF- β 1 treatment induced significantly higher differentiation of DHLFs to myofibroblasts compared with NHLFs, as evidenced by an increase in α -SMA transcript (Fig. 2G) and protein levels (Fig. 2H, I). Of importance, pretreatment of cells with RN1734 significantly inhibited TGF- β 1-induced differentiation in both NHLFs and DHLFs (Fig. 2H, I). These results demonstrate that DHLFs exhibit enhanced TRPV4 activity and associated enhanced differentiation potential.

PI3K acts upstream of TRPV4, whereas p38 and Rho are activated downstream of TRPV4 in response to TGF- β 1 in NHLF differentiation

Next, we sought to investigate the molecular mechanism by which TRPV4 is activated downstream of TGF- β 1 and how TRPV4 mediates fibroblast differentiation. First, we examined differentiation of NHLFs in the presence of a range of selective inhibitors that can block known differentiation pathways. We observed that PI3K inhibitor LY294002 (50 μ M), p38 MAPK inhibitor SB203580 (10 μ M), and Rho kinase inhibitor Y27632 (10 μ M) inhibited TGF- β 1-induced fibroblast differentiation in a dose-dependent manner (Fig. 3A–C). Furthermore, as pharmacologic inhibitors have several off-target effects, we knocked down PI3K α expression (Fig. 3D) and p38 expression (Fig. 3E) in NHLFs using specific PI3K α and p38 MAPK siRNAs, respectively, and analyzed their differentiation potential in response to TGF- β 1. PI3K siRNA-transfected cells showed $\sim$ 65% reduction in PI3K expression compared with nonspecific (NS) siRNA treatment (Fig. 3D) and a corresponding reduction in α -SMA. Similarly, p38 MAPK siRNA-transfected cells showed $\sim$ 80% reduction in p38 MAPK expression compared with nonspecific (NS) siRNA treatment (Fig. 3E) and an equivalent reduction in α -SMA expression, which suggests that both PI3K and p38 play a vital role in TGF- β 1-induced differentiation. In accordance, we observed that TGF- β 1 induced phosphorylation of AKT (indicative of PI3K activation; Fig. 3F), p38 MAPK phosphorylation (Fig. 3G), and activation of Rho (Fig. 3J). Time points for

TGF- β 1-induced p38 MAPK phosphorylation and Rho activation were picked on the basis of extensive time course experiments (data not shown). Of interest, TRPV4 inhibitor, RN1734, inhibited TGF- β 1-mediated p38 MAPK phosphorylation and Rho activation, but not Akt phosphorylation. These findings suggest that TGF- β 1 activates p38 MAPK and Rho downstream of TRPV4 and PI3K upstream of TRPV4. To explore whether PI3K acts upstream of TRPV4 in response to TGF- β 1, we expressed EGFP-tagged TRPV4 in NHLFs and its translocation to the membrane (indicative of TRPV4 activation) was analyzed in the presence or absence of LY294002. We found distinct translocation of EGFP-TRPV4 to the plasma membrane in response to TGF- β 1 in NHLFs, which was significantly inhibited in the presence of LY294002 (Fig. 3H, I), suggesting that TGF- β 1 activates TRPV4 *via* PI3K-dependent pathway.

TRPV4 regulates matrix accumulation in response to TGF- β 1 *via* up-regulation of fibrotic genes

Enhanced ECM build-up and surface stiffness are characteristics of lung remodeling as a consequence of fibroblast differentiation. To determine how TRPV4 regulates myofibroblast differentiation and airway remodeling in asthma, we first asked whether TRPV4 contributes to the enhanced matrix accumulation in response to TGF- β 1 by examining 2 important matrix proteins, α 1 type 1 collagen (collagen1A1) and FN. We found that TGF- β 1 promoted time-dependent up-regulation of both collagen1A1 and FN transcripts in a TRPV4-dependent manner (Fig. 4A, B). Although it is not surprising that we found increased transcript of collagen1A1, a common TGF- β 1-responsive fibrotic gene, it is intriguing that FN transcript was increased as well. Of more importance, both collagen1A1 and FN were sensitive to TRPV4 inhibition; therefore, we sought to confirm whether FN protein expression was augmented by TGF- β 1 *via* TRPV4 as well. We found significant increase in expression of FN protein by TGF- β 1, as determined by both immunofluorescence (Fig. 4C) and Western blotting (Fig. 4D, E), which was inhibited by RN1734. Furthermore, DHLFs exhibited higher basal collagen1A1 and FN transcripts (Fig. 4F, G) and FN protein (Fig. 4H, I) compared with NHLFs upon stimulation with TGF- β 1. In addition, FN expression induced by TGF- β 1 in both NHLFs and DHLFs was sensitive to TRPV4 inhibition by RN1734 (Fig. 4H, I).

To understand how TRPV4 mediates fibroblast differentiation and increases matrix accumulation, we analyzed the transcriptional expression of SM22, a fibrotic gene (in addition to α -SMA) and another profibrotic transcription factor, MRTF-A. TGF- β 1 is known to enhance the expression of several profibrotic markers in inducing differentiation (36). We observed an increase in SM22 transcript in response to TGF- β 1, which was attenuated by RN1734 (Fig. 5A). Furthermore, DHLFs expressed higher basal SM22 transcript than did NHLFs (Fig. 5B). We next focused on MRTF-A, a mechanosensitive transcription factor that is known to be activated in response to stress

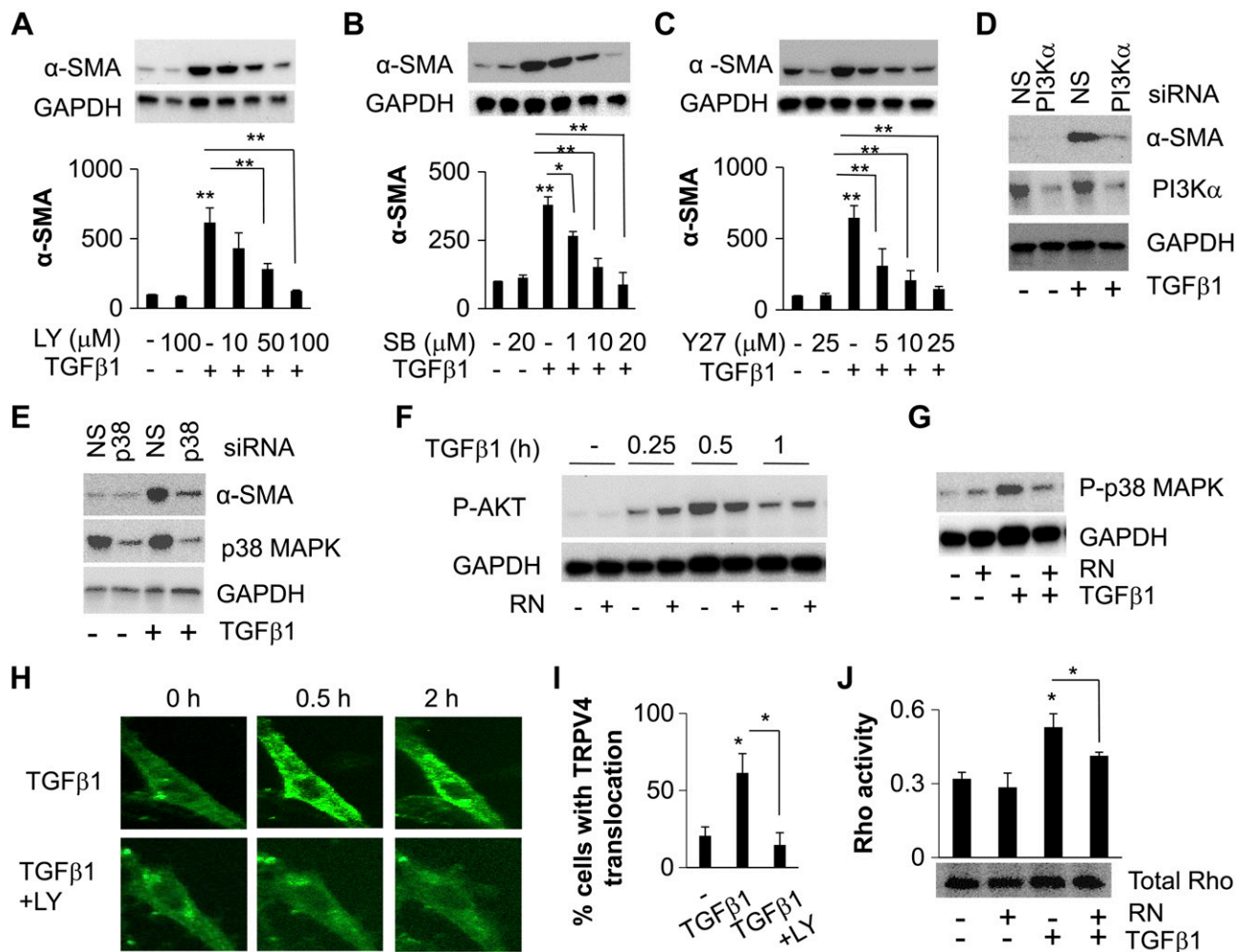


Figure 3. PI3K mediates TGF- β 1-dependent activation of TRPV4, whereas p38 and Rho are activated downstream of TRPV4. A–C) NHLF cells were preincubated with indicated concentrations of PI3K inhibitor (LY294002; A), p38 MAPK inhibitor (SB203580; B), and Rho kinase inhibitor (Y27632; C) for 30 min, followed by treatment with TGF- β 1 (2 ng/ml; 48 h), and α -SMA was determined by Western blotting. Note that all the 3 inhibitors blocked TGF- β 1-induced α -SMA induction. D, E) PI3K P110 α protein (D) and p38 MAPK (E) was knocked down in NHLFs by transfecting them with corresponding siRNA (100 nM). NHLFs transfected with nonspecific (NS) siRNA were used as control. Twenty-four hours after transfection, NHLFs were treated with TGF- β 1 (2 ng/ml; 48 h) and α -SMA expression was analyzed by Western blotting. F, G, J) Effect of TRPV4 antagonist on the activation of AKT, p38 MAPK, and Rho. NHLFs were preincubated with RN1734 (30 μ M) and treated with TGF- β 1 for indicated time points. Western blot analysis showing the phosphorylation of AKT (F) and phosphorylation of p38 MAPK (30 min; G) and Rho activation (J). Rho activity was measured by RhoA activation assay kit (Cytoskeleton) 60 min after TGF- β 1 stimulation. Total Rho in the lysates was determined by Western blotting. Blots were stripped and reprobed with GAPDH Ab and quantified by using AlphaView software. H, I, J) Fluorescence images showing the effect of PI3K inhibitor on TGF- β 1-mediated TRPV4 membrane translocation. NHLFs were transfected with GFP-tagged TRPV4 construct and translocation of TRPV4 to the membrane was analyzed in response to TGF- β 1 treatment (2 ng/ml) with or without LY (50 μ M) over time (0, 0.5, and 2 h). Number of cells with TRPV4-GFP at the plasma membrane was counted without knowledge of the treatment groups and expressed as percentage. Results are means $\pm$ SEM from at least 3 independent experiments (A–C, I, J). * P < 0.05; ** P < 0.01.

fiber formation *via* Rho (37). Of interest, TGF- β 1 enhanced MRTF-A expression in a TRPV4-sensitive manner, at both transcript and protein levels (Fig. 5C, D). Furthermore, DHLFs expressed higher basal MRTF-A expression (Fig. 5E). When activated, MRTF-A is localized to nucleus; therefore, we also analyzed MRTF-A expression in the nuclear fraction. We found that TGF- β 1 enhanced MRTF-A in the nuclear fraction, which was sensitive to TRPV4 inhibition (Fig. 5F). To determine whether MRTF-A is activated downstream of Rho, we pretreated NHLFs with Y27 (a selective Rho inhibitor), stimulated with TGF- β 1,

and analyzed MRTF-A expression. Indeed, TGF- β 1-mediated NHLF differentiation was significantly attenuated in the presence of Y27632 (Fig. 5G).

TRPV4 controls PAI-1 expression

Because matrix accumulation is a balance between matrix synthesis and degradation, and our results validated that TRPV4 enhances both fibrotic gene expression and matrix synthesis in response to TGF- β 1, we investigated whether TRPV4 can regulate matrix

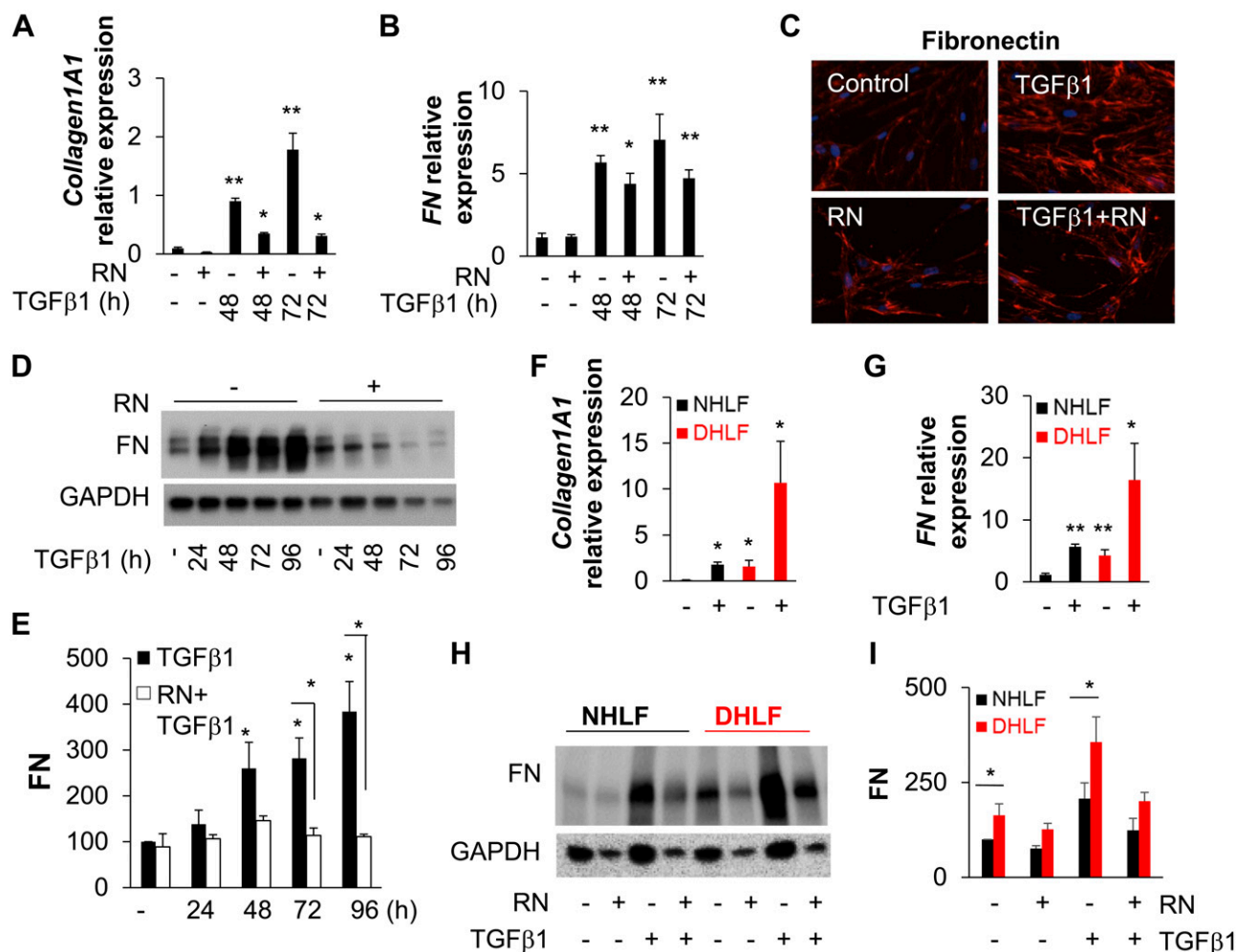


Figure 4. TRPV4 mediates matrix synthesis in fibroblasts. *A, B*) NHLFs were treated with TGF- β 1 (2 ng/ml; indicated time) with or without TRPV4 inhibitor RN1734 (30 μ M preincubation) and collagen 1A1 (*A*) and FN (*B*) transcripts were analyzed by real-time PCR. *C*) FN protein accumulation was analyzed by immunofluorescence in cells without permeabilizing with Triton X-100. *D, E*) FN protein expression was determined by Western blotting and quantified. *F–I*) DHLFs exhibit higher basal and TGF- β 1-induced matrix transcripts and protein levels compared with NHLFs. NHLFs and DHLFs were treated with TGF- β 1 (2 ng/ml; 72 h) and collagen 1A1 (*F*) and FN (*G*) transcripts were analyzed by real-time PCR. FN protein expression was determined by western blotting (*H*) and quantified in the presence or absence of RN1734 (30 μ M; *I*). Blots were stripped and reprobed with GAPDH Ab. Blots were quantified using AlphaView software (ProteinSimple). Results are means $\pm$ SEM from at least 3 independent experiments (*A, B, E, G, I*). * P < 0.05; ** P < 0.01.

degradation as well. Tissue-type plasminogen activator (*t*-PA) or urokinase-type PA (*u*-PA) converts plasminogen to the active enzyme, plasmin, which is involved in degradation of ECM components and PAI-1 functions as a major inhibitor of both *t*-PA and *u*-PA (38). Previous studies have demonstrated significant association between elevated PAI-1 levels and diminished lung function in patients with asthma (39); therefore, we analyzed whether TGF- β 1/TRPV4 promotes matrix accumulation by modulating PAI-1 levels. Of interest, we observed enhanced expression of PAI-1 by TGF- β 1 in a time-dependent manner at both the transcript and protein levels, which were inhibited by TRPV4 antagonist, RN1734 (**Fig. 6A, B, D, E**). In addition, we found that, similar to fibrotic genes, DHLFs express higher levels of PAI-1, which is also sensitive to TRPV4, compared with NHLFs (**Fig. 6C, F, G**).

Elucidation of the signaling pathway downstream of TGF- β 1/TRPV4

Our data demonstrated that Rho and p38 MAPK were activated by TGF- β 1 in a TRPV4-sensitive manner (**Fig. 3**); however, we do not know the hierarchical position of these signaling molecules in TGF- β 1/TRPV4-mediated fibrotic gene expression, PAI-1 induction, and FN protein levels. When we pretreated cells with p38 MAPK inhibitor SB203580, fibrotic gene expression levels were inhibited, whereas PAI-1 transcript and protein levels remained unchanged (**Fig. 7A, B, D, E**). In contrast, both fibrotic genes and PAI-1 levels were completely inhibited in the presence of a Rho inhibitor, Y27632 (**Fig. 7A, C, D, E**). These findings suggest that Rho acts upstream of both fibrotic gene induction and PAI-1, whereas p38 MAPK only regulates fibrotic gene expression. Of importance, this data suggests that TGF- β 1/TRPV4 regulates fibrotic

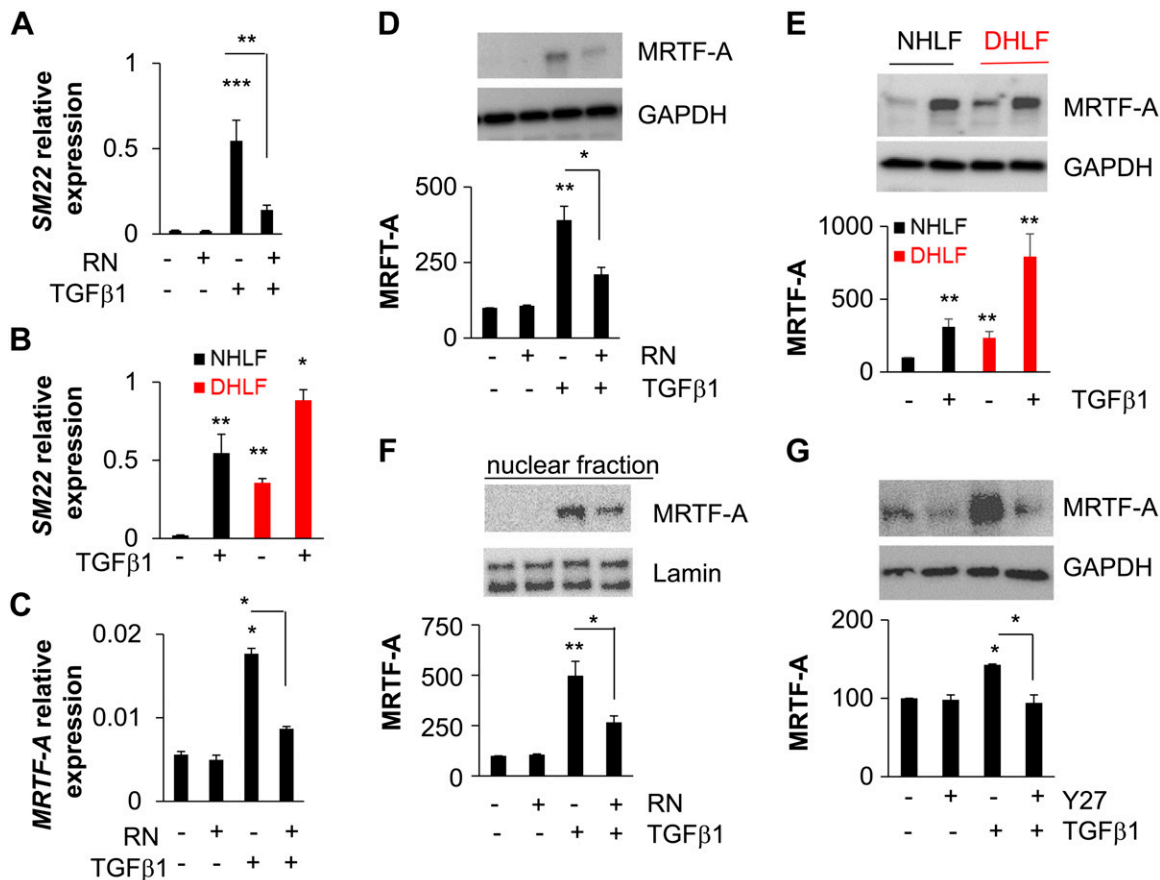


Figure 5. TRPV4 regulates TGF- β 1-mediated fibrotic gene expression. A–C) NHLFs were treated with TGF- β 1 (2 ng/ml; 48 h) with or without RN1734 (30 μ M) and the transcripts of SM22 (A, B) and MRTF-A (C) were analyzed by real-time PCR. D, F) MRTF-A protein was determined by Western blotting (D) and TRPV4-dependent MRTF-A nuclear translocation (F) to the membrane in response to TGF- β 1 (2 ng/ml; 24 h) in the presence or absence of TRPV4 inhibitor RN1734 (30 μ M) was analyzed by Western blotting in nuclear fractions. Blot was stripped and reprobed with lamin Ab to confirm equal loading. B, E) DHLFs exhibit higher basal and TGF- β 1-mediated SM22 mRNA and MRTF-A protein expression. NHLFs and DHLFs were treated with TGF- β 1 (2 ng/ml; 48 h) and (B) SM22 transcript was analyzed by real-time PCR (E) MRTF-A protein was determined by Western blotting. G) TGF- β 1-mediated MRTF-A expression is sensitive to Rho kinase inhibition. NHLFs were treated with TGF- β 1 (2 ng/ml; 48 h) with or without Y27632 (10 μ M) and MRTF-A protein was determined by Western blotting. Blots were stripped and reprobed with GAPDH Ab and were quantified by using AlphaView software. Graphs represent means $\pm$ SEM from at least 3 independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001.

gene expression and PAI-1 induction *via* 2 distinct pathways, both of which contribute to matrix (FN) synthesis and remodeling. In accordance, inhibition of both p38 MAPK and Rho kinase inhibited FN accumulation (Fig. 7B–D). Of note, Rho kinase inhibitor Y27632 prevented accumulation of FN more effectively, as it can block both PAI-1 and p38 MAPK-dependent fibrotic gene expression. In contrast, p38 inhibitor SB203580 partially inhibited FN increase, as it can only attenuate the p38 fibrotic gene induction pathway. Of interest, phosphorylation of p38 MAPK was attenuated by Rho kinase inhibitor (Fig. 7F), which suggests that p38 MAPK is downstream of Rho kinase. Taken together, these findings suggest that TRPV4 regulates matrix remodeling *via* 2 distinct p38 MAPK-dependent and -independent pathways downstream of Rho (Fig. 8).

DISCUSSION

Airway remodeling is the central feature of asthma, which is mediated by differentiated fibroblasts. Soluble

factor, TGF- β 1, has been identified as a major driving force for fibroblast to myofibroblast differentiation (36, 40, 41) *in vitro* and in tissue fibrosis of many organs (42, 43) *via* canonical and noncanonical pathways. Apart from soluble factors, the mechanical stiffness/rigidity offered by ECM is also a contributing factor in the differentiation of fibroblasts to myofibroblasts. In fact, both TGF- β 1 and mechanical signaling are critical for myofibroblast differentiation, though the mechanosensor is not known (41, 44). Recently, a mechanosensitive ion channel, TRPV4, has been implicated in the differentiation of cardiac (20) and lung (21) fibroblasts; however, the mechanism by which TRPV4 is activated or whether and how TRPV4 facilitates fibroblast differentiation and matrix remodeling, specifically in asthma, is not yet known. In the present study, we analyzed the role of TRPV4 in *D. farinae*-induced asthma *in vivo* by using TRPV4 KO mice and demonstrate that TRPV4 mediates fibroblast differentiation and matrix remodeling that contributes to pathologic airway remodeling in asthma. Our findings help to explain

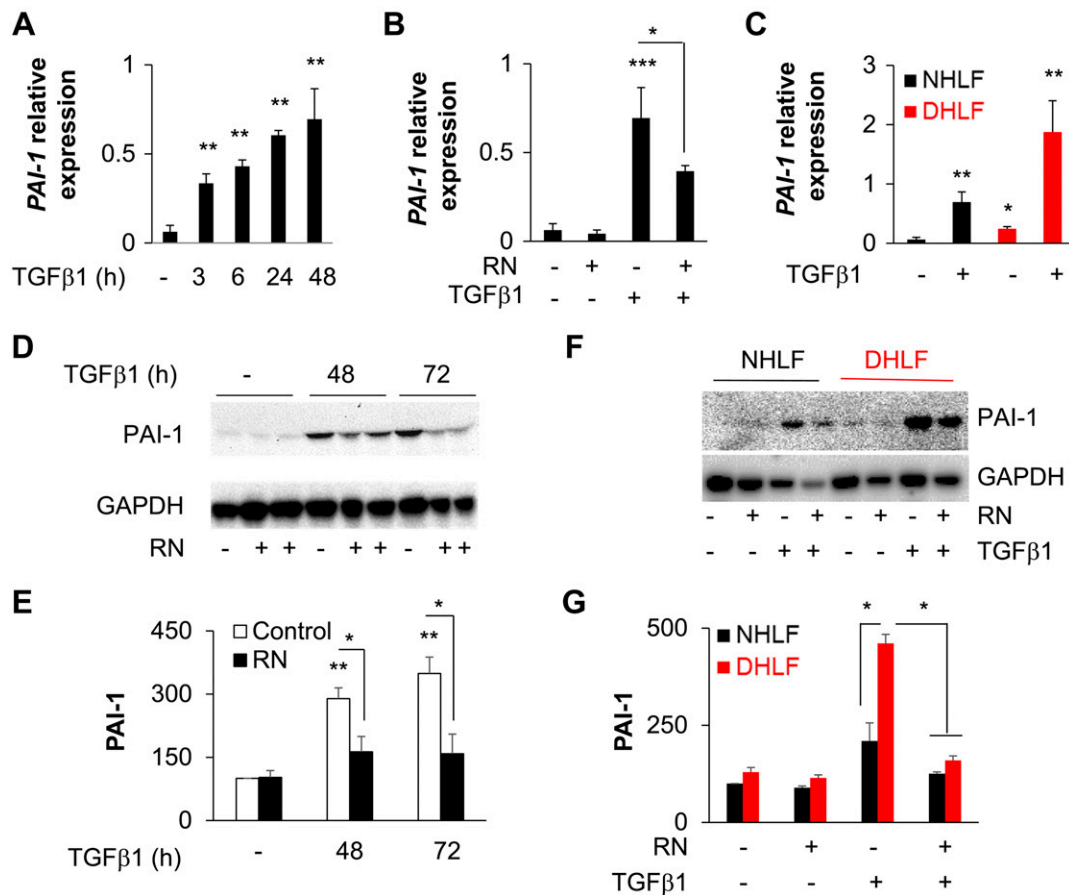


Figure 6. TRPV4 controls TGF- β 1-mediated PAI-1 expression. *A, B, D, E*) NHLFs were treated with TGF- β 1 (2 ng/ml; 48 h or indicated periods of time) with or without RN1734 (30 μ M) and (*A, B*) PAI-1 transcript was analyzed by real-time PCR, (*D, E*) PAI-1 protein was determined by Western blotting. *C, F, G*) DHLFs exhibit higher basal and TGF- β 1-mediated PAI-1 expression. NHLFs and DHLFs were treated with TGF- β 1 (2 ng/ml; 48 h) and (*C*) PAI-1 transcript was analyzed by real-time PCR; (*F, G*) PAI-1 protein was determined by Western blotting. Blots were stripped and reprobed with GAPDH Ab and were quantified by using AlphaView software. Graphs represent means $\pm$ SEM from at least 3 independent experiments (*A, C, E, G*). * P < 0.05; ** P < 0.01; *** P < 0.001.

the role of long-recognized, but not well understood, mechanical signaling in asthma. We found that, compared with WT mice, immune cell infiltration, number of goblet cells, matrix protein accumulation, T_H2 cytokines, and other remodeling features were reduced in the lungs of TRPV4 KO mice (Fig. 1 and Supplemental Fig. 1) in response to intranasal instillation of *D. farinae*. In asthmatics, susceptibility to injury, followed by abnormal repair responses, result in persistent activation of fibroblasts, which leads to subepithelial fibrosis (45). Fibroblasts are large, stellate cells that reside close to the basal epithelium in the lung. During wound healing or in an inflammatory environment, such as the asthmatic airway, these fibroblasts are activated/differentiated into hypersecretory myofibroblasts that facilitate wound healing *via* increased ECM protein synthesis and acquisition of a contractile phenotype (46–48). However, disproportionate production and prolonged survival of myofibroblasts can lead to pathologic fibrosis, a result of excessive secretion of ECM proteins (36, 40, 41). To address whether the preserved lung remodeling effects observed in TRPV4 KO mice were the result of reduced fibroblast differentiation, we isolated mLFs from WT and TRPV4 KO mice and analyzed their differentiation potential (α -SMA expression) as well as

their ability to synthesize ECM protein, FN, in response to TGF- β 1. WT fibroblasts exhibited robust TGF- β 1-induced α -SMA and FN protein expression in a time-dependent manner, which was attenuated in TRPV4 KO fibroblasts. Most of the previous studies have demonstrated increased collagen content as indicative of fibrosis and/or matrix remodeling; however, we found that, in addition to collagen, levels of fibronectin are increased significantly, which suggests that airway remodeling includes the remodeling of different ECM proteins. Our findings also suggest that TRPV4 plays a critical role in the lung remodeling response seen *in vivo* in response to *D. farinae* by regulating fibroblast differentiation. Indeed, fibroblasts isolated from patients with asthma (DHLFs) exhibited higher TRPV4 activity and basal α -SMA that were significantly enhanced in the presence of TGF- β 1 compared with NHLFs, which supports such possibility. Furthermore, TRPV4 inhibitor, RN1734, dose-dependently inhibited both NHLF and DHLF differentiation. TRPV4 has been implicated in cardiac fibroblast and lung fibroblast differentiation *in vitro*, and our findings here demonstrate that TRPV4 signaling contributes to enhanced fibroblast differentiation and lung remodeling in asthma.

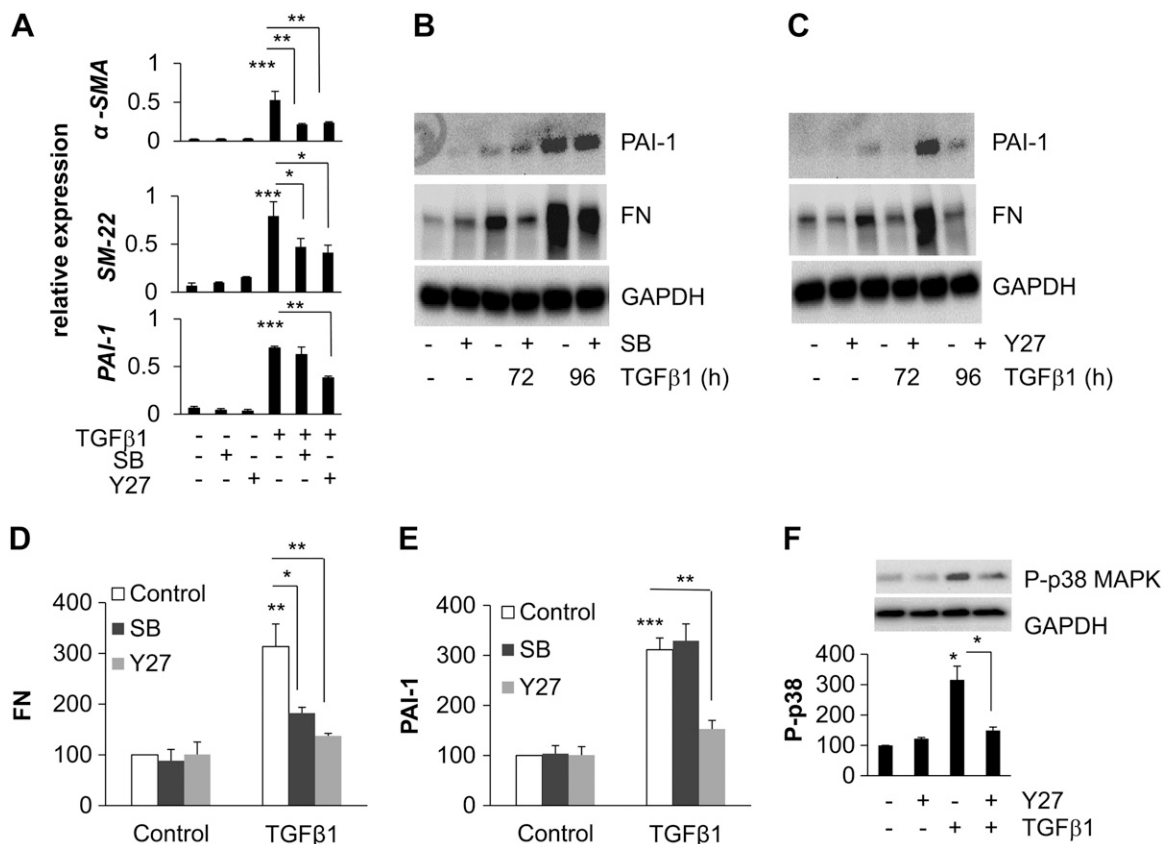


Figure 7. TRPV4-mediated signal transduction downstream of TGF-β1. NHLF cells were treated with TGF-β1 (2 ng/ml) for 48 h or indicated times in the presence or absence of SB203580 (10 μM), Y27632 (10 μM; A), and α-SMA, SM22, and PAI-1 transcripts were analyzed by real-time PCR (B, C, E). PAI-1 protein; (B, C, F) FN protein was determined by Western blotting. F) p38 MAPK is downstream of Rho kinase. NHLF cells were treated with TGF-β1 (2 ng/ml) for 30 min in the presence or absence of Y27632 (10 μM), and phosphorylation of p38 was determined and quantified. Blots were stripped and reprobed with GAPDH Ab and were quantified by using AlphaView software. Graphs represent means ± SEM from at least 3 independent experiments (A, D, E, F). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

In an attempt to identify the players involved, we found that TGF-β1 activated PI3K, Rho, and p38 MAPK during TGF-β1/TRPV4-mediated fibroblast differentiation. Of interest, TRPV4 antagonist, RN1734, inhibited only the activation of Rho and p38 MAPK, but not AKT, which suggests that the PI3K pathway may be acting upstream of TRPV4, whereas Rho and p38 MAPK are downstream of TRPV4. Furthermore, we found that TGF-β1-induced translocation of TRPV4 to the cell membrane, attenuated by PI3K inhibitor, LY294002, emphasized that TRPV4 is regulated by PI3K. Although TRPV4 has previously been implicated in TGF-β1-induced differentiation of cardiac and lung fibroblasts, there are no reports on how TGF-β1 activates TRPV4. To our knowledge, this is the first report to demonstrate that TRPV4 activity is regulated by PI3K in response to TGF-β1 in fibroblasts. In contrast, TRPV4 has been shown to activate the PI3K pathway and integrin activation in endothelial cells exposed to cyclic strain (27).

In an inflammatory environment, such as the asthmatic airway, myofibroblasts secrete excessive ECM proteins, which eventually leads to the remodeling of tissue and culminates in impaired lung function (2). Changes in the ECM composition alter tissue architecture and mechanical properties, which leads to

transformed cell behavior. Because ECM of the airway is dynamic, a shift in balance toward increased matrix deposition results in fibrosis and abnormal mechanical sensing; therefore, we next analyzed whether TRPV4 signaling regulates matrix remodeling in response to TGF-β1. We observed enhanced expression of matrix proteins collagen1A1 and FN transcripts in response to TGF-β1, which was significantly reduced by TRPV4 inhibition. As collagen1A1 induction by TGF-β1 and its role in fibroblast differentiation have been well established, we focused on FN transcription and protein expression in response to TGF-β1/TRPV4. Of note, we found that TGF-β1 treatment induced time-dependent accumulation of FN that was inhibited by TRPV4 antagonist. In agreement with a role for TRPV4 in matrix remodeling in asthma, DHLFs exhibited higher basal FN and higher TRPV4 activity. We then investigated the molecular mechanism by which TRPV4 enhances matrix production by TGF-β1. We focused on the MRTF-A pathway, which has been shown to convert mechanical stimuli in response to matrix stiffening into fibrogenic nuclear signal that promotes fibroblast to myofibroblast differentiation (49). Indeed, we found that TGF-β1 promoted transcription and nuclear

translocation of MRTF-A in NHLFs. Furthermore, inhibition of TRPV4 blocked TGF- β 1-induced MRTF-A transcript and protein expression, which suggests that TRPV4 acts upstream of MRTF-A and is involved in the transduction of mechanical signals from cell surface to MRTF-A. Transcription of fibrotic genes, including α -SMA, depends on nuclear complexes of serum response factor (SRF) (50) and MRTF-A (51), a coactivator, present in the cytosol anchored to monomeric G-actin. TGF- β 1-mediated actin polymerization releases this coactivator, which then translocates to the nucleus and associates with and activates SRF. Upon activation, SRF, a transcription factor, binds to serum response element of contractile genes and triggers SRF-dependent fibrotic gene expression (52). MRTF-A is implicated in α -SMA expression and is shown to be activated by the Rho pathway (37). Rho has been implicated earlier in the regulation of cell tension *via* enhancement of actin polymerization, which triggers translocation of MRTF-A to the nucleus (53). Therefore, we asked whether Rho, which is activated downstream of TRPV4, would regulate MRTF-A. We found that a Rho kinase pathway inhibitor significantly reduced MRTF-A expression in NHLFs. Next, we investigated the role of p38 MAPK in TGF- β 1/TRPV4-mediated matrix synthesis. We found that p38 MAPK inhibitor significantly inhibited expression of fibrotic genes α -SMA and SM-22 (Fig. 7) and MRTF-A protein expression (data not shown). Taken together, these findings clearly indicate that TRPV4 mediates fibroblast differentiation and matrix production *via* Rho-dependent p38 MAPK and MRTF-A pathways (Fig. 8).

However, airway remodeling in asthma includes an imbalance between ECM protein production and degradation (54), which suggests that it depends on the ratio of protease and antiprotease pathways that favor profibrotic balance. PAI-1 is a member of the serine protease inhibitor gene family and is the major physiologic inhibitor of urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), which are critical mediators of matrix degradation. Previous studies suggest that PAI-1 plays an important role in the pathogenesis of asthma by promoting airway inflammation and remodeling (39, 55–57); therefore, we investigated whether TRPV4 may enhance matrix deposition in response to TGF- β 1 by enhancing PAI-1 levels in addition to enhancing fibrotic gene expression. We observed an increase in PAI-1 transcript and protein in response to TGF- β 1. Of importance, TGF- β 1-induced expression of PAI-1 was significantly inhibited by TRPV4 antagonist, RN1734, which suggests that TRPV4 acts upstream of PAI-1. Furthermore, DHLFs exhibited higher basal and enhanced PAI-1 expression in response to TGF- β 1 compared with NHLFs.

Finally, we investigated the mechanisms by which TRPV4 regulates matrix accumulation (fibrotic gene expression; α -SMA and SM22) and matrix degradation (PAI-1 expression). Of interest, we found that whereas blocking of the Rho/Rho kinase pathway inhibits expression of both fibrotic genes and PAI-1, p38 MAPK inhibition attenuated only fibrotic gene expression without any effect on PAI-1. Furthermore, Rho pathway inhibition

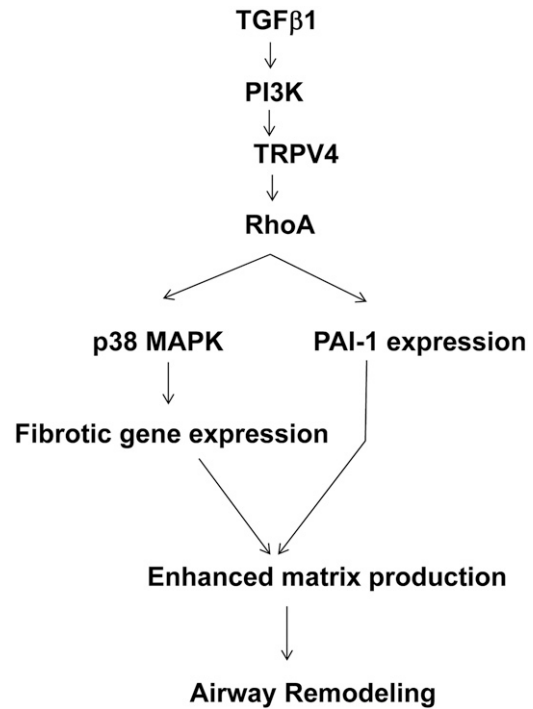


Figure 8. Hypothetical model depicting TRPV4 in the regulation of fibroblast differentiation and airway remodeling.

significantly abolished TGF- β 1/TRPV4-induced p38 MAPK phosphorylation. Taken together, these findings suggest an existence of 2 pathways: a Rho/p38 MAPK-dependent fibrotic gene expression and a Rho-dependent but p38 MAPK-independent PAI-1 pathway, both of which contribute to matrix accumulation and remodeling, respectively, during asthma. Of importance, p38 MAPK phosphorylation was inhibited by Rho kinase inhibitor, which suggests that the pathway is further bifurcated at Rho. Although our *in vivo* experiments suggest that TRPV4 KO mice are protected from *D. farinae*-induced asthma and that TRPV4 in fibroblasts contributes to enhanced matrix production, it is hard to tease out the contribution of TRPV4 from other immune cells in airway remodeling employing global TRPV4 KO mice and not fibroblast-specific conditional KO mice.

In summary, our results indicate that TRPV4 mediates fibroblast differentiation and enhances matrix accumulation in 2 separate but interdependent pathways: 1) by enhancing fibrotic gene expression and 2) by inhibiting matrix degradation by activating/enhancing PAI-1. Enhanced matrix accumulation *via* TRPV4-dependent pathways, in turn, may increase mechanical properties of the airway and promote fibrosis, generating a positive feedback loop and contributing to further airway remodeling. Our findings thus identify TRPV4 as a critical regulator of airway remodeling in asthma and we propose that TRPV4 is a novel therapeutic target for asthma and other pathologic lung remodeling. [F]

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AUTHOR CONTRIBUTIONS

F. Gombedza, V. Kondeti, N. Al-Azzam, S. Koppes, E. Duah, P. Patil, M. Hexter, and D. Phillips performed experiments and analyzed the data; C. K. Thodeti provided TRPV4 KO mice, designed some of the experiments, and edited the manuscript; and S. Paruchuri designed experiments, performed research, analyzed and interpreted data, and wrote the manuscript.

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Mechanosensitive transient receptor potential vanilloid 4 regulates *Dermatophagoides farinae*-induced airway remodeling via 2 distinct pathways modulating matrix synthesis and degradation

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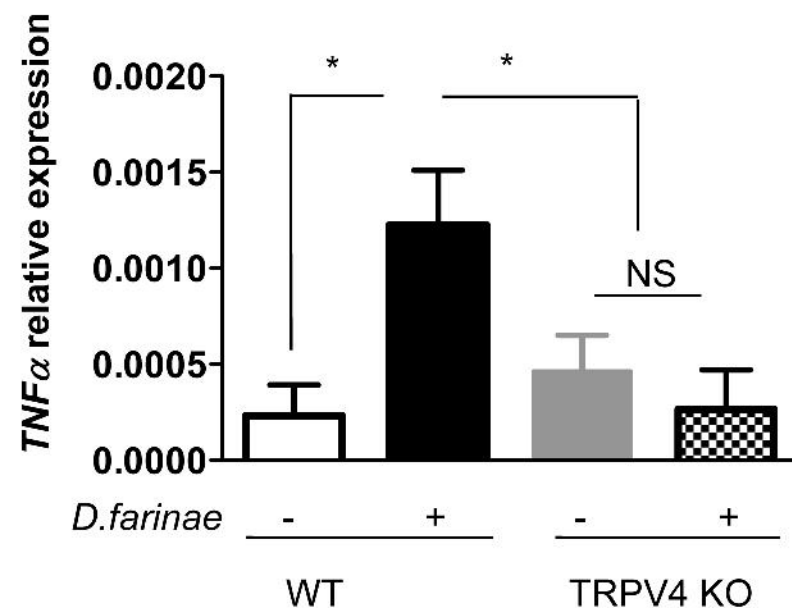
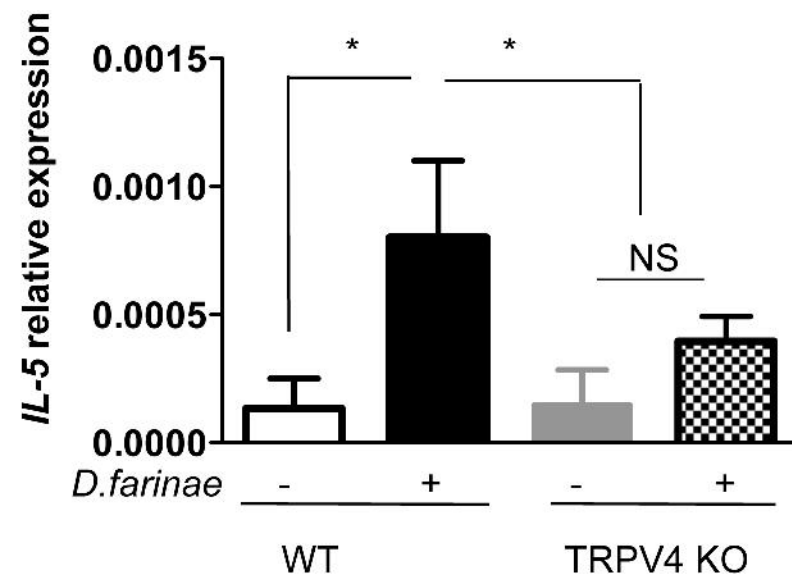
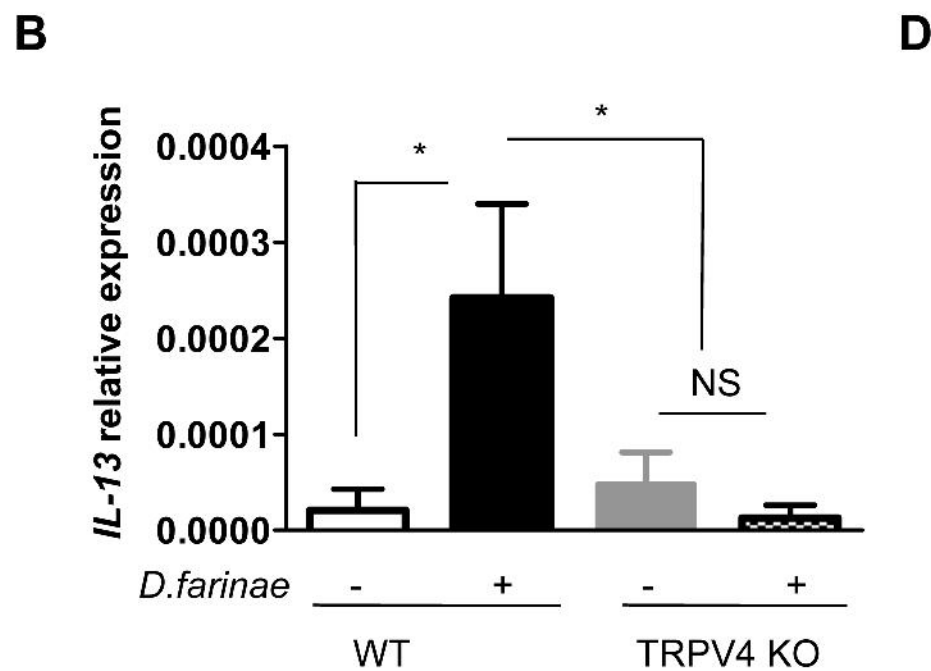
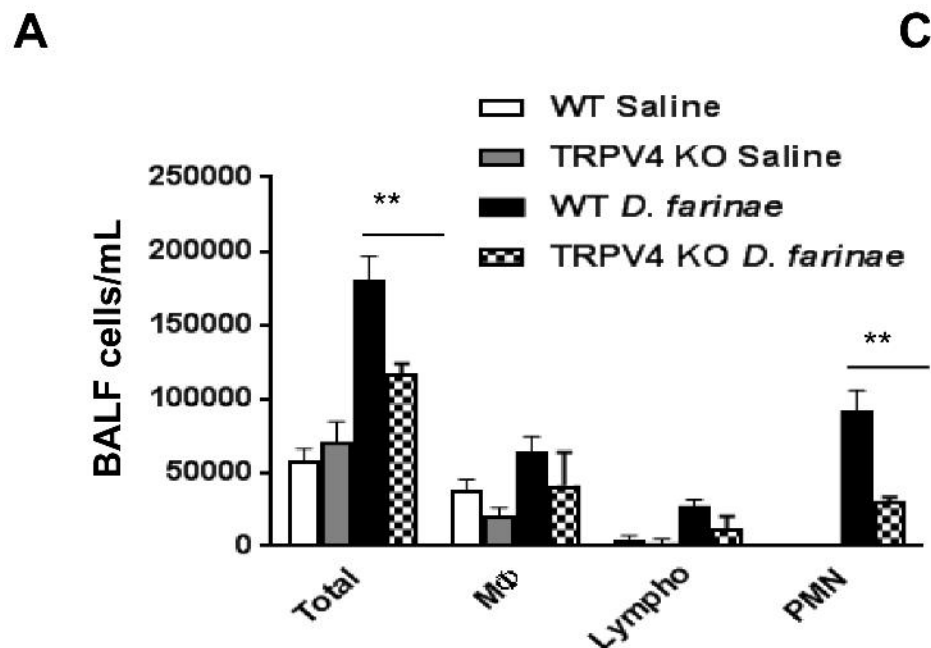
SUPPLEMENTAL DATA

FIGURE LEGENDS

Supplemental Figure 1. WT mice treated with *D. farinae* express enhanced inflammation and Th2 cytokines. Wild-type (WT) and TRPV4 KO mice were treated with saline or *D. farinae* (25 µg/animal) intranasally, 3 times a week for 5 weeks. Mice were sacrificed 24h after the final challenge. (A), Total cell count and differential cell counts from the BAL fluid. Expression of (B), IL-13 (C), IL-5 and (D) TNFα transcripts normalized to GAPDH as determined by real-time PCR of whole lung RNA extracted from the indicated groups. Results are mean ± SEM from three independent experiments.

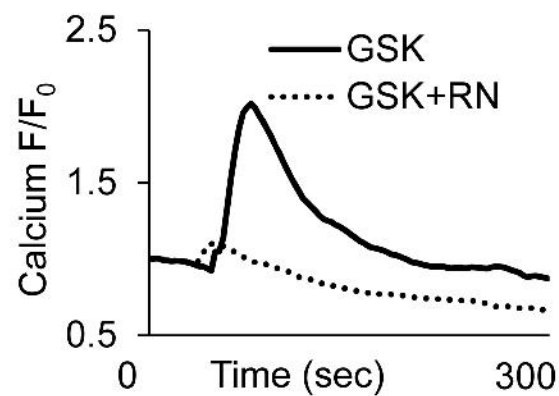
Supplemental Figure 2. RN intervention is selective to TRPV4. (A-C), Effect of RN treatment on TRPV4-dependent calcium flux and α-SMA incorporation into stress fibers in NHLF. NHLF were loaded with Fluo-4 and calcium flux was measured as described in methods section in response to a specific TRPV4 activator, GSK1016790A (100 nM) and (B), quantified. (C), NHLF were pre-treated with RN (30 µM), treated with TGFβ1 (2ng/ml; 48h) and α-SMA localization was determined by immuno-fluorescence (red). (D), GSK induces α-SMA expression. NHLF were treated with GSK (100 nM) or TGFβ1 (2ng/ml) for 48h and expression of α-SMA was analyzed by western blotting.

Supplemental Fig. 1

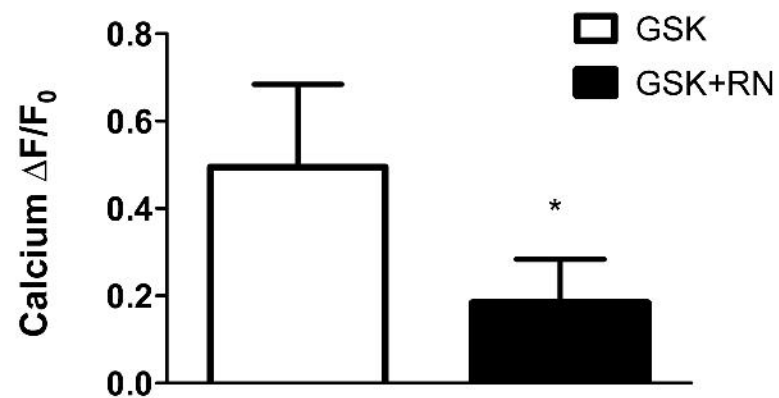


Supplemental Fig. 2

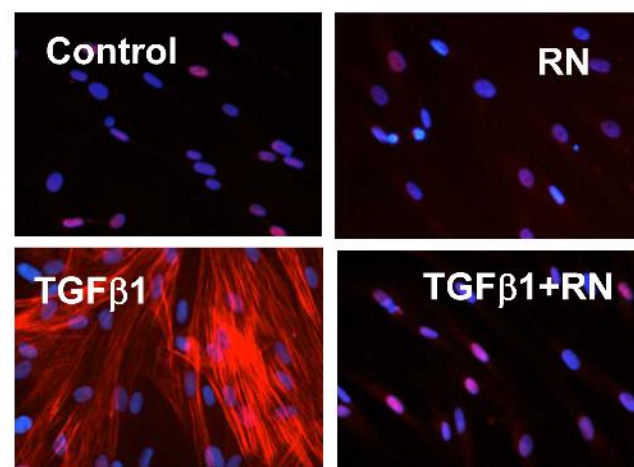
A



B



C



D

