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Transforming Growth Factor-β1 Decreases β2-Agonist–induced Relaxation in Human Airway Smooth Muscle

Christie A. Ojiaku

Elena Chung

Vishal Parikh

Jazmean K. Williams

Anthony Schwab

See next page for additional authors

Authors

Christie A. Ojiaku, Elena Chung, Vishal Parikh, Jazmean K. Williams, Anthony Schwab, Ana Lucia Fuentes, Maia L. Corpuz, Victoria Lui, Sam Paek, Natalia M. Bexiga, Shreya Narayan, Francisco J. Nunez, Kwangmi An, Rennolds S. Ostrom, Steven S. An, and Reynold A. Pannettieri Jr.

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Complete List of Authors:	Ojiaku, Christie; University of Pennsylvania, Department of Systems Pharmacology and Translational Therapeutics; Rutgers The State University of New Jersey, Rutgers Institute for Translational Medicine and Science Chung, Elena; Rutgers The State University of New Jersey, Rutgers Institute for Translational Medicine and Science Parikh, Vishal; Rutgers The State University of New Jersey, Rutgers Institute for Translational Medicine and Science Williams, Jazmean; Drexel University Schwab, Anthony; Rutgers The State University of New Jersey, Rutgers Institute for Translational Medicine and Science Fuentes, Ana Lucia; Rutgers The State University of New Jersey, Rutgers Institute for Translational Medicine and Science Corpuz, Maia; Chapman University School of Pharmacy, Department of Biomedical and Pharmaceutical Sciences Liu, Victoria; Johns Hopkins University Bloomberg School of Public Health, Department of Environmental Health and Engineering Paek, Sam; Johns Hopkins University Bloomberg School of Public Health, Department of Environmental Health and Engineering Bexiga, Natalia; University of Sao Paulo, Department of Pharmaceutical Biochemistry Technology; Johns Hopkins University Bloomberg School of Public Health, Department of Environmental Health and Engineering Narayan, Shreya; Johns Hopkins University Bloomberg School of Public Health, Department of Environmental Health and Engineering Nuñez, Francisco; Chapman University School of Pharmacy, Department of Biomedical and Pharmaceutical Sciences Ahn, Kwangmi; National Institutes of Health Ostrom, Rennolds; Chapman University School of Pharmacy, Department of Biomedical and Pharmaceutical Sciences An, Steven; Johns Hopkins University Bloomberg School of Public Health, Department of Environmental Health and Engineering, ; Johns Hopkins University, Department of Chemical and Biomolecular Engineering; Johns Hopkins School of Medicine, Department of Oncology Panettieri, Reynold; Rutgers Robert Wood Johnson Medical School, Medicine; Child Health Institute of New Jersey,
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TGF-β1 Decreases β2-Agonist-Induced Relaxation in Human Airway Smooth Muscle

Christie A. Ojiaku[1,2], Elena Chung[2], Vishal Parikh[2], Jazmean K. Williams[3], Anthony Schwab[2], Ana Lucia Fuentes[2], Maia L. Corpuz[4], Victoria Lui[5], Sam Paek[5], Natalia M. Bexiga[5,6], Shreya Narayan[5], Francisco J. Nunez[4], Kwangmi Ahn[7], Rennolds S. Ostrom[4], Steven S. An[5,8,9], and Reynold A. Panettieri, Jr[1,2]

[1]Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; and.
[2]Rutgers Institute for Translational Medicine and Science, Child Health Institute, Rutgers University, New Brunswick, New Jersey.
[3]Drexel University, Philadelphia, PA
[4]Department of Biomedical and Pharmaceutical Sciences, Chapman University School of Pharmacy, 9401 Jeronimo Road Irvine, CA, USA
[5]Department of Environmental Health and Engineering, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA
[6]Department of Pharmaceutical Biochemistry Technology, University of Sao Paulo, Sao Paulo, Brazil
[7]National Institutes of Health, Bethesda, MD, USA
[8]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, USA
[9]Department of Oncology, Johns Hopkins School of Medicine, Baltimore, MD, USA

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Address for Correspondence:
Reynold A. Panettieri, Jr. M.D.
Rutgers Institute for Translational Science & Medicine
89 French Street, Suite 4210
New Brunswick, NJ08901
Tel: 732-235-6404
Fax: 732-235-7178
E-mail: rp856@ca.rutgers.edu

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CAO, EC, VP, JKW, MC, RSO, SSA, and RAP contributed to the experimental concept and design. CAO, EC, VP, JKW, AS, AF, MC, VL, SP, NB, SN, and FJN performed the experiments. CAO, EC, JKW, MC, RSO, KA, SSA, and RAP contributed to the analysis and interpretation of the data. CAO wrote the manuscript. CAO, RSO, SSA, and RAP edited and reviewed the manuscript for important intellectual content.

ABSTRACT

Helper T effector cytokines implicated in asthma modulate the contractility of human airway smooth muscle (HASM) cells. We have reported recently that a profibrotic cytokine, transforming growth factor beta 1 (TGF-β1), induces HASM cell shortening and airway hyper-responsiveness (AHR). Here we assessed whether TGF-β1 affects the ability of HASM cells to relax in response to β2-agonists, a mainstay treatment for AHR in asthma. Overnight TGF-β1 treatment significantly impaired isoproterenol (ISO)-induced relaxation of carbachol-stimulated isolated HASM cells. This single-cell mechanical hypo-responsiveness to ISO was corroborated by sustained increases in myosin light chain (MLC) phosphorylation. In TGF-β1 treated HASM cells, ISO evoked markedly lower levels of intracellular cAMP. These attenuated cAMP levels were, in turn, restored with pharmacological and siRNA inhibition of PDE4 and Smad3, respectively. Most strikingly, TGF-β1 selectively induced PDE4D gene expression in HASM cells in a Smad2/3-dependent manner. Together these data suggest that TGF-β1 decreases HASM cell β2-agonist relaxation responses by modulating intracellular cAMP levels via a Smad2/3-dependent mechanism. Our findings further define the mechanisms underlying β2-agonist hypo-responsiveness in asthma, and suggest TGF-β1 as a potential therapeutic target to decrease asthma exacerbations in severe and treatment-resistant asthma.

KEYWORDS

Human airway smooth muscle, TGF-β1, relaxation, severe asthma, β2-agonists

INTRODUCTION

β2-agonist bronchodilators are a mainstay therapeutic used for acute and long-term control of asthma exacerbations. However, patients with severe asthma often respond poorly to β2-agonists, and increasing evidence demonstrates that frequent β2-agonist use leads to resistance and deterioration of asthma control (1, 2). Therefore, understanding the mechanisms mediating β2-agonist resistance is important for decreasing asthma-related morbidity and mortality.

Evidence suggests a link exists between β2-adrenergic receptor (β2AR) hypo-responsiveness and airway hyper-responsiveness (AHR), where increased levels of bronchoconstriction can decrease bronchodilator responsiveness (2, 3). Unsurprisingly, several cytokines modulate hyper-responsiveness and β2-agonist resistance in human airway smooth

muscle (HASM), the main regulator of bronchomotor tone (4, 5). We have previously reported that transforming growth factor β1 (TGF-β1)–a pro-fibrotic cytokine elevated in the airways of patients with asthma–augments agonist-induced contractile responses in HASM via a Smad3-dependent pathway (6). However, the role of TGF-β1 in modulating β2-agonist-induced relaxation responses in HASM remains unknown.

β2-agonists induce airway relaxation by binding to β2-adrenergic G-protein coupled receptors (GPCRs) on HASM cells, stimulating adenylyl cyclase (AC) enzyme activity (7). AC activation by the β2AR G_s alpha subunit elevates intracellular cyclic adenosine monophosphate (cAMP) levels, and increased cAMP leads to subsequent HASM cell relaxation by antagonizing HASM cell contractile pathways. HASM cell relaxation responses are also regulated by the action of prostaglandin E2 (PGE2), an arachidonic acid-derived mediator that exerts its effects via prostanoid EP receptors from the GPCR family (8). Stimulation of the G_s-coupled EP2 and EP4 receptor subtypes elevates intracellular cAMP levels via activation of AC, with EP4 receptor stimulation selectively leading to HASM cell relaxation (9, 10).

Intracellular cAMP levels in HASM cells are regulated by the balance between AC activation and cAMP-hydrolyzing phosphodiesterase (PDE) activity. While HASM cells express multiple PDE isoforms (11), functional studies have established PDE3 and PDE4 as the major cAMP hydrolyzing enzymes (12–14). PDE4, in particular, plays a pivotal role in HASM cell cAMP degradation and is more widely studied as a therapeutic target in airway disease (15). Of the four PDE4 encoding genes (16), evidence supports a critical role for PDE4D in mediating HASM cell contractile and relaxation responses (17–20). Increased PDE4D activity and expression is associated with decreased β2-agonist-induced cAMP generation in HASM from subjects with asthma (20). Mice deficient in PDE4D also exhibit a loss of responsiveness to cholinergic stimulation (10), suggesting the therapeutic potential of PDE4D inhibitors in asthma.

Previous studies investigating the role of TGF-β1 in decreased β2AR responses have been purely biochemical in nature and largely limited to human tracheal smooth muscle cells and human lung embryonic fibroblasts (21, 22). As these studies were conducted in the presence of PDE inhibitors, neither study assessed the potential of TGF-β1 to modulate downstream components of the cAMP signaling pathway via PDE4. Therefore, we aimed to elucidate the mechanisms by which TGF-β1 modulates β2-agonist-induced relaxation responses in HASM cells.

METHODS

Human Airway Smooth Muscle (HASM) Cell Culture

Human lungs from otherwise healthy, aborted transplant donors were received from the International Institute for the Advancement of Medicine (IIAM; Edison, NJ, USA) and the National Disease Research Interchange (NDRI; Philadelphia, PA, USA). HASM cells were isolated from the trachea and cultured as previously described (23).

Immunoblot Analysis

Confluent HASM cells were serum starved overnight prior to treatment and collected as previously described (24).

Magnetic Twisting Cytometry (MTC)

Dynamic changes in cell stiffness were measured as an indicator of the single-cell contraction and/or relaxation of isolated HASM cells as previously described (25, 26). Briefly, RGD-coated ferrimagnetic microbeads bound to the cytoskeleton were magnetized horizontally and then twisted in a vertically-aligned homogeneous magnetic field that varied sinusoidally in time (27). The ratio of specific torque to bead displacements is expressed here as the cell stiffness in units of Pascal per nm (Pa/nm).

Small Interfering RNA (siRNA) Transfection

In vitro siRNA knockdown was performed using a reverse transfection procedure as previously described (28). HASM cells were seeded onto cell culture plates for a final siRNA concentration of 10 μM.

Measurement of Cyclic AMP Levels

Following stimulation, cAMP levels were measured in lysed HASM cells using the Applied Biosystems cAMP-Screen® ELISA system according to manufacturer protocol. For kinetic measurement of cAMP production in live cells, HASM cells were infected with a recombinant BacMam virus expressing the cADDis cAMP sensor (Montana Molecular, Bozeman, MT) as previously described *(29)*. Cells were stimulated with agonist then fluorescence measured at 30 second intervals for 30 minutes. Data were fit to a single-site decay model using GraphPad Prism

7.0 (GraphPad Software Inc., San Diego, CA). Concentration-response curves were generated from each decay curve by multiplying the kinetic rate constant, k, with the plateau.

Quantitation of Phosphodiesterase (PDE) Gene Expression

RNA was isolated from HASM cells using the RNeasy Mini Kit (Qiagen Sciences, Inc., Germantown, MD, USA). cDNA was generated using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). Relative cDNA quantification was performed using TaqMan quantitative RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA) and the $\Delta\Delta C_t$ method, and expression was normalized to β-actin control.

Statistical Analysis

Unless otherwise stated, statistical analysis was conducted using GraphPad Prism software (La Jolla, CA, USA), with significance evaluated at a p-value of < 0.05. Significance was determined using Fisher's Least Significant Differences tests or multiple t-tests with Holm-Sidak correction. For MTC experiments involving multiple lung donor cell responses, statistical analysis was conducted using mixed effect models using SAS V.9.2 (SAS Institute Inc., Cary, NC) (30).

Materials

Compounds were purchased from Sigma Aldrich (St. Louis, MO, USA) [isoproterenol, prostaglandin E2, carbachol, perchloric acid], Selleck Chemicals (Houston, TX, USA) [roflumilast], Cayman Chemicals (Ann Arbor, MI, USA) [3-isobutyl-1-methylxanthine (IBMX)], and R&D Systems (Minneapolis, MN, USA) [TGF-β1, SB-431542]. Immunoblot antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA) [pMLC(3674S)] and EMT Millipore (Billerica, MA, USA) [MLC(MABT180)]. siRNA was purchased from Thermo Fisher Scientific (Waltham, MA, USA) [Smad3(VHS41114)] and Dharmacon (Lafayette, CO, USA) [Smad2(L-003561-00), Non-targeting Pool(D-001810-10-05)].

RESULTS

TGF-β1 Decreases β2-Agonist-Induced Relaxation in HASM Cells

To determine the extent to which TGF-β1 mediates resistance to β2AR-induced relaxation in HASM cells, we investigated contractile outcomes in TGF-β1-pretreated HASM cells stimulated acutely with the β-agonist isoproterenol (ISO) (Fig. 1). Single-cell relaxation responses were determined using magnetic twisting cytometry (MTC), a technique that measures changes in cell stiffness as a surrogate for agonist-induced force generation (26). TGF-β1 or vehicle pretreated cells were pre-contracted to carbachol and stimulated acutely with ISO. TGF-β1 significantly impaired ISO-induced single-cell relaxation in basal and carbachol-stimulated HASM cells as compared to vehicle control (Fig. 1A). No significant changes in cell stiffness were observed in non-stimulated vehicle controls for the duration of our measurements (data not shown) (25, 26). To further confirm TGF-β1's effects on HASM cell contractile responses, we investigated the phosphorylation of MLC–an essential component of agonist-induced HASM cell contraction–following overnight TGF-β1 treatment. TGF-β1 augmented basal and agonist-induced MLC phosphorylation in a similar manner to previously published literature (6). Following stimulation with ISO, MLC phosphorylation in TGF-β1-treated HASM cells remained significantly higher than that of vehicle control (Fig. 1B). Notably, the addition of the contractile agonist carbachol to TGF-β1 and ISO-treated HASM cells significantly increased MLC phosphorylation to levels above that in TGF-β1 and ISO-treated HASM cells.

TGF-β1 Blunts Agonist-Induced cAMP Levels

To elucidate the mechanism by which TGF-β1 reduces HASM cell relaxation responses, total cAMP levels were measured in lysed TGF-β1-treated HASM cells. In TGF-β1 treated cells, ISO- and PGE2-induced cAMP levels were decreased versus that of respective control (Fig. 2A). TGF-β1 treatment did not alter forskolin-stimulated cAMP levels (Fig. 2B), suggesting that AC function was not negatively affected by TGF-β1; there were no significant differences in forskolin-evoked cAMP levels in vehicle control and TGF-β1 treated HASM cells.

To further confirm these results, cAMP levels were monitored in live HASM cells pretreated with either vehicle or TGF-β1. In TGF-β1 treated cells, ISO-induced cAMP responses were 2.6-fold less potent and 1.7-fold less efficacious compared to the vehicle-treated control (Fig. 2C, E1A-B). TGF-β1 treatment appeared to decrease the potency of PGE2-stimulated cAMP responses, although this increase did not reach significance due to large variation of

PGE2 responses between donors (Fig. 2D, E1C-D). Forskolin-stimulated cAMP responses were unaffected by TGF-β1 treatment in live HASM cells (Fig. 3E, E1E-F).

PDE Inhibition Rescues ISO-Stimulated Responses in TGF-β1-Treated HASM Cells

Intracellular cAMP levels are primarily reduced via hydrolysis–an effect mediated by the action of PDEs in HASM cells (31). To determine whether TGF-β1 mediates β2-agonist hypo-responsiveness by modulating PDE-mediated cAMP hydrolysis, MLC phosphorylation and cAMP levels were measured in TGF-β1 and ISO-treated HASM cells in the presence or absence of the pan-PDE inhibitor IBMX (Fig. 3, E2A).

MLC phosphorylation in HASM cells was increased following TGF-β1 treatment, and levels remained higher than vehicle control following ISO stimulation (Fig. 3A, *left*). Treatment with IBMX, however, reduced MLC phosphorylation in TGF-β1-pre-treated, ISO-stimulated HASM cells to a level similar to that of vehicle control (Fig. 3A, *left*). In ISO-stimulated HASM cells, MLC phosphorylation levels were increased in TGF-β1 and carbachol-treated cells above those in TGF-β1-treated cells alone (Fig. 3A, *right*). IBMX treatment decreased MLC phosphorylation in TGF-β1 and carbachol-treated cells to a level similar to that of vehicle control (Fig. 3A, *right*).

We next investigated the role of PDE activity in TGF-β1-mediated decreases in ISO-induced cAMP (Fig. 3B). Vehicle or TGF-β1-treated HASM cells were pre-treated with IBMX prior to ISO stimulation. IBMX pretreatment significantly elevated ISO-induced cAMP levels in TGF-β1-treated HASM cells (Fig. 3B).

TGF-β1 Induces PDE4D Gene Expression in a Concentration-Dependent Manner

To determine the extent to which PDEs contribute to β2-agonist hypo-responsiveness in TGF-β1-treated HASM cells, we investigated the expression of HASM cell-specific PDEs in TGF-β1-treated HASM cells (Fig. E3) (29). TGF-β1 selectively increased PDE4D gene expression in a concentration-dependent manner (Fig. 4A, E3). Furthermore, inhibition of TβR-I receptor signaling with SB-431542 pretreatment blocked increased PDE4D gene expression evoked by TGF-β1.

To further determine the extent to which TGF-β1 modulates PDE4D to decrease β2-agonist-induced relaxation responses, cAMP accumulation, MLC phosphorylation, and cell

stiffness were measured in HASM cells treated with the PDE4 inhibitor roflumilast (Fig. 4B-D). Roflumilast pretreatment rescued blunted ISO-stimulated cAMP levels in TGF-β1-treated cells (Fig. 4B). In the presence of roflumilast, TGF-β1-induced MLC phosphorylation in ISO-stimulated cells showed little increase over vehicle control (Fig. 4C, E2B). Additionally, roflumilast pretreatment decreased augmented HASM cell stiffness in TGF-β1 and ISO-stimulated HASM cells (Fig. 4D).

TGF-β1-Decreases β2-Agonist-Induced Relaxation Responses in a Smad2/3-Dependent Manner

The canonical TGF-β1 signaling pathway involves the activation of Smad2/3–intracellular signaling proteins that mediate a variety of TGF-β1's effects on HASM cell signaling in asthma (32). To determine the role of Smad proteins in TGF-β1-mediated inhibition of HASM cell relaxation responses, we investigated TGF-β1's modulation of ISO-induced cAMP levels in Smad2/3 siRNA-transfected cells (Fig. 5). ISO-induced cAMP was significantly increased in Smad3 siRNA-transfected cells in the presence and absence of TGF-β1 treatment (Fig. 5A). TGF-β1 blunted ISO-induced cAMP levels in HASM cells transfected with non-targeting and Smad2 siRNA, but had little effect on ISO-induced cAMP levels in Smad3 siRNA-transfected HASM cells.

To determine the role of Smad signaling in TGF-β1-mediated induction of PDE4D gene expression, PDE4D gene expression was investigated in Smad2 or Smad3 siRNA-transfected HASM cells following overnight TGF-β1 treatment (Fig. 5B). Smad2 and Smad3 knockdown reduced PDE4D gene expression induced by TGF-β1 treatment of HASM cells (Fig. 5B).

DISCUSSION

In the present study, we demonstrate that TGF-β1 attenuates β2-agonist-induced relaxation responses in HASM cells. To date, TGF-β1 has been shown to negatively modulate β-adrenergic responses in multiple cell types (21, 22, 33, 34). Here, we demonstrate that TGF-β1 treatment – in the presence or absence of the contractile agonist carbachol – significantly attenuates ISO-induced HASM cell relaxation via increased cell stiffness and MLC phosphorylation (Fig. 1). Importantly – as β1 agonists have little bronchodilator effect in humans and HASM cell beta receptors are solely of the β2 subtype – this study selectively demonstrates the effects of TGF-β1 and the β-agonist ISO on β2AR-induced relaxation (35, 36). While

previous studies suggest that TGF-β1 modulates β2AR-mediated responses through a protein synthesis-dependent mechanism, the details by which this modulation occurs is not fully understood (21, 22). For the first time, we demonstrate that TGF-β1's effects on HASM cell relaxation responses occur via a Smad2/3 pathway that upregulates the expression of PDE4D. Collectively, our findings further establish TGF-β1 as a mediator of bronchodilator resistance via modulation of downstream cAMP pathway effects.

Previous studies suggest that TGF-β1 attenuates ISO-induced cAMP accumulation by negatively regulating β2AR number, protein, and gene expression (21, 22). However, our data suggest yet an additional mechanism for the attenuation of cAMP by TGF-β1. In our study, TGF-β1 blunted cAMP induced by both ISO and PGE2, a mediator that binds to the G_s/(G_i)-associated prostaglandin EP2 and EP4 G protein-coupled receptors to elevate intracellular cAMP levels (Fig. 2A, 2C, 2D) (37). Little is known regarding TGF-β1's effects on EP receptor expression in HASM, and it is unlikely that TGF-β1 blunts HASM cell cAMP by decreasing the expression of two independent G_s-coupled receptors.

Interestingly, other studies suggest a role for TGF-β1 in modulating G protein function. Treatment with pertussis toxin, an irreversible G_i inhibitor, blocked TGF-β1-induced PGE2 production in human lung fetal fibroblasts (38). Additionally, a report demonstrating an augmentation of cholera and pertussis toxin-induced ADP-ribosylation in TGF-β1-treated rat osteoblast-like cells suggests that TGF-β1 alters the abundance of both G_s and G_i proteins (39). TGF-β1 also modulates the expression of guanine nucleotide exchange factors (GEF) – proteins that regulate the activity of small G proteins – in various cells (40, 41). A study in murine fibroblasts suggests that TGF-β1 increases GTPase activity via a pertussis-sensitive mechanism (42). Further studies will be needed to investigate whether TGF-β1 modulates G protein expression or activity in HASM cells, and whether this potential modulation further affects HASM cell relaxation responses. However, our present results suggest that TGF-β1 – in addition to attenuating β2AR function – works downstream of the receptor level to impair ISO-stimulated cAMP levels.

We used forskolin – a direct activator of AC – as a tool to further investigate TGF-β1's downstream effects on the cAMP signaling pathway (43). In this study, TGF-β1 did not significantly alter forskolin-stimulated cAMP levels in HASM cells (Fig. 2B, 2E). Current literature suggests an unclear role for cytokines in modulating AC activity. In previous reports using human and guinea pig airway smooth muscle, TGF-β1 treatment induced little or modest

reductions in forskolin-stimulated cAMP accumulation (21, 34). Curiously, other reports demonstrate that chronic cytokine treatment sensitizes AC in HASM (44). In these studies, chronic incubation of HASM cells with the cytokine IL-1β or TNF-α caused a 2- to 3-fold increase in forskolin-stimulated cAMP (44, 45). It is posited that AC sensitization may be a feedback response to upregulate relaxation pathways in the face of cytokine-induced airway hyperresponsiveness (45). While TGF-β1 induces hyperresponsiveness in HASM cells (6), we did not find significant alteration of forskolin-stimulated cAMP in TGF-β1-treated HASM cells, (Fig. 2B). Thus, further studies will be needed to determine the effect of TGF-β1 on AC activation.

As TGF-β1 did not negatively regulate AC function in HASM cells, we next investigated the role of cAMP-hydrolyzing PDE enzymes in TGF-β1's attenuation of HASM cell relaxation responses. Previous reports suggest that TGF-β1 modulates PDE4 expression and activity. In human alveolar epithelial cells, TGF-β1 upregulated PDE4 mRNA, protein expression, and total cAMP-PDE activity (46). TGF-β1 has also been shown to mediate fibronectin, collagen I, and connective tissue growth factor induction in bronchial rings via a PDE4D-dependent mechanism (47). In human fetal lung fibroblasts, TGF-β1-mediated collagen gel contraction, fibronectin release, and fibroblast chemotaxis was inhibited in the presence of PDE4 pharmacological inhibitors (48). Therefore, we aimed to further investigate the role of PDE4 in the attenuation of ISO-induced cAMP by TGF-β1.

We demonstrate that TGF-β1 selectively induces PDE4D gene expression in HASM cells, and that PDE4D inhibition rescues attenuated ISO-induced cAMP levels in HASM cells (Fig. E3, 4A, 4B). While roflumilast only modestly enhanced ISO-mediated decreases in TGF-β1-induced MLC phosphorylation (Fig. 4C), roflumilast significantly enhanced ISO-induced, single-cell relaxation in TGF-β1-treated HASM cells (Fig. 4D). While discrepancies between biochemical and cell stiffness measurements in roflumilast-treated HASM cells are puzzling, studies suggest that both actomyosin cross-bridge cycling – regulated by MLC phosphorylation - and actin polymerization (49, 50) mediate HASM cell contractile responses. Reports demonstrate that TGF-β1 induces both MLC phosphorylation (6, 40) and actin polymerization (51, 52) in HASM cells. While the individual contributions of these pathways to HASM cell shortening remain unclear, both pathways are modulated by cAMP signaling (31, 53). Evidence suggests that PDEs shape compartmentalized cAMP signaling in the cell, where subcellular PDE localization mediates variations in cAMP-stimulated responses (16, 29, 54). As both PDE3 and

PDE4 hydrolyze cAMP in HASM, the observed discrepancy may result from the relative contribution of cAMP signaling to each pathway, driven by the spatially-mediated effects of PDE isoforms.

To further determine the mechanism by which TGF-β1 attenuates ISO-induced responses, we investigated the role of the canonical TGF-β1 signaling pathway via Smad2/3 in HASM cells (Fig. 5). In non-targeting and Smad2 siRNA-transfected cells, ISO-stimulated cAMP was decreased following TGF-β1 treatment (Fig. 5A). In Smad3 siRNA-transfected cells – however – TGF-β1 had little effect on ISO-induced cAMP. Surprisingly, ISO stimulation induced significantly higher cAMP levels in Smad3 siRNA-transfected cells than those observed in non-targeting siRNA-transfected cells.

This increase in cAMP may indicate that Smad3 knockdown attenuates baseline TGF-β1 receptor activity following the release of biologically active TGF-β1 in HASM cells (55). Alternatively, it is possible that Smad3 knockdown augments basal cAMP levels through its association with HASM cell microtubules. Smad3 has been reported to bind directly to microtubules in the absence of TGF-β1 signaling (56), and TGF-β1 has been shown to induce microtubule stability in a variety of cell types (57, 58). Therefore, impaired TGF-β1 signaling via Smad3 knockdown may exert destabilizing effects on microtubule stability.

Microtubule destabilization has been correlated with impaired cAMP accumulation in multiple cell types. The microtubule assembly inhibitor colchicine has been shown to induce cAMP generation in human leukocytes in a concentration-dependent manner (59). In human leukocyte and S49 lymphoma cell studies, multiple microtubule assembly inhibitors enhanced β-adrenergic and prostaglandin-stimulated cAMP accumulation in a time- and concentration-dependent manner, potentially by acting on microtubules that inhibit AC activity (60, 61). However, further studies are needed to determine the significance of the interaction between Smad3 and microtubules in HASM cells, and how this interaction may affect microtubule stability and cAMP generation.

In addition to modulating HASM cell cAMP levels, Smad2/3 knockdown also decreased TGF-β1-stimulated PDE4D gene expression (Fig. 5B). These findings were mirrored by a decrease in TGF-β1-stimulated PDE4D gene expression in HASM cells pre-treated with the TβR-I receptor inhibitor SB-431542 (Fig. 4A). SB-431542 is a highly selective inhibitor of the TβR-I receptor ALK5 (IC_{50} = 94 nM), and – to a lesser extent – the activin type I receptor ALK4, and the nodal type I receptor ALK7, which share highly-related kinase domains and

Smad2/3 proteins as substrates (62). SB-431542 selectively inhibits TGF-β1 signaling in HASM at concentrations as high as 10 μM – and exerts little effect on more divergent ALK family members that recognize bone morphogenic proteins – suggesting it to be an effective and selective inhibitor of Smad2/3 signaling in HASM (6, 62, 63). Together, these experiments suggest that TGF-β1-induced PDE4D gene expression is Smad2/3 activation-dependent.

In both Smad2 and Smad3 siRNA-transfected HASM cells, PDE4D gene expression in TGF-β1-treated cells was not significantly increased over vehicle control (Fig. 5B). These results are surprising given that Smad2 and Smad3 exert differential effects on β2-agonist-induced cAMP in TGF-β1-treated cells (Fig. 5A). However, these results support previous studies demonstrating that Smad2 and Smad3 can exert differential effects on cell function (6, 64, 65). It is possible that Smad3 selectively modulates PDE4D activity, while Smad2/3 mediate induction of PDE4D expression by TGF-β1. However, more studies will be needed to assess the potential role of Smad2/3 in PDE4D activation. Nonetheless, our collective findings demonstrate a role for TGF-β1 and Smad2/3 signaling in decreased HASM cell relaxation responses.

Due to the breadth and complexity of TGF-β1 signaling, there may be additional pathways by which TGF-β1 attenuates HASM cell cAMP levels that we did not investigate in this study. Other cytokines that attenuate HASM cell relaxation responses – such as IL-1β – attenuate ISO-induced cAMP via COX-2 induction and prostanoid release (66, 67). As TGF-β1 induces COX-2 expression in HASM cells (68), it is possible that prostanoid induction contributes to TGF-β1's impairment of relaxation responses. Further studies will be needed to determine the contribution of potential TGF-β1 signaling pathways in HASM cell relaxation responses.

In conclusion, our study further establishes TGF-β1 as a mediator of bronchodilator resistance in asthma via a Smad3-dependent pathway (Fig. 6). In light of our previous work on TGF-β1-induced hyperresponsiveness in HASM, these results further suggest TGF-β1 to be a promising therapeutic target to increase bronchodilator sensitivity and attenuate airway obstruction in asthma.

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FIGURE LEGENDS

Figure 1. TGF-β1 Decreases β2-Agonist-Induced Relaxation in HASM Cells. A) Single-cell relaxation of isoproterenol (ISO)-stimulated HASM cells in the presence or absence of TGF-β1 (10 ng/mL, 18 h) (N= 3 donors ± SEM). HASM cells were contracted with carbachol (CCh) for 5 min and subsequently relaxed with isoproterenol. CCh-stimulated stiffness was measured for the first 0-60 s, and changes in cell stiffness in response to ISO were measured continuously up to the indicated time (60-300 s). For each cell, stiffness was normalized to CCh-stimulated stiffness before ISO stimulation. **B)** Phosphorylated MLC following TGF-β1 (10 ng/mL, 18 h), CCh (20 μM; *bottom left*), and/or isoproterenol (ISO, 1 μM; *bottom right*) treatment (N=4-7 ± SEM). *Representative immunoblot of seven separate experiments. *P ≤ 0.05*

Figure 2. TGF-β1 Blunts Agonist-Induced cAMP Levels. A) HASM cells were pre-treated with TGF-β1 (10 ng/mL) overnight and acutely stimulated with ISO (1 μM, 5 min) (N=7 ± SEM; ISO 1 μM: 3684.2 ± 1170.0 pmol/well)**,** PGE2 (100 nM; 5 min) (N=4 donors ± SEM; PGE2: 40270.4 ± 25537.2 pmol/well), or **B)** Forskolin (10 μM; 15 min) (N=3 donors ± SEM; FSK 10 μM: 7192.4 pmol/well ± 3244.3) prior to lysis for cAMP level determination. **C)** Live HASM cells were pre-treated with TGF-β1 (10 ng/mL) overnight then acutely stimulated with various concentrations of this indicated drug and cAMP levels monitored using cADDis. Isoproterenol (vehicle $logEC_{50}$ -9.25 ± 0.258, E_{max} 0.0052 ± 0.00035; TGF-β1 $logEC_{50}$ -8.83 ± 0.433, E_{max} 0.0031 ± 0.00038). **D)** PGE2 (vehicle $logEC_{50}$ -9.08 ± 1.798, E_{max} 0.0026 ± 0.00065; TGF-β1 $logEC_{50}$ -8.37 ± 0.647, E_{max} 0.0021 ± 0.00048). **E)** Forskolin (vehicle $logEC_{50}$ -5.40 ± 1.547, E_{max} 0.010 ± 0.0012; TGF-β1 $logEC_{50}$ -5.44 ± 2.31, E_{max} 0.0074 ± 0.0132). Data is expressed as mean ± SEM of N=5 donors. **P ≤ 0.05 **P ≤ 0.01; ***P ≤ 0.001.*

Figure 3. PDE Inhibition Rescues ISO-Stimulated Responses in TGF-β1-Treated HASM Cells. A) MLC phosphorylation in HASM cells pre-treated with vehicle or TGF-β1 (10 ng/mL; 18h) and/or IBMX (500 μM, 30 min) prior to stimulation with CCh (20 μM; 12 min) and/or ISO (1 μM, 10 min) (N=4 ± SEM; Max: 23.2 fold change over vehicle ± 9.4). **B)** cAMP levels in TGF-β1 (10 ng/mL; 18h)-treated HASM cells pre-treated with vehicle (N=7± SEM; ISO 1 μM: 3684.2 ± 1170.0 pmol/well) or IBMX (500 μM, 30 min) (N=6± SEM; IBMX 1 μM ISO:

11927.4 ± 1599.3 pmol/well) prior to ISO (1 μM, 5 min) stimulation. N=4 donors ± SEM. *$P \leq 0.05$

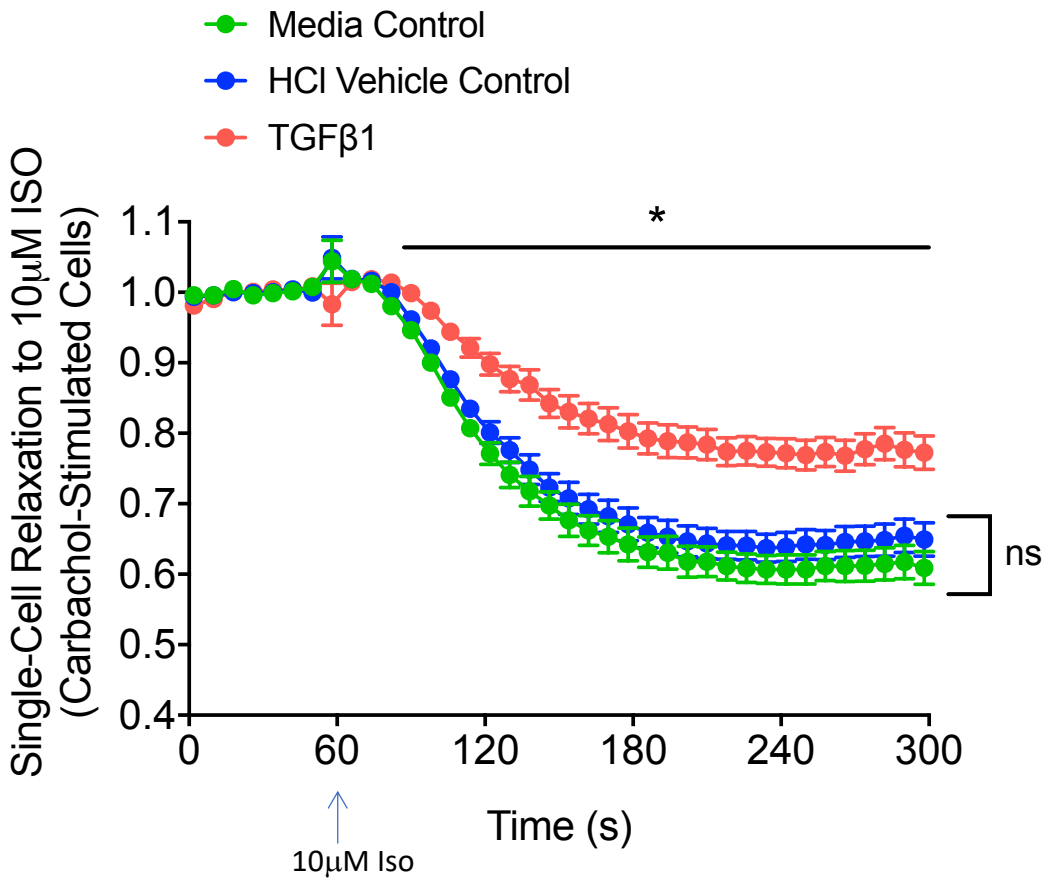
Figure 4. TGF-β1 Induces PDE4D Gene Expression in a Concentration-Dependent Manner. A) PDE4D gene expression in TGF-β1-treated (10 ng/ml; 18 h) HASM cells in the presence or absence of SB-431542 (5 μM; 1 h pretreatment) (N=3 donors ± SEM). **B)** cAMP levels in ISO-stimulated HASM cells treated with TGF-β1 (10 ng/mL; 18h) in the presence or absence of roflumilast (RF; 10 μM, 30 min) pretreatment (N=6 ± SEM; ISO μM**:** 1281.1 ± 406.6 pmol/well). **C)** MLC phosphorylation in TGF-β1 (10 ng/ml; 18 h)-treated HASM cells in the presence of roflumilast (RF; 10 μM, 30 min), CCh (20 μM, 12 min) and/or ISO (1 μM, 10 min) stimulation (N=6 donors ± SEM). **D)** Single-cell relaxation of TGF-β1 (10 ng/ml 18 h)-treated HASM cells in the presence or absence of roflumilast *(*RF; 10 μM, 30 min) (N=1 donor; N=223 ± SEM). *$P \leq 0.05$; *relative to control unless otherwise shown.*

Figure 5. TGF-β1-Decreases β2-Agonist-Induced Relaxation Responses in a Smad2/3-Dependent Manner. A) *Top:* cAMP levels in non-targeting (NT) or Smad2/3 siRNA-transfected HASM cells pre-treated with TGF-β1 (10 ng/mL, 18 h) and stimulated with CCh (20 μM; 10 min) and/or ISO (1 μM, 5 min) (N=4 donors ± SEM; Max: 15397.2 ± 3010.4 pmol/well). *Bottom:* Representative immunoblot of total Smad3 (*left,* 16% of NT siRNA control ± 15%, N=3) and total Smad2 (*right,* 10.7% of NT siRNA control ± 22.7%, N=3) protein expression in Smad2/3 siRNA transfected HASM cells. **B)** *Top:* PDE4D gene expression in non-targeting (NT)- or Smad2/3 siRNA-transfected HASM cells pre-treated with SB-431542 (5 μM, 30 min) prior to TGF-β1 (10 ng/mL) overnight treatment (N=3-4 donors ± SEM). *Bottom:* Representative immunoblot of total Smad3 (*left,* 20.3% of NT siRNA control ± 4.2%, N=3) and total Smad2 (*right,* 38.1% of NT siRNA control ± 23.4%, N=3) in Smad2/3 siRNA transfected HASM cells. *$P \leq 0.05$

Figure 6. Proposed Role of TGF-β1 in HASM Cell Contractile Responses in Asthma. TGF-β1 signaling augments basal and HASM cell shortening through a Smad3, ROCK-dependent pathway as previously described (6). In addition to modulating HASM cell contractile responses, Smad2/3 activation increases PDE4D gene expression, leading to increased cAMP hydrolysis and blunted HASM cell relaxation responses. TGF-β1, transforming growth factor beta 1; TβR-

I/II, TGF-β receptor I/II; ROCK, rho-associated protein kinase; RhoA, Ras homolog gene family, member A; MLCP, myosin light-chain phosphatase; MLCK, myosin light chain kinase; MLC20, 20-kDa myosin light chain 20; cAMP, cyclic adenosine monophosphate; 5'AMP, 5' adenosine monophosphate; PDE4D, phosphodiesterase 4D.

A


Media Control
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Single-Cell Relaxation to 10μM ISO
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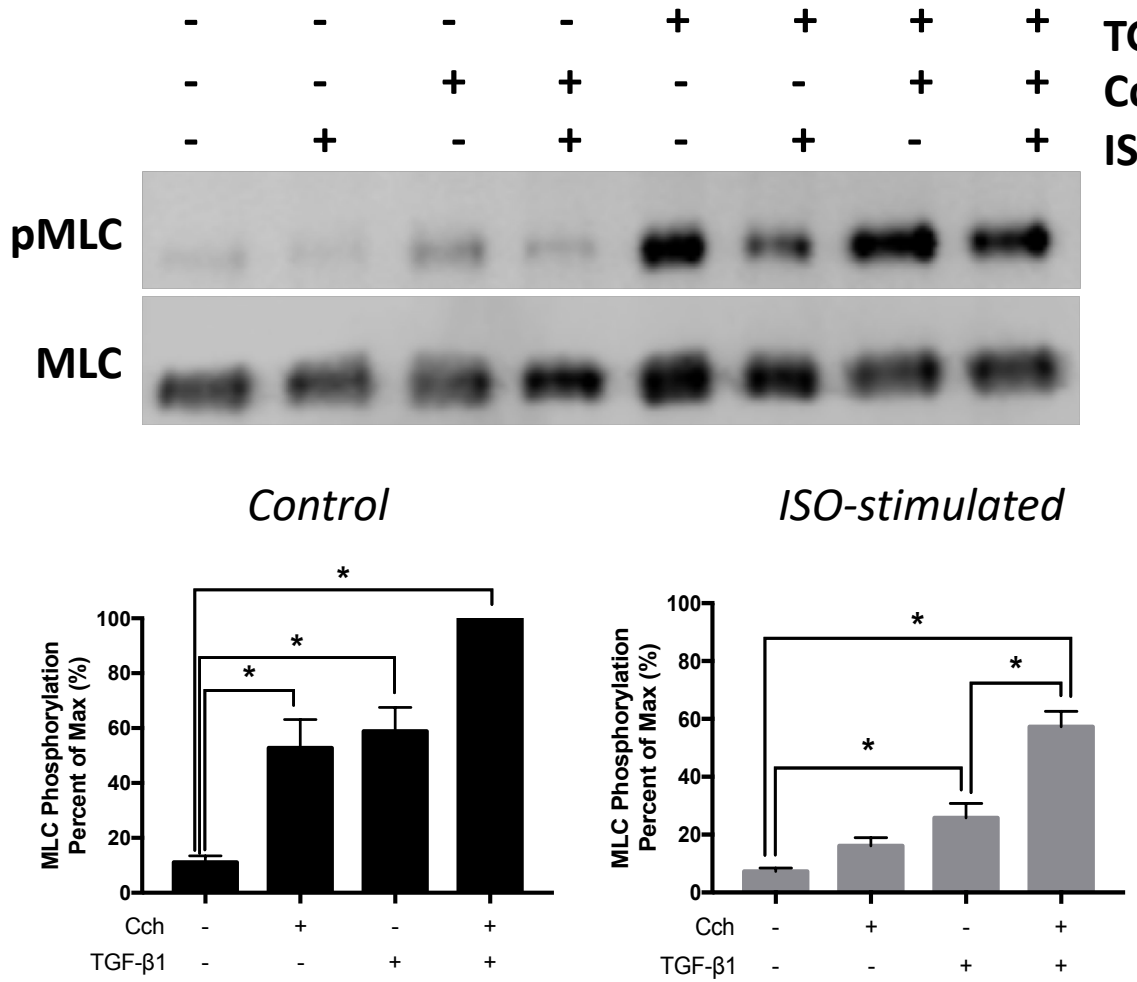
10μM Iso

B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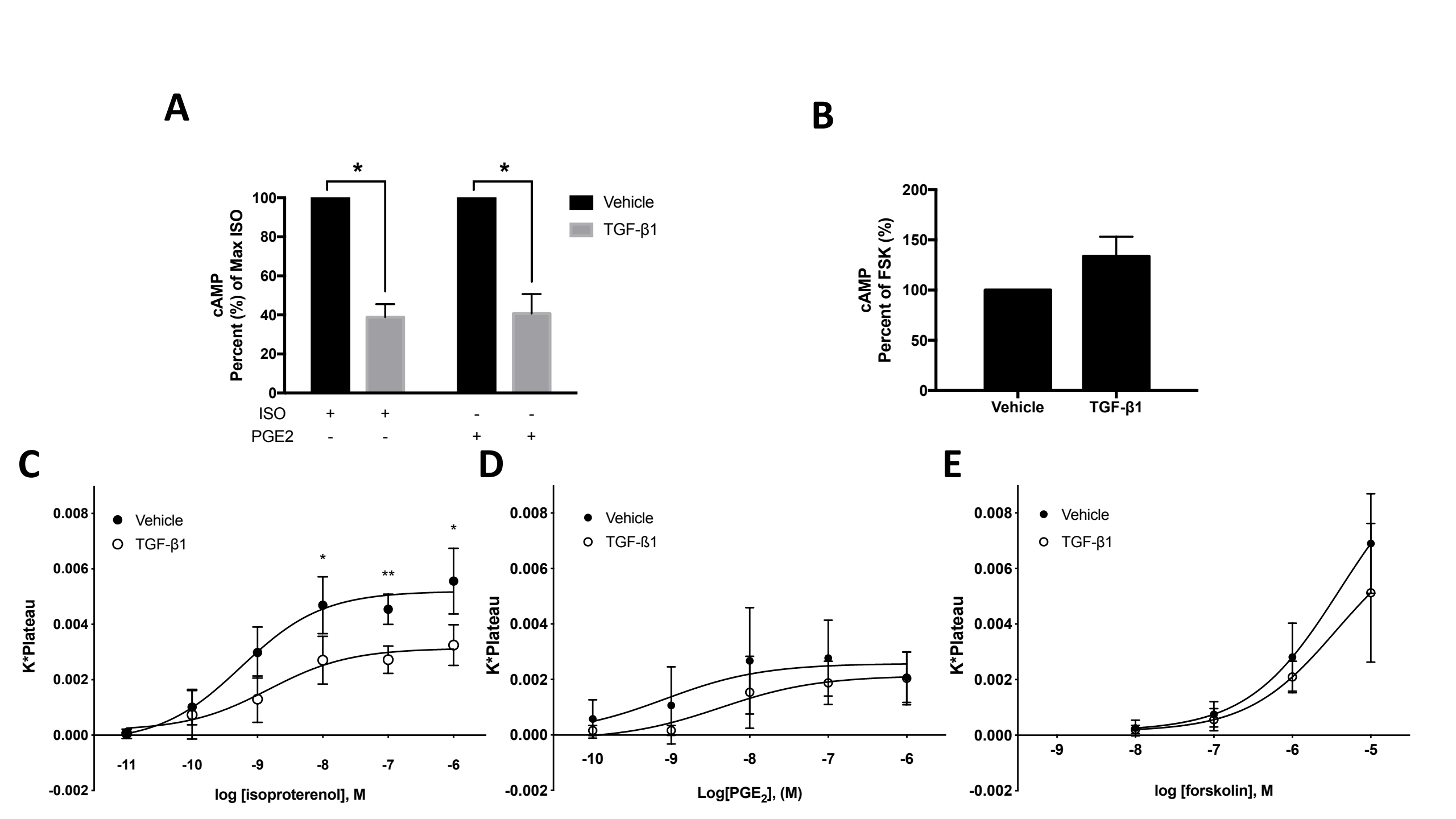
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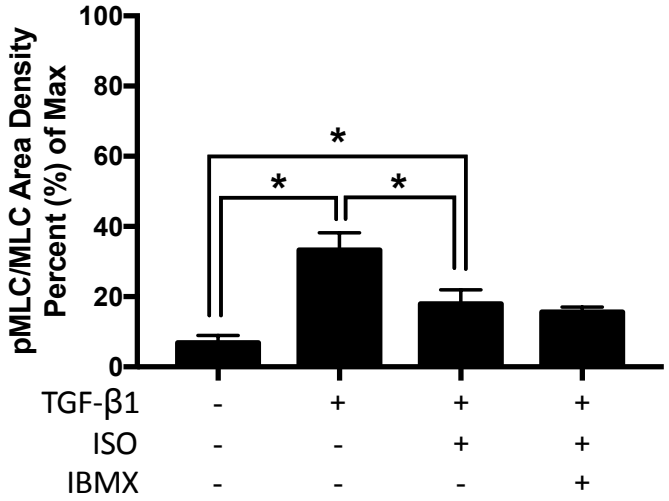
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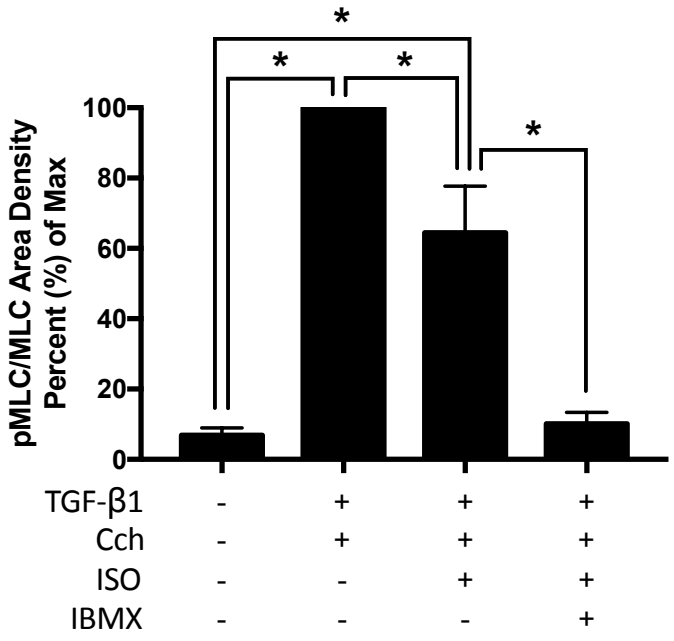
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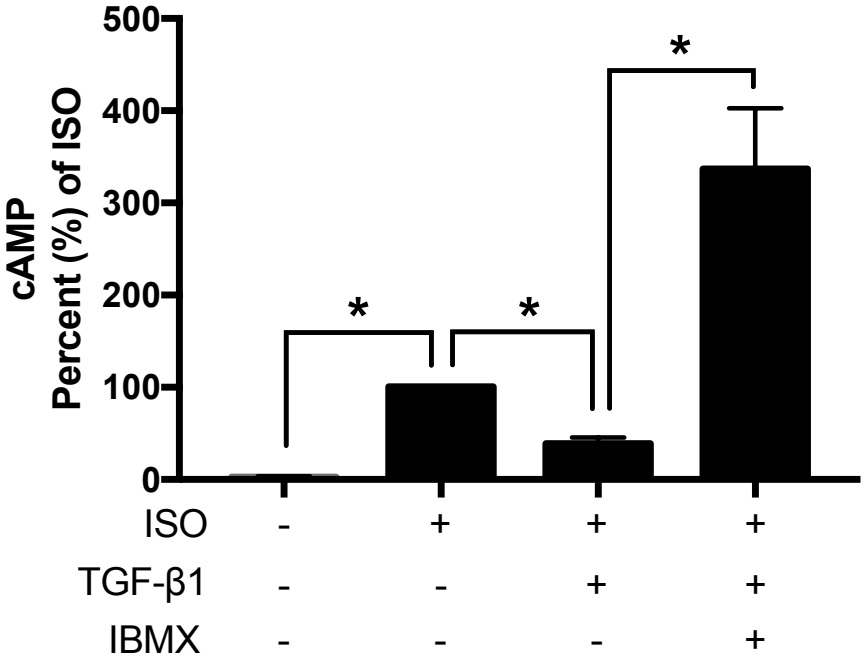
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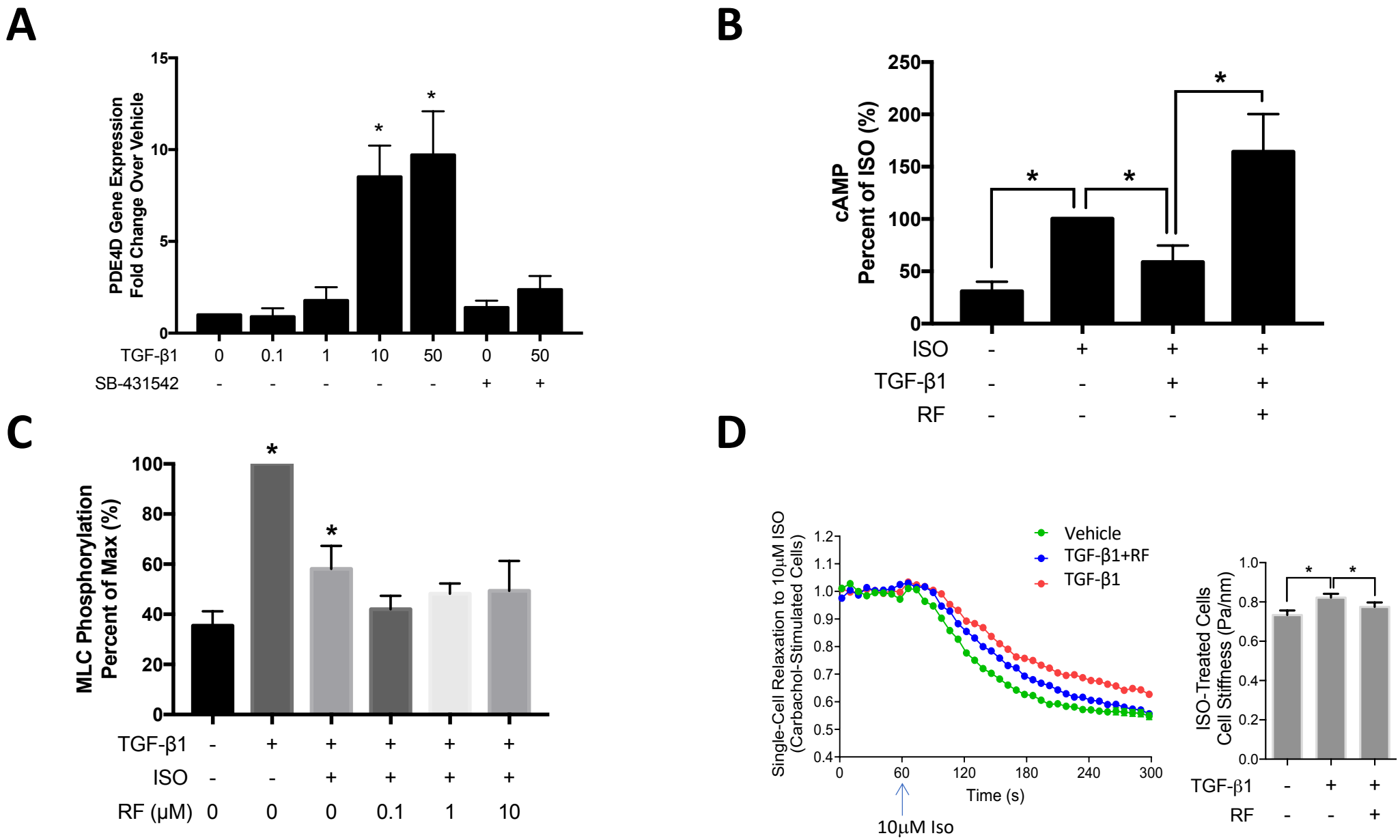
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TGF-β1 0 0.1 1 10 50 0 50
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cAMP
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ISO - + + +
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MLC Phosphorylation
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TGF-β1 - + + + + +
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D
Single-Cell Relaxation to 10μM ISO
(Carbachol-Stimulated Cells)
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Time (s)
10μM Iso
Vehicle
TGF-β1+RF
TGF-β1
ISO-Treated Cells
Cell Stiffness (Pa/nm)
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0.8
0.6
0.4
0.2
0.0
TGF-β1 - + +
RF - - +

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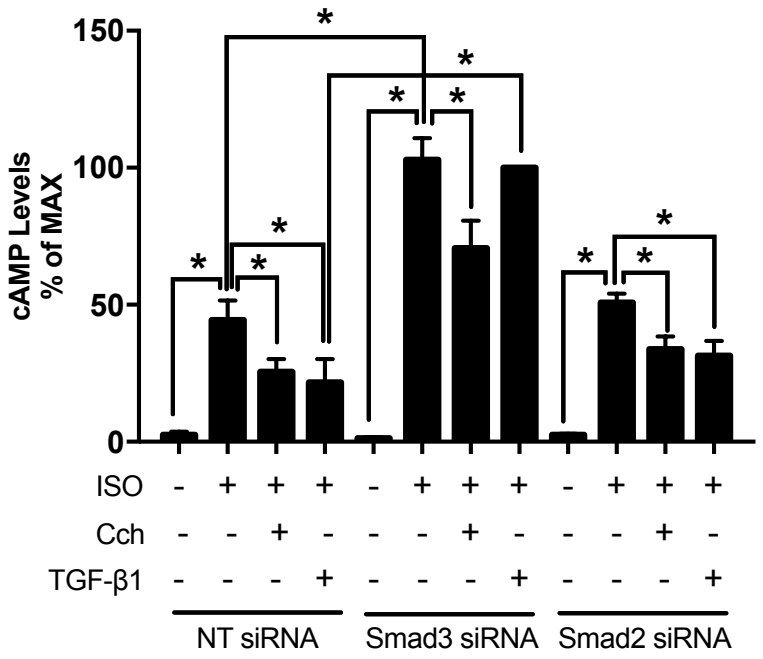
cAMP Levels
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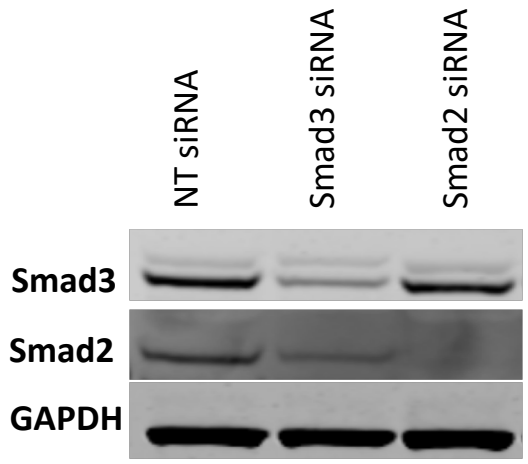
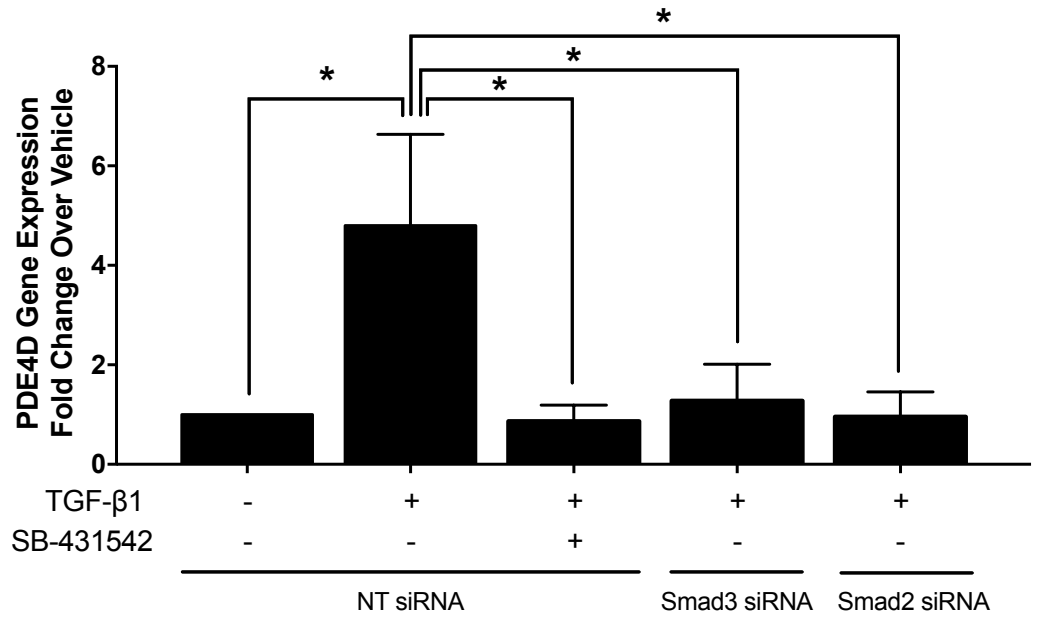
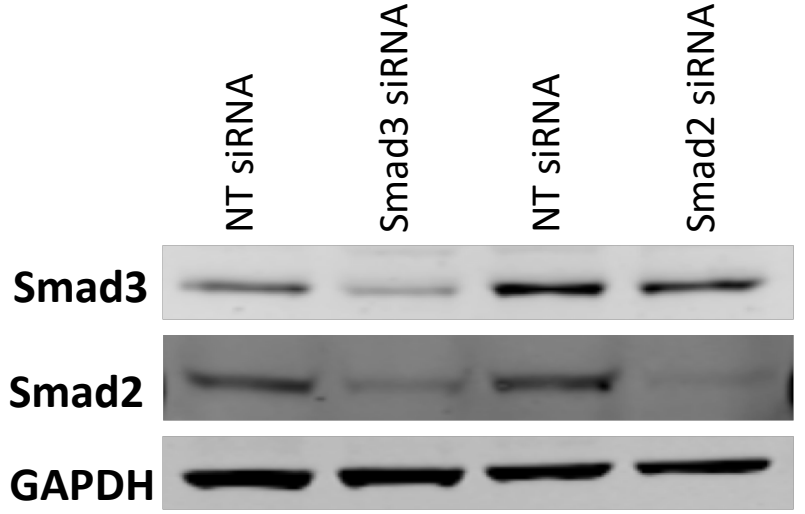
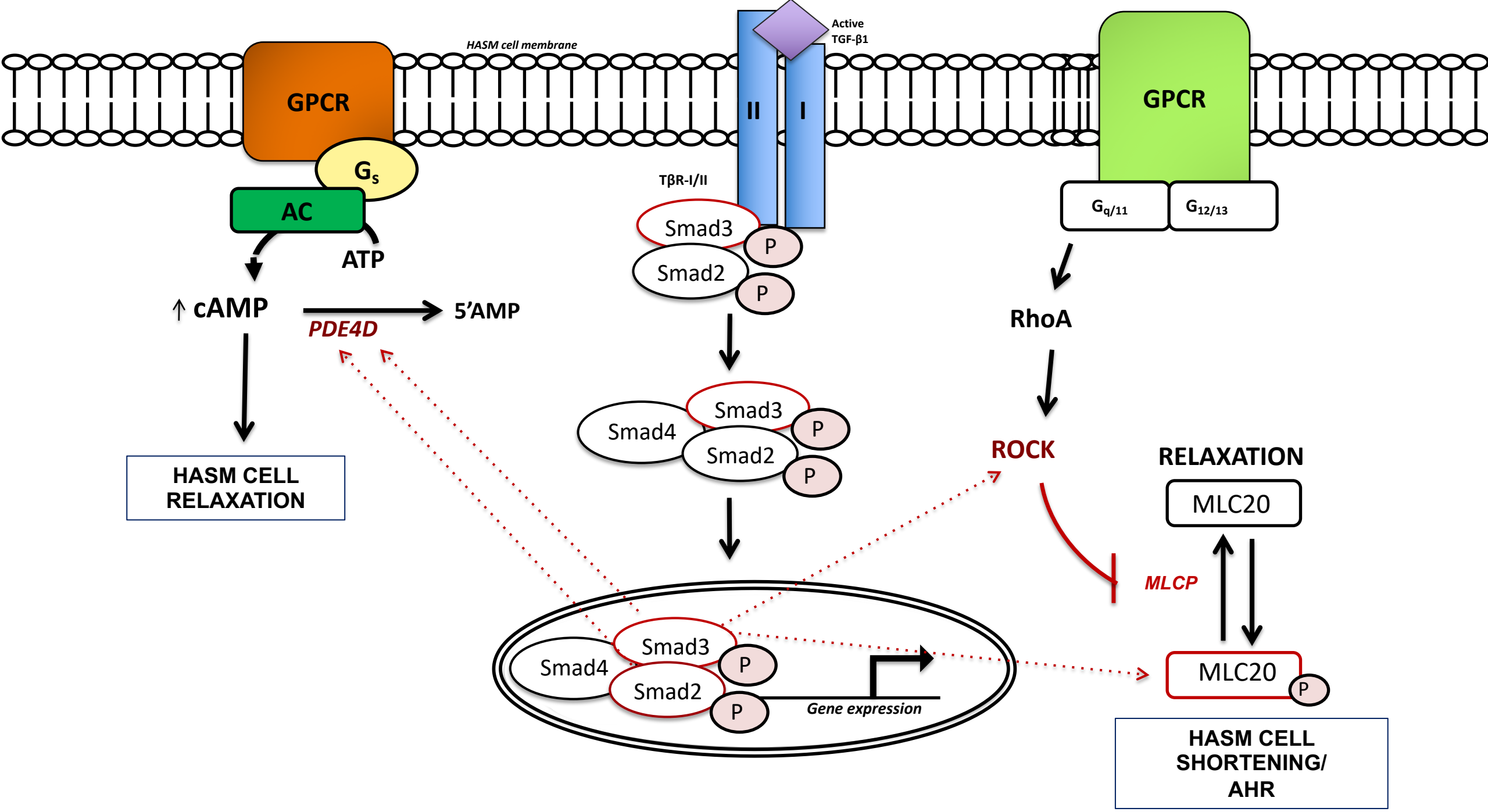
Cch
TGF-β1
NT siRNA
Smad3 siRNA
Smad2 siRNA


NT siRNA
Smad3 siRNA
Smad2 siRNA
Smad3
Smad2
GAPDH

B


PDE4D Gene Expression
Fold Change Over Vehicle
8
6
4
2
0
TGF-β1
SB-431542
NT siRNA
Smad3 siRNA
Smad2 siRNA


NT siRNA
Smad3 siRNA
NT siRNA
Smad2 siRNA
Smad3
Smad2
GAPDH


HASM cell membrane
Active TGF-β1
GPCR
G_s
AC
ATP
↑ cAMP
5'AMP
PDE4D
HASM CELL RELAXATION
TβR-I/II
II
I
Smad3
P
Smad2
P
Smad4
Smad3
P
Smad2
P
GPCR
$G_{q/11}$
$G_{12/13}$
RhoA
ROCK
RELAXATION
MLC20
MLCP
MLC20
P
Smad4
Smad3
P
Smad2
P
Gene expression
HASM CELL SHORTENING/ AHR

Supplementary Methods

Human Airway Smooth Muscle (HASM) Cell Culture

Human lungs from otherwise healthy, aborted transplant donors were received from the International Institute for the Advancement of Medicine (IIAM; Edison, NJ, USA) and the National Disease Research Interchange (NDRI; Philadelphia, PA, USA). All HASM cell cultures were derived from non-smokers with no prior documented history of respiratory disease (for additional data, see Supplemental Fig. 4). HASM cells were isolated from the trachea and cultured as previously described (1). HASM cells were used solely at subculture passages 1-4 due to strong native contractile protein expression (2). HASM cells were serum starved 24 h prior to treatment.

Immunoblot Analysis

Confluent HASM cells were serum starved overnight prior to treatment and collected as previously described (3). Briefly, HASM cells were serum-starved for 24 h prior to treatment, and perchloric acid was added to the cell media for a final concentration of 0.1%. Cells were then scraped, pelleted, and resuspended in RIPA lysis and sample buffer. Samples were then heated, subjected to SDS-PAGE, and then transferred to nitrocellulose membranes as previously described (3, 4). Phosphorylation of MLC was normalized to total MLC protein. Immunoblots are single experiments representative of at least three biological replicates.

Magnetic Twisting Cytometry (MTC)

Dynamic changes in cell stiffness were measured as an indicator of the single-cell contraction and/or relaxation of isolated HASM cells as previously described (5, 6). Briefly, RGD-coated ferrimagnetic microbeads (4.5 μm in diameter) bound to the cytoskeleton through cell surface integrin receptors were magnetized horizontally and then twisted in a vertically aligned homogeneous magnetic field that was varying sinusoidally in time. This sinusoidal twisting magnetic field caused both a rotation and a pivoting displacement of the bead: as the bead moves, the cell develops internal stresses which in turn resist bead motions (7).

To assess changes in cell stiffness, HASM cells were pre-contracted with carbachol (CCh) for 5 min and subsequently relaxed with isoproterenol. CCh-stimulated stiffness was measured for the first 0-60 s, and changes in cell stiffness in response to ISO were measured continuously up to the indicated time (60-300 s). For each cell, stiffness was normalized to CCh-stimulated stiffness before ISO stimulation. Lateral bead displacements in response to the resulting oscillatory torque

were detected with a spatial resolution of ~5 nm, and the ratio of specific torque to bead displacements was computed and expressed here as the cell stiffness in units of Pascal per nm (Pa/nm). Studies were conducted in the absence of a non-stimulated control as we have previously determined that a non-stimulated vehicle control does not appreciably change cell stiffness for the duration of our measurements (5, 6).

Small Interfering RNA (siRNA) Transfection

In vitro siRNA knockdown was performed using a reverse transfection procedure as previously described (8). Ham's F-12 media, siRNA, and HiPerFect Transfection Reagent (Qiagen #301705) were combined and incubated for 20 min at room temperature. Confluent HASM cells were trypsinized, pelleted, and resuspended in Ham's F-12 media. The HASM cell suspension was incubated with the siRNA mixture for 15 min prior to seeding on cell culture plates. After 6 h, complete cell culture media (Ham's F-12 medium supplemented with 100 U mL-1 penicillin, 0.1 mg mL-1, streptomycin, 2.5 mg mL-1 amphotericin B and 10% FBS) was added to the seeded cells in a 1:1 ratio for a final siRNA concentration of 10 μM. Media was replaced with complete media after 18 h, and cells were serum-starved and treated with TGF-β1 24 h prior to collection. Cells were collected 72 h post-transfection. Only experiments that successfully reduced target protein expression by ≥40% were included in the analysis.

Measurement of Cyclic AMP Levels

Following stimulation, cAMP levels were measured in lysed HASM cells using the Applied Biosystems cAMP-Screen® ELISA system according to manufacturer protocol. For kinetic measurement of cAMP production in live cells, HASM cells were infected with a recombinant BacMam virus expressing the cADDis cAMP sensor (Montana Molecular, Bozeman, MT) as previously described *(9)*. Cells were stimulated with agonist then fluorescence measured at 30 second intervals for 30 minutes. Data were fit to a single site decay model using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). Concentration-response curves were generated from each decay curve by multiplying the kinetic rate constant, k, with the plateau.

Quantitation of Phosphodiesterase (PDE) Gene Expression

RNA was isolated from HASM cells using the RNeasy Mini Kit (Qiagen Sciences, Inc., Germantown, MD, USA). cDNA was generated using SuperScript™ IV First-Strand Synthesis

System (Thermo Fisher Scientific, Waltham, MA, USA). Relative cDNA quantification for PDE isoforms was performed using TaqMan quantitative RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA) and the $\Delta\Delta C_t$ method. Gene expression assays were from Thermo Fisher and validated against –RT controls and known non-expressing cell lines (HEK293, HFL1, PC12, COS7).

Statistical Analysis

Unless otherwise stated, statistical analysis was conducted using GraphPad Prism software (La Jolla, CA, USA), with significance evaluated at a p-value of < 0.05. Significance was determined using Fisher's Least Significant Differences tests or multiple t-tests with Holm-Sidak correction. For MTC experiments involving multiple lung donor cell responses, statistical analysis was conducted using mixed effect models using SAS V.9.2 (SAS Institute Inc., Cary, NC) (10).

Materials

Compounds were purchased from Sigma Aldrich (St. Louis, MO, USA) [isoproterenol, prostaglandin E2, carbachol, perchloric acid], Selleck Chemicals (Houston, TX, USA) [roflumilast], Cayman Chemicals (Ann Arbor, MI, USA) [3-isobutyl-1-methylxanthine (IBMX)], and R&D Systems (Minneapolis, MN, USA) [TGF-β1, SB-431542]. Immunoblot antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA) [pMLC(3674S)] and EMT Millipore (Billerica, MA, USA) [MLC(MABT180)]. siRNA was purchased from Thermo Fisher Scientific (Waltham, MA, USA) [Smad3(VHS41114)] and Dharmacon (Lafayette, CO, USA) [Smad2(L-003561-00), Non-targeting Pool(D-001810-10-05)].

Supplemental References

E1. Panettieri R. Isolation and culture of human airway smooth muscle cells. *Methods Mol Med* 2001;56:155–60.

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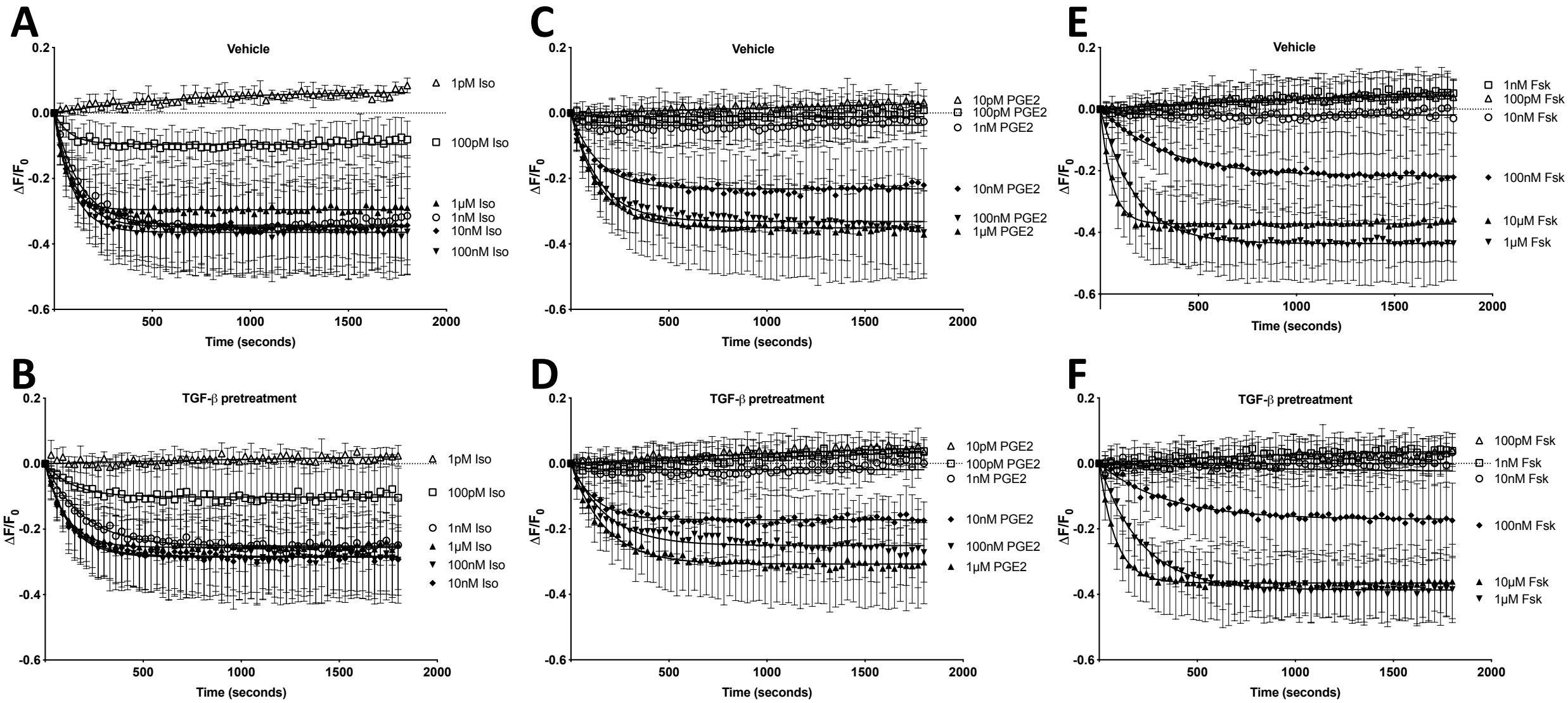
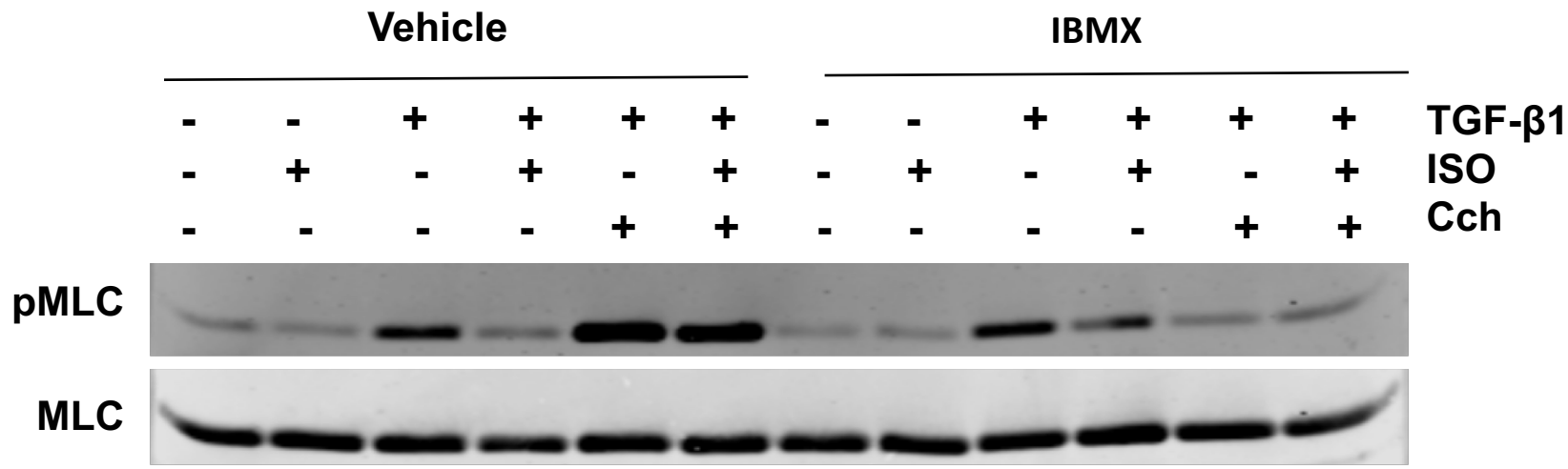
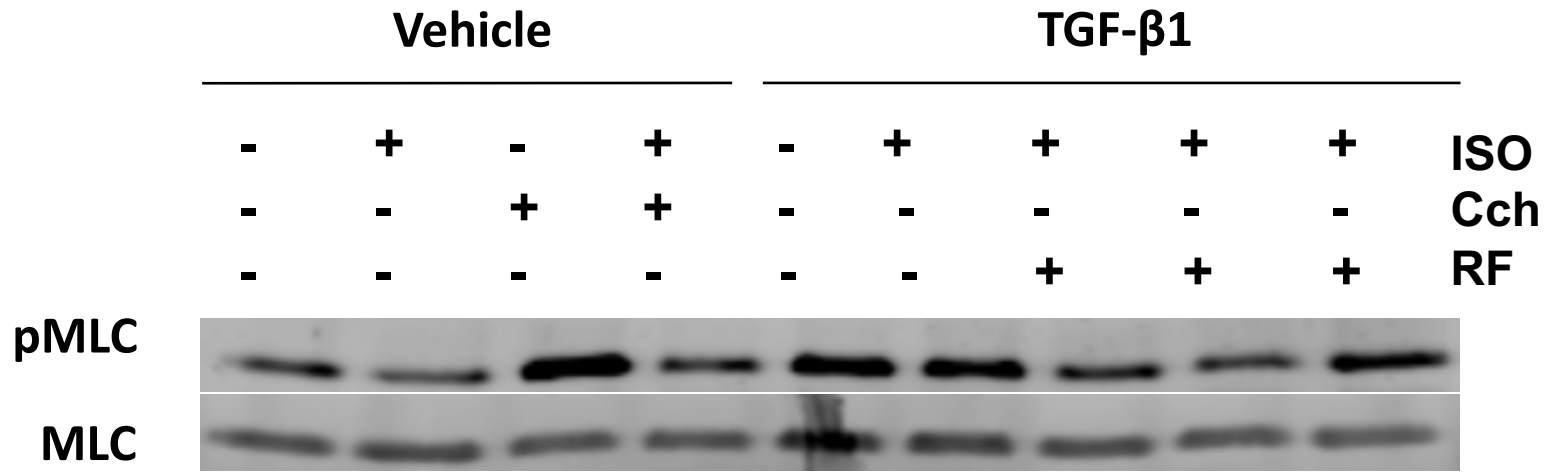
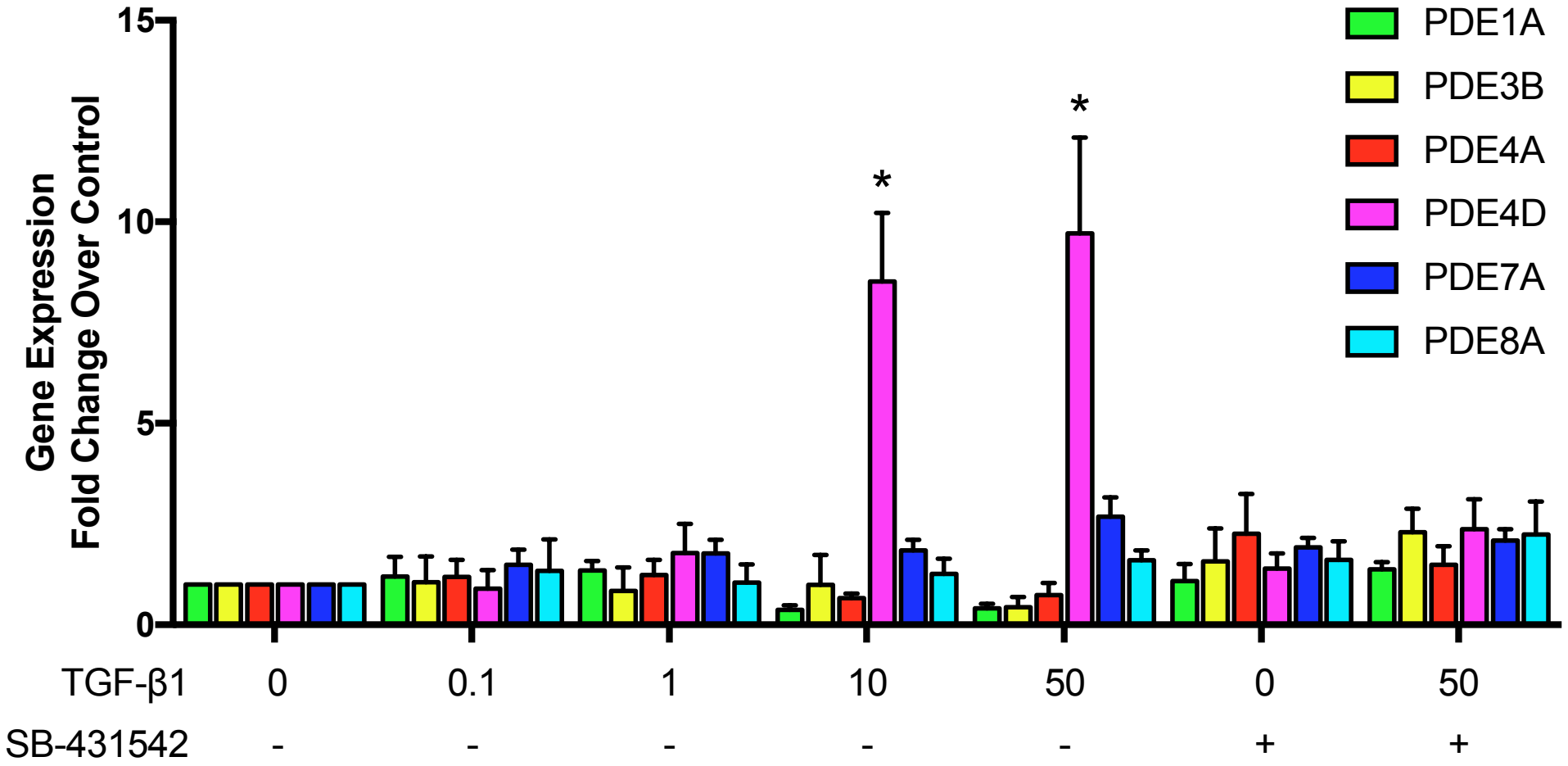

A
Vehicle
1pM Iso
100pM Iso
1μM Iso
1nM Iso
10nM Iso
100nM Iso
ΔF/F0
Time (seconds)
B
TGF-β pretreatment
1pM Iso
100pM Iso
1nM Iso
1μM Iso
100nM Iso
10nM Iso
ΔF/F0
Time (seconds)
C
Vehicle
10pM PGE2
100pM PGE2
1nM PGE2
10nM PGE2
100nM PGE2
1μM PGE2
ΔF/F0
Time (seconds)
D
TGF-β pretreatment
10pM PGE2
100pM PGE2
1nM PGE2
10nM PGE2
100nM PGE2
1μM PGE2
ΔF/F0
Time (seconds)
E
Vehicle
1nM Fsk
100pM Fsk
10nM Fsk
100nM Fsk
10μM Fsk
1μM Fsk
ΔF/F0
Time (seconds)
F
TGF-β pretreatment
100pM Fsk
1nM Fsk
10nM Fsk
100nM Fsk
10μM Fsk
1μM Fsk
ΔF/F0
Time (seconds)

A


Vehicle
IBMX
- - + + + + - - + + + +
TGF-β1
- + - + - + - + - + - +
ISO
- - - - + + - - - - + +
Cch
pMLC
MLC

B


Vehicle
TGF-β1
- + - + - + + + + +
ISO
- - + + - - - - -
Cch
- - - - - - + + +
RF
pMLC
MLC


Gene Expression
Fold Change Over Control
15
10
5
0
*
*
PDE1A
PDE3B
PDE4A
PDE4D
PDE7A
PDE8A
TGF-β1
0
0.1
1
10
50
0
50
SB-431542
-
-
-
-
-
+
+

DONOR CHARACTERISTICS

Sex, M/F	16/10
Age, yr	30.54 (13.82)
Race, C/B/H/NA	17/5/3/1
BMI, kg/m^2	28.96 (8.42)

Data are means (SD); *n* = 26 donors. M, male; F, female; C, Caucasian; B, Black; H, Hispanic; NA, Native American; BMI, body mass index