TGF-β1 Increases Expression of Contractile Genes in Human Pulmonary Arterial Smooth Muscle Cells by Potentiating Sphingosine-1-Phosphate Signaling

Yajing Ji¹, Erika M. Lisabeth¹, Richard R. Neubig¹,² *
¹Department of Pharmacology and Toxicology, Michigan State University,
East Lansing, Michigan, 48823
²Nicholas V. Perricone, MD, Division of Dermatology,
Department of Medicine,
College of Human Medicine
East Lansing, MI 48823

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Corresponding author:

R.R. Neubig, MD. Ph.D.

Department of Pharmacology & Toxicology

1355 Bogue Street, B440 Life Sciences Building

Michigan State University

East Lansing, MI 48823

Tel: 517-353-7145

Email: rneubig@msu.edu

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Abbreviations:

αSMA alpha smooth muscle actin
BMPR2 Bone morphogenetic protein receptor type 2
CNN1 Calponin 1
HPASMCs Human pulmonary arterial smooth muscle cells
MLC2 Myosin light chain 2
pMLC2 Phosphorylated myosin light chain 2
MRTF Myocardin-related transcription factor
PAH Pulmonary arterial hypertension
ROCK Rho-associated protein kinase
S1P Sphingosine-1-Phosphate
S1PR Sphingosine-1-Phosphate Receptor
SMAD Mothers against decapentaplegic homolog
SRF Serum response factor
SphK1 Sphingosine kinase 1
TGF-β Transforming growth factor β
Abstract

Pulmonary arterial hypertension (PAH) is characterized by elevated pulmonary arterial pressure and carries a very poor prognosis. Understanding PAH pathogenesis is needed to support the development of new therapeutic strategies. TGF-β drives vascular remodeling and increases vascular resistance by regulating differentiation and proliferation of smooth muscle cells (SMCs). Also, sphingosine-1-phosphate (S1P) has been implicated in PAH but the relation between these two signaling mechanisms is not well understood. Here, we characterize the signaling networks downstream of TGF-β in human pulmonary arterial smooth muscle cells (HPASMCs) which involves SMAD signaling as well as Rho GTPases. Activation of Rho GTPases regulates myocardin-related transcription factor (MRTF) and serum response factor (SRF) transcription activity and results in upregulation of contractile gene expression. Our genetic and pharmacologic data show that in HPASMCs, upregulation of alpha smooth muscle actin (αSMA) and calponin (CNN1) by TGF-β is dependent on both SMAD and Rho/MRTF-A/SRF transcriptional mechanisms.

The kinetics of TGF-β-induced myosin-light chain 2 (MLC2) phosphorylation, a measure of RhoA activation, is slow, as is regulation of the Rho/MRTF/SRF-induced αSMA expression. These results suggest that TGF-β1 activates Rho/pMLC2 through an indirect mechanism which was confirmed by sensitivity to cycloheximide treatment. As a potential mechanism for this indirect action, TGF-β1 upregulates mRNA for sphingosine kinase (SphK1), the enzyme that produces sphingosine-1-phosphate (S1P), an upstream Rho activator as well as mRNA levels of the S1P Receptor 3 (S1PR3). A SphK1 inhibitor and S1PR3 inhibitors (PF543 and TY52156/VPC23019) reduce TGF-β1-induced αSMA upregulation. Overall, we propose a model where TGF-β1 activates Rho/MRTF-A/SRF by potentiating an autocrine/paracrine S1P signaling mechanism through SphK1 and S1PR3.
Significance Statement

In human pulmonary arterial smooth muscle cells, TGF-β depends on S1P signaling to bridge the interaction between SMAD and Rho/MRTF signaling in regulating αSMA expression. The Rho/MRTF pathway is a signaling node in the αSMA regulatory network and is a potential therapeutic target for the treatment of PAH.
Introduction

Pulmonary arterial hypertension (PAH) is characterized by elevated mean pulmonary arterial blood pressure and right heart failure which often leads to death. In the US, PAH has a prevalence of 12.4 cases/million people (Burger, D'Albini, Raspa, & Pruett, 2016). PAH patients are diagnosed at a mean age of 50 ±14 and their three year-survival rate is 63% (Leopold & Maron, 2016; McGoon & Miller, 2012). The standard of care for PAH is focused on alleviating symptoms but fails to stop disease progression. To develop therapeutic approaches which halt PAH progression it is critical to better understand the cellular and molecular mechanisms underlying disease progression.

Both vasoconstriction and vascular remodeling contribute to increased vascular resistance in the pulmonary circulation, ultimately leading to elevated blood pressure (Vaillancourt, Ruffenach, Meloche, & Bonnet, 2015). Vasoconstriction results from the contraction of smooth muscle cells (SMCs) (Vaillancourt et al., 2015). Dysregulated proliferation, migration, and hypertrophy of SMCs contribute to vascular remodeling (Vaillancourt et al., 2015), which involves TGF-β signaling in PASMCs (Morrell et al., 2001). Also, TGF-β is elevated in the serum of PAH patients (Yan et al., 2016). TGF-β signaling is one of the main drivers of aberrant SMCs behavior in PAH (Rol, Kurakula, Happe, Bogaard, & Goumans, 2018).

Generally, smooth muscle cells are categorized into two mutually exclusive phenotypes. The contractile phenotype has high expression of contractile proteins, a lower proliferation rate and is less migratory (Gomez & Owens, 2012). The proliferative phenotype has low expression of contractile proteins and an elevated rate of proliferation and migration (Gomez & Owens, 2012). In PAH, PASMCs have both increased proliferation and elevated levels of contractile proteins such as αSMA (Zabini et al., 2018). Interestingly, TGF-β promotes differentiation of PASMCs isolated from non-diseased lungs but paradoxically drives proliferation of PASMCs isolated from PAH patients (Morrell et al., 2001). Further investigation of how TGF-β regulates the
proliferation and differentiation of PASMCs is important for understanding the mechanism of this dual proliferative and contractile SMC phenotype in PAH pathogenesis.

TGF-β regulates gene expression through SMAD2/3, which is generally considered to be the canonical signaling pathway (Xing et al., 2015). TGF-β also regulates gene expression through SMAD-independent mechanisms such as Rho/MRTF/SRF (Xing et al., 2015). This starts with the activation of RhoA subfamily small GTPases which, in turn, induces actin polymerization. Actin polymerization drives the transcriptional coactivator MRTF to translocate into the nucleus where MRTF binds to serum response factor (SRF) to regulate gene expression (Guo & Chen, 2012). The suites of genes regulated by SMAD and Rho/MRTF/SRF pathways overlap in the modulation of fibrosis and cellular migration by TGF-β, suggesting that these two transcriptional mechanisms may cooperate to regulate gene transcription (He & Dai, 2015). Both SMAD and Rho/MRTF/SRF upregulate the expression of contractile genes and markers of differentiation, such as αSMA (Guo & Chen, 2012). Elevated expression of contractile genes results in increased SMCs contractility (Bai et al., 2020), which could result in excessive vasoconstriction and SMC hypertrophy in PAH. During myofibroblast differentiation in mouse fibroblast 10T1/2 cells, SMAD3 interacts directly with an SRF-associated complex and mediates TGF-β-induced expression of SM22, another contractile protein (Qiu, Feng, & Li, 2003). It is unknown whether a similar mechanism applies to the transcriptional regulation of αSMA and CNN1 in HPASMCs.

It is also unclear how TGF-β activates Rho/MRTF/SRF. In human-derived fibroblasts, TGF-β increases the level of the Rho activator S1P by upregulating its synthetic enzyme SphK1 (Yamanaka et al., 2004). This suggests a model wherein TGF-β activates Rho signaling through S1P. S1P is a bioactive sphingolipid which binds to a GPCR family of S1P receptors, activates RhoA, and stimulates expression of αSMA and CNN1 in SMCs (Wamhoff, Lynch, Macdonald, & Owens, 2008). SMAD3 activation is responsible for SphK1 upregulation in C2C12 myoblasts (Cencetti, Bernacchioni, Nincheri, Donati, & Bruni, 2010). It is possible that TGF-β upregulates SphK1 levels through a SMAD pathway in HPASMCs. This in turn could promote S1P synthesis.
and activate Rho/MRTF/SRF in an autocrine/paracrine manner. Consistent with this, plasma S1P levels are increased in idiopathic PAH patients and in a rodent model of PAH (Gairhe et al., 2016). Genetic and pharmacologic inhibition of SphK1 activity is protective in PAH animal models (J. Chen et al., 2014; Gairhe et al., 2016), highlighting the importance of S1P signaling in PAH. Further clarifying the signaling interaction of between the TGF-β and the S1P pathways will increase our understanding of understanding of the molecular mechanisms underlying PAH pathogenesis.

In this study we explore crosstalk mechanisms between SMAD signaling and Rho/MRTF/SRF signaling in order to better understand how TGF-β modulates SMCs. We provide evidence that S1P signaling bridges the SMAD and Rho/MRTF/SRF pathways to co-regulate gene expression in HPASMCs. Our data also suggests that this is primarily mediated by S1PR3.

Materials and Methods

Cell Culture

Human Pulmonary Artery Smooth Muscle Cells (ThermoFisher, Waltham, MA #C0095C) were cultured in Medium 231 (ThermoFisher #M231500) supplemented with SMGS (smooth muscle growth supplement, ThermoFisher #S00725) and 1% Antibiotic-Antimycotic (ThermoFisher #15240062). HPASMCs (passage 6-8) were starved in 0.1% SMGS Medium 231 overnight prior to any experiments.

Compounds and Antibodies

Recombinant human TGF-β1 protein was purchased from Research And Diagnostic Systems, Inc. (Minneapolis, MN). Y-27632 (#S1049) was purchased from Selleckchem, Houston, TX. SIS3 (#15945), JTE-013 (#10009458), TY 52156 (#19119), VPC23019 (#13240) and PF-543 (#17034) were purchased from Cayman Chemical (Ann Arbor, MI). All compounds were
dissolved in DMSO and frozen at -20°C. Antibodies against MRTF-A (#sc21558) and MRTF-B (#sc98989) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against MRTF-A (#14760), Smad2/3 (#8685), pMLC2 (#3674) and MLC2 (#3672) were ordered from Cell Signaling (Danvers, MA). αSMA antibody (#7817) and pSMAD3 antibody (#52903) was purchased from Abcam (Cambridge, MA) and CNN1 antibody (13938-1-AP) was purchased from Proteintech (Rosemont, IL). All secondary antibodies [Donkey anti-Mouse680 (#C31216-02), Donkey anti-Mouse800 (#C90507-03), Donkey anti-Goat680 (#C41105-05), Donkey anti-Rabbit680 (#C40130-02) and Donkey anti-Rabbit800 (#C90129-05)] are all purchased from LI-COR (Lincoln, NE). The MRTF/SRF pathway inhibitor CCG-222740 (Hutchings et al., 2017) was obtained from the lab of Dr. Scott Larsen at the University of Michigan.

**siRNA Transfection**

ON-TARGETplus siRNA for MRTF-A (Dharmacon #L-015434-00-0010, Lafayette, CO), MRTF-B siRNA (Dharmacon #L-019279-00-0010) and non-targeting pool control (Dharmacon #D-001810-10-05) were used based on the manufacturer’s protocol. siRNAs were diluted in OptiMEM, mixed with DharmaFECT (Dharmacon #T-2001-01) and then mixed with fresh medium 231 with 5% SMGS at a final concentration of 25 nM. Cells were seeded at a density of ~80% confluence and were transfected overnight. The next day the cells were serum starved for 16-20 hours prior to the treatment with TGF-β.

**RT-qPCR**

HPASMCs were re-suspended in complete medium and 180,000 cells were seeded in each well of a 6-well plate. The cells were allowed to reach confluence (approximately four days) before being serum-starved in 0.1% SMGS Medium 231 overnight. Cells were treated as described in the figure legends and total cellular RNA was collected using the RNeasy kit (Qiagen, Hilden, Germany #74104) according to the manufacturer’s protocol. The High-Capacity cDNA RT kit (ThermoFisher #4368814) was used to reverse transcribe the RNA into cDNA following the
manufacturer’s protocol. SYBR Green PCR Master Mix (ThermoFisher #4309155) was used to perform qPCR following the manufacturer’s protocol on the Stratagene Mx3000P qPCR machine. Fold-change of gene expression was normalized to GAPDH and analyzed by the ΔΔC_T method. Primer sequences are listed in Supplemental Table S1.

Immunoblotting
HPASMCs were cultured and treated as described in the figure legends. Total cellular protein was collected in 2x Laemmli Sample Buffer (Biorad, Hercules, CA #1610737). After heating the samples at 100°C for 10 mins, protein samples were resolved on 10% (MRTF) or 12% (pMLC2/MLC2) polyacrylamide gels and transferred to PVDF Membranes (Millipore Sigma, Burlington, MA #IPFL00010). Blots were blocked in Odyssey Blocking buffer in PBS (LI-COR #927-40000) at room temperature for 1 h, then incubated with primary antibody at room temperature for 1 h or overnight at 4°C. Blots were washed three times for 5 min each with Tris-buffered saline with 0.1% Tween-20 (TBST) and then incubated with the appropriate secondary antibodies diluted 1:10,000 in blocking buffer at room temperature for 1 h. After 3 washes for 5 mins each with TBST, blots were imaged using a LI-COR Odyssey FC instrument and analyzed using Image Studio Lite software v.4.0.

Cell Proliferation
HPASMCs were cultured and were seeded in 96-well plates at a density of 10,000 cells/well. The next day, the cells were starved in 1% SMGS Medium 231 for 14~18 hours. Then the cells were cultured in 1% SMGS Medium 231 with or without 10 ng/mL TGF-β1 for two days. Cells were then fixed with 3.7% formaldehyde for 10 mins. After three washes with PBS for 5 mins, cells were stained with 500 ng/mL DAPI. Images were captured at the center of each well using a Cytation 3 automated microscope (Biotek). All images were blinded by an automated R script before quantifying the cell numbers with Image J.
Statistical Analysis

Data were analyzed through either Paired t-test or One-way Repeated Measures ANOVA followed by Dunnett's post-test using GraphPad Prism 7. Data are presented as the mean ± SD, and P < 0.05 is considered statistically significant.

Results

Both SMAD and Rho/MRTF/SRF pathways are necessary for TGF-β-induced contractile gene expression

To identify the mechanism by which TGF-β1 regulates expression of the contractile proteins αSMA and CNN1 in HPASMCs, we first inhibited phosphorylation of SMAD3 using SIS3. TGF-β1-induced αSMA and CNN1 expression were reduced to control levels by 10 μM SIS3 (Figure 1A). This suggests that phosphorylation of SMAD3 is important for TGF-β1-induced contractile gene expression. In order to test the role of Rho/MRTF/SRF in regulating contractile gene expression, we used the ROCK inhibitor Y27632 and the MRTF/SRF pathway inhibitor CCG-222740 (Hutchings et al., 2017). Y27632 and CCG-222740 reduced TGF-β1-induced expression of contractile genes by approximately 60% and 100% respectively (Figure 1A & 1B). Finally, siRNA-mediated silencing of MRTF-A reduced TGF-β1-induced protein levels of αSMA, however there was only a minor reduction upon MRTF-B silencing. (Figure 1C). Taken together, these data show that both SMAD and the Rho/MRTF-A/SRF pathways are required for TGF-β1-induced contractile gene expression.

TGF-β indirectly activates Rho signaling

In order to better understand how TGF-β1 activates Rho signaling in HPASMCs, we first measured the kinetics of TGF-β1-induced phosphorylation of myosin-light chain 2 (MLC2), a
commonly used readout of Rho activation (Yu et al., 2017). To assess the kinetics of TGF-β1-induced MLC2 phosphorylation, HPASMCs were treated with TGF-β1 for 1 to 9 hours; phosphorylated MLC2 (pMLC2) was increased only after 6-9 hours (Figure 2B). In contrast, S1P-induced MLC2 phosphorylation is maximal after 0.5-1 hours of treatment (Figure 2A). S1P is a GPCR agonist that signals through G\(_{12/13}\), which can rapidly activate RhoA and induce MLC2 phosphorylation (Ambesi & McKeown-Longo, 2009). The delayed kinetics of TGF-β1-induced MLC2 phosphorylation suggests that the action of TGF-β1 is through an indirect, perhaps transcriptional/translational signaling mechanism. Consistent with this hypothesis, cycloheximide blocked TGF-β1-induced MLC2 phosphorylation but did not suppress, and even slightly enhanced, S1P-induced MLC2 phosphorylation (Figure 2). Thus, TGF-β1-induced Rho signaling activation, as indirectly detected by pMLC2 levels, requires the translation of new proteins (Figure 2B). In contrast, TGF-β1-induced SMAD3 phosphorylation peaks at 1 h and is not blocked by cycloheximide, consistent with the expected direct activation of SMAD3 phosphorylation by the TGF-β receptor (Figure S1).

**TGF-β induces mRNA expression of SphK1**

Given our hypothesis that TGF-β1 induces Rho activation indirectly by upregulating Rho activators, we next wanted to identify which factors may be mediating this process. We found that TGF-β1 increases the mRNA level of Endothelin-1, Connective tissue growth factor (CTGF) and SphK1. However, in our preliminary experiments (data not shown), only S1P signaling inhibitors reduced TGF-β1-induced αSMA expression, so we focused the remainder of our studies on S1P signaling. We compared the kinetics by which TGF-β1 upregulates SphK1 with that of αSMA and CNN1 upregulation (Figure 3A). HPASMCs were treated with TGF-β1 for 1, 3, 6, 12 or 24 hours. αSMA mRNA was upregulated 3.8-fold by TGF-β1 at 12h, while SphK1 mRNA was increased 4- or 7-fold at 3 or 6h respectively. The peak of SphK1 mRNA level at 6h is approximately the same time when αSMA mRNA started to increase, supporting the idea that
increased S1P activated αSMA expression. This is similar to results observed in fibroblasts where TGF-β1 activates Rho signaling through S1P (Cencetti et al., 2010). The time course of the increase in CNN1 mRNA (Figure 3A) was more similar to that for SphK1 than for αSMA, but CNN1 mRNA had a delayed peak at 12h (8-fold) as compared to SphK1. CNN1 mRNA increased by 2.6-fold at 3h, which is earlier than TGF-β1-induced phosphorylation of MLC2 (Figure 2B). This suggests that TGF-β1 regulation of CNN1 expression may have a distinct mechanism from αSMA.

**TGF-β modulates the expression of S1P receptor 3**

In HPASMCs, 10 µM S1P induces approximately a 1.4-fold increase in αSMA protein levels (Figure S2). Surprisingly, with a lower concentration of S1P (1 µM), we did not observe induction of αSMA expression, which is inconsistent with the observation that S1P induces αSMA expression and differentiation in smooth muscle cells (Lockman et al., 2004). The blunted response of HPASMCs to S1P may be a result of different cell types having a different response to S1P, or it could be because primary cells may respond differently to S1P than cultured cell lines. In addition to the levels of S1P, the amount and composition of its receptors could also determine cellular responses to S1P. Consequently, we tested whether TGF-β1 also modulates the expression of S1P receptors in addition to SphK1. Based on the literature, HPASMCs express three subtypes of S1P receptors. Both S1PR2 and S1PR3 are coupled to G_{12/13} which, in-turn, will result in Rho activation (Wamhoff et al., 2008). We found that S1PR3 mRNA was elevated after 3 h of TGF-β1 treatment and was further increased after 12 h. S1PR1 and S1PR2 mRNA were unaffected by TGF-β1 (Figure 3B). The TGF-β1-upregulated S1PR3 mRNA levels was reduced by the SMAD3 phosphorylation inhibitor SIS3 from 2.7- to 1.6- fold over control (95% confidence interval of the difference is -2.5 to 0.3, Figure 3C). Although this effect was not
statistically significant due to variability, treatment with the SMAD inhibitor SIS3 reduced the stimulation of S1PR3 expression by about 65%.

**S1PR3 antagonists reduce TGF-β-induced αSMA expression but an S1PR2 antagonist does not**

To determine which S1P receptors might be functionally relevant for TGF-β1-induced αSMA and CNN1 expression, HPASMCs were treated with TGF-β1 for 24 hours along with the S1PR2 antagonist JTE013, the S1PR3 antagonist TY52156, or the dual S1PR1/3 antagonist VPC23019. Both the S1PR3 and the S1PR1/3 antagonists reduced TGF-β1-induced stimulation of αSMA and CNN1 mRNA levels after 24 hours of co-treatment (Figure 4A). There was no effect of the S1PR2 antagonist JTE013. αSMA protein levels were also reduced by the two antagonists (TY52156 and VPC23019) targeting the S1PR3 receptor (Figure 4B). Inhibition of CNN1 protein levels by these two antagonists was only modest and did not achieve statistical significance. The overlapping effect of S1PR3 and S1PR1/3 antagonists leads us to conclude that TGF-β1 regulates αSMA expression through S1P signaling mainly via S1PR3, while CNN1 expression may also be regulated by S1PR3-independent signaling mechanisms.

**SphK1 inhibitors reduce TGF-β-induced αSMA expression**

To test whether SphK1 regulates TGF-β-induced contractile genes expression, HPASMCs were treated with TGF-β1 for 24 hours with and without a SphK1 inhibitor, PF-543 at 10 µM (Figure 5). PF-543 decreased both the αSMA and CNN1 mRNA by 80% and 50% respectively. PF-543 trended towards decreasing αSMA protein from 2.6- to 1.9-fold of control, and CNN1 protein from 1.9- to 1.4-fold of control. While not statistically significant, this finding is similar to that for the S1PR3 receptor antagonists. In addition to SphK1, SphK2, another Sphingosine kinase, is also expressed in HPASMCs. However, the function of SphK2 is less well characterized than
SphK1. TGF-β treatment did not affect the mRNA level of SphK2 in HPASMCs (Figure S3). Several studies demonstrated that SphK2 may be able to compensate for the deficiency of SphK1 and in this case may maintain the S1P levels when SphK1 is silenced. It required deletion of SphK1 and SphK2 to completely abolish the production of S1P and inhibit the effect of S1P (Meng, Yuan, & Lee, 2011; Mizugishi et al., 2005; Xiong et al., 2013). This could explain the less significant inhibitory effect of PF-543 on TGF-β-induced αSMA expression, since PF-543 might not fully block the function of SphK2 at 10 µM (Yang, Jiang, Xu, Song, & Wang, 2019). Overall, these findings still suggest that SphK1 is important for regulating TGF-β-induced αSMA expression and that CNN1 expression is not completely dependent on S1P signaling.

Discussion

TGF-β signaling is enhanced in PAH patients (Yan et al., 2016) and transgenic mice overexpressing TGF-β1 spontaneously develop PAH (Calvier et al., 2019). The contributions and interactions of SMAD, Rho/MRTF and other mechanisms downstream of TGF-β that contribute to PASMC activation remain controversial (Calvier et al., 2019; Tang, Yang, Friesel, Vary, & Liaw, 2011; Zabini et al., 2018). Additionally, S1P plays an important role in PAH (Xing et al., 2015). Here, in HPASMCs, we investigated the interaction between TGF-β1 and S1P signaling to further define the signaling network downstream of TGF-β, with a focus on the SMAD and Rho/MRTF pathways.

SMAD signaling interacts with the Rho/MRTF/SRF pathway in multiple contexts. For example, in Monc-1 neural crest cells, RhoA directly regulates the phosphorylation of SMAD (S. Chen et al., 2006). In cardiac myoblasts, an MRTF-A/pSMAD complex serves as a transcriptional regulatory element controlling the expression of αSMA (Parmacek, 2010). In HPASMCs, the Zabini group reported that loss of SMAD3 disinhibits MRTF and drives the αSMA expression in PAH (Zabini et al., 2018). However, the Hansmann group did not observe reduced expression.
of SMAD3 in the lungs from SUGEN/Hypoxia rats and pulmonary arteries from PAH patients and claimed that TGF-β1 signaling drives αSMA expression in HPASMCs through canonical SMAD3 activation instead of by SMAD3 downregulation (Calvier et al., 2019). Our results suggest that both SMAD and Rho/MRTF/SRF signaling are involved in the regulation of αSMA and CNN1 in HPASMCs. We also show that MRTF-A, but not MRTF-B is required for regulating αSMA expression in HPASMCs. Based on these data we suggest a model wherein SMAD3 induces the activation of Rho/MRTF/SRF and regulates αSMA expression by potentiating the pathway of Rho activators such as S1P.

The interaction between TGF-β and S1P signaling has been characterized in fibrosis and cancer. In those contexts, TGF-β increases the expression of SphK1 and in turn the level of S1P in fibroblasts, which contributes to TGF-β-mediated modulation of gene expression. SphK1/S1P has been reported to mediate TGF-β1-induced proliferation in rat PASMCs (Wang et al., 2019), however, we did not observe a significant pro-proliferative effect of TGF-β1 in human PASMC (Figure S4). This highlights potential differences between the responses of rat and human PASMCs to the same stimuli. In addition to SphK1, TGF-β upregulates S1PR3 through the SMAD3 signaling axis in lung adenocarcinoma cell lines and in those cells, S1PR3-mediated signaling drives the lung carcinoma cells growth (Zhao et al., 2016). We found a similar interaction between TGF-β and S1P signaling in regulation of αSMA in HPASMCs; TGF-β1 elevates αSMA expression, which is reduced by inhibition of either SphK1 or S1PR3. However, it is important to note that while we saw statistically significant differences at the mRNA level, in some cases the differences at the protein level did not achieve statistical significance. TGF-β1 induced an approximately 2-3 folds increase in αSMA protein levels, which gives a narrow window to test the inhibitory effect of antagonists, and maybe contribute to the variability in some experiments. Despite this, we believe that the similar response to several
modulators targeting the S1P pathway, suggests that SphK1 and S1PR3 are important for regulating TGF-β1-induced αSMA expression.

We demonstrated the essentiality of S1PR3 in the upregulation of αSMA by TGF-β1 in HPASMCs. Meanwhile, PASMCs from PAH patients showed elevated S1PR2 levels (J. Chen et al., 2014). Silencing of S1PR2 or pharmacological inhibition of S1PR2 has been shown to ablate S1P-stimulated SMC proliferation (J. Chen et al., 2014). S1P regulates proliferation and differentiation of SMCs through different S1P receptors, which are coupled to different G proteins (Wamhoff et al., 2008). Thus, TGF-β-induced S1P levels could result in increased differentiation or proliferation of PASMCs based on the composition of S1P receptors of that the cells express. Overall, Sphingosine kinase and S1PR3 are two critical components of the S1P signaling pathway, this highlights the importance of S1P signaling in regulating αSMA expression and further supports their potential as PAH drug targets.

Our results showed that the S1PR3 antagonists markedly, but not completely, reduced the elevation of αSMA levels. One possibility is that TGF-β1 regulates αSMA expression through the SMAD pathway in parallel. Alternatively, TGF-β1 may also upregulate other Rho activators such as Endothelin-1 and CTGF, which in turn contribute to the αSMA elevation. Another observation we had is that CNN1 is regulated in a different manner from αSMA. CNN1 mRNA increased at a similar time course as SphK1 and earlier than TGF-β1-induced phosphorylation of MLC2. However, the upregulation of CNN1 mRNA at 24 hours was substantially suppressed by the ROCK inhibitor Y27632 (Figure 1A). The S1PR3 and Sphingosine kinases inhibitors inhibited the TGF-β1-induced CNN1 expression to a lesser extent. These suggest that the regulation of CNN1 expression relies on a Rho-dependent mechanism at later times but SMAD or other mechanisms early on during stimulation.
Phosphorylation of MLC2 causes contraction of SMCs. Additionally, increased expression of the contractile protein αSMA also contributes to the elevated vasoconstriction and SMC hypertrophy (Bai et al., 2020; Zabini et al., 2018). In this study we demonstrated the role of SphK1 and S1PR3 in TGF-β1-induced αSMA expression. We found that both SMAD3 and Rho/MRTF-A/SRF are important mediators of TGF-β1-induced αSMA expression in HPASMCs. We proposed a model, similar to what was demonstrated in fibroblasts, wherein TGF-β1 upregulated αSMA expressions in HPASMCs by potentiating S1P signaling. Our results show that SIS3 tends to inhibit TGF-β1 induced S1PR3 mRNA, although this observation did not reach statistical significance. While we lack evidence that strongly implicates the SMAD pathway in this model, our data suggests that TGF-β1 potentiates S1P signaling through SMAD pathway, which is activated at an earlier timepoint. In total, these findings suggest that both S1P and MRTF-A/SRF signaling are potential therapeutic targets to reduce vascular contraction and SMC hypertrophy in PAH.

**Authorship Contributions**

Participated in research design: Ji, Neubig, Lisabeth

Conducted experiments: Ji

Performed data analysis: Ji, Neubig, Lisabeth

Wrote or contributed to the writing of the manuscript: Ji, Neubig, Lisabeth

**References**


**Footnotes**

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**Conflict of Interest Statement**

Dr. Neubig is President and Co-founder of FibrosIX Inc. which holds an option on intellectual property for CCG-222740 and related compounds.

**Figure Legends**

**Figure 1.** TGF-β1-induced αSMA and CNN1 expression is dependent on both the SMAD pathway and the Rho/MRTF/SRF pathway.

HPASMCs were seeded into 6-well plates (for the siMRTF protein experiment, cells were seeded into 60 mm petri dish) and allowed to reach confluence. Then the cells were serum deprived in 0.1% SMGS Medium 231 overnight before being treated the next day. **A.** Cells
were pre-treated with 10 μM SMAD3 inhibitor SIS3 for 1h and then along with 10 ng/ml TGF-β1 for another 12h. Cells were also concurrently treated with 10 μM ROCK inhibitors Y-27632 and 10 ng/ml TGF-β1 for 24h. DMSO was used as a vehicle control. After treatment total cellular RNA was extracted and RT-qPCR was used to measure αSMA and CNN1 expression. B. HPASMCs were co-treated with 10 ng/ml TGF-β1 and 10 μM CCG-222740 or vehicle. Both mRNA and protein were collected to measure the expression levels of αSMA and CNN1. C. HPASMCs were transfected with 5 μM siMRTF-A or siMRTF-B as described in materials and methods. The cells were then starved as described above before being treated with 10 ng/ml TGF-β1 for 24 hours. Protein (N=3 Santa Cruz anti-MRTF-A, N=1 Cell Signaling anti-MRTF-A) levels of αSMA, MRTFs and GAPDH were examined. (N>=3. Mean with SD. Paired t-test * P<0.05, ** P<0.01)

**Figure 2. TGF-β1 indirectly activates the Rho signaling.**

HPASMCs were seeded into 6-well plates and allowed to reach confluence before being serum deprived in 0.1% SMGS Medium 231 overnight. The next day the cells were treated with 10 ng/mL cycloheximide or vehicle and were stimulated with 1 μM S1P for 0.5, 1, 3, or 6h, or 10 ng/ml TGF-β1 for 1, 3, 6, or 9 h. Protein was extracted and the levels of pMLC, total MLC, and GAPDH were measured. The ratio of pMLC/MLC is compared. (N=5. Data are represented as Mean with SD. Paired t test * P<0.05)

**Figure 3. Kinetics of TGF-β1-induced contractile genes and TGF-β1 modulation of S1P receptors.**

HPASMCs were seeded into 6-well plates and allowed to reach confluence before being serum deprived in 0.1% SMGS Medium 231 overnight. A. Cells were then treated with 10 ng/ml TGF-β1 for 1, 3, 6, 12, 24 h in low serum medium. Afterwards, mRNA was extracted and the levels of αSMA, CNN1, and SphK1 were measured by RT-qPCR. B. The mRNA levels of S1PR1, S1PR2 and S1PR3 were measured by RT-qPCR. C. HPASMCs were pretreated with 10 μM SIS3 for 1 hour and then treated with 10 ng/ml TGF-β1 for 12 hours in low serum medium. RT-qPCR was
used to assess the mRNA level of S1PR3. (N=5. Data are represented as Mean with SD. For Figure 3A, One-way Repeated Measures ANOVA followed by Dunnett’s post-test was performed. For Figure 3B, Paired t-test was conducted. * P<0.05, ** P<0.01)

**Figure 4. TGF-β1-induced αSMA and CNN1 expression is mediated by S1PR3 but not by S1PR2.**

HPASMCs were seeded into 6-well plates and were allowed to reach confluence before being serum deprived in 0.1% SMGS Medium 231 overnight. Cells were treated with 10 ng/ml TGF-β1 for 24 h and 5 μM JTE013 (S1PR2 antagonist), 1 μM TY52156 (S1PR3 antagonist), 2 μM VPC23019, or vehicle control. The protein and mRNA levels of αSMA and CNN1 were analyzed by western blot and RT-qPCR respectively. Both blots were probed on the same membrane, but the bands were cropped to include only relevant lanes. αSMA protein levels in TGF-β1 treated groups with or without TY52156 and VPC23019 show a mean difference of -0.47 with 95% CI of -0.93 to 0.01 and -0.94 with 95% CI of -1.58 to -0.31. (N>=3. Data are represented as Mean with SD. Paired t-test* P<0.05)

**Figure 5. TGF-β1-induced αSMA and CNN1 expression is reduced by SphK1 inhibitors.**

HPASMCs were seeded into 6-well plates and were allowed to reach confluence before being serum deprived in 0.1% SMGS Medium 231 overnight. Cells were treated with 10 ng/ml TGF-β1 for 24 h and 10 μM PF-543 or vehicle control. The protein and mRNA levels of αSMA and CNN1 were analyzed by western blot and RT-qPCR respectively. Both blots were probed on the same membrane, but the bands were cropped to include only relevant lanes. The αSMA protein levels in TGF-β1 treated groups with or without PF-543 show a mean difference of -0.6779 with 95% CI of -1.646 to 0.2898. (N>=3. Data are represented as Mean with SD. Paired t-test* P<0.05)

**Figure 6. Summary overview of the interaction of TGF-β and S1P signaling in regulation of αSMA expression in HPASMCs.**
Figure 3.

A.

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</table>

mRNA / GAPDH (Fold Change)

TGFβ1 (Hours)

B.

mRNA/GAPDH (Fold Change)

S1PR1  S1PR2  S1PR3

TGFβ1 (Hours)

C.

mRNA/GAPDH (Fold Change)

S1PR3

Control  TGFβ  TGFβ+SIS3

*
Figure 4. A.

RNA

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<tr>
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mRNA / GAPDH (Fold Change)

B.

Control  | TGFβ1 | TGFβ1+TY52156 |

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Protein

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<td>TGFβ+VPC23019</td>
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</table>
Figure 5. A.

RNA

mRNA / GAPDH (Fold Change)

Control  TGFβ  TGFβ+PF543  Control  TGFβ  TGFβ+PF543

αSMA  CNN1

*  *

Figure 5. B.

Protein

Control  TGFβ1  TGFβ1 PF543

αSMA  CNN1  GAPDH

Protein / GAPDH (Fold Change)

Control  TGFβ  TGFβ+PF543  Control  TGFβ  TGFβ+PF543

αSMA  CNN1