Title
The Role of Integrin αvβ6 in TGF-β Activation and Pulmonary Fibrosis

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Publication Date
2011

Peer reviewed|Thesis/dissertation

The Role of Integrin αvβ6 in TGF-β Activation and Pulmonary Fibrosis

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

DEDICATION AND ACKNOWLEDGMENTS

This thesis is dedicated to my wonderful husband, Craig Giacomini, for his never-ending love, support, and encouragement and to my parents, Jimmy and May Mok, and John and Kathleen Giacomini for their unconditional love and guidance throughout my life and career. I would like to thank my fantastic mentor, Dean Sheppard, for helping me grow as a scientist and for making research fun! Finally, I would like to thank my special family, friends, and labmates for making my graduate career a phenomenal experience.

THE ROLE OF INTEGRIN $\alpha_v\beta_6$ IN TGF-β ACTIVATION AND PULMONARY FIBROSIS

Marilyn Giacomini

ABSTRACT

Idiopathic pulmonary fibrosis (IPF), or scarring of the lungs, is an incurable progressive disease that has a 5-year mortality rate of 80%. The cytokine, Transforming Growth Factor Beta (TGF-β) is considered a central mediator in the pathogenesis of this fibrotic disorder. Virtually all cells secrete TGF-β as a latent complex that must be activated for the cytokine to exert its biological functions. We previously discovered that the integrin $\alpha_v\beta_6$ functions to activate TGF-β. However, the signals and mechanisms that regulate this process in epithelial cells are unclear. We discovered that the phospholipid, Sphingosine 1-Phosphate (S1P), induces $\alpha_v\beta_6$-mediated TGF-β activation by increasing Rho Kinase (ROCK)-mediated actomyosin contraction. Furthermore, we demonstrate that this process requires intracellular mechanical forces applied to the integrin and the generation of cellular tension. Interestingly, lung epithelial cells appear to exert force on latent TGF-β using sub-cortical actin rather than the actin stress fibers utilized by fibroblasts and other traditionally "contractile" cells. In addition, we show that aerosol delivery of small molecule ROCK inhibitors to

wild type mice is an effective method of blocking TGF-β signaling *in vivo.* Finally, we report six candidate biomarkers of inhibiting $\alpha_v\beta_6$-mediated TGF-β activation and demonstrate the potential of utilizing alveolar macrophages as a biosensor for monitoring this pathway. These findings enhance our understanding of the mechanisms that regulate $\alpha_v\beta_6$-mediated TGF-β activation, and they demonstrate the potential of blocking this pathway in vivo and monitoring the effectiveness of blockade, defining a potential strategy for developing improved treatments for pulmonary fibrosis.

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CHAPTER 1: INTEGRINS AND TRANSFORMING GROWTH FACTOR BETA

INTRODUCTION

A. Integrins as Adhesion Receptors

Integrins are a widely expressed large family of cell surface glycoproteins. These heterodimeric transmembrane type I receptors are composed of an alpha (α) and a beta (β) subunit that range between 120-180 kDa and 90-180 kDa in size, respectively. Integrin subunits generally have very short cytoplasmic tails that are about 40-70 amino acids in length (except β_4, which has 1088 amino acids). In humans, there are 18 α and 8 β subunits that non-covalently associate through their extracellular domains to form 24 distinct integrin pairs (Figure 1.1). These receptors require divalent cations to bind their ligands, and are classified according to the type of ligand they bind and the molecular interaction of this binding [1]. Because of their affinity for matrix proteins, integrins are the major receptors by which cells attach to the extracellular matrix (ECM). Through this function, integrins and their multiple ligands play key roles in a diverse array of biological processes and diseases, including development, immune responses, autoimmune disorders, cancer, and fibrosis [2].

B. Integrins as Signaling Receptors

In addition to their roles as cell adhesion molecules, integrins are ideally poised to mediate bidirectional signaling, or transmit signals across the plasma membrane in both directions. However, integrins are often expressed on cell surfaces in an inactive state and require specific intracellular interactions with numerous adaptor proteins, including talin, vinculin, and focal adhesion kinase for optimal activity (Figure 1.2) [3]. Upon activation, integrins cluster into focal adhesions, or large, dynamic protein complexes that are located in close proximity to adhesive substrates. These focal adhesions not only serve as the sites of structural attachment between the cytoskeleton and ECM, but they also provide a pathway for cells to communicate signals with the extracellular environment. In this fashion, integrins serve as transmembrane mechanical links between the intracellular environment and the extracellular matrix and function to modulate virtually all aspects of cell behavior, including motility, proliferation, survival, polarity, and differentiation [4].

C. Integrins as Mechanotransducers

Mechanotransduction, the process of converting mechanical stimuli into chemical and molecular signals, has been shown to be crucial in regulating a variety of cellular processes. Cells possess intricate sensory mechanisms that allow them to "feel" and respond to both external (e.g. the stiffness of the ECM)

and internal forces (e.g. cell contraction). For example, naïve mesenchymal stem cells cultured on matrices that mimicked the stiffness of brain, muscle, or bone tissue differentiate into neurons, myoblasts, or osteoblasts, respectively [5]. In another example, there is evidence that an increase in actomyosin contraction allows cancer cells with a more rounded morphology to invade and remodel ECM in a protease-independent manner. This increase in force generation enables the cells to deform collagen fibers and essentially "push" their way through the dense ECM [6]. Many studies have demonstrated that mechanical forces affect the dynamics, morphology, and behavior of a cell. Since integrins are intimately connected to the actin cytoskeleton, they are perfectly positioned to sense these forces and convert them into signals to modify cell behavior.

D. Integrins can Exert Force on Their Ligands

In addition to sensing mechanical stimuli, integrins themselves can exert force to mediate signaling pathways. In particular, they can activate ligand molecules by altering their conformation through an increase in tension. For example, the assembly of fibronectin into an insoluble fibrillar matrix is the result of tension exerted by the integrin $\alpha_5\beta_1$. Fibroblasts secrete fibronectin as an inactive soluble molecule; however, to form a fibrillar matrix, fibronectin must be assembled into activated insoluble fibrils. This process occurs through binding of fibronectin to $\alpha_5\beta_1$ via an arginine-glycine-aspartic acid (RGD) sequence. Upon Rho-mediated actomyosin contraction, cells expressing $\alpha_5\beta_1$ contract and exert

tension on fibronectin. This increase in cell tension stretches the molecule, causing a conformational change that exposes cryptic amino acid motifs embedded within the protein. Upon exposure of these sites, surrounding fibronectin molecules can then bind to form a fibronectin-fibronectin interaction, resulting in the assembly of the insoluble fibronectin matrix [7]. The pleiotropic cytokine, Transforming Growth Factor Beta (TGF-β), is another integrin ligand that can undergo biologically important conformational change in response to cellular tension exerted through integrins.

E. Transforming Growth Factor Beta

TGF-β and its signaling pathway are involved in regulating many cellular processes, including differentiation, proliferation, homeostasis, and apoptosis [8]. In mammalian cells, TGF-β exists in three isoforms (TGF-β1, 2, and 3). Virtually all cells secrete the cytokine as a large latent complex (LLC), which includes a latency-associated peptide (LAP), the mature TGF-β cytokine, and a latent TGF-β binding protein-1 (LTBP). Upon secretion, latent TGF-β binds to the ECM via LTBPs and is stored there until its activation.

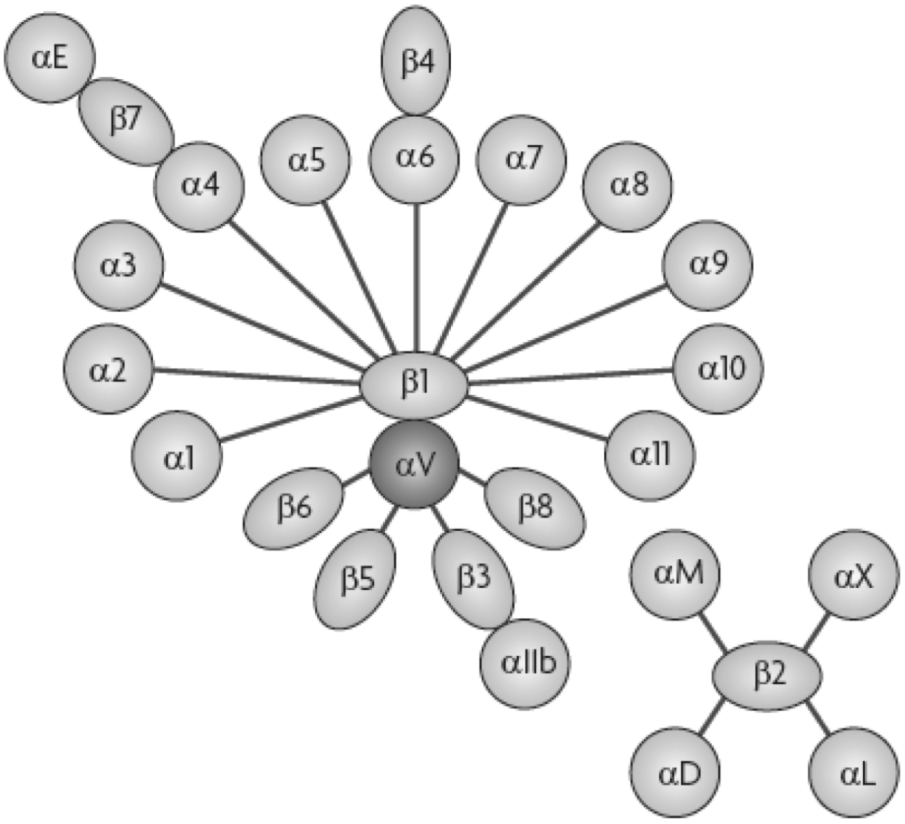
Latent TGF-β must be activated to exert its biological functions. While there are many ways to activate the cytokine *in vitro*, such as temperature and pH changes, exposure to oxidants, or certain proteases, the relevance of these processes *in vivo* is poorly understood [9]. Our laboratory and others have discovered that specific integrins that recognize an arginine-glycine-aspartic acid

(RGD)-binding site motif on their corresponding ligand molecules are also capable of directly binding to the RGD site on LAP-TGF-β isoforms 1 and 3 and activating the cytokine. These specific integrins have been demonstrated to play a major role in regulating TGF-β signaling *in vivo.*

F. RGD-binding Integrins can Activate TGF-β

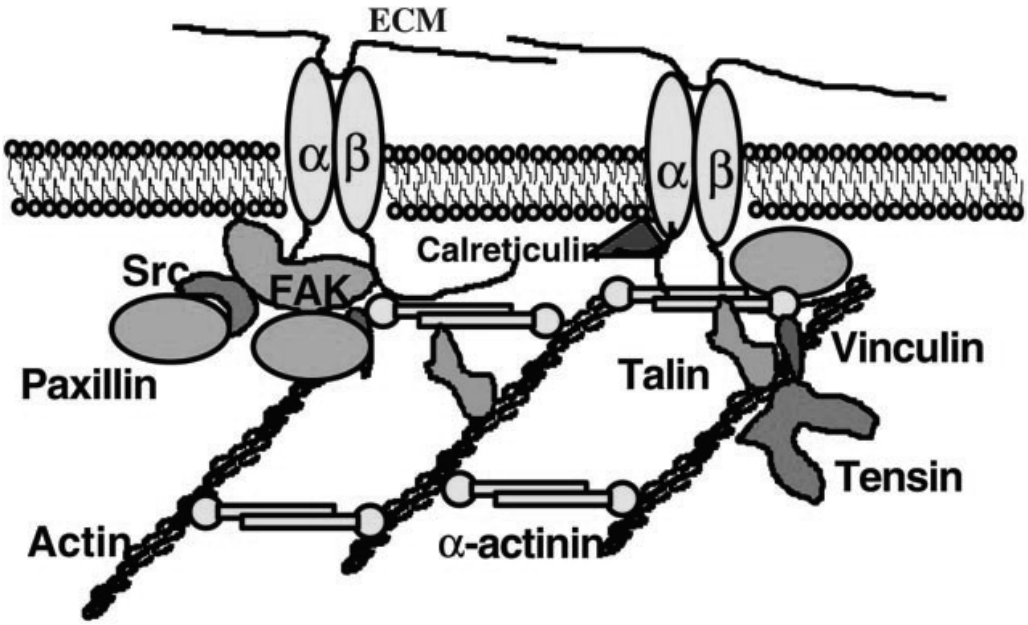
The importance of RGD-binding integrins in TGF-β activation is highlighted in transgenic mice that harbor a knock-in mutation of *Tgfb1*, so that the RGD binding site is mutated to RGE. These *Tgfb1*$^{RGE/RGE}$ mice exhibit multi-organ inflammation and autoimmunity, a phenotype that completely phenocopies that of *Tgfb1*$^{-/-}$ mice [10]. The phenotype of *Tgfb1*$^{RGE/RGE}$ mice is presumably due to a lack of binding, and thus, activation of TGF-β by RGD-binding integrins. There are eight RGD-binding integrins ($\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$, $\alpha_{IIb}\beta_3$), and only six of them ($\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_V\beta_6$, $\alpha_V\beta_8$ and $\alpha_8\beta_1$) have been found to directly bind to TGF-β1 and 3 (no RGD site on TGF-β2). However, only integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ have been definitively shown to play a role in regulating TGF-β bioactivity *in vivo*. Specifically, knockout mice of these integrins exhibit phenotypes that recapitulate *Tgfb1*$^{-/-}$ and *Tgfb3*$^{-/-}$ mice. For example, *Itgb6*$^{-/-}$ mice develop exaggerated inflammation in the skin and on mucosal surfaces, a phenotype that recapitulates that of *Tgfb1*$^{-/-}$ mice (although substantially less severe) [11]. Most *Itgb8*$^{-/-}$ embryos die at day 10 due to defects in vasculogenesis in the yolk sac, similar to *Tgfb1*$^{-/-}$ mice [12]. Furthermore, the *Itgb8*$^{-/-}$ embryos

that survive develop either cleft palate, like *Tgfb3*$^{-/-}$ mice, or abnormal brain vascular morphogenesis, like *Tgfb1*$^{-/-}$ and *Tgfb3*$^{-/-}$ mice [12, 13]. Finally, the phenotypes of *Tgfb1*$^{-/-}$ and *Tgfb3*$^{-/-}$ mice can be fully reproduced in the *Itgb6*$^{-/-}$*;Itgb8*$^{-/-}$ double knockout, or by treating surviving *Itgb8*$^{-/-}$ mice at birth with a β_6-blocking antibody [14]. These findings suggest that TGF-β activity *in vivo* is largely regulated by these two RGD-binding integrins.



Cox, D., *Nature Reviews, Drug Discovery*, (2010)

Figure 1.1. The Integrin Receptor Family. Integrins are heterodimers composed of an α and a β subunit. There are 24 known integrin pairs in humans. Each of the unique integrin pairs is depicted. Specifically, the α_v subunit associates with five β subunits, whereas the α_6 subunit only associates with the β_4 subunit.



Sheppard, D., *Physiological Review*, (2003)

Figure 1.2. Integrin-Actin Cytoskeleton Interactions. A simplified model of integrin signaling complexes. The model depicts adaptor proteins that link the integrin cytoplasmic tails to the actin cytoskeleton and a small subset of associated signaling proteins.

CHAPTER 2: INTEGRIN $\alpha_v\beta_6$-MEDIATED TGF-β ACTIVATION REQUIRES MECHANICAL FORCE AND CELLULAR TENSION

INTRODUCTION

A. Integrin $\alpha_v\beta_6$-mediated TGF-β Activation

Integrin $\alpha_v\beta_6$ is an epithelial-restricted integrin that has been shown to mediate cell adhesion to the ECM proteins fibronectin and tenascin-C *in vitro*. This integrin is normally expressed at low levels on skin and mucosal surfaces; however, expression dramatically increases in response to tissue injury or inflammation [11]. As discussed, the phenotype of *Itgb6*$^{-/-}$ mice suggests an interaction with TGF-β. In addition to exhibiting exaggerated inflammatory responses to injury, these mice are dramatically protected from developing diseases that are mediated by TGF-β. For example, in three models of acute lung injury (bleomycin, endotoxin, and ventilator-induced), *Itgb6*$^{-/-}$ mice were protected from developing pulmonary edema [15, 16]. *Itgb6*$^{-/-}$ mice are also protected from developing fibrosis in various organs, including pulmonary fibrosis induced by the anti-cancer drug, bleomycin, or by thoracic irradiation, renal fibrosis, and biliary fibrosis [9, 11, 17-19]. These findings support a role for $\alpha_v\beta_6$ in activating TGF-β, since previous studies demonstrated that mice systemically administered a TGF-β blocking antibody are also protected from

developing bleomycin-induced pulmonary fibrosis [20]. Furthermore, inhibitors of TGF-β or TGF-β signaling by soluble TGF-β receptor II have demonstrated beneficial effects against many fibrotic disorders, including corneal, renal, cardiac, and hepatic fibrosis [21-24]. Although substantial evidence exists that $\alpha_v\beta_6$ functions in the activation of TGF-β in epithelial cells, the signals and mechanism that regulate this process are poorly understood.

B. Integrin $\alpha_v\beta_6$ Activates TGF-β by Inducing a Conformational Change to the Latent TGF-β Complex

We previously demonstrated *in vitro* that $\alpha_v\beta_6$ activates TGF-β. Specifically, when β_6-expressing cells were co-cultured with TGF-β reporter cells expressing luciferase under the control of a TGF-β responsive promoter, increased luciferase activity was observed that was inhibited by antibodies to β_6 and TGF-β. In comparison, no activity was seen in the control mock-transfected cells.

Unlike TGF-β activation by other integrins, for example, $\alpha_v\beta_8$ activation involves protease degradation of the LAP and results in the release of active TGF-β from its large latent complex, direct cell-cell contact between integrin-expressing cells and neighboring cells expressing TGF-β receptors is required for TGF-β signaling to occur. In particular, luciferase activity is abolished when β_6-expressing cells and TGF-β reporter cells are cultured on opposite sides of a microporous filter [17]. Furthermore, luciferase activity diminishes when cells

simultaneously expressing both the integrin and a TGF-β reporter are diluted so that the cells no longer touch each other (unpublished data). Therefore, TGF-β activation by $\alpha_v\beta_6$ does not result in the release of freely diffusible active cytokine.

In addition to direct cell-cell contact, $\alpha_v\beta_6$-mediated TGF-β activation requires an intact actin cytoskeleton. For example, treatment of β_6-expressing cells with cytochalasin D, an inhibitor of actin polymerization, completely abrogates TGF-β activity. Furthermore, truncation mutagenesis of the β_6 cytoplasmic domain revealed mutants that retained their ability to bind to the LAP of TGF-β, but failed to activate the cytokine [17]. Again, this demonstrates that binding of $\alpha_v\beta_6$ to the RGD site of TGF-β1 and 3 alone is insufficient for activation of the cytokine and that cytoplasmic interactions with the actin cytoskeleton are required.

Finally, TGF-β activation by this integrin requires LTBP-1 and its interaction with TGF-β and the ECM. Specifically, β_6-expressing cells lacking functional LTBP-1 cannot activate TGF-β. However, reconstitution of these cells with wild type LTBP-1, or a fusion protein containing only the regions of LTBP-1 required for disulfide-linkage to LAP and for cross-linking of LTBP-1 to the ECM is sufficient to restore this ability [25]. This finding supports the idea that LTBP-1 is a mechanical tether for latent TGF-β to the ECM. Taken together, these findings suggest that a mechanical process requiring an intact actin cytoskeleton and interaction with the ECM may regulate $\alpha_v\beta_6$-mediated TGF-β activation. In particular, we hypothesized that TGF-β activation by this integrin

results in a conformational change to the latent molecule, much like the process of fibronectin matrix assembly, that allows exposure of the active portion to its cognate receptors on adjacent cells. The major focus of my research has been to elucidate the pathways and signals that mediate this intricate process.

Here, we report that the phospholipid, Sphingosine 1-phosphate (S1P), induces $\alpha_v\beta_6$-mediated TGF-β activation through an increase in Rho Kinase (ROCK)-mediated actomyosin contraction. We discovered that $\alpha_v\beta_6$-mediated TGF-β activation is blocked in the presence of a specific non-muscle myosin II inhibitor. Furthermore, this process results in a reorganization of the actin cytoskeleton to form cortical actin structures but not stress fibers, indicating a role for cortical actin in transmitting force by epithelial cells. The characterization of this pathway will likely improve our understanding of TGF-β biology in epithelial cells and may ultimately facilitate the development of novel therapies for diseases involving aberrant $\alpha_v\beta_6$-mediated TGF-β activation.

RESULTS

S1P Induces $\alpha_v\beta_6$-mediated TGF-β Activation via ROCK

Sphingosine 1-phosphate (S1P) is a G-protein coupled receptor agonist that signals through five receptors ($S1P_{1-5}$) to initiate various physiological processes, including immune cell trafficking, development, and organogenesis [26]. The phospholipid is abundantly stored in platelets and is released at sites of increased TGF-β signaling, such as inflamed or injured tissues. S1P has been implicated to play a role in tissue fibrosis. For example, S1P has been shown to induce several pro-fibrotic markers in retinal pigmented epithelial cells [27], and $S1P_2$ deficient mice were found to be protected from carbon tetrachloride-induced liver fibrosis [28]. Because of the role of TGF-β in fibrosis, we hypothesized that the development and progression of this disease involves the regulation of TGF-β signaling by S1P.

Smad2 is a protein in the TGF-β signaling pathway that is phosphorylated by TGF-β serine/threonine kinase receptors upon activation of the pathway. Once phosphorylated, pSmad2 associates with the transcription factor, Smad4, and translocates into the nucleus to regulate expression of TGF-β responsive genes. Thus, pSmad2 is an early readout of TGF-β activity. To determine if S1P induces $\alpha_v\beta_6$-mediated TGF-β activation, primary normal human bronchial epithelial cells (NHBE) were treated with increasing concentrations of the phospholipid in the presence or absence of a β_6-blocking antibody, and pSmad2 expression was

examined as readout of TGF-β activation. We found that S1P increased pSmad2 in a dose-dependent manner that was reduced in the presence of a β_6-blocking antibody (Figure 2.1a).

We previously reported in β_6-transfected mouse embryonic fibroblasts that thrombin and Lysophosphatidic Acid, ligands of other G-protein coupled receptors, also induce β_6-mediated TGF-β activation, and Rho Kinase (ROCK) mediates this process [16, 29]. To determine whether S1P induced β_6-mediated TGF-β activation also involves ROCK, NHBE were treated with S1P in the presence or absence of Y-27632, a non-specific ROCK inhibitor. As a control, active human recombinant TGF-β1 was added to the cells. We discovered that in the presence of this antagonist, S1P induced β_6-mediated TGF-β activation was blocked (Figure 2.1b).

The respiratory epithelium lining the upper airways is composed of multiple cell types, including goblet, basal, ciliated, and non-ciliated cells. More importantly, the epithelial cells are polarized, so that they have distinct apical, basal, and lateral domains. Each of these domains is separated by tight junctions, feature different compositions of lipids and proteins, and have specialized functions. Therefore, to obtain the best *in vitro* representation of the airway epithelium, we differentiated NHBE by culturing them under air-liquid interface, so that the apical side of the cell is exposed to air, and the basal side to growth media. Active human recombinant TGF-β1 was added to the cells as a control. We found that S1P also induces β_6-mediated TGF-β activation under this more physiologic condition (Figure 2.1c).

Integrin $\alpha_v\beta_6$-mediated TGF-β Activation Requires Actomyosin Contraction and Cellular Tension

As noted above, our evidence for an important role of the actin cytoskeleton along with the finding that $\alpha_v\beta_6$-mediated TGF-β activation requires physical tethering of the latent complex to the ECM by LTBP-1, suggests that this process might require application of physical force from the integrin-expressing epithelial cell to the tethered latent complex. Recently, a study using myofibroblasts, highly contractile cells that exert force on the ECM, suggested that these cells could activate latent TGF-β by exerting force through the integrin $\alpha_v\beta_5$. The study discovered that TGF-β activation occurs upon increased cell contraction, during which the generation of intracellular force induces the integrin to "pull" on latent TGF-β bound to both the receptor and ECM [30]. While this study demonstrated a novel potential mechanism of TGF-β activation, the role of $\alpha_v\beta_5$ integrin in activating TGF-β *in vivo* remains unclear, since *Itgb5*$^{-/-}$ mice do not exhibit any phenotype that indicates loss of TGF-β activity. Furthermore, $\alpha_v\beta_6$ is limited in its expression to epithelial cells and these cells are generally not thought of as contractile cells. Therefore, it was important to determine whether epithelial cells could activate TGF-β by mechanical deformation of the latent complex.

The RhoA/Rho Kinase pathway is well known to be involved in actomyosin contraction and reorganization of the actin cytoskeleton. Therefore, we hypothesized that $\alpha_v\beta_6$-mediated TGF-β activation is the result of increased

epithelial cell contraction. If this were the case, we would expect that myosin, in addition to actin, would play an important role in $\alpha_v\beta_6$-mediated TGF-β activation. To test this possibility, we treated NHBE cells with Blebbistatin, a specific non-muscle myosin II inhibitor, and examined effects on TGF-β activation in response to S1P. We also tested its effect on LPA induced $\alpha_v\beta_6$-mediated TGF-β activation. Blebbistatin treatment inhibited both S1P and LPA induced $\alpha_v\beta_6$-mediated TGF-β activation (Figure 2.2a,b).

Mechanical properties of the ECM and physical interactions with the cytoskeleton have been demonstrated to modulate numerous cellular events. For example, cells cultured on "soft" substrates exhibited reduced spreading and irregularly shaped focal adhesions, compared to cells cultured on rigid substrates. These observations demonstrate the ability of cells to "feel" and respond to mechanical properties in their surrounding environment, and suggests the involvement of forces generated by the actin-myosin cytoskeleton [31]. These polyacrylamide-based, collagen-coated substrates are chemically identical; yet by varying the concentration of bis-acrylamide, a wide range of substrate flexibility can be generated. Thus, this contraction assay allows the altering of only the physical property of the substrate, while maintaining a constant chemical environment. To determine if activation of the cytokine is due to an increase in cellular tension, we cultured NHBE on these polyacrylamide gel substrates at varying levels of stiffness. If generation of tension is required, TGF-β should not be activated on flexible substrates, which would be deformed by cell contraction and thus not allow transmission of force to bound latent complex. In contrast,

contraction of cells plated on rigid substrates should allow transmission of force to bound latent complex and facilitate its activation. We observed that NHBE cultured on a low rigidity substrate did not activate TGF-β upon stimulation with S1P, whereas cells cultured on a stiff substrate activated the cytokine, findings consistent with a role for physical tension and mechanical deformation in $\alpha_v\beta_6$-mediated TGF-β activation by epithelial cells (Figure 2.2c).

S1P and LPA Induce Cortical Actin Formation

Epithelial cells are not generally thought of as contractile cells. However, contraction of these cells has been demonstrated to be important in maintaining the integrity of epithelial protective barriers. Specifically, in response to local injury, cells in the epithelium can undergo apoptosis, potentially increasing the leakiness of the barrier, or creating gaps upon phagocytosis of those dead cells. To prevent disruption of the barrier, it has been demonstrated that dying cells send out signals to neighboring epithelial cells to increase actomyosin contraction. This increase in contractility has been shown to extrude the dying cell and prevent formation of gaps from forming in the epithelial barrier upon exiting of the dying cell [32]. In endothelial cells and fibroblasts, the actin cytoskeleton is organized into stress fibers upon increased cellular contraction. In contrast, a different form of actin organization, one that surrounds the cell periphery, called cortical actin, mediates contraction in epithelial cells [33]. Consistent with this finding, we found that treatment with S1P and LPA did not induce stress fiber formation in

NHBE cells, but reorganized actin into cortical structures (Figure 2.3a). We also observed cortical structures in S1P and LPA treated NHBE cells cultured at an air-liquid interface (Figure 2.3b).

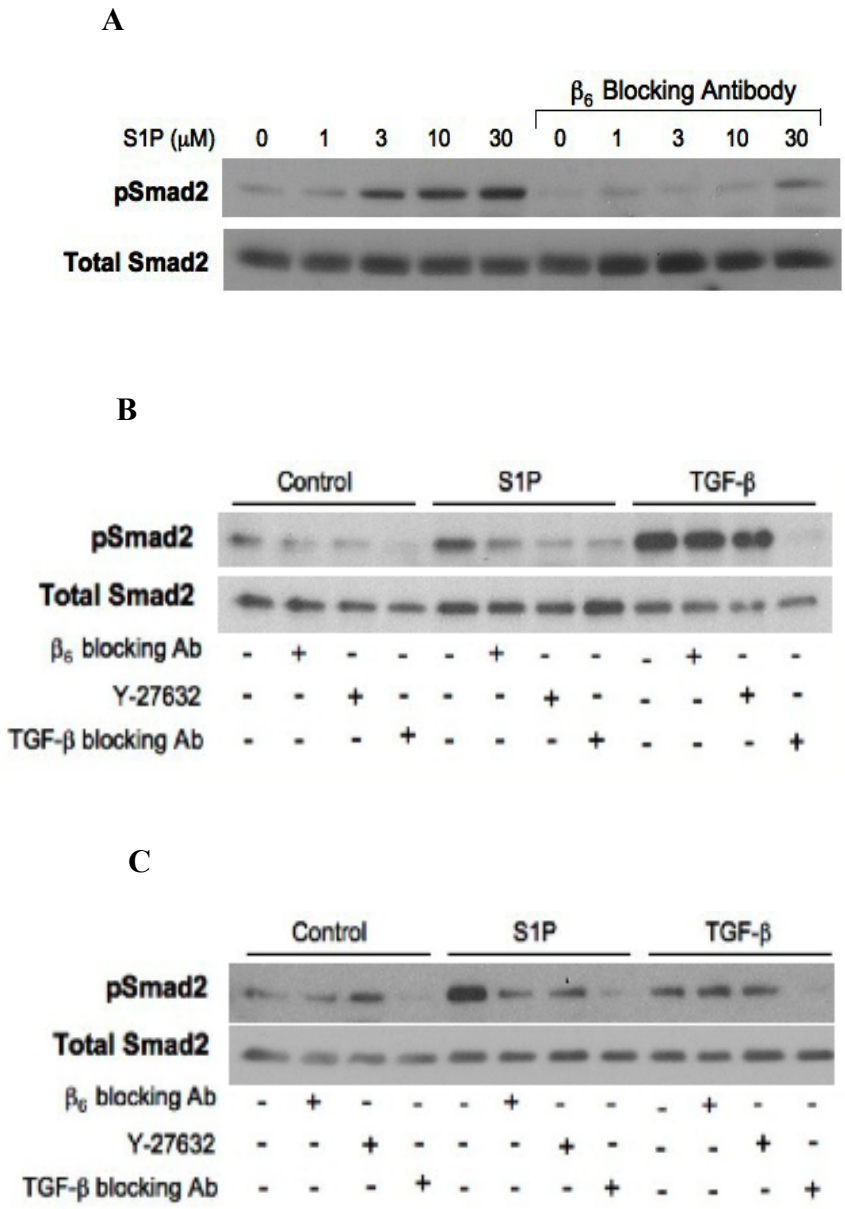


Figure 2.1. S1P induces $\alpha_v\beta_6$-mediated TGF-β activation via Rho Kinase. **a)** Western blot for pSmad2 or Total Smad2 from lysates of NHBE treated with methanol (control) or S1P (1-30 uM) in the presence or absence of an anti-β_6 antibody (3G9, 40 ug/mL. **b)** Western blots, as above, of lysates from NHBE treated with methanol (control) or S1P (30 uM) in the presence or absence of a Rho Kinase inhibitor (Y-27632, 100uM), 3G9 (40 ug/mL), or anti-TGF-β antibody (1D11, 40 ug/mL). **c)** Western blots of lysates of air-liquid interface cultures of NHBE treated with saline alone or S1P (30 uM) in the presence or absence of Y-27632 (100 uM) and 1D11 (40 ug/mL).

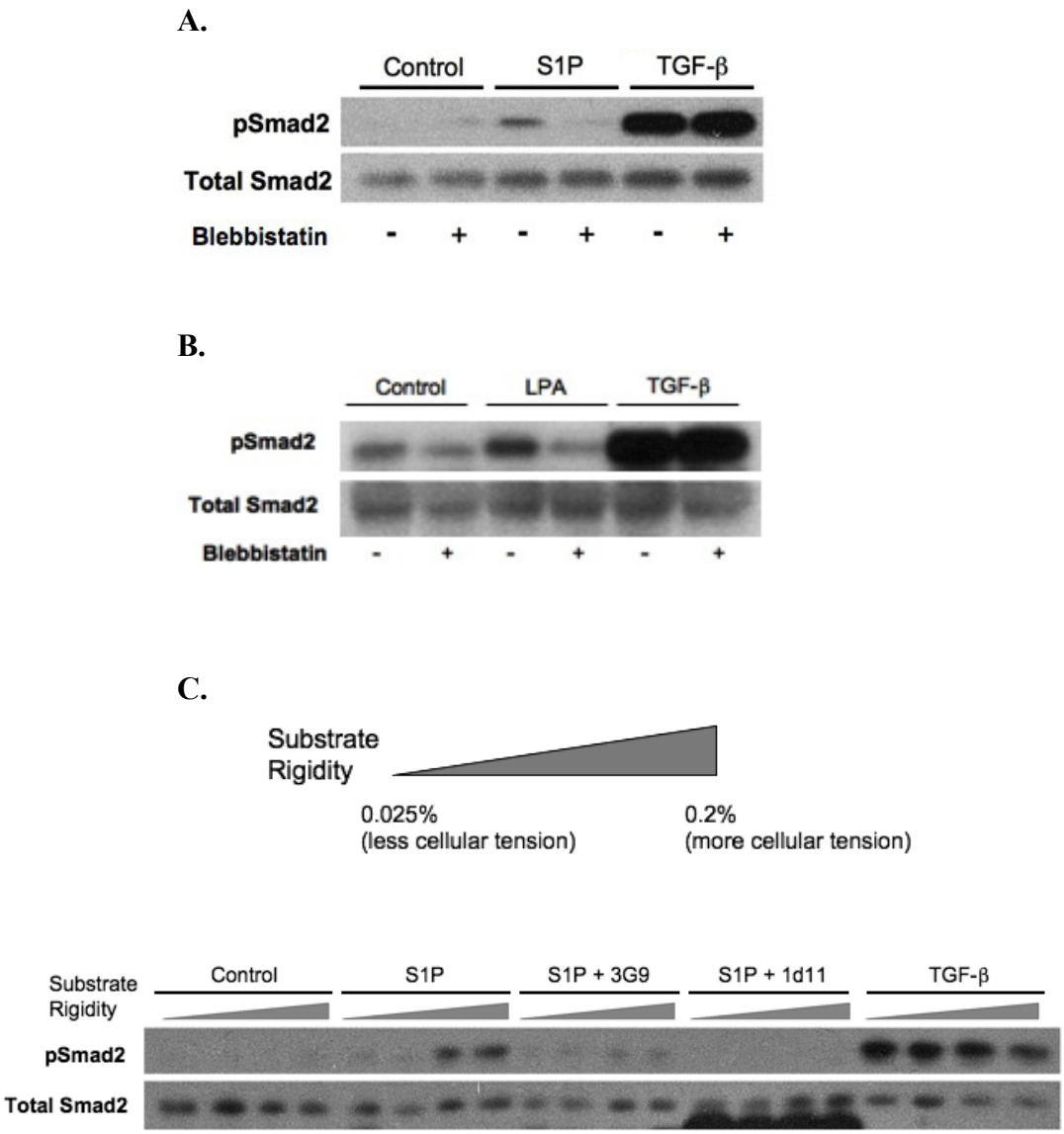
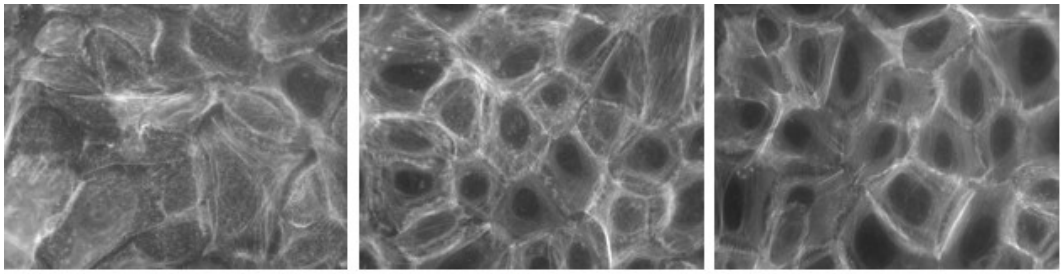


Figure 2.2. S1P and LPA induced $\alpha_v\beta_6$-mediated TGF-β activation requires cellular tension. **a)** Western blots for pSmad2 and Total Smad2 of lysates of NHBE cells treated with methanol (control) or S1P (30 uM) in the presence or absence of Blebbistatin (20 uM) and active TGF-β1 (15 pg/mL) **b)** Western blots, as above, of lysates of NHBE treated PBS (control) or LPA (50 uM) in the presence or absence of Blebbistatin (20 uM) and active TGF-β1 (15 pg/mL) **c)** Western blots of lysates from NHBE cultured on various rigidities of polyacrylamide flexible substrates and treated with methanol (control) or S1P (30 uM) in the presence or absence of 3G9 (40 ug/mL) and 1d11 (40 ug/mL).

A.



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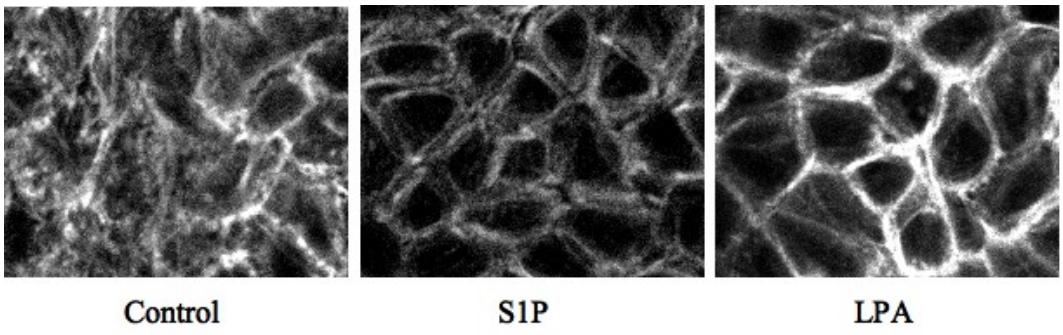


Figure 2.3. S1P and LPA induced β_6-mediated TGF-β activation results in cortical actin formation. **a)** Phalloidin staining of NHBE cells cultured in plastic dishes and treated with saline alone (control), S1P (30 uM) or LPA (50 uM) **b)** Phalloidin staining of air-liquid interface cultures of NHBE treated with saline alone, S1P (30 uM) or LPA (50 uM),

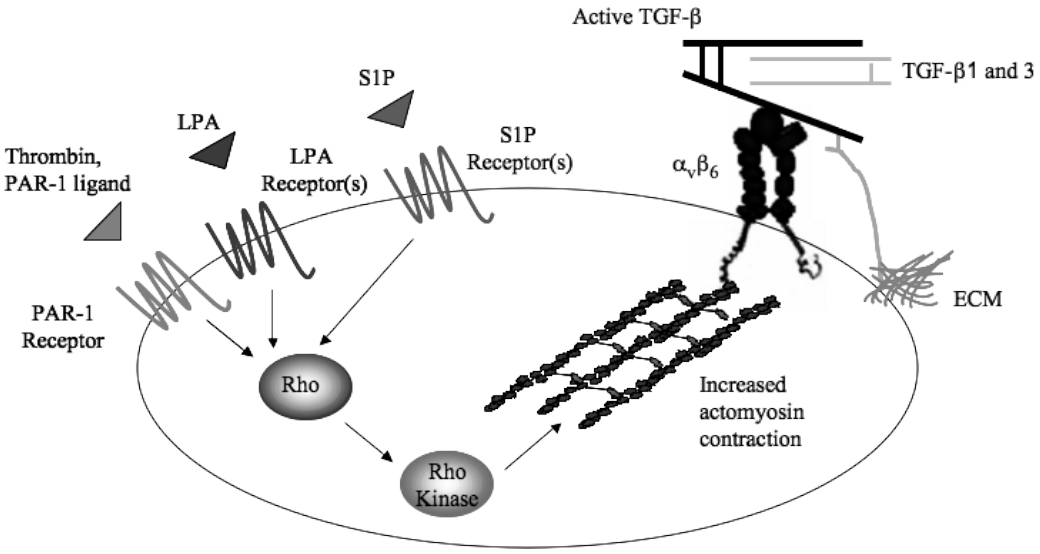


Figure 2.4. Model of Integrin $\alpha_v\beta_6$-mediated TGF-β activation.

DISCUSSION

The main objective of this study was to characterize the signals and pathways that govern $\alpha_v\beta_6$-mediated TGF-β activation. Because of its role in tissue fibrosis and inflammation, we hypothesized and subsequently demonstrated that the phospholipid, S1P, activates TGF-β through $\alpha_v\beta_6$. Previous work in the laboratory demonstrated that binding of the integrin $\alpha_v\beta_6$ was insufficient to activate latent TGF-β, suggesting that this process could be regulated. Indeed, ligands to certain G-protein coupled receptors could stimulate this process. Our finding that S1P induces $\alpha_v\beta_6$-mediated TGF-β activation, especially given the role of S1P at sites of tissue injury and inflammation, and its role as a fibrotic mediator in the eye and liver, further enhances our understanding of this process. This finding also suggests a role for S1P in pulmonary fibrosis and provides additional strategies for blocking this pathway in IPF, or other epithelial diseases that are a result of aberrant $\alpha_v\beta_6$-mediated TGF-β activation.

Many mechanisms of TGF-β activation have been described. However, the apparent role of the actin cytoskeleton along with the need for a functional LTBP-1 to tether the latent complex to the ECM, suggests that mechanical force regulates activation of latent TGF-β by the $\alpha_v\beta_6$ integrin. Since previous work has demonstrated that integrins can activate other ligands by exerting force to change their conformation, we hypothesized that $\alpha_v\beta_6$-mediated TGF-β activation occurs through mechanical force (Figure 2.4). The most compelling evidence that mechanical force and an increase in cellular tension is required for $\alpha_v\beta_6$-mediated

TGF-β activation is our finding that this process only occurs on substrates that are rigid enough to allow the cell to generate retractile force. Increased stiffness in the ECM in the presence of contractile agonists may thus constitute a positive feedback loop to promote the progression of fibrotic disorders by stimulating $\alpha_v\beta_6$-mediated TGF-β activation. Cytoskeletal tension itself is influenced by the stiffness of the ECM substrate, which may further amplify this positive feedback loop. Overall, such a positive feedback loop likely contributes to progression of tissue fibrosis, since excessive scar tissue and rigidity of the matrix may lower the threshold of cell contractile stimuli necessary for subsequent TGF-β activation.

MATERIAL AND METHODS

Cell lines, Antibodies, and Reagents

Normal human bronchial epithelial (NHBE) cells were obtained commercially (Lonza, passages 1-3). The cells were cultured according to the company guidelines. Briefly, they were cultured at 37^{o}C, 5% CO_2 with the media replaced every other day. Antibodies used were a β_6-blocking antibody, 3G9 (provided by Paul Weinreb and Shelia Violette (Biogen Idec)) and a mouse monoclonal TGF-β blocking antibody (clone 1D11; R&D Systems). Agonists used include S1P and LPA (Sigma Aldrich) and active human recombinant TGF-β1 (R&D Systems). Inhibitors used were a ROCK inhibitor, Y-27632, and a specific non-muscle myosin II inhibitor, Blebbistatin (Sigma Aldrich).

Western Blot

NHBE cells (250,000 per well) were seeded into a 48-well plate (Costar) in BEGM growth medium (Lonza). After 24 hours, the media was replaced with fresh media. After 96 hours, cells were pre-incubated with blocking antibodies or inhibitors for 30 minutes before agonists were added. The duration of the experiments was 2 hours. Cell lysates were harvested in 40 μL of 1X RIPA (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% Sodium Deoxycholate, 0.1% SDS) buffer containing 1X of Halt Protease and Phosphatase inhibitor (Thermo Scientific). Total protein is quantified using the Bradford Lowry assay (D_c assay, Biorad). Approximately 20-50 μg of protein is separated on an SDS-

PAGE gel and transferred onto a PVDF membrane (Hybond-ECL; Amersham Biosciences). The membrane is incubated with 3% nonfat dry milk in Tris-buffered saline with 0.05% Tween (TBST) (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4) for 1 hour before being probed with a primary rabbit polyclonal anti–pSmad2 (1:1000, Cell Signaling Technology) or a mouse monoclonal anti–Total Smad2 (1:1000, Cell Signaling Technology) for 2 hours at room temperature or 4^{o}C overnight. Membranes were probed with either an HRP-conjugated goat anti-rabbit (1:3000, Santa Cruz), or goat anti-mouse (1:3000, Santa Cruz) secondary antibody for 30 minutes at room temperature. Proteins were detected using ECL Plus (Amersham Biosciences). For examination of Total Smad2 levels, the blots were stripped using Western Restore stripping buffer (Pierce Biotechnology) for 15 minutes at room temperature.

Phalloidin Staining

NHBE cells (250,000 per well) were seeded onto rat tail collagen I (10 ug/mL, Sigma Aldrich) coated 12 mm coverslips (Fisher Scientific). The culture media is changed the next day and experiments were done on "day 3" for 2 hours. Cells were fixed with 1% paraformaldehyde for 5 minutes, permeabilized with 0.1% Triton-X for 1 minute, and then stained with rhodamine-phalloidin (Invitrogen, 1:40 diluted in 1% BSA) for 20 minutes. Coverslips were mounted onto slides using ProLong Gold Anti-Fade mounting medium (Invitrogen).

Air-liquid Interface Cultures

NHBE cells (500,000 per well) were seeded onto collagen I coated Transwells (Corning) in bronchial epithelial differentiation medium (BEDM), which consists of 50% Bronchial Epithelial Basal Medium (BEBM) and 50% Dulbecco's Modified Eagle Medium (DMEM, Mediatech Cellgro) supplemented with all of the included SingleQuots (Lonza) except: gentamycin/amphotericin, retinoic acid, and triiodothyronine. Retinoic acid solution was added to a final concentration of 50 nM. Media on the apical and basal sides of the transwell were changed every other day for 2 weeks, in which the media is then removed from the apical side. Media on the basal side is changed every other day for an additional 2 weeks. Experiments were done for 2 hours after pre-incubation for 30 minutes with inhibitors added to the basal side.

Polyacrylamide Flexible Substrates

NHBE cells (250,000 per well) were seeded onto polyacrylamide flexible substrates created on round glass coverslips (12 mm) according to the protocol described [31, 34]. Briefly, the surfaces of 12 mm coverslips were treated with 0.1 N NaOH, 3-aminopropyltrimethoxy silane and 0.5% gluteraldehyde. After "activating" the coverslips, 12 uL of polyacrylamide mixture (bis-acrylamide, acrylamide, 50 mM HEPES, pH=8.5, Ammonium Persulfate, Temed) was pipetted onto the coverslips. Another 12 mm coverslip was placed directly on top of the droplet. After polymerization, the top coverslip was removed and substrates were rinsed with 50 mM HEPES. The gels were then treated to UV

irradiation with 0.5 mg/mL Sulfo-SANPAH (Pierce). Collagen I (10 ug/mL) was used to coat the substrates overnight at 4^{o}C. Prior to seeding the cells, the substrates were UV treated for 15 minutes. NHBE were seeded and experiments were performed the next day.

CHAPTER 3: AEROSOL DELIVERY OF DRUGS TARGETING INTEGRIN-MEDIATED TGF-β ACTIVATION IN THE TREATMENT OF PULMONARY FIBROSIS

INTRODUCTION

A. Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF), or scarring of the lungs, is an incurable progressive disease that has a 5-year mortality rate of 80% [35]. While the disease often occurs in older patients with other co-morbid diseases, most patients with IPF die as a direct consequence of their lung fibrosis. Unfortunately, there is a lack of effective pharmacological treatments for IPF, with lung transplant representing the only "cure" for the disease. No drugs that are currently on the market for IPF either substantially slow its progression or improve patient symptoms. For example, corticosteroids, such as Prednisone, or immunosuppressants, such as azathioprine, are frequently used to reduce pulmonary inflammation but have not been shown to have a meaningful effect on clinically significant outcome variables in IPF. Furthermore, these drugs have a number of adverse side effects, such as increased risk for infection.

Pulmonary fibrosis is characterized by tissue damage, and the presence of excessive extracellular matrix deposits in the lung. These fibrous connective

tissue deposits impair oxygen exchange across the alveoli. Pulmonary fibrosis is a multifactorial group of diseases that can arise from various factors that inflict injury to the lungs, including drug-induced toxicity (e.g. antibiotics, chemotherapeutics, anti-arrythmics), viral or bacterial infections, autoimmune diseases, smoking, and prolonged exposure to occupational or environmental contaminants (e.g. asbestos, silica, hard metal dusts). However, for most patients with pulmonary fibrosis, the precise cause is unknown, and most of these patients have thus been described as having idiopathic pulmonary fibrosis (IPF). The molecular mechanisms that underlie IPF remain poorly understood. Elucidating these molecular processes is critical to discover improved pharmacological therapies for this devastating disease.

B. TGF-β in Fibrosis

Transforming Growth Factor Beta (TGF-β) has been well established as a central mediator of fibrotic diseases, and is presumed to play a central role in IPF. Generally, fibrosis is considered to arise as a consequence of the normal wound healing process gone awry. Normally, after injury to tissues, "activated" fibroblasts, or myofibroblasts, proliferate and migrate into the wounded areas. Once there, the myofibroblasts increase synthesis and deposition of ECM proteins, such as collagen and fibronectin. Ultimately, these cells become inactivated and scar tissue formation is halted. However, in tissue fibrosis, these cells remain highly contractile and continue to deposit excess amounts of matrix

proteins. Furthermore, normal degradation of ECM proteins is impaired, resulting in an accumulation of excess scar tissue. Various studies demonstrate that enhanced TGF-β signaling plays a major role in this process; however the cause of this hyperactivity is not fully understood [36].

TGF-β modulates multiple signaling pathways, which contribute to fibrosis. First, the cytokine induces the expression of ECM proteins, such as collagen and fibronectin, and also suppresses expression of matrix metalloproteinases that degrade ECM. Secondly, TGF-β induces differentiation of resident interstitial fibroblasts to activated myofibroblasts, cells that likely contribute to the abnormal matrix production in IPF [37]. Finally, TGF-β has been shown to mediate epithelial-to-mesenchymal transition (EMT), a process that has also been suggested to play a major role in the development of fibrosis in some epithelial organs. EMT occurs when epithelial cells detach from the basement membrane and downregulate epithelial markers while simultaneously increasing expression of mesenchymal markers. Lineage tracing data performed in the bleomycin-induced mouse model of pulmonary fibrosis demonstrated that approximately 30% of fibroblasts present in the lungs during fibrosis were of epithelial origin. This EMT process appears to also be dependent on integrin $\alpha_v\beta_6$ [38]. Consequently, understanding the signals and mechanism that regulate TGF-β bioactivity is crucial in finding a therapeutic intervention for IPF and in general, diseases involving aberrant TGF-β signaling.

We have previously discovered that integrin $\alpha_v\beta_6$ activates TGF-β and that β_6 knockout mice are protected from fibrotic disorders in various organs [17, 18].

As part of my thesis, I explored the possibility of administering small molecule inhibitors of the β_6-mediated TGF-β activation pathway as a treatment for pulmonary fibrosis. In collaboration with a medicinal chemist, I examined approved drugs on the market for new indications, and we developed new compounds that inhibit α_v integrins, including $\alpha_v\beta_6$. Lastly, I explored the possibility of administering these compounds via aerosol delivery. My research findings demonstrate the potential of developing drugs that target the β_6-mediated TGF-β activation pathway as a new therapeutic intervention in pulmonary fibrosis.

RESULTS

Macrophages as Biosensors of TGF-β Activation

Several challenges exist in testing pharmacological agents during IPF clinical trials. For example, IPF patients often present with similar histology and clinical symptoms, yet respond very differently to the same drug. A major goal in improving treatments for IPF involves identifying biomarkers that would allow monitoring of responsiveness to specific pharmacological agents [35]. To this end, we used DNA microarrays to profile gene expression on lung tissues under various conditions. In particular, we compared the gene expression patterns of whole lung tissue from primates treated with a control or β_6-blocking antibody with the expression patterns of alveolar macrophages from wildtype mice and β_6-knockout mice. Based on this analysis, five genes were chosen whose expression was dramatically affected by either loss or blockade of β_6 in both mice and primates. We found that expression of TREM-1, Osteopontin, and PAI-1 dramatically decreased upon integrin loss, while ALCAM and MMP-19 increased. To confirm these findings, we treated wild type mice with a range of two weekly systemic doses of a β_6-blocking antibody. The range of doses we chose included a dose below (0.3 mg/kg) what has been demonstrated to be effective at inhibiting bleomycin and radiation-induced pulmonary fibrosis (1 mg/kg) [39]. Twenty-four hours after the second dose, we purified alveolar macrophages from bronchoalveolar lavage fluid, obtained RNA from these cells

and evaluated gene expression by quantitative real-time polymerase chain reaction (qRT-PCR). We found these five genes were dramatically affected by blocking β_6 at the dose that has been shown to inhibit fibrosis (1 mg/kg) (Figure 3.1). Furthermore, we performed an ELISA examining pSmad2 expression in the macrophage lysates obtained from the same experiment. We found that pSmad2 also dramatically decreased upon treatment with the 1 and 3 mg/kg doses of the β_6-blocking antibody (Figure 3.2).

Novel Small Molecule Inhibitors of $\alpha_v\beta_6$-mediated TGF-β Activation

A humanized monoclonal blocking antibody against $\alpha_v\beta_6$ is currently in Phase II clinical trials, however, systemically inhibiting this molecule could result in potential adverse effects. In addition, the limitations and expense of utilizing a monoclonal antibody for long-term treatment may present as a challenge and inconvenience. Therefore, we explored the possibility of small molecule inhibitors of the β_6-mediated TGF-β activation pathway as a potentially more efficient and effective treatment for pulmonary fibrosis.

A class of small molecule inhibitors of $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_v\beta_6$ integrins has been described [40]. Among the compounds, EMD 527040 was determined to be the most potent and selective in blocking $\alpha_v\beta_6$ ($IC_{50} = 0.04 \pm 0.07$ nM) (Figure 3.3). In an acute model of biliary fibrosis, rats treated with this compound had reduced periductal collagen deposition by ~50% and improved liver architecture and function [41]. This compound exists as a mixture of two stereoisomers

(*(S,S)*-isomer and *(S,R)*-isomer)), and the activity of each isomer is unclear (Figure 3.3). In collaboration with Brad Backes, a medicinal chemist, we synthesized each diastereomer and tested their efficacy in blocking $\alpha_v\beta_6$ by using a cell adhesion based assay. We treated SW480-β_6 with either the *(S,S)*-isomer or the *(S,R)*-isomer and examined the ability of the cells to adhere to recombinant LAP (ligand of β_6) or bovine serum albumin (BSA; to detect non-specific binding). We found that the *(S,S)*-isomer was significantly more potent at blocking $\alpha_v\beta_6$-mediated adhesion and probably accounts for all of the reported activity of this compound (Figure 3.4).

Systemic administration of the ROCK inhibitor, Y-27632, has already been shown to protect mice from bleomycin-induced pulmonary fibrosis [42]. Furthermore, the ROCK inhibitor, Fasudil, is approved for human use in Japan for the treatment of cerebral vasospasm in patients. Fasudil is also in several active clinical trials in the United States and has been shown to have beneficial effects in patients with pulmonary hypertension, atherosclerosis, stroke, and angina. However, a major limitation of ROCK inhibitors is that they are vasodilators and systemic administration of the drug at concentrations that would completely block the enzyme would result in hypotension. Therefore, we developed better ROCK inhibitors that are more potent and have a shorter systemic half-life than existing ROCK inhibitors (Figure 3.5). The increased potency and rapid systemic catabolism of these compounds could allow them to be delivered locally into the alveoli (by aerosol), thereby enhancing their effects on alveolar epithelial cells while minimizing adverse effects on blood pressure.

To determine the effect of Fasudil and other ROCK inhibitors on blocking β_6-mediated TGF-β activation, we used a TGF-β bioassay that involves co-culturing a cell line expressing β_6 (SW480-β_6) with a mink lung epithelial reporter cell line, stably transfected with plasminogen activator inhibitor-1 (PAI-1), a TGF-β responsive promoter. All of the ROCK inhibitors blocked $\alpha_v\beta_6$-mediated TGF-β activation in a dose-dependent manner. However, Compound 10 and Y-39983 were more potent at blocking TGF-β activity than Fasudil (and Y-27632, a common ROCK inhibitor) (Figure 3.6a,b).

Aerosol Delivery of $\alpha_v\beta_6$-mediated TGF-β Activation Inhibitors

As mentioned above, systemic administration of ROCK inhibitors at high concentrations results in hypotension. To potentially minimize this effect, we tested whether high concentrations administered locally in the form of an aerosol would be effective at blocking TGF-β activation. We used a nose-only aerosol delivery system, administered a total of 6 mg of Fasudil to wild type mice over 30 minutes and examined pSmad2 expression in BAL purified alveolar macrophages. We found that Fasudil inhibited pSmad2 expression from macrophage lysates compared to delivery of vehicle alone (Figure 3.7a). We wanted to examine the effect of this dose on blood pressure in the mice, so we aerosolized the drug in alternating intervals of 5 minutes of drug exposure, then 2 minutes of blood pressure monitoring, for a total of 30 minutes of exposure to the drug. We found that mice treated with Fasudil at a dose that blocked pSmad2 expression

experienced a dramatic drop in blood pressure compared to vehicle treatment alone (Figure 3.7b). Aerosol delivery of Fasudil may be effective in blocking TGF-β activation, however, the dose that is effective has adverse effects on blood pressure, making this not a feasible therapeutic option. Thus, we tested the hemodynamic effects of aerosol delivery of a more potent ROCK inhibitor, Y-39983. We found that Y-39983 had a much smaller effect on blood pressure than did Fasudil on the three mice we treated (Figure 3.8a-c). However, we have yet to generate sufficient quantities of this compound to systematically evaluate its effectiveness in blocking alveolar TGF-β activation.

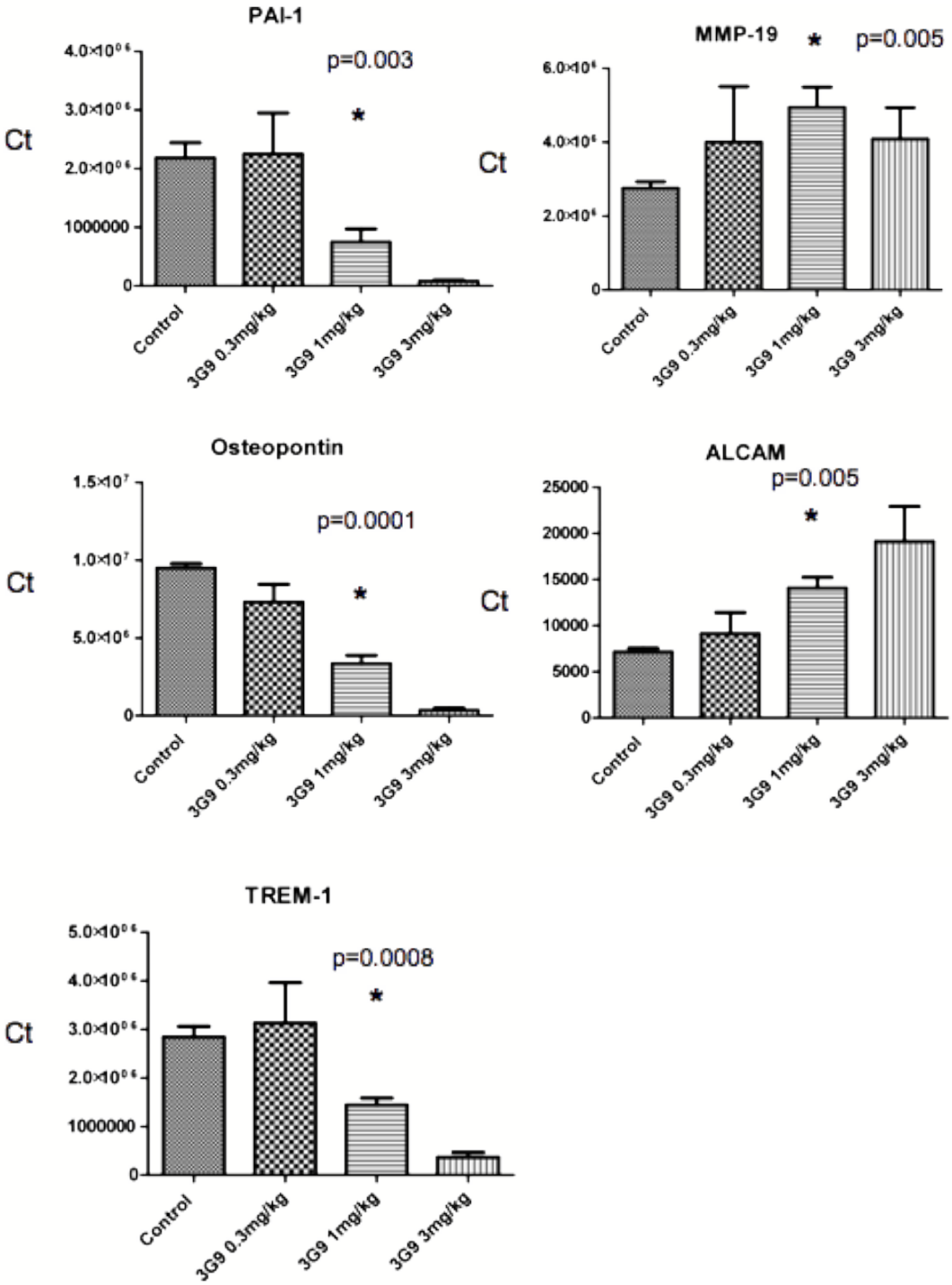


Figure 3.1. Gene copy number, by real time PCR, of each of the five transcripts selected as a signature for *in vivo* inhibition of $\alpha_v\beta_6$. Analysis was performed on RNA from alveolar macrophages purified from mice (five per group) treated with a range of two weekly doses of the β_6-blocking antibody, 3G9. Statistical difference was determined by comparing to control.

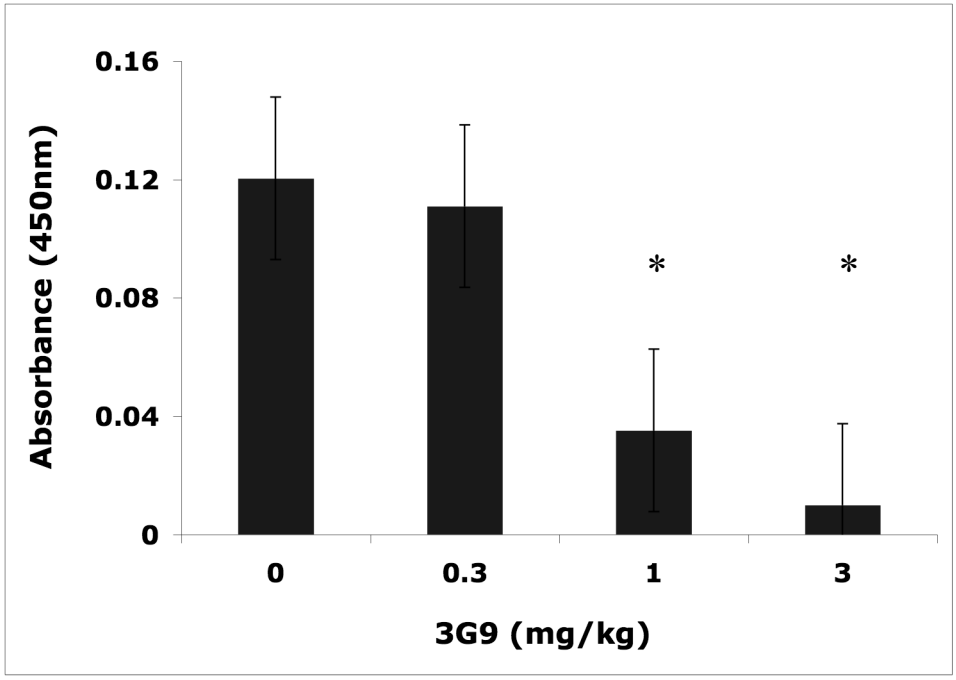
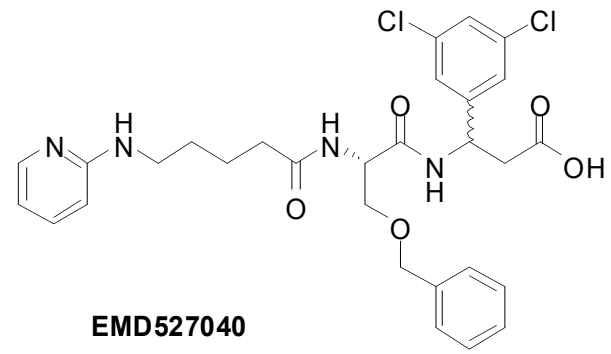
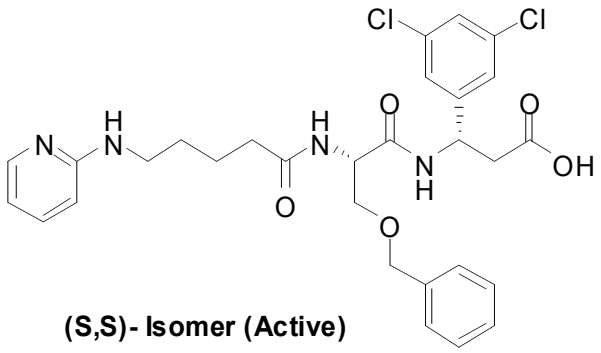


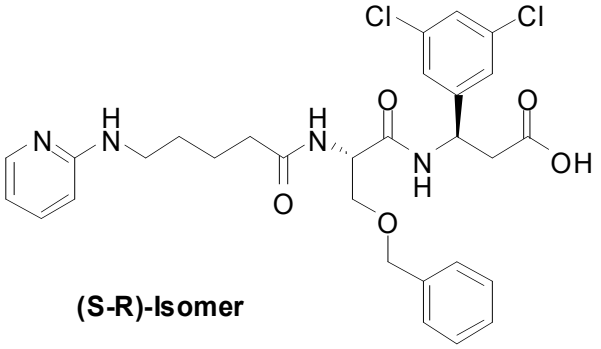
Figure 3.2. β_6-blocking antibody inhibits pSmad2 in alveolar macrophages. ELISA assay for pSmad2 from lysates of BAL purified alveolar macrophages obtained from mice treated IP with a range of two weekly doses of the $\alpha_v\beta_6$-blocking antibody 3G9 (or PBS as control).
* $p < 0.01$ compared to control.



EMD527040



(S,S)- Isomer (Active)



(S-R)-Isomer

Figure 3.3. Structures of the $\alpha_v\beta_6$ inhibitor, EMD527040 and its two diastereomers

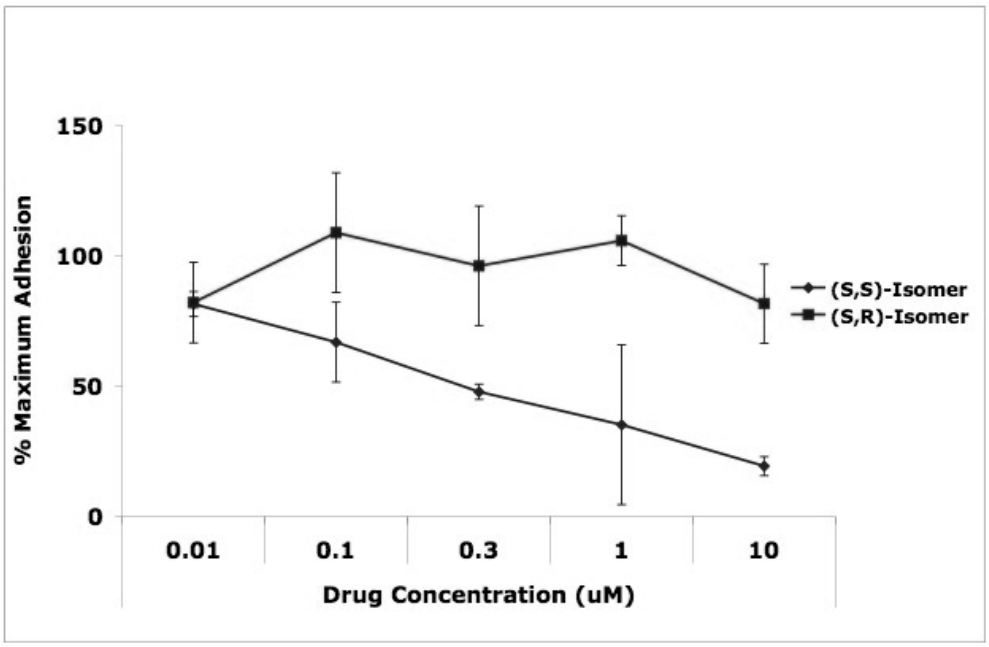


Figure 3.4. Concentration-response curves for inhibition of $\alpha_v\beta_6$-mediated adhesion to TGF-β1 LAP (1 ug/mL). SW480-β_6 cells treated with *(S,S)*-isomer or *(S,R)*-isomer of EMD 57040 at concentration 0.01-10 uM and allowed to adhere to wells coated with LAP for 1 hour. Adhesion was calculated from absorbance of crystal violet-stained cells.

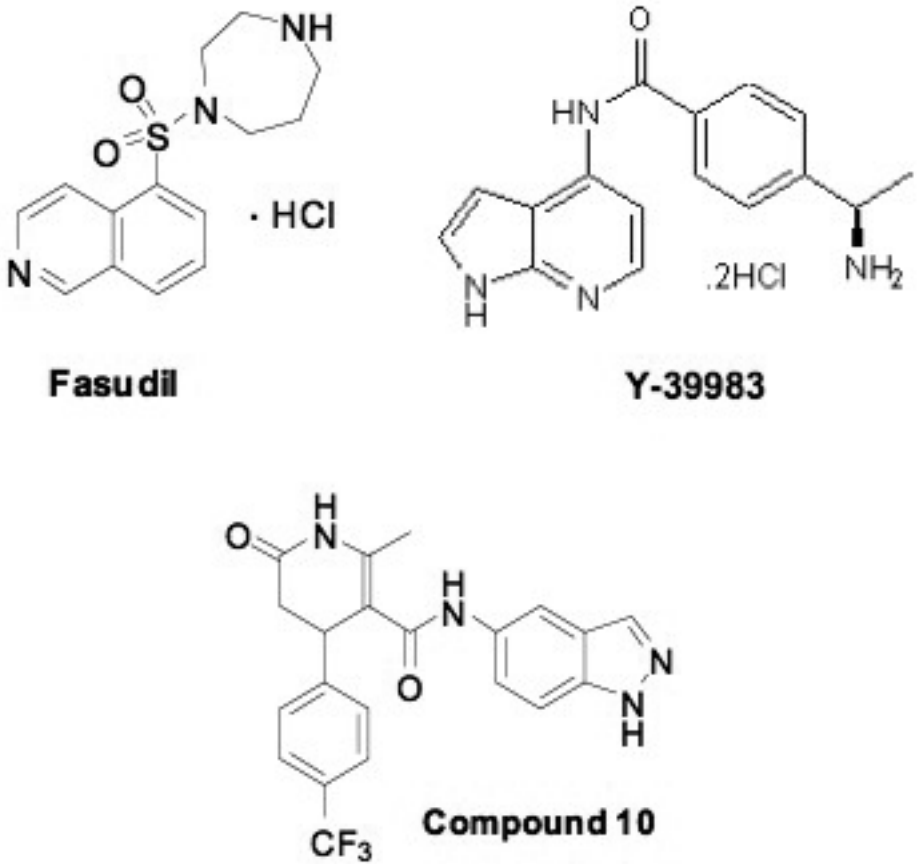
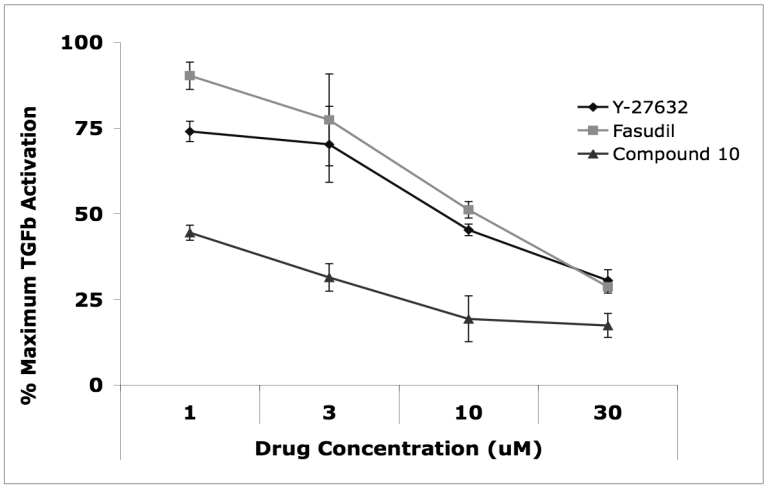


Figure 3.5. Structures of Rho Kinase inhibitors evaluated.

A.



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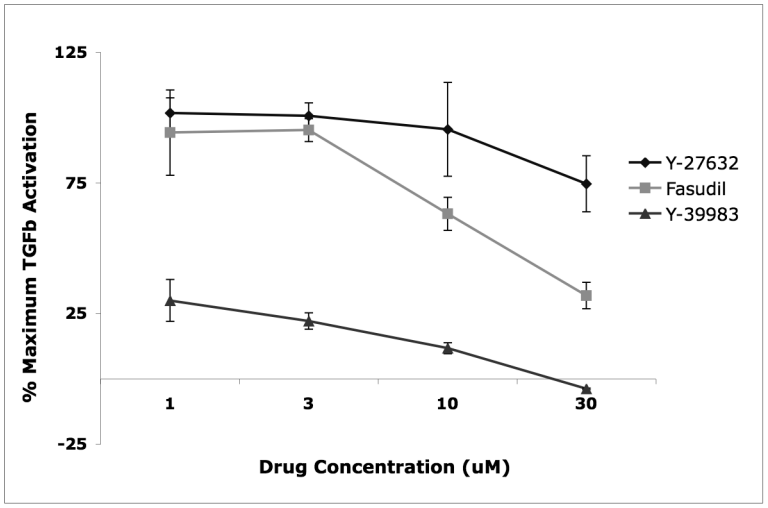
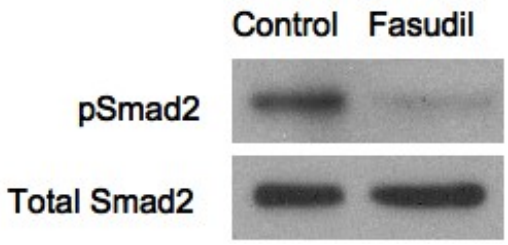


Figure 3.6. Concentration-dependent inhibition of $\alpha_v\beta_6$-mediated TGF-β activation. **a)** SW480-β_6 treated with Y-27632, Fasudil, or Compound 10 were co-cultured for 18 hours with reporter cells stably expressing a TGF-β sensitive portion of the PAI-1 promoter driving firefly luciferase in the presence or absence of a range of concentrations of each ROCK inhibitor. TGF-β activation was determined by luminescence from cell lystates. **b)** SW480-β_6 treated with Y-27632, Fasudil, Y-39983 were co-cultured for 18 hours with reporter cells stably expressing a TGF-β sensitive portion of the PAI-1 promoter driving firefly luciferase in the presence or absence of a range of concentrations of each ROCK inhibitor. TGF-β activation was determined by luminescence from cell lystates.

A.



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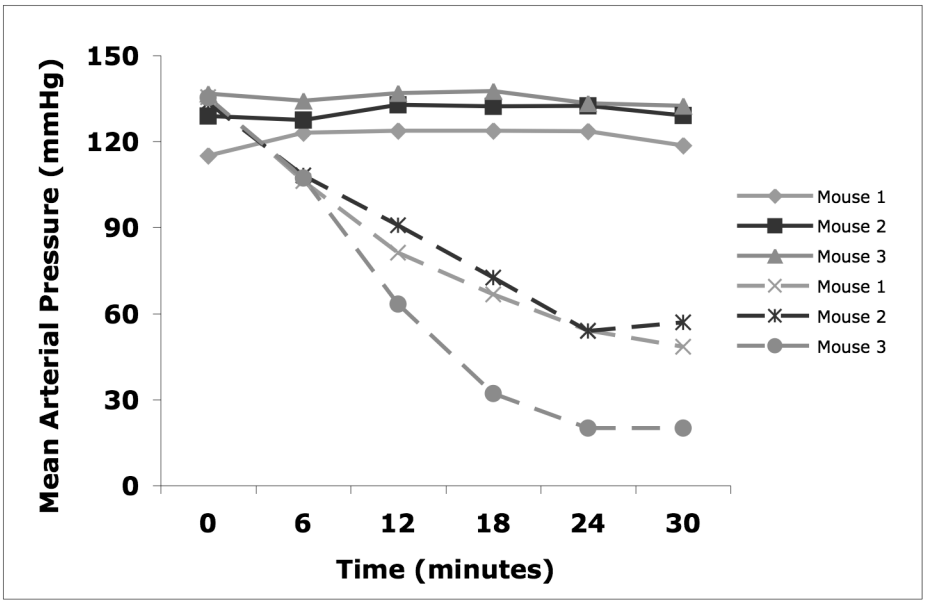
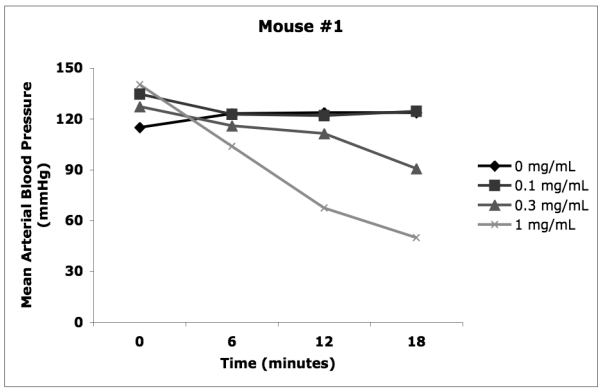
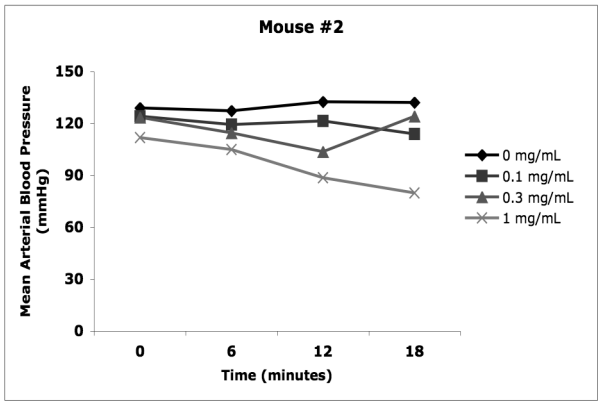


Figure 3.7. Aerosol delivery of Fasudil. **a)** Western blot of pSmad2 and Total Smad2 expression in lysates from BAL purified macrophages. **b)** Three mice were treated with vehicle for 30 minutes to obtain baseline blood pressure measurements. Afterwards, the same three mice were given a total of 6 mg Fasudil over 30 minutes. Solid line = Vehicle (water) treatment, Dashed line = Fasudil treatment

A



B



C

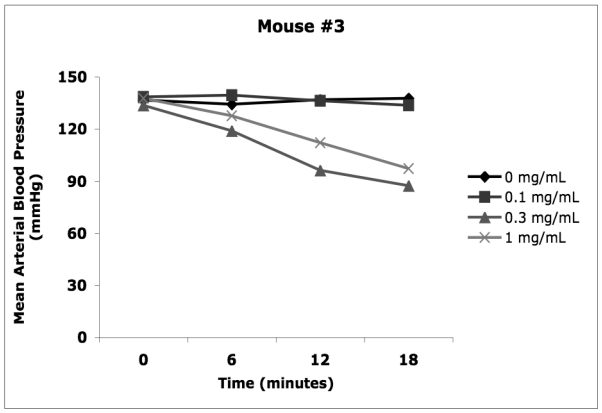


Figure 3.8. Blood pressure measurements in mice treated with Y-39983 via aerosol delivery. All mice were treated with vehicle for 18 minutes to obtain baseline blood pressure measurements. Afterwards, the mice were treated with Y-39983 for 18 minutes per drug concentration. **a)** Mouse 1 **b)** Mouse 2 **c)** Mouse 3. Black line = Vehicle (water), Red line = Y-39983 (0.1 mg/mL), Blue line = Y-39983 (0.3 mg/mL), Green line = Y-39983 (1 mg/mL).

DISCUSSION

The main objective of this study was to develop new drugs for the treatment of pulmonary fibrosis. Our previous finding that the integrin $\alpha_v\beta_6$ functions to activate TGF-β, a known mediator in fibrotic diseases, led us to examine the possibility of administering small molecule inhibitors of this pathway as a potential treatment. Specifically, we were interested in directly targeting the alveolar epithelium by aerosol delivery of modified compounds generated by our laboratory. Our findings highlight the potential of several compounds (*(S,S)*-isomer, Compound 10, and Y-39983) in blocking TGF-β activation by $\alpha_v\beta_6$. Although we have not yet tested the *in vivo* effectiveness of these compounds on blocking TGF-β signaling, EMD 527040 has already been demonstrated to have a protective effect on the progression of biliary fibrosis, and Y-39983 has been tested in axonal regeneration in cats and as a topical drug to lower intraocular pressure in rabbits and monkeys [43, 44].

We have also started testing drugs that target the α_v subunit. While blocking $\alpha_v\beta_6$ is sufficient to protect mice from developing pulmonary fibrosis, other α_v binding integrins that have been shown either *in vitro* or *in vivo* to activate TGF-β (e.g. $\alpha_v\beta_5$, $\alpha_v\beta_8$) could contribute to the disease. However, inhibiting the α_v subunit could have negative effects, since this would also block any integrin that had the α_v subunit as its binding partner. For example, *Itg*$\alpha v^{-/-}$ mice die during embryonic development or immediately after birth because of

defects in vascular development [45]. Again, our strategy to deliver these compounds via aerosol delivery may minimize the toxic effects of these drugs.

As mentioned above, Fasudil results in hypotension at very high concentrations. While this compound demonstrates beneficial effects in a wide range of studies and is currently in clinical trials, this generation of ROCK inhibitors is not very potent compared to newer generations of ROCK inhibitors (e.g. Y-39983). Although our studies demonstrate that Fasudil may not be a feasible candidate, our findings do highlight the potential of aerosol delivery of ROCK inhibitors as a therapeutic option, especially if compounds are designed to have potent local effects and minimal systemic toxicities.

In addition to developing new compounds and testing a new method of drug delivery for the treatment of pulmonary fibrosis, we generated a list of five genes that could serve as candidate biomarkers of the therapeutic efficacy of blocking β_6. Our finding with pSmad2 indicates that it may also be a useful readout of blocking TGF-β signaling *in vivo*. Furthermore, our efforts to use alveolar macrophages as a biosensor is advantageous, given the current lack of effective methods for monitoring therapy response in patients, and secondly, extracting macrophages from BAL can be performed repeatedly throughout drug treatment. This work has resulted in some promising findings that could help the field of pulmonary fibrosis research. In addition to discovering new biomarkers and a method of monitoring drug treatment, we have characterized more potent drugs that could lead to novel ways of treating an incurable disease.

MATERIAL AND METHODS

Cell Adhesion Assay

Untreated polystyrene 96-well flat-bottom microtiter plates (Costar) were coated with either recombinant LAP (1 ug/mL) or 1% BSA. Cells were seeded (50,000 cells per well) with added compounds and centrifuged (top side up) at 10 *g* for 5 min and then incubated for 1 hour at 37^{o}C. Media was removed and the plate was centrifuged (top side down) at 10 *g* for 5 min. Cells were fixed with 4% paraformaldehyde and stained with Crystal Violet for 10 minutes. After staining, cells were washed with water, 2% SDS was added and incubation proceeded at room temperature for 30 min. Absorption was measured at 550 nm.

TGF-β Co-culture Bioassay

As described previously with a few modifications, 50,000/well β_6-expressing test cells are co-cultured with 15,000/well mink lung epithelial reporter cells in triplicates in a 96-well plate [46]. Both cell types are cultured in 100 uL of 10% FBS + Dulbecco's Modified Medium (Gibco) with or without antibodies or inhibitors for 16-20 hours. Prior to adding 30 μL of 5X Cell Culture Lysis Buffer, cells are washed once with 1X Phosphate-Buffered Saline. After lysis buffer is added, the plate is vortexed and centrifuged at 1500 rpm for 5 minutes. To read luciferase activity, 25 uL of the supernatant is transferred into a 96-well white plate (Corning), where 100 μL/well of luciferase substrate (Promega) is

added. The relative luciferase units are read immediately following addition of substrate using the TECAN plate reader.

Aerosol Delivery of Drugs

Wild type mice were consciously restrained in a SCIREQ FLEXiware Aeroneb Nebulizer cone (one per mouse) (SCIREQ). They were exposed to either control vehicle or drug for 15-30 minutes at a 3L/min flow rate. Afterwards, the animals were placed back into their cages and monitored for any signs of distress or agitation.

Surgical Implantation of Blood Pressure Catheters

A PA-C10 radio-telemetry catheter (Data Sciences International) was implanted into each mouse one to two weeks prior to administration of ROCK inhibitors. Each mouse was anesthetized with isoflurane (4% induction, 2.5% maintenance, 2 L/min) and kept warm on a heated pad (T/Pump, Gaymar). Pre-surgical analgesia (ketoprofen 5 mg/kg SQ) was provided after isoflurane induction. Prior to making an incision, body hair was removed from the intended incision sites using Nair, and the incision sites were scrubbed three times with disinfectant. A 1.5 cm midline incision was made through the skin over the neck. The mandibular glands were carefully separated using sterile cotton tip applicators. The left gland was retracted using an elastic stay hook, and the left carotid artery was identified and isolated, taking care not to disturb the vagus nerve. Three pieces of 6-0 non-absorbable sutures was passed underneath the isolated carotid artery: a ligation

suture, temporary occlusion suture, and a suture to hold the catheter in place after artery cannulation. The ligation suture was positioned just proximal to the bifurcation of the interior and exterior carotid arteries and secured. The radio-telemetery catheter was placed into the carotid artery using an introducer (25 gauge needle with a bent bevel) and vessel cannulation forceps after temporary proximal occlusion of the carotid artery. The catheter was advanced until its sensor tip was located 2 mm into the aortic arch and secured in place. The temporary occlusion suture was then released. A small drop of Vetbond tissue adhesive was applied to the suture knots to prevent dehiscence. A subcutaneous pocket was produced through the neck incision by blunt dissection. This pocket was irrigated with warmed, sterile saline. One drop of local anesthetic (bupivicaine) was applied to the pocket site. The PA-C10 transmitter body was inserted into the pocket, flat side against the mouse body, and rounded side towards the skin. A small drop of Vetbond tissue adhesive was used to secure the transmitter in place. The skin was closed with 6-0 absorbable sutures. Once closed, the wound was sealed with Vetbond. Post-surgical anethesia was provided (ketoprofen 5 mg/kg sq 12-24 hours) for five days. Any wound infection was treated with topical Bacitracin Zinc-Polymixin S04 ointment.

Blood Pressure Measurements

One to two weeks post-catheter implantation, wild type mice are anesthetized with 2-3% isoflurane (to aid in their placement into the delivery cone). The mice are placed in a SCIREQ FLEXIWARE cone (one per mouse), which restrained

them from movement. Once the mice recovered from the isoflurane, the nebulizer was turned on at a flow rate of 3 L/min. The mice are exposed to the aerosolized ROCK inhibitors for < 15 min. Afterwards, the mice are placed back into their cages and monitored for any signs of distress or agitation. Blood pressure and heart rate are measured every 10 minutes in 30 second epochs.

Purifying Macrophages from Bronchoalveolar Lavage Fluid

Mice are euthanized with isoflurane. The trachea was exposed and a 27-gauge catheter was inserted. A syringe was connected to the catheter and 1 mL of 1% BSA diluted in PBS was injected into the trachea and then extracted. This process was performed five times. BAL fluid was collected and centrifuged at 1200 rpm for 5 minutes. The pellet was resuspended with 10% RPMI culture media and plated into a 6-well plate well. The cells are incubated in 37^{o}C for 30 minutes to allow the macrophages to attach. Afterwards, the well was washed 3 times with PBS, and cell lysates were harvested in 40 μL of 1X RIPA (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1% Sodium Deoxycholate, 0.1% SDS) buffer containing 1X of Halt Protease and Phosphatase inhibitor (Thermo Scientific).

Statistics

Statistical difference was determined using StatView. Groups were compared using ANOVA followed by a post-hoc analysis using the Tukey-Kramer method.

CHAPTER 4: CONCLUSION

Substantial research has demonstrated the importance of integrins in regulating biological processes and diseases. Specifically, research in the area of fibrosis has highlighted the importance of RGD-binding integrins and their activation of TGF-β, a central mediator of fibrosis in many organs. The central focus of my thesis work has been to study the molecular mechanisms that contribute to pulmonary fibrosis. In particular, we describe S1P induced $\alpha_v\beta_6$-mediated TGF-β activation as a novel pathway in this disease. We also describe novel inhibitors that we developed with the hope that aerosol delivery of these small molecules could provide a new therapeutic strategy for pulmonary fibrosis. Finally, we provide a list of candidate genes that may serve as effective biomarkers for monitoring blockade of β_6-mediated TGF-β activation *in vivo*, and demonstrate the potential of using macrophages as biosensors to monitor drug therapy.

We demonstrate that $\alpha_v\beta_6$-mediated TGF-β activation is a result of increased actomyosin contraction and requires the generation of cellular tension. While our finding with the flexible substrates is compelling, an ideal experiment would be to use optical tweezers to directly examine the amount of force a single integrin molecule exerts on its bound latent TGF-β for activation. Another experiment would be to possibly conjugate fluorophores to the latent TGF-β molecule and use fluorescence resonance energy transfer (FRET) to examine

conformational changes that may occur to TGF-β upon activation. These experiments are technically challenging, however, they would provide additional direct evidence that TGF-β activation by $\alpha_v\beta_6$ is regulated by mechanical force.

In addition to mechanical force, we demonstrated that ligands to the PAR-1, LPA, and S1P receptors stimulated $\alpha_v\beta_6$-mediated TGF-β activation by acting through ROCK. These findings provide additional strategies for inhibiting this pathway in pulmonary fibrosis. Drugs targeting S1P receptors have either already been approved, such as Fingolimod, an $S1P_1$ receptor agonist, or are in clinical trials. In addition, the orally active LPA_1 receptor antagonist, AM966, has been shown to reduce bleomycin-induced pulmonary fibrosis in mice [52]. Our findings suggest that other agonists that stimulate the RhoA/Rho kinase pathway in epithelial cells may also stimulate TGF-β activation by $\alpha_v\beta_6$. Since pulmonary fibrosis is a multifactorial disease, it is unlikely that treatment would involve just one drug. Therefore, developing drugs to multiple targets or integrins (e.g. α_v inhibitor) may be the most effective.

Integrins have been considered attractive therapeutic targets for pharmacological intervention because these widely expressed molecules function in a diverse array of biological processes. Pharmacological inhibitors have been approved targeting three integrins: (1) natalizumab, a monoclonal antibody against $\alpha_4\beta_1$, (2) tirofiban, eptifibatide, and abciximab, small molecule inhibitors and antibody fragment, respectively, targeting $\alpha_{IIB}\beta_3$, and (3) efalizumab, a monoclonal antibody targeting $\alpha_L\beta_2$. Antibodies or small molecule inhibitors targeting other integrins are also in various phases of clinical trials. While these

anti-integrin therapies have benefits in reducing disease activity, they also have been associated with some rare and serious side effects, including progressive multifocal leukoencephalopathy. Because of this, natalizumab has a black-box warning on its drug label, and efalizumab was withdrawn from the market in 2009 [47]. Nevertheless, developing inhibitors to integrins that mediate TGF-β activation is of great interest, especially since studies have reported increased expression of $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$ in diseases that involve TGF-β signaling [18, 39, 48-51]. However, to minimize the risks of anti-integrin drugs, or any type of therapy, determining drug efficacy throughout treatment is crucial.

A humanized blocking antibody to $\alpha_v\beta_6$ is currently in Phase II clinical trials. As seen in the examples above, systemic administration of those anti-integrin drugs can result in rare and fatal side effects. Therefore, the biomarkers we described, along with the use of macrophages, can help in monitoring the effectiveness of this treatment. Examining pSmad2 expression in macrophages may also be useful in monitoring the efficacy of drugs that generally block TGF-β signaling in the alveolar space. The β_6-blocking antibody has demonstrated great potential in preventing and even treating existing pulmonary fibrosis in murine models of the disease. However, finding a small molecule that can be locally administered into the lungs (e.g. aerosol delivery) may be a better long-term alternative. Because of this, we tested small molecule inhibitors to the integrin and to other parts of the $\alpha_v\beta_6$-mediated TGF-β activation pathway.

We discovered that aerosol delivery of the ROCK inhibitor, Fasudil, blocked TGF-β signaling in the alveolar space. The next experiment was to

determine whether aerosol delivery of ROCK inhibitors, or any other inhibitors that block $\alpha_v\beta_6$-mediated TGF-β activation, would protect mice from developing pulmonary fibrosis. Furthermore, if the results were promising, we wanted to determine if the drugs would treat existing fibrosis.

The work described in this thesis contributes to the area of pulmonary fibrosis research in several ways. We describe a novel pathway for regulating TGF-β bioactivity in this disease, and we demonstrate the potential of inhibiting this pathway using aerosol delivery as a therapeutic option. We also introduce a novel way of monitoring drug efficacy and TGF-β signaling in alveolar epithelial cells by using macrophages as biosensors. If successful, this work could lead to the development of new pharmacological agents for the treatment of pulmonary fibrosis.

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