UNIVERSITY OF CALIFORNIA, SAN DIEGO

Thy-1 Expression Inhibits Myofibroblastic Differentiation of Profibrotically Stimulated Rat Lung Fibroblasts

A Thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

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2014

The Thesis of Jeeyeon Kim is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2014

DEDICATION

This thesis is dedicated to my family:

To my father, Jongmoon, and my mother, Sunghee,

for their unconditional love and support

and

to my brother, Jaeheun, for his faith in me.

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ACKNOWLEDGEMENTS

Firstly, I would like to express my utmost gratitude to Dr. Hagood for giving me the opportunity to be a member of his lab. He was always available to provide guidance and support whenever it was needed, and his passion for research and dedication to the medical clinic was an important source of inspiration for my career goals in the science field. Thank you!

I would also like to thank Dr. Lu and Dr. Tour for their support as members of my thesis committee.

I would like to thank all the members of the Hagood lab, especially Dr. Celia Espinoza and Dr. Simon Wong for helping me to develop my research techniques and for providing me with the feedback and encouragement I needed to complete this thesis. I would also like to thank Dr. Carmen Taype de Roberts for helping in the design of qPCR primers seen in Table 1, as well as for helping me with statistical analysis. Thank you all!

Finally, I would like to thank all of my family and friends who have cheered me on as I worked towards this degree.

ABSTRACT OF THE THESIS

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Master of Science in Biology

University of California, San Diego, 2014

Professor James S. Hagood, Chair

Idiopathic pulmonary fibrosis (IPF) has been characterized by aberrant scarring of the lung tissue as a result of myofibroblastic differentiation of lung fibroblasts and epithelial cells. It has been estimated to affect up to 120,000 individuals in the United States alone, with no effective available treatment. In IPF, myofibroblasts localize within characteristic lesions termed fibroblastic foci,

triggering the deposition and excess accumulation of extracellular matrix (ECM) proteins. Previous evidence demonstrates stimulation of fibroblasts by profibrotic cytokines, such as transforming growth factor-β1, connective tissue growth factor, and endothelin-1 leads to a myofibroblastic phenotype. Myogenic markers such as α-SMA and MyoD, as well as collagen type 1, α2 have been shown to act as indicators of myofibroblastic differentiation in lung fibroblasts. Thy-1 is a cell surface glycoprotein expressed heterogeneously in fibroblast subsets which appears to function as a profibrotic suppressor.

RFL-6 cells, which are endogenously Thy-1 (-), were transfected with Thy-1 or empty vector, and used in this study to determine the level of myofibroblastic differentiation based on the degree of expression of myogenic markers following the addition of profibrotic mediators. Thy-1(-) RFL-6 cells were found to have significantly higher expression levels of myogenic and myofibroblastic genes and proteins compared to the levels of expression in Thy-1 transfected fibroblasts after profibrotic cytokine stimulation. Collagen gel contraction assays were used to demonstrate increased contractility in Thy-1(-) cells, supporting the hypothesis that cells lacking Thy-1 expression have a stronger myofibroblastic phenotype, and Thy-1 inhibits myofibroblastic differentiation.

INTRODUCTION

Idiopathic Pulmonary Fibrosis (IPF) and myofibroblastic differentiation

IPF is classified as a fibrotic lung disease which has been suggested to result from repetitive instances of alveolar injury followed by aberrant alveolar wound-healing mechanisms, leading to irreversible destruction of the lungs via scar formation (1, 2). This devastating disease affects about 3 to 6 cases per 100,000 individuals (7) and has an expected median survival rate of approximately 2.8 to 4.2 years (3). An effective treatment is yet to be developed. Severe and progressive pulmonary function impairment results in high mortality during the first 2 years following the diagnosis of IPF (4), giving it a worse prognosis than many types of cancer. Lung fibroblasts are mediators of fibrillar collagen deposition and normal wound repair. Myofibroblasts are a distinct population of contractile fibroblasts that are considered to be main sources of extracellular matrix (ECM) production in scar tissue. In typical wound healing, blood or platelet-rich plasma deposits in extravascular spaces and forms a fibrinous clot (5). Fibroblasts migrate to the site of injury, proliferate, and accumulate de novo deposition of ECM components like fibronectin and collagen while also undergoing myofibroblastic differentiation (5). This phenotypic change is thought to occur as a result of changes in the organization and mechanical characteristics of the ECM (6). In normal wound healing, the deposition of ECM components as a reaction to injury gradually subsides when tissue is returned to its non-injured state. In IPF, however, myofibroblasts persist and proliferate

within lesions termed fibroblastic foci, leading to excess and progressive formation of scar tissue (8, 9). Understanding the molecular basis of myofibroblastic differentiation is a critical first step in developing novel therapies for fibrotic diseases such as IPF.

Myofibroblastic phenotype inducers and pro-myofibroblastic markers

One of the best understood mechanisms of myofibroblastic origin is the induction of myofibroblast differentiation via activated profibrotic cytokines (14). Transforming growth factor (TGF)-β1 is a stimulator of myofibroblastic differentiation and has been known to be upregulated in IPF lungs (15). Latent TGF-β1 is secreted from cells together with its latency-associated peptide (LAP) and tethered to latent TGF-β1 binding proteins (LTBPs) within the ECM. Activation of latent TGF-β1 by a number of mechanisms leads to signaling cascades which result in increased ECM production and fibroblast-to-myofibroblast differentiation. Active TGF-β1 upregulates connective tissue growth factor (CTGF), a downstream profibrotic mediator of TGF-β1and one of the first members to be discovered as part of the CCN (Cyr61 CTGF NOV) protein family (14). In previous studies, CTGF displayed increased expression in fibrotic lung tissue of IPF (18). Endothelin-1 (ET-1) is also secreted by myofibroblasts and is considered to be upregulated mostly by upstream TGF-β1 within the profibrotic pathway (19). TGF-β1 induces a higher level of ET-1 gene expression and protein release through transcriptional regulation of the ET-1 gene promoter and so ET-1 has been suggested to act synergistically with TGF-β1 (20). CTGF acts

downstream to both ET-1 and TGF-β1, while ET-1 is regulated downstream of TGF-β1. Thus, these three mediators can interact as members of a common pathway to induce myofibroblastic differentiation.

Expression of alpha-smooth muscle actin (α-SMA) is a major marker of the myofibroblast phenotype. Myofibroblasts are contractile and contain α-SMA stress fibers, which allows for the development of higher contractile forces (14, 21, 22). The expression of α-SMA was shown to be significantly higher in IPF fibroblasts (14) and α-SMA has been used in a previous study to measure the degree of myofibroblastic differentiation following the activation of TGF-β1. Stimulation using CTGF and ET-1 also induced increased levels of α-SMA (13, 25). TGF-β1 also acts to increase collagen synthesis in fibroblasts via the promoter of collagen type 1 gene (23). There have been previous studies confirming the myofibroblast as an important source of collagen gene expression provided by in situ hybridization for pro-collagen type I mRNA (24). Finally, MyoD is a recognized transcription factor which is crucial for myogenic differentiation; it has also been associated with the tissue repair/fibrosis model (26). MyoD is considered as a master regulator of fibroblast-to-myofibroblast transition. Together, myofibroblastic differentiation can be observed through increased expression levels of α-SMA, collagen type 1, and MyoD both transcriptionally and post-transcriptionally.

Thy-1 expression and how it affects myofibroblastic differentiation

Thy-1, or CD90, is a glycosylphosphatidylinositol-linked cell surface glycoprotein which is heterogeneously expressed in fibroblasts from various tissues, including murine and human lung, as well as different subsets of neural cells and lymphocytes (10, 11, 13). Thy-1 has been shown to affect adhesion, signaling and cell mobility in fibroblasts, and the surface expression of Thy-1 promotes the focal adhesion and the formation of actin stress fibers, while also inhibiting migration (12, 27). It has been found in a previous study that in rat lung fibroblasts, activation of latent-TGF-β1 and increased expression levels α-SMA were noted in Thy-1(-) fibroblasts and a correlation was determined to exist between lung fibrogenesis and the loss of Thy-1 expression (14).

In this study, myofibroblastic differentiation was measured at the level of mRNA and protein by real-time qPCR and immunoblotting, respectively and by analyzing the contraction of collagen matrices *in vitro*. By utilizing the three profibrotic mediators TGF-β1, CTGF, and ET-1 as stimulators and by measuring the subsequent levels of expression multiple myogenic markers (α-SMA, collagen type 1, and MyoD) it can be hypothesized that Thy-1.2 (+) RFL-6 cells will have a very small likelihood of generating a myofibroblastic differentiation phenotype typically seen in IPF.

MATERIALS AND METHODS

Rat lung fibroblast culture, transfection, and cell sorting

Commercial rat fetal lung (RFL-6) fibroblasts were obtained at passage number 8 (American Type Culture Collection). These cells were cultured in Ham's F12K nutrient mixture (F12K) media containing 10% fetal bovine serum (FBS) and 0.5% gentamicin. RFL-6 cells were then transfected with vector pcDNA3.1 Zeo+ containing either the full-length murine Thy-1.2 cDNA (RFL-1 CD90.2) or empty vector (RFL-6 ev) using Lipofectamine 2000 transfection reagent (Invitrogen). Cells were then cultured in F12K with zeocin to select for transfected cells, and further sorted for presence of cell surface Thy-1 by flow cytometry using FITC rat anti-mouse CD90.2 and FITC Rat IgG1,κ isotype as a control.

RFL-6 fibroblast stimulation

RFL-6 Thy-1(-) cells (RFL-6 ev) and FACS-sorted Thy-1(+) cells (RFL-6 90.2) were grown in 75 cm^2 flasks to 70% confluence and then seeded in 6-well plates at a density of 4×10^5 cells per well. These cells were grown to 80% confluence and then made quiescent using F12K media with 0.1% FBS and 0.5% gentamicin. Fresh 0.1% FBS/F12K medium was added at 2 ml/well before treatment of the cells using TGF-β1 (PeproTech) at 5 ng/ml, CTGF (PeproTech) at 100 ng/ml, or ET-1 (American Peptide) at 100 nM. A negative control was used by adding 2 ml of 0.1% FBS/F12K to the respective wells. The plates were

then incubated at 37ºC and 5% CO_2 for 24h before cell lysates and RNA were extracted.

RNA extraction and cDNA synthesis

To extract RNA, TriPure isolation reagent (Roche) was added to each sample at 500 µl per well. The cells were scraped down using a cell scraper and samples were collected in 1.7 ml Eppendorf tubes. Total RNA isolation of these samples was performed using a Direct-zol RNA miniprep kit (Zymo Research). Isolated RNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific) at 260nm. Then, samples were reverse transcribed into cDNA using PrimeScript RT Mastermix (Takara). 500 ng of total RNA was used from each sample.

Myofibroblastic phenotype marker primers and real-time qPCR

Primers for myofibroblastic markers were designed by using the NCBI Primer-blast tool. The forward and reverse primers used are listed in Table 1. Real-time quantitative PCR was performed using iQ Universal SYBR Green Supermix (BioRad) and using an iCycler iQ (BioRad) real-time detection system. Each sample and gene mix was made in duplicates using 100 ng of cDNA and 300 nM primer to bring the total reaction volume up to 20 µl. The thermal cycling conditions were 95ºC for 30 s, 40 cycles of 95ºC for 15 s, and 60ºC for 30 s. Gene expression was relatively quantified using a comparative C_T method as

shown previously (37). All expression values of the samples were normalized using the values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Cell lysate extraction and immunoblotting

Cell lysates were extracted by washing the cells with cold 1x phosphate buffered saline (PBS) and using a mix of immunoprecipitation (IP) lysis buffer and protease/phosphatase cocktail inhibitor (Thermo Scientific). A volume of 200 µl mix was added per well at the end of the 24h stimulation. The cells were scraped down and collected into 1.7 ml Eppendorf tubes. The cell mixture was incubated at 4ºC for 30 min and centrifuged at 13,000 x *g* for 10 min before the supernatant was collected. The subsequent protein samples were quantified using a BCA protein assay kit (Thermo Scientific) and run through a VersaMax microplate reader set at 562 nm (Molecular Devices). Polyacrylamide gels were used in SDS-PAGE with an addition of 20 µg of sample per well. The proteins then underwent electrophoretic transfer from the gels on to PVDF membranes at 100V for 1.5h in an ice bath. 5% non-fat dry milk in 1x TBST (Tris-buffered saline/0.1% Tween-20) was used as a blocking solution for the membranes. Blocking was done at 25ºC with gentle rotation for 1h. To detect myofibroblastic markers, anti-α-SMA monoclonal antibody (Biocare Medical) at 1:1000 and anti-MyoD polyclonal antibody (GeneTex) at 1:1000 were used as primary antibodies, while anti-GAPDH antibody (GeneTex) at 1:7000 was used for normalization. The membranes were incubated with each antibody in 1x TBST overnight at 4ºC with agitation. After incubation with the primary antibodies, the membranes were

washed 4 times for 5 min each before adding the secondary antibody (anti-rat IgG-HRP antibody from Thermo Scientific) for 1.5h at 25ºC. The membranes were washed again and immunodetected by chemiluminescence using a 1:1 mixture of peroxide and luminol reagents (Millipore). The resulting films (Genesee Scientific) were scanned and the bands were quantified by ImageJ analysis system. The values were normalized for gel loading relative to GAPDH.

In vitro collagen gel contraction assay

An *in vitro* floating collagen matrix (FCM) contraction model was used to determine the contractile capacity of Thy-1(-) and Thy-1(+) cells. The cells were trypsinized using 0.25% trypsin/1 mM EDTA and counted and then resuspended in serum-free F12K medium. This mixture was added to collagen type 1 rat tail solution (Corning) at a final concentration of 2 mg/ml and adjusted to pH 7.4 with 0.35M NaOH. The cells were suspended in 24-well plates and the final count was 1×10^5 cells per well. The plates were incubated at 37ºC and 5% CO_2 for 2h for polymerization. The resulting gels were then stimulated with various conditions in 0.5 ml of serum free F12K medium (TGF-β1 at 5 ng/ml, CTGF at 100 ng/ml, and ET-1 at 100 nM, using 0.5 ml SF-F12K as a negative control). Each mediator was added in quadruplicates. After 2h of treatment, the gels were detached from the wells gently using a small sterile spatula. Gel contraction was photographed after 48h of detachment using a Gel Doc XR+ system with an Epi-white light setting (BioRad). The gel areas were measured using ImageJ and compared relative to the total single-well area of the 24-well plate.

Statistical data analysis

Data was statistically analyzed by paired t-tests using IBM SSStatistics and formatted into graphs using GraphPad Prism 6.0. Values were presented as the mean ± SEM, with at least two trials of each experiment. A *P* value of ≤0.05 was considered to be statistically significant.

RESULTS

Myofibroblastic marker mRNA expression is inhibited by Thy-1 expression

To determine the level of mRNA expression of myofibroblastic markers (collagen type 1α2 and α-SMA) in Thy-1.2 (-) and (+) RFL-6 cells after stimulation using profibrotic mediators TGF-β1, CTGF and ET-1, real-time quantitative RT-PCR (qRT-PCR) was performed. Primers designed and sequence-verified via NCBI PrimerBlast (29) were used (Table 1) and GAPDH was used to normalize data. There was a statistically significant increased expression of Col1α2 in Thy1.2 (-) cells for all mediators, with TGF-β1 showing the largest difference between Thy-1.2(+) and (-) cells (Fig. 1A). Expression of Col1a2 was calculated to be increased by 9.2-fold, 5.7-fold, and 8.7-fold for TGF-β1, CTGF and ET-1, respectively (Fig. 1A). Alpha-smooth muscle actin (α-SMA), or Acta2, was also used as a marker to measure the degree of myofibroblastic differentiation in previous studies (13). In this study, there was also an increased expression of Acta2 in Thy1.2 (-) cells as compared to the Thy1.2 (+) RFL-6 cells. Respectively, there was an increase of 11.5-fold, 5.9-fold and 9.9-fold for TGF-β1, CTGF and ET-1 (Fig. 1B). These values were also statistically significant. Through this experiment it was determined via gene expression levels of Col1α2 and α-SMA that expression of Thy-1.2 inhibits myofibroblastic differentiation of lung fibroblasts.

Thy-1 expression also regulates protein expression of characteristic myofibroblastic markers

Thy-1.2(-) and (+) RFL-6 cells were harvested for cell lysates following stimulation with the same mediators as seen in Fig.1 to determine the degree of expression of myofibroblastic differentiation markers at the post-transcriptional level. PVDF membranes were probed with two primary antibodies, MyoD and α-SMA, with GAPDH used to normalize conditional values. MyoD is a transcriptional and post-transcriptional regulator which has been shown to initiate the transition from fibroblasts to myofibroblasts (28). There were higher levels of protein MyoD in all mediators (TGF-β1, CTGF, ET-1), with a 5.1-fold increase in Thy1.2 (-) cells upon stimulation by TGF-β1 and ET-1 (Fig. 2A, lower panel). Little change was observed in the levels of MyoD expression for Thy1.2 (+) cells.

It has also been previously shown that differences in α-SMA protein levels were significantly higher in Thy-1.2(-) cells than Thy1.2(+) cells (13). It can also be seen here that Thy1.2 (-) cells had an increased expression of α-SMA after TGF-β1 stimulation by 2.4-fold, while a 1.9-fold increase occurred in RFL-6 cells lacking Thy-1 after being treated with CTGF and ET-1 (Fig. 2B). GAPDH protein levels were used for normalization. It was determined that post-transcriptional expression levels of certain myofibroblastic markers (MyoD and α-SMA) was increased in Thy-1.2 (-) cells compared to the levels seen in Thy-1.2 (+) cells, implying that myofibroblastic differentiation was more readily occurring in fibroblasts without Thy-1.

Fibroblasts expressing Thy-1 show a higher resistance to contraction of collagen matrices

A floating collagen matrix contraction assay was completed in a span of 48h to determine the effect of Thy-1 on lung fibroblast contractile activity. The same conditions were used as shown in Figures 1 and 2 (negative control, TGF-β1, CTGF and ET-1). Photos of the gels were taken after 2h of polymerization of the collagen gels and 48h stimulation using the aforementioned mediators and a histogram was created using the ImageJ system. By measuring the gels, it can be seen that Thy-1.2(+) RFL-6 cells had a lower contractile ability than Thy-1.2(-) cells (Fig. 3, upper panel). It was observed that Thy-1.2(-) cells showed a significantly higher level of contractile activity in TGF-β1 by 48.03%, CTGF by 30.64%, and ET-1 by 38.10% compared to the baseline gel area in cm^2 (Fig. 3, lower panel). This experiment demonstrated that fibroblasts lacking Thy-1 have higher contractile activity and results in an increased contraction of collagen over time than fibroblasts which express Thy-1.

DISCUSSION

In this study, rat lung fibroblasts were cultured in the presence of profibrotic cytokines to induce myofibroblastic differentiation in cells either transfected with or lacking Thy-1. After investigating expression of selected myofibroblastic markers at both mRNA and protein levels, it was determined that EV-transfected, Thy-1.2 (-) RFL-6 cells exhibited a significantly increased level of expression both in fibroblastic genes and proteins following stimulation by profibrotic mediators. Collagen matrix contraction was also measured to observe levels of contractility between the two cell types, and a higher level of contraction was seen in cells without the expression of Thy-1. Thy-1.2 (-) fibroblasts display a more myofibroblastic phenotype consistent with what is observed in IPF patients.

Fibroblast-to-myofibroblast differentiation occurs at a higher degree in Thy-1.2 (-) fibroblasts upon exposure to profibrotic cytokines

Primers specific to collagen type 1α2 and α-SMA genes were used to quantify the level of transcriptional expression in both Thy-1.2(+) and (-) fibroblasts. Profibrotic cytokines TGF-β1, CTGF, and ET-1 were used to stimulate myofibroblastic differentiation. In previous studies, expression of Col1α2 mRNA was measured after TGF-β1 induction of neonatal lung mesenchymal stem cells (MSCs) which produced an increased level of expression upon cytokine induction (30). Another study confirmed that when

localization of fibroblastic foci undergoing active ECM protein deposition and increased collagen matrix synthetic activity occurred, and Col1α2 gene expression was upregulated (24). This significantly higher expression level in collagen type 1α2 mRNA after TGF-β1 was induced could also be seen in Thy-1.2(-) fibroblasts, while Thy-1.2(+) cells did not show a change in gene expression even after profibrotic cytokines such as CTGF and ET-1 were added. A less significant difference between Thy-1.2(+) and Thy1.2(-) fibroblasts was seen in cells stimulated by CTGF and ET-1, which may be because both are regulated downstream of TGF-β1 (Fig. 1A). Nevertheless, the use of Col1α2 as a marker for myofibroblastic differentiation was effective in demonstrating a more profibrotic phenotype in Thy1.2(-) cells.

MyoD is a member of a transcription factor superfamily known as myogenic regulatory factors (MRFs) that regulates transcription by binding to a specific motif which is present in the promoters of skeletal muscle protein genes expressed in myofibroblasts (34, 35). A research group studying MyoD has shown activity being regulated by both transcriptional and post-transcriptional mechanisms (36). A separate study conducted by our lab displayed the presence of MyoD, via immunofluorescence, co-localized in the cytoplasm and nucleus in Thy-1(-) fibroblasts, indicating a baseline activation and nuclear translocation of MyoD in the fibroblasts lacking Thy-1 expression (13). In this experimental procedure, protein levels of MyoD was quantified via immunoblotting to determine the degree of myofibroblastic differentiation in Thy-1.2(-) and (+) fibroblasts. There was a significant increase in protein levels after TGF-β1,

CTGF, and ET-1 stimulation of Thy-1.2(-) RFL-6 cells, but no change in Thy1.2(-) cells (Fig. 2B).

α-SMA, a common marker for myofibroblastic differentiation, showed significant changes in both gene expression and protein levels in Thy-1.2(-) cells after stimulation with the mentioned mediators. CTGF, ET-1 and TGF-β1 demonstrated a statistically significant increased α-SMA expression in both mRNA and protein levels of Thy-1.2(-) RFL-6 fibroblasts (Fig. 1B, 2B), which was consistent with previous studies showing via immunofluorescence how different profibrotic cytokines, like TGF-β1, promoted the incorporation of α-SMA into stress fibers in Thy-1.2(-) cells (13). The greatest difference in α-SMA expression levels between the two cell types could be seen in TGF-β1-stimulated fibroblasts. While it is not definitely known how TGF-β1 regulates the α-SMA, it has been shown before that the Smad3 pathway is a possible mechanistic driver of myofibroblastic differentiation (33). This further confirmed via that myofibroblastic phenotype was more common in fibroblasts lacking Thy-1 expression.

Lung fibroblasts lacking an expression of Thy-1 have a greater tendency for collagen matrix contraction compared to Thy-1.2 (+) fibroblasts

The use of an *in vitro* floating collagen matrix assay measured the ability of fibroblasts to contract after profibrotic cytokine stimulation. This experiment was representative as a model for wound healing and fibrogenesis. The contraction of collagen gel was much more prominent in Thy-1.2(-) RFL-6 fibroblast conditions after 48h of addition of TGF-β1, CTGF, and ET-1 mediators

to the medium of the polymerized gels (Fig.3, upper panel). A more increased level of contraction was quantified by comparing the level of contractility to the total area each well of the 24-well plates. When the areas were measured, TGF-β1 induction showed the smallest quantified area of collagen matrix in Thy-1.2(-) cells, which correlated to the highest level of contractility (Fig. 3, lower panel). Floating collagen gel assays conducted in previous studies allowed the matrices to contract without an external mechanical load, which allowed TGF-β1 to promote matrix contraction directly as a cell agonist as well as regulating contractility indirectly by activating fibroblast-to-myofibroblast differentiation; this is the main reason why the floating assay was used in this study as opposed to the stressed collagen gel assays, which only promoted contraction via cell activation (31).

The contraction of collagen matrices in response to profibrotic stimulation over a period of 48h was important because it illustrated a temporal correlation with the gene expression of Col1α2 and α-SMA, as well as the increased levels of protein of MyoD and α-SMA. Previous studies have also correlated α-SMA and the production of contractile force in wound healing and fibrocontractive diseases (32). This evidence, in concert with the work completed in this study, determined an increased degree of myofibroblastic differentiation in Thy-1.2(-) fibroblasts characteristic in IPF and other fibrotic diseases.

Future Directions

While there has been meaningful progress in understanding the molecular functions of Thy-1 in this study, the mechanism by which Thy-1 affects myofibroblastic differentiation is still largely unexplored. For example, Thy-1 has yet to have a clearly defined ligand. It also lacks a transmembrane or cytoplasmic domain, which means Thy-1 must affect signaling through its association with other molecules. It has been found in previous studies that integrins, which are heterodimeric (α and β subunits) transmembrane receptors for the ECM proteins, modulate TGF-β1 activation and downstream effects. A previous study conducted in collaboration with our lab showed evidence of Thy-1-integrin αvβ5 interaction via the integrin-binding RGD-like motif (RLD) on Thy-1 (10,11). When this motif was mutated to RLE, Thy-1 lost its ability to bind integrin, which in turn allowed for the activation of latent TGF-β1, triggering myofibroblastic differentiation of lung fibroblasts (10).

To continue exploring the mechanisms of Thy-1, an RLE-mutated Thy1.2 vector may be transfected in RFL-6 fibroblasts to determine the impact of the RLD motif on Thy-1-integrin binding. The level of myofibroblastic differentiation can then be measured using the same methods from this study. It is important to study the interaction of Thy-1 with integrins to determine whether the myofibroblast-inhibitory effects of Thy-1 can be recapitulated by drugs which target or mimic its molecular interactions. Integrin inhibitors, such as the drug cilengitide, may also be considered in future studies to further understand the relationship between Thy-1 and integrins and how binding affects myofibroblastic

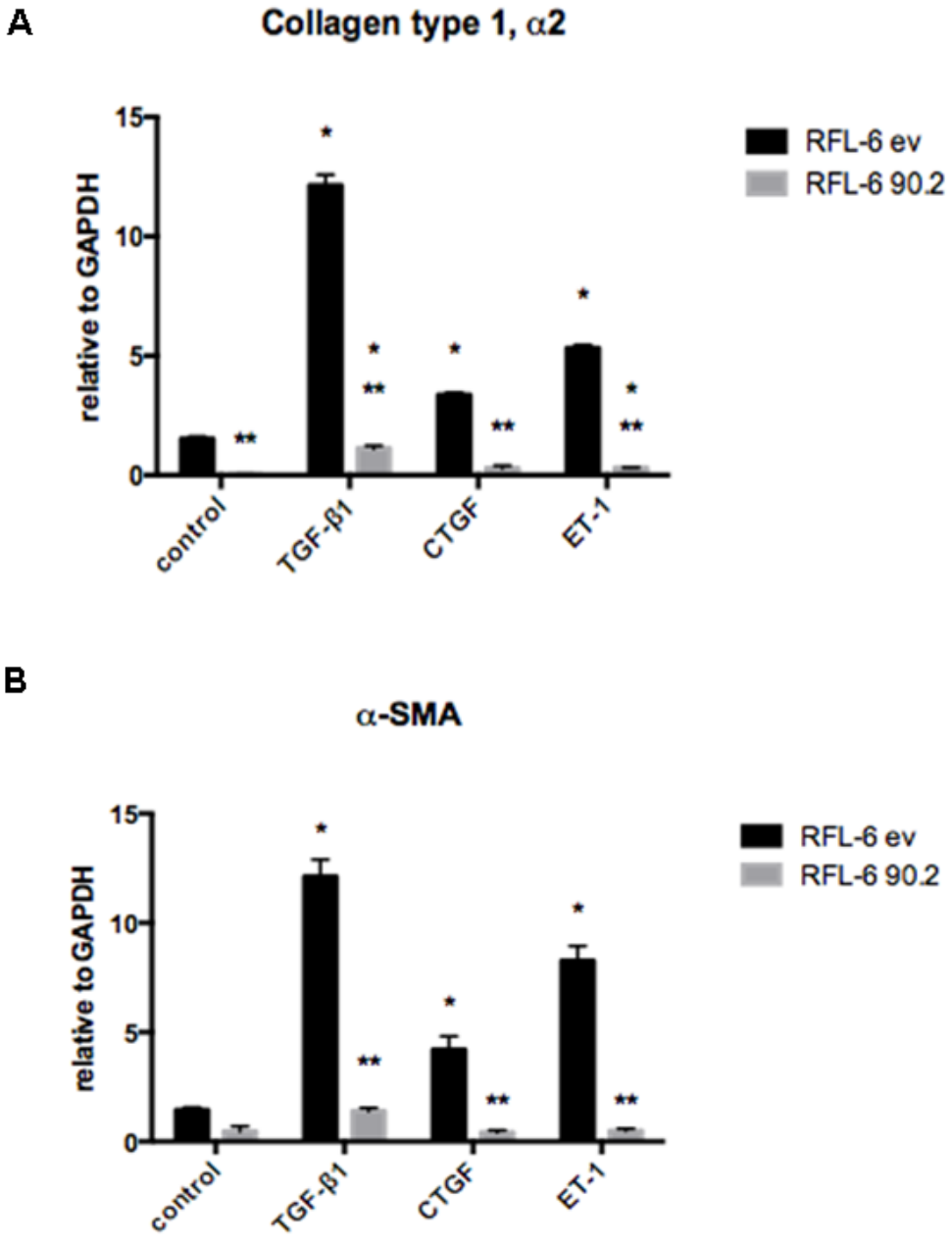
phenotype. The mechanism of Thy-1 in myofibroblastic differentiation, therefore, must be continually studied in order to develop future therapeutics for fibrotic disease.

FIGURES AND TABLES

Table 1. Primer sequences of myofibroblastic markers for qPCR analysis

Gene	Gene Bank Number	Sequence	Base pairs
GAPDH	NM_017008.4	5' – CAACTCCCTCAAGATTGTCAGCAA – 3'	118
		3' – GGCATGGACTGTGGTCATGA – 5'	
α-smooth muscle actin	NM_031004.2	5' – CACTGCTGCTTCCTCTCTTCTTC – 3'	133
		3' – CGCCGACTCCATTCCAATG – 5'	
collagen, type 1, α2	NM_053356.1	5' – GAGGCTTCTACAGGGCTGAC – 3'	198
		3' – TCCAGTAGTAATCGCTCTTCCAC – 5'	

Figure 1. Thy 1.2 (+) RFL-6 cells demonstrate transcriptionally decreased levels of expression of myofibroblastic markers (A) Collagen type 1, α2 (Col1a2). (B) α-SMA (Acta2). RFL-6 ev (Thy-1-) and 90.2 (Thy-1+) cells were exposed to TGF-β1, CTGF, ET-1 as described in the Methods for 24h. SFM was used as a negative control. Total RNA was extracted and purified using a commercial kit, and cDNA was synthesized as discussed in the Methods. Real-time qRT-PCR was performed using the designed primers from Table 1. Each sample was quantified in duplicates and histograms indicate the mean+SEM of three separate experiments using the values of GAPDH for normalization. Values with $P<0.05$ as compared to the negative control were denoted by * while values with $P<0.05$ as compared to Thy-1.2(-) samples were denoted by **.


A
Collagen type 1, α2
relative to GAPDH
15
10
5
0
control
TGF-β1
CTGF
ET-1
RFL-6 ev
RFL-6 90.2
B
α-SMA
relative to GAPDH
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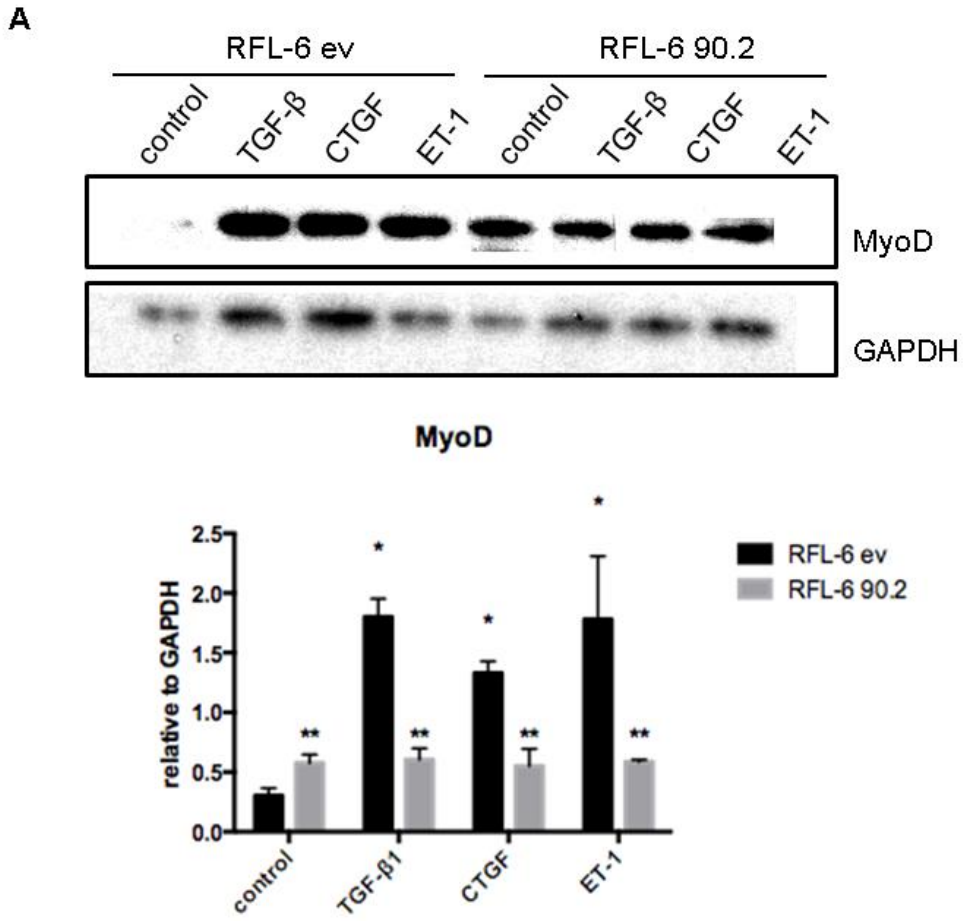

Figure 2. Decreased protein expression of myofibroblastic markers was measured in Thy 1.2(+) RFL-6 cells (A) Immunoblotting using anti-α-SMA antibody. (B) Immunoblotting using anti-MyoD antibody. Cells were subjected to stimulation as described in the Methods for 24h. Cell lysates were collected and run through SDS-PAGE gel and then proteins were transferred via immunoblotting as described in the Methods. The blots were exposed to photographic film and the resulting bands were quantified by scanning densitometry using ImageJ for expression levels of each primary antibody. Anti-GAPDH antibody was used for normalization and histograms in the lower panel indicate mean±SEM of two independent experiments performed. Values with $P<0.05$ as compared to the negative control were denoted by * while values with $P<0.05$ as compared to Thy-1.2(-) samples were denoted by **.

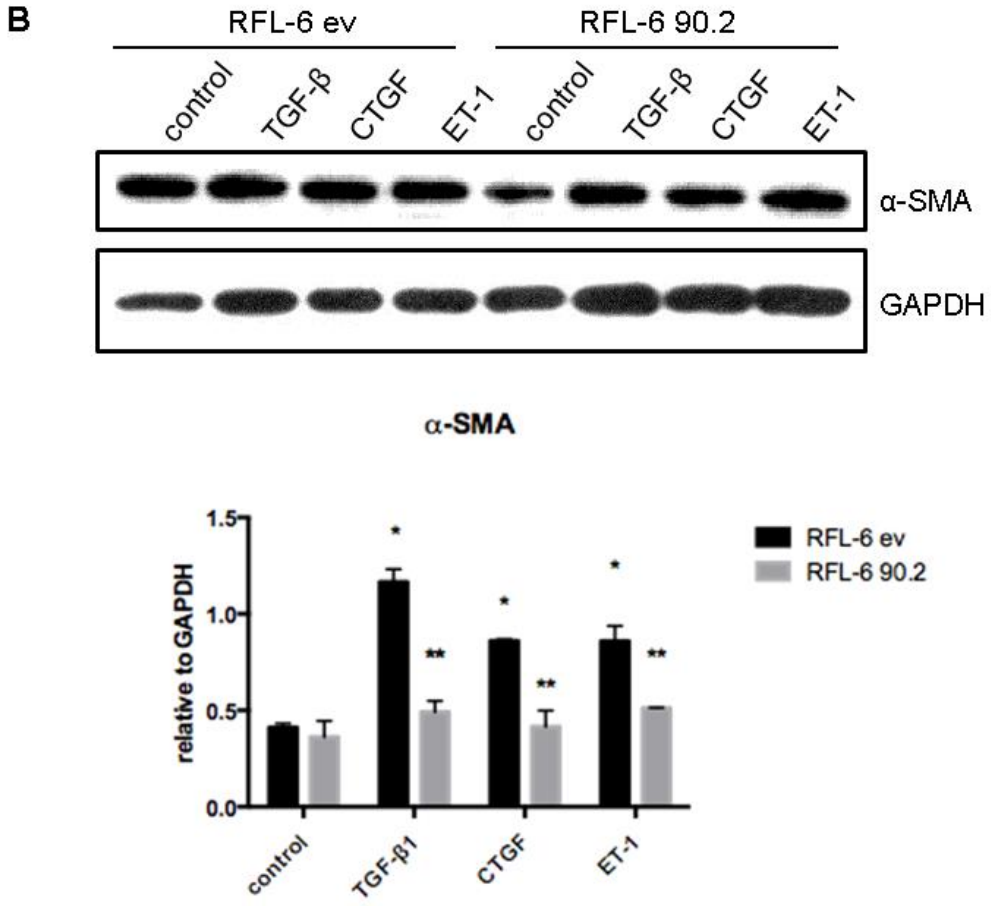

Figure 2. Decreased protein expression of myofibroblastic markers was measured in Thy 1.2(+) RFL-6 cells, Continued

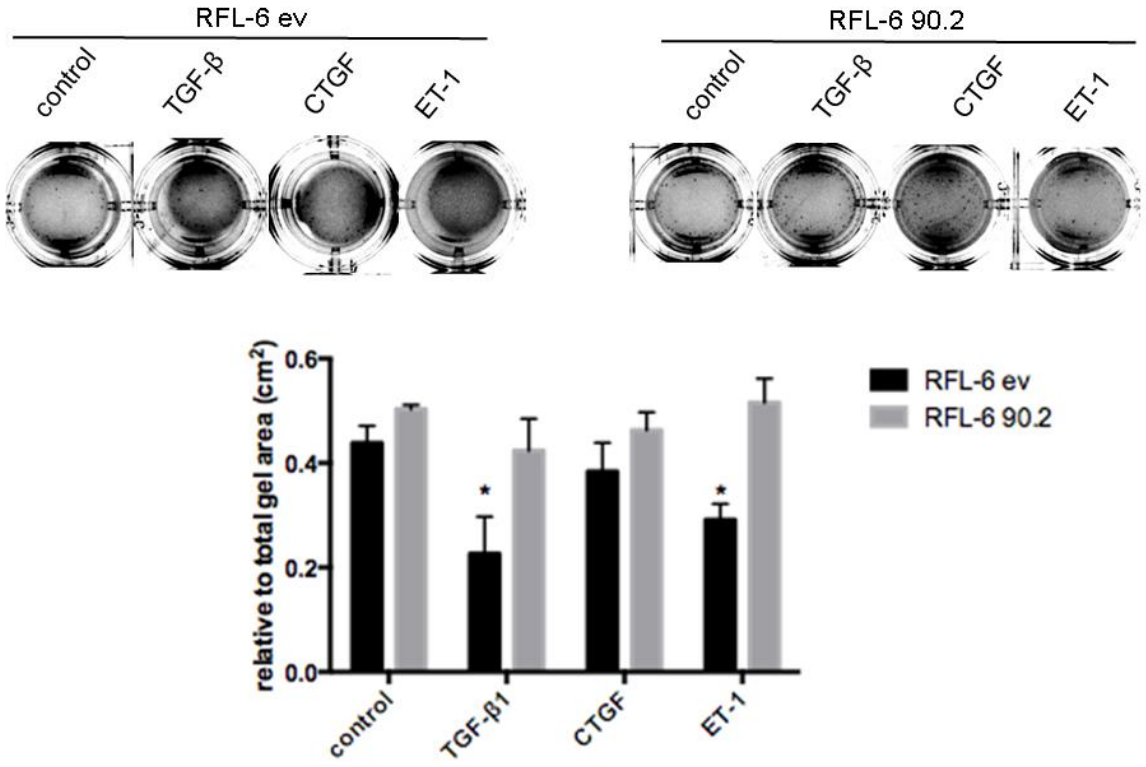

Figure 3. Greater contraction of floating collagen matrices in Thy-1(-) RFL-6 cells RFL-6 cells were polymerized in collagen gels and exposed to each stimulator for 48h as described in the Methods. Gel photos are shown in the top panel and histograms of the gel area in cm^2 compared to the total area of a single well of a 24-well plate (displayed in the lower panel) were representative of three individual experiments. Values with $P<0.05$ as compared to the negative control were denoted by * while values with $P<0.05$ as compared to Thy-1.2(-) samples were denoted by **.

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