Enhanced Myofibroblastic Differentiation and Survival in Thy-1(−) Lung Fibroblasts

Yan Y. Sanders, Pallavi Kumbla, and James S. Hagood

Departments of Pediatrics (Pulmonary Division), Cell Biology, and Pathology, University of Alabama–Birmingham, Birmingham, Alabama

Thy-1 is a glycosylphosphatidyl-inositol–linked cell surface glycoprotein whose exact biological role remains unclear. Differential expression of Thy-1 affects fibroblast proliferation and fibrogenic signaling. In idiopathic pulmonary fibrosis, the proliferating myofibroblasts within the fibroblastic foci are Thy-1(−), whereas normal lung fibroblasts are predominantly Thy-1(+). In this study, we used rat lung fibroblasts sorted for Thy-1 expression to examine myofibroblastic differentiation in response to fibrogenic stimuli. We examined the effects of transforming growth factor–β, endothelin-1, and connective tissue growth factor on the expression of myofibroblast proteins and myogenic regulatory factors by real-time RT-PCR and immunoblotting. Thy-1(−) cells have significantly higher myofibroblast and myogenic regulatory factor gene and protein expression compared with Thy-1(+) cells, confirmed by immunofluorescence. We also used floating collagen matrix contraction assays to assess the functional differentiation of the fibroblasts. At baseline and after stimulation with transforming growth factor–β and endothelin-1, Thy-1(−) cells caused significantly greater collagen contraction than did Thy-1(+) cells, supporting the hypothesis that Thy-1(−) cells are more fully differentiated myofibroblasts. Because apoptosis has been implicated in the regression of myofibroblasts, we examined the percentage of apoptotic cells in the contracted collagen matrices at baseline and after stimulation with fibrogenic agents. A significantly greater proportion of Thy-1(+) cells underwent apoptosis in all conditions compared with Thy-1(−) fibroblasts. Transfection of Thy-1 into Thy-1(−) cells inhibits collagen matrix contraction and reduces cell survival. Our data indicate that Thy-1 regulates myogenic gene expression, myofibroblastic differentiation, and survival in lung fibroblasts.

Keywords: lung; fibroblasts; myofibroblasts; contractility; apoptosis

CLINICAL RELEVANCE

The regulation of lung myofibroblast differentiation by Thy-1 has not been previously described and may provide novel therapeutic targets for fibrotic diseases.

Idiopathic pulmonary fibrosis (IPF) is an aggressive and pernicious pulmonary fibrotic disorder characterized by active fibrogenesis and accumulation of extracellular matrix (1). A distinct feature of IPF is the development of fibroblastic/myofibroblastic foci, which represent areas of active fibrosis and in which the myofibroblasts are thought to arise from fibroblasts (2). Myofibroblasts are transdifferentiated, contractile fibroblasts occurring in response to a variety of stimuli, such as transforming growth factor (TGF)-β and endothelin-1 (ET-1) (3). They express α-smooth muscle actin (α-SMA) and other muscle markers, such as myosin and desmin (4). Myofibroblasts are one of the main sources for extracellular matrix production in scar tissue. They are the major cellular component in IPF fibroblastic foci (3). Myofibroblasts also express a number of skeletal muscle genes, including myosin heavy chain isoforms (5). In recent studies, expression of a family of skeletal muscle–specific transcription factors called myogenic regulatory factors (MRFs) has been described in myofibroblasts (6). Members of this family include MyoD, myocardin, myf-5, and myogenin. MyoD is considered a master regulatory gene of muscle differentiation and is known to initiate the conversion of fibroblasts into myofibroblasts (7).

Fibroblasts in the lung and elsewhere consist of phenotypically diverse subpopulations. Differences in cytoskeletal arrangement, surface markers, cytokine expression profiles, and receptor expression have been reported (3). Thy-1(CD90) is a glycosylphophatidylinositol-linked outer membrane leaflet glycoprotein that is expressed on subsets of neural cells, lymphocytes, and fibroblasts (8). It is differentially expressed by subsets of normal fibroblasts and affects multiple aspects of the fibrogenic phenotype (9–11). Thy-1 can function as a cell adhesion molecule and modulates cellular signaling pathways (8, 12, 13). In normal human lung, the majority of fibroblasts are Thy-1(+), whereas in fibroblastic foci of IPF, the majority of myofibroblasts are Thy-1(−) (14). We and others showed that Thy-1(+) and Thy-1(−) subsets have distinct functional properties. In rat lung fibroblasts, the Thy-1(−) subset activates latent TGF-β and expresses α−SMA in response to fibrogenic stimuli, whereas the Thy-1(+) subset fails to do so (15).

TGF-β is a profibrotic cytokine; many studies have shown that it is a key factor in developing fibrosis in the lung and most other tissues (3, 16). It stimulates fibroblast proliferation, induces myofibroblast differentiation, and stimulates extracellular matrix production (15, 17). Blocking the action of TGF-β attenuates lung fibrosis (17), whereas transient overexpression in rat lung results in prolonged and severe pulmonary fibrosis (18). Studies show that connective tissue growth factor (CTGF) is secreted by fibroblasts when stimulated by TGF-β and is thought to be responsible for mediating multiple fibrogenic effects, such as cell proliferation, migration and adhesion and synthesis of extracellular matrix (19, 20). In pulmonary fibrosis and other fibrotic disorders, there is a dramatic increase in CTGF expression (21). We have demonstrated selective proliferation of Thy-1(−) fibroblasts in response to CTGF (22). ET-1 has also been observed with higher circulating levels and tissue content in patients with IPF (23). ET-1 is not only a potent vasoconstrictor; it is also a growth-promoting factor for smooth muscle cells (24) and stimulates collagen synthesis by fibroblasts (25). When the ET-1

(*Received in original form May 18, 2006 and in final form August 23, 2006*)

This work was supported in part by American Lung Association Career Investigator Award CI-018-N, Children's Center for Research and Innovation grant (J.S.H.), and Research Facilities Improvement Program Grant C06 RR 15,490 from the National Center for Research Resources.

Correspondence and requests for reprints should be addressed to James S. Hagood, Professor of Pediatrics (Pulmonary Division), University of Alabama – Birmingham, 1670 University Blvd., 648A VH Birmingham, AL 35294-0019. E-mail: jhagood@peds.uab.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 36. pp 226–235, 2007
Originally Published in Press as DOI: 10.1165/rcmb.2006-0178OC on September 7, 2006
Internet address: www.atsjournals.org

gene was overexpressed in mice, it resulted in the development of progressive pulmonary fibrosis and inflammation (26).

A key to normal wound healing is the removal of cells that are no longer needed. Studies have shown that this occurs at least in part by apoptosis (27). Failure of apoptosis is a prominent factor in developing hypertrophic scars in fibrotic diseases (28). Lung fibroblasts from pulmonary fibrosis are more resistant to apoptosis, which may result in increased cellularity and persistent deposition of extracellular matrix (29). In this study, we demonstrate by real-time PCR and immunoblotting that the Thy-1(−) fibroblasts have increased expression of multiple myogenic markers in response to fibrogenic cytokines. Thy-1(−) fibroblasts also demonstrate greater contractility in three-dimensional collagen matrices compared with Thy-1(+) fibroblasts and increased survival. Our findings suggest that the emergence of Thy-1(−) fibroblasts indicates a more fibrogenic phenotype that is more responsive to fibrogenic cytokines, with enhanced myofibroblastic differentiation and resistance to apoptosis.

MATERIALS AND METHODS

Rat Lung Fibroblast Purification, Cell Sorting, and Transfection

Primary lung fibroblasts isolated from Lewis rats were cultured as described previously (30) in Eagle's minimal essential medium (MEM) containing 10% FBS, 1% penicillin/streptomycin, and 2.5 mM Hepes and sorted for surface expression of Thy-1 by flow cytometry. These cells have been extensively characterized as fibroblasts (30). Thy-1(+) and Thy-1(−) subpopulation cultures were considered positive if surface expression was $>$ 90% positive or negative, respectively, for Thy-1. Rat fetal lung fibroblast (RFL6) cells were cultured in F-12K media containing 10% FBS and 1% penicillin/streptomycin. RFL6 cells were transfected with the mammalian expression vector pcDNA3.1 Zeo+ containing the full-length murine Thy-1.2 (CD90.2) cDNA (RFL6.CD90.2) or empty vector (RFL6.ev) as previously described using LT-1 transfection reagent (Mirus, Madison, WI), and the expression of Thy-1 at levels similar to those in naturally occurring Thy-1(+) fibroblasts was confirmed by FACS analysis (31).

Cell Treatments

Thy-1–sorted fibroblasts were seeded at a density of 3×10^4 cells/well in 6-well plates. Cells were grown to 80% confluence in culture dishes and made quiescent with 0.4% FBS MEM for 48 h. Fresh 0.4% FBS MEM was added before stimulation with TGF-β (R&D Systems, Minneapolis, MN) at 50 ng/ml; ET-1 (Bachem Bioscience Inc, King of Prussia, PA) was added at 100 nm; CTGF (Cell Sciences, Canton, MA) was added at 100 ng/ml; or an additional 5 μl 0.4% FBS MEM (negative control) was added at 37°C and 5% CO_2 for 4 h before total RNA extraction or preparation of cell lysates.

RNA Extraction and cDNA Synthesis

Total RNA isolation from cells was performed using RNeasy Mini Kits (Qiagen, Valencia, CA). Total RNA was quantified by spectrophotometry at 260 nm. cDNA synthesis was performed with 1 μg of total RNA from each sample using a cDNA synthesis kit (Clontech Lab, Palo Alto, CA).

Real-Time RT-PCR

Real-time RT-PCR was performed using iQ SYBR Green Supermix (BioRad, Palo Alto, CA) and using an iCycler iQ (BioRad) real-time detection system. Primers were designed by using Beacon Designer (BioRad) and synthesized commercially (Invitrogen, Carlsbad, CA). Primer sequences are shown in Table 1. Assays for each sample and primer set were performed in triplicate, with each reaction using 10 ng of cDNA and 0.5 μM primers in a total reaction volume of 25 μl. Thermal cycling conditions were 95°C for 3 min, 40 cycles of 95°C for 35 s, and 60°C for 30 s. The relative quantification of gene expression was determined using the comparative C_T method as described previously (32). Values obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used to normalize the expression data.

Collagen Matrix Contraction Assay

To determine the contractile capacity of Thy-1(+) and Thy-1(−) fibroblasts, a slightly modified *in vitro* floating collagen matrix contraction model was used (33). Briefly, Thy-1(+) and Thy-1(−) fibroblasts were trypsinized (0.25% trypsin/1 mM EDTA), centrifuged, and counted. MEM (10×; Invitrogen) was added to collagen solution (3 mg/ml) (Vitrogen-100; Celltrix, Santa Clara, CA). The resulting mixture was adjusted to pH 7.4 with 0.34 M NaOH. The final collagen cell solution contained 2 mg/ml collagen and 2×10^5 cells/ml. Prepared collagen-cell suspension (0.5 ml) was added in each well of a 24-well culture plate. Plates were incubated for 2 h at 37°C, which caused polymerization of collagen cell lattices. Finally, 0.5 ml of serum-free cell culture medium (MEM) was applied with or without mediators (TGF-β, 50 ng/ml; ET-1, 100 nm; CTGF, 100 ng/ml). Each condition was performed in quadruplicate. After 2 h, the gels were detached from the walls of the culture wells carefully with a thin sterile spatula. Contraction of the gel was photographed and measured using a Kodak image analysis system over a 48-h period. In a parallel stressed collagen matrix model, collagen gels were left attached to the walls of the culture wells during the 48-h stimulation period and detached for 2 h before the gel area was measured.

Apoptosis Assay

Cells were prepared as in the collagen matrix contraction assay. At the end of the 48-h incubation period, the gels for each treatment were collected in 60-mm dishes and digested in 1 ml of 2 mg/ml collagenase at 37°C for 60 min. After gel dissolution, 10% FBS/MEM was added to stop the reaction. Cells were collected by centrifugation and resuspended in 500 μl binding buffer from the Annexin V–FITC Apoptosis

TABLE 1. PCR PRIMER SEQUENCES

Gene Name	Gene Bank Number	Primer Sequence
GAPDH	X02231	S 5′-ATGCCGCCTGGAGAAACC-3′
		AS 5′-AGAATGGGAGTTGCTGTTGAAG-3′
MyoD	M84176	S 5′-GCCTGAGCAAAGTGAACGAG-3′
		AS 5′-CAGACCTTCAATGTAGCGGATG-3′
Sarcomeric myosin	S68736	S 5′-CACGCTGGATGCCGAGATC-3′
		AS 5′-ATGCCTTGGGTGTTCCTGTAG-3′
α-Actin	X06801	S 5′-CACTGCTGCTTCCTCTTCTTC-3′
		AS 5′-CGCCGACTCCATTCCAATG-3′
Desmin	X73524	S 5′-GTCCTACACCTGCGAGATTGATG-3′
		AS 5′-CGATGTTGTCCTGATAGCCACTG-3′
Myogenin	NM_017115	S 5′-GAAGGGAGGGAACGATAGGG-3′
		AS 5′-TGGCATCAGCACAGGAGAC-3′
Myocardin	AF 464910	S 5′-GCACATTCTCCAAGCCTCCAC-3′
		AS 5′-GTTCTTCTCCACCAGTTCCAAGG-3′

Definition of abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Detection Kit (Medical and Biological Laboratories Co., Ltd, Woburn, MA). Annexin V–FITC and propidium iodide were added (5 μl of each), followed by incubation for 5 min at room temperature in dark and quantification by flow cytometry.

Antibodies and Immunoblotting

Anti–α-SMA monoclonal antibody was from Biocarta US (San Diego, CA). Anti-myoD monoclonal antibody was from Stratagene (La Jolla, CA). Anti-sarcomeric myosin polyclonal antibody was a kind gift from Dr. Leslie Leinwand (University of Colorado-Boulder). Anti–β-actin polyclonal antibody was from Imgenex (San Diego, CA). At the end of the treatments, cells were washed with cold PBS twice and lysed with 2× SDS reducing sample buffer containing protease inhibitors. Cell lysates were collected in siliconized tubes and sonicated for 4 s. After centrifugation at 4,000 × *g* for 1 min at 4°C, cell lysates were aliquoted and stored at −80°C until use. Equal amounts of cell lysate were loaded on SDS-PAGE gels under reducing conditions. After electrophoresis, the proteins were electrophoretically transferred from the gel to nitrocellulose membranes at 100V for 2 h at 4°C. To block nonspecific protein binding sites, the membranes were incubated with 5% BSA in Tris-buffered saline/Tween-20 (0.1%) for 1 h at room temperature. Membranes were incubated with primary antibodies in Tris-buffered saline/Tween-20 (0.1%) overnight (anti–α-SMA at 1:10,000, anti-myoD at 1 μl/ml, anti-sarcomeric myosin at 1:10, and anti–β-actin at 0.25 μl/ml). Membranes were washed extensively before being incubated with appropriate peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunodetection was performed by chemiluminescence.

Densitometry

Immunoblots were scanned, and bands were quantified by the Kodak 1D image analysis system. Densitometric values were normalized to β-actin to control for gel loading.

Immunofluorescence Staining

Fibroblasts were cultured on coverslips, grown to 50% confluence, made quiescent with 0.4% FBS MEM for 48 h, and stimulated with TGF-β (10 ng/ml), ET-1 (10 nM), and CTGF (25 ng/ml) for 24 h. The cells were fixed with 3% formaldehyde for 30 min, permeabilized by a 60-s incubation in 0.1% Triton X-100, washed with sterile PBS, blocked in 10% normal goat serum, and incubated with anti–α-SMA (1:400) or anti-myoD (1:50) followed by Texas Red X-conjugated secondary antibody (1:200). Coverslips were washed and mounted using ProLong antifade reagent (Molecular Probes, Eugene, OR) on glass microscope slides and examined with a Nikon TE200U inverted fluorescence microscope, and images were acquired with a CoolSnap HQ 12 bit CCD digital camera (Photometrics, Tucson, AZ) and analyzed with MetaMorph software (Molecular Devices Corp., Sunnyvale, CA).

Statistical Analysis

Comparisons involving three or more groups were analyzed using one-way ANOVA (Newman-Keuls method for multiple comparisons). A P value ≤ 0.05 was used to determine statistical significance.

RESULTS

Thy-1(–) Cells Express Higher Levels of Characteristic Myofibroblastic Markers

To define the differential expression of myofibroblastic markers and MRFs in Thy-1(+) and Thy-1(–) fibroblasts in response to the pro-myofibroblastic stimuli TGF-β, ET-1, and CTGF, we performed real-time RT-PCR using RNA from stimulated sorted cells. Values for GAPDH were used to normalize the expression data. Thy-1(−) cells showed statistically higher expression of desmin and sarcomeric myosin heavy chain (Figure 1) when

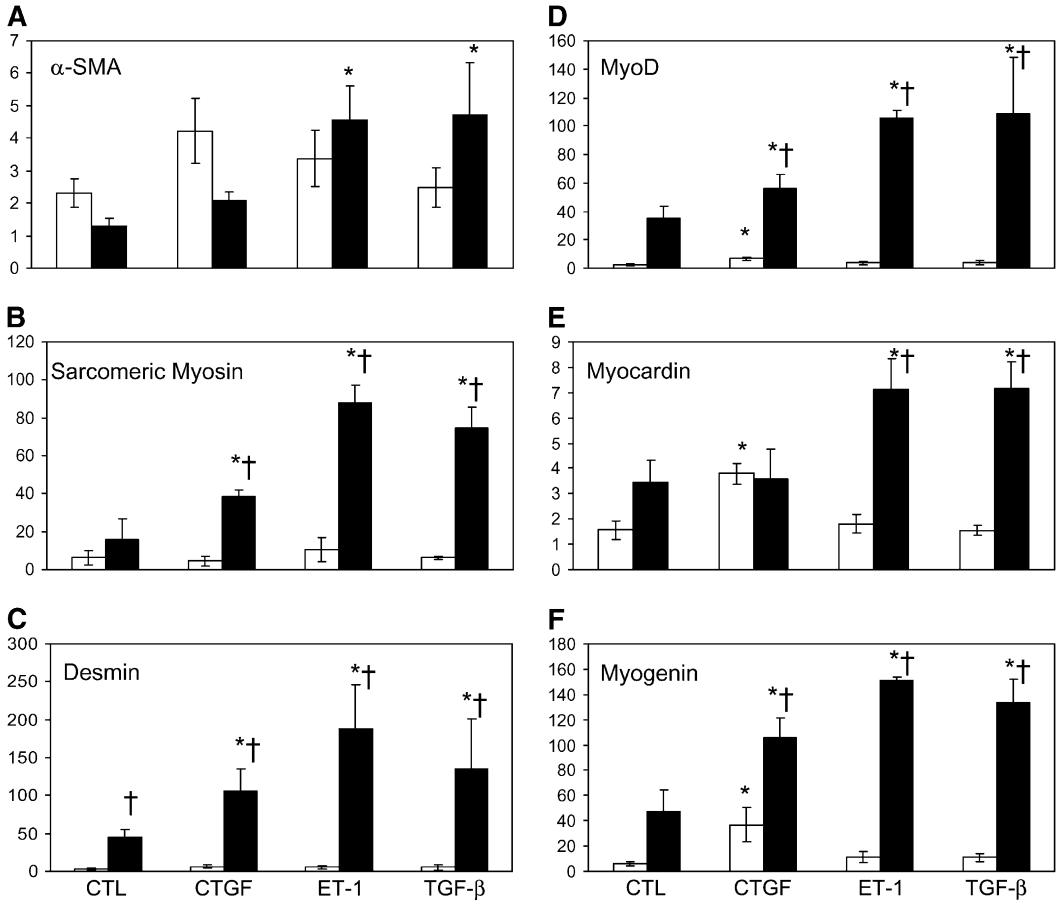

Figure 1. Histogram of real-time RT-PCR. (*A*) α-SMA. (*B*) Sarcomeric myosin. (*C*) Desmin. (*D*) MyoD. (*E*) Myocardin. (*F*) Myogenin. Rat lung fibroblasts were sorted into Thy-1(+) (*open bars*) and Thy-1(−) (*solid bars*). mRNA levels were measured by real-time RT-PCR. The cells were quiescent in 0.4% FBS-MEM for 48 h after reaching 80% confluence. Cells were treated with TGF-β (50 ng/ml), ET-1 (100 nm), CTGF (100 ng/ml), or 0.4% FBS-MEM (control) for 4 h. Experiments were performed in triplicate, and data are expressed as the ratio to GAPDH. $^{*}P < 0.05$ versus same cell subset control. $^{\dagger}P < 0.05$ versus Thy-1(+) under the same conditions.

stimulated with TGF-β, ET-1, and CTGF. Overall, Thy-1(−) cells have a higher baseline expression of these myofibroblastic markers and have stronger responses to the fibrogenic cytokines, especially TGF-β and ET-1 (Table 2). The MRFs (MyoD, myocardin, and myogenin) showed statistically higher expression in Thy-1(−) cells compared with Thy-1(+) cells after each stimulus, with the exception of myocardin in response to CTGF (Table 3). Both subpopulations express myf-5, but there was no significant difference in expression between the two (data not shown). Real-time RT-PCR for α-SMA did not show statistically different mRNA expression in Thy-1(+) and Thy-1(−) cells at baseline. However, upon stimulation with ET-1 or TGF-β, α-SMA gene expression increased in Thy-1(−) cells to a greater extent than in Thy-1(+) (Figure 1). Differences in α-SMA protein levels and organization of stress fibers were more striking (*see below*). The identity of the real-time PCR products was confirmed by sequencing.

After identifying differential expression of myofibroblastic genes and MRFs in Thy-1(+) and Thy-1(−) rat lung fibroblasts, we determined the protein levels of specific molecules. α-SMA is a widely used marker for myofibroblast differentiation and an important part of the contractile apparatus. Myosin is another critical element in the contractile apparatus. After normalization to β-actin expression, under basal conditions, Thy-1(−) cells expressed 1.6-fold more α-SMA than Thy-1(+) cells (Figure 2A). When stimulated with TGF-β, ET-1, and CTGF, Thy-1(−) cells showed significantly higher expression of α-SMA compared with Thy-1(+) cells (Figure 2A). Increased sarcomeric myosin protein was also demonstrated in the Thy-1(−) cells under basal conditions and much higher expression after stimulation with TGF-β, ET-1, and CTGF, compared with the same conditions in Thy-1(+) cells (Figure 2B). MyoD, which initiates the conversion of fibroblasts into myofibroblasts (34), is regulated by transcriptional and post-transcriptional mechanisms. It is highly phosphorylated in growing myofibroblasts (35). Our real-time RT-PCR showed that MyoD mRNA was significantly higher in Thy-1(−) cells than Thy-1(+) cells (Figure 1). Immunoblots indicate that Thy-1(−) cells have demonstrated not only increased levels of MyoD protein but also slightly retarded migration of MyoD in electrophoresis gels consistent with phosphorylation (Figure 2C).

Immunofluorescence

α-SMA is organized into stress fibers in contractile cells. MyoD functions as a transcription factor and thus undergoes nuclear translocation when activated. To determine the functional significance of differences in protein expression, we performed immunofluorescence microscopy for α-SMA and MyoD on sorted fibroblasts at baseline and after TGF-β stimulation for 48 h. Figure 3A shows thick, bundled α-SMA stress fibers at baseline in Thy-1(−) fibroblasts, which increase in response to TGF-β. In contrast, Thy-1(+) fibroblasts have thinner, more loosely organized α-SMA stress fibers that do not change significantly with TGF-β stimulation. Thy-1(−) cells have increased expression of cytoplasmic and nuclear (turquoise staining, indicating colocalization of blue nuclear dye and green MyoD antibody) MyoD at baseline compared with Thy-1(+) cells, which demonstrates nuclear translocation of MyoD only in response to TGF-β stimulation. To determine whether the observed differences are attributable to Thy-1 expression or some other difference in the sorted primary fibroblast subsets, we evaluated RFL-6 (Thy-1(−)) fibroblasts transfected with normal murine Thy-1 (CD90.2) or empty vector (ev). Figure 3B indicates that α-SMA is expressed as well-organized stress fibers in the majority of Thy-1(−) RFL-6.ev cells and that there is baseline and TGF-β–induced nuclear localization of the myogenic regulator MyoD, which is suppressed in RFL-6 cells transfected with Thy-1 (CD90.2).

Contraction of Fibroblast-Populated Collagen Matrices Correlates with the Myogenic Gene Expression Profile

To investigate the effects of Thy-1 on lung myofibroblast contractile activity, we performed the floating collagen matrix contraction assay in response to pro-myofibroblastic mediators. Unstimulated Thy-1(−) cells began to show collagen gel contraction after 24 h, whereas the Thy-1(+) cells showed minimal contraction (Figure 4A). In the presence of TGF-β and ET-1, both subpopulations showed significant contraction compared with control; however, Thy-1(−) cells showed significantly greater contraction when stimulated by TGF-β. There was no significant gel contraction in response to exposure to CTGF in either cell type compared with its control. After 48 h, Thy-1(−) cells caused significantly greater gel contraction than Thy-1(+) cells in all conditions (Figure 4B). Both cell subsets are capable of contracting collagen gels under appropriate stimuli. TGF-β caused 50% additional contraction in gel area in Thy-1(−) cells compared with control, and ET-1 caused 33% additional contraction. For Thy-1(+) cells, TGF-β stimulated additional 40% contraction compared with control, and ET-1 stimulated an additional 30%. There was no detectable change in contraction noted with CTGF treatment in either subset. In all conditions, Thy-1(−) fibroblasts caused greater collagen gel contraction than Thy-1(+) cells. There was no further obvious gel contraction in either group of cells beyond 48 h (up to 5 d; data not shown). In a stressed collagen matrix model, in which gels were left attached to the culture dishes during 48 h of stimulation and then released, findings were similar to those obtained in floating matrices (data not shown). Empty-vector or Thy-1 (CD90)-transfected RFL-6

TABLE 2. EXPRESSION RATIO (TO GAPDH) OF MYOFIBROBLASTIC MARKERS IN Thy-1(+) AND Thy-1(−) FIBROBLASTS BY REAL-TIME PCR

	Desmin			Sarcomeric Myosin			α-SMA		
Stimulus	Thy-1(+)	Thy-1(−)	Ratio (−/+)	Thy-1(+)	Thy-1(−)	Ratio (−/+)	Thy-1(+)	Thy-1(−)	Ratio (−/+)
Control	3.6	45†	12.5	6.3	15.6	2.5	2.30	1.29	0.56
CTGF	6.3*	106*†	16.8	4.6	38.3*†	8.3	4.22	2.06	0.49
ET-1	5.2*	188*†	36.2	10.7	87.8*†	8.2	3.37	4.55*	1.35
TGF-β	5.6*	135*†	24.1	6.1	74.7*†	12.2	2.49	4.71*	1.89

Definition of abbreviations: α-SMA, α-smooth muscle actin; CTGF, connective tissue growth factor; ET-1, endothelin-1; TGF-β, transforming growth factor-β.

* $P < 0.05$ versus control.

† $P < 0.05$ versus same condition in Thy-1(+).

TABLE 3. EXPRESSION RATIO (TO GAPDH) OF MYOGENIC REGULATORY FACTORS IN Thy-1(+) AND Thy-1(−) FIBROBLASTS BY REAL-TIME PCR

	MyoD			Myocardin			Myogenin		
Stimulus	Thy-1(+)	Thy-1(−)	Ratio (−/+)	Thy-1(+)	Thy-1(−)	Ratio (−/+)	Thy-1(+)	Thy-1 (−)	Ratio (−/+)
Control	1.9	27.5†	14.5	1.6	3.5	2.2	6.1	47.1†	7.7
CTGF	7.1*	43.6*†	6.1	3.7*	3.6	0.97	37*†	92.4*†	2.5
ET-1	3.2	94.0*†	29.4	1.8	7.1*†	3.9	11.4	134.8*†	11.8
TGF-β	2.9	84.6*†	29.2	1.5	7.1*†	4.7	11.2	108.1*†	9.65

For definition of abbreviations *see* Table 2.
* $P < 0.05$ versus control of the same group.
† $P < 0.05$ versus same condition of Thy-1(+).

cells were incubated in floating collagen matrices in the presence or absence of TGF-β. Although very little gel contraction was noted in either subset at baseline, Thy-1 transfection completely inhibited the TGF-β–induced gel contraction seen in empty-vector–transfected fibroblasts (shown in Figure E1 in the online supplement).

Thy-1(−) Fibroblasts Are Resistant to Apoptosis in Response to Collagen Contraction

Previous studies have shown that normal fibroblasts, when seeded into three-dimensional collagen matrices and allowed to contract, were induced to undergo apoptosis (36, 37). Here we determined whether the Thy-1(+) and Thy-1(−) fibroblasts would be equally susceptible to this phenomenon. Apoptosis was induced by serum starvation in contracting collagen gels for 48 h. Flow cytometry analysis (Figure 5) showed that serum-starved Thy-1(+) cells in contracting gels underwent significant cell death (92.51% total of apoptotic, necrotic, and dead cells), with only 7.49% cells being viable after 48 h. In contrast, Thy-1(−) cells had increased survival after 48 h compared with the Thy-1(+) cells, with 28.69% viable cells at the end of 48 h ($P < 0.001$) (Figure 6A). For empty-vector transfected RFL-6 cells, which are Thy-1(−), 44.32% remained viable after 48 h, whereas in Thy-1–transfected RFL-6 cells, surviving cells decreased to 28.93% ($P = 0.05$) (Figure 6B). Fibrogenic mediators had variable effects on survival and apoptosis in contracting collagen gels (Table 4), but in all cases the proportion of surviving cells was higher in cells lacking Thy-1. TGF-β provided a slight

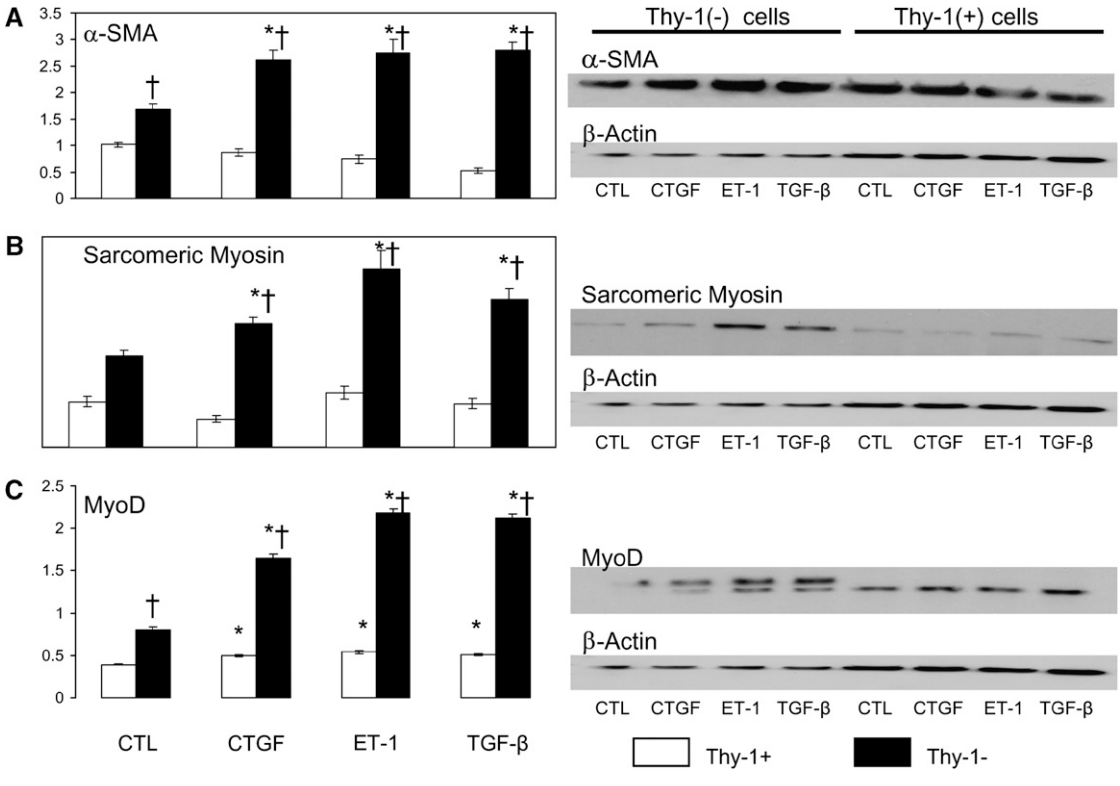

Figure 2. Immunoblots. (*A*) α-SMA. (*B*) Sarcomeric myosin. (*C*) MyoD. Thy-1(+) (*white bars*) and Thy-1(−) (*black bars*) rat lung fibroblasts were made quiescent in 0.4% FBS-MEM for 48 h, followed by the indicated treatments. Cell lysates were collected and subjected to electrophoresis in 10% SDS-PAGE gels under reducing conditions. Levels of α-SMA (*A*), sarcomeric myosin (*B*), and MyoD (*C*) expression were detected by immunoblotting as described in MATERIALS AND METHODS. Blots were stripped and reprobed with β-actin for normalization. Relative protein levels were determined by scanning densitometry and normalized to β-actin. Histograms indicate the means (± SD) of three independent experiments. *$P < 0.05$ versus same cell subset control. †$P < 0.05$ versus Thy-1(+) under the same conditions.

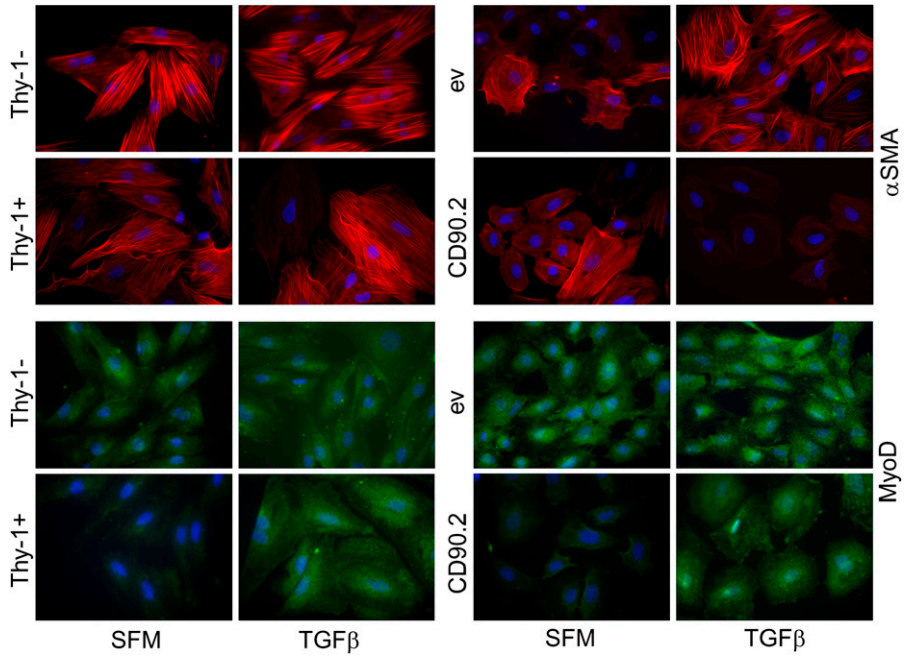

Figure 3. Immunofluorescence for α-SMA (*red, upper eight panels*) and MyoD (*green, lower eight panels*) in Thy-1(+) and Thy-1(−) primary lung fibroblasts (*left panels*) or in RFL-6 cells transfected with empty vector (ev) or Thy-1.2 (CD90) (*right panels*) as described in MATERIALS AND METHODS. Fibroblasts were cultured in serum-free medium or stimulated with TGF-β (5 ng/ml × 48 h). Hoechst (blue) staining was used to visualize nuclei.

survival advantage to Thy-1(−) primary fibroblasts ($P = 0.012$) but not to empty vector-transfected RFL-6 cells.

DISCUSSION

Fibroblasts express marked heterogeneity in morphologic and functional properties. In this study, we investigated differences in myofibroblastic transdifferentiation of rat lung Thy-1(+) and Thy-1(−) fibroblast subpopulations. The data indicate that in the absence of Thy-1 surface expression, rat lung fibroblasts are fully differentiated myofibroblasts under the stimulation of profibrotic cytokines. These findings are consistent with our previous data showing that in the bleomycin-induced mouse lung fibrosis model and in human IPF, absence of Thy-1 expression correlates with a more profibrotic myofibroblast phenotype (14).

These findings differ from those reported for fibroblasts derived from other tissues. Koumas and colleagues reported that human Thy-1(+) uterine myometrial fibroblasts express α-SMA

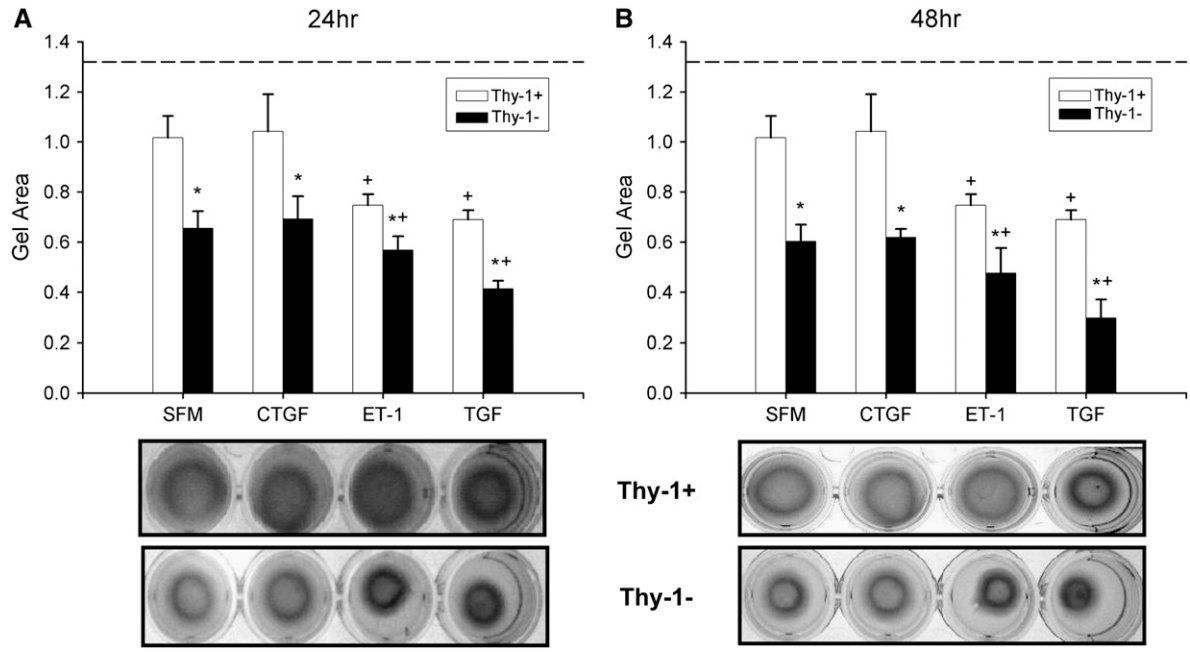

Figure 4. Histograms of gel area (means ± SD) and representative gel photos of floating fibroblast-populated collagen matrices after 24 h (*A*) and 48 h (*B*) of stimulation with serum-free medium, CTGF, ET-1, or TGF-β as described in MATERIALS AND METHODS. *$P < 0.001$ versus mean area of Thy-1(+) under same conditions. †$P < 0.001$ versus same subset serum-free medium control. The *dashed line* represents the starting gel area of 1.33 cm^2.

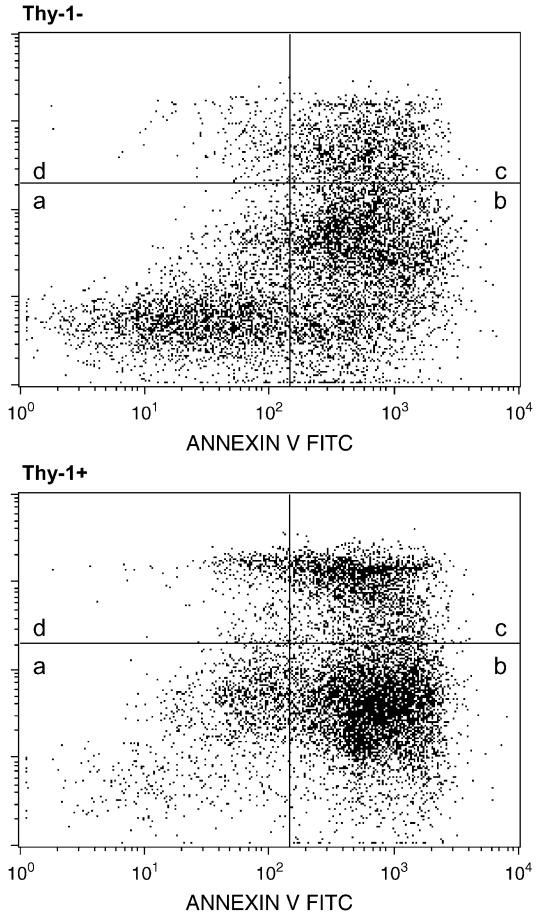

Figure 5. Representative flow cytometry dot plots of Thy-1(+) and Thy-1(−) rat lung fibroblasts cultured in floating collagen matrices followed by digestion in 2 mg/ml collagenase at 37°C for 60 min. Digestions were stopped by adding 10% FBS-MEM. Cells were collected and washed followed by Annexin V-FITC apoptosis detection kit. (*a*) Live cells. (*b*) Early apoptotic cells. (*c*) Late apoptotic cells. (*d*) Necrotic cells. *See* Table 4 for percentages of live and apoptotic cells in all conditions and Figure 6 for graphic representation.

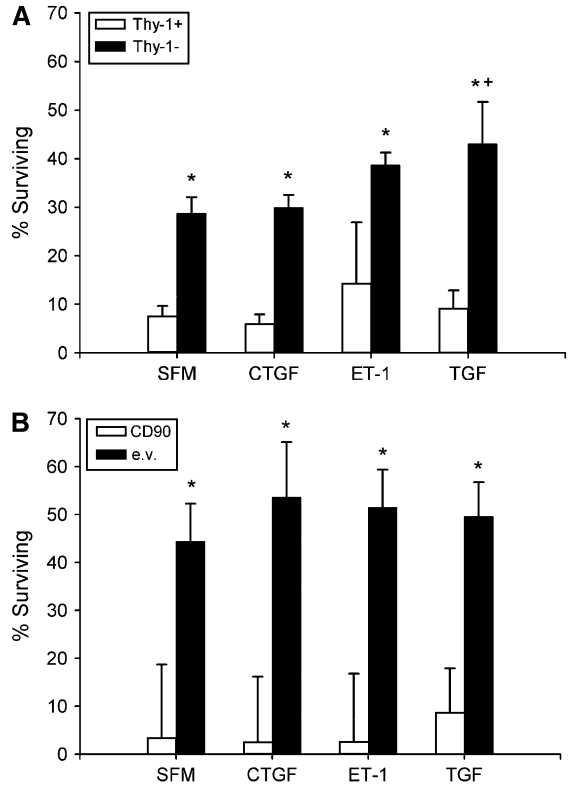

Figure 6. Percentage (mean ± SD) of fibroblasts surviving after 48-h culture in floating collagen matrices exposed to serum-free medium, CTGF, ET-1, or TGF-β as described in MATERIALS AND METHODS. (*A*) Sorted primary rat lung fibroblasts. (*B*) RFL-6 cells transfected with empty vector (ev) or Thy-1.2 (CD90). *$P \leq 0.05$ versus mean percent surviving Thy-1(+) fibroblasts under the same conditions. †$P = 0.012$ versus same subset serum-free medium control.

in a constitutive, heterogeneous manner not seen in human myometrial Thy-1(−) fibroblasts (38). They also reported that Thy-1(+) human myometrial and orbital fibroblasts were capable of myofibroblastic differentiation after treatment with TGF-β, whereas the Thy-1(−) subpopulation in these tissues differentiated to lipofibroblasts (38). Together with the data reported here, these findings suggest that fibroblastic phenotypes and differentiation in response to stimuli are heterogeneous across differing tissues. These do not seem to be merely species-specific differences because we have previously demonstrated myofibroblastic differentiation of human lung fibroblasts associated with loss of Thy-1 (14).

α-SMA is the most widely used marker of myofibroblasts. We found no significant differences in α-SMA expression by real-time RT-PCR between Thy-1(−) and Thy-1(+) fibroblasts, but α-SMA expression was increased by ET-1 and TGF-β only in Thy-1(−) cells. Skeletal muscle sarcomeric myosin and desmin, which are major components of the myocontractile apparatus, are expressed at higher levels in Thy-1(−) fibroblasts. At the protein level, Thy-1(−) fibroblasts have higher levels of α-SMA and higher baseline incorporation into stress fibers. CTGF, ET-1, and TGF-β increase α-SMA and sarcomeric myosin protein only in Thy-1(−) fibroblasts (Figure 2), and TGF-β promotes α-SMA incorporation into stress fibers in Thy-1(−) but not in Thy-1(+) fibroblasts. In fact, TGF-β seems to decrease stress fiber incorporation of α-SMA in Thy-1–transfected cells (Figure 3, *upper right panels*). The mechanism for the differential effects of TGF-β on α-SMA is under investigation but likely involves non-Smad signaling because Smad activation in response to active TGF-β has been shown to be equivalent in Thy-1(−) and Thy-1(+) fibroblasts (15).

These results present a more complete characterization of myofibroblastic differentiation than those usually reported. In addition to α-SMA, sarcomeric myosin, and desmin, we examined the expression of myogenic regulatory factors (MyoD, myogenin, and myocardin) in the differentiation of myofibroblasts after stimulation with fibrogenic cytokines. MRFs are members of a basic helix-loop-helix transcription factor superfamily that regulates transcription by binding to the E-box motif (consensus sequence CANNTG, where "N" is any nucleotide), which is present in the promoters of many of the sarcomeric protein genes expressed in myofibroblasts (6). In this family, MyoD is a critical factor upstream of myogenin in skeletal muscle development and in regulating muscle gene expression in fibroblasts (7). One group induced exogenous MyoD expression while blocking new protein synthesis in MyoD−/−/Myf5−/− mouse embryo fibroblasts and identified 88 genes up-regulated and 28 genes

TABLE 4. PERCENTAGE OF LIVE AND APOPTOTIC CELLS IN CONTRACTING COLLAGEN GELS AFTER 48 H

Cell Type/Stimulus	% Apoptotic	% Live
Thy-1(−)		
Control	51.28	46.83
ET-1	56.56	40.95
CTGF	50.22	47.80
TGF-β	45.73	50.89
Thy-1(+)		
Control	86.19	4.99
ET-1	88.41	4.7
CTGF	89.21	4.52
TGF-β	88.47	6.92
RFL-6.ev		
Control	58.5	44.32
ET-1	54.62	40.66
CTGF	56.89	39.62
TGF-β	49.98	44.34
RFL-6.CD90.2		
Control	70.43	27.42
ET-1	65.55	31.87
CTGF	69.50	28.27
TGF-β	62.65	34.65

For definition of abbreviations *see* Table 2.

downregulated by MyoD (39). MyoD activity is regulated by transcriptional and post-transcriptional mechanisms. It is highly phosphorylated in growing myoblasts and undergoes substantial dephosphorylation during differentiation (35). Myogenin is required for the full differentiation and cell cycle withdrawal of precommitted skeletal myoblasts (34). It is required to trigger the formation of myotubes and subsequent muscle development (40). Myocardin is a serum response factor coactivator expressed specifically in cardiac and smooth muscle cells and is necessary for cardiac and smooth muscle gene expression (41). Previous studies showed that the balance of myogenic factors is critical for the establishment and maintenance of the muscle phenotype, with different levels of MRFs affecting selection of specific muscle genes for activation or repression (7). Because the MRFs are involved in the expression of muscle-specific gene programs in multiple cell types, analyzing expression of these genes at the mRNA and protein level provides a powerful tool for identifying fibroblasts undergoing myofibroblast differentiation. After stimulation with fibrogenic cytokines, we found that MyoD, myogenin, and myocardin had statistically higher expression in Thy-1(−) fibroblasts than in Thy(+) fibroblasts. Additionally, our studies suggest differential post-translational regulation of MyoD in Thy-1(−) versus Thy-1(+) fibroblasts (Figure 2C) and differential subcellular localization (Figure 3, *lower panels*). In Thy-1(−) and empty-vector–transfected cells, MyoD is seen in cytoplasm and nucleus (as indicated by "turquoise" staining of nuclei, indicating colocalization of blue nuclear dye and green MyoD antibody), indicating that there is baseline activation and nuclear translocation of MyoD in the absence of Thy-1. In Thy-1(+) and Thy-1–transfected cells, MyoD expression and nuclear translocation is seen only in response to exogenous TGF-β.

The ability of fibroblasts to reorganize and contract collagen matrices *in vitro* has been used to model events in wound healing and fibrogenesis. In the "floating" model, the freshly polymerized collagen matrix containing fibroblasts is released from the culture dish and allowed to float in medium, and contraction occurs without an external mechanical load (33). In the "attached" model, the matrix remains attached to the culture dish during contraction, allowing the development of isometric tension. In the two-step "stressed" or "stress-relaxation" model, mechanical loading is allowed to develop as in the attached model, but the matrix is released and allowed to contract at the end of the assay (33). In floating gels TGF-β promotes matrix contraction directly as a cell agonist and indirectly by promoting myofibroblast differentiation (42), whereas in stressed gels the latter mechanism predominates. The fibroblastic foci of IPF, which are clearly correlated to poor outcome, are felt to represent active early-phase "wound healing," in which fibroblast migration, matrix attachment, proliferation, and myofibroblast differentiation occur (43). Floating gels are though to recapitulate such early healing, as compared with the stressed matrix model, which more closely matches the late fibrocontractive phase, such as that which occurs in honeycomb lesions (33). Our data indicate that lung fibroblasts lacking Thy-1 expression are more responsive to myofibroblast contractile stimuli when cultured in floating three-dimensional collagen matrices (Figure 4), which is consistent with our prior findings of Thy-1(−) myofibroblasts in the fibroblastic foci of IPF (14). We also cultured fibroblasts in the stressed collagen matrix model and found that Thy-1(−) cells were consistently more contractile (data not shown). The observed increase in contractility in response to profibrotic/contractile mediators correlates temporally with the upregulation of MRF levels (Figures 1 and 2), supporting an important role for the MRFs in the establishment of the contractile myofibroblast phenotype. In contrast, although the Thy-1(+) lung fibroblasts demonstrate some myofibroblastic characteristics, such as α-SMA expression, they express significantly lower levels of myogenic and MRF genes under identical stimuli and are less contractile, indicating that they are not fully differentiated myofibroblasts. The phenomenon of multiple levels of transdifferentiation of myofibroblasts characterized by expression of different myofibroblastic proteins has been previously described (4). The differing regulation of α-SMA and MyoD at baseline and in response to TGF-β after transfection with Thy-1 suggests that specific signaling pathways associated with Thy-1 are linked to the ability of the cells to differentiate into myofibroblasts. Given that this phenomenon seems to be regulated in a tissue-specific manner, further studies are necessary to determine the mechanisms by which Thy-1 modulates myofibroblastic differentiation.

Myofibroblasts are present in normal wound healing but persist and fail to undergo apoptosis in pathologic situations such as lung fibrosis (44). In addition to modeling the early phases of wound healing, floating collagen gels are important in exploring resistance to apoptosis, which is a feature of profibrotic fibroblasts in IPF, scleroderma, and other fibrotic disorders (45). Our findings indicate that Thy-1(–) myofibroblasts are resistant to cell death associated with collagen gel contraction, suggesting that this subpopulation of cells may have a survival advantage in fibrotic lesions. This is also in keeping with our previous *in vivo* findings (14). The importance of Thy-1–regulated signaling in this process is supported by our finding that transfection of Thy-1 into Thy-1(–) RFL-6 cells leads to increased apoptosis and decreased survival in floating collagen matrices (Figure 6 and Table 4). Resistance to apoptosis is a possible mechanism for the persistence of Thy-1(–) myofibroblasts in fibroblastic foci.

In this study, we provide evidence that Thy-1(–) lung fibroblasts are more completely differentiated as myofibroblasts, with a myogenic gene expression profile at baseline and in response to fibrogenic mediators, correlating with enhanced contractility. Thy-1(–) lung myofibroblasts are also more resistant to apoptosis during collagen matrix contraction. Myofibroblasts with enhanced survival within sites of tissue injury may contribute to prolonged and excessive accumulation of extracellular matrix, promoting extensive remodeling in lungs undergoing fibrogenesis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

***Acknowledgments*:** The authors thank Leslie Leinwand (University of Colorado, Boulder) for the sarcomeric MyHC antibody and for invaluable assistance with study design; Dr. Leinwand and Namasivayam Ambalavanan for assistance with data interpretation; and Mark MacEwen, Tanya Rege, and Kaiyu Yuan for technical assistance.

References

1. Katzenstein AL, Myers JL. Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. *Am J Respir Crit Care Med* 1998; 157:1301–1315.
2. Kuhn C, Boldt J, King TE, Crouch E, Vartio T, McDonald JA. An immunohistochemical study of architectural remodeling and connective tissue synthesis in pulmonary fibrosis. *Am Rev Respir Dis* 1989; 140:1693–1703.
3. Phan SH. Fibroblast phenotypes in pulmonary fibrosis. *Am J Respir Cell Mol Biol* 2003;29:S87–S92.
4. Gabbiani G. The myofibroblast in wound healing and fibrocontractive diseases. *J Pathol* 2003;200:500–503.
5. van der Ven PF, Furst DO. Expression of sarcomeric proteins and assembly of myofibrils in the putative myofibroblast cell line BHK-21/C13. *J Muscle Res Cell Motil* 1998;19:767–775.
6. Rice NA, Leinwand LA. Skeletal myosin heavy chain function in cultured lung myofibroblasts. *J Cell Biol* 2003;163:119–129.
7. Tapscott SJ. The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription. *Development* 2005;132:2685–2695.
8. Rege TA, Hagood JS. Thy-1 as a regulator of cell-cell and cell-matrix interactions in axon regeneration, apoptosis, adhesion, migration, cancer, and fibrosis. *FASEB J* 2006;20:1045–1054.
9. Hagood JS, Mangalwadi A, Guo B, MacEwen MW, Salazar L, Fuller GM. Concordant and discordant interleukin-1-mediated signaling in lung fibroblast thy-1 subpopulations. *Am J Respir Cell Mol Biol* 2002; 26:702–708.
10. Hagood JS, Miller PJ, Lasky JA, Tousson A, Guo B, Fuller GM, McIntosh JC. Differential expression of platelet-derived growth factor-alpha receptor by Thy-1(-) and Thy-1(+) lung fibroblasts. *Am J Physiol* 1999;277:L218–L224.
11. Phipps RP, Penney DP, Keng P, Quill H, Paxhia A, Derdak S, Felch ME. Characterization of two major populations of lung fibroblasts: distinguishing morphology and discordant display of Thy 1 and class II MHC. *Am J Respir Cell Mol Biol* 1989;1:65–74.
12. Saalbach A, Hildebrandt G, Haustein UF, Anderegg U. The Thy-1/Thy-1 ligand interaction is involved in binding of melanoma cells to activated Thy-1-positive microvascular endothelial cells. *Microvasc Res* 2002;64: 86–93.
13. Saalbach A, Haustein UF, Anderegg U. A ligand of human thy-1 is localized on polymorphonuclear leukocytes and monocytes and mediates the binding to activated thy-1-positive microvascular endothelial cells and fibroblasts. *J Invest Dermatol* 2000;115:882–888.
14. Hagood JS, Prabhakaran P, Kumbla P, Salazar L, MacEwen MW, Barker TH, Ortiz LA, Schoeb T, Siegal GP, Alexander CB, *et al.* Loss of fibroblast Thy-1 expression correlates with lung fibrogenesis. *Am J Pathol* 2005;167:365–379.
15. Zhou Y, Hagood JS, Murphy-Ullrich JE. Thy-1 expression regulates the ability of rat lung fibroblasts to activate transforming growth factor-{beta} in response to fibrogenic stimuli. *Am J Pathol* 2004;165:659–669.
16. White ES, Lazar MH, Thannickal VJ. Pathogenetic mechanisms in usual interstitial pneumonia/idiopathic pulmonary fibrosis. *J Pathol* 2003; 201:343–354.
17. Pittet JF, Griffiths MJ, Geiser T, Kaminski N, Dalton SL, Huang X, Brown LA, Gotwals PJ, Koteliansky VE, Matthay MA, *et al.* TGF-beta is a critical mediator of acute lung injury. *J Clin Invest* 2001;107: 1537–1544.
18. Sime PJ, Xing Z, Graham FL, Csaky KG, Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. *J Clin Invest* 1997;100: 768–776.
19. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev* 1997;8:171–179.
20. Allen JT, Knight RA, Bloor CA, Spiteri MA. Enhanced insulin-like growth factor binding protein-related protein 2 (Connective tissue growth factor) expression in patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. *Am J Respir Cell Mol Biol* 1999;21:693–700.
21. Howell DC, Goldsack NR, Marshall RP, McAnulty RJ, Starke R, Purdy G, Laurent GJ, Chambers RC. Direct thrombin inhibition reduces lung collagen, accumulation, and connective tissue growth factor mRNA levels in bleomycin-induced pulmonary fibrosis. *Am J Pathol* 2001;159:1383–1395.
22. Hagood JS, Lasky JA, Nesbitt JE, Segarini P. Differential expression, surface binding, and response to connective tissue growth factor in lung fibroblast subpopulations. *Chest* 2001;120:S64–S66.
23. Uguccioni M, Pulsatelli L, Grigolo B, Facchini A, Fasano L, Cinti C, Fabbri M, Gasbarrini G, Meliconi R. Endothelin-1 in idiopathic pulmonary fibrosis. *J Clin Pathol* 1995;48:330–334.
24. Panettieri RA Jr, Goldie RG, Rigby PJ, Eszterhas AJ, Hay DW. Endothelin-1-induced potentiation of human airway smooth muscle proliferation: an ETA receptor-mediated phenomenon. *Br J Pharmacol* 1996;118:191–197.
25. Shi-Wen X, Denton CP, Dashwood MR, Holmes AM, Bou-Gharios G, Pearson JD, Black CM, Abraham DJ. Fibroblast matrix gene expression and connective tissue remodeling: role of endothelin-1. *J Invest Dermatol* 2001;116:417–425.
26. Hocher B, Schwarz A, Fagan KA, Thone-Reineke C, El-Hag K, Kusserow H, Elitok S, Bauer C, Neumayer HH, Rodman DM, *et al.* Pulmonary fibrosis and chronic lung inflammation in ET-1 transgenic mice. *Am J Respir Cell Mol Biol* 2000;23:19–26.
27. Desmouliere A, Redard M, Darby I, Gabbiani G. Apoptosis mediates the decrease in cellularity during the transition between granulation tissue and scar. *Am J Pathol* 1995;146:56–66.
28. Moulin V, Larochelle S, Langlois C, Thibault I, Lopez-Valle CA, Roy M. Normal skin wound and hypertrophic scar myofibroblasts have differential responses to apoptotic inductors. *J Cell Physiol* 2004; 198:350–358.
29. Ramos C, Montano M, Garcia-Alvarez J, Ruiz V, Uhal BD, Selman M, Pardo A. Fibroblasts from idiopathic pulmonary fibrosis and normal lungs differ in growth rate, apoptosis, and tissue inhibitor of metalloproteinases expression. *Am J Respir Cell Mol Biol* 2001;24:591–598.
30. McIntosh JC, Hagood JS, Richardson TL, Simecka JW. Thy1 (+) and (-) lung fibrosis subpopulations in LEW and F344 rats. *Eur Respir J* 1994;7:2131–2138.
31. Barker TH, Pallero MA, MacEwen MW, Tilden SG, Woods A, Murphy-Ullrich JE, Hagood JS. Thrombospondin-1-induced focal adhesion disassembly in fibroblasts requires Thy-1 surface expression, lipid raft integrity, and Src activation. *J Biol Chem* 2004;279:23510–23516.
32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408.
33. Grinnell F, Ho CH, Lin YC, Skuta G. Differences in the regulation of fibroblast contraction of floating versus stressed collagen matrices. *J Biol Chem* 1999;274:918–923.
34. Riou P, Vandromme M, Gazzolo L. Human T-cell leukemia virus type 1 tax protein inhibits the expression of the basic helix-loop-helix transcription factor MyoD in muscle cells: maintenance of proliferation and repression of differentiation. *Cell Growth Differ* 2001;12:613–622.
35. Kitzmann M, Vandromme M, Schaeffer V, Carnac G, Labbe JC, Lamb N, Fernandez A. cdk1- and cdk2-mediated phosphorylation of MyoD Ser200 in growing C2 myoblasts: role in modulating MyoD half-life and myogenic activity. *Mol Cell Biol* 1999;19:3167–3176.
36. Pei Y, Sherry DM, McDermott AM. Thy-1 distinguishes human corneal fibroblasts and myofibroblasts from keratocytes. *Exp Eye Res* 2004; 79:705–712.
37. Fluck J, Querfeld C, Cremer A, Niland S, Krieg T, Sollberg S. Normal human primary fibroblasts undergo apoptosis in three-dimensional contractile collagen gels. *J Invest Dermatol* 1998;110:153–157.
38. Koumas L, Smith TJ, Feldon S, Blumberg N, Phipps RP. Thy-1 expression in human fibroblast subsets defines myofibroblastic or lipofibroblastic phenotypes. *Am J Pathol* 2003;163:1291–1300.
39. Bergstrom DA, Penn BH, Strand A, Perry RL, Rudnicki MA, Tapscott SJ. Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. *Mol Cell* 2002;9:587–600.
40. Venuti JM, Morris JH, Vivian JL, Olson EN, Klein WH. Myogenin is required for late but not early aspects of myogenesis during mouse development. *J Cell Biol* 1995;128:563–576.
41. Wang Z, Wang DZ, Pipes GC, Olson EN. Myocardin is a master regulator of smooth muscle gene expression. *Proc Natl Acad Sci USA* 2003; 100:7129–7134.

42. Grinnell F, Ho CH. Transforming growth factor beta stimulates fibroblast-collagen matrix contraction by different mechanisms in mechanically loaded and unloaded matrices. *Exp Cell Res* 2002;273:248–255.
43. Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* 2001;134:136–151.
44. Frankel SK, Cosgrove GP, Cha SI, Cool CD, Wynes MW, Edelman BL, Brown KK, Riches DW. TNF-alpha sensitizes normal and fibrotic human lung fibroblasts to Fas-induced apoptosis. *Am J Respir Cell Mol Biol* 2006;34:293–304.
45. Xia H, Nho RS, Kahm J, Kleidon J, Henke CA. Focal adhesion kinase is upstream of phosphatidylinositol 3-kinase/Akt in regulating fibroblast survival in response to contraction of type I collagen matrices via a beta 1 integrin viability signaling pathway. *J Biol Chem* 2004;279:33024–33034.