Thy-1 Signals through PPAR γ to Promote Lipofibroblast Differentiation in the Developing Lung

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Thy-1 is a glycosylphosphatidylinositol-linked cell-surface glycoprotein present on a subset of lung fibroblasts, which plays an important role in postnatal alveolarization. In the present study, we define the role of Thy-1 in pulmonary lipofibroblast differentiation and in the regulation of lipid homeostasis via peroxisome proliferator-activated receptor- γ (PPAR γ). Thy-1 was associated with interstitial cells containing lipid droplets *in vivo*. The transfection of Thy-1 into Thy-1 (–) fibroblasts increased triglyceride content, fatty-acid uptake, and the expression of the lipofibroblast marker adipocyte differentiation-related protein. Thy-1 (+) fibroblasts exhibited 2.4-fold higher PPAR γ activity, and the inhibition or activation of PPAR γ reduced and increased triglyceride content, respectively. Thy-1 (–) fibroblasts were not responsive to either of the PPAR γ agonists ciglitazone or prostaglandin J₂, supporting the importance of Thy-1 in signaling via PPAR γ . Thy-1 (+) fibroblasts expressed significantly higher concentrations of fatty-acid transporter protein-3 mRNA, and demonstrated higher rates of fatty-acid uptake and increased triglyceride content. The inhibition of fatty-acid transporter protein function reduced Thy-1 (+) fibroblast lipid content. The expression of Thy-1 in C57BL/6 lung fibroblasts increased during the neonatal period, coinciding with the onset of alveolarization. Thy-1 promoted lipofibroblast differentiation via the expression of PPAR γ , stimulated lipid accumulation via fatty-acid esterification, and enhanced the fatty-acid uptake mediated by fatty-acid transporter proteins. Thy-1 is important in the regulation of lipofibroblast differentiation in the developing lung.

Keywords: Thy-1; lipofibroblast; PPAR γ ; lipid metabolism

Beginning at 35 weeks after conception and continuing for up to 8 years after birth, the alveolar stage of lung development is marked by the sequential division of larger airspaces into smaller ones by secondary alveolar septa (1, 2). From term birth until adulthood, the 5-fold increase in the number of alveoli and the

CLINICAL RELEVANCE

Pulmonary lipofibroblasts are important in alveolar development, and the peroxisome proliferator-activated receptor- γ (PPAR γ) is known to induce lipofibroblast differentiation. However, little is known about the factors that activate PPAR γ in pulmonary fibroblasts. This study demonstrates that pulmonary fibroblast Thy-1 expression induces lipofibroblast differentiation through PPAR γ , and describes a mechanism by which pulmonary fibroblasts accumulate neutral lipid.

20-fold increase in alveolar surface area are critical in meeting the increasing oxygenation and ventilation needs of human growth and activity (2, 3). Premature birth, exposure to high concentrations of inspired oxygen, and mechanical ventilation lead to impaired alveolar development and chronic lung disease (4). The impaired lung function of chronic lung disease results in significant morbidity, mortality, and healthcare costs from early life through adulthood (5–8). Understanding how the different tissues of the lung behave during normal and impaired alveolar development is a key first step toward restoring alveolarization and attenuating the burden of respiratory disease after premature birth.

Pulmonary lipofibroblasts comprise an incompletely characterized population of lipid-containing interstitial cells that play an important role in alveolarization. Hyperoxia, hypoxia, and nicotine exposure stimulate lipofibroblasts to myofibroblast differentiation and impair alveolar development (9–12). Peroxisome proliferator-activated receptor- γ (PPAR γ) signaling is important in lipofibroblast differentiation (13, 14), and PPAR γ agonists can reverse lipofibroblast to myofibroblast differentiation and restore alveolar development in murine and rat models (10, 15). The only described signaling pathway upstream of PPAR γ that promotes fibroblast lipid accumulation is the parathyroid-related peptide-leptin axis (16), although a recent report questioned the importance of lipofibroblast leptin signaling (17). To understand the key events in alveolar development, a broader understanding of the endogenous factors that promote lipofibroblast differentiation is needed.

Thy-1 is a glycosylphosphatidylinositol-linked glycoprotein critical in the determination of pulmonary fibroblast phenotype (18, 19). Pulmonary fibroblast Thy-1 expression has been associated with the presence of lipid inclusions (20, 21), and the loss of pulmonary fibroblast Thy-1 expression causes myofibroblast differentiation (18, 22–24). Although the lungs of *Thy-1*^{–/–} and wild-type mice are similar at birth, postnatal alveolar septation is impaired in the *Thy-1*^{–/–} mouse (25). Whether Thy-1 stimulates or is merely associated with lipid accumulation in pulmonary fibroblasts remains unknown. Because Thy-1 is a key regulator of pulmonary fibroblast phenotype and important in alveolar development, we hypothesized that Thy-1 signaling stimulates lipofibroblast differentiation through PPAR γ , and

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that fibroblast Thy-1 expression increases at the onset of alveolarization.

MATERIALS AND METHODS

Please see the online supplement for more detailed descriptions of materials and methods.

Animal Use

C57BL/6 wild-type (WT) and *Thy-1*^{-/-} mice on a C57BL/6 background (Dr. Roger Morris, King's College, London, UK) were used, with approval by our institutional Animal Care and Use Committees.

Lung Histology and Thy-1 Expression

The lungs of mice at serial gestational ages from Postnatal Day (PND) 1 to 8 weeks were immunostained for Thy-1. Snap-frozen lungs of mice at PND5 and PND14 were immunostained for Thy-1, costained with oil-red-O, and evaluated by fluorescence microscopy.

Lung Triglyceride Content

Lungs of WT and *Thy-1*^{-/-} PND5 and PND14 mice were stained with oil-red-O, and the numbers of lipid inclusions in five ×40 fields were counted. The neutral lipid contents of lung homogenates from WT and *Thy-1*^{-/-} mice were analyzed using the Wako triglyceride assay (Wako, Richmond, VA).

Cells and Tissue Culture Conditions

Sorted fibroblasts. PND14 WT fibroblasts were isolated and sorted for Thy-1, as described in the online supplement. Fibroblasts were analyzed by FACS for the maintenance of Thy-1 expression and used at a total passage number of six.

Transfected fibroblasts. To isolate the effects of Thy-1 on fibroblast phenotype, a previously described transfection model was used (26). Fetal rat lung fibroblasts, which are entirely Thy-1-negative (RFL6; American Type Culture Collection, Manassas, VA), were transfected with a control vector (RFL6.EV) or the murine Thy-1.2 vector (RFL6.CD90).

Both sorted fibroblasts and Thy-1-transfected fibroblasts were used for tissue-culture experiments. To determine the effects of Thy-1 on PPAR γ activity and lipid metabolism, ciglitazone, prostaglandin J₂, the PPAR γ inhibitor GW9662, and the fatty-acid transporter inhibitor phloretin were used. Thy-1 knockdown was achieved in Thy-1-transfected fibroblasts, using Thy-1 Silencer Select silencing (si) RNA (Invitrogen, Carlsbad, CA) or control siRNA.

PPAR γ Luciferase Assay

A previously described PPAR γ luciferase reporter plasmid (27) and renilla luciferase control plasmid were transfected into RFL6.CD90 and RFL6.EV cells. After overnight transfection, cells were grown in 10% FBS-containing media for 48 hours, and the cell lysate was collected for analysis.

Fibroblast Triglyceride Content

Thy-1 sorted or transfected fibroblasts were serum-starved for 24 hours, and then grown in 10% FBS containing media for 48 hours. Cells were collected, washed, and lysed. The lysate was centrifuged, its volume was normalized for the number of cells, and the supernatant was analyzed for triglyceride content.

Fatty-Acid Uptake

To quantitate fatty-acid uptake, sorted and transfected fibroblasts were serum-starved for 24 hours. The media were aspirated, and fatty-acid uptake was quantitated using the QBT Fatty Acid Uptake Assay (Molecular Devices, Sunnyvale, CA).

Quantitative PCR and Western Blotting

Thy-1-transfected fibroblasts were used to assess how Thy-1 alters the mRNA and protein expression of lipofibroblast-related genes. Taqman RT-PCR was performed using the validated primers listed in the online supplement. The protein expression of adipocyte differentiation-related protein, PPAR γ , and β -actin was determined by Western blotting.

Metabolic Activity

The metabolic activity of transfected RFL6 cells was determined using the WST-1 assay (Roche, Branford, CT), according to the manufacturer's instructions.

Statistical Analysis

Statistical comparisons between groups were performed using the Student's *t* test or one-way ANOVA according to the Holm-Sidak method. *P* < 0.05 was considered significant.

RESULTS

Fibroblast Thy-1 Expression Increases at the Onset of Alveolarization

We previously reported that whole-lung Thy-1 mRNA increased with development, but the localization of Thy-1 expression to pulmonary fibroblasts remained unclear (25). By immunohistochemistry, Thy-1 stained principally in the interstitium, and the number of brown/purple-staining cells increased during development (Figure 1A). Pulmonary fibroblasts from mice at sequential ages were assessed for Thy-1 expression by FACS. Isolated fibroblasts were negative for the macrophage markers CD11b and F4/80, the epithelial marker E-cadherin, and the endothelial marker CD31 (see Figure E1 in the online supplement). The percentage of fibroblasts expressing Thy-1 increased in a bimodal pattern from fewer than 5% positive fibroblasts at post-fertilization day 18.5 (E18.5) to 70% in adulthood (Figure 1B). These data show that pulmonary fibroblast Thy-1 expression increases coincident with the onset of alveolarization.

Thy-1 Expression Is Associated with Neutral Lipid Accumulation in Primary Lung Fibroblasts

Previous studies reported the association of Thy-1 with pulmonary fibroblast lipid droplets, but the lipid content was not quantitated. Thy-1 colocalized with lipid droplets in oil-red-O-stained PND5 lung sections (Figure 1C). Lung fibroblasts (PND14) sorted for Thy-1 maintained Thy-1 expression with passaging (Figure 2A). Thy-1 (+) sorted fibroblasts contained 111% more triglyceride than Thy-1 (-) sorted fibroblasts (Figure 2B), demonstrating that in pulmonary fibroblasts, the expression of Thy-1 is associated with increased fibroblast lipid content.

Thy-1 Expression Stimulates Neutral Lipid Accumulation in Pulmonary Fibroblasts

A heterologous expression model was used to ascertain the role of Thy-1 fibroblast lipid accumulation. Thy-1-transfected fibroblasts (RFL6.CD90) contained substantially more and larger lipid droplets than empty vector-transfected fibroblasts (RFL6.EV) (Figure 2C). RFL6.CD90 fibroblasts maintained their expression with passaging, and contained 331% more triglyceride than RFL6.EV fibroblasts (Figures 2D and 2E). The knockdown of Thy-1 in RFL6.CD90 fibroblasts using Thy-1 siRNA reduced triglyceride content by 52%, compared with control siRNA-transfected fibroblasts (Figures 2F and 2G), indicating that Thy-1 signaling stimulates neutral lipid accumulation in pulmonary fibroblasts.

Figure 1. Pulmonary fibroblast Thy-1 expression is developmentally regulated, and Thy-1 colocalizes with interstitial lipid droplets. (A) Immunohistochemistry for Thy-1 reveals that expression in pulmonary interstitial cells increases throughout alveolarization. Scale bar, 50 μ m. (B) Quantitative analysis of pulmonary fibroblast Thy-1 expression, using flow cytometry, reveals a bimodal increase in fibroblast Thy-1 expression throughout alveolar development. (C) In wild-type (WT) Postnatal Day (PND) 5 murine lung tissue, Thy-1 (+) interstitial cells (green) contain lipid droplets (red). Magnification, $\times 67$. * $P < 0.05$, compared with previous value. ** $P < 0.01$, compared with previous value.

The *Thy-1*^{-/-} Mouse Does Not Have Reduced Numbers of Interstitial Lipid Droplets

Whether the *in vitro* finding that Thy-1 (+) fibroblasts contain larger and more numerous lipid droplets is also true *in vivo* remains unknown. On PND5, *Thy-1*^{-/-} murine lungs contained more lipid droplets than did WT lungs, but on PND14, the numbers of lipid droplets were similar (Figures E2A–E2E). The total lung lipid content of *Thy-1*^{-/-} mice on PND14 is less than for WT mice (Figure E2F). Lipid droplets were not present in alveolar Type II cells, as seen in other models of dysregulated pulmonary fibroblast lipid metabolism (Figure E3) (28). The deletion of Thy-1 in the *Thy-1*^{-/-} mouse is not pulmonary fibroblast-specific, and *in vitro* findings from isolated fibroblasts may not represent *in vivo* conditions.

Thy-1 Increases PPAR γ Expression, Activity, and PPAR γ -Mediated Neutral Lipid Accumulation

PPAR γ is a well-described regulator of lipofibroblast differentiation, but whether Thy-1 alters PPAR γ expression or activity remains unknown. By overexpressing Thy-1 in RFL6 fibroblasts (RFL6.CD90), PPAR γ mRNA expression was increased 88%, and protein expression increased 93%, compared with Thy-1-deficient RFL6.EV fibroblasts (Figures 3A and 3B). Adipocyte differentiation-related peptide (ADRP) is both a downstream target of PPAR γ and a lipofibroblast marker. ADRP may play an independent role in pulmonary fibroblast lipid accumulation, insofar as 3T3 fibroblasts overexpressing ADRP accumulate lipid in a PPAR γ -independent manner (29). ADRP mRNA expression was increased 80%, and protein content increased 930%, in RFL6.CD90 fibroblasts compared with RFL6.EV fibroblasts (Figures 3C and 3D). As assessed by a PPAR γ luciferase assay, PPAR γ signaling was increased by 142% in RFL6.CD90 cells compared with RFL6.EV cells (Figure 3F). The PPAR γ ligand ciglitazone increased triglyceride content by 80% in a dose-dependent manner in RFL6.CD90 fibroblasts, but no increase in lipid content was evident in RFL6.EV fibroblasts (Figure 3G). The inhibition of PPAR γ signaling with GW9662 reduced triglyceride content by 62% in RFL6.CD90 fibroblasts (Figure 3H). To test whether the lack of ciglitazone response in RFL6.EV fibroblasts was attributable to reduced PPAR γ expression or an inhibition of

PPAR γ signaling, the PPAR γ response element ligand prostaglandin J₂ (PGJ₂) was used. PGJ₂ did not increase triglyceride content in RFL6.EV fibroblasts until a threshold concentration of 8–10 nM was achieved, at which point the triglyceride content increased by 710% (Figure 3I). Cell viability was not altered by the reported concentrations of ciglitazone, GW9662, or PGJ₂. Doses of ciglitazone (1 μ M and greater) and combinations of ciglitazone and GW9662 reduced cell viability. These data demonstrate that Thy-1 increases the expression of PPAR γ , increases PPAR γ signaling, and increases PPAR γ -mediated neutral lipid accumulation.

Thy-1 Increases Expression of the PPAR γ Heterodimer Retinoid X Receptor- α

PPAR γ must heterodimerize with retinoic-acid receptors to activate the PPAR γ response element (30), and the retinoid X receptor- α (RXR α) is important in alveolar development (31). RXR α mRNA was increased by 38% in RFL6.CD90 cells compared with RFL6.EV cells (Figure E4A). RXR α can also heterodimerize with the liver X receptor α (LXR α), which regulates many lipid metabolic processes (30), and which causes keratinocyte lipid accumulation (32). The expression of LXR α was decreased by 61% in RFL6.CD90 fibroblasts, compared with RFL6.EV fibroblasts (Figure S4AB). These data suggest that Thy-1 (+) fibroblasts possess a greater abundance of the PPAR γ -RXR α heterodimer.

Thy-1/PPAR γ Signaling Increases Fibroblast Lipid Content through Increased Fatty-Acid Transporter Protein Expression and Increased Fatty-Acid Esterification

Although both PPAR γ (10, 13, 14) and ADRP (29) play a role in the development of the lipofibroblast phenotype, the mechanism by which lipid enters and accumulates in fibroblasts remains unknown. One potential mechanism involves the hydrolysis of triglyceride at the cell membrane and the transport of liberated fatty acids. Lipoprotein lipase is expressed by lipofibroblasts (33), and its transcription is positively regulated by PPAR γ (30). However, lipoprotein lipase mRNA was not detected in RFL6.EV or RFL6.CD90 fibroblasts by quantitative PCR. Another potential mechanism of lipid accumulation in

Figure 2. Thy-1 induces neutral lipid accumulation in pulmonary fibroblasts. (A) Isolated murine lung fibroblasts sorted for Thy-1 (black) maintain Thy-1 expression *in vitro*. (B) Thy-1 (+) murine lung fibroblasts (solid bar) have significantly higher lipid content than Thy-1 (-) fibroblasts (open bar). (C) By oil-red-O staining, empty vector (EV)-transfected rat fetal lung fibroblasts (RFL6) have very rare lipid droplets, whereas RFL6 fibroblasts transfected with Thy-1 (CD90) have more numerous and larger lipid droplets. (D) RFL6.CD90 (black) and RFL6.EV (white) fibroblasts maintain Thy-1 expression in culture. (E) RFL6.CD90 fibroblasts (solid bar) contain significantly more triglyceride than RFL6.EV fibroblasts (open bar). (F) Knockdown of Thy-1 expression (gray) in RFL6.CD90 fibroblasts results in a reduction of Thy-1 expression. (G) Thy-1 knockdown (light gray bar) by silencing RNA (siRNA) results in a reduction of fibroblast triglyceride content, compared with control siRNA (dark gray bar) and no siRNA (solid bar). * $P < 0.05$. *** $P < 0.001$. FL1-H is fluorescent intensity in channel 1.

Thy-1 (+) fibroblasts involves the uptake of free fatty acids liberated by adjacent cells (potentially endothelial cells). RFL6.CD90 fibroblasts took up 40% more free fatty acid than did RFL6.EV fibroblasts (Figure 4A), and the siRNA knockdown of Thy-1 in RFL6.CD90 fibroblasts reduced fatty-acid uptake (Figure 4B), indicating that the presence of Thy-1 increases the transport of free fatty acids into pulmonary fibroblasts.

Fatty acids can enter cells via either active transport or facilitated diffusion. Active transport occurs via fatty-acid translocase (CD36). CD36 is expressed by alveolar Type II cells (34), and is regulated by PPAR γ (30). However, in RFL6.CD90 fibroblasts, CD36 was detected by neither quantitative PCR nor FACS (with gating based on an isotype control; data not shown). Fatty-acid transporter proteins allow for the facilitated diffusion of fatty acids, and are positively regulated by PPAR γ (35). Two of these proteins are expressed in the lung: solute carrier family 27, member 1 and member 3 (SLC27A1 and SLC27A3) (36, 37). SLC27A1 mRNA expression was not statistically different between RFL6.CD90 and RFL6.EV fibroblasts, whereas SLC27A3 mRNA was 82% higher in RFL6.CD90 fibroblasts (Figures 4C and 4D). This finding suggests that fatty-acid transport through SLC27A3 at least partly accounts for differences in fatty-acid uptake between RFL6.CD90 and

RFL6.EV fibroblasts. To test whether fatty-acid transporter proteins, including SLC27A3, are important mediators of fatty-acid uptake in Thy-1 (+) fibroblasts, fatty-acid uptake was measured in the presence of increasing concentrations of phloretin, an inhibitor of these transporters. Phloretin decreased fatty-acid uptake by 53% (Figure 4E), and triglyceride content by 77% (Figure 4F), in RFL6.CD90 fibroblasts. Toxicity was evident with doses of phloretin at 50 μ M and above. These findings support the concept that fatty-acid transporter proteins, which are inhibited by phloretin, are likely to mediate increased fatty-acid uptake and influence the increased lipid content in Thy-1 (+) pulmonary fibroblasts.

Because fatty-acid transporter proteins facilitate only the passive transport of fatty acids, a gradient is required for fatty-acid transport. Two processes could generate this gradient: fatty-acid oxidation or fatty-acid esterification. To test for increased fatty-acid oxidation in Thy-1 (+) fibroblasts, we determined metabolic activity under serum-free conditions (i.e., the same conditions used in the fatty-acid uptake assay). The WST-1 assay assesses metabolic activity by photometrically measuring the mitochondrial production of nicotinamide adenine dinucleotide phosphate-reduced. RFL6.CD90 fibroblasts cleaved 24% less WST-1, indicating reduced metabolic activity in RFL6.CD90 fibroblasts compared with

Figure 3. Thy-1 up-regulates peroxisome proliferator-activated receptor- γ (PPAR γ) and adipocyte differentiation-related peptide (ADRP) expression in pulmonary fibroblasts, and induces pulmonary fibroblast neutral lipid accumulation through PPAR γ . (A) PPAR γ transcription is increased in RFL6.CD90 fibroblasts (solid bar), compared with RFL6.EV fibroblasts (open bar). (B) RFL6.CD90 fibroblasts express more PPAR γ protein than do RFL6.EV fibroblasts. (C) mRNA expression of the downstream target of PPAR γ and the lipofibroblast marker ADRP is increased in RFL6.CD90 fibroblasts. (D) RFL6.CD90 fibroblasts express more ADRP than do RFL6.EV fibroblasts. (E) Western blot of ADRP, PPAR γ , and loading control (β -actin). Intervening lanes were removed. (F) PPAR γ luciferase assay demonstrates increased PPAR γ signaling in RFL6.CD90 fibroblasts. (G) Activation of PPAR γ with the PPAR γ ligand ciglitazone induces a dose-dependent increase in triglyceride accumulation in RFL6.CD90 but not RFL6.EV fibroblasts (open bars). (H) Inhibition of PPAR γ signaling with GW9662 reduces triglyceride content of RFL6.CD90 fibroblasts. (I) Prostaglandin I_2 (PGJ₂), a PPAR γ response-element activator, significantly increases triglyceride accumulation in RFL6.EV fibroblasts after a threshold concentration between 8–10 nM is surpassed. * P < 0.05, ** P < 0.01, and † P < 0.001, condition versus control samples.

RFL6.EV fibroblasts (Figure 5A). Fatty-acid esterification involves the creation of an ester moiety from a fatty-acid carboxyl group and a glycerol hydroxyl group. We assessed the expression of two key fatty-acid esterifying enzymes: cytidine diphospho (CDP)-diacylglycerol synthase (CDS1) and monoacylglycerol acyltransferase (MOGAT1). The expression of CDS1 was increased by 167% (Figure 5B), and the expression of MOGAT1 was increased by 22%, in Thy-1-transfected cells (Figure 5C). These data suggest that increased fatty-acid esterification into triacylglycerol, but not increased fatty-acid oxidation, creates the diffusion gradient by which fatty acids enter Thy-1 (+) pulmonary fibroblasts.

DISCUSSION

In this study, we show that Thy-1 stimulates lipofibroblast differentiation through the activation of PPAR γ . The overexpression of Thy-1 in Thy-1 (–) pulmonary fibroblasts increased the expression of PPAR γ and its heterodimer RXR α , increased the expression of the lipofibroblast marker ADRP, and increased fibroblast lipid content. The inhibition of PPAR γ reduced the lipid content of Thy-1 (+) fibroblasts. These findings are consistent with previous observations that PPAR γ signaling promotes a lipofibroblast phenotype (9, 11), and demonstrate the novel

finding that PPAR γ signaling is modulated by lung fibroblast Thy-1 expression.

The expression of Thy-1 by lung fibroblasts increased at the onset of alveolarization, perhaps indicating an important role in alveolarization. This pattern is consistent with the observation that alveolarization is delayed in *Thy-1*^{–/–} mice (25). However, our findings differ from those in previous reports that indicate an earlier appearance of the pulmonary lipofibroblast (38, 39), suggesting that the Thy-1 (+) lipofibroblast represents a distinct cell population. This assertion is supported by two observations. First, Awonsonu and colleagues found that not all lipofibroblasts are Thy-1 (+) (38). Second, whereas the earlier-appearing lipofibroblast population was shown to be important in surfactant production *in vitro* (40, 41), *Thy-1*^{–/–} mouse pups do not exhibit respiratory distress or lung histology consistent with surfactant deficiency (10, 25, 42). The prevention of lipofibroblast-to-myofibroblast differentiation by the prenatal administration of rosiglitazone increased the numbers of lamellar bodies in alveolar Type II cells in rats on PND1 (43), supporting the assertion that earlier-appearing lipofibroblasts may play a role in surfactant production, but the later-appearing Thy-1 (+) lipofibroblasts do not.

Despite *in vitro* evidence for the role of Thy-1 in lipofibroblast differentiation, *Thy-1*^{–/–} mice do not have lower numbers of lipid

Figure 4. Thy-1 increases pulmonary fibroblast free fatty-acid uptake by increased expression and activity of fatty-acid transporter proteins. (A) RFL6.CD90 fibroblasts (solid circles) take up more free fatty acid than do EV fibroblasts (open circles). (B) The knockdown of Thy-1 expression in RFL6.CD90 fibroblasts (solid triangles) reduces fatty-acid uptake to concentrations comparable to those of RFL6.EV fibroblasts (open circles). (C) Similar mRNA expression of fatty-acid transporter protein SLC27A1 was evident between RFL6.EV fibroblasts (open bars) and RFL6.CD90 fibroblasts (solid bars). (D) Fatty-acid transporter protein-3 (SLC27A3) has higher expression in RFL6.CD90 fibroblasts. (E) Compared with vehicle-treated RFL6.CD90 fibroblasts (circle), the fatty-acid transporter protein inhibitor phloretin reduces fatty-acid uptake at concentrations of 0.05 μ M (triangles), 0.5 μ M (squares), and 5 μ M (diamonds). (F) Phloretin reduces triglyceride content of RFL6.CD90 fibroblasts. * P < 0.05, ** P < 0.01, *** P < 0.001, and $^{\dagger}P$ < 0.001, condition versus control samples.

droplets *in vivo*. Because all cells in this murine model are deficient in Thy-1, we cannot isolate its role in fibroblast lipid accumulation *in vivo* from the effects exerted by neighboring cells. Alveolar Type II cells also express Thy-1 (44), and the epithelium overlying Thy-1 (–) fibroblasts in idiopathic pulmonary fibrosis demonstrates an increased expression of Thy-1 (45). Because alveolar epithelial–mesenchymal signaling is important in lipofibroblast differentiation and function (18), and the potential effects of Thy-1 on epithelial cell functions are unknown, a fibroblast-

specific Thy-1 knockout mouse would be required to determine the cell-specific effects of Thy-1 on fibroblast phenotype *in vivo*.

The present study supports the concept that Thy-1 (+) fibroblasts increase lipid content via facilitated diffusion via fatty-acid transporter proteins. Thy-1 (+) fibroblasts demonstrate greater fatty-acid uptake and a higher expression of the fatty-acid transporter protein SLC27A3. The inhibition of fatty-acid transporter proteins with phloretin decreases both fatty-acid transport

Figure 5. Thy-1 (+) fibroblasts have greater expression of fatty-acid esterifying enzymes and lower metabolic activity than Thy-1 (–) fibroblasts. (A) Under serum-starved conditions, RFL6.CD90 fibroblasts (solid bar) are less metabolically active than RFL6.EV fibroblasts (open bar), as assessed by WST-1 assay. (B) Transcription of the fatty-acid esterifying enzyme cytidine diphospho (CDP)-diacylglycerol synthase is increased in RFL6.CD90 fibroblasts. (C) There is little difference in the mRNA expression of the upstream esterifying enzyme monoacylglycerol O-acyltransferase-1. * P < 0.05. ** P < 0.01.

Figure 6. Proposed mechanism by which Thy-1 increases pulmonary fibroblast lipid content. Thy-1 increases the transcription of PPAR γ and retinoic X receptor- α (RXR α), and also activates PPAR γ . PPAR γ up-regulates the expression of ADRP, which lies at the cytosol-lipid interface of lipid droplets. Thy-1, perhaps through PPAR γ -regulated transcription, also increases the expression of CDP-diacylglycerol synthase (CDS1), which promotes the esterification of free fatty acids to triglyceride. Thy-1, likely through PPAR γ , up-regulates the expression of SLC27A3, which allows the diffusion of fatty acids into fibroblasts. These fatty acids are esterified to triacylglycerols and stored in lipid droplets. References are noted in parentheses. MOGAT1, monoacylglycerol acyltransferase-1.

and triglyceride content in Thy-1 (+) fibroblasts. the increased expression of fatty-acid esterifying enzyme CDP-diacylglycerol synthase in Thy-1 (+) fibroblasts may create the necessary fatty-acid diffusion gradient. This mechanism of lipid accumulation is distinct from that of adipocytes (37) and keratinocytes (32), and demonstrates the unique biology of the Thy-1 (+) lipofibroblast. Pulmonary fibroblasts seem to have biological characteristics distinct from myometrial or orbital fibroblasts, because the expression of Thy-1 in those tissues does not seem to stimulate lipid accumulation (46–48). A schematic depiction of the proposed mechanism for lipid accumulation in Thy-1 (+) pulmonary lipofibroblasts is presented in Figure 6.

In conclusion, we have demonstrated that Thy-1 promotes lipofibroblast differentiation via PPAR γ , that it acts to increase fibroblast triglyceride content via fatty-acid transporter proteins, and that its developmental regulation is consistent with an important role in alveolar development. The Thy-1 (+) lipofibroblast represents a unique subpopulation, and the manipulation of Thy-1 expression or associated signaling pathways could improve alveolar development after premature births.

Author disclosures are available with the text of this article at www.atsjournals.org.

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