Thy-1 Expression in Human Fibroblast Subsets Defines Myofibroblastic or Lipofibroblastic Phenotypes

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Fibroblasts represent a dynamic population of cells, exhibiting functional heterogeneity within and among tissues. Fibroblast heterogeneity also results from phenotypic differences and may arise from activation or differentiation processes taking place in the cells. We previously reported that human fibroblasts were heterogeneous with respect to surface Thy-1 expression and that separation into Thy-1⁺ and Thy-1⁻ subsets resulted in functionally distinct subpopulations, leading to the concept of fibroblast subset specialization. In this report we investigated whether Thy-1⁺ and/or Thy-1⁻ fibroblasts were capable of differentiating into myofibroblasts or lipofibroblasts. Fibroblast subsets were used from human myometrium and orbit to test this hypothesis. Only Thy-1⁺ human myometrial and orbital fibroblasts were capable of myofibroblast differentiation after treatment with TGF β or platelet concentrate supernatant, assessed by α smooth muscle actin expression. Interestingly, only Thy-1⁻, but not Thy-1⁺ subsets differentiated to lipofibroblasts, as determined by the accumulation of cytoplasmic lipid droplets after treatment with 15-deoxy- $\Delta^{12,14}$ -PGJ₂ or ciglitazone. We propose that fibroblast Thy-1 display pre-determines lineage to a contractile or lipid-like phenotype in the human myometrium and orbit. This additional distinction between Thy-1⁺ and Thy-1⁻ human fibroblast subtypes has important consequences in normal tissue homeostasis and in pathogenesis of orbital and myometrial diseases characterized by persistent myofibroblasts or fat accumulation, such as occurs in Graves' ophthalmopathy, tissue fibrosis, abnormal wound healing, and scarring. (*Am J Pathol* 2003, 163:1291-1300)

Existence of fibroblast heterogeneity has been well documented over the past 25 years with respect to a variety of parameters. These included differences in proliferation rates,^{1,2} responses to prostaglandin E₂,^{3,4} collagen synthe-

sis,^{5,6} cytokine production,⁷ cytokine receptors,⁸ and C1q receptors.⁹ Beside differential functional attributes, there also exists phenotypic diversity within fibroblast populations. Differences in morphology,¹⁰ and cell size^{11,12} have also been reported in fibroblast strains from various tissues. Phenotypic heterogeneity may further suggest different functional properties between fibroblast subpopulations.

Additional evidence of phenotypic heterogeneity among fibroblasts suggests that heterogeneity may also arise as a consequence of activation or differentiation processes occurring in the cells. The concept of fibroblast plasticity proposes that fibroblasts can be induced to acquire features of other types of mesenchymal cells. Hence, relatively undifferentiated fibroblasts can adopt a particular phenotype according to physiological requirements and to the microenvironmental factors to which they are exposed.¹³ In particular, a subpopulation of fibroblasts has been reported to express characteristics of smooth muscle differentiation.^{14,15} These cells are termed myofibroblasts and display prominent cytoplasmic actin filaments (stress fibers), characterized by the expression of α smooth muscle actin (α SMA), typically absent from fibroblasts, but present in smooth muscle cells.¹⁶ Myofibroblasts were initially identified in granulation tissue where they were considered responsible for the contractile forces that close wound margins.¹⁷ Myofibroblasts have been observed in both normal and pathological situations and have a central role in wound healing and fibrotic responses.¹⁸ Normal connective tissues containing myofibroblasts are ones where function of the organ requires contractile or traction forces, such as in ovarian follicles, the uterus, the pulmonary septa, and the periodontal space.¹⁸ Through appropriate stimulation, cultured fibroblasts have the capacity to differentiate into distinct morphological and biochemical cell types, a condition that may reflect modulations seen *in vivo*.¹⁹ Specifically, fibroblasts were induced to acquire a myofibroblastic phenotype *in vitro*, and this was evidenced by the expression of α SMA.^{20,21}

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The identification of lipid-Interstitial cells and non-lipid interstitial cells further supports the concept of phenotypic fibroblast heterogeneity.^{22,23} Adipocyte differentiation and abnormal fat accumulation may be the result of a pathological condition, as in thyroid-associated ophthalmopathy (TAO; also known as Graves' ophthalmopathy), and is thus an important factor in the disease process. Fibroblasts can differentiate into lipid-containing cells or lipofibroblasts *in vitro* given the appropriate stimuli. Peroxisome proliferator-activated receptor γ (PPAR γ) is a critical transcription factor in the regulation of adipogenesis. A natural ligand to PPAR γ , the prostaglandin called 15-deoxy- $\Delta^{12,14}$ -PGJ₂, has recently been identified and is a potent inducer of adipocyte formation.^{24–26} Previously, we demonstrated that human orbital fibroblasts derived from the adipose/connective tissue depot exhibit phenotypic diversity, where lipid droplet formation was observed in a subpopulation of fibroblasts representing pre-adipocytes.^{27,28}

In the past decade, we established that the surface receptor Thy-1 is heterogeneously expressed in fibroblast strains from many tissues. These included mouse lung and spleen,^{7,10} human lung, myometrium, and orbit.^{29–31} Thy-1 is a cell-surface glycoprotein, whose function remains ill-defined.³² Furthermore, using Thy-1 as the discriminatory cell surface marker, we successfully separated mouse lung and spleen and human myometrial and orbital primary fibroblast strains into *bona fide* Thy-1⁺ and Thy-1[−] subsets, and these were shown to display distinct functional properties.^{7,10,30,33} In particular, human myometrial Thy-1⁺ and Thy-1[−] subsets exhibited differences in cytokine and prostaglandin production, CD40 expression, and cyclooxygenase localization.^{30,34} Human orbital fibroblast subpopulations were heterogeneous with respect to cytokine and prostaglandin production and MHC class-II expression.³³ Differences in functional properties suggest fibroblast subset specialization and attribute unique roles to fibroblast subpopulations in tissue homeostasis and pathogenesis.

In the current manuscript we sought to determine whether, in addition to delineating functional heterogeneity, Thy-1 expression also defined phenotypic heterogeneity in human fibroblast populations. To provide support for this concept, studies were conducted using human Thy-1⁺ and Thy-1[−] fibroblast subsets from two otherwise unrelated tissues, the human myometrium and orbit. To address different phenotypic attributes, we tested if fibroblast subsets were capable of differentiating along the myofibroblastic or lipofibroblastic pathway. Herein, it is demonstrated that Thy-1 can be used as the discriminatory marker to define lineage to a smooth muscle-like or adipocyte-like phenotype in human myometrium and orbit. These new studies further define human Thy-1⁺ and Thy-1[−] fibroblast subset specialization.

Materials and Methods

Tissue Collection and Fibroblast Strain Derivation

Myometrial and orbital tissue samples were obtained as previously described.^{35,36} Briefly, myometrial tissue biop-

sies were collected from women undergoing gynecological procedures for benign conditions. All women had regular menstrual cycles (25 to 35 days), were pre-menopausal (20 to 42-years-old), and did not receive any form of hormonal treatment 3 months before the procedure. Written informed consent was received from all patients and ethical approval was obtained. Primary orbital fibroblasts were isolated from surgical explants as described.³¹ TAO fibroblasts were obtained from individuals undergoing decompressive surgery.

Fibroblast cultures were established by standard explant techniques as previously described.³⁷ Cells were examined for fibroblast markers using immunostaining and exhibited a morphology consistent with the fibroblast phenotype. They expressed vimentin and collagen, but not cytokeratin (epithelial cell marker), Factor VIII (endothelial cell marker), nor CD45 (bone marrow-derived cell marker).³¹ All cells used in experiments were early passage (passages 4–12). Myometrial fibroblast strains were cultured in RPMI 1640 media and Graves' fibroblast strains in minimal essential medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 0.1 mmol/L non-essential amino acids, and 0.048 mg/ml gentamicin (Life Technologies). Fibroblasts were passaged every ~7 days, after reaching confluence by dissociating monolayers with 1:1 0.05% trypsin:0.1% ethylenediamine tetraacetate solution (EDTA) (Life Technologies), and were re-seeded at 5×10^5 cells per 75-mm² tissue culture flask (Costar, Cambridge, MA).

Fibroblast Subset Separation

As previously described, fibroblast subset separation into Thy-1⁺ and Thy-1[−] subsets was accomplished by three to four rounds of magnetic bead selection.^{30,33} Human myometrial and Graves' orbit Thy-1⁺ and Thy-1[−] fibroblast subpopulations had a stable Thy-1 phenotype in culture, as was determined by flow cytometry before each experiment. Myometrial fibroblast subsets were >99% Thy-1⁺ and >99% Thy-1[−], while Graves' fibroblast subsets were >99% Thy-1⁺ and >97% Thy-1[−].

Preparation of Platelet Concentrate Supernatant (PCS)

Supernatant was derived from platelet concentrates (PCS) prepared for clinical use by the American Red Cross. Briefly, approximately 500 ml of human whole blood is collected into 63 ml of CP2D solution (containing sodium citrate, citric acid, sodium phosphate, and glucose) in plastic bags and stored at room temperature for up to 8 hours. Platelet-rich plasma is made by mechanically expressing the supernatant into an integrally attached plastic bag after centrifugation (all centrifugations at 20 to 24°C) at $2500 \times g$ for 3.5 minutes. The platelet-rich plasma is then centrifuged at $4300 \times g$ for 6 minutes to form a platelet pellet. Residual plasma is expressed to leave approximately 45 to 65 ml for resuspension of the platelets by gentle mechanical agitation after a 1-hour

rest period. This final platelet concentrate typically contains about 1 to 2×10^6 platelets per μl . After storage for 5 days at room temperature with gentle agitation the platelet concentrate is centrifuged at $5000 \times g$ for 6 minutes and samples of platelet concentrate supernatant are taken for use in the experiments described.

Immunohistochemistry for αSMA Expression

Human fibroblast subsets were seeded in glass chamber slides (BD Biosciences, San Diego, CA) with 10% FBS. Cells were serum-starved with 0.5% FBS for 72 hours and then left untreated or treated with recombinant human TGF β 1 (5 ng/ml; R&D Systems, Minneapolis, MN), IFN γ (500 U/ml; R&D Systems), human platelet concentrate supernatant (PCS; 1:50 from pooled platelet bags), or combination treatments in 0.1% FBS media. αSMA expression was assayed at 24 hours, 2 days, 4 days, and 6 days after treatment using immunohistochemistry. The expression of αSMA was also assessed in the absence of serum (data were not shown) and the results were similar as when αSMA was assessed in 0.1% serum. The 0.1% final serum concentration was chosen for the bulk of the studies because at the 6-day time point the fibroblast viability was reduced when cultured in serum-free conditions. For immunohistochemistry, fibroblasts were fixed with 2% paraformaldehyde. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and non-specific binding was blocked with 5% normal horse serum (Vector Labs, Burlingame, CA). Cells were then stained with a mouse monoclonal anti-human αSMA antibody (1:800; Sigma, St. Louis, MO) or with isotype control mouse IgG2a (Caltag, Burlingame, CA) overnight at 4°C. Biotinylated horse anti-mouse IgG (heavy and light chain) (1:200; Vector Labs) was added as a secondary antibody and streptavidin-horseradish peroxidase (1:1000; Jackson ImmunoResearch Labs, West Grove, PA) was added as a substrate. Samples were visualized by adding an aminoethyl-carbachol (AEC) chromogen (Zymed, South San Francisco, CA) and cover-slipped using Immu-mount (Thermo Shandon, Pittsburgh, PA). Cells were counterstained with hematoxylin.

Western Blot Analysis

Fibroblast subsets were treated as above, and then harvested using a lysis buffer and a protease inhibitor cocktail (Sigma, St. Louis, MO). Human lung fibroblasts treated with TGF β 1 for 72 hours were used as a positive control. Protein was quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Equal amounts of protein from each sample were loaded onto 10% denaturing acrylamide gels and protein was electrophoretically transferred onto nitrocellulose membranes. Blots were blocked with 10% dry nonfat milk in PBS-T (phosphate-buffered saline, 0.1% Tween) for 2 hours. The mouse monoclonal anti-human αSMA antibody (1:4000) was added in 5% blocking solution for 1 hour. Sheep anti-mouse Ig-horseradish peroxidase (1:2000 dilution in 5% blocking buffer; Amersham Biosciences, Piscataway, NJ) was added as a

secondary antibody for 1 hour. Blots were developed using an enhanced chemiluminescence kit (Amersham Biosciences).

Lipid Droplet Staining

Fibroblast subsets were seeded in glass chamber slides with 10% FBS. They were treated with 15-deoxy- $\Delta^{12,14}$ -PGJ $_2$ (referred to as 15-day-PGJ $_2$; Biomol, Plymouth Meeting, PA) or the PPAR γ agonist ciglitazone (Biomol) at a concentration of 10 $\mu\text{mol/L}$ in 10% FBS media. DMSO was used as vehicle control. Treatments were added fresh every 48 hours and lipid droplet formation was assayed at 4 and 6 days by Oil Red O staining. Cells were fixed with 10% formalin for 10 minutes, washed with water, then 60% isopropanol, and Oil Red O working solution was added for 20 minutes. Slides were rinsed with 60% isopropanol, then water, counterstained with hematoxylin and cover-slipped using Immu-mount.

Immunofluorescence Analysis for αSMA and Thy-1 Expression

Human parental myometrial and orbital fibroblast strains were analyzed for αSMA and Thy-1 expression simultaneously. αSMA was assayed as described above, using the mouse monoclonal anti-human αSMA antibody or mlgG2a overnight at 4°C. Secondary antibody horse anti-mouse IgG-biotin was used and then streptavidin-phycoerythrin (PE) (1:100; Jackson ImmunoResearch Labs) was added as a substrate. Cells were then double-stained with a mouse monoclonal anti-human Thy-1-fluorescein isothiocyanate (FITC)-labeled antibody (1:100; Serotec, Oxford, UK) or its isotype mlgG1-FITC (BD Biosciences). After washing, slides were cover-slipped using fluoro-mount and analyzed. For fluorescence analysis, a long pass cube was used for PE and a narrow band pass cube for FITC detection.

Results

αSMA Is Only Expressed in Thy-1 $^+$ Cells in Human Parental Myometrial and Orbital Fibroblasts

We first investigated the hypothesis that Thy-1 expression determined differentiation to a myofibroblastic phenotype in human fibroblast populations. To evaluate this possibility, αSMA and Thy-1 expression were assessed simultaneously in four parental human myometrial and four parental Graves' ophthalmopathy fibroblast strains using double immunofluorescence analysis. Expression of Thy-1 in these strains was previously assessed by flow cytometry to be heterogeneous, ranging from 41 to 94% in myometrial strains and from 55 to 95% in orbital strains. The Thy-1 phenotype was stable in culture. Parental fibroblast strains were treated with TGF β , a known inducer of αSMA and myofibroblast differentiation in cultured fibroblasts,^{38,39} and then double-stained for αSMA and

Figure 1. α SMA is only expressed in Thy-1⁺ human myometrial fibroblasts. Primary myometrial fibroblasts were double-stained by immunofluorescence for α SMA (PE; red) and Thy-1 (FITC; green) after a 4-day TGF β treatment (5 ng/ml), as described in Materials and Methods. Isotype controls for α SMA and Thy-1 were mIgG2a and mIgG1 respectively, and stained negative for PE or FITC. Fibroblasts within the parental culture stained positive for both α SMA and Thy-1. Fibroblasts negative for Thy-1 were also negative for α SMA, and they are indicated in the phase picture by **arrows**. Original magnification, $\times 20$.

Thy-1 as described in Materials and Methods. α SMA was visualized with the PE (red) fluorochrome and Thy-1 was directly conjugated to FITC (green). Isotype controls for α SMA (mIgG2a) and Thy-1 (mIgG1) stained negatively for PE or FITC, respectively (Figures 1 and 2). Intracellular or alternatively diffuse external staining was observed for Thy-1. It is clearly shown in Figures 1 and 2, that myometrial and orbital fibroblasts expressing Thy-1 were also positively stained for α SMA. Accordingly, fibroblasts that lacked Thy-1 expression (indicated by arrows in the phase picture) were also negative for α SMA induction in both human myometrium and orbit. These results indicated that only Thy-1⁺ fibroblasts were responsible for α SMA induction and hence differentiation to a myofibroblastic phenotype.

Figure 2. α SMA is only expressed in Thy-1⁺ human orbital fibroblasts. Primary Graves' orbital fibroblasts were treated with TGF β for 4 days and then double-stained for α SMA by PE labeling (red) and for Thy-1 with FITC (green), as described in Materials and Methods. Isotype controls mIgG2a and mIgG1 stained negative for PE or FITC. Fibroblasts within the parental orbital culture that expressed Thy-1 were also positive for α SMA. Fibroblasts negative for Thy-1 did not stain for α SMA, and they are indicated in the phase picture by **arrows**. Original magnification, $\times 20$.

A. α SMA, Thy-1⁺ myometrial subset

B. α SMA, Thy-1⁻ myometrial subset

Figure 3. TGF β and platelet concentrated supernatant (PCS)-induced α SMA expression in Thy-1⁺, but not Thy-1⁻ human myometrial fibroblasts; IFN γ antagonizes α SMA induction. Myometrial fibroblasts were left unstimulated or treated with TGF β (5 ng/ml), PCS (1:50), IFN γ (500 U/ml), TGF β with IFN γ or PCS with IFN γ for 4 days. Cells were then stained for α SMA as described in Materials and Methods. **A:** Unstimulated Thy-1⁺ myometrial fibroblasts express constitutive α SMA in a heterogeneous fashion. TGF β and PCS induce α SMA expression in Thy-1⁺ fibroblasts, and this up-regulation is reduced by IFN γ . Original magnification, $\times 40$. **B:** Thy-1⁻ myometrial fibroblasts do not express α SMA, even after TGF β or PCS treatments. Original magnification, $\times 20$.

TGF β and Human Platelet Concentrate Supernatant (PCS) Induced α SMA Expression in Thy-1⁺, but Not Thy-1⁻ Human Myometrial Fibroblasts; IFN γ Antagonizes α SMA Induction

We next investigated a myometrial parental strain where 41% of the fibroblasts were Thy-1⁺. Separation into subsets based on Thy-1 expression was accomplished using magnetic bead selection.³⁰ This resulted in two fibroblast subsets, one of which contained $>99\%$ Thy-1⁺ cells and the other displaying $<1\%$ Thy-1⁺ cells. To evaluate presence of a myofibroblastic phenotype, fibroblast subsets were assessed for α SMA, a characteristic actin isoform also expressed by smooth muscle cells and myofibroblasts.¹⁶ Human Thy-1⁺ and Thy-1⁻ myometrial fibroblast subpopulations were then subjected to selected treatments, and α SMA assessed after 4 days by immunohistochemistry. TGF β was used as a known inducer of α SMA expression and the myofibroblastic phenotype. Subsets were also treated with human platelet concentrate supernatant (PCS), since platelets are abundant in areas of injury and wound healing. IFN γ is a prototypic

Th1/Type 1 cytokine that has been demonstrated to inhibit TGF β /SMAD signaling,⁴⁰ and in addition to modulate α SMA expression in cultured fibroblasts.¹⁴ IFN γ was therefore included in the TGF β and PCS treatments to determine its effects on myometrial myofibroblast differentiation. As demonstrated in Figure 3, only the Thy-1⁺, but not the Thy-1⁻ subset, was capable of expressing α SMA. Interestingly, α SMA was expressed at various levels within unstimulated Thy-1⁺ myometrial fibroblasts (Figure 3A). This expression was dramatically up-regulated with TGF β and PCS treatments and was reduced to basal levels when IFN γ was added in combination. Thy-1⁻ myometrial fibroblasts failed to induce α SMA when subjected to the same treatments (Figure 3B).

α SMA Expression Is Up-Regulated in TGF β and PCS-Treated Thy-1⁺, but Not Thy-1⁻ Human Orbital Fibroblasts; IFN γ Inhibits α SMA Up-Regulation

To support the finding that myofibroblast differentiation was unique to the Thy-1⁺ fibroblast subset across tissues, α SMA expression was next examined in human orbital Thy-1⁺ and Thy-1⁻ fibroblast subsets, derived from a parental orbital strain that was initially 65% Thy-1⁺. Subsets were generated as previously described,³³ resulting in one subpopulation expressing >99% Thy-1⁺ cells, and the other consisting of <3% Thy-1⁺ cells. Human Thy-1⁺ and Thy-1⁻ orbital fibroblast subpopulations were then subjected to the same treatments as described above for myometrial subsets, and α SMA expression was documented after 4 days using immunocytochemistry. The results were consistent with those observed in myometrial subsets, with the exception that unstimulated Thy-1⁺ orbital fibroblasts did not constitutively express α SMA. This difference in basal α SMA expression between myometrial and orbital Thy-1⁺ fibroblasts further supports fibroblast heterogeneity across tissues. As shown in Figure 4A, TGF β and PCS treatments induced α SMA expression only in Thy-1⁺ orbital fibroblasts, and IFN γ completely inhibited this up-regulation. Furthermore, Thy-1⁻ orbital fibroblasts were not capable of α SMA induction after TGF β or PCS treatments (Figure 4B).

α SMA Protein Induction in Human Myometrial and Orbital Human Fibroblast Subsets

Western blot analysis for α SMA expression by both human myometrial and orbital Thy-1⁺ and Thy-1⁻ fibroblast subsets was next performed, to quantitate the amount of α SMA. Fibroblast subsets were treated with TGF β or PCS for 24 hours, 48 hours, 4 days, or 6 days. Some fibroblasts were also incubated with IFN γ to assess its inhibitory effects. Human lung fibroblasts treated with TGF β for 72 hours were used as positive control for induction of α SMA. Fibroblasts were harvested in lysis buffer and equal amounts of protein were subjected to Western blot analysis for α SMA (Figures 5 and 6). These results con-

Figure 4. α SMA expression is up-regulated in TGF β - and PCS-treated Thy-1⁺, but not Thy-1⁻ human orbital fibroblasts; IFN γ inhibits α SMA up-regulation. Graves' orbital fibroblasts were left unstimulated or treated with TGF β (5 ng/ml), PCS (1:50), IFN γ (500 U/ml), TGF β with IFN γ , or PCS with IFN γ for 4 days. Cells were then stained for α SMA as described in Materials and Methods. **A:** Unstimulated Thy-1⁺ orbital fibroblasts do not express α SMA. After TGF β and PCS treatments, α SMA expression is induced and this up-regulation is completely inhibited by IFN γ . Original magnification, $\times 40$. **B:** Thy-1⁻ orbital fibroblasts are not positive for α SMA staining when treated with TGF β or PCS. Original magnification, $\times 40$.

firmed observations with immunohistochemical techniques. Myometrial and orbital Thy-1⁻ fibroblasts only expressed a very faint band for α SMA at any of the indicated time points after TGF β or PCS treatments, which was not suggestive of significant levels of α SMA (Figures 5 and 6). Myometrial Thy-1⁺ fibroblasts express modest amounts of α SMA constitutively, and this expression was up-regulated with TGF β as early as 24 hours after treatment, and was sustained through 6 days (Figure 5A). IFN γ reduced the α SMA expression induced by TGF β to the same level as unstimulated fibroblasts. PCS treatment resulted in α SMA up-regulation at 4 days and 6 days. IFN γ decreased the PCS-induced α SMA expression to basal levels (Figure 6A). Human orbital Thy-1⁺ fibroblasts did not express α SMA when untreated, but TGF β treatment resulted in α SMA induction at 48 hours, which peaked to a great extent at 4 days and was sustained at 6 days (Figure 5B). It is clearly shown that IFN γ completely blocked the ability of TGF β to induce α SMA expression (Figure 5B). Induction of α SMA by PCS was less prominent when compared to TGF β treatment, but was still up-regulated compared to unstimulated cells (Figure 6B). The PCS-induced α SMA was also totally inhibited by IFN γ .

A. Myometrial fibroblasts

B. Orbital fibroblasts

Figure 5. TGF β induces α SMA protein expression in Thy-1⁺ myometrial and orbital fibroblast subsets and up-regulation is inhibited by IFN γ . Myometrial and orbital fibroblasts were left unstimulated or treated with TGF β (5 ng/ml) for 24 hours, 48 hours, 4 days, and 6 days, with or without IFN γ . Cells were harvested in lysis buffer and subjected to Western blot analysis, as described in Materials and Methods. Human lung fibroblasts treated with TGF β for 72 hours, were used as positive control indicated by (+). Unstimulated cells are designated by “untx”. **A:** Myometrial Thy-1⁺ fibroblasts express constitutive α SMA, which is up-regulated as early as 24 hours with TGF β treatment, and is sustained through 6 days. IFN γ reduces the induced α SMA to basal levels. Thy-1⁻ myometrial fibroblasts do not express α SMA under the same treatments. **B:** α SMA in Thy-1⁺ orbital fibroblasts is up-regulated at 48 hours and peaks to high levels at 4 and 6 days after TGF β treatment. IFN γ completely blocks α SMA induction. Thy-1⁻ orbital fibroblasts do not display α SMA expression.

Differentiation to a Lipofibroblastic Phenotype Is Restricted to Thy-1⁻ Fibroblast Subsets in Human Myometrium and Orbit

To evaluate a second important phenotypic attribute that can result after fibroblast differentiation, we examined the potential of human Thy-1⁺ and Thy-1⁻ myometrial and orbital fibroblasts to develop into lipofibroblasts. Human Thy-1⁺ and Thy-1⁻ myometrial and orbital fibroblasts were exposed to adipogenic stimuli and then assessed for their capacity to produce lipid droplets by Oil Red O staining. Fibroblast subsets were incubated with the PPAR γ agonist ciglitazone or with the natural PPAR γ ligand, 15-day-PGJ₂, for 4 or 6 days. It was very exciting to observe that only Thy-1⁻ myometrial and orbital fibroblast subsets were capable of developing lipid droplets in their cytoplasm after ciglitazone or 15-day-PGJ₂ treatments (Figure 7, A and B). Thy-1⁺ fibroblasts were not induced to acquire a lipofibroblastic phenotype when exposed to the same conditions (Figure 7, A and B). Even though we do not know any details about the nature of the accumulating lipids in the Thy-1⁻ lipofibroblasts, in other cells undergoing fatty differentiation triglyceride accumulation is a prominent component.

Discussion

Fibroblast heterogeneity applies to both phenotypic and functional properties exhibited by a fibroblast population within or across tissues. In the current study, we investigated phenotypic differences that occurred in human

A. Myometrial fibroblasts

B. Orbital fibroblasts

Figure 6. PCS up-regulates α SMA protein in Thy-1⁺ myometrial and orbital fibroblasts and induction is reduced by IFN γ . Myometrial and orbital fibroblasts were left unstimulated or treated with PCS (1:50) for 24 hours, 48 hours, 4 days, and 6 days, with or without IFN γ . Cells lysates were subjected to Western blot analysis, as described in Materials and Methods. Human lung fibroblasts treated with TGF β for 72 hours, were used as positive control indicated by (+). Unstimulated cells are designated by “untx”. **A:** PCS induces α SMA in myometrial Thy-1⁺ fibroblasts after 4 and 6 days of treatment. IFN γ reduces the induced α SMA to basal levels. Thy-1⁻ myometrial fibroblasts do not express α SMA under PCS treatments. **B:** α SMA in Thy-1⁺ orbital fibroblasts peaks at 4 and 6 days after PCS treatment. IFN γ inhibits α SMA induction. Thy-1⁻ orbital fibroblasts do not express α SMA under the same conditions.

Thy-1⁺ and Thy-1⁻ fibroblast subpopulations after a differentiation or activation process. Herein, reported for the first time, is that Thy-1 expression by human fibroblast subsets determines differentiation to a myofibroblastic or lipofibroblastic phenotype. These results further support functional heterogeneity in human myometrial and orbital fibroblast subsets.^{30,33} The data presented herein, support that fibroblasts expressing Thy-1 have the potential of becoming myofibroblasts, while fibroblasts that lack Thy-1 surface expression represent a pool of pre-adipocytes and can differentiate into lipofibroblasts. It is possible that different fibroblastic phenotypes may exert specific functional properties in defined anatomical locations, for example within human myometrium or orbit.

Some studies in the literature suggest that fibroblasts might be heterogeneous between organs, hence the emerging concept of “tissue-specific” fibroblasts; this was previously demonstrated by functional diversity between fibroblasts derived from different tissues.^{41,42} Little is known about reproductive tract fibroblasts, but orbital fibroblasts are also different from fibroblasts derived from other tissues.^{7,30,36,43} Since each organ exhibits unique functions to maintain normal physiology, it is expected that fibroblasts derived from different tissues will be functionally diverse. Our observations in the present study support the concept of “tissue-specific” fibroblasts, since human Thy-1⁺ myometrial fibroblasts express α SMA in a constitutive, heterogeneous manner, a feature missing from human orbital Thy-1⁺ fibroblasts. The difference in basal *in vitro* α SMA expression between myometrial and orbital Thy-1⁺ fibroblasts further supports fibroblast heterogeneity across tissues, and suggests that myofibroblasts may be needed in human myometrium to maintain

A. Myometrial fibroblasts

B. Orbital fibroblasts

Figure 7. Differentiation to a lipofibroblastic phenotype is restricted to Thy-1[−] fibroblasts from human myometrium and orbit. Human myometrial and orbital fibroblast subsets were treated with DMSO (vehicle control) or 15-day-PGJ₂ (10 μ mol/L) every other day for 6 days. Cells were then stained for cytoplasmic lipid droplets by Oil Red O. **A:** Only Thy-1[−] myometrial fibroblasts acquire lipid droplets after treatment with 15-day-PGJ₂. **B:** Thy-1[−], but not Thy-1⁺ orbital fibroblasts accumulate lipid droplets in their cytoplasm when treated with 15-day-PGJ₂.

normal homeostatic functions and tissue physiology.^{44,45} Fibroblasts from other normal connective tissues that require contraction or tissue remodeling for organ function have been reported to express constitutive α SMA in a subpopulation of cells, such as from ovarian follicles,

the uterus, mammary glands, the pulmonary septa, and the periodontal space.^{14,18}

We demonstrate that TGF β , a known inducer of myofibroblast differentiation, up-regulates α SMA expression only in Thy-1⁺ myometrial and orbital fibroblasts, distin-

guishing them in this phenotypic characteristic from Thy-1[−] fibroblasts. Interestingly, we also observed an induction of the myofibroblastic phenotype when Thy-1⁺ subsets were treated with PCS. This finding is novel and has important implications in the role of Thy-1⁺ fibroblasts in wound healing processes, both in human myometrium and orbit, where platelets are key instigators. Myofibroblasts in the eye orbit are present as a result of pathology, such as occurs in abnormal wound healing and fibrosis.¹³ In addition to wound healing and fibrotic processes, myometrial myofibroblasts may be responsible for normal functions, since α SMA expression has been linked to contractility. Our recent finding that platelets release CD40 ligand (L), implicates an interaction between fibroblasts and platelets through the CD40-CD40L pathway,⁴⁶ another established conduit by which myometrial and orbital fibroblasts can be activated.^{30,33,47,48} We thus suggest that platelets first induce myofibroblast differentiation in the healing wound, and these activated, differentiated fibroblasts enhance the healing process by secreting appropriate mediators. Even though we have not yet identified which substances in PCS induce myofibroblast differentiation, TGF β appears to be a candidate mediator. In the future we would like to pinpoint the active moieties in the PCS resulting in α SMA induction. Since sustained activation and thus chronic presence of myofibroblasts in the organs studied may lead to pathological conditions, like abnormal wound healing and/or fibrosis, it is imperative to be able to recognize the potential of a particular tissue to differentiate along the myofibroblastic pathway, demonstrated herein to be achievable by fibroblast Thy-1 display.

The role of IFN γ as an inhibitor of TGF β -induced α SMA formation has been described in the literature for rat palatal and human dermal fibroblasts.^{18,49} We clearly demonstrate that the same pattern occurs in human Thy-1⁺ myometrial and orbital fibroblasts, where IFN γ not only blocks TGF β -induced, but also PCS-induced α SMA expression. We speculate that TGF β in the PCS induces myofibroblast differentiation, but other factors such as PDGF or CD40L may also play a role. Also, thrombospondin-1 is released by platelets and can activate TGF β ,^{50,51} suggesting additional means by which PCS might induce myofibroblast formation. Identifying a factor so potent in inhibiting myofibroblast formation, such as IFN γ , may lead to potential therapeutics in pathological conditions associated with α SMA expressing stromal cells, such as hypertrophic scars, fibromatoses, and scleroderma.^{18,52}

Of additional importance to disease pathogenesis is the accumulation of fatty tissue within an organ. This is usually the result of injury or disease and occurs in radiation-induced lung fibrosis,^{29,53,54} the liver,⁵⁵ kidney,⁴¹ and in the eye orbit.⁵⁶ Fibroblasts are key effector cells in Graves' ophthalmopathy, responsible for the connective tissue remodeling, and are a rich source of inflammatory mediators. We have recognized in the past that a subset of orbital fibroblasts can be induced to differentiate into mature adipocytes *in vitro*.^{27,28} When incubated in a serum-free medium containing several factors, including those enhancing cAMP, a small fraction of these cells

undergo a dramatic change in morphology and accumulate cytoplasmic lipid droplets. Furthermore, this subpopulation failed to display Thy-1 on their surface.²⁸ We hypothesized that lack of Thy-1 expression in human fibroblasts determined a lipofibroblastic phenotype. We clearly demonstrate herein that only Thy-1[−] human myometrial and orbital fibroblasts are capable of lipofibroblast differentiation, extending our previous observations.²⁸ The potential to accumulate lipid droplets has immense implications for the Thy-1[−] orbital fibroblast in Graves' disease, where increases in orbital connective tissue and fat are responsible for most of the detrimental manifestations of ophthalmopathy. Even though Thy-1[−] myometrial fibroblasts can form lipid droplets *in vitro*, fat accumulation in human myometrium appears to be a rare occurrence. Since a much more complex microenvironment exists *in vivo*, it is possible that lack of lipid-like cells in the myometrium may result from adipogenesis inhibition through regional cytokine and extracellular matrix profiles. Indeed, mediators implicated in maintaining myofibroblast formation such as TGF β and TNF α , have the capacity to impair adipogenesis.^{18,57,58} Extracellular matrix components also play important functional roles in their microenvironment. For example, fibronectin and thrombospondin-1 are both essential for TGF β -induced myofibroblast formation^{39,59} and their presence may affect the outcome of differentiation. Decorin on the other hand, a known inhibitor of TGF β , may act to suppress myofibroblast differentiation.⁶⁰ Interestingly, an essential ECM factor for myofibroblast formation, fibronectin, also exerts a negative regulatory role on adipogenesis,⁶¹ perhaps ensuring the development of one phenotype *in vivo* and not the other. It will be interesting to determine how these phenotypes are regulated in each tissue *in vivo*. The balance of regional mediator production may play crucial roles in the outcome of differentiation in each tissue. It seems likely that in the myometrium, the local microenvironment may not be such to support adipogenesis.

Increasing research on fibroblasts has made clearer the concept of subset specialization. Our current report supports the existence of subset specialization in the human myometrium and orbit with respect to their regional differentiation potential. It is possible that fibroblasts acquire a new gene repertoire once differentiated to either myofibroblastic or lipofibroblastic phenotype, resulting in the production of a new cytokine profile that suits their acquired function. Our results suggest that it is possible to pre-determine which fibroblast subpopulation can adopt a particular phenotype before the actual differentiation process, by means of their surface Thy-1 expression. The percentage of Thy-1⁺ fibroblasts within a particular tissue or organ may thus provide evidence of differentiation pathway potential. This finding may prove a useful diagnostic tool in disorders where lipid-forming cells or persistent myofibroblast presence affects the outcome and severity of the disease, such as in Graves' disease, tissue fibrosis, abnormal wound healing, and hypertrophic scars.

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