Fibrocytes are a distinct population of fibroblast-like progenitor cells in peripheral blood that have recently been shown to possess plasticity to differentiate along mesenchymal lineages, including commitment to myofibroblast and adipocyte cells. Here, we demonstrated that transforming growth factor (TGF) \( \beta 1 \) drives fibrocyte-to-myofibroblast differentiation through activating Smad2/3 and SAPK/JNK MAPK pathways, which in turn stimulates \( \alpha \)-smooth muscle actin expression. We determined that SAPK/JNK signaling acts in a positive feedback loop to modulate Smad2/3 nuclear availability and Smad2/3-dependent transcription. Conversely, fibrocyte-to-adipocyte differentiation is driven by the peroxisome proliferator-activated receptor (PPAR) \( \gamma \) agonist troglitazone, which is associated with cytoplasmic lipid accumulation and induction of aP2.

Differentiation of Human Circulating Fibrocytes as Mediated by Transforming Growth Factor-\( \beta \) and Peroxisome Proliferator-activated Receptor \( \gamma \)

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A large body of evidence now supports the existence of adult stem cells. Although they are predominantly found in the bone marrow, they have also been isolated from muscle, adipose tissue, connective tissue, and in peripheral circulation. Adult stem cells are characterized by their ability to differentiate along multiple lineages, giving rise to fully differentiated cells with distinct function. Multiple signaling networks orchestrate the development and differentiation of adult stem cells into functional mesenchymal, neuronal, and epithelial lineages.

We recently characterized the plasticity of an adult progenitor cell found in circulation, termed fibrocytes, which can differentiate into myofibroblast, osteoblast, and adipocyte lineages (1, 2). Fibrocytes are a distinct population of fibroblast-like cells in peripheral blood with a unique cell surface phenotype, expressing CD45RO, CXCR4, and collagen I (3, 4). They are distinct from tissue fibroblasts, monocyte/macrophages, T and B lymphocytes, dendritic cells, or their precursors, as well as epithelial and endothelial cells (5, 6).

Known functions of fibrocytes include potent stimulation of \( T \) cells in antigen-specific immunity (7), wound healing following injury (4), as well as pathologic fibrosis in response to local inflammation (2).

The human transforming growth factor-\( \beta \) isoforms constitute extracellular signaling molecules that have pleiotropic functions. Transforming growth factor-\( \beta 1 \) (TGF\( \beta 1 \)) serves as a key fibrogenic mediator that initiates signaling by binding to type I and type II receptor kinases (T\( \beta R \)). This activates receptor-associated Smads such as Smad2 and Smad3, allowing them to complex with Smad4 for translocation to the nucleus. Alternatively, T\( \beta R \) can also signal through other pathways, the most prominent being mitogen-activated protein kinase (MAPK) (8, 9). Members of the MAPK family include ERK as well as two stress-activated protein kinases (SAPK): the c-Jun N-terminal kinase (JNK) and the p38 pathway. The mechanism whereby TGF\( \beta 1 \) induces activation of MAPK pathways was recently elucidated and involved activation through the upstream mediators Ras, RhoA, and TGF-activated kinase 1 (TAK1) (10, 11).

Adipocyte differentiation is a complex process regulated by extracellular hormones and cytokines. Appropriate environmental exposure leads to up-regulation of specific transcription factors that serve as master regulators in the activation of adipocyte-specific genes, such as leptin and aP2 (12). One such transcription factor is peroxisome proliferator-activated receptor \( \gamma \) (PPAR\( \gamma \)). Following binding to natural ligands (i.e. 15-deoxy-PGJ\(_2\)) or synthetic agonists (i.e. troliglizone), PPAR\( \gamma \) becomes activated and forms a heterodimer with RXR\( \alpha \) (13, 14). The complex then translocates to the nucleus and binds to specific PPAR response elements (PPREs) in the promoter.

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region of its target genes, such as aP2, and contributes to differentiation (15).

In the present study, we examined the role of TGFβ1 in activating distinct signaling pathways that lead to the stimulation of α-smooth muscle actin (SMA) expression during myofibroblast differentiation. Under different environmental cues, fibrocytes can differentiate into adipocytes, a process that involves activation of the PPARγ pathway leading to induction of aP2 expression. TGFβ1 signaling in fibrocytes activates both Smad 2/3 and MAP kinases, specifically the ERK1/2 and SAPK/JNK pathways. PPARγ agonists can negatively regulate this process, through a PPARγ-independent pathway. A blockade of the SAPK/JNK pathway either by troglitazone or by chemical treatment (JNK inhibitor) inhibited TGFβ1-induced aSMA expression. Conversely, we found that TGFβ1 signaling suppressed PPARγ activity and aP2 expression. TGFβ1-induced SAPK/JNK phosphorylation leads to downstream signaling that negatively affects the transactivation activity of PPARγ. Taken together, SAPK/JNK signaling affected the divergence of adipogenic and myofibrogenic differentiation of fibrocytes. This is important because it provides a therapeutic target whereby through the use of a synthetic PPARγ agonist we are able to block a key TGFβ1-mediated pro-fibrotic effect, which has exciting implications for therapy of currently untreated fibrotic diseases.

EXPERIMENTAL PROCEDURES

Fibrocyte Isolation—Fibrocytes were harvested from peripheral blood mononuclear cells according to previously published methods (2, 3). Briefly, peripheral blood mononuclear cells were isolated from human leukopheresis packs by gradient centrifugation over Ficoll-Paque (Amersham Biosciences). Following culturing on fibronectin-coated flasks for 72 h in Dulbecco’s modified Eagle’s medium with 20% fetal bovine serum and 4% L-glutamine, non-adherent cells were removed. The adherent cells were supplemented with new media and remained incubated for 7–10 days. The cells were then gently detached from flasks by incubation with Accutase (Innovative Cell Technologies, San Diego, CA) for 10 min at 37 °C. This crude fibrocyte preparation was purified of contaminating monocytes/macrophages, T cells, and B cells by magnetic immunodepletion using anti-CD14; pan-T, anti-CD2; and pan-B, anti-CD19 Dynabeads, respectively (Dynal Inc., Brown Deer, WI).

Myofibroblast and Adipocyte Differentiation—For myofibroblast differentiation, enriched fibrocytes were treated with serum-depleted Dulbecco’s modified Eagle’s medium supplemented with 10 ng/ml TGFβ1. Culture media was changed every 48–72 h. For adipocyte differentiation, fibrocytes were treated with PBM culture media (Cambrex, Charles City, IA) supplemented with 10 μM troglitazone. Culture media was changed every 48 h. All reagents with the exception of TGFβ1 (R&D Systems, Minneapolis, MN) were purchased from Sigma.

Oil Red O Staining—Cells were stained with Oil Red O to assess for lipid accumulation, as previously described (16). Briefly, cells are washed with 1× phosphate-buffered saline, fixed in 10% formalin solution for 1 h at room temperature, rinsed twice with phosphate-buffered saline, followed by wash with 60% isopropyl alcohol for 5 min. Cells are then stained with Oil Red O solution for 90 min, followed by a gentle rinse with water. Cells are counterstained with hematoxylin for 3 min and washed with water before microscopic visualization.

Real Time RT-PCR—Total RNA was prepared using TRIzol (Invitrogen) as previously described (1, 2). For RT-PCR analysis, 2.0 μg of RNA was converted to cDNA utilizing random hexamer primers and reverse transcriptase from Maloney murine leukemia virus. Real time PCR was performed using an ABI Prism 7700 sequence detector and SDS analysis software (PE Applied Biosystems, Foster City, CA). The following specific oligonucleotide primers were used in experiments: aSMA, forward, 5′-CGGCTTTTCTGTGGTTATG-3′, reverse, 5′-CCCTCGATGGATGGGAAA-3′; aP2, forward, 5′-GGAAAATCAACCCATAAAGA-3′, reverse, 5′-GGAAGTGACGCCCTTCATGAC-3′.

Immunocytochemistry—Fibrocyte-derived myofibroblast or adipocyte cell monolayers were fixed in 4% paraformaldehyde for 2 h and then stained for aSMA or aP2 antibodies using the Vectastain ABC system (Vector Laboratories, Burlingame, CA). Briefly, cells were incubated with a 1:1 mixture of 3% hydrogen peroxide in methanol. Nonspecific binding sites were blocked with PowerBlock (Biogenex, San Ramon, CA) for 30 min, washed, and overlaid with either control or species-specific anti-human antibody. Slides were then rinsed and overlaid with secondary biotinylated goat anti-rabbit IgG and incubated for 30 min. After washing twice with phosphate-buffered saline, slides were overlaid with Vectastain ABC systems peroxidase-conjugated streptavidin and incubated for 30 min. 3,3′-Diaminobenzidine tetrahydrochloride reagent was used for chromogenic localization of antibody. After optimal color development, sections were immersed in sterile water, counterstained with Mayer’s hematoxylin, neutralized in 10% ammonia, and coverslipped with Permount solution after drying overnight.

Last, for immunostaining experiments that examined the role of TGFβ1 in activating Smad2/3, we treated fibrocytes with 10 ng/ml TGFβ1 for 60 min. The cells were stained with either appropriate isotype control or Smad2/3 antibody to monitor for nuclear localization of Smad2/3 proteins.

Immunoblot Analysis—Immunoblotting was performed on 50 μg of protein from cellular nuclear/cytoplasmic fractions as previously described (1, 2). In brief, cell monolayer in flasks were washed with Hanks’ balanced salt solution and then treated with lysis buffer, after which lysates were heated at 100 °C for 10 min and clarified by centrifugation. Equal amounts of protein (50 μg) were then loaded onto 10% gradient gels (Amersham Biosciences) and electrophoresed at 100 V until the dye-front reached the end of the gel. The gels were transferred to nitrocellulose membrane using the manufacturer’s suggested protocol. The membranes are blocked using 5% (w/v) evaporated milk in Tris-buffered saline containing 0.001% (v/v) Tween 20. The membranes were probed with primary antibody overnight using antibodies against the targets listed: ERK1/2, pERK1/2, SAPK/JNK, pSAPK/JNK, p38 MAPK, pp38 MAPK (all from Cell Signaling Technology, Danver, MA), aSMA (R&D Systems), PPARγ (Upstate, Billerica, MA), aP2 (Hycult Biotechnology, Uden, Netherlands), and GAPDH.
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Appropriate horseradish peroxidase-conjugated secondary antibodies were used based on species-specific requirements; final chemiluminescence detection is based on the ECL kit (Amersham Biosciences) per the manufacturer’s protocol.

Vector Transfection—Fibrocytes were transfected using Amaxa Nucleofection Technology™ (Amaxa, Koeln, Germany). Cells were re-suspended in solution from a nucleofector kit, also available as part of the Amaxa cell optimization kit, following Amaxa guidelines for cell line transfection (see Amaxa literature). We have optimized this kit for use with fibrocytes. Briefly, 100 μl of 5 × 10^6 fibrocyte suspension mixed with 2 μg of cDNA was transferred to the provided cuvette and nucleofected with an Amaxa Nucleofector apparatus (Amaxa). Cells were transfected using the U023 pulsing parameter and immediately transferred into wells containing 37 °C pre-warmed serum-starved culture medium in 6-well plates. After transfection, cells were cultured from 8 h before treatment with appropriate parameters. PPRE (17) and Smad binding element (SBE) luciferase reporter gene plasmid as well as PPARγ over-expression vector (pCMV-PPARγ) were obtained from Panomics Inc. (Fremont, CA). PPRE (PPRE3X-Lux) and SBE (SBE3X-Lux) luciferase reporter constructs were used to monitor PPARγ and SMAD transactivation activities, respectively, with each vector containing multiple repeat-specific consensus binding sites. Twelve hours after transfection, fibrocytes were subjected to treatment based on specific experimental design. Cells were then harvested and luminescence was measured in a microplate luminometer.

RNA Interference—RNA duplex siRNA for JNK1 was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A control scramble siRNA duplex was also used. 8 × 10^6 cells were plated into a 6-well plate with fresh medium without antibiotics 24 h before testing. siRNA transfection was performed according to the manufacturer’s protocol, except for modifications as noted below. Briefly, 160 pmol of siRNA was used per 100 μl of siRNA duplex medium. The cells were incubated for 3.5 h with siRNA duplex solution, followed by treatment in growth media containing 15% fetal calf serum. After siRNA transfection, the cells were incubated for three additional days at 37 °C before additional treatment and analysis. To assess and optimize transfection efficiency, GAPDH siRNA was used to examine appropriate GAPDH gene silencing in fibrocytes.

Statistical Analysis—Differences between groups were compared using either the Mann-Whitney U test if the data were not normally distributed, or the Student’s t test if the observations were consistent with a sample from a normally distributed population. Data were analyzed on an IBM PC computer using GraphPad Prism 4 version 4.00 for Windows (GraphPad Software, San Diego CA). Results were determined to be statistically significant if p < 0.05 unless otherwise specified.

RESULTS

Fibrocytes Possess the Ability to Differentiate to Myofibroblasts and Adipocytes—Fibrocytes were extracted from peripheral blood mononuclear cells (Fig. 1A). After 10 days of expansion in vitro, contaminating T cells, B cells, and monocytes were removed using negative selection with magnetic beads (1, 2). The purified fibrocyte population expressed CXCR4, Col I, and CD45RO. These cells do not express myofibroblast marker αSMA or the mature adipocyte marker, adipocyte lipid-binding protein (aP2, also known as FABP4). To induce fibrocyte differentiation to myofibroblasts, the cells were cultured in media supplemented with TGFβ1 (10 ng/ml) for 3 weeks. To evaluate the phenotype of fibrocytes following differentiation, we performed immunocytochemical staining for the mature myofibroblast protein αSMA. As compared with the isotype control, the majority of fibrocytes treated with TGFβ1 markedly

FIGURE 1. Differentiation of human circulating fibrocytes to myofibroblasts and adipocytes. A, morphology of purified fibrocytes following in vitro expansion for 2 weeks. B, fibrocyte differentiation to myofibroblasts. Cultures of fibrocytes in chamber slides were treated with 10 ng/ml TGFβ1 for 3 weeks (culture media changed every 48 h). The cells are stained with either αSMA or appropriate isotype control antibody. All slides were also counterstained with hematoxylin. C, fibrocyte differentiation to adipocytes. Cultures of fibrocytes in chamber slides were treated with 10 μM troglitazone for 3 weeks (culture media changed every 48 h). Fibrocyte-derived adipocytes transformed into cells of rounder morphology are shown in the top left panel. Adipogenesis is associated with intracellular lipid accumulation as evident by positive Oil Red O staining (top right panel). The cells are stained with either appropriate isotype control (bottom left panel) or aP2 (bottom right panel) antibodies. All slides are also counterstained with hematoxylin. Positively stained cells for αSMA and aP2 appear as reddish brown.

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expressed cytoplasmic \( \alpha \text{SMA} \) (Fig. 1B). No appreciable change in cell morphology was seen with myofibroblasts as compared with untreated fibrocytes. In cells maintained in media without TGF\( \beta 1 \) supplementation, a small population of cells (\( \sim 15\% \)) underwent spontaneous differentiation to myofibroblast with associated expression of \( \alpha \text{SMA} \).

We previously demonstrated the ability of fibrocytes to undergo differentiation to adipocytes when exposed to cyclic treatment of adipogenic induction media containing indomethacin, dexamethasone, 3-isomethylxanthine, and insulin (1). An up-regulation of PPAR\( \gamma \) was seen in fibrocyte-derived adipocytes. Here we demonstrate that treatment of fibrocytes with a synthetic PPAR\( \gamma \) ligand, troglitazone (TGZ), induced similar fibrocyte to adipocyte differentiation. Following 21 days in culture, the cells were observed to accumulate lipids in intracellular vacuoles, as evidence by extensive Oil Red O Staining (Fig. 1C). Unlike differentiation to myofibroblasts, fibrocytes treated with TGZ transformed into cells of rounder morphology (Fig. 1C). To confirm differentiation into adipocytes, we performed immunocytochemical staining for the mature adipocyte marker \( \alpha \text{P2} \). As compared with isotype controls, fibrocyte-derived adipocytes stained extensively for \( \alpha \text{P2} \), localized in proximity to intracellular lipid vesicles (Fig. 1C). Whereas a small population of fibrocytes underwent spontaneous differentiation of \( \alpha \text{SMA} \) expressing myofibroblasts, untreated cells did not express \( \alpha \text{P2} \) even after 6 weeks (data not shown). Not all treated cells underwent differentiation to adipocytes, as only \( \sim 45\% \) of the cells committed to the adipose lineage. No increase in adipogenic differentiation was observed even with extended treatment.

**TGF\( \beta 1 \) Induces \( \alpha \text{SMA} \) Expression in Fibrocytes and Promotes Its Differentiation to Myofibroblast**—We next examined the role of TGF\( \beta 1 \) in induction of \( \alpha \text{SMA} \) expression. We assessed the kinetics and dose-dependence effect of TGF\( \beta 1 \) activation in fibrocytes. Fibrocytes were treated with varying concentrations of TGF\( \beta 1 \) (0.3–100 ng/ml) for different time periods (1–48 h). Semi-quantitative RT-PCR analysis showed that TGF\( \beta 1 \), at concentrations of 10–100 ng/ml, was able to induce \( \alpha \text{SMA} \) transcription (Fig. 2A). The maximal induction was observed with 10 ng/ml TGF\( \beta 1 \), with \( \alpha \text{SMA} \) mRNA level increasing 7-fold as compared with unstimulated cells. We also performed kinetic evaluation of \( \alpha \text{SMA} \) expression using TGF\( \beta 1 \) (10 ng/ml). The \( \alpha \text{SMA} \) mRNA transcript was elevated within 8 h after treatment, with an increase of 4.1-fold (Fig. 2B). It was increased to a maximal of 7.3-fold by 24 h. Sustained increase in TGF\( \beta 1 \)-stimulated \( \alpha \text{SMA} \) transcripts were seen even at 48 h.

To confirm our findings, we next looked at protein expression of \( \alpha \text{SMA} \) following TGF\( \beta 1 \) treatment. Fibrocytes were treated with TGF\( \beta 1 \) (10 ng/ml) for 1–72 h, and protein whole cell extracts were obtained. When we analyzed for \( \alpha \text{SMA} \) expression on Western blot, we observed a time-dependent increase in the expression of \( \alpha \text{SMA} \) protein, with significant levels seen at 48 and 72 h following treatment (Fig. 2C). The augmented \( \alpha \text{SMA} \) protein level was sustained up to 4 days following treatment.

**Fibrocyte Differentiation to Adipocytes Is Dependent on Activation of PPAR\( \gamma \) and Associated with an Induction of \( \alpha \text{P2} \)**

Expression—We had previously demonstrated that fibrocytes differentiate into adipocytes when cells were exposed to a permissive microenvironmental niche (1). In the current study, we examined in greater detail the role of PPAR\( \gamma \) in mediating this process. PPAR\( \gamma \) is well documented as a critical transcription factor in the regulation of adipogenic differentiation. Upon activation, PPAR\( \gamma \) binds to common consensus PPRE sites on promoters of specific adipocyte genes in the nucleus, including that of \( \alpha \text{P2} \) (15, 18). \( \alpha \text{P2} \) is often used as a mature adipocyte marker because its expression parallels the degree of adipogenic differentiation. To activate PPAR\( \gamma \) in fibrocytes, we treated the cells with troglitazone (10 \( \mu \)M) for varying lengths of time (1–72 h) and monitored the kinetics of \( \alpha \text{P2} \) transcript activation using semi-quantitative RT-PCR. When compared with untreated cells, an up-regulation of the \( \alpha \text{P2} \) mRNA transcript was seen within 12 h, with maximal expression of 13.8-fold at 24 h, and with a sustained response at 48 h (Fig. 3A).

To verify the role of PPAR\( \gamma \) in \( \alpha \text{P2} \) induction in fibrocytes, we examined protein expression of PPAR\( \gamma \) and \( \alpha \text{P2} \) following TGZ treatment. Both nuclear and cytoplasmic protein extracts were prepared for Western blot analysis. A low basal level of PPAR\( \gamma \) was detected in unstimulated fibrocytes (Fig. 3B, lane 1). Following TGZ treatment, there was a significant time-dependent increase in accumulation of PPAR\( \gamma \) in the nucleus of cells within 4 h (lane 3) and with maximal stimulation 24 h (lane 4). As for \( \alpha \text{P2} \), no basal expression was seen in untreated cells (Fig. 3B). In response to PPAR\( \gamma \) activation, we observed a significant, but delayed, increase in \( \alpha \text{P2} \) level starting at 24 h (Fig. 3B). The
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FIGURE 3. Fibrocyte to adipocyte differentiation is regulated through a PPARγ-dependent mechanism, leading to activation of aP2 expression. A, time-dependent stimulation of aP2 expression by troglitazone. Fibrocytes were serum starved, then treated up to 48 h with 10 μM TGZ. Transcription analysis was performed using quantitative RT-PCR. B, nuclear translocation of PPARγ following TGZ treatment with an associated increase in cytoplasmic aP2 protein level. C, the PPARγ antagonist GW9662 inhibited the effect of TGZ in activation of aP2 expression. D, fibrocytes were transfected with a PPRE-luciferase reporter gene plasmid (PPRE3x-Lux). Increase in PPARγ transactivation activity was observed following treatment with TGZ (10 μM) or transfection with constitutive PPARγ overexpression vector. Luciferase assays were performed in duplicate and normalized to Renilla. Each bar represents the mean luciferase activity (as relative light units (RLU) per mg of protein ± S.E.).

As further verification, we examined the ability of TGZ to promote PPARγ transactivation activity in fibrocytes. We first transfected the cells with a luciferase reporter gene containing 3 PPREs (PPRE3x-Lux) (17), which was used as an index of PPARγ transactivation. They were then either treated with TGZ or were co-transfected with a construct encoding PPARγ under the constitutive cytomegalovirus promoter (pCMV-PPARγ). PPRE-driven luciferase activity was then monitored and normalized to Renilla luciferase. As shown in Fig. 3D, use of null vector (lane 2) did not result in spontaneous activation. Conversely, TGZ was able to induce a 5.9-fold increase in luciferase reporter activity (lane 6). Co-transfection with the pCMV-PPARγ expression construct also resulted in robust up-regulation (7.8-fold increase) of PPARγ transactivation in the absence of TGZ (lane 7). Taken together, these results strongly suggest the role of PPARγ as a key regulatory molecule in the activation of fibrocyte-to-adipocyte differentiation.

TGFβ1-mediated Induction of αSMA Expression Is Negatively Regulated by Troglitazone via a PPARγ-independent Mechanism—We have now demonstrated the ability of fibrocytes to differentiate along two different lineages. This plasticity is dependent on the presence of specific growth factors such as TGFβ1 for myofibroblast differentiation or PPARγ signaling for adipocyte differentiation. Here, we examined the effects of TGZ on TGFβ1-mediated myofibroblast differentiation. Fibrocytes were treated with TGFβ1 (1–100 ng/ml) in the presence or absence of TGZ (10–30 μM). Semi-quantitative RT-PCR analysis showed that TGZ, at a concentration of 30–100 μM, was able to suppress the induction of αSMA transcription activated by TGFβ1 (Fig. 4, A, lanes 8 and 9; B, lane 7). Interestingly, whereas TGZ at 10 μM was sufficient to activate fibrocyte adipogenesis, a higher concentration was needed to effectively suppress the expression of αSMA.

We next tested whether the effects of TGZ in inhibiting αSMA was mediated through the activation of PPARγ. Although it is well documented that PPARγ ligands can influence transcription of genes in a PPAR-dependent manner, recent research has revealed that these drugs also elicit “nongenomic” effects (19). For example, PPARγ ligands can rapidly induce phosphorylation of members of the MAPK family or phosphatidylinositol 3-kinase pathway (20). To examine this, we pretreated fibrocytes with the PPARγ antagonist GW9662 before treatment with TGFβ1 or TGZ, either alone or in combination. As shown in Fig. 4B, GW9662 (3–10 μM) did not reverse the inhibitory effects of TGZ on αSMA transcription expression (lanes 8 and 9). Even higher concentrations of GW9662 did not improve on this effect (date not shown).

We also looked at the effect of TGZ on αSMA protein expression. Again, fibrocytes were pretreated with or without GW9662, then incubated in TGFβ1 (10 μM) and/or TGZ (30 μM) for 72 h. The expression of αSMA was assessed by Western blot. Consistent with mRNA findings, treatment with TGZ inhibited TGFβ1-induced αSMA expression (Fig. 4C, lane 5). The inhibitory effect of TGZ was not reversed by GW9662 (Fig. 4C, lane 6). Taken together, these findings suggest that, contrary to PPARγ-mediated induction of aP2, the effect of TGZ on αSMA expression is mediated by nongenomic PPARγ-independent effects.

TGFβ1 Activates Regulatory Smads2/3 and MAPK Pathways—We now focused in detail on the molecular mechanisms of interaction between TGZ and TGFβ1 during myofibroblast differentiation. To do this, we first dissected the exact mechanisms whereby TGFβ1 signaling leads to αSMA expression. We examined the two signaling pathways, Smads and MAPK, which have been reported by others to be associated with TβR activation in other cells (8, 20, 21). First, we evaluated whether TGFβ1 activates Smad2/3 in fibrocytes. The regulatory
Smad2/3 have two major phosphorylation sites, at linker regions and at C-terminal regions (22). Treatment with TGF1 (10 ng/ml) led to phosphorylation of Smad2/3 at the C-terminal SSXS region (Ser-465/467) but not at the linker region (data not shown). Phosphorylation of Smad2/3 was detected within 60 min and remained activated at 2 h. The total protein levels of total ERK and SAPK/JNK were not significantly affected by TGF1 treatment, which peaked at 30 min and demonstrated persistent activation at 2 h. The total protein levels of total ERK and SAPK/JNK were not significantly affected by TGF1 treatment.

We next investigated whether MAP kinases were intermediates in a TGF1-initiated signaling pathway leading to transcriptional activation of αSMA. We have shown that TGF1 induced a time-dependent activation of Smad2/3, kinetics of which lagged temporally behind ERK and SAPK/JNK activation. Considering the possibility that the MAPK pathway may influence Smad signaling, we evaluated whether a blockade of ERK1/2 or SAPK/JNK pathways altered activation of Smads or αSMA expression. We first pretreated the fibrocytes with PD98059 (20 μM), an ERK kinase (MEK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase)) inhibitor, prior to addition of TGF1. We then extracted cytoplasmic and nuclear protein extracts for Western blot analysis. Whereas PD98059 pretreatment led to marked suppression of ERK phosphorylation (Fig. 6A, lane 6), there was no change in TGF1-induced phosphorylation of Smad2/3 in either the cytoplasm or nucleus (lanes 2 and 6). There was also no difference in αSMA protein expression in cells pretreated with TGF1 treatment, which peaked at 30 min and demonstrated persistent activation at 2 h. The total protein levels of total ERK and SAPK/JNK were not significantly affected by TGF1 treatment.

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PD98059 (lanes 2 and 6), suggesting that the ERK1/2 MAPK pathway was not involved in TGF-\( \beta \)-induced \( \alpha \)SMA expression.

We next evaluated whether the JNK pathway may be implicated. Here, we pretreated the cells with a SAPK/JNK inhibitor, SP600125 (10 \( \mu \)M), prior to incubating cells with TGF-\( \beta \). Unlike PD98059, use of SP600125 led to inhibition of the TGF-\( \beta \)-mediated stimulation of \( \alpha \)SMA protein (Fig. 6B, lane 5). Whereas SAPK/JNK blockade did not influence the level of cytoplasmic pSmad2/3, it did markedly reduce the level of pSmad2/3 in the nucleus (Fig. 6B, lane 6). This was associated with significant loss in \( \alpha \)SMA expression. These results suggest that whereas SAPK/JNK signaling did not affect the ability of TGF-\( \beta \) to activate cytoplasmic Smad2/3, it did alter the level of activated Smad2/3 in the nucleus.

To rule out the possibility that importance of SAPK/JNK signaling is not due exclusively to a pharmacologic inhibitor, we used RNA interference to specifically knockdown endogenous JNK protein in fibrocytes. Fibrocytes were treated with siRNA oligo specific for JNK1 and the effect on JNK protein level was compared with that of a nonspecific siRNA oligo control or transfection reagent alone. Loss of JNK1 protein leads to reduced \( \alpha \)SMA expression.

Lastly, we assessed the effect of JNK blockade on Smad transactivation activity. A TGF-\( \beta \) reporter construct (SBE3\( x \)-Luc) containing a luciferase gene controlled by a TGF-\( \beta \)-inducible promoter (with SBE) was used to monitor TGF-\( \beta \)-induced changes in gene expression in fibrocytes. Transfection of SBE3\( x \)-Luc into the cells resulted in a strong induction (15.1-fold) of luciferase activity in response to TGF-\( \beta \) (Fig. 6D, lane 4). We then performed experiments where we chemically blocked either ERK1/2 or SAPK/JNK pathways using PD98059 or SP600125, respectively, before stimulating the cells with TGF-\( \beta \). TGF-\( \beta \)-driven luciferase activity was then monitored.

FIGURE 6. SAPK/JNK MAP kinases are intermediates in a TGF-\( \beta \)-initiated signaling pathway leading to transcriptional activation of \( \alpha \)SMA. A, chemical blockade using the ERK1/2 inhibitor PD98059 did not affect TGF-\( \beta \)-induced phosphorylation of Smad2/3 or \( \alpha \)SMA protein expression. Exposure to TGF-\( \beta \) attenuated TGF-\( \beta \)-induced \( \alpha \)SMA expression, an effect not reversed by PD98059 pretreatment. B, pretreatment using the SAPK/JNK inhibitor SP600125 markedly reduces the level of phosphorylated Smad2/3 in the nucleus and resulted in a significant loss in \( \alpha \)SMA expression, an effect similarly seen with treatment using TGF-\( \beta \). C, siRNA knockdown of JNK1 leads to loss of JNK1 protein. Fibrocytes were treated with siRNA oligo specific for JNK1 and their effect on JNK1 protein levels were compared with that of a nonspecific siRNA oligo control or transfection reagent alone. Loss of JNK1 protein leads to reduced \( \alpha \)SMA expression. D, transfection with a TGF-\( \beta \) reporter construct (p3SBE-Lux). Unlike PD98059 pretreatment, use of a JNK inhibitor significantly inhibited TGF-\( \beta \)-driven luciferase activity in fibrocytes.
and SAPK/JNK activation in fibrocytes. Previous studies have shown that PPARγ ligands such as TGZ, beyond activating PPARγ, can also regulate activity of MAP kinases (19, 20). The activation of MAPK pathways by PPAR agonists have been shown to exert either anti-fibrotic or pro-fibrotic influence depending on the cellular context (20, 23–25). We have already shown that contrary to PPARγ-mediated induction of aP2 the effect of TGZ on αSMA expression was mediated by its PPARγ-independent actions. We now examine if this inhibition is mediated through MAP kinase signaling.

Interestingly, ERK1/2 was not only activated by TGFβ1 (Fig. 6A, lane 2), but also by TGZ (lane 3). TGZ induced ERK phosphorylation without a change in total ERK1/2 level. TGFβ1 and TGZ together induced a higher level of phosphorylated ERK1/2 than either treatment alone (lane 4). To determine whether TGZ inhibition of TGFβ1-mediated αSMA expression involves ERK signaling, we performed pathway-specific inhibition studies using the ERK inhibitor PD98059 (20 μM). Pretreatment with PD98059 effectively decreased the level of pERK1/2 (lane 5–8). We suspect that if TGZ inhibition of αSMA is mediated through ERK activation, blockade of ERK signaling would abolish the inhibitory effect of TGZ treatment. This, however, was not observed. Whereas exposure of TGZ did result in loss of αSMA expression (lane 4), PD98059 pretreatment failed to reverse this effect (lane 7). Treatment with TGZ also resulted in a lower level of pSmads in the nucleus (lane 4), which also failed to reverse with chemical inhibition of ERK signaling (lane 7). Taken together, these results suggest that whereas TGZ activated ERK MAP kinase, this pathway was not involved in regulation of TGFβ1-mediated αSMA expression.

We next looked at the SAPK/JNK pathway. We showed that TGFβ1 activated the SAPK/JNK pathway (Fig. 6B, lane 2). To determine whether TGZ modulates its effect through regulation of TGFβ1-activated SAPK/JNK signaling, we performed pathway-specific inhibition experiments using the JNK inhibitor SP600125. TGZ alone did not activate SAPK/JNK, unlike its ability to activate the ERK pathway (lane 3). Instead, treatment with TGZ interfered with the ability of TGFβ1 to activate SAPK/JNK (lane 4). This outcome, mediated upstream of SAPK/JNK, is in effect similar to chemical blockade of SAPK/JNK (lane 6). Because abrogation of SAPK/JNK activity through chemical inhibition is associated with diminished Smad2/3 activation in the nucleus (lane 6), it was not surprising to see that TGZ treatment also provoked similar reduction in nuclear Smad activation (lane 4). Consequently, treatment with SAPK/JNK inhibitor or TGZ both resulted in loss of αSMA expression as stimulated by TGFβ1 (lanes 4 and 6; as compared with lane 2). Taken together, the effect of TGZ is mediated through its repression of SAPK/JNK activation. Because full induction of αSMA requires cooperative TGFβ1-mediated signaling through SAPK/JNK and Smad2/3, disruption of SAPK/JNK signaling may be one mechanism whereby TGZ exerts its important effects on expression of αSMA.

Fibrocyte Differentiation to Adipocyte Is Negatively Regulated by c-Jun N-terminal Kinase—We have demonstrated the ability of TGZ to initiate PPARγ-driven differentiation of fibrocytes to adipocytes. TGZ activation of PPARγ-independent signaling was also shown to negatively regulate the differentiation of fibrocytes to myofibroblasts. However, it remains unknown whether TGFβ1 plays a reciprocal role in modulating fibrocyte commitment to adipogenesis. To examine this issue, fibrocytes were treated with TGZ, with or without TGFβ1, for 48 h (i.e. the time point shown to induce maximal aP2 expression). TGFβ1 treatment prevented cell rounding and lipid accumulation and conferred a more densely packed, spindly morphology to the cells (data not shown). In addition, as compared with TGZ alone, the presence of TGFβ1 resulted in marked reduction in cytoplasmic aP2 expression (Fig. 7A, lane 4). No detectable level of aP2 protein was seen in either untreated cells or in cells treated with TGFβ1 alone.

A number of previous studies have suggested that the PPARγ and TGFβ1 pathways cross-talk to regulate adipogenesis of fat tissue preadipocytes. Whereas results have been conflicting, MAP kinases have been implicated to play an important role in mediating this downstream TGFβ effect. To dissect the potential involvement of MAP kinases in regulation of fibrocyte adipogenesis, we again utilized our ability to chemically block each individual MAP kinase pathway. We inhibited ERK1/2 or SAPK/JNK pathways through pretreatment with either PD98059 or SP600125, respectively. With TGZ alone, we observed PPARγ translocation to the nucleus, followed by induction of aP2 expression (Fig. 7B, lane 2). Neither TGFβ1 nor PD98059 alone induced spontaneous aP2 expression (lanes 3 and 5). When TGFβ1 was added, induction of aP2 expression by TGZ was significantly repressed (lane 4). Blockade of ERK1/2 using PD98059 failed to reverse TGFβ1-mediated inhibition of aP2 expression (lane 7). These findings suggest that the ERK1/2 MAP kinase pathway is not involved in this negative regulatory process.

We next examined whether the JNK pathway may be implicated. We targeted SAPK/JNK blockade using the inhibitor SP600125. Contrasting the effect with PD98059, SP600125 pretreatment led to the reversal of TGFβ1-mediated inhibition of aP2 (Fig. 7C, lane 7). To confirm this finding, we again used RNA interference to specifically knockdown endogenous JNK protein in fibrocytes. Fibrocytes were treated with siRNA oligo specific for JNK1 or control and the effect of JNK protein on TGFβ1 signaling was examined. Consistent with pharmacologic inhibition with SP600125, the knockdown of endogenous JNK protein resulted in abrogation of TGFβ1 inhibition on aP2 expression (Fig. 7D).

Because induction of aP2 transcription is PPARγ-dependent and requires translocation of activated PPARγ into the nucleus, an increase in the level of nuclear PPARγ was noted following TGZ induction. Interestingly, despite repression of aP2 by TGFβ1 or its reversal with JNK inhibition, no significant change in the level of nuclear PPARγ was seen with either treatment as compared with TGZ alone (Fig. 7C, lanes 4 and 6). These findings suggest that TGFβ1-mediated activation of SAPK/JNK signaling does not impact PPARγ activation or nuclear translocation directly. Instead, SAPK/JNK activation may lead to downstream modulation of PPARγ DNA binding or transactivation activity.

To examine this premise, we assessed the effect of SAPK/JNK activation on PPARγ transactivation activity. We transfected cells with PPREα-Lux for measurement as index of
PPARα transactivation. The cells were then treated with TGZ, in the presence or absence of TGFβ1. The measured PPRE-driven luciferase activity was monitored and normalized to Renilla luciferase. As expected, treatment with TGZ resulted in a strong induction of luciferase activity (Fig. 7E, lane 4). Addition of TGFβ1 markedly attenuated this effect (lane 5). When the SAPK/JNK pathway was inhibited with SP600125, this led to a reversal of TGFβ1-mediated inhibition of aP2 expression (lane 8). Similar pretreatment with the ERK inhibitor PD98059 failed to reverse the negative regulatory effect by TGFβ1. Altogether, these results suggest that whereas TGFβ1 activates both ERK and SAPK/JNK signaling, only the SAPK/JNK pathway may be important in the regulatory ability of TGFβ1 to dampen the fibrobyte adipogenic process.

**DISCUSSION**

In the present study, we demonstrated that adult fibrocytes are circulating progenitors that possess the ability to differentiate along different mesenchymal lineages. We showed that TGFβ1 was a key molecule that promotes the differentiation of fibrocyte toward myofibroblast lineage, whereas activation of PPARγ was critical for fibrocyte-to-adipocyte differentiation. Signaling pathways activated by TGFβ1, including Smad2/3 and SAPK/JNK MAPK, collaborate to induce αSMA transcription, whereas TGZ-mediated PPARγ activation leads to intracellular lipid accumulation and induction of aP2 expression.

Interestingly, treatment with TGZ negatively influenced TGFβ1-stimulated αSMA expression. This is modulated through attenuation of the SAPK/JNK activity, leading to decreased Smad2/3 levels and transactivation activity. Conversely, TGFβ1 was demonstrated to have reciprocal inhibition on the differentiation of fibrocytes to adipocytes. Through activation of SAPK/JNK MAP signaling, which is normally suppressed during adipogenesis, this results in the disruption of PPARγ-dependent induction of aP2 expression. Taken together, SAPK/JNK signaling seemed to affect the divergence of adipogenic and myofibrogenic lineages derived from fibrocyte progenitors, and ultimately, changed the commitment of cells directed into these lineages (Fig. 8).

In bone marrow-derived stem cells, the ability of TGFβ1 to regulate mesenchymal differentiation is well documented (26–28). The role of TGFβ1 in fibrobyte lineage determination was previously unknown. Recent studies have demonstrated the pivotal role of fibrocytes in contributing to a number of pathophysiologic fibrotic processes. These include aberrant pulmonary fibrosis (2, 29), vascular intimal hyperplasia (30), as well as renal-related fibrotic diseases (31). We showed that TGFβ1 leads to activation of both Smad2/3 and MAPK, with cross-talk between the two pathways.

Communication between MAPK and Smads have previously been reported for other cell lines (8, 31–34). Activation of
MAPKs by TGFβ1 was previously described to occur either with slow kinetics, possibly resulting from Smad-dependent transcription responses, or with rapid kinetics such as in fibrocytes. In the latter case, the rapid activation (5–30 min) of MAPK phosphorylation strongly suggests independence from Smad-driven transcription. Following activation, MAP kinases can modify Smad signaling by phosphorylation of Smad2/3, which may affect its capacity for nuclear translocation. Both ERK (35) and SAPK/JNK (22, 36) have been shown to phosphorylate the linker region of Smad2 and Smad3, which inhibits their nuclear translocation. In our study, inhibition of SAPK/JNK was associated with decreased levels of Smad2/3 in the nucleus as well as reduced transactivation activity. SAPK/JNK-mediated modification of Smad2/3 could have either altered their ability to form heterocomplex with Smad4 or impact their translocation into the nucleus.

We next examined the mechanism whereby TGZ inhibits TGFβ1 signaling in fibrocytes. Depending on cell type, PPARγ agonists can modulate MAPK signaling by different mechanisms. One simple possibility was that the agonist directly interfered with TGFβ1 signaling, preventing cells from receiving profibrotic signals. In some studies, PPARγ agonists were found to inhibit Smad2/3 phosphorylation either through direct interaction with TGFβ receptor or with native (non-phosphorylated) Smads. In our study, cytoplasmic phosphorylation of Smad2/3 was not affected by TGZ. Instead, a drop in level of activated Smads in the nucleus with decreased Smad transactivation activity was seen following TGZ exposure. Specifically, the inhibitory effect of TGZ on TGFβ1 signaling was dependent on the ability of TGZ to repress PPAR/JNK activation and decreased level of mature adipocyte marker aP2. We have already showed that TGFβ1 activated both ERK1/2 and SAPK/JNK pathways. However, only SAPK/JNK activation reversed TGZ-induced PPARγ activity. In previous studies, a number of signaling effectors, including MAPK family members, have been shown to phosphorylate PPARγ (41–43). PPARγ phosphorylation can affect its activity, including reduction in the sensitivity of PPARγ to its cognate ligands or affect its translocation or transactivation activity. This inhibition of adipogenesis by SAPK/JNK has also been reported by others (44–46). Interestingly, Hirosumi and colleagues (45) reported abnormally elevated JNK activity in obese mice. Gene disruption of JNK1, a principal JNK isoform, alleviated dietary as well as genetic obesity, and protected the animals from the development of obesity-induced insulin resistance (45).

Lastly, although beyond the scope of this paper, a number of different mechanisms can modulate the effect of SAPK/JNK activation on PPARγ activity. SAPK/JNK MAPK phosphorylation of PPARγ can potentially alter PPARγ protein stability, ability of PPARγ to bind to DNA, or change in ability of PPARγ to interact with other transcription intermediary proteins (i.e. coactivator binding to PPARγ-RXR complex). Alternatively, a number of transcription factors are downstream substrates for SAPK/JNK MAPK, including c-Jun, which is the main component of AP-1 complexes. Activation of AP-1 can facilitate binding to specific AP-1 recognition sites and modulate transactivation of target genes, including those of the adipose differentiation program. This possibility warrants additional investigation because in a recent study, Fu and associates (47)
demonstrated that introduction of dominant-negative AP1 led to reversal of TGFβ1 suppression of PPARγ promoter activity.

In summary, the present study demonstrates that a complex signaling circuit involving PPARγ, Smads, and MAPK activation serve to modulate lineage plasticity of circulating fibrocytes. Within the context of the local microenvironmental niche, the delicate balance of PPARγ and TGFβ1 activation drives the selection of defined differentiation pathways to converge on a specific gene program. When deregulated, changes in an individual signaling pathway may contribute to impaired differentiation and allow for the development of abnormal fibrosis or fat formation. We now have exciting molecular targets whereby pharmacologic attempts can be made to modulate signaling parameters that can ultimately regulate fibrocyte cell fate in early development toward or away from a specific mesenchymal lineage.

REFERENCES