Peroxisome Proliferator-activated Receptor γ Induces a Phenotypic Switch from Activated to Quiescent Hepatic Stellate Cells*

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Depletion of peroxisome proliferator-activated receptor γ (PPARγ) accompanies myofibroblastic transdifferentiation of hepatic stellate cells (HSC), the primary cellular event underlying liver fibrogenesis. The treatment of activated HSC in vitro or in vivo with synthetic PPARγ ligands suppresses the fibrogenic activity of HSC. However, it is uncertain whether PPARγ is indeed a molecular target of this effect, because the ligands are also known to have receptor-independent actions. To test this question, the present study examined the effects of forced expression of PPARγ via an adenoviral vector on morphologic and biochemical features of culture-activated HSC. The vector-mediated expression of PPARγ itself is sufficient to reverse the morphology of activated HSC to the quiescent phenotype with retracted cytoplasm, prominent dendritic processes, reduced stress fibers, and accumulation of retinyl palmitate. These effects are abrogated by concomitant expression of a dominant negative mutant of PPARγ that prevents transactivation of but not binding to the PPAR response element. PPARγ expression also inhibits the activation markers such as the expression of α-smooth muscle actin, type I collagen, and transforming growth factor β1; DNA synthesis; and JunD binding to the activator protein-1 (AP-1) site and AP-1 promoter activity. Inhibited JunD activity by PPARγ is not due to reduced JunD expression or JNK activity or to a competition for p300. But it is due to a JunD-PPARγ interaction as demonstrated by co-immunoprecipitation and glutathione S-transferase pull-down analysis. Further, the use of deletion constructs reveals that the DNA binding region of PPARγ is the JunD interaction domain. In summary, our results demonstrate that the restoration of PPARγ reverses the activated HSC to the quiescent phenotype and suppresses AP-1 activity via a physical interaction between PPARγ and JunD.

Hepatic stellate cells (HSC) are vitamin A-storing pericytes in the subendothelial space of the liver. Upon injury to the liver, HSC become transdifferentiated into myofibroblastic cells to participate in wound healing (1). This transdifferentiation is characterized by reduced vitamin A content, increased cell proliferation and migration, enhanced matrix protein expression, and induced expression of α-smooth muscle actin (1). This response of HSC constitutes the normal, reparative homeostatic response of the liver to injury. However, dysregulation of HSC leads to excessive accumulation of extracellular matrices, resulting in liver fibrosis and cirrhosis. No curative medical treatments are available for cirrhosis except liver transplantation, and a precise understanding of transdifferentiation of HSC is the prerequisite for eventual identification of “dysregulation” and future developments of specific therapeutic modalities for the disease. To this end, much investigative effort has been made to characterize transcriptional regulation that underlies HSC transdifferentiation. Such examples include identification of Kruppel-like factor 6, a differentially expressed zinc finger protein in activated HSC in vitro and in vivo (2). This transcription factor binds to the GC box sites of TGFβ1, TGFβ3 receptor type I and II (2), urokinase-type plasminogen activator (3), and α1(I) procollagen (2) and induces transcription of these fibrogenic genes. The myofibroblast phenotype seen in activated HSC is best characterized by induction of α-smooth muscle actin that is mediated by c-Myb binding to an E-box element in its promoter (4). The significance of this mode of regulation is supported by the demonstration of prevention of the myofibroblastic phenotypic switch by the treatment of HSC with antisense oligonucleotides for c-Myb (4). Sustained NF-κB activation confers activated HSC their proliferative and antiapoptotic status that may be important in progressive liver fibrogenesis (5). NF-κB may also mediate inflammatory responses by HSC via induction of chemokines and adhesion molecules (6, 7). Increased activator protein-1 (AP-1) activity is essential for induction of matrix metalloproteinase-8 (8), tissue inhibitor of matrix metalloproteinase-1, and interleukin-6 (9) gene transcription in activated HSC, where JunD is shown to play a pivotal role (9).

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† The abbreviations used are: HSC, hepatic stellate cell(s); PPARγ, peroxisome proliferator-activated receptor γ; PPARE, peroxisome proliferator-activated receptor response element; AP, activator protein, TGFβ3 and TGFβ1, transforming growth factor β3 and β1, respectively; RXRα, retinoid X receptor α; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; MOI, multiplicity of infection; DME, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; GFP, green fluorescent protein; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; HPLC, high pressure liquid chromatography.

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A complexity in the understanding of HSC differentiation is underscored by different cellular phenotypes that HSC are shown to express. In addition to the myofibroblastic phenotype exhibited by activated HSC, they also express MyoD, the myogenic transcription factor specific for skeletal muscle (9). Neuronal markers such as GFAP (10), N-CAM (6), nestin (11), and synaptophysin (12) are also expressed in HSC, suggesting the neural phenotype and that N-CAM and nestin are induced in activated HSC. Activated HSC express leptin (13), an adipocyte-specific gene, raising an intriguing possibility that HSC may also share the adipocytic phenotype. In fact, the quiescent HSC is laden with lipids including triglycerides, cholesterol, and phospholipids in addition to retinyl esters (14). In support of this notion, peroxisome proliferator-activated receptor γ (PPARγ), one of the key transcription factors for adipocyte differentiation (15), is expressed in the quiescent HSC (16-18), and its expression and activity decrease in HSC activation in vitro (16, 17) and in vivo (16). Further, the treatment of culture-activated HSC with the natural or synthetic ligands for PPARγ suppresses many functional parameters of the cell activation, including cell proliferation (17), expression of collagen, TGFβ, α-smooth muscle actin, monocyte chemotactic protein-1 genes, and chemotaxis (16, 18). More importantly, the treatment of the animal models of liver fibrosis with the PPARγ ligands ameliorates not only induction of fibrosis but also progression of preexisting fibrosis (17). Thus, these findings support the hypothesis that the maintenance of the quiescent state of HSC requires PPARγ and depletion of this adipogenic transcription factor underlies activation of HSC that can be circumvented by the ligand treatment. However, the ligands for PPARγ are also known to have receptor-independent effects. Using the embryonic stem cells from PPARγ null mice, neither macrophage differentiation nor anti-inflammatory effects of synthetic PPARγ ligands are shown to be dependent on PPARγ (19). Indeed, the PPARγ ligand 15-deoxyprostaglandin J2 suppresses NF-κB activation by directly inhibiting IκB kinase in a PPARγ-independent manner (20). Troglitazone also selectively induces early growth response-1 gene independently of PPARγ (21). Mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) are activated in activated HSC and to determine its effects on the cell activation. Our results demonstrate that the restoration of PPARγ and deletion of this adipogenic transcription factor underlies activation of HSC that can be circumvented by the ligand treatment. However, the ligands for PPARγ are also known to have receptor-independent effects. Using the embryonic stem cells from PPARγ null mice, neither macrophage differentiation nor anti-inflammatory effects of synthetic PPARγ ligands are shown to be dependent on PPARγ (19). Indeed, the PPARγ ligand 15-deoxyprostaglandin J2 suppresses NF-κB activation by directly inhibiting IκB kinase in a PPARγ-independent manner (20). Troglitazone also selectively induces early growth response-1 gene independently of PPARγ (21). Mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) are activated in activated HSC and to determine its effects on the cell activation. Our results demonstrate that the restoration of PPARγ and deletion of this adipogenic transcription factor underlies activation of HSC that can be circumvented by the ligand treatment. However, the ligands for PPARγ are also known to have receptor-independent effects. Using the embryonic stem cells from PPARγ null mice, neither macrophage differentiation nor anti-inflammatory effects of synthetic PPARγ ligands are shown to be dependent on PPARγ (19). Indeed, the PPARγ ligand 15-deoxyprostaglandin J2 suppresses NF-κB activation by directly inhibiting IκB kinase in a PPARγ-independent manner (20). Troglitazone also selectively induces early growth response-1 gene independently of PPARγ (21). Mitogen-activated protein kinases such as c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) are activated in activated HSC and to determine its effects on the cell activation. Our results demonstrate that the restoration of PPARγ and deletion of this adipogenic transcription factor underlies activation of HSC that can be circumvented by the ligand treatment.
**PPARγ and Hepatic Stellate Cells**

FoeB, JunD, or PPARγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added and incubated for an additional 20 min after the 30-min incubation.

**Transient Transfection and Reporter Gene Assay**—To determine whether PPARγ expressed by the adenoviral vector induces the PPAR response element (PPRE) promoter activity, HSCs were transiently transfected with a PPRE-luciferase construct (tk-PPRE × 3-luciferase) using Targefect F-2 (Advanced Targeting Systems, San Diego, CA). To examine the effect of PPARγ expression on AP-1 activity, HSCs transduced with Ad.PPARγ or Ad.GFP were transiently transfected with an AP-1 luciferase construct containing seven repeats of TGACTAA from the 12-O-tetradecanoylphorbol-13-acetate-responsive element (Stratagene, La Jolla, CA). We also examined the effects of CBP△300 expression on the PPRE-mediated effect on AP-1 by cotransfecting with Ad.PPARγ and pCDNA3-myc-hexaderniphillin (a gift kind from Dr. Stallcup, University of Southern California). For the determination of transfection efficiency, Renilla phRL-TK vector was used (Promega, Madison, WI). For transfection, 10-day cultures of HSC or HSC line in 6-well plates (70,000 cells/well; 3 days after infection with a viral vector) were incubated with 2 μg of each reporter construct, 0.02 μg of Renilla phRL-TK, and 2 μl of F-2 reagent in 1 ml of serum-free high glucose DMEM. Two h later, 1 ml of DMEM with 10% FBS was added to achieve the final FBS content of 5% for overnight incubation. On the next day, the medium was changed to DMEM with 10% FBS, and the cells were incubated for another 30 h. The cell lysate was collected for the determination of both Renilla and Firefly luciferase activities using the Dual-Luciferase reporter assay system (Promega). The results were normalized by Renilla luciferase activity.

**Morphological Analysis**—To investigate the effects of PPARγ on the HSC morphology, 7-day cultured HSCs were transduced with Ad.GFP, Ad.PPARγ plus Ad.GFP, or Ad.PPARγ plus Ad.dn.PPARγ using a total MOI of 100 and virus and cultured for an additional 5 days. The cells were examined under a fluorescence microscope and photographed for documentation. The cells were also fixed in 3% paraformaldehyde in a phosphate buffer for 1 h and washed with a phosphate buffer. Stress fibers were subsequently stained with rhodamine-labeled phalloidin for 30 min and washed with a phosphate buffer, and differential interference contrast images were acquired to assess the effects of PPARγ expression.

**Retinyl Palmitate Measurement**—To investigate the effect of PPARγ on the formation of intracellular lipid droplets, the cells were cultured with retinol (5 μM), palmitate (100 μM), or both for 48 h and stained with Oil Red O solution (in 60% isopropanol alcohol) for 20 min followed by counterstaining with hematoxylin. The stained slides were examined and photographed using an inverted light microscope. For chemical quantification of retinyl palmitate (a predominant vitamin A form stored in HSC), lipids were extracted with methanol and hexane from HSC treated with both retinol and palmitate (27). Retinyl palmitate in the hexane-extractable lipid phase was analyzed by reverse-phase high pressure liquid chromatography (HPLC) with the wavelength detector at 203 nm. The hexane-extractable lipid phase was analyzed by reverse-phase high pressure liquid chromatography (HPLC) with the wavelength detector at 303 nm and 204 nm.

**RESULTS**

**Forced Expression of PPARγ and PPRE Transactivation by Ad.PPARγ in Activated HSC**—We first validated the forced expression of PPARγ in culture-activated HSC transduced with Ad.PPARγ by Western blot analysis. Ad.GFP-transduced HSC as a control had no detectable PPARγ (Fig. 1A), confirming the previous finding in our and other laboratories that activation of HSC results in depletion of this transcription factor (16). As expected, Ad.PPARγ infection indeed resulted in the expression of PPARγ in HSC (Fig. 1A). Further, the forced PPARγ expression increased PPRE promoter activity by 250-fold as determined by transfection of HSC with a PPRE-luciferase reporter gene containing three copies of the PPRE linked to the thymidine kinase promoter (Fig. 1B).

**Morphological Changes Induced by PPARγ Expression**—We examined whether the determination of PPARγ expression affected the morphological features of activated HSC. Culture-activated HSC transduced with Ad.GFP exhibited a large, flattened polygonal shape, the typical cell morphology of culture-activated primary HSC (Fig. 2A). Infection with Ad.PPARγ resulted in retracted and reduced cytoplasm with the reappearance of dendritic processes resembling the more quiescent HSC phenotype (Fig. 2B). Using an MOI of 50, the expression of the wild-type PPARγ (Ad.PPARγ) reproduced the similar morphological changes (Fig. 2C), and this effect was completely blocked by the co-infection of the cells with an adenoviral vector expressing a dominant negative mutant of PPARγ (Ad.dn.PPARγ, MOI 50) (Fig. 2D). For the control, we used an MOI of 50 of Ad.GFP, maintaining the total viral MOI of 100. Further, we tested the effect of PPARγ expression on actin cytoskeleton. The stress fibers were labeled with rhodamine-phalloidin. Ad.GFP-transduced cells showed prominent actin filaments, whereas Ad.PPARγ-transduced cells showed prominent stress fibers.
stress fibers in a pattern typically observed in activated HSC (Fig. 2F). The cells expressing PPARγ showed an aberrant pattern of reduced stress fibers (Fig. 2F). This change in the stress fibers was also blocked by the co-infection with Ad.dn.PPARγ (Fig. 2H). In summary, these results demonstrate that the forced expression of PPARγ reverses the morphology of activated HSC to that of more quiescent HSC, and this effect is mediated by trans-activation of PPRE promoters as dn.PPARγ effectively abrogates the effect.

**PPARγ Expression Decreases HSC Proliferation**—One of the most obvious and important parameters of HSC activation is increased DNA synthesis. We have examined next whether the forced PPARγ expression inhibits this parameter. Forced expression of PPARγ in activated HSC caused a 70% inhibition in HSC DNA synthesis compared with that found in Ad.GFP-infected HSC controls (Fig. 3A).

**PPARγ Expression Inhibits Expression of HSC Activation Marker Genes**—To further validate the reversal of HSC activation at the functional level, we next examined the effects of PPARγ expression on mRNA expression of α1(I) procollagen and TGFβ1, the two bona fide activation markers expressed by HSC (28, 29). Real time PCR analysis revealed that α1(I) procollagen and TGFβ1 mRNA levels were significantly reduced by 60 and 40%, respectively, in Ad.PPARγ-transduced HSC as compared with Ad.GFP-transduced cells, whereas the PPARγ mRNA level showed an expected robust increase (Fig. 3B). We also performed immunoblot analyses for α-smooth muscle actin and type I collagen and demonstrated that the expression of both proteins were inhibited in Ad.PPARγ-transduced cells compared with Ad.GFP-transduced cells (Fig. 3C). Densitometric and statistical analyses of three sets of blots revealed that the expression of α-smooth muscle actin and type I collagen proteins in Ad.PPARγ-transduced cells was reduced to 31.7 ± 28.3% (p < 0.05) and 30.0 ± 15.7% (p < 0.05) of those in Ad.GFP-transduced cells, respectively. These results unequivocally confirm inhibition of myofibroblastic activation of HSC by forced PPARγ expression.

**PPARγ Expression Allows HSC to Restore Their Ability to Accumulate Retinyl Esters**—One of the characteristic functions of the quiescent HSC is the storage of vitamin A, which is significantly diminished in activated HSC (30). Thus, we examined whether the forced expression of PPARγ by Ad.PPARγ restored the ability of HSC to accumulate retinyl esters. For this, we have added retinol (5 μM), palmitate (100 μM), or both to the cultures of Ad.PPARγ- or Ad.GFP-transduced HSC. In Ad.GFP-transduced cells, the addition of retinol or/and palmitate did not significantly modify the lipid staining (Fig. 3D, left panel). Ad.PPARγ infection alone slightly increased the lipid staining regardless of whether they were treated with retinol...
Fig. 3. PPARγ inhibits activation markers and restores the capacity to accumulate retinyl esters. A, Ad. PPARγ infection inhibits DNA synthesis as determined by incorporation of [3H]thyminidine. Note that PPARγ expression causes a 70% inhibition of DNA synthesis, *, p < 0.05 (n = 3). B, Ad.PPARγ infection causes an expected increase in PPARγ mRNA while inhibiting mRNA expression of α1(I) procollagen (+60%) and TGFβ1 (−40%) in culture-activated HSC as determined by Taqman PCR, *, p < 0.05 (n = 3). C, expression of α-smooth muscle actin (α-SMA) and type I collagen (Collagen) are inhibited in Ad.PPARγ-transduced HSC compared with Ad.GFP-transduced cells as assessed by immunoblot analysis. The equal protein loading is depicted by protein staining shown below each blot. D, accumulation of intracellular lipids is slightly increased by PPARγ expression (Ad.PPARγ + Ad.GFP) in the absence or presence of retinol or palmitate (top three panels of the middle column) as determined by staining with Oil Red O. Treatment of these cells with both retinol and palmitate results in marked accumulation of lipids, resembling quiescent HSC (bottom, middle column). These effects on intracellular lipids are partially blocked by the concomitant infection with Ad.dn.PPARγ (right column).

or palmitate alone (Fig. 3D, middle panel). However, the addition of both retinol and palmitate caused a conspicuous increase in the Oil Red O staining in these cells (Fig. 3D, middle bottom panel). The staining looked brownish in some cells due probably to microvesicular nature of lipid/retinoid storage in HSC. This type of lipid accumulation resembled the quiescent HSC cultured only for 1 day on plastic. The lipid accumulation caused by PPARγ was partially blocked by the expression of
To validate that the accumulation of lipids really reflected increased storage of retinyl palmitate, the major species of stored retinyl esters in HSC in vivo (23), we analyzed the content of the retinyl ester in extracted lipids using reverse phase HPLC. Indeed, this analysis demonstrated a 3-fold increase in the content of retinyl palmitate in Ad.PPARγ-transduced HSC (9.0 ± 0.92 μg/10^6 cells) as compared with Ad.GFP-transduced cells (2.9 ± 1.23 μg/10^6 cells) under the treatment of retinol and palmitate.

Reduced AP-1 Binding in PPARγ-expressing HSC—The results presented so far convincingly demonstrate that the forced expression of PPARγ in culture-activated HSC induces the reversal of the cell phenotype to that of quiescent HSC at both morphologic and biochemical levels. In order to investigate this effect at a more molecular level and to explore the mechanisms involved, we assessed the effects of the forced PPARγ expression on AP-1 binding, the parameter that is known to characterize and underlie many biochemical aspects of activated HSC (31). Nuclear extracts were prepared from Ad.GFP- and Ad.PPARγ-transduced HSC, and an electrophoretic mobility gel shift assay was performed. As a positive control, PPARγ binding to the PPRE (ARE7) probe is increased in nuclear extracts prepared from HSC transduced with Ad.PPARγ as assessed by an electrophoretic mobility gel shift assay. Note that Ad.GFP-transduced cells have no appreciable binding, confirming the depletion of PPARγ in culture-activated HSC. The last lane shows supershift with anti-PPARγ antibody (Ab). B, in contrast to PPARγ binding, AP-1 binding is nearly absent in the quiescent HSC (1-day HSC) and increases as HSC become activated on day 3 and day 7 in culture (first panel). AP-1 binding is reduced in HSC transduced with Ad.PPARγ (middle panel). The last lanes of the middle and right panels show successful supershift of the AP-1 binding complex with anti-JunD antibody but not with anti-c-Fos or anti-FosB antibody (second and third lanes of the right panel). C, AP-1 promoter activity is decreased in Ad.PPARγ-transduced HSC line as demonstrated by transient transfection experiments using the AP-1 promoter luciferase construct. The data were standardized by the transfection efficiency as determined by Renilla luciferase activity. *, p < 0.05 (n = 6). D, overexpression of CBP/p300 does not prevent PPARγ-induced suppression of AP-1 promoter activity. The HSC line was co-transfected with a pCMX or PPARγ expression vector in the absence or presence of a p300 expression vector. Note that this experiment confirms the inhibition of AP-1 promoter activity by PPARγ (pCMX versus PPARγ). Overexpression of p300 increases AP-1 promoter activity in both pCMX- and PPARγ-transfected cells. However, the magnitude of PPARγ-mediated inhibition of AP-1 is not altered by p300 overexpression. *, p < 0.05 (n = 3).
The major components of AP-1 complexes in activated HSC were previously reported to be JunD, c-Fos, and FosB (33). A supershift analysis using antibodies against c-Fos, FosB, and JunD revealed that JunD was the main component of the AP-1 complex shown to bind to the DNA (Fig. 4B). Thus, these results demonstrate that PPARγ decreases JunD binding to the AP-1 site and suggest that this may be of potential significance in inhibiting DNA synthesis and expression of activation marker genes such as TGFβ1 and α(I)I procollagen.

**PPARγ Expression Suppresses AP-1 Promoter Activity**—Next, we examined whether the decreased AP-1 binding by PPARγ is associated with a decreased AP-1 promoter activity in Ad.PPARγ-transduced HSC. Transient transfection using an AP-1 promoter luciferase construct showed that the AP-1 promoter activity was indeed reduced by 40% in the HSC line transduced with Ad.PPARγ as compared with Ad.GFP-transduced cells (Fig. 4C).

Overexpression of p300 Co-activator Does Not Ameliorate PPARγ-mediated Inhibition of AP-1 Promoter Activity—Expression of one transcription factor may affect the activity of another trans-acting factor via a competition for a shared co-activator. In fact, p300 is a co-activator important for optimal transactivation of the promoter dependent on AP-1 (34) as well as that of the PPARγ-driven PPRE promoter (35, 36). Therefore, PPARγ-mediated inhibition of AP-1 activity may be a consequence of a competition for the limiting level of p300. To test this possibility, HSC cell line was transiently co-transfected with the AP-1 luciferase construct, a PPARγ expression vector or empty vector in the presence or absence of a p300 expression vector. In this experiment, we first confirmed that expression of PPARγ inhibited AP-1 promoter activity (Fig. 4D, first two bars). Expression of p300 increased AP-1 promoter activity regardless of whether PPARγ was co-expressed, indicating that p300 indeed serves as a co-activator for AP-1 (Fig. 4D, last two bars). However, the expression of p300 did not affect the magnitude of inhibition of AP-1 promoter caused by PPARγ (Fig. 4D). Therefore, the expression of p300 did not rescue the cells from the suppressive effect of PPARγ on AP-1 promoter activity, suggesting that p300 was not the limiting factor for AP-1 transcription in the presence of PPARγ.

**Inhibited AP-1 Binding Is Not Due to a Change in JunD Expression or JNK Activity**—To determine the mechanisms underlying inhibited AP-1 DNA binding in Ad.PPARγ-transduced HSC, we examined whether JunD mRNA or protein level was diminished in the cells. As shown in Fig. 5A, neither JunD mRNA nor JunD protein level was altered in Ad.PPARγ-transduced cells. Since JNK activity is required for phosphorylation of JunD for its activation, we tested next whether JNK activity was altered by forced PPARγ expression. JNK activity, as assessed by phosphorylation of GST-c-Jun, was not reduced in Ad.PPARγ-transduced HSC (Fig. 5B). Thus, these results suggest that expression and activation of JunD are not decreased by PPARγ expression in activated HSC.

**The Direct Addition of PPARγ Dose-dependently Decreases JunD Binding to AP-1 Site**—To test the possibility of direct inhibition of JunD binding by PPARγ, we tested whether in vitro translated PPARγ inhibited the binding of in vitro translated JunD to the AP-1 site. As depicted in Fig. 6A, the addition of PPARγ dose-dependently reduced JunD-mediated AP-1 binding. To confirm this effect using nuclear extracts from culture-activated HSC as an abundant source of JunD protein, an increasing amount of in vitro translated PPARγ was added to the extracts. This addition also decreased endogenous JunD binding in a dose-dependent fashion (Fig. 6B). These results suggested that PPARγ directly interacted with JunD to inhibit its binding to the AP-1 site.

![Fig. 5. Neither JunD expression nor JNK activity is altered by PPARγ](image-url)

**Neither PPARγ-selective Ligand nor RXRa Enhances the Inhibitory Effect of PPARγ on JunD Binding**—Next, we determined whether the addition of a PPARγ-selective ligand (GW1929) potentiated the inhibitory effect of PPARγ on the binding of endogenous JunD to the AP-1 site. As shown in Fig. 6C (lane 2 versus lane 4), no additive effects of GW1929 were observed. These findings were also confirmed by an experiment using in vitro translated JunD (Fig. 6D, lane 2 versus lane 4). Since RXRa is a heterodimeric partner of PPARγ, we also assessed whether RXRa further decreased PPARγ-mediated JunD binding to the AP-1 site. Interestingly, RXRa alone decreased the binding of endogenous JunD (Fig. 6C, lane 1 versus lane 5) but not that of in vitro translated JunD (Fig. 6D, lane 1 versus lane 5) to the AP-1 site, suggesting the requirement of additional endogenous factor(s) for RXRa-mediated inhibition of AP-1 binding. However, the addition of RXRa together with PPARγ did not promote the inhibitory effect of PPARγ on JunD binding (Fig. 6, C and D, lane 2 versus lane 6).

**PPARγ and a Dominant Negative Mutant of PPARγ (dn.PPARγ) Equally Inhibit AP-1 Binding**—We next tested whether dn.PPARγ that blocked the morphologic effects of the wild type PPARγ could modify the inhibitory effect of PPARγ on AP-1 binding. Here adenoviral-mediated expression of PPARγ was again shown to inhibit AP-1 binding (data not shown). Interestingly, overexpression of dn.PPARγ alone rendered the same inhibitory effect. Using 50 MOI each, the wild type PPARγ caused a moderate inhibition of AP-1 binding, and this was further suppressed by the addition of Ad.dn.PPARγ (data not shown). Both PPARγ and dn.PPARγ bound to the ARE7 probe with the same efficiency confirming the original finding that the mutations in the AF-2 region of PPARγ did not affect DNA binding but rather inhibited PPRE transactivation (25). These results demonstrate that dn.PPARγ equally inhibits AP-1 binding, and this inhibition does not appear to involve the AF-2 region.

**PPARγ Physically Interacts with JunD**—The direct interaction between PPARγ and JunD was suggested by the aforementioned results, and this notion was tested by co-immunoprecipitation and GST pull-down assays. For the former, in vitro translated PPARγ and JunD were incubated, PPARγ was immunoprecipitated with the specific antibody, and the precipi-
The addition of increasing amounts of in vitro translated PPARγ dose-dependently decreases the binding of in vitro translated JunD (A) or endogenous JunD in HSC nuclear extracts (NE) (B) to the AP-1 probe as assessed by an electrophoretic mobility gel shift assay. The addition of in vitro translated RXRs or the PPARγ-specific ligand (GW1929) failed to promote the inhibition of the binding of endogenous JunD (C) or in vitro translated JunD (D) to the AP-1 probe. RXRs alone decreases the AP-1 binding by endogenous JunD (lane 4 of C) but not that by in vitro translated JunD (lane 5 of D).

Fig. 6. The addition of increasing amounts of in vitro translated PPARγ dose-dependently decreases the binding of in vitro translated JunD (A) or endogenous JunD in HSC nuclear extracts (NE) (B) to the AP-1 probe as assessed by an electrophoretic mobility gel shift assay. The addition of in vitro translated RXRs or the PPARγ-specific ligand (GW1929) failed to promote the inhibition of the binding of endogenous JunD (C) or in vitro translated JunD (D) to the AP-1 probe. RXRs alone decreases the AP-1 binding by endogenous JunD (lane 4 of C) but not that by in vitro translated JunD (lane 5 of D).

Fig. 7. PPARγ interacts with JunD protein. A, in vitro translated JunD and PPARγ were incubated together, and PPARγ was immunoprecipitated from the mixture. The precipitates were subjected to an immunoblot analysis for JunD. Note the presence of a JunD band in the second lane, whereas JunD immunoblots for the precipitate of the mixture containing no PPARγ (PCMX) and immunoprecipitation (IP) with nonimmune IgG showed no band. The first lane shows a positive control where in vitro translated JunD was immunoprecipitated with anti-JunD antibody followed by immunoblotting with the same antibody. B, for the GST pull-down assay, in vitro translated, 35S-labeled JunD was detected when GST-PPARγ was pulled down with GSH beads from the incubation mixture of a JunD expression vector and the GST-PPARγ fusion protein (lane 5) but not from the mixture of the empty vector and the fusion protein (lane 3) or that of the JunD vector with no fusion protein (lane 4). C, full-length and five truncated GST-PPARγ fusion proteins were incubated with in vitro translated 35S-labeled JunD. 35S-labeled JunD was detected when region 1–203 (lane 5) or region 1–138 (lane 4) was used. Further, the 204–506 region did not show any 35S-labeled JunD (lane 7) band, suggesting that the region between 139 and 203 of PPARγ (C region; DNA binding region) is the primary region for the interaction with JunD.

Assessment of the JunD-interacting Domain Using GST-PPARγ Deletion Mutants—To identify the domain of PPARγ that interacts with JunD, 35S-labeled JunD was incubated with the following PPARγ deletion mutants: full-length, the A/B region only (residues 1–138); A/B and C regions (resides 1–203); A/B, C, and D regions (residues 1–311); D and E regions (residues 204–506); and E region only (residues 312–506). The pull-down results demonstrated that JunD interacted with the 1–203 or 1–311 mutant but not with the 1–138 mutant, suggesting that the C region (residues 139–203) is the primary domain for the interaction (lanes 5 and 6). The E region (residues 312–506) of PPARγ sometimes showed a much weaker interaction with JunD (data not shown).

DISCUSSION

The present study is the first to test whether forced expression of PPARγ, a nuclear receptor that is depleted in activated HSC, reversed the activated phenotype of the cells to the quiescent phenotype. Indeed, the adenovirus-mediated restoration of PPARγ resulted in the reappearance of morphologic features of quiescent HSC and inhibition of functional parameters for HSC activation such as increased DNA synthesis, the expression of α-smooth muscle actin, type I collagen, and TGFβ1. More importantly, the forced PPARγ expression restored the ability of the cells to accumulate retinyl palmitate, the unique and specific function of quiescent HSC in vitro. These results support our underlying hypothesis that PPARγ is required for the maintenance of the quiescent phenotype of HSC much like its requirement for adipocyte differentiation. This hypothesis was based on several lines of experimental evidence.
cytic fibroblasts can be differentiated into mature adipocytes when cultured in the condition that promotes induction of transcription factors that are essential for induction of adipocyte-specific genes. One such factor is PPARγ that is considered as a master regulator for adipogenesis (15). This adipogenic differentiation is inhibited by growth factors (platelet-derived growth factor and TGFα/epidermal growth factor) or cytokines (tumor necrosis factor α and leptin) that appear to impair the activities of PPARγ or other adipogenic transcription factors (37). Interestingly, these same soluble factors are also implicated in activation of HSC, and this activation is characterized by transdifferentiation of lipid-rich quiescent HSC to lipid-devoid myofibroblastic cells (38, 39). This analogy between adipocyte differentiation and HSC transdifferentiation is further supported by the fact that HSC express adipocyte- or preadipocyte-specific genes such as leptin (13). In fact, three laboratories including our own reported in 1999 that in vitro (16, 18) or in vivo (16, 17) activated HSC had reduced PPARγ levels and activities, and the treatment of activated HSC in vitro with the natural or synthetic ligands of PPARγ inhibited the diverse fibrogenic parameters of the cell activation (16). These studies were followed by an in vivo study by Galli et al. (17), who demonstrated effective prevention of the initiation of liver fibrosis and progression of preexisting liver fibrosis using toxic (dimethyl nitrosamine or carbon tetrachloride) and cholestatic (bile duct ligation) animal liver fibrosis models and two PPARγ ligands (pioglitazone and rosiglitazone) (17). Thus, these findings collectively point toward the role of PPARγ as an anti-fibrogenic regulator in HSC that becomes depleted in activation of the cells. Indeed, the results presented in the current study definitively demonstrate the direct actions of PPARγ to reverse the activated phenotype without the use of exogenous ligands that are known to have receptor-independent effects as discussed in the Introduction (19–22). Further, our findings have far reaching implications beyond the therapeutic significance of PPARγ for liver fibrosis. They address the fundamental question of whether HSC transdifferentiation is analogous to adipocyte differentiation. Our previous analysis of the expression of PPARγ isoforms by an RNase protection assay demonstrated detection of the ubiquitous γ1 but not the adipocyte-specific γ2 isoform, indicating that HSC may not completely share the adipocyte phenotype (16). However, our present findings support a conclusion that similar regulatory mechanisms to those in adipocyte differentiation may exist in HSC transdifferentiation.

Another interesting finding from our study was that PPARγ facilitated both PPRE-dependent and -independent regulation of HSC. The morphologic reversal to the quiescent characteristics was dependent on PPRE promoter transactivation, since dn.PPARγ that exerts its effects at the promoter level blocked this morphologic effect. On the other hand, PPARγ-mediated inhibition of AP-1 promoter activity appeared to be at the level upstream of transcription and to be due to a direct interaction between PPARγ and JunD and consequent inhibition of JunD binding to the AP-1 site. There are numerous examples for this mode of negative cross-coupling between transcription factors. RAR and RXR are shown to inhibit AP-1 promoter activity via their ligand-dependent interactions with c-Jun (40) at their ligand binding domain. We also demonstrated inhibition of JunD binding to DNA by RXR when nuclear extracts but not in vitro translated JunD was used as the source of JunD. This differential effect is due probably to the presence or absence of an RXR ligand. In terms of PPARγ-mediated interactions, it is shown to physically interact with Smad3 to serve as a mechanism underlying ligand-mediated inhibition of expression of connective tissue growth factor in TGFβ-treated aortic smooth muscle cells (41). Further, PPARγ interacts with Sp1 and inhibits Sp1-mediated transcription of thromboxane receptor gene in vascular smooth muscle cells (42). PPARγ has most recently been shown to interact with NF-κB via PGC-2, an AF-1-specific co-activator for PPARγ. This complex formation inhibits PPARγ binding to DNA, and this mode of cross-coupling is suggested to play a role in shifting cellular differentiation of pluripotent mesenchymal stem cells to osteoblasts from adipocytes (43). They further showed that the DNA binding region of PPARγ was the interacting domain for p65. Our results with the deletion mutants also demonstrate that the same region was responsible for the interaction with JunD. However, in our study, in vitro translated PPARγ and JunD physically interacted without the presence of ligands or co-activators. Even when we used HSC nuclear extracts as the source of JunD, the inhibition of AP-1 binding by in vitro translated PPARγ was not promoted by the addition of the PPARγ-selective ligand or in vitro translated RXR, suggesting that the mechanisms of the protein-protein interaction in our experimental setting are different from previous studies and do not require either a heterodimer formation or activation of the complex with a ligand. Further, our finding that dn.PPARγ equally inhibited AP-1 binding suggests that this inhibition does not require AF-2-specific co-activators. Inhibition of JunD binding by PPARγ shown in our study has direct physiological implications in regulation of HSC activation, since JunD/AP-1 activity is shown to be important in expression of TGFβ1 (40), collagen (44), matrix metalloproteinase (8), tissue inhibitor of matrix metalloproteinase-1, and interleukin-6 (9) gene transcription. Indeed, suppressed JunD binding by adenovirus-mediated expression of PPARγ resulted in inhibition of both TGFβ1 and α1(I) procollagen gene expression in HSC (Fig. 3B).

The most striking phenotypic change facilitated by PPARγ was the restoration of the ability of HSC to accumulate retinyl palmitate when retinol and palmitate were presented to the cells. Since Ad.PPARγ infection alone or the concomitant addition of palmitate only slightly induced lipid accumulation, it appears unlikely that adipogenic differentiation was fully attained by these experimental conditions. Rather, these results suggest that PPARγ expression promoted the HSC-specific function of storing retinyl esters. It is interesting that this phenotype was partially blocked with dn.PPARγ, suggesting that induction of PPRE-driven genes may be partly involved. The understanding of the mechanisms underlying this effect requires further analysis of the genes involved in retinol esterification in HSC. Similarly, the morphologic reversal to the quiescent phenotype by PPARγ was dependent on the PPRE activity, suggesting the involvement of genes regulated by PPRE in attaining the morphologic effects. Further analysis of the genes involved in cytoskeletal organization and cell adhesion will be required to better understand the molecular mechanisms of the morphologic effects.

In summary, our results demonstrate that culture-activated HSC can be phenotypically and functionally reversed to the cells with the quiescent phenotype by forced expression of PPARγ. These results conceptually support the importance of PPARγ in the maintenance of the quiescent HSC phenotype. We also demonstrate a physical interaction of PPARγ with JunD but not alterations in JunD expression or JNK activity as part of the mechanisms underlying inhibition of JunD binding to DNA and AP-1 promoter activity. Our findings support the notion that PPARγ itself is the important effector molecule for controlling transdifferentiation of HSC. Whether and how other adipogenic transcriptional programs are involved in regulation of HSC are intriguing questions that need to be explored in the future.
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REFERENCES

1. Hautekeete, M. L., and Geerts, A. (1997) *Virchows Arch.* 430, 195–207
2. Kim, Y., Ratzu, V., Choi, S. G., Lalazar, A., Theiss, G., Dang, Q., Kim, S. J., and Friedman, S. L. (1998) *J. Biol. Chem.* 273, 33750–33758
3. Kojima, S., Hayashi, S., Shimokado, K., Suzuki, Y., Shimada, J., Crippa, M. P., and Friedman, S. L. (2000) *Blood* 96, 1309–1316
4. Bock, M., Kim, D. S., Houglum, K., Hassanein, T., and Chojkier, M. (2000) *Am. J. Physiol.* 278, G321–G328
5. Lang, A., Schoonhoven, R., Tovia, S., Brenner, D. A., and Rippe, R. A. (2000) *J. Hepatol.* 33, 48–58
6. Hellerbrand, C., Jobin, C., Licato, L. L., Sartor, R. B., and Brenner, D. A. (1998) *Am. J. Physiol.* 275, G269–G278
7. Knittel, T., Dinter, C., Kohold, D., Neubauer, K., Mehe, M., Eichhirst, S., and Ramadori, G. (1999) *Am. J. Pathol.* 154, 153–167
8. Smart, D. E., Vincent, K. J., Arthur, M. J., Eckelberg, O., Castellazzi, M., Mann, J., and Mann, D. A. (2001) *J. Biol. Chem.* 276, 24414–24421
9. Vincent, K. J., Jones, E., Arthur, M. J., Smart, D. E., Trim, J., Wright, M. C., and Mann, D. A. (2001) *Cytometry* 49, 713–719
10. Neubauer, K., Knittel, T., Aurisch, S., Fellner, P., and Ramadori, G. (1996) *J. Hepatol.* 24, 719–730
11. Niki, T., Pekny, M., Hellemans, K., Bleser, P. D., Berg, K. V., Vaeyens, F., Quartier, E., Schuit, F., and Geerts, A. (1999) *Hepatology* 29, 520–527
12. Cassiman, D., van Pottel, J., De Vos, R., Van Lommel, F., Desmet, V., Yap, S. H., and Reskams, T. (1999) *Am. J. Pathol.* 155, 1831–1839
13. Potter, J. J., Womack, L., Mezey, E., and Anania, F. A. (1998) *Biochem. Biophys. Res. Commun.* 244, 178–182
14. Yamada, M., Blaner, W. S., Soprano, D. R., Dixon, J. L., Kjeldbye, H. M., and Goodman, D. S. (1987) *Hepatology* 7, 1224–1229
15. Spiegelman, B. M., and Flier, J. S. (1996) *Cell* 87, 377–389
16. Miyahara, T., Schrum, L., Rippe, R., Xiong, S., Yee, H. F., Jr., Motomura, K., Anania, F. A., Wilseon, T. M., and Tsukamoto, H. (2000) *J. Biol. Chem.* 275, 35715–35722
17. Galli, A., Crabb, D. W., Ceni, E., Salzano, R., Mello, T., Svegliati-Baroni, G., Ridolfi, F., Trozzi, L., Surrenti, C., and Casini, A. (2002) *Gastroenterology* 122, 1924–1940
18. Marra, F., Elsen, E., Romanelli, R. G., Caligiuri, A., Pastacaldi, S., Batignani, G., Bonacchi, A., Caporale, R., Laffi, G., Pinzani, M., and Gentilini, P. (2000) *Gastroenterology* 119, 466–478
19. Chawla, A., Barak, Y., Nagy, L., Liu, D., Tontonoz, P., and Evans, R. M. (2001) *Nat. Med.* 7, 48–52
20. Straus, D. S., Pascual, G., Li, M., Welch, J. S., Ricote, M., Hsiang, C. H., Sengenathinalagy, L. L., Ghosh, G., and Glass, C. K. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 4844–4849
21. Baeke, S. J., Wilson, L. C., Hsi, L. C., and Eling, T. E. (2003) *J. Biol. Chem.* 278, 5845–5853
22. Lennon, A. M., Ramauge, M., Desourroux, A., and Pierre, M. (2002) *J. Biol. Chem.* 277, 29681–29685
23. Tsukamoto, H., Cheng, S., and Blaner, W. S. (1996) *Am. J. Physiol.* 270, G581–G586
24. Xiong, S., Yavrom, S., Hazra, S., Wu, D., and She, H. (2001) *Hepatology* 34, 520A (abstr.)
25. Gurnell, M., Wentworth, J. M., Agarwal, A., Adams, M., Collingwood, T. N., Provenzano, C., Browne, P. O., Rajanayagam, O., Burris, T. P., Schwahe, J. W., Lazar, M. A., and Chatterjee, V. K. (2000) *J. Biol. Chem.* 275, 5754–5759
26. Ohata, M., Liu, M., Satre, M., and Tsukamoto, H. (1997) *Am. J. Physiol.* 272, G589–G596
27. Bhat, P. V., and Lacroix, A. (1983) *J. Chromatogr.* 272, 269–278
28. Gressner, A. M., and Bachem, M. G. (1990) *Semin. Liver Dis.* 10, 30–46
29. Bissell, D. M., Wang, S. S., Jarnagin, W. R., and Roll, F. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 447–455
30. Friedman, S. L., Wei, S., and Blaner, W. S. (1993) *Am. J. Physiol.* 264, G947–G952
31. Mann, D. A., and Smart, D. E. (2002) *Gastroenterology* 122, 48–52
32. Juge-Aubry, C., Pernin, A., Vezien, E., Fruchart, J. C., and Auwerx, J. (1999) *J. Biol. Chem.* 274, 7681–7688
33. Gressner, A. M. (1996) *Kidney Int. Suppl.* 53, S39–S48
34. Jankeczy, R., and Hunter, T. (1998) *Nature* 391, 22–23
35. Mirukami, J., and Taniguchi, T. (1997) *Biochem. Biophys. Res. Commun.* 240, 61–64
36. Gressner, A. M., Zhou, G., Fajas, L., Raspe, E., Fruchart, J. C., and Auwerx, J. (1999) *J. Biol. Chem.* 274, 7681–7688
37. Saxena, N. K., Ikeda, K., Rockey, D. C., Friedman, S. L., and Anania, F. A. (2002) *Hepatology* 35, 762–771
38. Saile, B., Matthes, N., Knittel, T., and Ramadori, G. (1999) *Hepatology* 30, 196–202
39. Salbert, G., Fanjul, A., Pecherita, F. J., Lu, X. P., Kim, S. J., Tran, P., and Pfahl, M. (1993) *Mol. Endocrinol.* 7, 1347–1356
40. Fu, M., Zhang, J., Zhu, X., Myles, D. E., Willson, T. M., Liu, X., and Chen, Y. E. (2001) *J. Biol. Chem.* 276, 43888–43894
41. Sugawara, A., Uruno, A., Kudo, M., Ikeda, Y., Sato, K., Taniyama, Y., Ito, S., and Takeuchi, K. (2002) *J. Biol. Chem.* 277, 9676–9683
42. MIkikawa, I., Takada, L., Yano, K., Ohtake, F., Ogawa, S., Yamauchi, T., Kodawaki, T., Takeuchi, Y., Shiroya, H., Gotoh, Y., Matsumoto, K., and Takibe, S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 224–230
43. Chung, K. Y., Agarwal, A., Uitto, J., and Mauriel, A. (1996) *J. Biol. Chem.* 271, 3272–3278