Original Paper

Peroxisome Proliferator-Activated Receptor Gamma Negatively Regulates the Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells Toward Myofibroblasts in Liver Fibrogenesis

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Key Words

Peroxisome proliferator-activated receptor gamma • Bone marrow-derived mesenchymal stem cells • Differentiation • Fibrogenesis • Transforming growth factor β 1

Abstract

Background/Aims: Bone marrow-derived mesenchymal stem cells (BMSCs) have been confirmed to have capacity to differentiate toward hepatic myofibroblasts, which contribute to fibrogenesis in chronic liver diseases. Peroxisome proliferator-activated receptor gamma (PPAR γ), a ligand-activated transcription factor, has gained a great deal of recent attention as it is involved in fibrosis and cell differentiation. However, whether it regulates the differentiation of BMSCs toward myofibroblasts remains to be defined. **Methods:** Carbon tetrachloride or bile duct ligation was used to induce mouse liver fibrosis. Expressions of PPAR γ , α -smooth muscle actin, collagen α 1 (I) and collagen α 1 (III) were detected by real-time RT-PCR and Western blot or immunofluorescence assay. **Results:** PPAR γ expression was decreased in mouse fibrotic liver. In addition, PPAR γ was declined during the differentiation of BMSCs toward myofibroblasts induced by transforming growth factor β 1. Activation of PPAR γ stimulated by natural or synthetic ligands suppressed the differentiation of BMSCs. Additionally, knock down of PPAR γ by siRNA contributed to BMSC differentiation toward myofibroblasts. Furthermore, PPAR γ activation by natural ligand significantly inhibited the differentiation of BMSCs toward myofibroblasts in liver fibrogenesis and alleviated liver fibrosis. **Conclusions:** PPAR γ negatively regulates the differentiation of BMSCs toward myofibroblasts, which highlights a further mechanism implicated in the BMSC differentiation.

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Introduction

Liver fibrosis is characterized by overproduction of collagen, especially collagen $\alpha 1$ (I) (Col $\alpha 1$ (I)) and collagen $\alpha 1$ (III) (Col $\alpha 1$ (III)), and other extracellular matrix (ECM) components [1, 2]. Myofibroblasts, characterized by the cytoskeletal protein α -smooth muscle actin (α -SMA), are known to be the predominant cells to generate Col $\alpha 1$ (I) and Col $\alpha 1$ (III). In addition to hepatic stellate cells (HSCs), portal fibroblasts, circulating fibrocytes and epithelial-mesenchymal transition (EMT), bone marrow (BM)-derived cells have recently been considered as another origin of hepatic myofibroblasts [2-4]. In particular, by using lethally irradiated female mice which receive male BM transplants, and tracking BM-derived cells through in situ hybridization for the Y chromosome, Russo et al. [5] found that 70% of hepatic myofibroblast populations are from BM-derived mesenchymal stem cells (BMSCs). In keeping with this finding, we also proved that a significant proportion of hepatic myofibroblasts is of BMSC-differentiation origin with a model of lethally irradiated mice receiving BM transplants from enhanced green fluorescent protein (EGFP) transgenic mice [6]. Considering the importance of BMSCs in liver fibrosis, identification of the molecular mechanisms underlying BMSC differentiation toward myofibroblast may represent an effective strategy for the treatment of fibrotic liver disease.

Transforming growth factor $\beta 1$ (TGF- $\beta 1$) is a key profibrotic cytokine in the fibrogenesis [7-10], and can induce the differentiation of several sorts of fibroblasts toward myofibroblasts *in vitro*, such as fibroblasts from skin, lung, kidney and heart origin [11-14]. Our previous study has demonstrated that sphingosine kinase/sphingosine 1-phosphate (S1P)/S1P receptor axis is involved in the differentiation of BMSCs toward myofibroblasts induced by TGF- $\beta 1$ during liver fibrosis [9]. Nevertheless, it is still not clearly illustrated whether other molecules implicated in this process and the underlying mechanisms.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily [15], with three subtypes, PPAR α , PPAR β/δ and PPAR γ , relate to multiple functions, including metabolism, immune responses and cellular proliferation [16]. PPAR γ is mainly known to regulate adipocyte differentiation and fatty-acid uptake and storage [17-19]. Some clinical studies reported that synthetic PPAR γ agonists, such as thiazolidinedione, have beneficial effects in patients with type 2 diabetes [20, 21]. Besides its function in metabolism, a new action of PPAR γ in fibrosis has been revealed. It has been reported that activation of PPAR γ by specific agonists could ameliorate fibrosis in several organs, such as lung, kidney and heart [22]. In particular, several recent studies have illustrated that PPAR γ participates in the myofibroblast differentiation [11, 13, 23, 24]. However, whether PPAR γ is link to regulate the differentiation of BMSCs toward hepatic myofibroblasts and its molecular mechanisms remain obscure.

Based on the scientific background, the aim of this study is to elucidate the function of PPAR γ in BMSCs differentiation toward myofibroblasts in liver fibrogenesis. Here, we showed that PPAR γ expression was reduced in mouse liver fibrogenesis, as well as in the differentiation of primary mouse BMSCs toward myofibroblasts induced by TGF- $\beta 1$ *in vitro*. In addition, knock down of PPAR γ by siRNA contributed to the differentiation of BMSCs. Furthermore, activation of PPAR γ by ligands inhibited BMSCs differentiation toward myofibroblasts *in vitro* and *in vivo*, and ameliorated liver fibrosis. These observations strongly indicate that PPAR γ negatively governs the differentiation of BMSCs toward myofibroblasts, suggesting that PPAR γ -targeted therapy could be a potential strategy in the treatment of liver fibrosis.

Materials and Methods

Reagents

α -MEM was from Invitrogen (Grand Island, NY). Fetal bovine serum was from Hyclone/Thermo Scientific (Victoria, Australia). TGF- $\beta 1$ came from PeproTech (London, UK). 15d-PGJ $_2$ was from Cayman Chemi-

cal (Ann Arbor, MI). Troglitazone and ciglitazone were from Biomol (Tebu, France). Antibodies against PPAR γ , Col a1 (I), Col a1 (III) and tubulin were from Santa Cruz Biotechnology (CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was from Cell Signalling (Beverly, MA). PCR reagents were from Applied Biosystems (Foster City, CA). Anti- α -SMA antibody, bovine serum albumin (BSA) and other common reagents were from Sigma-Aldrich (St. Louis, MO).

BMSCs preparation

BM cells were isolated from bone marrow by flushing the tibias and femurs of ICR mice aged 3 weeks (Laboratory Animal Center, Capital Medical University) with culture medium using a 25-gauge needle. The cells were then passed through 70-mm nylon mesh and were washed three times with PBS containing 2% fetal bovine serum. BMSCs cultured as described previously [5]. In brief, BM cells were cultured with BMSCs culture medium (α -MEM) containing 20% fetal bovine serum for 1 week at 37°C in 5% CO $_2$. To remove the nonadherent cells, the culture medium was replaced twice a week. α -MEM containing 15% fetal bovine serum was used to culture BMSCs after the first subculture. BMSCs are characterized by the phenotypes as positive for CD44, CD105 and CD166, but negative for CD14, CD34 and CD45 in flow cytometry analysis [6]. BMSCs of passage 3 to passage 5 were used in the experiments. All animal work was performed under the ethical guidelines of the Ethics Committee of Capital Medical University.

Mouse models of liver fibrosis

Mouse models of chronic liver fibrosis were performed by injection of carbon tetrachloride (CCl $_4$) or bile duct ligation (BDL). ICR mice aged 6 weeks received intraperitoneal injections of 1 μ L/g body weight of a CCl $_4$ /olive oil (OO) mixture, 1:9 v/v, twice per week. 15d-PGJ $_2$ (0.3mg/kg body weight) or saline was administrated the day before CCl $_4$ or OO treatment, and then twice per week before CCl $_4$ or OO treatment for indicated times. The mice were sacrificed at 1, 3 days or at 1, 2, 4, or 8 weeks of CCl $_4$ treatment (n = 7 per group).

Another group of adult ICR mice received BDL operation as described previously [25]. In brief, mice were anesthetized to receive a midline laparotomy to expose the common bile duct which was ligated three times. One ligature was located in the distal portion of the bile duct and another two were placed in the proximal portion. Then, the bile duct was cut between the ligatures followed by closing the abdomen in layers. Sham-operated mice underwent a laparotomy with exposure, but no performance of ligation of the common bile duct, which used as controls. Mice were sacrificed at 3 days or at 1, 2 weeks of BDL (n = 7 per group).

BM transplantation

ICR mice received lethal irradiation (8 Grays), and then immediately received transplantation by a tail-vein injection of 1.5×10^7 whole BM cells obtained from 3-week-old EGFP transgenic mice. Four weeks later, mice received intraperitoneal injections of CCl $_4$ twice per week for 4 weeks. 15d-PGJ $_2$ (0.3 mg/kg body weight) or saline was applied the day before CCl $_4$ or OO injection, and twice per week before CCl $_4$ or OO treatment for 4 weeks (n = 7 per group).

Quantitative analysis of liver fibrosis

Liver tissues were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Samples were cut into 5 μ m slices and sections were stained for collagen visualization with Sirius Red.

Immunofluorescence and high content analysis

BMSCs with or without treatments were fixed in 4% paraformaldehyde in PBS for 30 minutes. Then cells were washed twice with PBS, permeabilized in 0.5% Triton X-100 for 15 minutes, blocked with 2% BSA for 1 hour, and incubated with antibodies to α -SMA (1:1000), Col a1(I) (1:100), Col a1(III) (1:200) and PPAR γ (1:100) overnight, followed by secondary antibody conjugated with Cy3 (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. Cells were washed twice with PBS after incubation with PBS containing 10 μ g/mL DAPI for 5 minutes, and 200 μ L PBS were left in each well. For negative controls, cells were processed the same way, except the primary antibody was omitted. The plates were imaged on a Thermo Scientific Cell Insight personal cell imaging (PCI) platform (Cellomics, Inc., Thermo Fisher Scientific Inc., Waltham, MA), with a $\times 10$ objective using the Thermo Scientific Cellomics iDEV Software.

High content analysis was performed as described previously [26]. In brief, at least 3,000 cells in thirty-six fields were automatically acquired by the software. The fluorescence intensity in each well was analyzed using Cellomics Cell Health Profiling BioApplication software.

Immunofluorescent detection for α -SMA in liver tissue was performed with a Vector M.O.M. (mouse-on-mouse) immunodetection kit (Vector Laboratories) [9]. Prepared liver sections were 5 μ m and antibody to α -SMA was a 1:1000 dilution.

Western blot analysis

Western blot analysis of α -SMA, Col α 1(I), Col α 1(III) and PPAR γ in BMSCs or liver tissue were performed with 50 μ g of protein extract using primary antibodies of α -SMA (1:1000), Col α 1(I) (1:100), Col α 1(III) (1:200) and PPAR γ (1:500). The bands were displayed using ODYSSEY and quantified by Odyssey v3.0 software. Results were normalized relative to the GAPDH (1:1000) or tubulin (1:1000) expression to correct for variations in protein loading and transfer.

RNA interference

The siRNA sequence specifically targeting mouse PPAR γ was synthesized by Dharmacon: L-040712-00-0005 (Thermo Scientific, Lafayette, CO). 40% to 50% confluent BMSCs were prepared. Transient transfection of siRNA (40 nmol/L) was performed by using Invitrogen Lipofectamine RNAiMAX, as recommended by the manufacturer. Control cells were treated with 40 nmol/L RNAi Negative Control Duplexes (scramble siRNA). Cells were collected to perform real-time RT-PCR and Western blot analysis after 48 hours.

Real-time RT-PCR

Total RNA was extracted from frozen liver specimens or cultured BMSCs with or without treatments, using an RNeasy kit (Qiagen, Hilden, Germany). Real-time RT-PCR was performed with an ABI Prism 7300 sequence-detecting system (Life Technologies, Foster City, CA), as described previously [6]. Primers (MWG Biotech, Ebersberg, Germany) used for real-time RT-PCR were as follows: 18s rRNA, sense, 5'-GTA ACC CGT TGA ACC CCA TT-3', and anti-sense, 5'-CCA TCC AAT CGG TAG TAG CG-3'; α -SMA: sense, 5'-ATG CTC CCA GCG CTG TTT T-3', and anti-sense, 5'-TTC CAA CCA TTA CTC CCT GAT GT-3'; Col α 1(I): sense, 5'-AGG GCG AGT GCT GTG CTT T-3', and anti-sense, 5'-CCC TCG ACT CCT ACA TCT TCT GA-3'; Col α 1(III): sense, 5'-TGA AAC CCC AGC AAA ACA AAA-3', and anti-sense, 5'-TCA CTT GCA CTG GTT GAT AAG ATT AA-3'; TGF- β 1: sense, 5'-TGC GCT TGC AGA GAT TAA AA-3', and anti-sense, 5'-TCA CTG GAG TTG TAC GGC AG-3'; PPAR γ : sense, 5'-GCC CAC CAA CTT CGG AAT C-3', and anti-sense, 5'-TGC GAG TGG TCT TCC ATC AC-3'.

Statistical analysis

All results were confirmed in three independent experiments. Data are expressed as mean $\pm$ SEM and were analyzed by Student's t-test or ANOVA for analysis of variance. Statistical significance was defined as $P < 0.05$.

Results

PPAR γ is down-regulated in mouse chronic liver fibrogenesis

There are recent reports that expression of PPAR γ is decreased in fibrogenesis [13, 27, 28]. We assessed PPAR γ content in liver tissue of mouse chronic liver fibrosis models induced by CCl $_4$ administration or BDL operation. The results showed that PPAR γ expression in both mRNA and protein levels were markedly reduced in CCl $_4$ administration group compared with OO-treated group (Fig. 1A and B). These findings were consistent with the results in BDL-induced liver fibrogenesis (Fig. 1C and D).

The expressions of main ECM, Col α 1 (I) and Col α 1 (III), were up-regulated in mouse liver fibrogenesis [29]. Here, we undertook correlation analysis between PPAR γ and the two molecules in liver tissue. Results presented in Fig. 1E and F demonstrated that the mRNA level of PPAR γ had negative correlations with Col α 1 (I) and Col α 1 (III), respectively (correlation coefficient $r = -0.503$, $P = 0.024$; $r = -0.525$, $P = 0.025$, respectively). In addition, the expression of α -SMA, a marker of myofibroblasts, which produces ECM predominantly,

Fig. 1. PPAR γ is involved in the chronic liver fibrogenesis. Real time RT-PCR was performed to determine mRNA expression of PPAR γ in mouse fibrotic liver induced by CCl $_4$ treatment (A) or BDL operation (C) (n = 7 per group). PPAR γ protein expressions in CCl $_4$ treatment (4 weeks, B) or BDL operation (2 weeks, D) were detected by Western blot. The correlations between mRNA expression of PPAR γ and Col α 1 (I) (E), Col α 1 (III) (F), α -SMA (G) or TGF- β 1 (H) in liver tissue. * P < 0.05, compared with olive oil (OO) or sham group.

was obviously increased as well after liver injury [29]. We also found a negative correlation between the mRNA levels of PPAR γ and α -SMA (correlation coefficient r = -0.505, P = 0.039) (Fig. 1G). Furthermore, there was a significant negative correlation between mRNA levels of PPAR γ and TGF- β 1, a key profibrotic cytokine and contributing to BMSC differentiation toward myofibroblasts in fibrotic liver [9] (correlation coefficient r = -0.610, P = 0.009) (Fig. 1H). Collectively, these results suggested PPAR γ might play an essential role in liver fibrogenesis.

PPAR γ is down-regulated in the differentiation of BMSCs toward myofibroblasts

In addition to representing the extent of liver fibrosis, presence of α -SMA and increased levels of Col α 1 (I) and Col α 1 (III) also denote the differentiation of BMSCs toward myofibroblasts [9]. Given that PPAR γ was negatively correlated with these molecules and TGF- β 1 in liver tissue, we then sought to determine the effects of PPAR γ on the differentiation of BMSCs. In this regard, we explored the abundance of PPAR γ in the context of BMSC

Fig. 2. PPAR γ is implicated in the differentiation of BMSCs toward myofibroblasts. (A) Immunofluorescence analysis of PPAR γ (red) in BMSCs with (+) or without (-) TGF- β 1 (10 ng/mL). (B) PPAR γ mRNA in differentiated BMSCs induced by indicated doses of TGF- β 1 for 24 hours. (C) Protein level of PPAR γ in BMSCs treated with 10 ng/mL TGF- β 1 for 24 hours. Correlations between PPAR γ and α -SMA (D), Col α 1 (I) (E) or Col α 1(III) (F) in BMSCs. All results were confirmed in three independent experiments. * P < 0.05, compared with control. Scale bars, 50 μ m.

differentiation toward myofibroblasts *in vitro*. Immunofluorescent staining showed that the degree of positive staining for PPAR γ was much weakened when BMSCs were induced differentiation toward myofibroblasts by TGF- β 1 (Fig. 2A). Furthermore, the mRNA level of PPAR γ was strikingly decreased during differentiation of BMSCs triggered by TGF- β 1 in a dose-dependent manner (Fig. 2B). In parallel, PPAR γ expression in protein level was attenuated, too (Fig. 2C). It was noteworthy that there were also inverse correlations between mRNA expressions of PPAR γ and α -SMA, Col α 1 (I) or Col α 1 (III) in BMSCs (Fig. 2D-F) (correlation coefficient $r = -0.691$, $P = 0.000046$; $r = -0.792$, $P = 0.000001$; $r = -0.737$, $P = 0.000008$, respectively). These observations highlight the link between PPAR γ and the differentiation of BMSCs toward myofibroblasts.

PPAR γ negatively modulates the differentiation of BMSCs toward myofibroblasts

To further characterize the function of PPAR γ in BMSC differentiation, we used an endogenous ligand (15d-PGJ $_2$) and two synthetic ligands (troglitazone and ciglitazone) of PPAR γ . As illustrated in Fig. 3A, α -SMA mRNA in differentiated BMSCs triggered by TGF- β 1

Fig. 3. Activated PPAR γ negatively regulates α -SMA expression in BMSCs. BMSCs were exposed to 5 μ M/L endogenous PPAR γ ligand (15d-PGJ₂) or 10 μ M/L PPAR γ synthetic agonists (troglitazone (Trog) or ciglitazone (Cig)) for 1 hour, then challenged by 10 ng/mL TGF- β 1 for 24 hours. Real time RT-PCR was performed to detect α -SMA mRNA (A). α -SMA protein expression was evaluated by Western blot (B). The intensity of each band with α -SMA antibody was quantified and normalized to that with anti- β -tubulin antibody (C). (H) Immunofluorescence analysis of α -SMA (red), and total fluorescence intensity of α -SMA protein was counted with high content analysis (G). Cells were transfected with scrambled siRNA or PPAR γ siRNA for 48 hours. Protein level of PPAR γ was detected by Western blot (E) and mRNA expression of PPAR γ and α -SMA was measured by real-time RT-PCR (D and F). All results were confirmed in three independent experiments. * P < 0.05, compared with control. # P < 0.05, compared to TGF- β 1 group. Scale bars, 50 μ m.

was obviously diminished when pretreatment with either natural or synthetic ligands of PPAR γ . In accord, α -SMA protein expression induced by TGF- β 1 was down-regulated by 15d-PGJ₂, troglitazone or ciglitazone (Fig. 3B and C). Furthermore, siRNA technology was

Fig. 4. PPAR γ activation suppresses Col α 1(I) expression in BMSCs. BMSCs were incubated with 5 μ mol/L 15d-PGJ₂, 10 μ mol/L troglitazone (Trog) or 10 μ mol/L ciglitazone (Cig) for 1 hour, followed by treatment of 10 ng/mL TGF- β 1 for 24 hours. mRNA expression of Col α 1(I) was examined by real-time RT-PCR (A). Col α 1(I) protein expression was detected by Western blot (B), immunofluorescence analysis (red) (E) and high content analysis (D). Arrows in (E) indicated that Col α 1(I) secreted into the medium. (C) Cells were transfected with scrambled siRNA or PPAR γ siRNA, and Col α 1(I) mRNA was evaluated 48 hours later. All results were confirmed in three independent experiments. * P < 0.05, compared with control; # P < 0.05, compared with TGF- β 1 group. Scale bars, 50 μ m.

performed to knock-down of PPAR γ . Transfection of BMSCs with PPAR γ -siRNA decreased the mRNA expression of PPAR γ by 70% (Fig. 3D). Meanwhile, Western blot analysis indicated PPAR γ protein expression was also significantly reduced after knock-down of PPAR γ (Fig. 3E). The results showed that PPAR γ knock-down enhanced α -SMA mRNA expression, whereas scrambled siRNA (SCR siRNA) had no effects (Fig. 3F).

To further confirm the function of PPAR γ in BMSC differentiation, we performed immunofluorescent staining for α -SMA to reveal morphological changes in BMSCs. Isolated BMSCs showed very weak expression of α -SMA under normal condition. TGF- β 1 treatment resulted in the appearance of numerous bundles of actin microfilaments and dramatically increased the expression of α -SMA in BMSCs (Fig. 3H). However, when BMSCs were pretreated with ligands of PPAR γ , the ability of TGF- β 1 to enhance the expression of α -SMA was markedly blunted (Fig. 3H). Additionally, the total fluorescence intensity of α -SMA examined by high content analysis demonstrated that exposure to 15d-PGJ₂, troglitazone or ciglitazone resulted in reducing α -SMA protein level in differentiated BMSCs initiated by TGF- β 1 (Fig. 3G).

Fig. 5. Activation of PPAR γ represses expression of Col α 1 (III) in BMSCs. BMSCs were pretreated with 15d-PGJ₂ (5 μ mol/L), troglitazone (Trog, 10 μ mol/L) or ciglitazone (Cig, 10 μ mol/L) for 1 hour, then cells were stimulated with TGF- β 1 (10 ng/mL) for 24 hours. Col α 1 (III) mRNA expression was assessed by real-time RT-PCR (A). Western blot (B), immunofluorescence analysis (red) (E) and high content analysis (D) were used to evaluate Col α 1 (III) protein. Col α 1 (III) secreted into the medium was shown as arrows in (E). (C) BMSCs were transfected with scrambled siRNA or PPAR γ siRNA for 48 hours, and Col α 1 (I) mRNA was evaluated by real-time RT-PCR. All results were confirmed in three independent experiments. * P < 0.05, compared with control; # P < 0.05, compared with TGF- β 1 group. Scale bars, 50 μ m.

Myofibroblasts are the predominant collagen-producing cells in liver fibrosis. Up-regulation of Col α 1 (I) and Col α 1 (III) is an essential event during BMSC differentiation toward myofibroblasts. Therefore, we sought to characterize the effects of PPAR γ on the expression of Col α 1 (I) and Col α 1 (III) in BMSCs. As expected, all three PPAR γ agonists, 15d-PGJ₂, troglitazone or ciglitazone blunted the increases of Col α 1(I) and Col α 1(III) mRNA expression in BMSCs treated with TGF- β 1 (Fig. 4A and 5A). Western blot analysis exhibited similar results in protein level (Fig. 4B and 5B). In accord with α -SMA, mRNA expressions of Col α 1 (I) and Col α 1 (III) were also increased in PPAR γ -knock-down cells (Fig. 4C and 5C).

Furthermore, immunofluorescence was also performed to study the effects of PPAR γ on the protein expressions of Col α 1 (I) and Col α 1 (III) in BMSCs. The results showed that there were strong immunoreactivities for Col α 1 (I) and Col α 1 (III) in BMSCs after treatment with TGF- β 1 (Fig. 4E and 5E), and collagen that was secreted into the medium was also detected by immunofluorescence (Fig. 4E and 5E, arrow). Manipulation of PPAR γ ligands reduced the degree of positive staining for Col α 1 (I) and Col α 1 (III) induced by TGF- β 1 (Fig. 4E and

Fig. 6. Activated PPAR γ inhibits BMSCs differentiation toward myofibroblasts during liver injury. (A) Mouse liver fibrosis was induced by injection of CCl₄ twice per week for different time periods with or without 15d-PGJ₂ administration (n = 7 per group). mRNA of α -SMA in liver tissues was detected with real-time RT-PCR. (B-D) ICR mice were lethally irradiated and received whole BM transplants from EGFP transgenic mice, followed by CCl₄ injection twice per week for 4 weeks in the presence or absence of 15d-PGJ₂ treatment (n = 7 per group). The proportion of myofibroblasts from BMSCs was evaluated using Image-Pro Plus software (B). Representative immunofluorescence images of α -SMA (red), EGFP (green) and merged fields to track myofibroblasts of BMSC origin (C and D). Insets in (C) were immunofluorescence images for olive oil (OO)-treated livers, and in (D) were livers of OO group with 15d-PGJ₂. The nuclei were stained with DAPI (blue). * $P < 0.05$ compared with OO group. # $P < 0.05$, compared with CCl₄ group without 15d-PGJ₂. Scale bars, 50 μ m.

5E). High content analysis indicated that the increased total fluorescence intensities of Col α 1 (I) and Col α 1 (III) were attenuated by 15d-PGJ₂, roglitazone or ciglitazone (Fig. 4D and 5D). Altogether, these findings showed that PPAR γ negatively governed BMSCs differentiation toward myofibroblasts.

Activated PPAR γ inhibits the differentiation of BMSCs toward myofibroblasts in mouse fibrotic liver

During liver injury, myofibroblasts are increased and its marker, α -SMA, is up-regulated to contribute to liver fibrogenesis [29]. In the present study, we found that treatment with the natural ligand of PPAR γ , 15d-PGJ₂, reduced α -SMA mRNA in liver fibrosis induced by different

Fig. 7. PPAR γ activation suppresses collagen expression in liver fibrosis. Different time periods of CCl $_4$ was used to induce mouse liver fibrosis with or without 15d-PGJ $_2$ administration (n = 7 per group). (A) Expression of Col α 1 (I) and Col α 1 (I-II) mRNA levels in liver tissues were measured by real-time RT-PCR. (B) Representative images of Sirius Red staining in the absence or presence of 15d-PGJ $_2$ after CCl $_4$ administration. * $P < 0.05$ compared with olive oil (OO) group. # $P < 0.05$, compared with CCl $_4$ group without 15d-PGJ $_2$. Scale bars, 50 μ m.

periods of CCl $_4$ injection (Fig. 6A). Given that myofibroblasts are mostly BMSC-derived, we further determined the effect of PPAR γ on BMSC differentiation toward myofibroblasts *in vivo*. BM in the irradiated mice was reconstituted by transplanting BM cells from EGFP transgenic mice, and then the proportion of BMSC-derived myofibroblasts was measured. As shown in Fig. 6C, after CCl $_4$ treatment, there was a strong expression of α -SMA colocalizing with EGFP in the fibrotic areas, which indicated they were BMSC origin. However, 15d-PGJ $_2$ significantly inhibited the differentiation of BMSCs toward myofibroblasts in liver fibrogenesis (Fig. 6D), and markedly decreased the proportion of BMSC-derived myofibroblasts compared with CCl $_4$ group without 15d-PGJ $_2$ treatment (Fig. 6B). These results indicated that PPAR γ activation could suppress BMSCs differentiate toward myofibroblasts *in vivo*.

Activated PPAR γ attenuates hepatic fibrosis

Col α 1 (I) and Col α 1 (III) are the main ECM in fibrotic liver tissue [1, 2]. After injection of CCl $_4$, there were dynamic changes in mRNA expressions of Col α 1 (I) and Col α 1 (III) in liver tissue, which markedly up-regulated in a way correlated with the progression of liver fibrosis (Fig. 7A). As expected, PPAR γ activation by 15d-PGJ $_2$ markedly decreased these mRNA levels (Fig. 7A). In addition, hepatic collagen deposition was further evaluated by morphometric analysis with Sirius red staining. As shown in Fig. 7B, collagen deposition was markedly attenuated after 15d-PGJ $_2$ administration. These data suggest activation of PPAR γ had anti-fibrotic properties.

Discussion

Liver fibrosis is a common pathological course of chronic hepatic diseases. So far, there is no effective and safe strategy for the prevention or inhibition of fibrogenesis in clinical practice. Differentiation of BMSCs toward myofibroblasts is an essential event for liver fibrogenesis, thus, elucidating the regulatory mechanisms in this process may shed light on the treatment of liver fibrosis. In the current study, we delineated the potential role of PPAR γ in the differentiation of primary mouse BMSCs toward myofibroblasts. We found PPAR γ negatively regulated BMSC differentiation toward myofibroblasts in liver fibrogenesis. Expression of PPAR γ was reduced in fibrotic liver tissue. In addition, differentiation of BMSCs was accompanied by the decrease of PPAR γ expression. Activated PPAR γ by specific ligands could attenuate BMSC differentiation *in vitro* and *in vivo*, and alleviate liver fibrosis. These results critically revealed a mechanism underlying differentiation of BMSCs toward myofibroblasts.

Accumulating evidence suggests that levels of PPAR γ were dramatically reduced in the process of fibrosis [13, 27, 28, 30, 31], whereas overexpression of PPAR γ could ameliorate the degree of fibrosis [32]. In the present study, the abundance of PPAR γ was evidently reduced in fibrogenesis, similar to the reports in rats that PPAR γ expression was down-regulated in fibrotic liver tissue induced by 2 or 8 weeks of CCl₄ combined with ethyl alcohol treatment or caused by 10 days of BDL operation [28, 33, 34]. In addition, mRNA expressions of PPAR γ and those fibrotic related molecules, such as TGF- β 1, α -SMA, Col α 1 (I) and Col α 1 (III), were negative correlations. Intriguingly, PPAR γ and plasminogen activator inhibitor-1 (PAI-1), associated with fibrosis and implicated in its pathogenesis, were inversely correlated in skin [13]. In heart, PPAR γ was also negatively correlated with tenascin-x, which was exclusively expressed in fibroblasts that can mediate fibrosis [35]. These results emphasize the importance of PPAR γ in the pathogenesis of fibrosis in different tissues.

Several studies reveal that PPAR γ was down-regulated in the differentiation of sorts of fibroblasts and myocytes toward myofibroblasts [11, 13, 36]. In our study, expression of PPAR γ was remarkably decreased during BMSC differentiation toward myofibroblasts induced by TGF- β 1, similar to our previous report that expression of PPAR γ almost couldn't be detected in human hepatic myofibroblasts [37]. In addition, the down-regulation of PPAR γ by specific siRNA had favorable effect on the differentiation of BMSCs. In accordance, enhanced myofibroblast formation potential was observed in PPAR γ -deficient mouse fibroblasts [38]. BMSCs are multipotent cells with the potential to differentiate toward a variety of mesenchymal cells, such as adipocytes [39]. The decreased expression of PPAR γ , a transcription factor that related to fat-formation, might make BMSCs lost potential to differentiate toward adipocytes, but contribute to the differentiation toward myofibroblasts in the present study. This hypothesis is in agreement with the results in HSCs, another type fat-storing cell, that significant reduced expression of PPAR γ was paralleled by activation of HSCs [32, 40-42]. It has been reported that the *necdin-wnt* pathway and a combination of methyl-CpG binding protein 2 (MeCP2), enhancer of zeste homolog 2 (EZH2) and miR132 cause epigenetic PPAR γ repression and result in HSC activation [43, 44]. In addition, PPAR γ expression could be repressed by TGF- β 1 through increasing the binding of histone deacetylase 1 (HDAC1) and decreasing the levels of acetylated histone3 (AcH3) at the PPAR γ promoter or via smad binding elements in the PPAR γ gene promoter to negatively regulate the promoter activity of PPAR γ gene in cardiac fibroblasts or HSCs [11, 24]. However, the precise regulatory mechanism of PPAR γ expression in BMSCs is scarce and should be valued in further research.

PPAR γ needs to be activated by ligands to exert effects. Results presented here showed that either natural or synthetic ligands of PPAR γ could suppress BMSC differentiate toward myofibroblasts induced by TGF- β 1 *in vitro*, with alleviation of α -SMA, Col α 1 (I) and Col α 1 (III) expression. More importantly, we found activation of PPAR γ by natural ligand could inhibit the differentiation of BMSCs toward myofibroblasts in liver fibrogenesis and decreased the proportion of BMSC-derived myofibroblasts. These results provide strong evidence for

that activated PPAR γ can negatively regulate BMSC differentiate toward myofibroblasts. In particular, serum concentrations of 15d-PGJ $_2$ in Caucasian patients with ongoing hepatic fibrogenesis and Chinese patients with hepatocellular carcinoma are elevated, which suggests that increased 15d-PGJ $_2$ might trigger PPAR γ activity to attempt to execute its beneficial effects [45]. Additionally, PPAR γ activation by agonists attenuates skin and renal fibroblast differentiation [12, 46] and inhibits HSC activation [32, 42]. It is noteworthy that effects of activated PPAR γ by different agonists may have diverse and complex roles in regulating cell differentiation. For instance, Da et al. [47] reported that PPAR γ ligand, pioglitazone, did not suppress cultured HSC activation by assessment expression of α -SMA and Col α 1(I).

Our previous result has indicated that sphingosine kinase/S1P/S1P receptor axis participates in the differentiation of BMSCs toward myofibroblasts [9]. In the present study, we found PPAR γ negatively regulates BMSC differentiation toward myofibroblasts. There are several reports have linked PPAR γ with S1P signaling pathway. In renal mesangial cells, synthetic PPAR γ agonists thiazolidinediones up-regulate sphingosine kinase and intracellular S1P resulting in decrease expression of CTGF to causes an anti-fibrotic effect [48]. In addition, thiazolidinediones could increase the abundance of S1P receptor [49]. In turn, PPAR γ is an intracellular target for S1P and S1P could activate PPAR γ coactivator 1 α (PGC-1 α) [50, 51]. These findings might partially explain the effect of PPAR γ on BMSC differentiation toward myofibroblasts and its inhibitory function in liver fibrosis. However, the relationship between PPAR γ and S1P pathway remains controversial. In 3T3-L1 preadipocytes, S1P down-regulates the expression of PPAR γ [52], which reflects the complexity of signaling cascades in a biological system.

An important issue of PPAR γ is that PPAR γ carries out its function requiring translocation from cytoplasm to nucleus, such as in the study of PPAR γ governs the differentiation of skin fibroblasts [46]. Besides regulating target gene expression directly, PPAR γ could interact with other cell signaling pathways, including fibrotic-related events in cells. It has been reported that PPAR γ is capable of binding to Smad3 and p-Smad3, and prevents nuclear accumulation of p-Smad3, resulting in disruption of TGF- β 1 signaling [53]. Activated PPAR γ by rosiglitazone reduced the induction of the early-immediate transcription factor Egr-1, a mediator of non-Smad TGF- β 1 signaling, to exhibit its antifibrotic function [54]. Furthermore, albumin may be a downstream effector of PPAR γ in phenotypic switch of pancreatic stellate cells (PSCs) [55], and PPAR γ activation might ameliorate renal fibrotic lesions via hepatocyte growth factor (HGF) [56]. However, whether these molecular mechanisms mediate the effects of PPAR γ on BMSC differentiation toward myofibroblasts need to be further explored.

In summary, our results demonstrate that PPAR γ is a negative regulator in BMSC differentiation toward myofibroblasts in liver fibrogenesis. Decrease in PPAR γ expression is accompanied by the differentiation of BMSCs toward myofibroblasts, and activated PPAR γ by agonists inhibits BMSC differentiation *in vitro* and *in vivo*, and causes an attenuation of liver fibrosis. Consequently, these findings expand our knowledge on the regulatory mechanisms of BMSC differentiation, and make PPAR γ to be a promising target for treatment of liver fibrotic diseases.

Abbreviations

PPAR γ (peroxisome proliferator-activated receptor gamma); BMSCs (bone marrow-derived mesenchymal stem cells); α -SMA (α -smooth muscle actin); 15d-PGJ $_2$ (15-Deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$); TGF- β 1 (transforming growth factor β 1).

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Disclosure Statement

The authors declared that they have no conflicts of interest related to this work.

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