Down-regulation of DNA Methyltransferase 1 by Peroxisome Proliferator-Activated Receptor Gamma in Hepatic Stellate Cells.

By

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Submitted to the Graduate Faculty of

Center of Pharmacogenetics in partial fulfillment

of the requirements for the degree of

Master of Sciences

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH
SCHOOL OF PHARMACY

This dissertation was presented

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It was defended on

July 26, 2012

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ABSTRACT

DNA methyltransferase (DNMT) 1 is a critical enzyme which is mainly responsible for conversion of unmethylated DNA into hemimethylated DNA. Previous studies have suggested that enzymes related to DNA methylation play a very important role in liver fibrosis. In this study, we identified a significant over-expression of DNMT1 in activated hepatic stellate cells (HSCs), which suggests a vital function of DNMT1 in HSCs transactivation. Interestingly, ligand activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) in activated HSCs led to efficient down-regulation of DNMT1 expression. Furthermore, the PPAR-γ ligand rosiglitazone (RSG) dramatically down-regulated the expression of DNMT1 and up-regulated the expression of PPAR-γ during the transactivation of primary rat HSCs, suggesting an important role of PPAR-γ in HSC transactivation. Reporter assays showed that ligand activation of PPAR-γ inhibited transcriptional activation of the human DNMT1 gene promoter and such inhibition was rescued by transfection of nuclear factor-kappaB (NF-κB) expression vector pCMV-p50 and pCMV-p65, which indicates that PPAR-γ suppress DNMT1 expression via negatively interfering with NF-κB binding activity on DNMT1 promoter. Conclusion: These studies suggest that PPAR-γ may play a role in inhibiting HSCs transactivation by suppressing expression of DNMT1.

1. CHAPTER ONE: INTRODUCTION

1.1 Purpose of Study

The purpose of this study is aiming at elucidating the role of DNMT1 in HSC activation and the potential of PPAR-γ activation as a novel approach in inhibiting the expression of DNMT1 for the treatment of liver fibrosis.

1.2 Background

1.2.1 Liver Fibrosis

Fibrosis is a severe systemic disease characterized by excessive accumulation of fibrous connective tissues, such as collagen[1]. Fibroproliferative diseases occur throughout the human body; lungs, kidneys, and liver are among the most common affected organs. The chronic and severe progression of fibrosis results in the failure of the physiological functions of tissues. Liver fibrosis has been extensively investigated because its long-lasting progression can result in hepatocellular carcinoma (HCC), which is the fifth most common cancer worldwide[2,3]. Hence, it is important to understand the biological mechanisms involved in liver fibrogenesis to develop effective treatments.

Liver fibrogenesis is a dynamic wound-healing process characterized by excessive production and deposition of extracellular matrix (ECM), mainly type I and III collagen, in the liver interval space between sinusoidal endothelial cells and hepatocytes[4]. As a result, ECM proteins progressively accumulate in liver parenchyma and form permanent fibrous scars distorting the normal hepatic architecture[5].

To date, remarkable progress has been made in understanding the pathophysiology of liver fibrosis at the cellular level. It has been established that the activation of hepatic stellate cells (HSCs), a type of nonparenchymal cell resident in the space of Disse, is the essential event in liver fibrogenesis, playing a pivotal role through

transdifferentiating into myofibroblasts[4]. Many studies have suggested that the HSC activation involve a number of cytokines and chemokines and the interaction between HSCs and other cell types[6]. It is believed that Kupffer cells, resident macrophages of liver sinusoids, are first activated by several types of liver injuries. The proinflammatory response triggers the activation of HSCs resulting in excessive proliferation of myofibroblasts and stimulation of ECM synthesis, eventually leading to liver fibrosis[1,7].

1.2.2 Hepatic Stellate Cells (HSCs)

HSCs represent approximately 15% of the cells in normal liver. Under physiological conditions, HSC stay as quiescent and store up to 85% of the body's vitamin A as lipid droplets[7,8]. When freshly isolated and cultured, quiescent HSCs display several characteristics including a low proliferative rate, low fibrogenic potential, little cytokine secretion, and lack of contractile[7,9].

Upon liver injury of various causes, HSCs undergo a process termed activation or transdifferentiation, whereby they change from non-proliferating state to a highly proliferative, fibrogenic, proinflammatory, and contractile myofibroblasts resulting in the overproduction of collagen-rich ECM[9]. During the activation, HSCs lose vitamin lipid droplets. Additionally, the migrating and fast-proliferating features of these activated HSCs perpetuate hepatic inflammation at the sites of liver injury[8,10].

Potential therapeutic targets for the treatment of liver fibrosis have been suggested according to the different features of activated HSCs (Figure. 1). These features include:

a) Activation and accumulation of activated HSCs at the sites of liver injury;
b) Alteration of the balance of collagen synthesis and degradation, leading to net collagen accumulation by HSCs[5].
c) Contractile response to vasoactive molecules, leading to increases in portal vasoresistance.

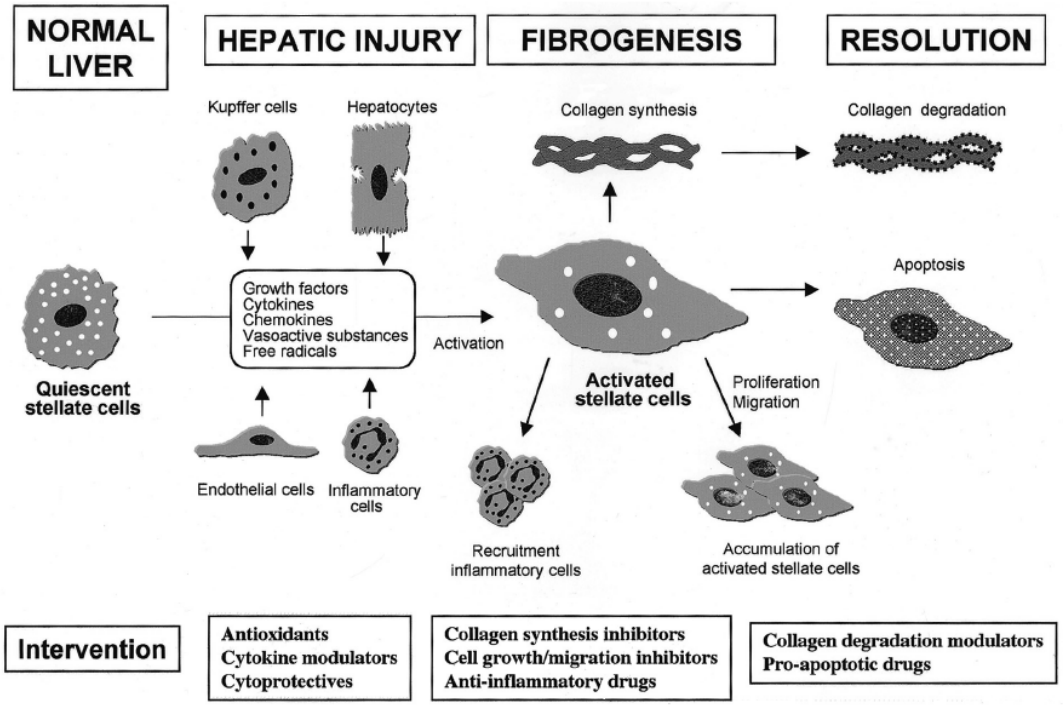


Figure 1 Biological actions of hepatic stellate cells (HSCs) during liver fibrogenesis and potential sites for therapeutic intervention.

Activation of HSCs is the primary culprit in liver fibrogenesis. Studies with rat HSCs have shown that low levels of messenger RNA (mRNA) encoding procollagen I and III are observed in quiescent HSCs, whereas these genes are significantly upregulated in activated HSCs both in vitro and in vivo. In activated HSCs, dramatic increases in the mRNA stability of procollagens might be one of the most important factors among other mechanisms[7,11].

Therefore, strategies that inhibition HSCs activation might be of importance in attenuating fibrogenic response. In the last decades, a number of studies have examined the cellular and molecular mechanisms underlying HSC activation: The initiation phase of this process is due primarily to paracrine stimuli from injured neighboring cells, which include hepatocytes, Kupffer cells, sinusoidal endothelial cells, platelets, and infiltrating inflammatory cells[7,12,13]. Lipid peroxides produced by hepatocytes and Kupffer cells lead to oxidative stress and promote HSC activation[14,15]. Moreover, cytokines released by damaged neighboring cells can also activate HSCs, through stimulating various signaling pathways such as Sp1, c-myb, nuclear factor κB (NF-κB), c-jun/AP1, and STAT-1, which regulate the expression of a number of genes

that are involved in the progression of liver fibrosis[16-19]. Activated HSCs perpetuate their own activation through several autocrine loops, including the secretion of TGF-β1 and an up-regulation of its receptors[20].

1.2.3 Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ)

The peroxisome proliferator-activated receptors (PPARs) belong to the superfamily of steroid/thyroid hormone nuclear receptors. Four isoforms of PPARs have been identified, namely PPARα, β, γ and δ, which regulate transcription of a number of target genes controlling numerous important physiological processes[21]. PPAR-γ has been extensively studied and shown to be implicated in various biological functions in various types of cells. Specifically, ligand activation of PPAR-γ can result in growth inhibition and/or apoptosis of fibroblasts[22].

PPAR-γ interacts with co-repressor proteins and inhibits gene expression in the absence of ligands. Upon activation by cognate ligands, PPAR-γ releases the co-repressor and translocates from cytoplasm to the nucleus where it recruits co-activator proteins and forms a heterodimer with retinoid X receptor-α (RXR-α). This multi-protein complex then binds to peroxisome proliferator response elements (PPRE), leading to transcriptional activation[23].

Much attention has been paid to the anti-inflammatory and protective effects of PPAR-γ during wound-healing. PPAR-γ regulates gene transcription involved in the tissue repair process of many organs, thereby ameliorating inflammation, oxidative stress, and matrix remolding[24]. Furthermore, studies have suggested that PPAR-γ might be an important molecular target in modulating in liver fibrosis. Its transcriptional dysfunction has been shown to be critically involved in liver fibrosis[25].

PPAR-γ has been identified to be a key transcription factor in controlling HSC activation and the associated changes in phenotypes. Expression of PPAR-γ protein is

dramatically reduced in activated HSCs both in vitro (human and rat cell lines) and in vivo (isolated rat HSCs)[26-28]. Mechanistic investigations have shown that PPAR-γ plays an important role in maintaining the quiescence status of HSCs through regulation of a panel of adipogenic transcription factors, including CCAAT/enhancer binding protein (C/EBP), liver X receptor-α (LXR-α), and sterol regulatory element-binding proteins-1c (SREBP-1c), all of which are rapidly diminished during HSC transdifferentiation[29].

It is known that PPAR-γ expression is downregulated during HSC activation, while PPAR-γ-cognate ligands inhibit profibrogenic and proinflammatory activities in HSCs, decrease collagen accumulation rate, and enhance collagenase activity in the injured liver[26,28]. Specifically, the treatment of culture-activated HSC with the natural or synthetic ligands for PPAR-γ suppresses many key functional parameters of the cell activation, including cell proliferation, expression of collagen, TGF-β1, α-smooth muscle actin (α-SMA), monocyte chemotactic protein-1(MCP-1) genes, and chemotaxis[26,28,31]. Importantly, treatment with the PPAR ligands ameliorates both induction of fibrosis and progression of preexisting fibrosis in various animal models[29]. These effects were reproducible when exposing activated HSCs to a variety of different PPAR-γ agonists and were abrogated in HSCs treated with a PPAR-γ antagonist[30], suggesting that the effects are mediated PPAR-γ.

Moreover, PPAR-γ regulation on adipogenic transcription in HSCs also occurs at the chromatin-packaging level. Studies showed that the DNA methylation inhibitor 5'-azacytidine (5-azadC) blocked HSC myofibroblastic transdifferentiation and prohibited the diminished expression of PPAR-γ in activated HSCs[31].

In summary, the above studies suggest an important role of PPAR-γ in regulating HSCs activation and the anti-fibrotic role of PPAR-γ ligands. In fact, forced ectopic expression of PPAR-γ expression in activated HSCs and PPAR-γ agonists treatment have exhibited dramatic therapeutic benefits for experimental liver fibrosis in various animal models[32-34].

1.2.4 DNA Methylation & DNA Methyltransferase (DNMT) 1

In mammals, DNA methylation occurs in the cytosine residues at the C5 position, primarily in the CG dinucleotides and occasionally at non-CG sites[35]. DNA methylation is a key process in epigenetic regulation of the expression of many gene[36, 37], parental imprinting regulation[38 - 40], X chromosome inactivation stabilization[41-44], and maintenance of the genome integrity through protection against endogenous retroviruses and transposons[45,46].

DNMT1, localized at DNA replication foci during the S phase[47], was the first mammalian DNA methyltransferase enzyme to be cloned[48] and biochemically characterized[49]. DNMT1 shows a preference for hemimethylated DNA over unmethylated DNA[50,51]. A role of DNMT1 as a maintenance methyltransferase has been well demonstrated, as well as its responsibilities on the preservation of the methylation patterns during cell divisions by methylation of hemimethylated CG dinucleotides produced by DNA replication[49].

Recently, the progression of liver fibrosis has been reported to be associated with hypermethylation of DNA[52]. HSCs activation is inhibited by 5-azadC, a DNA methylation inhibitor[53]. The loss of expression of IκBa and PPAR-γ, genes that are believed to maintain the quiescent phenotype of HSCs, which occurs during transdifferentiation, is also prevented by 5-azadC, which suppress the acquisition of proinflammatory and profibrogenic characteristics in activated HSCs[54]. Furthermore, several studies using heterozygous mice revealed that the inhibitor of DNA methyltransferase Dnmt1, 5'-azadC, also ameliorated renal fibrosis via inhibiting proliferation of myofibroblasts[55,56]. These results suggest a pivotal role of DNA hypermethylation in the progression of both liver and renal fibrosis[57].

1.2.5 NF-κB

In the past few decades, nuclear factor-kappaB (NF-κB) has been characterized to be one of the most crucial transcription factors that are involved in the regulation of various important genes. It has been demonstrated that NF-κB pathway is involved in several important events such as the control of cell survival, differentiation, and proliferation[58]. In addition, NF-κB activation has been shown to be implicated in obesity[59], malignancy[60] and other diseases59[59]. Subsequent studies have shown that increased activation of NF-κB leads to enhanced expression of various proinflammatory mediators[61],[62].

NF-κB activation is controlled by a number of positive and negative regulatory elements. The most common regulation pathway is the IκB regulation. In the ‘‘resting’’ state, NF-κB dimers stay as an inactive from in the cytoplasm through association with IκB proteins. Upon activation via various types of stimuli, the IκB kinase complex becomes activated, leading to phosphorylation, ubiquitination, and degradation of IκB proteins. Once IκB is detached from NF-κB complex, the remaining NF-κB dimers translocate to the nucleus, bind to specific DNA sequences, and promote transcription of target genes[58].

In hepatic fibrogenesis, NF-κB is activated during the transdifferentiation of HSCs[63], and NF-κB responsive genes are induced in the activated HSCs[64]. It has been demonstrated that activation of HSCs is closely associated with the transcription factor NF-κB activation, even though a causal relationship between NF-kB and HSCs activation is not fully understood. NF-κB induces the transcription of several genes involved in liver injury and activation of HSCs, such as MIP-2, MCP-1, CINC, ICAM- and IL-6[63]. NF-κB has also been shown to have protective function of TNFα-mediated apoptosis in several cell types[65,66]. In addition, diminution of IκBa is critically involved in transdifferentiation since this results in reprogramming of basal NF-κB activity to have a elevated level that drive the expression of a number of proinflammatory genes and render the cells resistant to apoptosis[67,68].

Connection has been built between PPAR-γ and NF-κB. For example, treatment

with a PPAR-γ ligand, rosiglitazone, results in a rapid and consistent suppression of the proinflammatory transcription factor NF-κB in MNCs[69].

Furthermore, recent studies have suggested that mouse DNMT1 is a target for the zinc finger protein Sp1[70], which also serves as a broad transcriptional co-regulator for NF-κB. The disruption of Sp1/NF-κB mediated DNMT1 transactivation in human acute myeloid leukemia (AML) can effectively cause DNMT1 down-regulation and DNA hypomethylation and transcription of methylation-silenced genes[71]. Based on the above studies, we hypothesize that PPAR-γ negatively regulates DNMT1 expression through inhibiting NF-κB signaling pathway.

1.3 Key Words

Hepatic Stellate Cells (HSCs); DNA Methyltrasferase (DNMT) 1; Peroxisome proliferator-activated receptor gamma (PPAR-γ); nuclear factor-kappaB (NF-κB); gene regulation.

2. CHAPTER TWO: MATERIAL AND METHODS

2.1 Materials

Rosiglitazone were purchased from Sigma (St Louis, Mo). All products for cell culture were purchased from Invitrogen (Carlsbad, Calif). pGL3 and pCMX-βgal were described previously[72,73]. phDNMT1-Luc was generated following lab protocol. Expression plasmids, Expression plasmids, PPAR-γ expression vector was kindly provided by Dr. Wen Xie (University of Pittsburgh, Pittsburgh, PA). NF-κB expression vector p50 & p65 was kindly provided by Dr. Xian Ming Chen (Creighton University Medical Center, Omaha, NE).

2.2 Cell Culture

LX-2 and CV-1 cells were cultured in DMEM medium supplemented with 10% FBS, streptomycin (100μg/mL) and penicillin (100U/mL). When treated with Rosiglitazone, LX-2 and CV-1 cells were cultured in DMEM medium supplemented with 1% FBS. Isolated Rat primary HSCs were cultured in DMEM supplemented with 10% FBS, streptomycin (100μg/mL) and penicillin (100U/mL).

2.3 Animals and HSCs Isolation

Retired male Sprague-Dawley rats were used for primary HSC isolation. HSCs were isolated via *in situ* proteinase/collagenase perfusion followed by density gradient centrifugation as described[74]. Primary cells were used at 5–7 days and were more than 95% pure. Cells were grown on standard tissue culture plastic dishes in DMEM with 10% fetal bovine serum and antibiotics.

2.4 Plasmid Construction

Human DNMT1 promoter containing fragment (-3190kb) was amplified by PCR with the oligonucleeotides 5`-CGGGTACCCGGGGATGTACCAAACGGAGAG-3` (forward primer) and 5`- CGCTCGAGCGACACAGCAAGACCCCATCTC-3` (reverse primer) using a human genomic chromosome clone (provided by Dr. Jiang Li, University of Pittsburgh, Pittsburgh, PA) as template. The fragment was cloned into pGL3-basic vector (Promega, Fitchburg, WI) after digestion with enzymes XhoI and KpnI (Biolab, Conyers, GA), and the resulting plasmid was named as phDNMT1-Luc.

2.5 RT PCR and Real-time PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen, Carlsbad, CA) and the first-strand cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). PCR amplification of DNMT1 was then performed as described by product SOP protocol.

Real time PCR assay of PPAR-γ and DNMT1 was performed with lab SOP protocol. Real-time PCR analysis was performed by use of SYBR Green-based assays with the ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification was performed under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min. Transcript abundance, normalized to β-glucuronidase expression, was expressed as fold increase over a calibrated sample.

2.6 Western Blot Analysis for DNMT1

Protein extraction and Western Blot analysis were performed as described by supplemental SOP protocol. Rabbit polyclonal IgG anti-DNMT1 antibody and secondary Goat anti rabbit IgG-HRP antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ECL chemiluminescence kit were purchased from Amersham Biosciences (Piscataway, NJ).

2.7 Transient Transfection and Luciferase Assay

CV-1 cells were grown to 60% to 70% confluence in 96-well plates. Cells were transiently transfected using InstantFect (Synthesized by Dr. Xiang Gao, University of Pittsburgh, PA) with luciferase reporters in the presence or absence of pGL3. The DNMT1 promoter fragment was cloned into pGL3-Basic luciferase vector (Pomega, Madison, WI). pGL3 was added to ensure identical amounts of DNA in each well.

Cells were transfected for overnight with 0.1 μg of this construct. Transfection efficiency was monitored by cotransfection of pCMX-βgal plasmid and transfection of EGFL plasmid in separate wells. Cell extracts were prepared after transfection, and the luciferase and β-galactosidase assays were performed as described, and luciferase activity was normalized β-galactosidase activity. Transfection experiments were performed at least 3 times in triplicate. Data were represented as fold induction over reporter gene alone.

2.8 Statistical Analysis

All data are expressed as means_SEM unless otherwise stated. Comparisons between 2 groups were made with unpaired Student's *t* test. Comparisons between 3 or more groups were made with ANOVA followed by Tukey–Kramer post hoc analysis. In all cases, $P<0.05$ was considered statistically significant.

3. CHAPTER THREE: RESULTS

3.1 Enhanced expression of DNMT1 in activated HSCs.

Recent studies have suggested a number of important functions of DNA methylation in HSCs activation in liver and kidney fibrosis[55,57]. Further study in kidney fibrosis has indicated a pivotal function of DNMT1 in HSCs activation[55].Thus, we hypothesized that DNMT1 plays a vital role in HSCs activation, and the up-regulated expression of DNMT1 facilitates the subsequent expression of pro-fibrosis-related genes in activated HSCs.

Then, in order to determine the importance of DNMT1 gene expression in HSCs activation, we first examined the DNMT1 mRNA expression in quiescent HSCs

which were isolated from rat liver tissue. Meanwhile, isolated quiescent HSCs were cultured in DMEM medium with 10%FBS for 7 days. After 7days, cultured-activated primary rat HSCs were examined by Real-Time PCR assay to examine the mRNA expression level. Figure 2 shows that the DNMT1 mRNA level is significantly up-regulated in quiescent HSCs compared to activated HSCs, which demonstrated that HSCs activation is associated with upregulation of DNMT1 gene expression. This suggested an important role of DNMT1 in HSCs activation.

To further support our observation, we collected the liver tissue from long-term and short-term CCl4 treated mice, and examined the DNMT1 mRNA expression by Real-Time PCR. Normally, short-term treatment of CCl4 leads to liver centrizonal necrosis and steatosis[75], while prolonged administration results in liver fibrosis, and eventually cirrhosis[76]. Results demonstrate that DNMT1 expression is significantly enhanced in long-term treatment group when compared to control group, while no difference is detected in the short-term treatment group, indicating that DNMT1 up-regulation may contribute to liver fibrosis development instead of acute inflammatory response (Figure 3).

All the above results have suggested a vital role of up-regulated DNMT1 in HSCs activation.

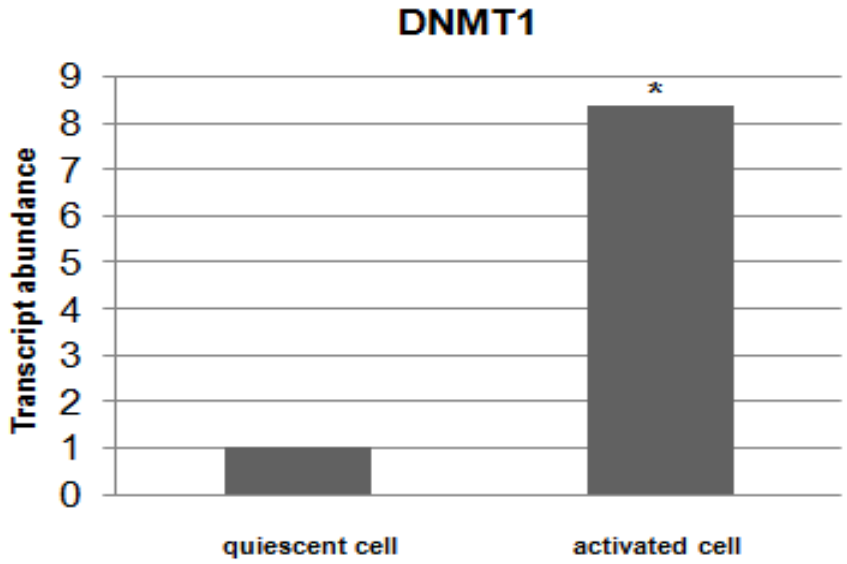
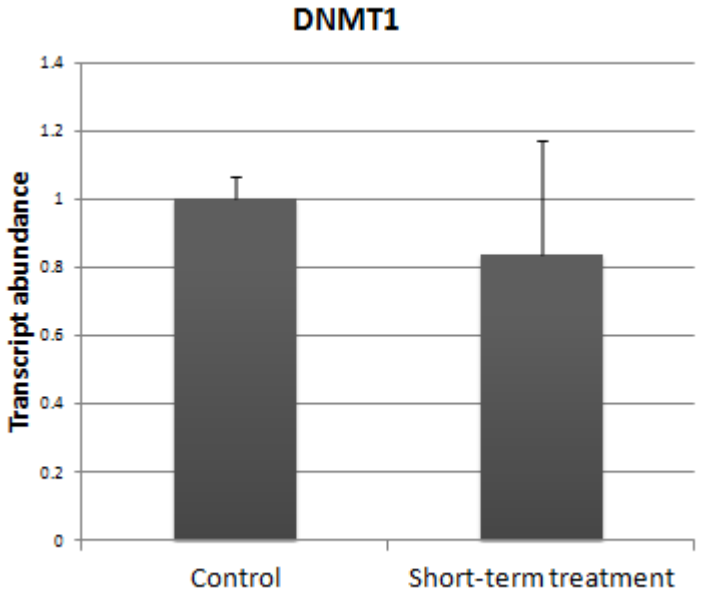
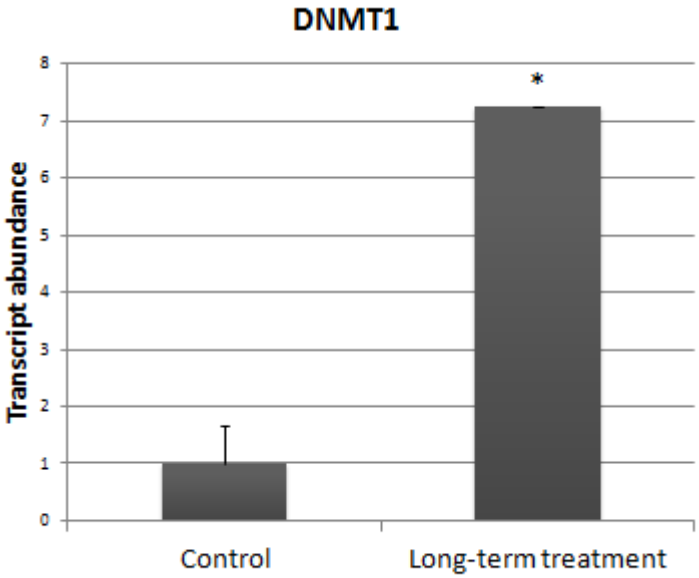


Figure 2, Expression of DNMT1 during HSCs trans-activation. Isolated rat HSCs were cultured in complete medium overnight following isolation. The mRNA of DNMT1 in quiescent HSCs was collected and analyzed with real-time RT-PCR. In a separate experiment, cells were cultured in 10% FBS DMEM medium for 7 days to allow transactivation. mRNA expression of DNMT1 in activated HSCs was similarly examined by real-time RT-PCR. *P < 0.01 vs. control group.





Figures 3, DNMT1 expression in liver of mice receiving short-term and long-term treatment of CCl4. Liver tissues were homogenized and DNMT1 mRNA expression was examined by RT-PCR. **P < 0.01 vs. control group.

3.2 Rosiglitazone (RSG) treatment down-regulates DNMT1 expression in HSCs.

Previous Studies showed that DNA methylation inhibitor 5'-azacytidine (5-azadC) blocked HSC myofibroblastic transdifferentiation and prohibited the diminished expression of PPAR-γ in activated HSCs[77]. Hence, evidences suggested a potential correlation between PPAR-γ and DNA methylation. Since our data have shown that there is negative correlation between PPAR-γ and DNMT1 expression, thereby, we hypothesized that PPAR-γ activation may play a direct role in the regulation of DNMT1 expression.

To investigate the biological function of PPAR-γ in DNMT1 regulation, we examined whether RSG activation of PPAR-γ can down-regulate DNMT1 expression

in activated HSCs. We selected LX-2 cell line because it has been described to have comparable features with human HSCs[78]. RSG is a member of the thiazolidinedione class of drugs, which selectively bind to PPAR-γ[79]. We tested the mRNA expression of DNMT1 in LX-2 cells after RSG treatment. Figure 4 shows that RSG treatment results in a significantly decreased expression of DNMT1 mRNA in a concentration-dependent manner. Interestingly, in cultured-activated primary rat HSCs, the treatment groups with RSG exhibited a dramatic suppression of DNMT1 gene expression in activated HSCs when compared to DMSO-treated group (Figure 5).

To further confirm manipulation of DNMT1 gene expression by PPAR-γ activation, Western Blot assay was conducted to examine the DNMT1 protein expression level in HSCs. As shown in Figure 6, DNMT1 (183kD) protein expression level were inhibited in a concentration-dependent manner in LX-2 cells after RSG treatment, which is consistent with mRNA study.

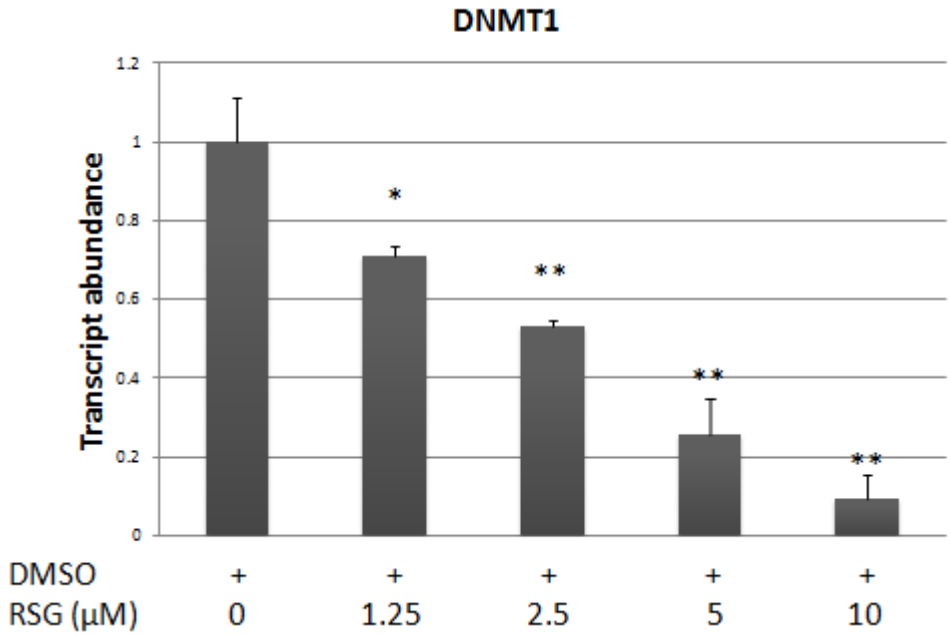


Figure 4, Regulation of DNMT1 expression by RSG treatment in LX-2. LX-2 were grown to 70% confluence in the complete medium and then cultured in the conditioned medium in the presence of indicated concentrations of RSG or vehicle DMSO (0.1%). After 24hrs, the mRNA was extracted and DNMT1 expression was analyzed by real-time RT-PCR. *P < 0.05, **P < 0.01 vs. DMSO. Data are mean±SEM from 3 assays performed in triplicate.

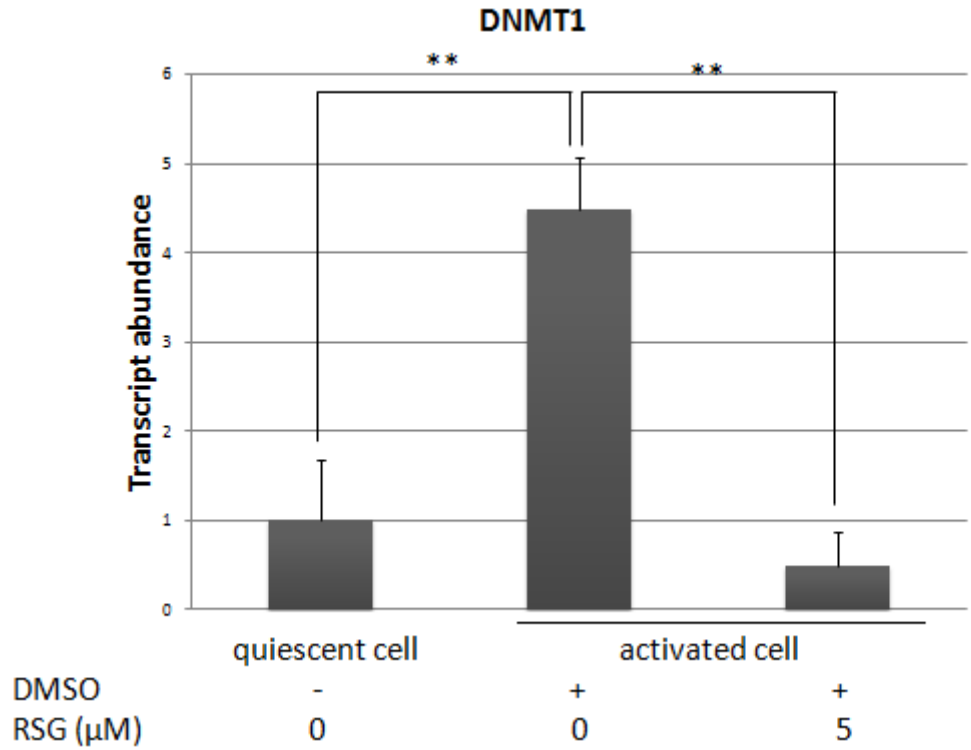


Figure 5, Regulation of DNMT1 expression with RSG treatment during HSCs activation. Isolated rat HSCs were cultured in complete medium for 24hrs following isolation. Cells were then cultured in conditional medium in the presence of RSG or vehicle DMSO (0.1%) for 7 days. The ligand-containing medium was changed every 48hrs. The expression levels of DNMT1 mRNA were examined via real-time RT-PCR at day 1 and day 7, respectively. *P < 0.05, **P < 0.01 vs. expression level in quiescent HSCs. Data are mean±SEM from two assays performed in duplicate.

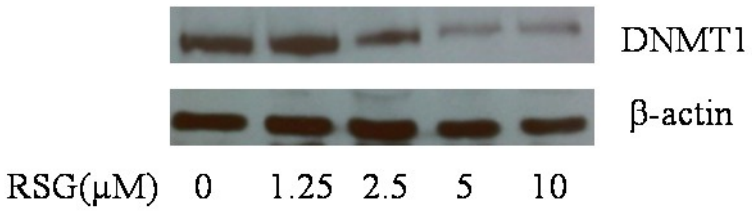


Figure 6, Regulation of DNMT1 protein expression with RSG treatment. DNMT1 expression was examined via Western Blot analysis 24 h following RSG treatment. β-actin was used as a loading control.

3.3 PPAR-γ treatment rescues the expression of PPAR-γ during HSCs activation

Recent study has suggested that once the HSCs are activated, the activated phenotype can be reversed into quiescent phenotype by forced expression of PPAR-γ via an adenoviral vector[80]. Thereby, we hypothesized whether the treatment of RSG during HSCs activation can recover the expression of PPAR-γ. Again, we tested the mRNA expression level of PPAR-γ from isolated rat HSCs. Figure 7 suggests that once quiescent HSCs become activated, the expression of PPAR-γ is highly suppressed, which is consistent with previous report[80]. We then compared the expression of PPAR-γ in activated rat HSCs from RSG treated group and DMSO treated group. As shown in figure 7, treatment of RSG during HSCs activation can rescue the PPAR-γ expression in culture-activated primary rat HSCs. This observation suggests an important role of RSG at the early-stage of liver fibrosis: RSG treatment can effectively inhibit DNMT1 expression and recover the PPAR-γ expression in activated HSCs.

In addition, we hypothesized whether the treatment of RSG could rescue the expression of PPAR-γ in fully activated human HSCs. At first, we tested the effect of PPAR-γ recovery efficiency after RSG 24hrs treatment. As shown in Figure 8, we failed to observe any changes of PPAR-γ expression in LX-2 cells. Thus, extended treatments were performed as 48hrs and 96hrs. Once again, no significant changes on PPAR-γ gene expression have been observed after the treatment (Figure 8). Thereby, the observations suggest that RSG treatment may not be able to rescue the expression of PPAR-γ in activated HSCs. We believe this may due to the low expression level of PPAR-γ presented in the activated HSCs, which leads to a minimized effect of PPAR-γ ligand activation.

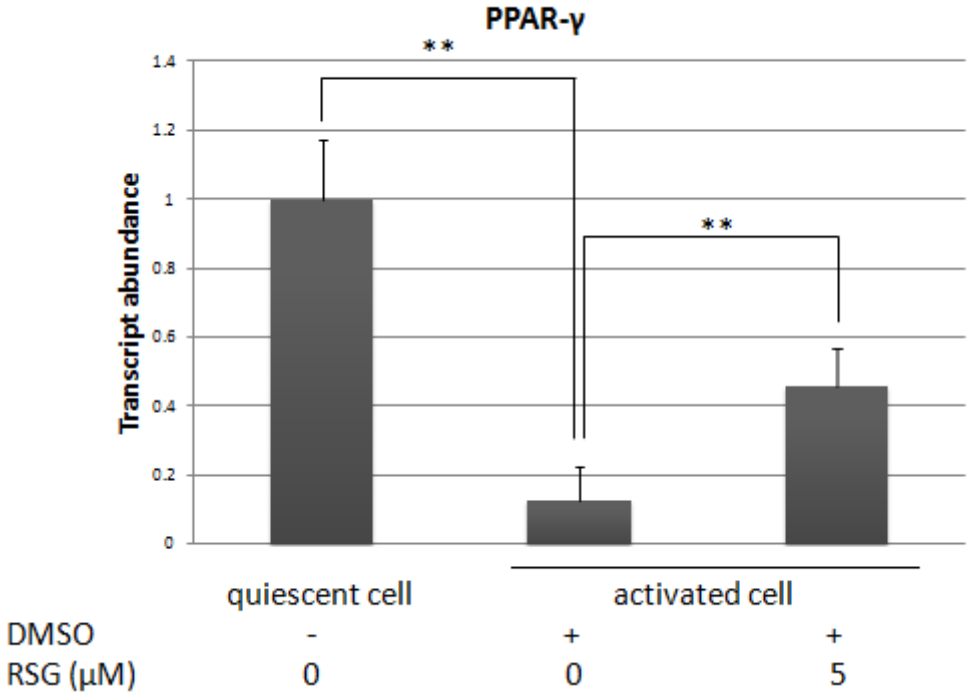
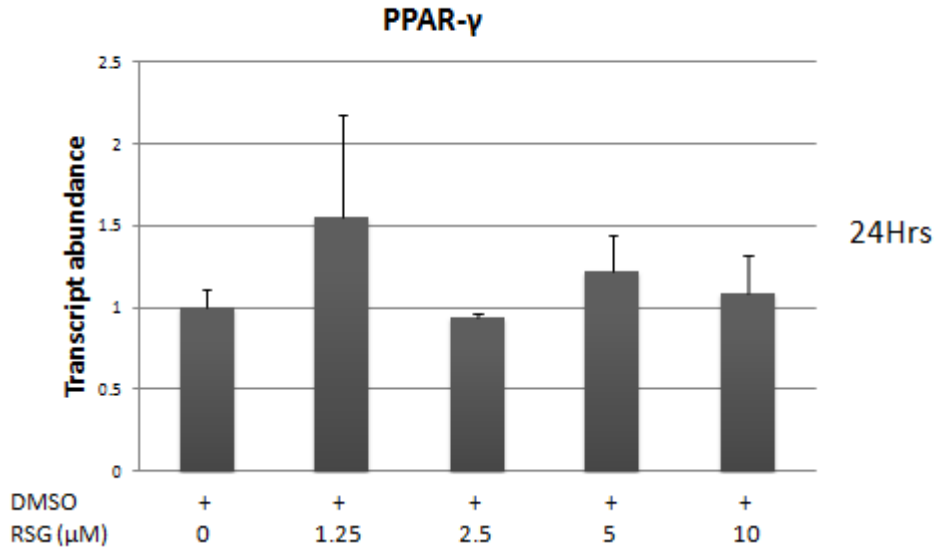


Figure 7, Regulation of PPAR-γ expression with RSG treatment during HSCs activation. Isolated rat HSCs were cultured in complete medium for 24hrs following isolation. Cells were then cultured in conditional medium in the presence of RSG or vehicle DMSO (0.1%) for 7 days. The ligand-containing medium was changed every 48hrs. The expression levels of PPAR-γ mRNA were examined via real-time RT-PCR at day 1 and day 7, respectively. *P < 0.05, **P < 0.01 vs. expression level in quiescent HSCs. Data are mean±SEM from two assays performed in duplicate.



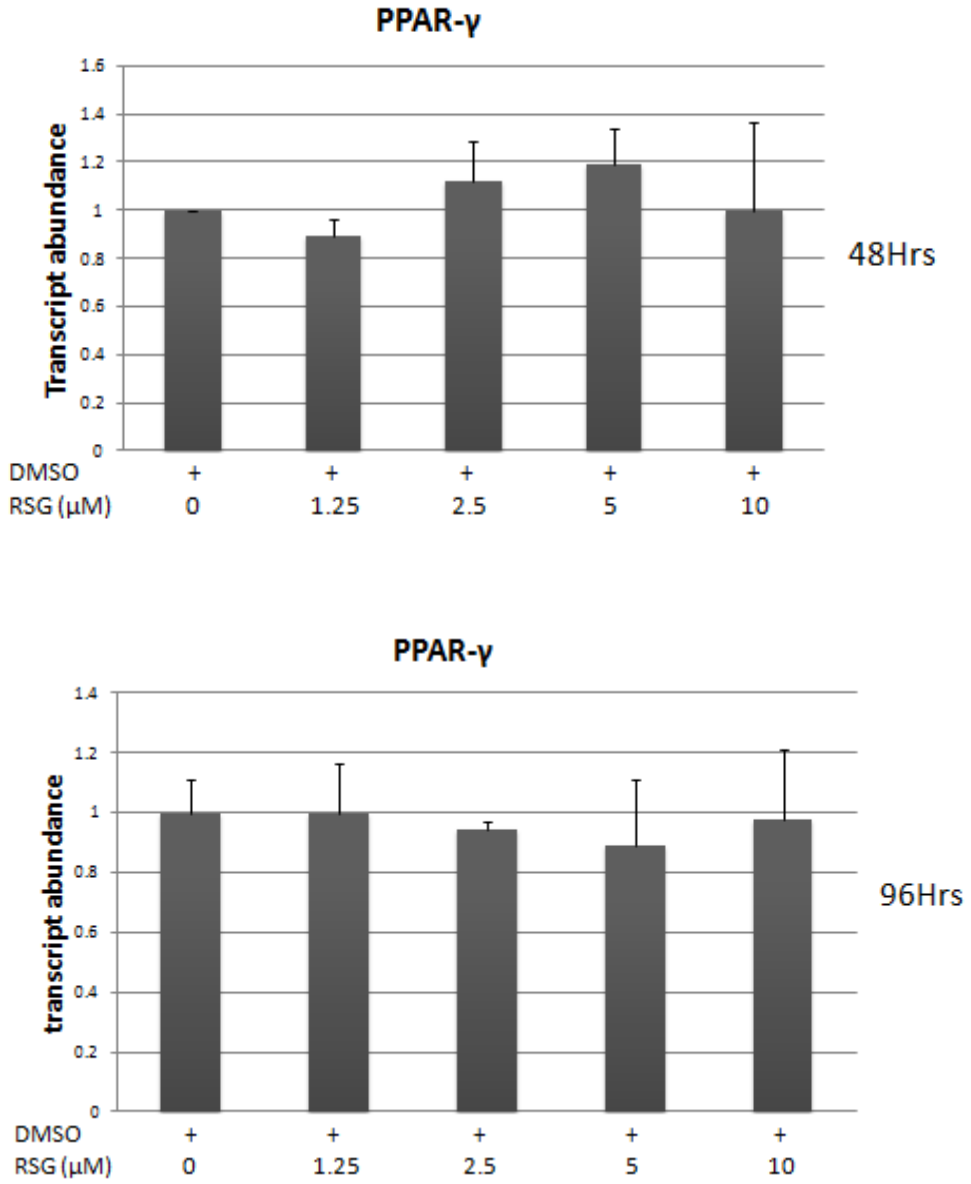


Figure 8, Effect of RGS treatment on PPAR-γ gene expression in LX-2. LX-2 were grown to 70% confluence in the complete medium and then cultured in the conditioned medium in the presence of indicated concentrations of RSG or vehicle DMSO (0.1%). The total mRNA was extracted after different time periods as shown in the figures and PPAR-γ expression was analyzed by real-time RT-PCR. *P < 0.05, **P < 0.01 vs. DMSO. Data are mean±SEM from 3 assays performed in triplicate.

3.4 PPAR-γ represses transcriptional activation of the human DNMT1 promoter

Down-regulation of DNMT1 mRNA expression under RSG treatment suggests that activation of PPAR-γ modulates DNMT1 expression at transcriptional level. We then hypothesized that ligand activation of PPAR-γ repress DNMT1 expression via exerting its inhibitory activity on DNMT1 promoter. To verify our hypothesis, we constructed a luciferase reporter expression plasmid (phDNMT1-Luc) that is driven by human DNMT1 promoter. As normally described, the promoter is predicted to locate within the 3kb upstream from transcriptional starting site[70]. Thus, we cloned the 3kb gene sequence of human DNMT1 promoter and cloned into pGL3 luciferase reporter vector.

Afterwards, CV-1 cells were co-transfected with constructed phDNMT1-Luc and PPAR-γ expression plasmid, and then treated with RSG for another 24hrs. Also, we tested the transfection efficiency by EGFL reporter vector. Fluorescence examination suggested that our transfection efficiency had reached 30%-40%, which shows a convincing transfection efficiency to support our further study. As shown by Figure 9, the treatment of RSG shows a significant inhibitory effect on DNMT1 promoter activity in a concentration-dependent manner. Hence, this observation has indicated that ligand activation of PPAR-γ inhibits DNMT1 expression via repressing DNMT1 promoter activity.

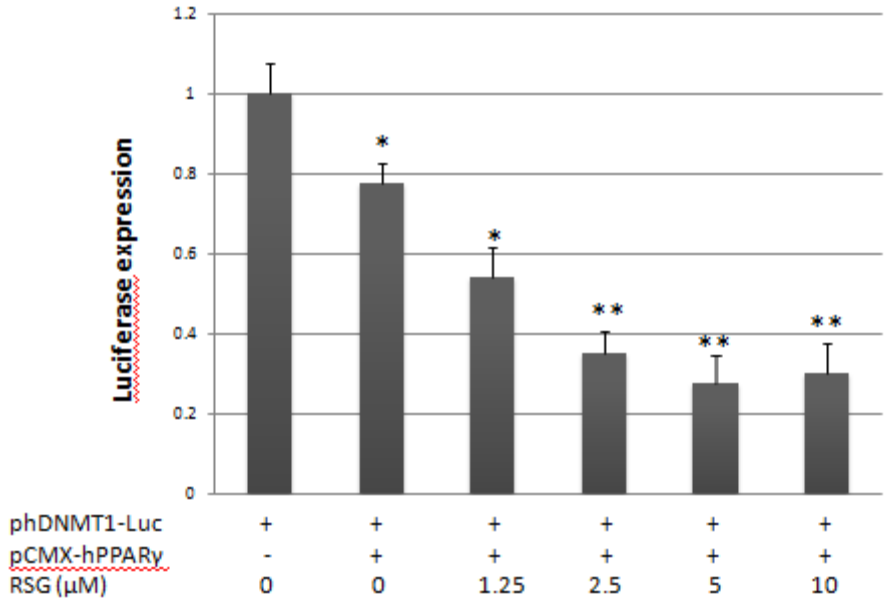


Figure 9, DNMT1 promoter transcriptional activity under RSG treatment. CV-1 cells were transiently co-transfected with phDNMT1-Luc and PPAR-γ expression vector at a molar ratio of 3:1. Five hours later the transfection medium was replaced with complete medium and cells were incubated for 12 hours. Cells were then cultured in the conditioned medium in the presence of indicated concentration of RSG or vehicle DMSO (0.1%) for 24hrs. Luciferase assay was then performed. Data shown in the panels represent mean (SD) from triplicate assays. *P < 0.05, **P<0.01 vs. the cells treated with DMSO.

3.5 RSG reduces the NF-κB binding activity

Since we have observed that ligand activation of PPAR-γ can effectively suppress the activity of DNMT1 promoter, we then conducted further studies to search for possible molecular mechanisms involved in this regulation. After searching DNMT1 promoter binding sites, we failed to identify any PPAR-γ binding consensus sequence. Instead, a few potential NF-κB binding sites have been predicted in the promoter. It has been shown that NF-κB is one of the most important transcriptional factors involved in regulation of DNMT1 expression[71]. It was postulated that PPAR-γ can inhibit activity of NF-κB through competition of limited availability of co-factors[81]. We thereby hypothesized that inhibition of DNMT1 expression is mediated by reduction of

NF-κB activity upon PPAR-γ activation. Thus, we co-transfected the phDNMT1-Luc, PPAR-γ expression plasmid and p50 & p65 NF-κB expression vectors into CV-1 cells, and examined the luciferase activeity after RSG treatmenr for 24hrs. As shown in Figure 10, the group with NF-κB over-expression has weakened the inhibitory effect of DNMT1 promoter activity by RSG treatment. The observation verified our hypothesis that PPAR-γ suppresses the DNMT1 promoter activity by interfere the NF-κB binding activity.

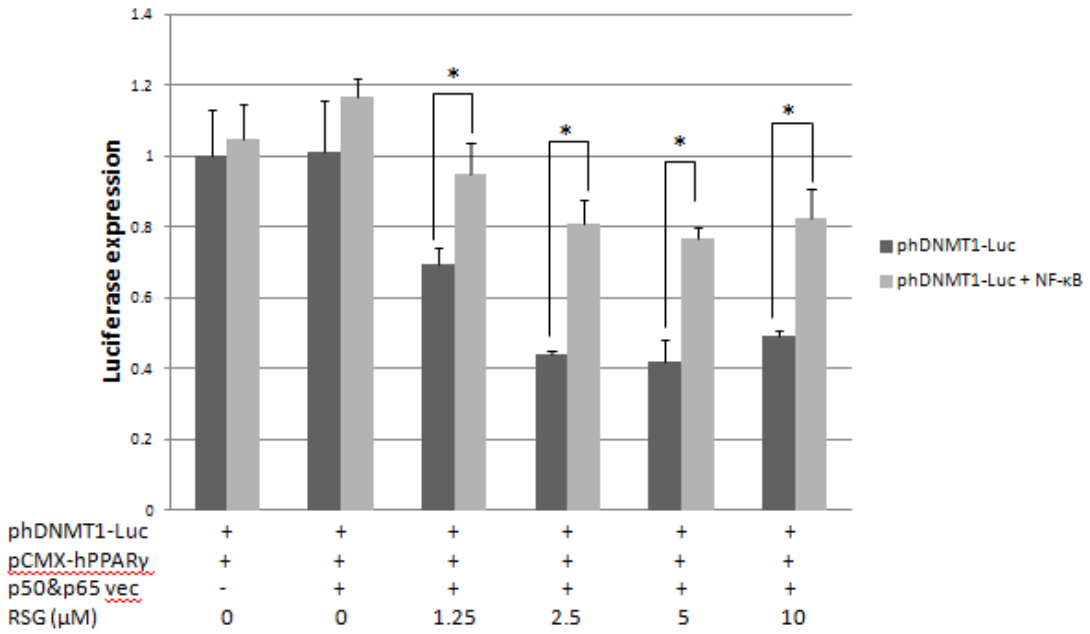


Figure 10, DNMT1 promoter transcriptional activity under RSG treatment with or without NF-κB over-expression. The darker bars represent groups transfected with only phDNMT1-Luc, while the lighter bars represent groups co-transfected with both phDNMT1-Luc and NF-κB. CV-1 cells were transiently co-transfected with phDNMT1-Luc, PPAR-γ expression vector and NF-κB expression vector p50 & p65 at a molar ratio of 3:1:1, respectively. Five hours later the transfection medium was replaced with complete medium and cells were incubated for 12 hours. Cells were then cultured in the conditioned medium in the presence of indicated concentration of RSG or vehicle DMSO (0.1%) for 24hrs. Luciferase assay was then performed. Data shown in the panels represent mean (SD) from triplicate assays. **P < 0.01 vs. the cells treated with DMSO.

4. CHAPTER FOUR: DISCUSSIONS

In this study, we have clearly demonstrated increased expression of DNMT1 in activated HSCs, which further provides additional important information about DNA methylation function in the HSCs activation. As one of the most important maintenance methyltransferase, DNMT1 exhibits vital functions in silencing genes, such as PPAR-γ. To date, most information about DNA methylation and DNMT1 are related to cancer oncogenesis, only few recent studies have studied the role of DNA methylation in liver fibrosis[57]. MeCP2 has been demonstrated to be a critical epigenetic regulator of liver fibrosis and HSCs activation[53]. Thus, our study provides more evidence on a role of DNA methylation in HSCs, which involves DNMT1.

PPAR-γ, as one of the most studied nuclear receptors, has been studied extensively in liver fibrosis, but limited information is available on its function on DNA methyltransferase genes. In this study, we demonstrated the ligand activation of PPAR-γ leads to down-regulation of DNMT1 expression in activated HSCs. Moreover, PPAR-γ showed more effect on DNMT1 expression in HSCs than other nuclear receptors such as RAR and FXR (data not shown).

As mentioned in previous studies, DNA methylation plays a pivotal role in HSCs activation[57]. Animal studies demonstrated that DNMT1 over-expression is involved in liver fibrosis rather than liver centrizonal necrosis or steatosis. Our study also shows that DNMT1 expression is over-expressed during HSCs activation. Therefore, it would be very beneficial if the expression of DNMT1 can be suppressed before quiescent HSCs become activated. Interestingly, we have shown that PPAR-γ activation can prevent up-regulation of DNMT1 during HSCs activation.

In addition, it is very exciting to notice that Rosiglitazone mediated PPAR-γ activation can significantly recover the PPAR-γ expression during HSCs activation. This observation has suggested the importance of PPAR-γ ligand treatment at the

early-stage of liver fibrosis due to PPAR-γ function recovery and inhibitory effect on DNMT1 expression.

The mechanism of DNMT1 down-regulation by PPAR-γ activation is not fully understood. Computational analysis did not reveal any PPAR-γ binding consensus sequence in either rat or human DNMT1 promoter region, suggesting that it is unlikely that PPAR-γ inhibits DNMT1 expression via directly interacting with DNMT1 promoter. Previous study has suggested that PPAR-γ may inhibit gene expression via interference of AP-1, STAT, and NF-κB signaling pathways via competition for essential cofactors[81]. In our study, we demonstrated the fact that PPAR-γ down-regulates DNMT1 gene expression by interfering NF-κB binding activity on DNMT1 promoter, and we suggest that a potential biological mechanism involved in this observation: via interfering NF-κB binding activity by competing co-factors of NF-κB. PPAR-γ reduces the binding activity of NF-κB on DNMT1 promoter. Also, other studies suggest several possible signaling pathways, for instance, AP-1 signaling through inhibition of c-Jun or c-Fos binding to an AP-1 site may also act as a potential regulator[82]. Also, people have suggested that in a PPAR-γ dependent or independent way, PPAR-γ ligand 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 can interfere the NF-κB binding activity by inhibit the degradation of IκBα enzyme, which results in the enhancement of IκBα in the cells, and leads to the impaired binding activity by NF-κB[83,84]. Therefore, more studies are needed to better illustrate the role of interaction of PPAR-γ with NF-κB transcriptional factors and its essential co-factors in PPAR-γ mediated inhibition of DNMT1 expression in HSCs.

Results from this study have provided a novel mechanism of PPAR-γ ligands effect on liver fibrosis, which may guide future therapeutical design for liver fibrosis treatment.

5. ACKNOWLEDGEMENT

This thesis could not have been written without the help from many people.

Firstly, I would like to thank my mentor, Dr. Song Li. I could not have imagined a better advisor and mentor for my MS study, and I hope to work with him for a very long time. He taught me how to do research, how to keep up a good reading habit, and most important: how to be a mature and responsible adult.

Next, I would like to thank all the Members of the Li's Lab and everyone in Center of pharmacogenetics throughout the years. They have always been there when I needed their help.

6. APPENDIX: PROTOCOL

6.1 mRNA extraction and Real-time PCR

Day 1: Extraction of mRNA and reverse transcription:

1. Remove culture from 6-well plate.
2. Add 1mL Trizol into each well of 6-well plate, mix and incubate at room temperature for 5min. Transfer the solution into Ep tubes.
3. Add 200μL Chloroform, shake with hands for 15s, and incubate at room temperature for 2-3 min until the two layers separated. Centrifuge at 4℃, 12000 rpm for 15min.
4. Transfer the colorless supernatant to another tube. (Take out the supernatant from top to bottom, slowly pipet up. Usually 450-500μL can be taken out. Some protein may between the layers, don't take the protein!) Add the same volume of isopropanol, and then shake with hands for 1 min. Centrifuge at 4℃, 12000 rpm

for 15 min.

5. Pour out the supernatant, and add 75% alcohol (~800μL to wash), shake a few times. Centrifuge at 4℃, 12000 rpm for 15 min.
6. Remove the supernatant (pour out, be gentle), Centrifuge at 4℃, 12000 rpm for 5 min. Pipet to remove residual supernatant. Air-dry 5-10 min until pellets become transparent.
7. Add 15μL water (autoclaved ultra-pure water), to each tube, mix and transfer 11μL sample to another tube (0.3mL small tubes in line)
8. Add 1μL dNTP and 1μL Oligo dT primer (for binding to poly-A tail) to each sample. (make master-mixture of dNTP and Oligo dT is helpful to decrease variation)
9. Incubate for 5min at 55-60℃, and then put the tubes on ice immediately, then wait at least 2min.(This step is to let AAA bind to TTT. Poly-A might be separated with Oligo dT if temperature drops slowly)
10. 5*FS (first-strand cDNA) buffer 4μL, 0.1M DTT 2.5μL (proteinase inhibitor, prevernt degradation of reverse transcriptase), Superscript III 0.5μL (reverse transcriptase), and then add 7μL mixture to each tube. (Total volume of each sample 11+1+1+7=20μL. Make master mixture for sample number+1)
11. RT (reverse transcription): 1.5hrs (program: A9). Continue with Real Time PCR or store the sample in -20℃.

Day2: Real-time PCR

1. Dilute the samples 4-folds with water.
2. 20μL Syber Green system: (2*master mix 10μL, water 7uL, primer 1μL)*(sample number+1), +sample 2μL. Master mix contains enzyme, dNTP, buffer and Syber Green. Be careful: never touch the bottom or upper side of the plate with finger.
3. Cover the film, press each edge with flat plate and let it attach tightly.
4. Centrifuge for twice. For the first time, centrifuge briefly and shake by tapping the edges and centrifuge for 5min, 2500 rpm.
5. Put the plate in Real-time machine. Set up: the name, volume of the sample and

location, save. Choose the “dissociation curve”.

6.2 Western Blot

SDS-page preparation:

1. Clean the apparatus
2. Join the two glasses (thick & thin) together and fit them into plastic holder (Bio-rad), then in stand
3. Prepare polyacrylamide gel:

Resolving gel:

40% acrylamide mix

10% SDS

10% APS (make fresh each time)

1.5 M Tris, pH 8.8 (resolving gel)

TEMED

Then mix and load to frame, and add isopropanol above that: to remove bubbles

Stacking gel:

40% acrylamide mix

10% SDS

10% APS (make fresh each time)

1.0 M Tris, pH 6.8 (stacking gel)

TEMED

Pour out the isopropanol before adding stacking gel, then load the stacking gel and put comb on top of it.

Wait at least 30min to check if it is solidfied, and then wrap it in plastic bag and put it in 4℃

Sample preparation:

1. Remove medium, wash with PBS 1X twice
2. Add loading & lyses buffer
3. Scratch and mix with the back side of the tips
4. Transfer to eppendorf tubes and put the eppendorf tubes on heating block. Keep the temperature above 95℃ for 10min, open the lid every 3min to prevent water pop.
5. Put the tubes on ice for use or store in -20℃

Sample loading:

1. Prepare electrophoresis buffer
2. Fill the buffer full in inner chamber, fill outer chamber half-full
3. Take out the comb, and load the samples into the holes of the resolving gel
4. Continue filling buffer to full
5. Close lid and run for 80v when in resolving gel,after cross the stacking gel, change to 120v.

Transfer gel to membrane:

1. Prepare filter paper and membrane by cutting to appropriate size; for each gel prepare two filter paper and one membrane
2. Take membrane with blunt-end forceps carefully (don't touch membrane with hands); put the membrane into methanol for 1-2min
3. Then put into transfer buffer and soak until the gel electrophoresis is ready. For filter papers, soak in transfer buffer with membrane
4. After gel electrophoresis finished, cut gel with clean razor blade to appropriate size
5. Use the forceps to put the gel on top of membrane carefully, and cover them with filter paper up and down
6. Take this "sandwich" to transfer cassette: semi-dry transfer method

7. Cover the lid and run 15v for 35 min (wet cassette with transfer buffer and swipe by tissue before cover the lid)

Staining:

1. Take out membrane into DD H_2O, wash and remove water
2. Pour little of red staining buffer, shake for a while, and check the bands to see if we have equal amount of proteins loading.
3. Wash membrane with PBST to get rid of staining buffer and color, change PBST every 10min, and shake for at least 30min until color fades out.

Blocking:

1. Prepare blocking buffer (5g non-fat milk into 100ml PBST)
2. Put membrane into blocking buffer and shake for 1hr.

Applying primary antibody

1. Dilute primary antibody 1:1000 for DNMT1 antibody, 1:4000 for β-actin into blocking buffer, total volume 15ml
2. Put membrane into the shaking cases and shake overnight at 4℃.

Applying secondary antibody

1. Take out membrane, and wash with PBST 3 time for 5min each.
2. Dilute secondary antibody 1:10000 in PBST 20ml
3. Put the membrane into the shaking cases and shake for 1hr.
4. Remove PBST and dry with tissue

Photo image:

1. Put membrane onto plastic sheet in the Western Blot cassette
2. Spray image enzyme substrate onto the membrane
3. Wrap plastic sheet on membrane and remove the bubbles
4. Cover cassette , go to the dark room

5. Take one negative film and put it on top of the plastic wrap, seal the cassette for 3min and exposure in developing machine. Adjust image quality by extend or shorten nagetive film exposure time.

6.3 Transformation

A. make competent cell

1. Inoculate one colony from LB plate into 2ml LB liquid medium. Shake at 37℃ overnight
2. Inoculate 1ml overnight cell culture into 100ml LB medium (in a 500ml flask).
3. Chill the culture on ice for 15min. also make sure the 0.1M $CaCl_2$ solution and 0.1M $CaCl_2$ plus 15% glycerol are on ice
4. Centrifuge the cells for 10min at 3300g at 4℃.
5. Discard the medium and resuspend the cell pellet in 30-40 ml cold 0.1M $CaCl_2$.
6. Keep the cells on ice for 30 min
7. Centrifuge the cells as above
8. Remove the supernatant, and resuspend the cell pellet in 6ml 0.1M $CaCl_2$ solution plus 15% glycerol
9. Pipet 0.4-0.5 ml of the cell suspension into sterile 1.5ml micro-centrifuge tubes. Freeze these tubes on dry ice and then transfer them to -80℃ freezer.

B. transformation protocol using heat shock

1. Take competent E.coli cells from -80℃, and turn on water bath to 42℃.
2. Put competent cells in a 1.5 ml tube. For transforming a DNA construct, use 50µl of competent cells. For transforming a ligation, use 100µl of competent cells.
3. Keep tubes on ice.
4. Add 50ng of circular DNA into E.coli cells. Incubate on ice for 10 min. to thaw competent cells.
5. Put tubes with DNA and E.coli into water bath at 42℃ for 45 seconds.

6. Put tubes back on ice for 2min to reduce damage to the E.coli cells
7. Add 1ml of LB (with no antibiotic added). Incubate tubes for 1 hour at 37℃.
8. Spread about 100μl of the resulting culture on LB plates (with Ampicillin antibiotic added) grow overnight.
9. Pick colonies about 12-16 hrs later.

6.4 phDNMT1-Luc vector ligation

1. Prepare insert DNMT1 3Kb DNA fragment using PCR methods. Design appropriate primes and use human genomic DNA as template.
2. Select restriction enzymes for insert and vector, and determine the appropriate reaction buffers.
3. Combine the following in a microfuge tube (30μL total volume):
   2μg DNA (insert or vector)
   1μL each restriction enzyme
   3μL 10x buffer
   3μL10x BSA (if recommended)
   xμL H_2O (to bring total volume to 30μL)
4. Incubate tubes at 37℃ for 1 hour
5. Run all of the samples on a gel to check whether the restriction enzyme cutting is successful, and then purify the DNA using Invitrogen Gel Extraction Kit.
6. Ligation: combine the following in a microgfuge tube (10μL total volume):
   1μL Vector DNA
   5μL Insert DNA
   1μL 10x Ligase Buffer
   1μL T4 DNA Ligase
   2μL H_2O (to bring total volume to 10μL)
   Tip: try different vector to insert ratios to optimize ligation reaction
7. Incubate at 16℃ for 2 hours, or at 4℃ overnight (follow the manufacturer's instructions).

8. Transformation and check colonies

6.5 Transfection

1. Prepare the following solution with 0.7µl X 4 =2.8µl "Instantfect" on a 96 well plate, and then add 100µl Opti-MEM without serum
2. Make up DNA working solution: add 1.32µg DNA in 100µl Opti-MEM without serum
3. Mix up the above two solution, let them stay in the hood for at least 15 min.
4. Wash CV-1 cells with PBS without Ca^{2+}, Mg^{2+}, and then removes the PBS.
5. Digest CV-1 with 1X Trypsin-EDTA, incubate the plate at 37℃ for 3-5 min until cells get loose.
6. Resuspend in 5ml DMEM, and spin wth 1500g X 5min
7. Remove the supernatant carefully; add 3ml Opit-MEM and 3ml DMEM to mix cells into suspension.
8. Add 200µl cell suspension to the well with DNA and "Instantfect", and then mix well. Next, aliquote 100µl into each well.
9. Incubate at 37℃ for 5hrs, and then remove the medium. Change to complete DMEM medium and culture overnight.

6.6 Luciferase assay

Preparation of luciferase lysis buffer

96well/96 samples

96 x 0.15 = 14.4 ml, so we prepare 16ml for 96 well plate

16 x 0.001 =16µl of 1M DTT

16 x 0.008 =128µl of IM $MgCl_2$

16 x 0.04 =640µl of 0.1M EGTA (pH 8.0)

16 x 0.0016 =25.6µl of 0.25M PMSF

β-gal assay buffer base

To make 4L

Na_2HPO_4 (Sigma S0876): 34.08g

KCl (Fisher P217-3): 2.9824g

0.5M $MgCl_2$: 8ml

Add MQH_2O to 4L, no pH adjustment

Preparation of β-gal assay buffer

Volume calculation the same as luciferase (each plate use 10ml, calculate total volume and add 2ml.)

V x 3.5µl of beta-macarporthaol

V x 0.0025g of ONPG

Add 12ml base buffer base

Lyse cell

150µl of lysis buffer added, shake for 5-10 min

50µl to clear plate/flat bottom (Corning from Fisher 07-200-90), add 100µl of β-gal buffer, shake, and incubate at 37℃, measure β-gal activity.

50µl to white/flat bottom plate (Corning from Fisher 07-200-591), add 100µl luciferase buffer, shake, and measure luciferase activity.

Luciferase assay buffer base

To make 1L 4x solution:

Tricine (Sigma T9784): 14.34g

Magnesium Carbonate (Sigma M0125): 2.08g

$MgSO_4$ (Sigma M5921): 2.63g

0.5M EDTA: 800µl

Use H_2O to bring the volume to 1L

Preparation of Luciferase assay buffer

96 x 0.1 =9.6ml, so we prepare 12ml

Total volume (V)

V x 0.25 of 4x base

V x 0.69 of MQ H_2O

V x 0.005 of 0.1M ATP

V x 0.005 of 1M DTT

V x 0.05 of 0.01M Luciferin in methanol (D-luciferin Firefly, Biosynth L-8200: methanol, Fisher A412-4)

V x 1.5µl of 0.1g/ml co-enzyme A in water (co-enzynme A Calbiochem #234101, 1g)

6.7 Rat Hepatic Stellate Cell Isolation

1. Prepare 50 ml GBSS without NaCl and HBSS with Ca^{2+} containing 50mg protease and 25mg collagenase type IV. Then warm up in 37℃ water bath
2. Anesthetize rat (put rat in chamber with isoflurane for 3-5 min), then fix its limbs with needles
3. Open abdomen, being careful not to puncture diaphragm, cut and move gut to the outside.
4. Perfuse the liver via portal vein canula. With 37℃ HBSS at 10/min flow rate. The color of liver changed while perfusing.

5. Recirculate HBSS solution containing digestion enzymes, first 10rcf/min until 35ml, change the rate to 3rcf/min. Total last time should be 30 min or more.
6. Collect and cut the liver into suspension with scissors and forceps (add HBSS without Ca^{2+} to suspend)
7. Then filter suspension into 4 tubes, add HBSS without $Ca^{2+}$to bring up the volume to 50ml in each tube. Afterwards, centrifuge under the condition of 1400rcf/min, 7min, 4℃
8. Genteelly pour out the supernatant, be careful not to disturb the cell pellets
9. Prepare Nycodenz solution 14.7g +51ml GBSS without NaCl
10. Prepare 0.3% BSA: 5ml 3% BSA + 45ml HBSS without Ca^{2+}
11. Suspend pellet with 10ml 0.3% BSA + 8ml Nycodenz, shake with hands genteelly, and split the suspension into six 15-ml tubes.
12. Add 2-3ml HBSS above the suspension very slowly and carefully to bring the total volume to 14ml, make sure the HBSS is clear above the suspensions.
13. Then centrifuge at 1400rcf/min, 4℃ for 25mins
14. Suck out the clear HBSS layer we added at last step very carefully
15. We can white cells pellets floating in the first layer of suspension, so use the pipet to take out all the cells at this layer. Then, resuspend in HBSS and centrifuge at 1400rcf/min 4℃ for 7min
16. Remove supernatant and split the remaining into 4-6 dishes with complete DMEM medium and culture overnight
17. Change the medium into fresh complete medium at the second day.

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