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Original Article

Epigenetic histone methylation regulates MMP-1 expression in myofibroblastic hepatic stellate cell

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Abstract: Objective: To investigated the epigenetic regulation of histone H3 lysine 9 dimethylation (H3K9me2) and histone H3 lysine 4 dimethylation (H3K4me2) of matrix metalloproteinases-1 (MMP-1) during primary hepatic stellate cells (HSC) differentiation. Methods: We used a pathological mouse's liver to model fibrosis that was induced by a carbon tetrachloride (CCl_4) treatment for 8 weeks. Levels of α-smooth muscle actin (α-SMA) and collagen I were determined by western blot. The expression of MMP-1 and tissue inhibitor of metalloproteinase-1 (TIMP-1) were determined by western blot and immunohistochemistry. The rat's HSC were isolated in situ perfusion of collagenase and combined with a density gradient centrifugation, where they were then cultured in vitro and identified by immunofluorescence staining and western blot. The changes of MMP-1, TIMP-1, H3K4me2 and H3K9me2 during the activation of HSC were detected by western blot. Chromatin immunoprecipitation assays (ChIP) assessed the binding of H3K4me2 and H3K9me2 to MMP-1 during the HSC differentiation. Results: Histopathological changes in the CCl_4-treated group were significantly higher than that of CCl_4-untreated group. The expression levels of α-SMA and collagen I proteins were also significantly increased by CCl_4 compared with the CCl_4-untreated group ($P < 0.05$). Immunohistochemistry and western blot analysis MMP-1 and TIMP-1 protein expression in fibrotic group were markedly increased compared with normal groups ($P < 0.05$). Immunofluorescence staining and western blot revealed that culturing 1 days and 9 days were quieted HSC (qHSC) and activated HSCs (aHSC) respectively. Results showed that the expression of the H3K4me2, MMP-1, TIMP-1 increased and H3K9me2 decreased in aHSC compared with qHSC ($P < 0.05$). ChIP revealed that increased H3K4me2 and decreased H3K9me2 in MMP-1 promoter accompanied the changes in expression of the MMP-1 ($P < 0.05$). Conclusions: Taken together, these results show the functional role of epigenetic chromatin histone H3K4me2 and H3K9me2 in HSC induced MMP-1 expression.

Keywords: HSC, MMP-1, H3K4me2, H3K9me2

Introduction

MMP-1 is a member of endopeptidases, which are expressed in embryonic development and remain in actively growing adult tissues [1]. In developed tissues, MMP-1 is dormant, but it can be re-activated in the cases of tissue injury, infection, and tumor growth [2]. Chronic infection and injury usually result in tissue fibrosis, characterized by the differentiation of normal cells into myofibroblasts, the accumulation of extracellular matrix (ECM) and the production of MMP-1 [3]. Liver fibrosis is mostly mediated by HSC, the pericytes in liver, which lodge underneath sinusoidal endothelia in loosely-packed ECM, a unique region called "the space of Disse". In a normal liver, qHSC produce the majority of hepatic ECM and store most retinoids [4, 5]. In response to stimuli factors, HSC express many MMP-1 in acute hepatic damage. It is generally accepted that MMP-1 in tissue injury and wound healing function by releasing growth factors that promote cell migration and cellular differentiation [6]. As the consequence of chronic liver injury, including hepatic viral infection and alcohol overconsumption, qHSC

undergo progressive differentiation, called "activation". Through this process, they are converted into myofibroblast-like cells as marked by increased expression of α-smooth muscle actin (α-SMA), collagen I, tissue inhibitor of metalloproteinase-1 (TIMP-1) and interstitial ECM fibers, such as dismin and are subject to enhanced contractibility [4, 5]. The gene expression of MMP-1 appears in the early stage of liver fibrosis before the deposition of fibrosis in the liver. However, little progress has been made in uncovering the mechanism underlying MMP-1 promotion in tissue fibrosis. Recent studies have shown that an aberrant histone methylation profile on a genome-wide scale has been associated with many liver diseases, such as liver fibrosis, liver cirrhosis and hepatic carcinoma.

Histone methylation is one of the most important epigenetic mechanisms for the transcriptional regulation of gene expression. Lysine residues in histone proteins, such as H3K4-9 and H3K27, are favorable sites for mono-, di-, and trimethylation (me1, me2 and me3, respectively) [7, 8]. The degree of methylation at a specific Lys residue in a histone octamer influences the recruitment of effector proteins, thereby affecting chromatin structure and regulating the transcription of down-stream genes. Depending on the modified lysine, the presence of a methyl group can lead to gene activation (e.g., H3K4me2), or gene repression (e.g., H3K9me2) [9, 10]. Previous reports have studied the role of H3K4 methyltransferase ASH1 in HSC, which increased the H3K4me3 associated with the proximal promoter of collagen I, α-SMA, TIMP1, and transforming growth factor-β1 (TGF-β1) genes in aHSC not in qHSC [11]. Additionally, a recent study has demonstrated and supported the functional role of epigenetic chromatin histone H3Kme in bile duct ligation (BDL)-induced TGF-β1 expression [12]. However, these were not comprehensive studies and they did not explore the H3K4me2 and H3K9me2 in MMP-1 during HSC spontaneous activation.

To understand the epigenetic mechanism of the aberrant expression of MMP-1 during HSC differentiation, we used both in vivo and in vitro models to illustrate their reactions. We first confirmed the concept of the aberrant expression of MMP-1 in a model rat. Then, we recapitulated the key features of MMP-1 expression in vitro using rat primary HSC. In the present study, we investigated variations in H3K9me2 and H3K4me2 during the activation of rat primary HSC. We examined whether key signaling pathways controlling the MMP-1 expression are defective during HSC differentiation. Upon researching, we discovered that increasing MMP-1 genes correlates to the decreased H3K9me2 and increased H3K4me2 in myofibroblastic HSC. This data shows that the aberrant of MMP-1 is likely mediated by an epigenetic switch.

Materials and methods

Chronic CCl_4 liver injury model

The rats (Wistar, obtained from Guizhou Laboratory Animal Engineering Technology Center) were divided into two groups: (1) control, (2) fibrosis (n=8). Rats were injected with carbon tetrachloride at 0.3 ml per 100 g of body weight, or mineral oil was alternatively used as the control, and then sacrificed on 8 weeks. Livers were collected for immunohistological and western blot studies. All animals received humane care in compliant use of laboratory animals in research.

Histological and immunohistochemistry evaluation

The wounded liver tissues were harvested, fixed in 10% formalin and later embedded in paraffin. The tissue blocks were then cut into 5 cm sections, transferred to glass slides and stained with H&E or Masson-Trichrome. Morphologic alterations in the liver tissues were examined using light microscopy and were photo documented. For immunostaining, formalin-fixed sections were dewaxed in cleared and dehydrated in alcohol. Antigen retrieval and nonspecific blocking was as described. Slides were incubated overnight at 4°C with anti-MMP1, TIMP1 at 1:100 dilution in phosphate-buffered saline (PBS) containing 20% swine serum, then washed in PBS and incubated with biotinylated swine antirabbit at 1:200 for 2 hours. After PBS washing slides were incubated with streptavidin biotin-peroxidase complex, and incubated at room temperature for 45 minutes. MMP1-positive cells and TIMP1-positive cells by were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB).

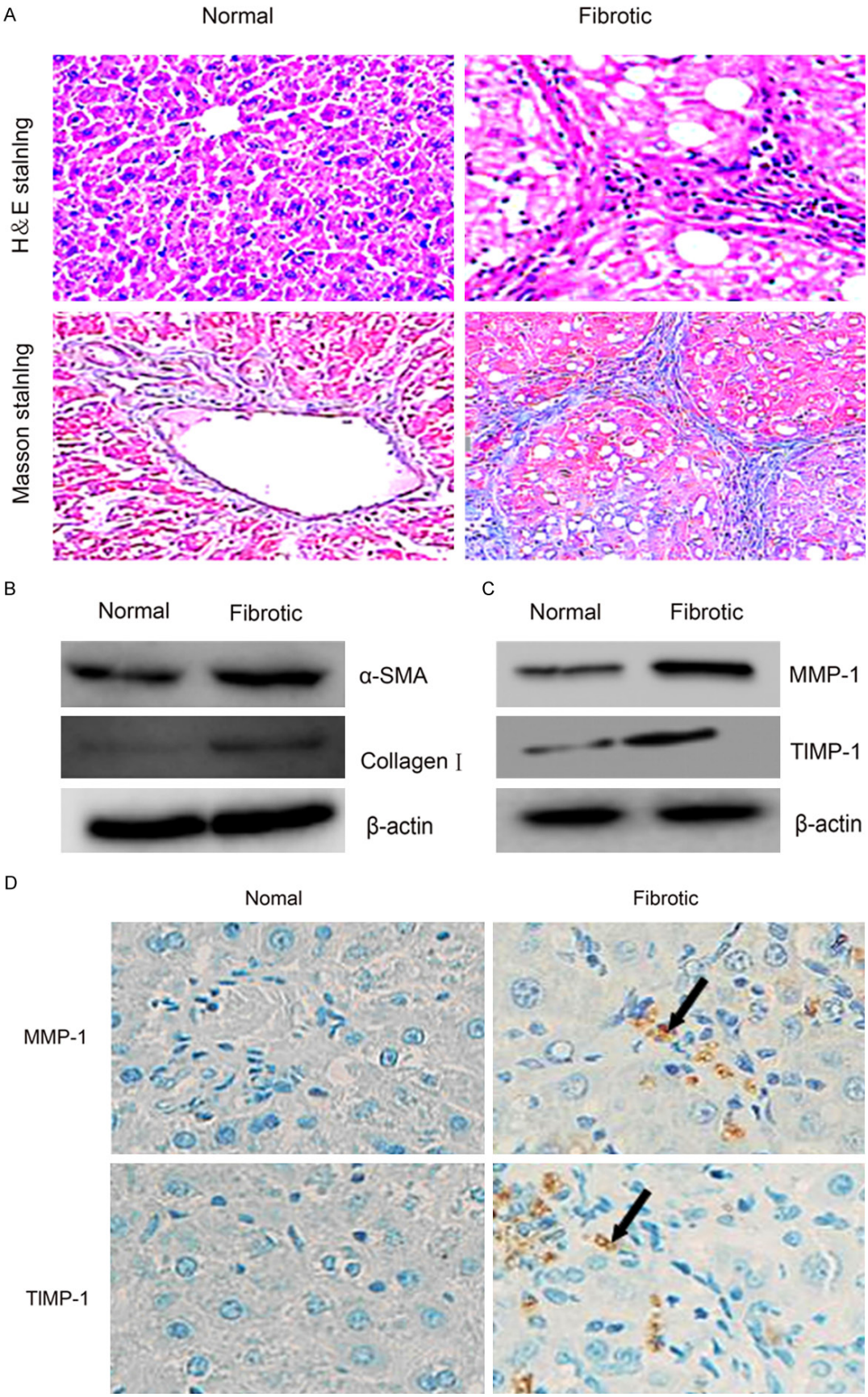
A
Normal
Fibrotic
H&E staining
Masson staining
B
Normal
Fibrotic
α-SMA
Collagen I
β-actin
C
Normal
Fibrotic
MMP-1
TIMP-1
β-actin
D
Nomal
Fibrotic
MMP-1
TIMP-1

Figure 1. MMP-1 and TIMP-1 are induced in fibrosis. A. Liver fibrosis was measured by H&E staining and Masson staining. B. Liver fibrosis was also confirmed by increased expression of collagen I and α-SMA as measured by western blot. C. Expression of MMP-1 and TIMP-1 in CCl_4-injured rat liver and normal control liver. D. Representative liver sections showing MMP-1 and TIMP-1 immunohistochemical staining in CCl_4-injured rat liver and normal control liver. The arrows show the positive cells. All photomicrographs are at 400 × magnification.

Isolation of rat HSC

HSC were isolated from retired normal male and female, Wister rats. Liver tissue was digested with collagenase IV and the cell suspension was subsequently separated by using a 25% Optiprep gradient (Axis-Shield PoC AS). HSC were seeded onto plastic (NEST), cultured in Dulbecco's modified Eagle's medium (Hyclone) and supplemented with 20% fetal bovine serum (Gibico), pyruvate, glutamine, penicillin, and streptomycin. They were maintained in an incubator at 37°C with 5% CO_2. Cells at day 2 of in vitro culture were treated as qHSCt and day 9 cultured were regarded as aHSC.

Immunofluorescence stain

HSC were placed and cultured on culture slides. The slides were incubated in 5% BSA-PBS for 1 hour at room temperature. Primary antibody for dismin or α-SMA, diluted 1:50 in 1% BSA-PBS, was applied and incubated overnight at 4°C in a humidified chamber with a light shield. On the next day, the slides were incubated with 500 μl of 1% BSA-PBS containing secondary antibodies conjugated with FITC (1: 200 diluted) for 1 hour at room temperature in the humidified chamber. After washing, the slides were incubated with DAPI for 5 minutes. The slides were washed with PBS for 10 minutes three times between each step. Specimens stained with DAPI and FITC were examined using Epi-fluorescence microscopy, equipped with filters for FITC and ultra violet (× 200).

Western blot

HSC cells or rat liver were lysed in a lysis buffer. The lysates were resolved through SDS-PAGE and transferred to nitrocellulose membrane. Blots were blocked with TBS/Tween 20 (0.1% T-TBS) containing 5% milk protein before being subjected to overnight incubation with the primary antibodies. Primary antibodies raised against MMP1, TIMP1, H3K4me2, H3K9-me2, collagen I and α-SMA were used at 1: 2000 dilution and β-actin was used at 1:600 dilution. Membranes were washed in T-TBS and incubated with antirabbit horseradish peroxidase (HRP)-conjugate antibodies at a 1:5000 dilution rate for 1.5 hours. Blots were washed and antigen detected by ECL.

Chromatin immunoprecipitation assay

HSC were cross-linked by 1% formaldehyde for 10 minutes at room temperature. Then cells were washed with ice cold PBS twice and scraped into ice cold PBS containing protease inhibitor cocktails (milipore), collected by 10 minutes of centrifugation at 3000 × g at 4°C. Cell pellets were re-suspended in a SDS lysis buffer and incubated on ice for 10 minutes. Sonication was then carried out to get chromatin fragments between 200-1000 bp. After that, the mixture was centrifuged at 10,000 × g for 10 minutes, at 4°C. The chromatin supernatant preparation was transferred to a new tube and aliquoted for subsequent experiment. For each assay, the chromatin preparation was first incubated overnight at 4°C with 5 μg of specific antibodies [anti-H3K9me2, anti-H3K4me2] with IgG control, or anti-RNA Polymerase II. The next day, immune complexes were collected on protein-A agarose beads, and the beads were washed to remove nonspecific binding. DNA was eluted from the beads, crosslinks were reversed, and DNA was extracted. ChIP-enriched samples were analyzed in triplicate by qPCR using primer MMP1 promoters (forward 5'-TGATTTATTCCCTGCTAC-TGAA-3' and reverse 5'-GCTCCAACATCCTGT-CCAAG-3'). Results were expressed as either fold over control or a percentage of control.

Statistical analysis

The data set is expressed as mean ± standard error mean (SEM) and analyzed using SigmaPlot 17 graphing and statistical analysis software. The student's t-test was used for two groups or ANOVA with Dunnet post tests for multiple groups. Differences in values were considered significant if $p < 0.05$.

Results

Liver fibrogenesis and promotion of MMP-1

First, we surveyed the concept of MMP-1 promotion in liver fibrosis over a standard 8 weeks,

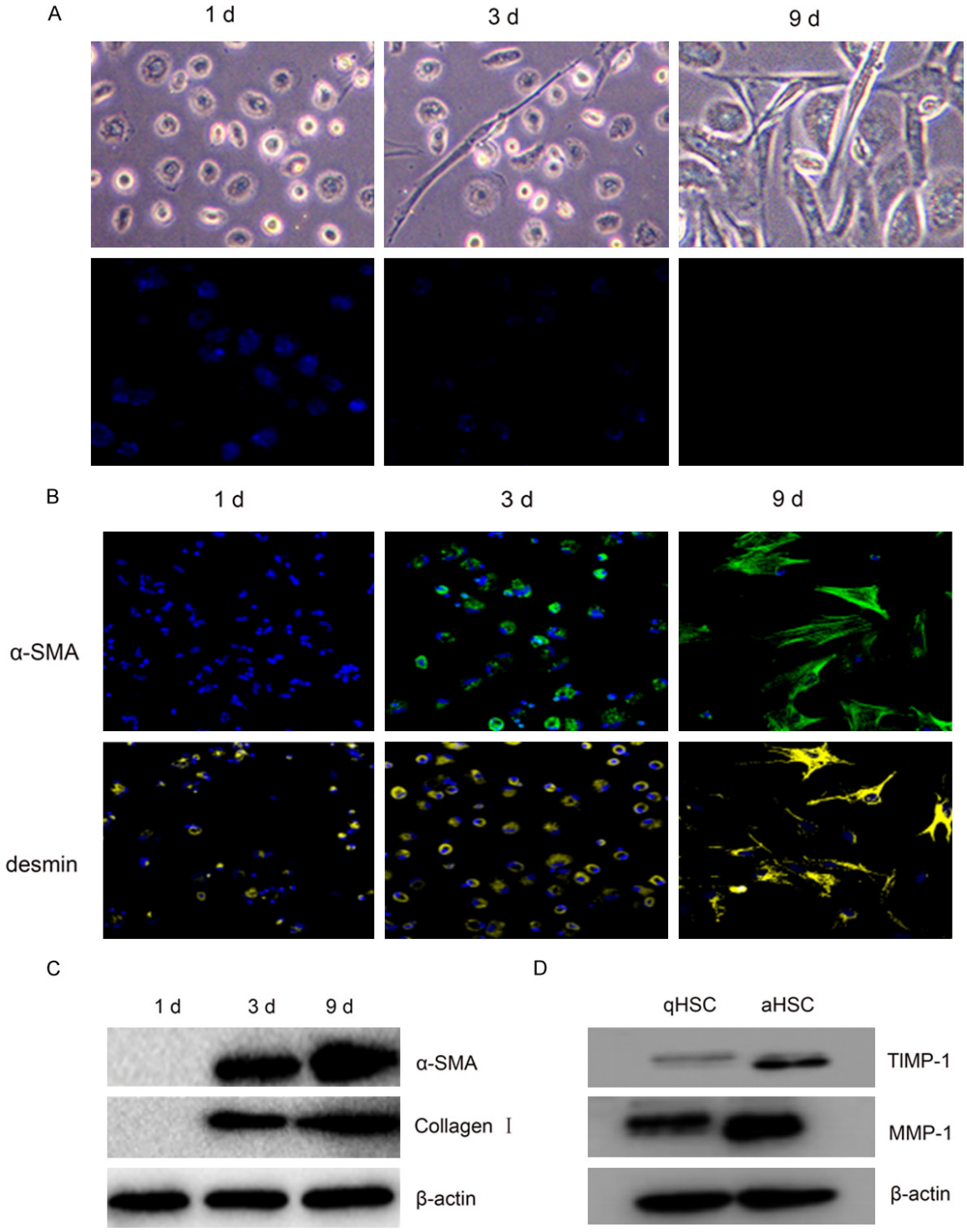

Figure 2. Induction of MMP-1 during HSC differentiation in vitro. Freshly isolated rat HSC after recovery for two to three days still retain some features of a quiescent phenotype. In continuous culture on plastic up to nine to ten days, the cells transdifferentiate into aHSC. A. Phenotypes and autofluorescence of quiescent (day two), intermediate (day four) and complete activation (day nine). B. HSC were subjected to immunofluorescence staining for α-SMA (green, a marker for aHSC) and desmin (yellow, a marker for HSC). C. Differentiation was monitored by increased expression of collagen I and α-SMA. D. In quiescent (day one, qHSC) and activated (day nine, aHSC) HSC were examined for MMP-1 and TIMP-1 by western blot analysis.

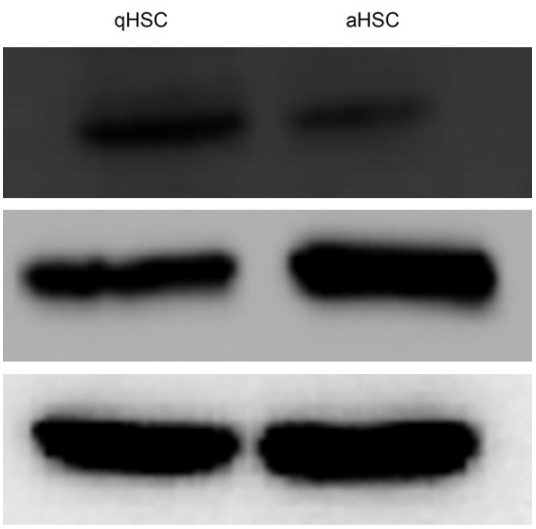

Figure 3. Expression profile of histone modification in qHSC and aHSC. Forty μg whole cell protein extract from three separate preparations of primary rat qHSC or aHSC were separated by SDS-PAGE and immunoblotted for H3K9me2 and H3K4me2 histone modifications. Equal loading was assessed using total histone H3 control.

shown by the formation of polygonal fibrotic septa in Masson staining. Upon evaluation, liver injury was indicated by H&E staining (**Figure 1A**), and validated by increased protein level of α-SMA and collagen I (**Figure 1B**). Expression of MMP-1 and TIMP-1 in the liver tissues were measured by western blot (**Figure 1C**) and Immunohistochemistry (**Figure 1D**). As expected, massive MMP-1 and TIMP-1 in liver parenchyma were observed in the fibrotic liver, but were absent in the normal mice (**Figure 1D**). In the liver, HSC are the major sources of both ECM and MMP-1. Such close proximity between enzymes (MMP-1) and substrates (ECM) suggests that HSC have a potential role in hepatic injury.

MMP-1 induction in differentiation of HSC in vitro

To address the mechanism enabling an increase in MMP-1 and TIMP-1 in liver fibrosis, we developed an in vitro system which recapitulated the key characteristics of MMP-1 and TIMP-1 expression in fibrotic livers. Freshly isolated rat HSC remained quiescent for 1 to 2 days, during which the cells appear as star-like phenotypes with condensed nuclei and cell bodies (**Figure 2A**), displaying ample vitamin -A droplets (visualized under UV excitation) (**Figure 2B**). After being cultured on plastic for 9 to 10 days, qHSC are morphologically and biochemically transformed into myofibroblast-like cells, as indicated by the loss of vitamin-A droplets, buildup of filamentous actin fibers, gain of contractibility, and cell size expansion. We also show α-SMA and desmin levels in all samples as confirmation of HSC (**Figure 2C**). To examine their potentials to express MMP-1 and TIMP-1, we embedded the qHSC and aHSC on plastic. As shown in **Figure 2D**, qHSC barely produced MMP-1 and TIMP-1, and greatly increased in aHSC.

Histone modification in HSC

A similar pattern for MMP-1 and TIMP-1 production in HSC implies that a common regulation may apply to both MMP-1 and TIMP-1 genes, a process that is at least in part regulated epigenetically. We initially assessed global levels of histone methylation signatures in qHSC versus in aHSC, and discovered major shifts in the histone marks tested. H3K9me2 decreased and H3K4me2 increased in aHSC as compared to that of qHSC (**Figure 3**). H3K9me2 at promoter confers transcriptional repression of gene expression and H3K4me2 is opposite. Previous studies have shown that H3K4me3 regulates TIMP-1 during HSC differentiation. We therefore carried out a ChIP assay for H3K4me2 and H3K9me2 on the MMP-1 gene.

H3K9me2 levels are decreased at the MMP-1 promoter in myofibroblastic HSC

To examine the hypothesis of epigenetic promotion of MMP-1 during HSC differentiation, we then investigated the assembly of transcription machinery on MMP-1 promoters. Using quantitative ChIP assays, we measured the H3K9me2 levels at the MMP-1 promoter, discovering that they were significantly impaired in the aHSC, further demonstrating the loose nucleosome in MMP-1 genes in the aHSC. (**Figure 4**).

H3K4me2 levels are increased at the MMP-1 promoter in myofibroblastic HSC

To examine the hypothesis of epigenetic promotion of MMP-1 during HSC differentiation, we then investigated the assembly of tran-s cription machinery on MMP-1 promoters. Using quantitative ChIP assays, we measured the

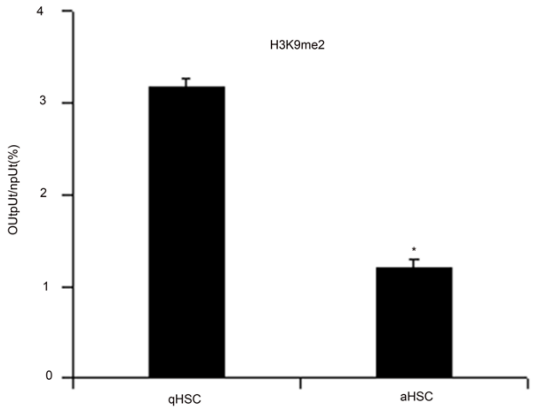

Figure 4. In qHSC and aHSC, basal levels of H3K-9me2 in the promoter of MMP-1 gene were measured by ChIP assay and enrichments of target DNA were analyzed with qPCR using respective primers specific for the MMP-1 promoter. Data represent the output/input ratios. *P < 0.05 by Student t tests for 3 independent.

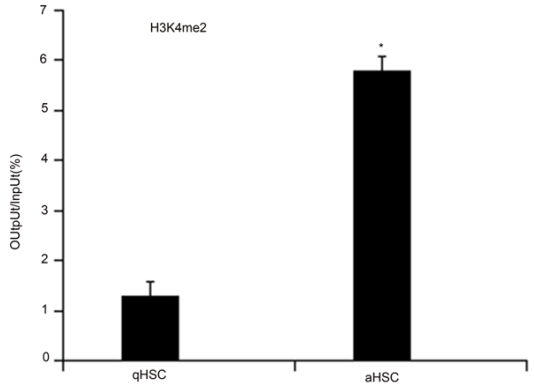

Figure 5. In qHSC and aHSC, basal levels of H3K-4me2 in the promoter of MMP-1 gene were measured by ChIP assay and enrichments of target DNA were analyzed with qPCR using respective primers specific for the MMP-1 promoter. Data represent the output/input ratios. *P < 0.05 by Student t tests for 3 independent.

H3K4me2 levels at the MMP-1 promoter, finding they were significantly enriched in the aHSC, further demonstrating the loose nucleosome in MMP-1 genes in the aHSC (**Figure 5**).

Discussion

Liver fibrosis results from continuous damage to the liver, by means of viral hepatitis, alcohol abuse, drugs and metabolic diseases. The end stage of liver fibrosis, cirrhosis, is characterized by and increased deposition and altered composition of the ECM. Accumulation of ECM, including fibrillar collagens, fibronectin, laminin, hyaluronan, and proteoglycans, results from both increased synthesis and decreased degradation. HSC are regarded as the main ECM-producing cells in the injured liver. But indirect evidence that indicates insufficient ECM degradation as a contributing factor in the pathological accumulation of specific matrix-degrading enzymes in this process is still controversial.

MMP-1 form a group of neutral proteinases requiring zinc ions for activity. The activity is stringently regulated by complex mechanisms at the levels of gene expression, proenzyme activation, and inhibition of active enzyme. After secretion in zymogen form, MMP-1 are activated by proteolytic enzymes or autocatalytic cleavage. MMP-1 activity is modulated by various types of serum and tissue inhibitor, including that of TIMP-1.

The increased collagenase activity in the early stages of liver fibrosis, which had been observed by a large number of studies, initially could not be correlated with the results of established gene expression research. Recently, many researchers had reported that during fibrogenesis the expression of MMP-1 was limited, whereas that of TIMP-1 increased [13, 14]. Initially, we had considered that MMP-1 decreased and TIMP-1 increased. However, contrary to our expectation, our observation suggested that both MMP-1 and TIMP-1 increased in rat hepatic fibrosis induced by chronic CCl_4 intoxication. We further demonstrated that cells with a strong MMP-1 presence and TIMP-1 were observed mainly at the interface between the resolving fibrous septa and the parenchyma by immunohistochemical staining. However, we postulated that an increase in MMP-1 gene expression, even if the increase is very slight, may be necessary for the destruction of the tissue in order to deposit newly formed matrix.

Our observations led to our next hypotheses: 1) That MMP-1 gene expression appears the process of experimental hepatic fibrosis, and that it decreased during the process of cirrhosis. 2) The amount of expressed TIMP-1 was much more than the amount of expressed MMP-1, resulting in MMP-1 activity inhibited by TIMP-1. The pathophysiological expression of MMP-1 in tissues is subject to tight control at

various levels, from the extracellular stimuli, including multiple cytokines and the surrounding ECM to the status of the cells in their epigenetic. The HSC is a pivotal key player that produces ECM, secreting MMP-1 for fibrolysis after receiving the signal stimuli. However, the apparent dilemma is establishing whether aHSC are producing MMP-1 and ECM simultaneously.

In this study we isolated rat primary HSC undergoing spontaneous activation when plated on uncoated plastic. Growth of HSC on plastic led to a phenotypic response known as activation, which paralleled closely the response of these cells to injury *in vivo.* We investigated the expression and activity of MMP-1 between the qHSC and aHSC. The results showed that the aHSC expressed high levels of ECM and MMP-1, while the activity of MMP-1 decreased. The decreased activity of ECM-removing MMP-1 was mainly due to an increased expression of its specific inhibitor-(TIMP-1).

The mechanism of MMP-1gene expression in HSC should be further investigated. The molecular mechanism of the epigenetic regulation of the MMP-1 gene has not been studied in HSC. Histone modification plays a key role in gene transcription by inducing changes in chromatin structure. In general, euchromatin states lead to gene expression, while heterochromatin states facilitate gene silencing. Early studies showed that H3K9me2 is largely associated with heterochromatin and gene silencing, while H3K4me2 is linked to euchromatin, where actively transcribed genes are located [7, 8]. We examined the global levels of H3K4me2 and H3K9me2, both in rat primary qHSC and in aHSC by western blot. We observed that there were higher H3K4me2 and lower H3K9me2 levels in aHSC. Thus, our findings suggest that H3K4me2 and H3K9me2 status involved in HSC trans-differentitation may be associated with the early-stages of liver fibrosis. Despite the data implicating epigenetic events relevant to liver fibrosis, the histone methylation related to MMP-1 expression has not been elucidated. We performed changes in key epigenetic chromatin marks, including histone H3K4me2 and H3K9me2 levels, at MMP-1 gene promoter. Our current results showed for the first time that, in the aHSC, H3K9me2 levels were decreased in MMP-1 promoter, compared with that of qHSC, and this inversely correlated with increased expression of these genes. This suggests that a loss of the repressive H3K9me2 mark can increase the expression of profibrogenic genes under pathologic conditions. We also showed that upregulation of H3K4 me2 marks, usually associated with active chromatin, occurs in parallel with the down regulation of H3K9me2 on MMP-1 promoter, suggesting that this further contributes to increased gene expression. Recent studies showed that H3K4me2 correlates with transcriptionally competent chromatin and is associated with active genes. This supports our observation that MMP-1 expression was positively correlated with increased H3K4me2 in the promoter.

In the current study, the observed increases in H3K4me2 and decreases in H3K9me2, occurring at the MMP-1 promoter to enhance MMP-1 expression in aHSC. Furthermore, the HMTs and histone demethylases (HDMs) may play cooperative roles in this process. Additional studies are needed to assess these factors, including the role of HMTs that mediate H3K4me2 and H3K9 me2.

Our current results showed that decrease of H3K9me2 and increase of H3K4me2 are involved in the upregulation of MMP-1 expression in aHSC.

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Disclosure of conflict of interest

None.

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