Liver fibrosis is a reversible wound-healing process that occurs in almost all patients with chronic liver diseases (CLDs). The sustained liver injuries in CLDs cause multiple cells and cytokines act in a dynamic and interactive way to comprise the mechanisms of fibrogenesis. Though early-stage liver fibrosis is usually silent in symptoms, progression to cirrhosis may cause almost all kinds of hepatic complications, including portal hypertension, ascites, hepatic encephalopathy and impaired metabolic disturbance, therefore it is largely responsible for the mortality of hepatopathy (Mas et al. 2009; Hernandez-Gea and Friedman 2011).

Activation and differentiation of hepatic stellate cells (HSCs) are core events in liver fibrosis (Fig. 1). HSCs are quiescent cells in the perisinusoidal space in liver, which facilitate hepatocytes interactions via releasing soluble inflammatory factors and producing extracellular matrix. The quiescent cells may develop adipogenic or myogenic characteristics during the differentiation process (Tsukamoto 2007). The distinct directions of differentiation are determined by the imbalance between clusters of adipogenic genes and myogenic genes. The expression of adipogenic genes is down-regulated under the stimulus of ischemia, drugs or inflammation. Peroxisome proliferator-activated receptor gamma (PPAR-γ) has a predominant leadership among the adipogenic genes. In this process, suppression of PPAR-γ expression was demonstrated to be regulated in complicated epigenetic ways, which influences the secretion of chemokines and proteins from HSCs (Miyahara et al. 2000). In the competition between adipogenic genes and myogenic genes, myogenic genes gain advantage, which induces the differentiation of adipogenic HSCs to myogenic HSCs. Myogenic HSCs can further differentiate to myofi-
broblasts. Myofibroblasts possess pro-fibrogenic potential and actively secrete the extracellular matrix (ECM), including \(\alpha\)-smooth-muscle actin (\(\alpha\)-SMA) and fibrillar collagens (collagen I and III) (Tsukamoto et al. 2011). In addition, HSC is the main source of tissue inhibitors of metalloproteinases (TIMPs), which may decrease ECM degradation through suppression of the matrix metalloproteinases (MMPs) activities (Ramachandran and Iredale 2012). The altered balance between ECM synthesis and degradation finally leads to fibrogenesis.

Many genes and cytokines in HSCs have been demonstrated to be involved in the pathogenesis of liver fibrosis (Tsukamoto 2007). However, the exact regulating mechanisms of these genes are largely unknown. Recent studies have highlighted the regulatory effect of epigenetic modifications at gene expression level. Increasing modification manners in fibrogenesis processes are brought to our attention, consisting of DNA methylation, histone modification, RNA interfering and chromatin structure change (Fig. 2), but the exact correlation between liver fibrosis development and the abnormal epigenetic modifications of fibrosis related genes remains unclear. Moreover, there is still no conclusive statement on the vital function of specific nucleic acid sequences and structures in gene promoters, such as CpG-islands and histone binding sites. This article therefore emphasizes that epigenetic modification in HSCs, a key player in liver fibrosis, should be reanalyzed, as summarized in Fig. 3.

**DNA methylation, the widely recognized factor contributing to liver fibrosis**

Methylation of CpG islands in gene promoters is an important regulating pattern of gene expression. CpG islands are CpG dinucleotide-rich areas located mainly in the gene promoter regions. DNA methylation can add methyl groups to CpG island, decrease gene transcription activity and may account for gene inactivation in the pathogenesis process (Yu et al. 2002). Among various kinds of epigenetic modifications, many studies have focused on DNA methylation. In the past decade, researches on hepatocellular carcinoma (HCC) related genes have successfully demonstrated that the expression of sets of genes are significantly down-regulated or silenced by the abnormal methylation of CpG islands in gene promoters in early HCCs. Multiple data from these studies in liver tissue demonstrate that except for the process from hepatic cirrhosis (HC) to HCC, there is an obvious change in the methylation rate during the process from normal to HC (Yu et al. 2002; Li et al. 2004; Nishida et al. 2012). For example, in a qualitative investigation using methylation-specific PCR (MSP) to detect various gene methylation status, the hypermethylation rate of promoter was found to be 15% in liver cirrhosis tissue and 0% in normal liver tissue (Zhang et al. 2008). Furthermore, a direct quantitative investigation showed the methylation rate of several hepatopathy related genes are distinct between HC patients and normal controls: p16INK4a (24% and 0%), GSTP1 (32% and 0%), MGMT (12% and 0%) and DAP-K (100% and 31%) (Harder et al. 2008). All these unintentionally discovered results have revealed it is still under doubt whether or not abnormal methylation on fibrosis-related genes is related to liver fibrosis.

Studies on fibrosis-related genes have become a popular topic in recent years. Promoter methylation states of several genes associated with DNA repair were detected in cirrhosis. Results showed the methylation rate of MGMT and hMSH3 gene promoters was high, but that of hMLH1 and MSI gene promoters was very low (Park et al. 2006). Researchers have also found some genes such as NOR1E1A (Ras-binding protein which belongs to a group of tumor
Fig. 2. Three main possible epigenetic modifications: methylation, histone modification and complex formation. They may either singly or simultaneously happen in liver fibrosis-related genes. Promoters are the vital targeting sites of these manners.

Fig. 3. Epigenetic modification contributes to liver fibrosis in various mechanisms of fibrogenesis. HSC has a crucial role.
Epigenetic modifications determine cell differentiation in liver fibrosis

—the most in-depth study

Studies upon most of the fibrosis-related genes have been conducted simply in a preliminary level. But the potential regulating mechanisms of genes contributing to HSC differentiation have been clarified.

Even though there are still arguments over the origin of HSC, subsequent differentiation mechanism is generally accepted. Genes determining the adipogenic or myogenic characteristics hold a balance during the process. Under normal circumstances, adipogenic genes are dominant compared with myogenic genes in liver cells, and the quiescent HSCs differentiate to adipogenic HSCs (Miyahara et al. 2000). However, situation changes when liver injury occurs. Adipogenic genes (such as PPAR-γ, enhancer binding protein and inhibitor of nuclear factor-κB protein-α) are down-regulated or silenced in response to liver injury (Elsharkawy et al. 1999; Miyahara et al. 2000; Oakley et al. 2003; She et al. 2005), and HSC differentiation is taken over by myogenic genes (such as collagens I and III, TIMP-1, α-SMA, interleukin-6) (Bataller and Brenner 2005; She et al. 2005), and HSC differentiation is taken over by myogenic genes in liver cells, and the quiescent HSCs differentiate to adipogenic HSCs (Miyahara et al. 2000). However, situation changes when liver injury occurs. Adipogenic genes (such as PPAR-γ, enhancer binding protein and inhibitor of nuclear factor-κB protein-α) are down-regulated or silenced in response to liver injury (Elsharkawy et al. 1999; Miyahara et al. 2000; Oakley et al. 2003; She et al. 2005), and HSC differentiation is taken over by myogenic genes (such as collagens I and III, TIMP-1, α-SMA, interleukin-6) (Bataller and Brenner 2005; Friedman 2008).

Two vital factors are involved in the cell differentiation process. First, PPAR-γ, the master of adipogenic genes, which is intrinsically a negative regulator of expression of type I collagen and myogenic phenotypic characteristics in liver cells. The basal function of PPAR-γ is to maintain the adipogenic phenotype characteristics of HSCs (Miyahara et al. 2000). The suppression of PPAR-γ is demonstrated to be a fatal action which is mainly regulated by epigenetic modifications. Second, methyl-CpG binding protein 2 (MeCP2), which participates in the regulation of PPAR-γ, can also be recruited to promoters in other master genes (such as inhibitor of nuclear factor-κB protein-α gene) and important modulating genes (such as the histone named H3K27 and methyltransferases named ASH1) (Tao et al. 2011; Perugorria et al. 2012). The epigenetic modulation dominated by PPAR-γ and MeCP2 mediates HSC differentiation in the following pathways.

CpG islands in promoters are the vital targeting sites in MeCP2 relay epigenetic modifications of PPAR-γ

Depletion of microRNA132 is regarded as the source of epigenetic change. MeCP2 gene is suppressed in normal conditions, but depletion of microRNA132 may make it over-expressed. The redundant MeCP2 induces generation of enhancer of zeste homolog 2 (EZH2) and hypermethylated of lysine 27 histone 3 (H3K27). Hypermethylated H3K27 may create repressive chromatin structure in the 3′ end of PPAR-γ and suppress its transcription. Besides, gene bank indicates that there are CpG islands existing in PPAR-γ promoter. MeCP2 can bind to CpG islands directly and promote the methylation of H3K9. It provides H3K9 the ability to recruit a transcriptional repressor HP1α and bind to the promoter, exon1, exon2 and 5′ end of PPAR-γ. All those activities mentioned above cooperate in an epigenetic pathway and lead to the suppression of PPAR-γ expression (Mann et al. 2010; Tsukamoto et al. 2011).

“GN” box in Wnt10b gene promoter helps PPAR-γ suppression through the Necdin-Wnt pathway

Necdin gene is expressed in neurons, skeletal and smooth muscle cells. Necdin protein can prevent HSCs from developing adipogenic characters, while sustain the forming of myogenic characters which are favorable to the fibrogenesis (Tseng et al. 2005; Zhu et al. 2010). Canonical wingless-related MMTV integration site 10b (Wnt10b), downstream of neclin, has a “GN” box in its promoter. “GN” box refers to multiple G nucleotide clusters intervened with single or double nucleotides of A, T or C. Necdin combines with the “GN” box and promotes the Wnt10b expression (Matsumoto et al. 2001). The expressed Wnt10b enriches MeCP2 to the PPAR-γ promoter, followed by methylation of a series of histones mentioned in the former pathway. As a final result, the HSCs lose adipogenic characters. Silencing of neclin was proved to be likely to inhibit Wnt10b expression and block the whole pathway, while the increase of exogenous canonical Wnt3a can neutralize this blocking effect (Zhu et al. 2010).

The two described manners well explained the epigenetic modification net of PPAR-γ gene (Fig. 4). However, we are surprised to find that the direct methylation of PPAR-γ promoter is not included. Gene bank indicates that, several CpG islands locate on PPAR-γ gene promoter sequence. Therefore, there is a possibility that DNA methylation, the only known regulatory modification of DNA itself, may play a potential role in the regulation of PPAR-γ gene transcription. Also, the change may be associated with the alternation of DNA methyltransferase (DNMT) in the microenvironment (Kanai 2010).

MeCP2-ASH1-relay methylation of H3K4 promoter

A recent research tested the alternation of three H3K4 methyltransferases: MLL5, Set1 and ASH1 (Perugorria et
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al. 2012). All of them were prominently up-regulated during myogenic HSC differentiation, and ASH1 was interestingly found to be positively regulated by MeCP2. In the following pathway, ASH1 promoted the methylation of H3K4 promoter. As a result, there was increased methylated H3K4 recruited to the promoters of α-SMA, collagen I, TIMP-1 and TGFβ-1 genes, whose products are closely related with liver fibrosis (Perugorria et al. 2012).

Inspiringly, there have been new genes or pathways discovered constantly, such as Ras GTPase activating-like protein 1 (RASAL1). The methylation of RASAL1 is also shown under control of MeCP2 (Tao et al. 2011), which may regard as another driver for HSC differentiation. The continuous perfection of MeCP2 related pathway is accelerating our understanding of cell differentiation and fibrogenesis.

When we try to extend researches to humans, the availability of specimens becomes a major problem. Only limited researches conduct primary culture of HSC and complete an in vitro examination within a cell line (Perugorria et al. 2012), because generally accepted extraction methods require massive liver tissue of more than 50 g. Accordingly, we can only use human excisional tissues in surgery, while incapable of acquiring clinical samples in large quantities. Therefore it cannot be applied to clinical diagnosis. This situation puts up a requirement for better extraction means and detecting methods in the future.

Epigenetic modifications in HSC promote oxidative stress and inflammation

Firstly, several COX derived products can bind to PPAR-γ and alter transcription of downstream genes (Forman et al. 1997). Then a variety of cytokines can be released by HSCs, regulating gene expression and cell characteristic at an early stage of fibrogenesis by means of paracrine (Heymann et al. 2009). These cytokines can enhance the oxidative stress and inflammation, which in return mediate cell differentiation and permeates through whole fibrogenesis process. The modulating mechanisms of genes coding cytokines in HSCs are underlined (Ghatak et al. 2011; Ramachandran and Iredale 2012).

Epigenetic modification of IκBα, the most important inflammation chemokine, is partially regulated by MeCP2. Rather than trigger the downstream histones methylation in PPAR-γ regulating pathway, MeCP2 recruits C-promoter binding factor-1 (CBF-1) to form co-suppressor complex and negatively regulates the expression of IκBα. Cross-linked CHIP helped directly confirmed the interaction between MeCP2 and IκBα. In order to investigate the mechanism, Mann and coworkers cultured HSC derived myofibroblast in vitro and further demonstrated the MeCP2 dose-dependent activation of IκBα promoter (Mann et al. 2006). Down-regulation of IκBα expression enhances the basal activity of nuclear factor-kappa B (NF-κB). NF-κB promotes the secretion of pro-inflammation chemokines by
activating HSC. This pathway is thus responsible for inflammatory action and oxidative stress in HSCs, and achieves partially epigenetic control in the process of liver fibrosis.

In another vital epigenetic pathways, histone hyper-acetylation and DNA hypomethylation are demonstrated to affect gene transcription by inhibition of ubiquitin-proteasome pathway (Bardag-Gorce 2009). This proteasome pathway is independent of the activation of NF-κB, and may lead to the similar consequences-inflammatory cytokine infiltration such as IL-18, activation of cell differentiation and further damage to liver cells (Joshi-Barve et al. 2003).

Furthermore, Martínez-Gras et al. (2011) demonstrated that the decrease of PPAR-γ expression in PBMCs was associated with the balance between inflammation and anti-inflammation in schizophrenia (Martínez-Gras et al. 2011). This phenomenon also indicated that PPAR-γ might have its potential role in the regulation of inflammation in PBMCs, independent of indirect way through HSCs. If we can clarify the connection between epigenetic modification in PBMC and HSC, we can detect the changes in PBMC to reflect the changes in liver cells, which may make non-invasive diagnosis to reality. But as of now, no related study has been done.

**Epigenetic regulation of MMP gene contributed to ECM accumulation**

Extracellular matrix (ECM) is a family of insoluble proteins distributed throughout the liver mesenchyma, liver cells and the basement membrane of blood vessels, mainly composed of collagen, noncollagenous glycoproteins and proteoglycan. Currently, dysregulation of fibrinolytic factors is treated not only a late-stage process, but also one of the underlying risk factors for obstructing ECM degradation (Kang and Mars 2011).

The epigenetic regulation on MMP in HSCs alters the degradation of ECM. Qin and Han (2010) showed that massive MMP expressed in HSCs was blocked in acute hepatopathy. The degradation function mediated by MMPs was therefore attenuated, which leads to ECM depositing. However, the whole downstream signal pathway was completed, implying the inhibition happens at the MMP gene level instead of the following signal pathway. Further study showed that impaired histone deacetylation in the process of HSC differentiation may lead to accumulation of histone deacetylase-4, which can suppress the activity of MMP gene promoter in quiescent HSC (Qin and Han 2010). In fact, this epigenetic modification suggests a histone deacetylase-4 dependent gene promoter silencing mechanism, which finally leads to MMP silence and ECM piling up.

Another key factor regulating ECM degradation is TIMP, which is mainly expressed in HSCs in the liver. Previous study demonstrated its epigenetic regulating effect in prostate tumor and ovarian tumor (Velino et al. 2010). Furthermore, methylation is involved in inhibiting TIMP-3 gene expression in HBV related HCC (Lai and Lo 2005). Considering the direct regulating function of TIMP-3 to MMPs and ECM, methylation of TIMP-3 is highly predicted existing in liver fibrosis. Studies have also showed that the H3K4 methyltransferase ASH1 can recruit to the regulatory regions of TIMP-1 and further mediate cell differentiation (Perugorria et al. 2012). However, direct epigenetic modification evidence of TIMP on liver fibrosis still needs further investigation.

**Correlation between epigenetic modifications and telomerase shows potential value in transgenerational inheritance**

Studies on epigenetics have demonstrated that epigenetic modifications target on the whole genome, including genes regulating cell differentiation, tissue generation, sensitivity to environmental injury, biochemical metabolism and even evolution (Liu and Tollefsbol 2008). Similar to gene polymorphism, epigenetic modification can be transgenerational inherited to the next generation through mitosis and meiosis, making an influence on the risk of liver fibrosis (Skinner 2011).

However, in liver fibrosis, evidence regarding the issue of epigenetic modification and transgenerational inheritance is limited. Prominent achievements include two points: telomere length and adaptation to liver injury. Telomere expression is restricted in human cells, but shortening or abnormal activation of telomere plays an important role in maintenance of genome stability and cell proliferation. Study on 120 HC patients and 10 normal persons showed the CpG islands methylation rate of HC patients was relatively high (15% and 0%). In the 15% of hypermethylated patients, 55.6% of them had a higher activity of telomerase, compared with 6.35% in normal (Zhang et al. 2008). This comparison clarifies the fact that the telomere length between liver fibrosis patients and normal persons is distinct and related with the methylation status of individuals. Regarding the issue of inheritance, another investigation compared telomere length between normal people and “special” people, who have sibship with hepatopathy patients (Diaz de Leon et al. 2010). The outcome showed “special” people have shorter telomere than normal person. Combination of the two researches can be considered as an evidence of illustrating the correlation between telomere length inheritance and gene methylation status. The second point of the achievements was got from study conducting on mice models (Mann et al. 2009). The parental generation mice were exposed or unexposed to CCL4, and the fibrogenesis sensibility of their offspring were compared when exposed to CCL4 under the same condition. Interestingly, offspring of mice exposed to CCL4 developed a tolerance to liver injury and experienced a lower degree of fibrosis compared with control (Mann et al. 2009). In addition, no distinction in tissue injury and inflammation was observed, which implied the adaptation was selectively expressed in the process of liver fibrosis. They further
analyzed gene expression in this process and found that postnatal protective and anti-fibrotic genes PPAR-γ and SMAD7 were over-expressed, while the fibrogenesis-favorable TGF-β gene was down-regulated. The tolerance became more obvious when the grand-parent and parent generation were both induced liver injury (Mann et al. 2009; Zimmer and Lammert 2011).

The two achievements mentioned above provide epigenetic evidence for the inheritance of liver fibrosis. However, as an important integral part, gene epigenetic modification, especially DNA methylation in transgenerational inheritance, still needs further investigation.

Clinical application using epigenetic modifications

The research of epigenetic modification of gene promoter has aroused general interest in the field of HSC and liver fibrosis. DNA methylation detection techniques are diversely and widely used, commonly including MSP, methyl-fluorescence detection, direct Sanger sequencing, high resolution melting (HRM) and pyrosequencing (Piperi and Papavassiliou 2011). In addition, effective methods with high sensitivity and reliability have been developed, such as the highly qualitative and quantitative bisulfite genomic sequencing method (Li and Tollefsbol 2011). Along with the development of methylation technique, the exploration of innovative diagnostic and therapeutic strategies becomes more feasible.

The pathogenesis of liver fibrosis turns gradually clear in recent years. Numerous fibrosis-related genes and transcription factors have been identified and make it possible to reverse early stage liver fibrosis. So, the primary target of researches on liver fibrosis is to discover accurate methods for early diagnosis. The widely used liver biopsy detection can distinguish occurrence and stages of fibrosis clearly. However, concerns on unexpected dangers and further complications caused by the invasive test give rise to low attendance rate. On the other hand, some researches attest that epigenetic alterations are prior to proteomic deviations. Unlike gene mutations in the subset of liver cells, the epigenetic modification influences are in large scale, which can regulate the genes in HSCs, peripheral blood cells and so on (Skinner 2011). Notion is also taken into consideration that compared with mRNA and protein whose expression profiles are easily affected by the microenvironment, DNA methylation is stably preserved in DNA double strands by covalent bonds. DNA methylation can be detected veraciously and may thus be regarded as believable diagnostic indicators (Kanai 2010). Moreover, epigenetic modification is often independent of nucleic acid sequence alteration, which means the examination of epigenetic state and host gene mutations can both be detected and further offset the shortage of simply one aspect testing.

After realizing early diagnosis, inhibiting and even reversing the process of liver fibrosis are keenly awaited. Lee and coworkers demonstrated the availability of IL-10 and α-melanocyte-stimulating hormone gene therapy, which, however, were limited to laboratory experiments (Hung et al. 2005; Lee et al. 2006). Compared to working on gene mutation, it is more realistic to regulate epigenetic changes. Um et al. (2011) presented that DNA methyltransferase inhibitors, including 5-azacytidine and 5-aza-20-deoxycytidine, may help to eliminate DNA hypermethylation rate as an epigenetic therapy. A recent study investigated the relationship between a methyl-deficient diet and promoter methylation status of 164 genes which altered multiple vital processes including lipid and glucose metabolism, DNA damage and repair, apoptosis, the development of fibrosis, and liver tissue remodeling (Tryndyak et al. 2011). Although there were both increased and decreased changes of CpG island methylation rates, the number of hypomethylated genes was substantially greater than the number of hypermethylated genes. Moreover, diet may artificially regulate the methylation level (Tryndyak et al. 2011). A recent study also provided the advanced step from Necdin-Wnt pathway mechanism to clinical application (Yang et al. 2012). They demonstrated the herbal prescription Yang-Gan-Wan (YGW), which has been known for its protective effects on liver, can decrease MeCP2 expression and prevent it from recruiting to PPAR-γ promoter. In addition, the possible active components rosmarinic acid and baicalin were found to suppress the Necdin-Wnt signaling in HSCs (Yang et al. 2012). Interfering epigenetic alterations of relevant genes may become a candidate therapeutic target to reverse liver fibrosis in the future.

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Conflict of Interest

The authors declare no conflict of interest.

References


