

Reversibility and heritability of liver fibrosis: Implications for research and therapy

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Abstract

Liver fibrosis continues to be a major health problem worldwide due to lack of effective therapy. If the etiology cannot be eliminated, liver fibrosis progresses to cirrhosis and eventually to liver failure or malignancy; both are associated with a fatal outcome. Liver transplantation, the only curative therapy, is still mostly unavailable. Liver fibrosis was shown to be a reversible process; however, complete reversibility remains debatable. Recently, the molecular markers of liver fibrosis were shown to be transmitted across generations. Epigenetic mechanisms including DNA

methylation, histone posttranslational modifications and noncoding RNA have emerged as major determinants of gene expression during liver fibrogenesis and carcinogenesis. Furthermore, epigenetic mechanisms have been shown to be transmitted through mitosis and meiosis to daughter cells and subsequent generations. However, the exact epigenetic regulation of complete liver fibrosis resolution and inheritance has not been fully elucidated. This communication will highlight the recent advances in the search for delineating the mechanisms governing resolution of liver fibrosis and the potential for multigenerational and transgenerational transmission of fibrosis markers. The fact that epigenetic changes, unlike genetic mutations, are reversible and can be modulated pharmacologically underscores the unique opportunity to develop effective therapy to completely reverse liver fibrosis, to prevent the development of malignancy and to regulate heritability of fibrosis phenotype.

Key words: Epigenetics; Epimutations; Inheritance; Liver cirrhosis; Hepatic stellate cells; Histone modification; DNA methylation; MicroRNA; Long noncoding RNA; Transcription regulation

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Core tip: Liver fibrosis, if untreated, progresses to cirrhosis and liver failure or malignancy. Liver fibrosis is reversible and potentially heritable process. Heritability and complete reversibility of liver fibrosis although debatable are of profound importance for research and therapy. Epigenetic mechanisms regulate both processes and hold the key for deciphering their regulatory mechanisms and providing new and much needed therapeutic options.

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LIVER FIBROSIS: CONTINUED SAGA

Liver fibrosis and cirrhosis are major causes of morbidity and mortality worldwide and result from different etiologies of chronic liver injury. Liver fibrosis is considered the first step in the progression to cirrhosis and eventually to the development of hepatocellular carcinoma. Although removal of the underlying injurious agent as with antiviral treatment in chronic viral hepatitis may reduce the progression of liver fibrosis, however, patients who don't respond to treatment and those who had advanced fibrosis before treatment starts will eventually need liver transplantation as the only effective treatment currently available. Nevertheless, the unavailability of adequate donor pool and the associated morbidity and mortality of the procedure underscores the need for novel effective antifibrotic therapy^[1].

Fibrosis is a dynamic process resulting from a wound-healing response involving pathways of fibrogenesis and inflammation^[2]. Activation of hepatic stellate cells (HSCs) to myofibroblasts that synthesize and secrete excessive amounts of collagen I and III takes a central role in the fibrogenesis process^[3,4]. Fibrosis accumulation reflects the imbalance between matrix production and degradation and the major determinant of progressive fibrosis is failure to degrade the increased interstitial matrix^[5]. Failure to remove the injurious agent (*e.g.*, hepatitis B or C virus infection in humans or repeated administration of hepatotoxic agents in animals) results in advanced fibrosis and cirrhosis^[6]. In cirrhosis, hepatocytes form dysplastic nodules and finally hepatocellular carcinoma (HCC) lesions^[7].

Epigenetic mechanisms have recently emerged as major determinants of gene expression during HSC activation and deactivation^[8] and have also been described in HCC development^[7]. A brief overview of the general epigenetic regulatory mechanisms as related to liver cirrhosis is essential because it highlights common paradigms of controlling transcription activity during fibrosis progression and resolution and will help to identify the main cellular and molecular mechanisms that mediate fibrosis resolution and heritability.

EPIGENETIC REGULATION OF LIVER FIBROSIS

Epigenetics are the regulatory mechanisms responsible for alteration in gene expression without a change in nucleotide sequence. It is agreed that epigenetic changes are reversible and are heritable^[9-11]. Epigenetic mechanisms regulate gene expression in response to

environmental cues and in cell-specific context. Most importantly, many of these epigenetic modifications are maintained not only during the life span of the individual cell but have been shown to be transmitted during cell division and between generations. The important implication is that environmental cues not only influence phenotypes but is also heritable to subsequent generations^[12].

Epigenetic mechanisms are comprised of 3 major mechanisms including DNA methylation, post-translational modifications of the amino acid tails of histones and non-coding RNAs of which microRNAs (miRNA) and long noncoding RNAs (lncRNA) are the best characterized (reviewed in refs^[10,11]).

DNA METHYLATION AND LIVER FIBROSIS

DNA methylation in eukaryotes involves addition of a methyl group to the carbon 5 position of the cytosine ring in the context of the sequence 5'-CG-3', which is also referred to as a CpG dinucleotide. CpG dinucleotides are unevenly distributed throughout the genome, usually found in dense (> 55%) stretches of approximately 500-2000 bp which are called CpG islands. CpG islands are often but not always found in promoter regions. The rest of the genome, such as the intergenic and the intronic regions, is considered to be CpG poor. In healthy cells, CpG poor regions are usually methylated whereas CpG islands are generally hypomethylated, with a few exceptions including the inactive X chromosome^[13]. As a broad rule, methylation of cytosine in CpG islands leads to silencing of the gene, whereas unmethylated CpG tend to be found in housekeeping/expressed genes (Figure 1).

Methylation of cytosines represents a stable, heritable and reversible mark that is generally associated with transcriptional repression by one of two mechanisms. First, the methyl group on cytosine can physically prevent transcription factor binding thus repressing gene transcription. Second, methylated DNA recruits methyl CpG binding proteins (MeCPs and MBDs) together with co-repressor molecules^[14,15]. For example, one of the MBDs proteins, methyl CpG binding protein 2 (MeCP2), has been shown to be involved in silencing of peroxisome proliferator-activated receptor gamma (PPAR γ) gene expression in liver fibrosis^[16,17]. When found within promoters, DNA methylation prevents the reactivation of silent genes, even when the repressive histone marks are reversed. This allows the daughter cells to retain the same expression pattern as the precursor cells and is important for many cellular processes including the silencing of repetitive elements, X-inactivation, imprinting, and development^[12,18,19]. During the development of cancer, many CpG islands, such as those in the promoter of tumor suppressor genes undergo hypermethylation while those CpG poor

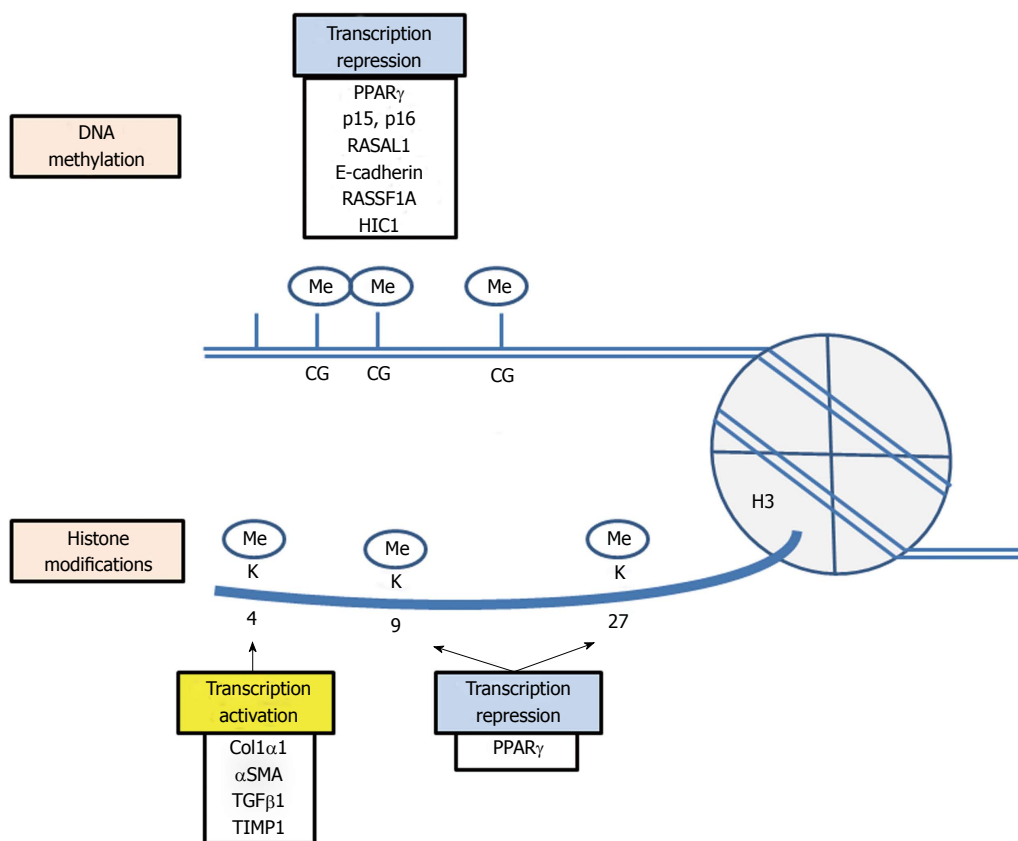


Figure 1 Summary of DNA methylation and histone post translational modifications of liver fibrosis-related genes.

promoters of oncogenes or intergenic regions and repetitive elements become hypomethylated. This alteration in DNA methylation pattern leads to changes in chromatin structure causing the silencing of tumor suppressor genes, activation of oncogenes and instability of the genome, respectively^[20].

DNA methylation is catalyzed by three different DNA methyltransferases (DNMTs) encoded by different genes on distinct chromosomes: DNMT1, DNMT3a, and DNMT3b. This covalent modification uses S-adenosyl-methionine as the methyl donor^[8]. DNMT3a and DNMT3b function act as “*de novo* methyltransferases” that establish DNA methylation patterns during embryonic development and during carcinogenesis. DNMT1, the “maintenance” methyltransferase conducts maintenance methylation. However, because of the inefficiency of DNMT1 in maintaining the methylation of many CpG dense regions, the *de novo* activities of DNMT3a and DNMT3b are also necessary in somatic cells in order to reestablish the methylation patterns so that they are not lost due to the inefficient activity of DNMT1 (For detailed reviews^[21,22]). DNA demethylation occurs through a series of chemical reactions as there is no known mechanism in mammalian cells that can cleave the strong covalent carbon-to-carbon bond that connects cytosine to a methyl group (reviewed in^[18]).

Few studies have evaluated the status of DNA methylation in experimental liver fibrosis and in human cirrhosis (Figure 1)^[12,17,23-25]. The main findings

in these studies are that progression of liver fibrosis is associated with increased DNA methyltransferase DNMT3a enzyme and hypermethylation of the cell cycle genes (p15 and p16), tumor suppressor genes [RASAL1, E-cadherin and hypermethylated-in-cancer 1 (HIC-1)]^[25] and the antifibrotic gene PPAR_γ^[12,24]. There is also promoter hypomethylation of the inflammatory cytokine secreted phosphoprotein 1 (Spp1, Osteopontin)^[23,26-28]. DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-azadC), was shown to inhibit HSC proliferation and transdifferentiation, to block the decreased expressions of RASAL1^[17] and to prevent loss of the antifibrogenic PPAR_γ expression^[24]. Table 1 summarizes the main findings of these studies.

HISTONE POSTTRANSLATIONAL MODIFICATIONS AND LIVER FIBROSIS

In the nucleus, DNA is packaged into chromatin as repeating units of nucleosomes. Nucleosomes are composed of 146 base pair of DNA wrapped around histone octamers (composed of two copies of histone H2A, H2B, H3, and H4). Histone proteins contain a globular domain and an amino-terminal tail which possess large number and type of modified residues. There are at least eight types of modifications found on histone including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP

Table 1 Summary of studies evaluating DNA methylation in liver fibrosis

Ref.	Model	Findings
Komatsu <i>et al</i> ^[23]	Mouse, early fibrosis (2 wk CCl ₄)	global and Spp1 promoter hypomethylation
Tao <i>et al</i> ^[17]	Rat (12 wk CCl ₄) and HSC-T6 cell line	5-aza-2'-deoxycytidine inhibited HSC proliferation and blocked the decreased expressions of RASAL1
Mann <i>et al</i> ^[24]	Rat (5 wk CCl ₄) HSC and human HSC line	5-aza-2'-deoxycytidine blocked HSC transdifferentiation and prevented loss of PPAR γ expression
Zeybel <i>et al</i> ^[12]	Human NAFLD	PPAR γ promoter hypermethylation in severe more than mild fibrosis
Oh <i>et al</i> ^[25]	Human cirrhotic and HCC tissues	Increased DNMT3a mRNA and hypermethylated cell cycle and tumor suppressor genes in cirrhotic but less than in HCC tissues

NAFLD: Non-alcoholic fatty-liver disease; HCC: Hepatocellular carcinoma.

Table 2 Summary of studies evaluating lysine methylation in liver fibrosis

Histone mark	Function	Ref.
H3K9me3	MeCP2 binds to the 5' end of PPAR γ and promotes methylation of H3K9. MeCP2 stimulates expression of EZH2	Mann <i>et al</i> ^[16]
H3K27me3	and methylation of H3K27 to form a repressive chromatin structure in the 3' exons of PPAR γ	
H3K9me2	Repression of I κ B α promoter. 5-azadC treatment of aHSCs increased expression of I κ B α and protein	Mann <i>et al</i> ^[24]
H3K4	ASH1 (H3K4 methyltransferase) binds to promoters and 5' end of α SMA, collagen I, TIMP-1 and TGF β -1 in aHSCs resulting in transcriptional activation of gene expression	Perugorria <i>et al</i> ^[33]
H3K4	ASH1 and EZH2 lysin methyltransferases that regulate H3K4 and H3K27 methylation, respectively were	Atta <i>et al</i> ^[32]
H3K27	upregulated during liver fibrosis progression and downregulated during fibrosis resolution	

ribosylation, deamination and proline isomerization and more than 60 modifications have been described (reviewed in^[10]). It is the combination of site and type of modifications that gives rise to the "histone code" (Figure 1). Many of these modifications function either by disrupting chromatin contacts or by affecting the recruitment of nonhistone proteins to chromatin and in this way regulate important biological functions including transcription, repair and replication of DNA^[8,10,29].

Histone acetylation is associated with transcription activation and is dynamically regulated by the competing enzymatic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which mediate its addition and removal, respectively. Histone acetylation is believed to enhance transcription by neutralizing the basic charges of lysine residues and recruiting bromodomain-containing proteins, including other HATs and chromatin remodeling enzymes, that prevent chromatin compaction^[10,30].

Histones can become methylated and this process is mediated by histone methyltransferases (HMTs). Methylation of histone proteins can also be reversed by histone demethylases (reviewed in^[10]). In contrast to histone acetylation, the impact of histone lysine methylation on gene expression is dependent on the specific lysine residue. For example, histone 3 lysine 4 (H3K4) and H3K36 methylation are associated with transcriptionally permissive chromatin, whereas H3K9 and H3K27 methylation are markers of transcriptionally silent chromatin. In addition, single lysine residues are variably methylated to mono-, di-, and trimethylated states. This can be contrasted with addition of a single acetyl group. Some lysine residues can be modified

by either methylation or acetylation, but never both together. The different histone methylation states are functionally relevant. Active promoters are enriched in trimethylated H3K4, while enhancer elements are enriched in monomethylated H3K4^[31].

Lysine methylation, in particular, is the most extensively studied post-translational modifications in the context of HSC activation (Table 2). Lysine methylation was shown to regulate liver fibrosis in several studies^[16,32,33] (Figure 1). For example, ASH1 (H3K4 methyltransferase) was found to be upregulated during progression of fibrosis^[32] and to bind to promoters and 5' end of α SMA, collagen I, TIMP-1 and TGF β -1 in activated HSCs (aHSCs) resulting in transcriptional activation of gene expression^[33]. EZH2, the enzyme responsible for H3K27 methylation, is also upregulated during progression of fibrosis^[32] and it methylates and represses PPAR γ ^[16]. H3K9 dimethylation is involved in repression of the inhibitory protein I κ B α that sequester NF κ B in an inactive state in the cytoplasm in activated HSCs^[24]. Upregulation of the transcription factor NF κ B has important role in development of liver fibrosis^[34,35].

NON-CODING RNA AND LIVER FIBROSIS

Non-coding RNAs are grouped into two major classes based on the transcript size; small ncRNAs and long ncRNAs. Of the several types of small non-coding RNAs, micro ribonucleic acids (miRNA) are the best characterized and shown to be involved in the regulation of liver fibrosis^[36]. MicroRNAs (miRNAs) are single stranded noncoding RNA molecules of about 22 nucleotides. MicroRNAs modulate gene expression

through imperfect base pairing with the 3'-untranslated region (3'-UTR) of target mRNA, resulting in the inhibition of translation (repression) or the promotion of mRNA degradation. Each miRNA might bind to a number of mRNAs transcripts and in turn one mRNA could be targeted by more than one miRNA, which creates substantial complexity in their capacity to modulated fundamental biological processes (reviewed in^[36-40]).

Furthermore, it is now well established that miRNAs genes are regulated by and can regulate other epigenetic modifications. Several studies have documented that an aberrant pattern of methylation of CpG islands near or within miRNA genes or aberrant chromatin modifications result in dysregulated expression of miRNAs and in pathogenic alterations including tumorigenesis. Additionally, epigenetic drugs including DNA methyltransferase and histone deacetylase inhibitors have both been used to control miRNA expression. Similarly, miRNA can regulate epigenetic modifications by directly targeting the post-transcriptional regulation of DNA and histone modifying enzymes^[14].

Since miRNAs are essentially involved in the regulation of most cellular processes both in health and in disease and that one miRNA can regulate a large number of mRNA transcripts, it is not surprising that several miRNAs have been shown to be involved in liver fibrosis and in activation of HSCs. Specifically, a large body of evidence has demonstrated that many different types of miRNAs play an important role in the progression of liver fibrosis through the regulation of proliferation and apoptosis of HSCs (reviewed in refs^[39,41,42]).

Mechanistically, these studies documented that several miRNAs targets the mRNA 3'-UTR of several key factors of fibrogenesis such as Col1 α 1^[43], transforming growth factor beta receptor II (TGF- β R II)^[44], SPRY2 (sprouty2, potent inhibitor of the MAPK pathway), hepatocyte nuclear factor 4 alpha (HNF4 α)^[45], Retinoid X receptor α (RXR α)^[46] and long MeCP2 transcripts^[16]. For example, several studies have shown that miR-29b is downregulated during HSC activation, its expression is negatively correlated with degree of liver fibrosis in humans and overexpression of miR-29b attenuated the increased expression of Col1 α 1 and Col1 α 2, α -SMA, discoidin domain receptor (DDR2), fibronectin-1 (FN1), integrin β 1 (ITGB1), and platelet-derived growth factor receptor- β (PDGFR- β) mRNAs, suppressed c-fos mRNA and inhibited HSC activation^[43,47,48].

Moreover, recent investigations have documented that miRNAs (such as miR-155) not only target multiple cellular pathways during hepatic fibrogenesis but can also be used to treat hepatitis C virus infection, an important underlying etiologic factor for liver fibrosis, as in case of miR-122^[49,50]. Studies showed that miR-155, which is down-regulated in activated HSC, directly bind to the 3'UTR of mRNAs of T cell factor 4 (TCF4) and angiotensin II receptor type 1 (AGTR1). TCF4 is transcriptional factor that promotes epithelial-mesenchymal transition (EMT),

an important process that contributes to liver fibrosis. AGTR1 contributes to fibroblast activation through enhancement of ERK1 signaling pathway in hepatic fibrosis^[50]. Serum miR-122 has been shown to serve as a biomarker of liver injury in chronic viral hepatitis, non-alcoholic fatty-liver disease, drug-induced liver disease^[49,51] and in carbon tetrachloride-induced mouse model of liver fibrosis^[52]. Because miR-122 has been shown to be an essential host factor for hepatitis C virus (HCV) infection, an antisense oligonucleotide that sequesters mature miR-122 was developed to inhibit its function^[49,53]. Indeed, a miR-122 inhibitor (miravirsin) was among the first miRNA-based molecules to enter phase 2 clinical trial for the treatment of chronic HCV infection^[53]. This extensive role of miRNAs constitutes novel targets for diagnostic biomarkers and molecular therapy^[38]. Comprehensive analysis of all reported miRNAs is beyond the scope of this editorial; however, a summary of most recently reported miRNAs involved in liver fibrosis is shown in Table 3.

In contrast to miRNAs, lncRNAs are mRNA-like transcripts ranging in length from 200 nucleotides to about 100 kilobases and act directly on genes. The primary function of lncRNAs is regulation of protein-coding gene expression of fundamental biological processes through diverse mechanisms. They have been associated with a spectrum of biological processes including epigenetics, alternative splicing, alternation of protein localization, precursors of small RNAs and miRNAs silencing (reviewed in refs^[40,54]).

Because the regulatory mechanisms of the lncRNAs are still being explored, their role in liver fibrosis is just being unfolded. Only recently, He *et al*^[55] showed that the levels of a lncRNA encoded by maternally expressed gene 3 (MEG3) were remarkably decreased in CCl₄-induced mouse liver fibrosis and human fibrotic livers. It has been shown previously that MEG3 was downregulated in liver cancer. The authors also showed that the expression of MEG3 was downregulated in human hepatic stellate cell lines (LX-2 cells) in response to TGF- β 1 stimulation in dose and time-dependent manner. Importantly, hypermethylation of MEG3 promoter was identified by methylation-specific PCR and MEG3 expression was robustly increased by the inhibition of methylation with either 5-azadC, or siRNA to DNA methyltransferase 1 (DNMT1) in TGF- β 1-induced HSC lines. Importantly, overexpression of MEG3 resulted in caspase-3-dependent apoptosis in TGF- β 1-treated LX-2 cells. These results suggest that lncRNA may play important role in the pathogenesis of liver fibrosis.

REVERSIBILITY OF LIVER FIBROSIS

It has been held for long time that liver fibrosis/cirrhosis is an irreversible process. This concept has been challenged with evidence that both advanced fibrosis/cirrhosis undergo at least partial resolution

Table 3 Summary of recent studies evaluating role of miRNA in liver fibrosis

miRNA	Function	Ref.
miR-15b/16	Downregulated in aHSCs. Restoring miR-16 and miR-15b reduced Bcl-2, and increased expression of caspases 3, 8, and 9 and aHSC apoptosis	Guo <i>et al</i> ^[87]
miR-19b	Downregulated in aHSCs and in human fibrotic liver. miR-19b binds to the 3'-UTR of TGF- β RIL. miR-19b mimic decreases expression of TGF- β RIL, collagen and α -SMA and increases quiescent characteristics	Lakner <i>et al</i> ^[44]
miR-21	Upregulated in PDGF-BB-aHSCs through PTEN/Akt pathway	Wei <i>et al</i> ^[88]
miR-21	Upregulated in cirrhotic patients and rats. Targets the 3'-UTR of SPRY2 and HNF4 α mRNAs in human and rat. Downregulating miR-21 suppressed ERK1 signaling, inhibited HSC activation, and blocked EMT in TGF β 1-treated hepatocytes	Zhao <i>et al</i> ^[45]
miR-27a/b	Upregulated in cultured-aHSCs. Downregulation of miR-27a/b induced quiescent HSC phenotype. Target Retinoid X receptor α (RXR α)	Ji <i>et al</i> ^[46]
miR-29a/b/c	Decreased expression in murine and human fibrotic livers. Serum expression negatively correlated with degree of liver fibrosis in humans	Roderburg <i>et al</i> ^[48]
miR-29b	miR-29b overexpression suppressed Col1 α 1 mRNA and protein	Ogawa <i>et al</i> ^[43]
miR-29b	Downregulated during HSC activation in primary culture. miR-29 overexpression attenuated the increased expression of Col1 α 1 and Col1 α 2, α -SMA, DDR2, FN1, ITGB1, and PDGFR- β mRNAs, inhibited HSC activation and suppressed c-fos mRNA	Sekiya <i>et al</i> ^[47]
miR-122	Downregulated in HCV-induced liver fibrosis. Expression inversely correlated with fibrosis stage	Morita <i>et al</i> ^[89]
miR-132	Downregulated in aHSCs. In quiescent HSCs miR-132 binds and represses MeCP2 transcripts that carry an extended 3'-UTR that includes the miR132 recognition motif. MeCP promotes methylation of H3K27 of PPAR γ through stimulation of EZH2	Mann <i>et al</i> ^[16]
miR-150, miR-194	Both are reduced in aHSCs. miR-150 targets c-myc and miR-194 targets rac-1. Both inhibit HSC activation and ECM production <i>via</i> inhibition of c-myc and rac-1	Venugopal <i>et al</i> ^[90]
miR-155	Decreased expression in aHSCs, sera and liver tissues of cirrhotic patients. MiR-155 interacts with 3'-UTR of TCF4 and AGTR1 mRNAs involved in EMT and ERK1 pathway, respectively	Dai <i>et al</i> ^[50]
miR-199/200	Expression correlated to progression of liver fibrosis	Murakami <i>et al</i> ^[91]
miR-214-5p	Upregulated in human and mouse fibrotic liver	Iizuka <i>et al</i> ^[92]
miR-221/222	Upregulated in human liver in a fibrosis progression-dependent manner	Ogawa <i>et al</i> ^[93]
miR-335	Downregulated during HSC activation. Restoring expression of miR-335 inhibited HSC migration and reduced α -SMA and collagen type I	Chen <i>et al</i> ^[94]

HSC: Hepatic stellate cell; 3'-UTR: 3'-Untranslated region.

following withdrawal of the injurious stimulus^[56]. Extensive experimental studies using murine model of liver cirrhosis^[32,57-60] and clinical data from advanced human cirrhosis^[61-63] showed that withdrawal of the etiological source of the chronic injury results in decrease of pro-inflammatory and fibrogenic cytokines, increased collagenase activity, decreased ECM production, and the apoptosis of aHSCs^[64-67]. Multiple epigenetic mechanisms have been shown to regulate the process of fibrosis progression and resolution as discussed above^[32].

Although partial resolution of liver fibrosis has been well documented, reversion to a complete normal liver architecture remains controversial^[68]. Since HSCs are the critical cells involved in liver fibrosis, researchers have investigated the fate of aHSCs that do not undergo apoptosis or become senescent after removal of liver injury^[69]. Troeger *et al*^[69] showed that gene expression of fibrosis markers, Col1 α 1 and TIMP-1 gradually decrease in a large proportion of HSCs during recovery from CCl₄-induced fibrosis, suggesting that HSCs in the recovering liver are undergoing deactivation. In order to determine whether deactivated HSCs have the same phenotypic biology as naïve HSCs that never been activated before, the authors compared fibrogenic responses between treatment-naïve quiescent HSCs and HSCs that had been isolated 45 d after the last CCl₄ treatment and undergone reversion to a quiescent phenotype. Both types of HSCs were exposed to

different but well-established fibrogenic stimuli including TGF β , PDGF, and 10% FBS for 48 h in order to exclude adaptations to previous CCl₄ repeated injury. They demonstrated that reverted HSC demonstrated a higher level of activation and profibrotic gene expression (Col1 α 1, α SMA) compared to treatment naïve, quiescent HSCs. This suggests that deactivated HSCs remain in a primed state of readiness and are able to respond more vigorously than quiescent stellate cells to further episodes of liver injury. This is consistent with an incomplete return of reverted HSCs to quiescence^[70].

In a similar study, investigators demonstrate that half of the genetically labeled aHSCs in CCl₄-induced liver fibrosis in mice escape apoptosis during regression of liver fibrosis, down-regulate fibrogenic genes, and acquire a phenotype similar to, but distinct from, quiescent HSCs in their ability to more rapidly reactivate into myofibroblasts in response to fibrogenic stimuli and strongly contribute to liver fibrosis. Inactivation of HSCs was shown to be associated with up-regulation of the anti-apoptotic genes Hspa1a/b, which participate in the survival of HSCs in culture and *in vivo*^[71].

These studies clearly demonstrate that there are existing mechanisms that are responsible for keeping reverted HSCs in incomplete quiescent state and are in a ready state for reactivation. Consequently several questions arise. What is the significance of incomplete resolution of liver fibrosis? Does incomplete

resolution of liver cirrhosis pose a specific risk for future activation or predisposition for tumorigenesis? Can pharmacologic intervention, including epigenetic modulation, enhance complete resolution of liver cirrhosis? Further research is required to understand the molecular mechanisms underlying incomplete reversibility of deactivated HSCs.

HERITABILITY OF EPIGENETIC PHENOTYPE

There is a growing body of literature suggesting that epigenetic disruptions (AKA epimutations)^[9] developed in response to environmental stimuli (*e.g.*, drugs, infections, *etc.*) can be passed on through cell divisions and generations^[72]. This process is termed “*epigenetic inheritance*” and has been defined as the inheritance of a phenotype in a manner that is independent of the DNA sequence and that remains self-perpetuating in the absence of the initial stimulus that caused the phenotype in the parental cell or organism. However, the detailed mechanisms by which this epigenetic information is transferred remain to be elucidated^[72].

Epimutations that occur in somatic cells can propagate to all daughter cells via mitosis, but they would be very unlikely to be transmitted to subsequent generations. However, if epimutations are sustained in cells of the germ line, the potential exists for these defects to be transmitted to subsequent generations, although it requires that such epimutations survive through the process of developmental reprogramming aimed at erasing (and hence correcting) the epigenetic memory of their parent-of-origin^[72,73].

More recently however, studies have indicated that DNA methylation associated with certain regions of the genome avoids the otherwise global reprogramming events^[74,75]. It has been known that sperm chromatin is protamine-dominant, and only a small amount of histones are retained. However, recent data, suggest that a small proportion of the sperm genome remains complexed with histones in a non-random manner such that certain genes and/or regions of the genome (*e.g.*, imprinted gene clusters and non-coding RNA clusters) are consistently associated with histones in all sperm cells. In addition, sperm-borne RNAs, including RNAs that are associated with the sperm plasma membrane and sperm chromatin, also represent an integral part of the sperm epigenome because these RNA molecules can be delivered into the oocyte during fertilization^[74-76]. Therefore, it is conceptually possible that environmentally-induced epimutations may be retained throughout development of the germ line and could therefore form the basis of transgenerational epigenetic inheritance^[73,77-79].

Epigenetic inheritance has been subdivided into multigenerational and transgenerational inheritance. While multigenerational means that an exposure that directly influences multiple generations, on the other

hand, transgenerational means that transmission between generations, but not involving direct exposure. Thus for the unequivocal transgenerational transmission of an adult onset disease phenotype through the germ-line requires assessment of the F3 generation for embryonic exposure, and F2 generation for postnatal exposure (reviewed in^[80]).

DNA methylation is the primary epigenetic mechanism involved in transgenerational transmission of epimutations. The evidence for the heritability of DNA methylation patterns is now well established because there is a well-defined molecular mechanism by which DNA methylation patterns are transmitted through DNA replication during either mitosis or meiosis^[73,81]. It has been shown that DNA methylation patterns are propagated through cell division via the semiconservative copying of the parental-strand methylation pattern onto the progeny DNA strand. This provides a way of passing epigenetic information between cell generations^[21,82].

In other epigenetic mechanisms such as patterns of histone modifications and ncRNA, there is anecdotal evidence that these marks are heritable through mitosis or meiosis, however no well-defined molecular mechanism has been delineated to explain the manner in which patterns of any of these epigenetic marks may be faithfully propagated through DNA replication^[31,83,84] (reviewed in^[72,73]).

More recently, it has been proposed that ncRNAs should have multiple molecular mechanisms to participate in environmental epigenetic transgenerational inheritance^[75]. The first mechanism by which ncRNAs can be regulated epigenetically is through methylation of gene from which ncRNA may originate causing repression of ncRNA expression. The second mechanism is by methylation-induced masking or disruption of the ncRNA recognition motif in target genes causing failure of binding of ncRNA to its target gene or mRNA. Alternatively, ncRNAs can indirectly regulate epigenetic states by affecting transcriptional or translational activity, or by affecting the stability of mRNAs encoding epigenetic factors. Additionally, single nucleotide polymorphisms in genes that encode miRNAs can affect their processing and target binding, along with cancer risk, response to treatment, and disease progression^[75].

HERITABILITY OF EPIGENETIC CHANGES IN LIVER FIBROSIS

Published reports on the inheritance, whether multigenerational or transgenerational, of epigenetic changes in liver fibrosis are rare. An important study investigating heritable multigenerational influence of liver injury on epigenetic pattern of liver fibrogenesis-related genes was reported recently^[12]. The authors induced transient liver injury in four groups of male rats whose fathers and grandfathers both, one of them, or neither had received the same liver injury,

thus creating four different ancestral exposures to liver injury. They showed that male rats whose father and grandfather both had liver injury had the least fibrosis, whereas those whose ancestors had no injury had the greatest fibrosis. The groups that only one of their ancestors had liver injury had intermediate grades of liver fibrosis. These differences were attributed to reduced PPAR γ promoter methylation but increased TGF- β 1 promoter methylation in the group with paternal history of fibrosis compared with the group that have no history of injury. They were also able to demonstrate that PPAR γ promoter methylation was reproduced in the sperm of untreated recipient rats when serum from injured rats was adoptively transferred into naive rats. However, attenuation from fibrosis was restricted to the liver, as the male offspring of treated rats had no difference in the amount of fibrosis in a kidney model of injury in contrast to what was observed in the liver damage model. Moreover, there were no differences in methylation of the PPAR γ promoter in the spleen, only in the liver^[12,85]. Given the fact that most DNA methylation is globally erased during spermatogenesis and again during embryonic development and thus these methylation marks may not be responsible for the fibrosis attenuation effect. The investigators reasoned that chromatin modifications that affect DNA methylation in the sperm may be implicated. They found that histone variant H2A.Z and H3K27 are incorporated into chromatin at the PPAR- γ promoter at increased levels from sperm of rats recovering from fibrosis relative to controls. Interestingly, they found the same effect when they subjected sperm from uninjured rats to repeated serum transfers from rats that had liver injury with CCL₄ for 4 wk. It has been reported that histone variant H2A.Z protects genes from DNA methylation^[86]. Nevertheless, the mechanistic association of these chromatin modifications with the adaptive phenotype in offspring and the nature of the serum soluble factor that influences the sperm epigenome remain to be determined^[12].

IMPLICATIONS FOR RESEARCH AND THERAPY: FINAL REMARKS

Liver fibrosis, if untreated, progresses to cirrhosis which predisposes to liver failure or hepatocellular carcinoma. Without liver transplantation, both conditions are fatal. Epigenetic mechanisms underlie the critical molecular changes that govern fibrogenesis and carcinogenesis and can be transmitted to daughter cells following mitosis. Experimental and clinical studies have shown that these epigenetic changes undergo reversion concomitantly with the resolution of liver fibrosis.

Nevertheless, studies have also revealed that resolution of liver fibrosis may be incomplete because a subset of the reverted HSCs, the principal fibrosis-

producing cell type in the liver, does not completely reach the quiescent status of the naive HSCs. This subset of incompletely reverted HSCs was found to readily be activated in response to recurring fibrogenic stimuli. The clinical implications of this finding have not yet been fully delineated. Does incomplete resolution of liver fibrosis predisposes the patient to an accelerated course of fibrogenesis once exposed to future liver injury? Or does incomplete resolution predisposes for the development of liver malignancy whether exposed to a new injurious agent or not?

The potential impact of multigenerational/transgenerational transmission of epimutations is very significant. A single exposure to a disruptive environmental influence has the potential to impact the function of cells, tissues and organs in descendants for multiple generations thereafter. It is therefore critical that an understanding of the manner in which multigenerational/transgenerational epimutations are incurred and subsequently transmitted is achieved in order to develop strategies to avoid or preclude their incurrence or transmission in the future^[73]. Moreover, there are key experimental gaps in our knowledge of the epigenetic mechanisms that regulate multigenerational transmission of fibrogenic signals such as the durability of epigenetic changes in the affected animal or across generations and the effect of gender on transmission. These and other unresolved questions require further investigations.

The challenge to decipher the epigenetic mechanisms that underlie both reversibility and heritability of liver fibrosis cannot be overemphasized. Fortunately, unlike genetic mutations, epigenetic mechanisms are reversible and can be modulated pharmacologically. Thus, researcher should seize this opportunity to develop effective therapies that not only completely reverse liver fibrosis but that also prevent the risk of future development of liver malignancy and that regulate heritability of fibrosis phenotype.

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