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In vivo Experimental Models for hepatotoxin Induced Fibrosis – A Toxicological View

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Abstract

Liver fibrosis is common sequel of chronic liver injury regardless of any etiology. The experimental animal models of hepatic fibrosis remain hallmark for the evaluation of antifibrotic agents prior to clinical studies. Accumulating evidence indicates that carbon tetrachloride, N-nitrosodimethylamine, thioacetamide, ethanol and bile duct ligation models are commonly employed and these models have been extensively studied for their potency of inducing hepatic fibrosis and for the evaluation of various antifibrotic agents in experimental animals. However, none of these experimental models reproduces exactly human liver fibrosis, each model has its own limitation and disadvantages i.e., development of ascites, extra hepatic toxicity, mortality etc. The development, progression and resolution of hepatic fibrosis induced by these hepatotoxins in experimental animals varying on the routes of their administration, frequency (intermittent/chronic) of administration, dose, species, and strain. Nevertheless, over past three decades knowledge gained from these hepatotoxin induced fibrosis models has advanced our understanding of the pathophysiology, molecular mechanisms and wide spectrum of signaling pathways associated with hepatic fibrosis. This article reviews some of the most studied hepatic fibrosis experimental models.

Keywords: Carbon Tetrachloride, Ethanol, Liver Fibrosis, Oxidative Stress, Thioacetamide

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1. Introduction

Hepatic fibrosis is characterized by the accumulation of extracellular matrix (ECM) proteins resulting from chronic liver injury (Lee *et al.*, 2015). The excess deposition of ECM disrupts the normal architecture of the liver resulting in pathophysiological damage to the organ. If left untreated, fibrosis can progress to cirrhosis, ultimately leading to organ failure and death. Reports suggesting that globally up to 45% of deaths can be attributed to chronic liver injury and fibrosis (Mormone *et al.*, 2011). There are several models have previously employed for the induction of hepatic fibrosis in experimental animals to understand the physiopathological mechanism of fibrosis and to evaluate the efficacy of synthetic antifibrotic drugs or plant derived principles. Over the past three decades, studies have identified a number of potentially important experimental fibrosis models by induction of carbon tetrachloride (CCl_4), *N*-nitrosodimethylamine (DMN), thioacetamide (TAA), ethanol (EtOH), etc. All of these models were extensively studied for the evaluation of anti-fibrotic efficacy of various hepatoprotective agents and herbal extracts. Hence, in this review we focused on well studied *in vivo* experimental models of hepatic fibrosis and their mechanism of fibrosis induction is discussed.

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2. *In vivo* Models for the Study of Hepatic Fibrosis

Several well-established profibrogenic agents have been identified by their ability to induce hepatic fibrogenesis *in vivo*. The most frequently used approach to induce toxin mediated experimental liver fibrosis are CCl_4, DMN, TAA, and EtOH in mice or rats and these are reported in the literature as classical models for the study of hepatic fibrosis. Several common experimental models of liver fibrosis were summarized (Table 1) and their advantages and disadvantages discussed hereunder.

2.1 Carbon Tetrachloride

Carbon tetrachloride (CCl_4; CAS No. 56-23-5) induced liver fibrosis has long been used as an animal model for liver fibrosis (Zhao *et al.,* 2014; Pérez, 1983). CCl4-induced liver fibrosis, biochemical alterations and histopathological changes resemble human liver cirrhosis (Jeong *et al.,* 2005). Hence, this model has been suggested to useful in studying the effects of hepatoprotective drugs with therapeutic potential to be used in humans (Zhao *et al.,* 2014). Several experimental studies have demonstrated that CCl_4 administration causes generation of highly reactive intermediate *i.e.,* CCl_3 by the microsomal

Table 1. Well studied hepatic fibrosis models in rodents

Model	Species	Dose	Duration	Fibrosis pattern	Reference
CCl_4 – i.p.	Mice	300 or 1000 µl/kg. bw.	Weekly twice for 12 weeks	Extensive centrilobular fibrosis with fibrotic septa and collagen accumulation	[4]
CCl_4 – i.p.	Rats	2 ml/kg (50% dissolved in olive oil)	Weekly twice for 10 weeks	Collagen accumulation and induction of profibrogenic cytokines	[5]
CCl_4 – i.p.	Rats	500 µl/100 gm. bw. (1:1 with olive oil)	Weekly twice for 6 weeks	Acidophilic degeneration, stretching perivascular fibrosis	[6]
CCl_4 – i.g.	Rats	0.08 ml/ml of olive oil	9 weeks	Extensive collagen deposition, portal bridging fibrosis	[7]
CCl_4 – p.o.	Mice	0.04 cc of a 40% CCl_4 solution (with olive oil)	Weekly thrice for 17 weeks	Extensive collagen deposition, pseudo lobular formation, bridging fibrosis (Ishak score-3-4)	[8]
DMN – i.p.	Rats	10 mg/kg. bw.	First 3 consecutive days of a week for 3 weeks	Collagen accumulation, bridging fibrosis and necrosis, micronodular cirrhosis	[9]
DMN – i.p.	Rats	10 mg/kg. bw.	3 consecutive days of a week 1, 2 or 4	Bridging fibrosis from central vein to portal triad or to another vein, central vein surrounded by collagen fibers	[10]
DMN – i.p.	Rats	10 µg/gm bw.	Once a day for 3 consecutive days in a week for 4 or 6 weeks	Knodell scores increased due to peri-portal or portal-portal fibrotic septa.	[11]
TAA – p.o.	Rats	0.03% in distilled water	daily for 12 weeks	Proliferation of bile duct, accumulation of collagen bundles around lobules results in huge fibrotic septa	[12]
TAA – i.p.	Rats	350 mg/kg. bw.	3 consecutive days/week for 12 weeks	Deposition of fibrous tissue in portal areas leading to formation of fibrotic septa	[13]
TAA – i.p.	Rats	200 mg/kg	Weekly twice for 6 weeks	Accumulation of collagen bundles and large fibrous septa	[14]
TAA–i.p.	Rats	200 mg/kg. bw.	12 weeks	High levels of collagen deposition and bridging fibrosis.	[15]
Dietary	Rats	2 g cholesterol, 0.5 g cholic acid, Corn oil (5 ml/kg. bw, 15% fructose solution + Cl_4 micro dose (0.5 ml/kg. bw)	8 weeks	Steatosis, steatohepatitis and ibrosis	[16]

enzyme CYP2E1 during its biotransformation in liver. This reactive intermediate is reported to responsible for the paranchymal cell damage and activation of hepatic stellate cells (HSCs) leading to accumulation of ECM and fibrosis (Fu *et al.,* 2008; Burk *et al.,* 1984). When this intermittent CCl_4 insult is repeated, successive rounds of wound healing occur prior to resolution of the previous one resulting in fibrosis accumulation.

In CCl_4 models, evolution of liver injury starts progressively from the first week of administration with significant fibrosis after 2-4 weeks, severe bridging fibrosis after 5-7 weeks, and cirrhosis from 8th week onwards (Constandinou *et al.,* 2005). Therefore, several studies were reported with 6-7 weeks treatment regimen of CCl_4 administration (1 or 2 ml/kg, b.w., twice a week) for the development pathological alterations such as formation of large fibrosis septa, pseudolobe separation and collagen accumulation. These lesions are considered as the hallmarks of hepatic fibrosis (Ma *et al.,* 2015). Extensive literature survey reveal that several studies employed administration of CCl_4 at 2 ml/kg., b.w., twice a week for 6 or 7 weeks period to induce significant hepatic fibrosis in rats or in mice. In such studies, fibrotic changes (early fibrosis) were observed between second and third week after CCl_4 administration. While early central fibrosis and central fibrosis with centro-central fibrosis bridges were observed after 3 and 4 weeks respectively (Ma *et al.,* 2015; Clichici *et al.,* 2015).

It has been suggested that CCl_4-induced fibrosis is good tool to predict a "true" antifibrotic drug effect (Schuppan *et al.,* 2003). Therefore, CCl_4 has employed as an experimental tool to induce hepatic fibrosis for the evaluation of antifibrotic properties of drugs and antioxidant plant principles such as curcumin (Zhao *et al.,* 2014), silymarin (Tsai *et al.,* 2008), silibinin (Muriel *et al.,* 2005), resveratrol (Chávez et al., 2008), morin (Heeba and Mahmoud, 2014), and synthetic antioxidants such as N-acetylcysteine (Galicia-Moreno *et al.,* 2009), melatonin (San-Miguel *et al.,* 2015). Interestingly, most of these studies were reported that CCl_4 minced either in corn or olive oil and administered intraperitoneally. On the contrary, it also been reported previously that olive oil itself posses the protective ability against the CCl_4-induced hepatic fibrosis in rats (Fang *et al.,* 2008). Hence, in future care should be taken on these grounds. In addition to fibrotic lesions, CCl_4 also has an ability to induce micro or macro vesicular hepatic steatosis (Fujii *et al.,* 2010). CCl_4-induced mortality and weight loss during its administration also been reported. To minimize mortality, subsequent doses given were dependent on the amount of weight gained or lost by individual rats during the previous week (Rivera *et al.,* 2001).

2.2 *N*-nitrosodimethylamine (DMN)

N-nitrosodimethylamine ($C_2H_6N_2O$; CAS No. 62-75-9) is a prominent member of nitrosamine family that has been demonstrated as a potent hepatotoxin, carcinogen and mutagen (Magee, 1956). Intermittent DMN administration has been shown to induce necrosis and bridging fibrosis in the liver tissue of rats associated with the collapse of parenchymal framework of the liver (Ezhilarasan *et al.,* 2012; Ezhilarasan and Karthikeyan, 2016) and their lesions are said to resemble the features of human liver fibrosis (Jézéquel *et al.,* 1987). Accumulating evidence have confirm that intermittent intraperitoneal (i.p.) injection at 10 mg/kg of DMN for the first three days of a week over the period of 3 to 4 weeks were frequently used model to induce fibrosis in rats, the mechanism of hepatic fibrosis induction was associated with activation of HSCs, Kupffer cells and expression of profibrotic cytokines (Ezhilarasan *et al.,* 2012; George, 2003; Vendemiale *et al.,* 2001).

The microsomal enzyme CYP2E1 is said to responsible for biological activation and degradation of DMN. During DMN biotransformation in liver produces formaldehyde, methanol and alkylating intermediates that react with tissue macromolecules such as nucleic acids and proteins to form methylated macromolecules (Kang *et al.,* 2007). The generation of such reactive intermediates in liver has been implicated in the activation of HSCs and progression of hepatic fibrosis. Liedtke *et al.* suggest that DMN has the capability of inducing profibrotic mechanism and also has strong mutagenesis and carcinogenesis inducing properties. Hence, the analysis of underlying profibrotic mechanisms in this experimental model could be more complex because of overlapping or even mutated signaling pathways (Liedtke *et al.,* 2013). However, repetitive low-dose administration of DMN has been shown to be a good reproducible model to induce experimental fibrosis in rats. It causes an initial central hemorrhagic necrosis, with rapid formation of septa, fully established ascites (usually starts during the second week of DMN administration), fibrosis and micronodular cirrhosis three weeks after its

administration in rats and hence this model is quite often advocated to induce hepatic fibrosis (Ezhilarasan *et al.,* 2012; Ezhilarasan and Karthikeyan, 2016).

The hepatic fibrosis induced by DMN in rats has been widely used for evaluation on molecular mechanisms of fibrosis and cirrhosis and for investigation of anti-fibrotic properties of drugs and herbal extracts (Chen *et al.,* 2012; Hu and Liu, 2006). In very recent studies, DMN used as an experimental tool to induce hepatic fibrosis for the evaluation of antifibrotic efficacy of various plant derived hepatoprotective principles such as black tea extracts (Weerawatanakorn *et al.,* 2015), resveratrol (Ahmad and Ahmad, 2014), hesperidin (Elshazly and Mahmoud, 2014), puerarin (Xu *et al.,* 2013), sinapic acid (Shin *et al.,* 2013), silibinin Ezhilarasan and Karthikeyan, 2016), salvianolic Acid B (Li *et al.,* 2012), in experimental animals. Due to the potent toxic nature of DMN, various pathological alterations apart from fibrosis have also been documented with this model, particularly mortality, ascites, induction of patchy, centrilobular and piecemeal necrosis, micro or macrovesicular cirrhosis are the most common disadvantages associated with this model (Ezhilarasan and Karthikeyan, 2016). Despite its toxic potential this model is being used till date, as a profibrogenic agent in the recent experimental studies (Ezhilarasan *et al.,* 2012; Weerawatanakorn *et al.,* 2015; Ahmad and Ahmad, 2014; Elshazly and Mahmoud, 2014; Xu *et al.,* 2013; Shin *et al.,* 2013; Li *et al.,* 2012).

2.3 Thioacetamide (TAA)

Thioacetamide (CH_3CSNH_2; CAS No. 62-55-5) is a well-known hepatotoxin. In addition to DMN and CCl_4, TAA also widely used model for the induction of liver fibrosis in experimental animals. TAA commonly administered either in drinking water or through i.p. injections (Heindryckx *et al.,* 2009). Studies reveal that TAA itself is not hepatotoxic, however, its reactive metabolites covalently bind to proteins and lipids thereby causing oxidative stress and hepatic fibrosis (Koen *et al.,* 2013; Staňková *et al.,* 2010). During biotransformation in liver TAA converted to TAA-S oxide subsequently transformed into thioacetamide S, S-dioxide by the mixed function oxidase enzymes with or without being further oxidized to form reactive intermediates that exert their toxic effect on liver fibrosis (Spira and Raw, 2000).

Thioacetamide-induced liver fibrosis in various mouse strains with wide variations in the routes of administration, doses and duration of treatment has been reported. Repeated administration of TAA, over a period of 10-15 weeks results in the progression of significant fibrosis in mice (Salguero *et al.,* 2008). Administration of TAA at 100-200 mg/kg, i.p., and 200 mg/l in drinking water, p.o., induced hepatic fibrosis in experimental animals and their lesions are said to closely resemble alcoholic fibrogenesis (Salguero *et al.,* 2008; Wasmuth *et al.,* 2009; Schnur *et al.,* 2004). Further, this model has high reproducible results with low mortality and less toxic than DMN and CCl4-induced fibrosis models. When compared with CCl_4 model, TAA treatment induces pronounced periportal infiltration and ductal proliferation. Main disadvantage reported include long latency of fibrogenic effects and the development of cholangiocarcinoma, cirrhosis and hepatocellular carcinoma have also been described in rats after long time exposure to TAA (Spira *et al.,* 2002).

2.4 Ethanol (EtOH)

Chronic ethanol (C_2H_5-OH; CAS No. 64-17-5) consumption is known to accelerate the progression of liver fibrosis. Several animal models of ethanol-induced hepatotoxicity have been established previously for the study of hepatic fibrosis. Among these Lieber-DeCarli model of liquid diet is one of the standard, commonly employed for the study of alcohol-induced hepatic fibrosis in experimental animals. In this model, alcohol isocalorically substitutes for carbohydrates up to 50%. In rats and mice the maximum ethanol intake is gradually attained is more than 30% of the caloric intake. However, the development of micro or macrovesicular steatosis is common disadvantage associated with models Lieber and DeCarli, 1989; Lieber and DeCarli, 1994).

In clinical studies, alcohol consumption has been shown to increase apoptosis of hepatocytes, oxidative stress and hepatic fibrosis in patients with chronic liver diseases (Rigamonti *et al.,* 2003). The apoptotic bodies from injured hepatocytes and reactive intermediates have been implicated in the activation of HSCs and fibrosis. Moreover, studies have demonstrated the role of acetaldehyde in alcohol induced hepatic fibrosis. These studies confirm the role acetaldehyde in the induction of type I collagen synthesis in HSCs not in hepatocyte (Casini *et al.,* 1991; Moshage *et al.,* 1990). The acetaldehyde has been reported to originate from the hepatocytes since alcohol dehydrogenase is not detected in HSCs. It is likely that acetaldehyde originates in hepatocytes and enters stellate cells in a paracrine fashion

(Fontana *et al.,* 1997). The pivotal role of profibrogenic cytokine *i.e.,* transforming growth factor-β (TGF-β) in alcohol-induced hepatic fibrosis also reported previously (Meyer *et al.,* 2010). Thus the acetaldehyde, the primary metabolite of ethanol, can induce the transcription of type I collagen directly as well as indirectly by upregulating the synthesis TGF-β. Generation of reactive oxygen species in hepatocytes due to ethanol biotransformation is said to responsible for the activation of HSCs and production of ECM during the progression of hepatic fibrosis (Figure 1).

2.5 Dietary Models

Diets high in saturated fat and fructose have been implicated in the development of obesity and non-alcoholic steatohepatitis (NASH) in humans. This strategy has been applied in the experimental animal for the development of NASH and hepatic fibrosis. These dietary models would be appropriate for the study of NASH-associated hepatic fibrosis. Charlton *et al.* recently reported the fast food diet mouse model (FFD) of NASH with micro doses of CCl_4 which recapitulates the

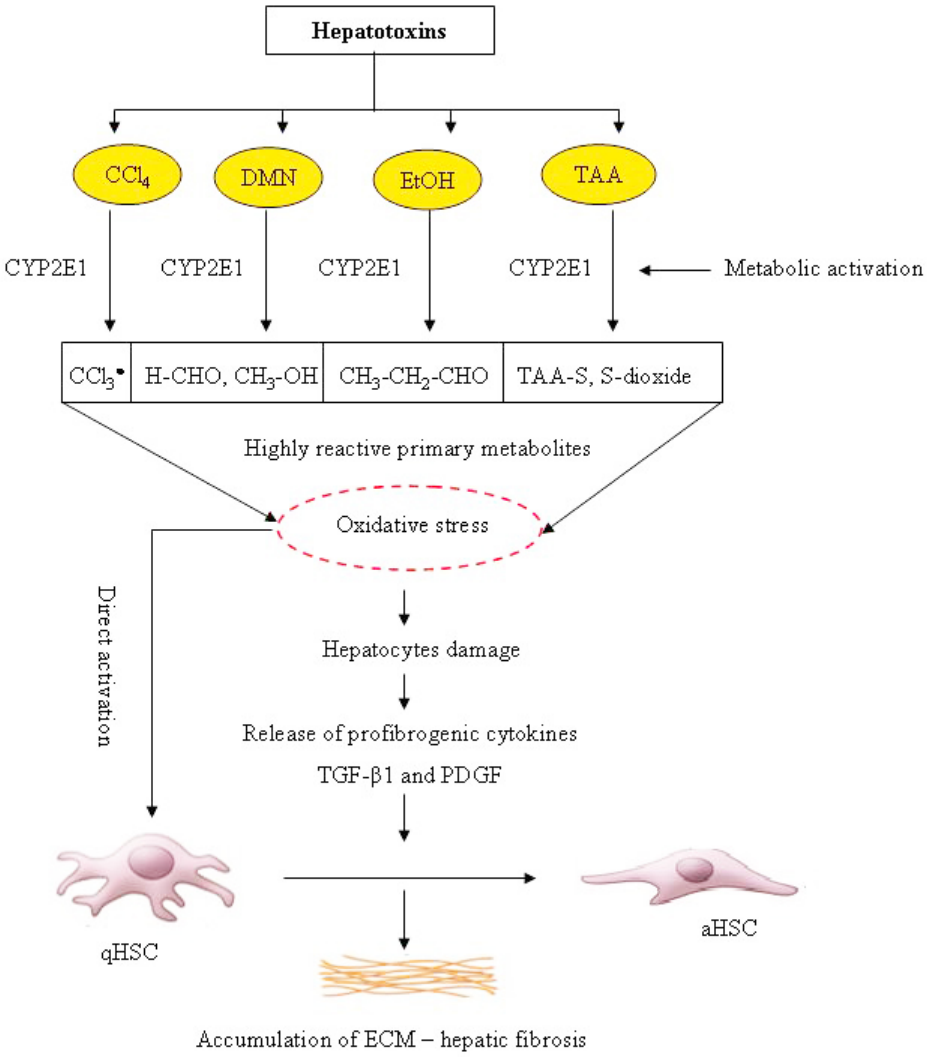


Figure 1. Schematic depiction of hepatic fibrosis induced by the profibrogenic agents.
CCl_4-carbontetrachloride, DAMN-dimethylnitrosamine; EtOH-ethanol; TAA-thiocetamide; MFO-mixed function oxidases; CCl_3-carbontrichloride radical; H-CHO-formaldihyde; CH_3-OH-methanol; CH_3-CH_2-CHO-acetaldehyde; TAA-S,S-dioxide - thioacetamide S, S-dioxide; TGF-β 1; PDGF-platelet derived growth factor; qHSC and aHSC-quiescent and activated hepatic stellate cells respectively; ECM-extracellaler matrix.

features of NASH with progressive fibrosis (Charlton *et al.,* 2011). The FFD comprise of cholesterol, cholic acid, corn oil and 15% fructose. In a recent study with this model observed the development of non-alcoholic fatty liver disease (NAFLD) histological features including steatosis, inflammation and extensive fibrosis in 8 weeks suggest that this model can be used for the development of NAFLD therapeutics and liver anti-fibrotics (Chheda *et al.,* 2014).

2.6 D-galactosamine

Studies are also documented D-galactosamine-induced fibrosis models. In a rat model, portal fibrosis and bile duct proliferation has been described after the administration of D-galactosamine-induced at a dose of 300 mg/kg., i.p., once in a week for 2 weeks (Kume *et al.,* 2006). Multiple injections of D-galactosamine induce liver fibrosis is also reported, in such studies, rats were administered 10, 20, 30, 40, 60, 80, 100 and 140 i.p. injections of D-galactosamine (500 mg/kg b.w., three times/week). The paradigm of fibrosis include expansion of portal tracts, prominent bile ductular proliferation and gradual formation of fibrous septa with deposition of ECM proteins such as fibronectin, collagen types I, III, and IV and laminin were noticed after 20 to 40 injections (Jonker *et al.,* 1994). However, this fibrosis model was less studied when compared to other models.

3. Selection of an in vivo experimental fibrosis model

The pathogenesis and evolution of chronic liver injury vary according to multiple factors such as etiologic agent, duration and frequency of administration (continuous/intermittent), animal model, strain, dose, mortality induction and various other pathophysiological alterations induced by the toxin other than hepatic fibrosis. Generally, the selection of an experimental model should appropriate for the intention of the particular study. For instance, for the study of oxidative stress induced necrosis and fibrosis one can opt for DMN or CCl_4-induced models While, for the study of secondary biliary fibrosis, the bile duct ligation method would be appropriate. However, the classical hepatic fibrosis experimental model is selected based on its high reproducibility, low induction of extra-hepatic toxicity, mortality and biochemical and histopathological patterns that resemble human hepatic fibrosis.

4. Future Directions

Currently, there are several well established experimental animal models are available for the study of hepatic fibrosis that are induced by hepatotoxins. In addition to these models, diet based hepatotoxin induced fibrosis models have also gained attention recently for the study of NASH associated hepatic fibrosis and transgenic mouse models also used for the study of hepatic fibrosis, these models offer to study the specific molecular signaling and mechanism associated with hepatic fibrosis in a clear and defined manner. For instance, several transgenic mice were developed for such studies especially TGF-β and PDGF transgenic mice, used for the study of hepatic fibrosis. While, *in vitro* models also serve as an excellent tools to evaluate and understand molecular pathogenesis of hepatic fibrosis, which permit high-throughput testing, cost effective and yield high reproducible results. Accordingly, human primary HSCs, LX-2 cells (human HSC cell line), rat primary HSCs and rat HSCs cell line etc., are recommended for the studies of hepatic fibrosis *in vitro*.

5. Conclusion

Overwhelming evidence indicate the fact that over the past 3 decades numerous models have been employed for their ability to induce hepatic fibrosis in experimental animals. Undoubtedly, intermittent or chronic administration of these hepatotoxins plays significant role in the development of oxidative stress consequently, progression of hepatic fibrosis. All these models suggested for manifesting characteristic features of pathology of human hepatic fibrosis. Hence, in this context, several studies were used these toxins as a profibrogenic agents aimed to evaluate the antioxidant and anti-fibrotic properties of drugs and came out with promising results. However, none of these experimental models reproduces exactly human liver fibrosis, each model has its own limitation and disadvantages. Certainly, the development of hepatic fibrosis in experimental animals may vary depending on

the routes of drug/toxicant administration, frequency (intermittent/chronic) of administration, dose, species, and strain. Nevertheless, knowledge gained from these models has greatly advanced our understanding of the molecular mechanisms and various signaling pathways associated with hepatic fibrosis.

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