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REVIEW

In Vivo and In Vitro Models to Study Liver Fibrosis: Mechanisms and Limitations

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SUMMARY

Because the development and progression of liver fibrosis differ based on the etiology, it is important to select an appropriate liver fibrosis model according to the purpose of study and type of disease. To study liver fibrosis, many in vivo animal and in vitro models have been developed. This review summarizes and analyzes the various in vivo and in vitro liver fibrosis models and their implications and limitations.

Liver fibrosis is a common result of liver injury owing to various kinds of chronic liver diseases. A deeper understanding of the pathophysiology of liver fibrosis and identifying potential therapeutic targets of liver fibrosis is important because liver fibrosis may progress to advanced liver diseases, such as cirrhosis and hepatocellular carcinoma. Despite numerous studies, the underlying mechanisms of liver fibrosis remain unclear. Mechanisms of the development and progression of liver fibrosis differ according to etiologies. Therefore, appropriate liver fibrosis models should be selected according to the purpose of the study and the type of underlying disease. Many in vivo animal and in vitro models have been developed to study liver fibrosis. However, there are no perfect preclinical models for liver fibrosis. In this review, we summarize the current in vivo and in vitro models for studying liver fibrosis and highlight emerging in vitro models, including organoids and liver-on-a-chip models. In addition, we discuss the mechanisms and limitations of each model. ***(Cell Mol Gastroenterol Hepatol 2023;16:355–367; https://doi.org/10.1016/j.jcmgh.2023.05.010)***

Keywords: Liver Fibrosis; In Vivo; In Vitro; Experimental Model.

Liver fibrosis is fibrous scar formation by extracellular matrix (ECM) accumulation resulting from chronic liver inflammation caused by conditions including chronic viral hepatitis B and C, autoimmune hepatitis, alcoholic liver disease (ALD), primary biliary cholangitis, primary sclerosing cholangitis (PSC), and nonalcoholic fatty liver disease (NAFLD).[1,2] Liver fibrosis may progress to cirrhosis and further to hepatocellular carcinoma (HCC).[3] Treating underlying liver disease may ameliorate liver fibrosis, even at the cirrhosis stage.[4,5] Although many targets for liver fibrosis were investigated, effective medications have not been developed.[6] Therefore, exploring the precise pathophysiologies of liver fibrosis is crucial for better understanding and for discovering new therapeutic targets. Various animal models are used in preclinical studies on liver fibrosis. Because no model is perfect, the selection of relevant animal models to study target fibrotic diseases is crucial. A better understanding of the pathogenesis of each animal model and its implications and limitations is essential. In this review, we summarized the currently available in vivo and in vitro models for studying liver fibrosis and discussed emerging in vitro models.

In Vivo Models of Experimental Liver Fibrosis

In vivo animal models are the gold standard in studying liver fibrosis. The main effectors that produce ECM in liver fibrosis are activated hepatic stellate cells (HSCs). Various types of liver cells are involved in HSC activation in liver fibrosis. These cells include immune cells (monocyte-derived macrophages, Kupffer cells, B cells, and T cells), cholangiocytes, liver sinusoidal endothelial cells (LSECs), and hepatocytes. These cells produce inflammatory and fibrotic cytokines (transforming growth factor-β [TGF-β], platelet-derived growth factor [PDGF], connective tissue growth factor [CTGF], interleukin 1β, and C-C motif chemokine ligand 2) and mediators (reactive oxygen species and nitric oxide) to affect HSC activation (Figure 1*A*). Because various cell types are involved in HSC activation and fibrosis, simple in vitro cell culture models cannot entirely recapitulate the disease course of liver fibrosis. To study different etiologies of liver fibrosis, hepatotoxin, cholestasis, and nonalcoholic

Abbreviations used in this paper: **ALD, alcoholic liver disease; CCl_4, carbon tetrachloride; CDAA, choline-deficient L-amino acid-defined; CD-HFD, choline-deficient high-fat diet; CYP2E1, cytochrome P450 2E1; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DMN, dimethylnitrosamine; ECM, extracellular matrix; HCC, hepatocellular carcinoma; HFD, high-fat diet; HSC, hepatic stellate cell; IL, interleukin; iPSC, induced pluripotent stem cell; LSEC, liver sinusoidal endothelial cell; MCD, methionine- and choline-deficient; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PCLS, precision-cut liver slices; PDGF, platelet-derived growth factor; PSC, primary sclerosing cholangitis; scRNA-seq, single-cell RNA sequencing; snRNA-seq, single nuclear RNA sequencing; TAA, thioacetamide; TGF-β, transforming growth factor-β; 3D, 3-dimensional.**

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steatohepatitis (NASH)-induced liver fibrosis models are commonly used (Table 1), and each model shows different patterns of fibrosis (Figure 1*B*).

Hepatotoxin-Induced Liver Fibrosis Models

Carbon tetrachloride. Carbon tetrachloride (CCl_4) is the most popular compound used for rodent liver fibrosis models. CCl_4 is metabolized to trichloromethyl radical and trichloromethyl peroxide by cytochrome P450 2E1 (CYP2E1), directly injuring hepatocytes and endothelial cells.[30–33] CCl_4 activates Kupffer cells and HSCs that induce liver fibrosis. Because CYP2E1 is expressed predominantly in pericentral zone 3 hepatocytes, hepatotoxic CCl_4 metabolites are generated and induce hepatocyte injury and ECM deposition in the pericentral area. Liver injury caused by a single injection of CCl_4 is recovered quickly. To maintain this injury and ECM deposition, repeated injections are required. The researchers should be aware that CCl_4 can cause mortality in mice and that the animal should weigh at least 20 g before initiating CCl_4 injection. In CCl_4-induced liver fibrosis, serial processes and mechanisms contribute to repeated hepatocyte injury, inflammation, and ECM production, followed by resolution and hepatocyte proliferation, making data interpretation complicated. Although CCl_4 is not used currently in daily life, it was used as a solvent in industries and is distributed in nature. Because CCl_4 injures hepatocytes, this model may be proposed to study the mechanism of hepatocyte injury–induced liver fibrosis in chronic hepatitis B and C. However, hepatocyte injury in the CCl_4 model does not persist, and, instead, pericentral and central–central bridging fibrosis occurs. This finding does not match the pathogenesis of chronic hepatitis B and C, and oral and intraperitoneal administration of CCl_4 are common; however, an inhalational model develops cirrhosis and ascites. The inhalation model is useful for studying end-stage liver fibrosis.[34] Notably, CCl_4 treatment cessation rapidly inactivates HSCs, which are associated with fibrosis regression.[35] Activated HSCs express collagen Type I Alpha 1 Chain, α-smooth muscle actin, and tissue inhibitor of metalloproteinase 1. HSCs reexpress their quiescent markers glial fibrillary acidic protein, peroxisome proliferator-activated receptor gamma, and bone morphogenic protein and activin membrane-bound inhibitor after resolution. Gamma-aminobutyric acid type A receptor subunit alpha3 can be used as an inactivated HSC marker.[35,36] Thus, the CCl_4 model is a useful model for studying the resolution mechanism. Given its high reproducibility, many researchers use this model as a primary model to study liver fibrosis.

Thioacetamide. Thioacetamide (TAA) is the second most commonly used fibrosis-inducing hepatotoxin in rodents.[37] TAA itself is nontoxic, but its metabolites, TAA sulfoxide and TAA sulfdioxide, converted by CYP2E1, are hepatotoxic.[38] Toxic metabolites induce oxidative stress for centrilobular necrosis and inflammation, thus activating HSCs and inducing fibrosis.[39] The TAA model promotes hepatocyte damage in zones 1 and 3, and develop portal–portal and portal–central bridging fibrosis, respectively. Hepatocyte injury is progressive and persistent. Fibrosis regression is lesser after TAA discontinuation than that after CCl_4 discontinuation. This model is suitable for studying fibrosis regression by treatment, but inappropriate for studying spontaneous regression. TAA administration through drinking water induces continuous liver damage, which may mimic human chronic hepatitis B and C better than the CCl_4 model.

Dimethylnitrosamine. Dimethylnitrosamine (DMN) is a nitrosamine, a known carcinogen, which develops liver fibrosis in rodents and is used commonly in Asian laboratories for preclinical fibrosis studies. DMN induces iron deposition, fat accumulation, centrilobular congestion, and hemorrhagic necrosis.[40,41] Fibrosis progression induces porta–portal and portal–central bridging fibrosis by enhancing collagen cross-linking, in which type III collagen is dominant compared with type I.[42] The DMN model develops severe fibrosis and displays increased expression of α-smooth muscle actin,[43] making it useful in studying advanced fibrosis mechanism.

NASH-Induced Fibrosis

NAFLD is becoming a major cause of chronic liver disease.[44] NASH develops in 20%–25% of NAFLD patients. Patients with NASH may develop fibrosis, and some of them progress to cirrhosis.[45] Because fibrosis is the most important prognostic factor,[46,47] a preclinical NAFLD-fibrosis model is crucial for NAFLD research. Several NAFLD rodent models are available; however, each model has limitations.

A high-fat diet (HFD) containing 40%–60% of fat calories is suitable for studying obesity, insulin resistance, and simple steatosis. Because saturated fat promotes NASH progression better than unsaturated fat,[48] a HFD mainly contains saturated fat as the main source.[49] Although a HFD increases serum alanine aminotransferase levels and inflammatory gene expression after 2–6 months of feeding, it requires approximately 50 weeks to develop mild fibrosis.[50] Modified HFD models can be used. Fructose, which is enriched in sodas and candies, increases hepatic de novo lipogenesis and inhibits fatty acid β-oxidation.[51] Administration of a HFD and fructose-/glucose-containing drinking water for 4–6 months induces steatosis, necroinflammation, insulin resistance, and fibrosis.[52,53] A HFD supplemented with 0.2%–2% cholesterol also is used because it promotes inflammation and fibrosis.[54–56] The feeding of a HFD supplemented with fructose and cholesterol for 6 months induces NASH with hepatocellular ballooning, progressive fibrosis, and features of the metabolic syndrome.[57] A recent systematic review showed that a HFD with a high-fructose model resembles human NAFLD.[58] A hybrid model using a HFD and CCl_4 is another option to study NASH fibrosis. CCl_4 treatment may not be relevant to human NASH unless to study the interaction with environmental toxins. The transcriptomic analysis of this model showed similar gene expression patterns to human NASH.[59] Thus, this model is a relevant preclinical model for NASH studies. A Western diet with high sugar water and low-dose CCl_4 rapidly developed advanced fibrosis (12 weeks) and HCC (24 weeks).[59]

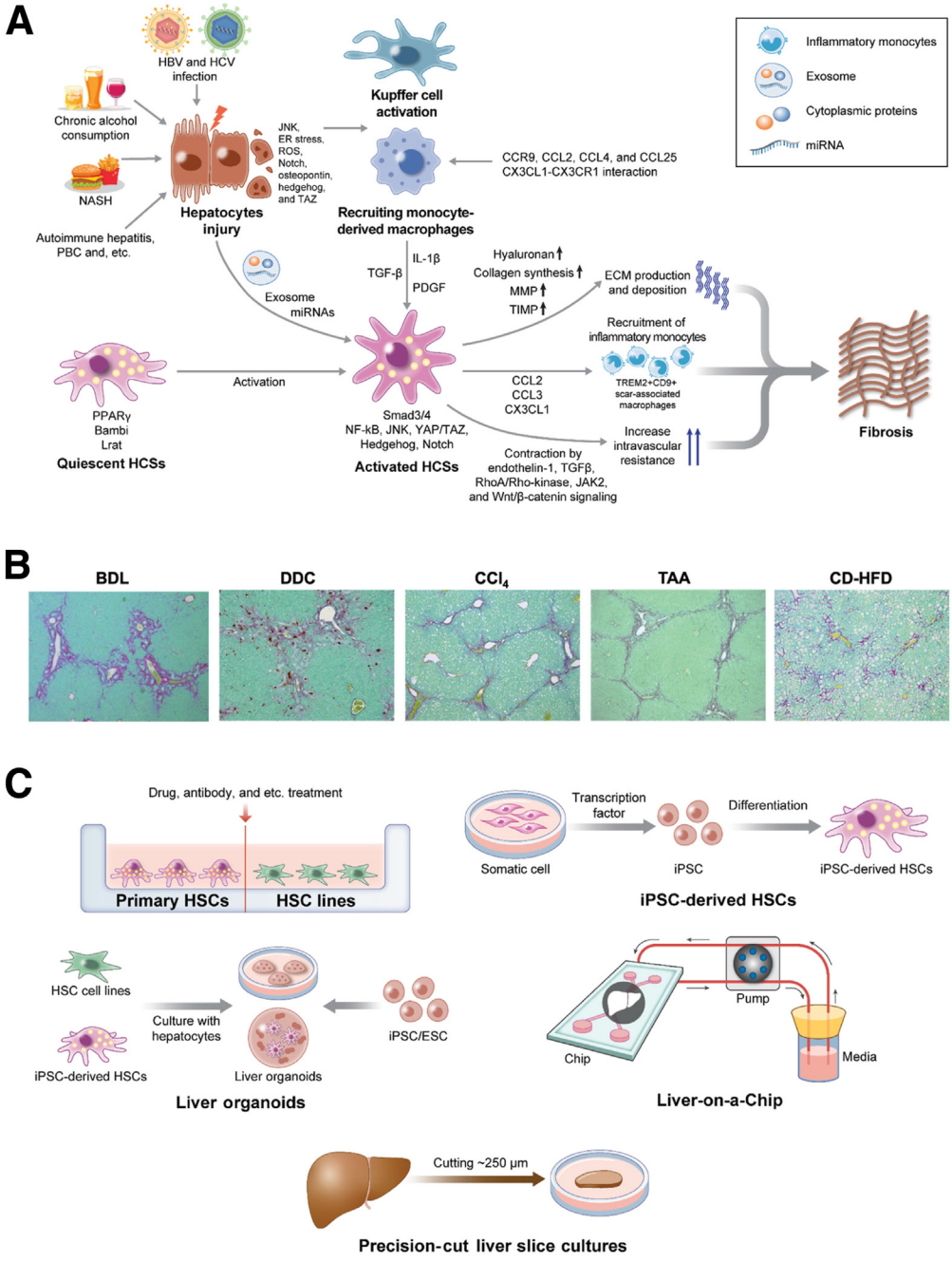

Figure 1. Mechanism of liver fibrosis and in vivo/in vitro models for styding liver fibrosis. (*A*) Molecular mechanism of liver fibrosis. By chronic liver injury, hepatocytes activate the signaling related to Janus kinase (JNK), Notch, osteopontin, hedgehog, and TAZ,[7–9] and produce exosomes containing microRNAs (miRNAs) for HSC activation.[10] Inflammation activates Kupffer cells,[11] and recruits monocyte-derived macrophages via C-C motif chemokine receptor (CCR)9, C-C motif chemokine ligand (CCL)2, CCL4, and CCL25.[12–15] The C-X3-C motif chemokine ligand 1 (CX3CL1)–C-X3-C motif chemokine receptor 1 (CX3CR1) interaction regulates macrophage survival, differentiation, and polarization.[16–18] Hepatic macrophages activate HSCs by producing TGF-β, PDGF, and interleukin (IL)1β.[19] Quiescent HSCs store vitamin A–containing lipid droplets.[20] Activated HSCs produce collagens and other ECM and express inflammatory chemokines CCL2, CCL3, and CX3CL1 to recruit inflammatory monocytes.[21,22–25] HSC-derived matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) contribute to ECM maintenance, remodeling, and fibrosis.[26] Activated HSCs contribute to contractile force in sinusoids to increase intravascular resistance, leading to portal hypertension, which is influenced by endothelin-1, TGF-β, RhoA/Rho-kinase, JAK2, and Wnt/β-catenin signaling.[27–29] (*B*) Representative histology of liver fibrosis models. Sirius red staining for bile duct ligation (BDL), DDC, CCl_4, TAA, and CD-HFD models. (*C*) Summary of in vitro model for studying liver fibrosis. Primary HSC. HSC line. iPSC. Liver organoid. Liver-on-a-Chip. Precision-cut liver slice cultures. ER, endoplasmic reticulum; HBV, hepatitis B virus; HCV, hepatitis C virus; HSC, hepatic stellate cell; NF-κB, nuclear factor-κB; PBC, primary biliary cholangitis; ROS, reactive oxygen species.

Table 1. In Vivo Animal Models of Liver Fibrosis

Models	Methods	Duration	Advantages	Limitations	References
CCl_4	IP injection Inhalation	4–12 wk	Simplicity (IP) High reproducibility, induction of portal hypertension (inhalation)	High toxicity Risk of peritonitis (IP injection) Requirement of special equipment (inhalation)	11–13
TAA	IP injection Oral administration	6–8 wk (IP injection) 2–4 mo (oral)	Simplicity High reproducibility	Long time to induce liver fibrosis Highly toxic	15, 16
DMN	IP injection	4–8 wk	Simplicity Inducing significant fibrosis	More suitable to study HCC Risk of carcinogenesis in researcher	17, 18
HFD	Feeding	2–6 mo (for fatty liver) 50 wk (for fibrosis)	Inducing obesity and insulin resistance	Long time to induce liver fibrosis	27
HFD with glucose/fructose water ± cholesterol	Feeding	16 wk to 12 mo	Significant feature of metabolic syndrome	Long time to induce liver fibrosis	29, 30, 32, 33
MCD diet	Feeding	5–8 wk	Short time to induce fibrosis	Reduces body weight No feature of metabolic syndrome	38, 39
CDAA diet	Feeding	More than 20 wk	Body weight gain with insulin resistance Useful to study NASH-induced HCC	Hindrance of studying liver fibrosis by HCC development	40
CD-HFD	Feeding	6–24 wk	Human-relevant NASH fibrosis	Long time to induce liver fibrosis	41, 43, 46, 47
CDA-HFD	Feeding	12 wk	NASH with severe fibrosis with short duration	No feature of obesity or insulin resistance	49
BDL	Surgery	3 wk	Relevant to cholestasis Useful to study relationship between gut microbiome and fibrosis	High surgery skill level required High mortality rate	52–56
DDC diet	Feeding	4–8 wk	Representing chronic cholangiopathy Not requiring surgical expertise	Not reducing bile flow	58, 59
$Mdr2^{-/-}$ mice	Genetically modified mice	Age 8–12 wk	Representing chronic cholangiopathy Not requiring surgical expertise	Developing cholangiocarcinoma at age 4–6 mo Mouse genetic variability in the process of straining C57BL/6 and Balb/c	61–66
Hepatocyte-specific $TAK1^{-/-}$ mice	Genetically modified mice	1 month old	Early development of liver fibrosis	Hindrance of studying liver fibrosis by HCC development	67, 68
$ARE\text{-}Del^{-/-}$ mice	Genetically modified mice	20 weeks of age	Relevant to PBC	Limited data	69
Alcohol	Mixed with water or liquid diets Intragastric feeding	10 days (NIAAA) 4–12 wk (Lieber–Decarli) 4 wk to 4 mo (Tsukamoto–French)	Suitable for studying alcoholic liver disease	Difficult to induce liver fibrosis Need for second hit to induce fibrosis	73–78, 81, 82

ARE, adenylate uridylate-rich element; BDL, bile duct ligation; CDA-HFD, choline-deficient, L-amino acid-defined, high-fat diet; HCC, hepatocellular carcinoma; IP, intraperitoneal; NIAAA, National Institute on Alcohol Abuse and Alcoholism; PBC, primary biliary cholangitis; TAK, transforming growth factor-β–activated kinase.

Methionine- and choline-deficient (MCD) diets contain high sucrose proportions and moderate amounts of fat, but are methionine and choline deficient. The deficiency of these essential components prevents the export of lipids from hepatocytes, resulting in hepatic lipid accumulation, impaired β-oxidation, and reactive oxygen species production.[60] The MCD diet induces NASH in 3 weeks and fibrosis in 5–8 weeks.[61] However, this diet does not show systemic metabolic phenotypes, such as body weight gain, dyslipidemia, and insulin resistance.[62] Because of its irrelevant systemic metabolic state, the MCD model is less common in the liver research field in the United States. The choline-deficient L-amino acid-defined (CDAA) diet is another choline-deficient, methionine-supplemented NASH diet, which produces low choline amounts, allowing rodents to survive longer. CDAA diet feeding, after 6 months, results in body weight gain and mild insulin resistance with fibrosis.[63] A longer CDAA diet feeding (84 weeks) develops HCC; thus, this model is useful to study NASH-induced HCC.[63] In contrast, a choline-deficient HFD (CD-HFD) model induces major features of NASH steatosis, inflammation, and fibrosis with systemic metabolic features—body weight gain and insulin resistance. A CD-HFD causes pericellular fibrosis in 6 weeks, bridging fibrosis in 24 weeks, and HCC in 12–15 months.[64,65] In patients with the metabolic syndrome, systemic choline levels are decreased as a result of an altered gut microbiome that promotes the conversion of choline to methylamine and reduces plasma levels of phosphatidyl choline.[66] Decreased systemic choline levels are associated with NAFLD fibrosis, and dietary choline supplementation improves NAFLD.[67–71] We prefer to use the CD-HFD model for NASH-fibrosis studies and consider it the human-relevant NASH-fibrosis model. Contrarily, some laboratories use a choline-deficient L-amino acid–defined HFD that is a choline-deficient, low-methionine (0.1%)–supplemented diet. This model develops NASH, fibrosis, and HCC more rapidly than the CD-HFD model, but does not show insulin resistance and weight gain.[72] This model is distinct from the CD-HFD model and researchers need to select models for their research directions.

Leptin-deficient ob/ob mice develop obesity, insulin resistance, and fatty liver by hyperphagia.[73] Because leptin signaling is required for HSC activation, these mice are resistant to liver fibrosis and are not suitable for studying fibrosis.

Biliary Fibrosis Models

Biliary fibrosis is a result of cholestasis, associated with defects in cellular secretion of bile or mechanical obstruction of the bile duct.[74] Bile duct ligation is a surgical model that ligates the extrahepatic bile duct.[75] After surgery, serum aminotransferase levels are increased for 2–3 weeks[76] and bilirubin level is increased for 7 days.[77] The biliary obstruction induces ductular reactions by increased biliary epithelial cell proliferation, HSC activation, and profibrotic gene expression.[78,79] Periportal fibrosis begins in 10 days. Portal–portal bridging fibrosis develops 3 weeks after bile duct ligation.[75] This model induces leaky gut and bacterial translocation and is suitable for studying the gut–liver axis in fibrosis. However, undeveloped surgical skills increase the mortality rate and vitamin K supplementation may increase the survival rate.[80]

The 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet is another model for cholestatic liver fibrosis. DDC feeding induces porphyrin secretion into the bile duct and the formation of porphyrin crystals and plugging in bile ducts, leading to biliary fibrosis.[81,82] The DDC diet induces onion skin–like fibrosis in the periductal area after 4–8 weeks of feeding. Although this model represents chronic cholangiopathy, bile flow is not reduced.[81,83]

The Mdr2$^{-/-}$ mouse model is a genetically modified mouse model that resembles human PSCs. *Mdr2* is a homolog of the human *ABCB4* gene coding for the canalicular transporter that regulates biliary phospholipid excretion.[84,85] Mdr2$^{-/-}$ mice have a defect in phospholipid secretion into bile, leading to periportal fibrosis.[86] Mdr2$^{-/-}$ mice show increased profibrogenic gene expression at 2 weeks of age.[87] Progressive biliary fibrosis develops at 4–8 weeks of age. HCC develops after 4–6 months of age.[88,89] However, its genetic background, FVB/N, differs from that of the popular laboratory mouse strains C57BL/6 and Balb/c.

Another genetically modified liver fibrosis mouse model is a mouse with hepatocyte deletion of the TGF-β–activated kinase 1, a mitogen-activated protein kinase kinase kinase activated by interleukin 1, TGF-β, and Toll-like receptor ligands.[90] Hepatocyte-specific TGF-β–activated kinase 1$^{-/-}$ mice spontaneously develop pericellular and periportal liver fibrosis from 1 month of age, followed by HCC formation at 6 months of age.[91]

Adenylate uridylate-rich element–Del$^{-/-}$ mice can be used as primary biliary cholangitis model, especially in female mice.[92] Adenylate uridylate-rich element–Del$^{-/-}$ mice show increased expression of interferon-γ resulting in biliary epithelial injury. Fibrosis is observed at 20 weeks of age and this model is ideal to investigate primary biliary cholangitis.

Alcohol-Induced Fibrosis Models

ALD encompasses diseases from steatosis to severe forms, including alcoholic hepatitis and cirrhosis, resulting from alcohol misuse.[93] Chronic alcohol consumption induces steatosis, and 20%–40% of these patients develop fibrosis.[93] Although alcohol metabolism and preference are differ between human beings and rodents, partly because of different CYP2E1 activity,[30,94] several ALD models are used; however, these models basically do not develop fibrosis.

A chronic ethanol-feeding (Lieber–Decarli) model is a traditional model.[95] The feeding of approximately 5% ethanol-containing liquid diet for 4–12 weeks induces steatosis, inflammation, and aminotransferase increase, but no fibrosis.[96,97] The intragastric ethanol (Tsukamoto–French) model continuously feeds a liquid ethanol diet to rodents, which then develop severe liver injury and inflammation compared with the Lieber–Decarli model, but fibrosis is mild.[98,99] The intragastric model could induce fibrosis in combination with a HFD or Western diet.[100,101] Accessibility is a limitation of this model because it

requires a special surgical technique and each rodent requires an infusion pump in a separate cage.[30] The chronic-binge ethanol feeding (National Institute on Alcohol Abuse and Alcoholism) model is a simple model.[102] Mice are fed with 5% ethanol liquid diet for 10 days followed by a single ethanol administration on day 11. This model induces marked aminotransferase increase and steatosis, but no fibrosis.[103] The combination of alcohol feeding with low-dose CCl_4 treatment induces liver fibrosis.[104,105] Collectively, the combination models in rodents induce fibrosis, but alcohol treatment alone does not. The cause of fibrosis, either by ethanol or other supplemental substances, thus is difficult to determine. Researchers should be aware that an adaptation period is needed to shift to feeding high concentrations of ethanol from low concentrations.

In Vitro Experimental Models for Studying Liver Fibrosis

Because various cell types contribute to liver fibrosis, in vivo models are crucial for understanding the disease mechanism. However, complex mechanisms often complicate the interpretation of in vivo models. In contrast, in vitro cell culture models are more simple and are useful for understanding the molecular mechanisms of HSC activation (Table 2 and Figure 1*C*).

Primary HSCs

Primary HSCs are the gold standard in vitro model to investigate liver fibrosis pathogenesis and are isolated from human and rodent livers. In situ collagenase–pronase digestion via hepatic vessels followed by density gradient centrifugation can purify high-quality HSCs.[106] Because primary HSCs from the healthy liver are quiescent and their densities are low as a result of lipid droplets,[107] density gradient centrifugation effectively separates quiescent HSCs from other liver cells.[106,108] Isolated HSCs are cultured on plastic dishes and activated in a time-dependent manner. Day 1 quiescent HSCs are oval-shaped, containing lipid droplets with vitamin A.[20] Days 4–5 HSCs change their morphology to star-shaped pseudopodia and lose lipid droplets.[109] Fully activated HSCs on day 14 become myofibroblast-like cells without lipid droplets.

Researchers can use HSCs in different activation states. Day 1 HCSs from the normal liver are quiescent and are suitable for studying the function of quiescent HSCs and the mechanism of HSC activation during spontaneous activation or with stimulation with profibrogenic factors, such as TGF-β and PDGF. We can study the function of activated HSCs after 3–7 days of culture. These cells are sensitized and their response to profibrogenic factors is altered compared with that of quiescent HSCs. When activated HSCs are used, we can use culture-activated HSCs or in vivo–activated HSCs

Table 2. In Vitro Models of Liver Fibrosis

Models	Characteristics	Advantages	Limitations	References
Primary HSCs	Isolation from human and rodent liver	Fresh HSCs such as in vivo state Observation from quiescent HSCs to myofibroblasts	Complexity of the isolation process Difficulty in isolating activated HSCs Contamination of macrophage Limited life span	83, 85, 87, 89
HSC lines	Deriving from primary HSCs Immortalization by transformation with SV40T, expression of TERT, subjection to UV light or acquirement during culturing	Easy access with high scalability Cost effectiveness Efficiency of transfection	Fully activated state Different response	91, 92
iPSC-derived HSCs	Induction of HSCs from PSCs using stepwise culture with various factors	Providing enough quiescent HSCs	Including immature HSCs or non-HSC lineages Nonstandardized protocol	94–96
Liver organoids	Induction from adult or fetal liver tissue, or PSCs	3D spatial organization Disease modeling Personalized medicine	Limited cell maturation Expression of fetal markers (PSCs) Limited source of tissue (liver tissue)	98, 99, 101, 102
Liver-on-a-chip	Culture cells in microfluidic chips with precision control of fluid flow	In vitro culture with physiological liver environment Disease modeling Suitable for testing drug toxicity	Complex methods to culture cells on a chip Requirement of a perfusion system	105–109
Precision-cut liver slice cultures	Culture thinly sliced liver tissue	Easy and human relevant model	Short duration of viability	110–115

SV40T, simian virus 40 large T-antigen; TERT, telomerase reverse transcriptase.

isolated from mice with liver fibrosis. Because activated HSCs lose lipid droplets, gradient centrifugation is not effective. HSC isolation from NASH fibrosis has a similar issue because hepatocytes store lipid droplets, which prevents the separation of the HSC fraction from fat-accumulated hepatocytes. Another limitation is the contamination of inflamed liver macrophages. Additional macrophage depletion by magnetic-activated cell sorting, fluorescent-activated cell sorting with genetic labeling (eg, Coll-GFP Tg mice, L-rat Cre-Tomato mice), or by targeting autofluorescence of vitamin A after isolation, or in vivo Kupffer cell depletion by liposomal clodronate will increase the purity of HSCs.[109,110] Because in vitro culturing does not reproduce the in vivo HSC activation process, the investigation of in vivo–activated HSCs may provide additional insights into HSC biology. Another consideration is the in vivo ECM conditions, which are crucial for HSC biology. Matrigel and collagen coatings mimic the in vivo ECM scaffold[111] and mechanical stiffness, respectively, which helps in understanding the physiological HSC activation process.[112] Thus, primary HSCs are the standard in vitro model in the liver fibrosis research field.

HSC Lines

Despite several limitations in using HSC lines that lose the original morphology and function of primary HSCs,[113] HSC lines are used as alternatives to primary HSCs for in vitro experiments. HSC lines are easily accessible, highly scalable, and cost effective. Various HSC lines, such as LX-2, HSC-T6, LI90, and GRX, are currently used. HSC lines are myofibroblast-like shaped without lipid droplets, which are considered fully activated.[113] LX-2 cells show 98.7% transcriptional similarity with primary HSCs.[114] Although the efficiency of gene transfection to primary HSCs is low, it is high for LX-2 cells. Thus, LX-2 cells are useful for studies requiring genetic modifications. Although HSC lines have limitations because of their activated state and different responses to fibrogenic stimuli, their study advances our understanding of HSC biology.

Induced Pluripotent Stem Cell–Derived HSCs

Induced pluripotent stem cells (iPSCs) can be a source for various types of liver cells, including HSCs.[115] Several protocols were reported to develop iPSC-HSCs.[116–118] Day 12 iPSC-HSCs have similar gene expression profiles to primary HSCs. Human iPSC-HSCs increase the expression of fibrotic markers in response to TGF-β, lipopolysaccharide, or fetal bovine serum, and are used for studying spontaneous culture activation from the quiescent state.[118] iPSC-derived HSCs are also used for high-throughput drug screening to identify therapeutic agents for liver fibrosis.[118] However, these cells may include immature undifferentiated cells and some cells that differentiate into non-HSC lineages and may have different characteristics from primary HSCs. Because each protocol has minor differences, it may be necessary to standardize the use of these cells. Nonetheless, iPSC-HSCs are powerful tools for human-based in vitro study.

Liver Organoids

Three-dimensional (3D) organoids are better physiological modalities for disease modeling, drug screening, and regenerative medicine than monolayer culture systems.[119,120] Organoids maintain 3D cell-to-cell and cell-to-ECM interactions, which help in studying liver fibrosis in physiological settings. Liver organoids are introduced using adult or fetal liver tissues or stem cells.[120,121] A 3D co-culturing system of hepatocytes and HSCs (rat hepatocytes and HSC-T6; HepaRG cells and primary human HSCs) was established.[122,123] These organoids are suitable for studying drug toxicity (eg, acetaminophen) and drug-induced fibrosis (eg, methotrexate and alcohol). iPSCs are another approach for generating hepatic organoids. Ouchi et al[124] established human iPSC-derived liver organoids containing epithelial and mesenchymal lineage cells, differentiated by treating retinoid and Matrigel scaffolds. This organoid contained hepatocyte-, Kupffer cell–, and HSC-like cells, and developed fatty liver and fibrotic responses after fatty acid challenge. Fibroblast growth factor 19 treatment reduced fat accumulation and fibrosis-like phenotype in the organoid. Liver organoids can be used in the study of genetic disorders. For studying congenital hepatic fibrosis pathogenesis, Guan et al[125] developed an organoid model differentiated from human iPSCs with a mutation in autosomal-recessive polycystic kidney disease edited using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology. This model is useful for studying the pathogenesis of congenital hepatic fibrosis. Enlarged bile ducts, ECM deposition, and myofibroblast expansion are observed in the autosomal-recessive polycystic kidney disease–mutant liver organoids. Thus, iPSC-derived liver organoids can be an ex vivo NAFLD-fibrosis model and used for validating drug efficacy and toxicity and for studying genetic fibrotic disorders in a 3D environment.[121]

Liver-on-a-Chip

The organ-on-a-chip system is an in vitro model for culturing cells in microfluidic chips with control of fluid flow, mimicking the in vivo physiological liver environment.[126–128]

The microfluidic chip comprises top and bottom channels separated by an ECM-coated porous membrane, mimicking a hepatic sinusoid-like architecture. The liver-on-a-chip model places hepatocytes in the top channel and LSECs and other liver nonparenchymal cells (eg, HSCs and Kupffer cells) in the bottom channel. The use of human-originated primary cells reproduces human-relevant metabolic states, cell sensitivity to substances, and physiological cell plasticity. Continuous media flow that maintains proper oxygen and nutrient concentrations and washes out metabolites and cell debris and relevant shear stress further mimics the physiological liver microenvironment with longer culture periods of up to 4 weeks. Co-culturing organ- and species-specific endothelial cells help to maintain better hepatocyte metabolism, albumin production capacity, and alcohol and drug metabolism. Fatty acids and ethanol challenges induce lipotoxicity and

ethanol-induced liver damage, fat accumulation, and HSC activation, which can be human-relevant ex vivo models for studying NAFLD, ALD, and fibrosis, and for testing drugs for these diseases.[129,130] This model may become an indispensable preclinical ex vivo model for validating drug safety and hepatotoxicity.[131]

Precision-Cut Liver Slice Cultures

Precision-cut liver slices (PCLS) are another 3D culture method that uses thin liver-sliced tissues (~250 μm) from animal and human specimens.[132] To prepare liver-sliced tissues, various instruments and equipment are required, including a tissue slicer, mechanical drill, and incubation cabinet.[133] The PCLS model maintains the 3D structure, physiological ECM, and cell–cell interactions. PCLS from normal liver tissues can be stimulated with TGF-β or PDGF to test fibrogenesis. In contrast, PCLS from fibrotic livers can be used to test antifibrotic drugs. Fibrosis in PCLS can regress when treated with effective drugs.[134–137] A limitation is the short duration (24-48 hours) of cell viability resulting from hypoxia and down-regulated hepatocyte functions. The PCLS model is a human-relevant ex vivo model for investigating the mechanism of liver fibrosis and for testing drug efficacies.

Emerging Tools to Study HSC Heterogeneity

Single–cell RNA sequencing (scRNA-seq) is used to examine whole transcriptomics at the single-cell level, determining the heterogeneity of liver cells in normal and fibrotic livers.[138] This approach reveals unique liver cell subpopulations, including scar-associated $TREM2^+CD9^+$ macrophages, $ACKR1^+$ and $PLVAP^+$ endothelial cells, and $PDGFR\alpha^+$ collagen-producing myofibroblasts. Human HSCs are separated into 2 subpopulations: $GPC3^+$ HSCs and DBH^+ HSCs.[139] Unique liver subpopulations can be identified through this method.

The general scRNA-seq workflow requires a single-cell suspension from fresh liver tissues. To obtain high-quality data, high-cell viability and yield are crucial and require immediate tissue dissociation and appropriate cell isolation skills. To overcome this limitation, stored frozen tissues can be used for single nuclear RNA sequencing (snRNA-seq), which may show less variation among investigators, but still needs proper optimization of nuclear preparation.[140,141] scRNA-seq is good for analyzing nonparenchymal cell populations, especially immune cell populations, but is not suitable for HSCs, LSECs, hepatocytes, and cholangiocytes.[142] In contrast, snRNA-seq has the advantage of analyzing HSCs, LSECs, hepatocytes, and cholangiocytes. Although studies have attempted to understand gene profiles in different hepatic zones using zonation markers (zone 1, arginase 1; zone 3, cytochrome P450 family 2 subfamily E member 1), one limitation is that sc/snRNA-seq technology loses spatial information. Recent advancements in spatial transcriptomic and proteomic approaches (eg, NanoString Geo-MX, 10× Visium, CyTOF, and CODEX) could overcome these limitations. Although spatial proteomic analysis can be analyzed at the single-cell level, spatial transcriptomics are still based on regions of interest, not at the single-cell level. However, adding sc/snRNA-seq data can complement single-cell information on spatial analysis. Thus, appropriate uses and combinations of scRNA-seq, snRNA-seq, and spatial transcriptomic/proteomic analyses can enhance further understanding of liver cell heterogeneity in liver fibrosis.

Of Mice and Men: Future Prospective of Preclinical Models

In vitro cell culture models and the validation of results from in vitro results in animal models are the gold standard approaches for elucidating the mechanisms underlying liver fibrosis. This approach can be used to discover and test effective drugs for liver fibrosis treatment. Although many antifibrotic drug candidates have been investigated, most drugs have not been recommended for clinical trials or unsatisfactory results are observed after clinical trials.[143,21] There may be a large gap in the pathophysiology of liver fibrosis between rodents and human beings. Enzyme activities crucial for drug and alcohol metabolism (hepatic CYP enzymes) are higher in human beings than those in rodents. The sensitivity of human immune cells and HSCs to fibrotic factors and lipopolysaccharide differ between human beings and rodents (eg, the human body is highly sensitive to lipopolysaccharide). The human gut microbiome is dominant in gram-negative bacteria, whereas the mouse gut microbiome is gram-positive dominant. Genetically, human beings are heterogeneous; however, most laboratory mice are inbred. All of these factors prevent the development of human-relevant liver fibrosis models.[144] Therefore, we attempted to identify common mechanisms among various in vitro and in vivo models, while considering that diverse etiologies of human fibrosis have distinct mechanisms that should not be overlooked. One option is to use 3D multicellular culture systems with human-relevant cells to create an experimental platform for mini-human livers.[145] Combining human-relevant in vitro cell culture and in vivo animal models will help explore novel mechanisms for liver fibrosis and test novel drugs before moving to clinical trials.

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