Review Article

Animal Models for Fibrotic Liver Diseases: What We Have, What We Need, and What Is under Development

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Abstract

Liver fibrosis is part of the wound-healing response to liver damage of various origins and represents a major health problem. Although our understanding of the pathogenesis of liver fibrosis has grown considerably over the last 20 years, effective antifibrotic therapies are still lacking. The use of animal models is crucial for determining mechanisms underlying initiation, progression, and resolution of fibrosis and for developing novel therapies. To date, no animal model can recapitulate all the hepatic and extra-hepatic features of liver disease. In this review, we will discuss the current rodent models of liver injuries. We will then focus on the available ways to target specifically particular compounds of fibrogenesis and on the new models of liver diseases like the humanized liver mouse model.

Keywords: Liver; Fibrosis; Hepatic stellate cell; Animal models; Cell tracking.

Abbreviations: 2OA-BSA-α-GalCer, 2-octynoic acid coupled to bovine serum albumin and α-galactosylceramide; Ad-2D6, adenovirus Ad5 expressing human cytochrome P450 2D6; AE2, anion exchanger 2; AIH, autoimmune hepatitis; Alb, albumin; ALD, alcoholic liver disease; ANIT, α-naphtylisothiocyanate; ASH, alcoholic steatohepatitis; αSMA, alpha-smooth muscle actin; ATG7, autophagy-related protein 7; CBDL, common bile duct ligation; CCl_4, carbon tetrachloride; CDAA, choline-deficient, L-amino acid-defined; CRISPR, clustered regularly interspaced short palindromic repeats; CYP, cytochrome P450; DDC, 3,5-diethylcarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; ECM, extracellular matrix; Fah, fumarylacetoacetate hydrolase; GFAP, glial fibrillary acid protein; GFP, green fluorescent protein; GSPC, Gold Standard Publication Checklist; HA, hemagglutinin autoantigen; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; HFD, high fat diet; HSC, hepatic stellate cell; HSV-Tk, herpes simplex virus-Thymidine kinase; ip, intraperitoneal; KO, knockout; LDLR, low-density lipoprotein receptor; Lrat, lecithin-retinol acyltransferase; MCDD, methionine- and choline-deficient diet; MDR, multi-drug resistance; MRP2, multi-drug resistance protein 2; NAFL, non-alcoholic fatty liver; NASH, non-alcoholic steatohepatitis; NK, natural killer; nSREBP-1c, nuclear sterol regulatory element-binding protein 1c; OLETF, Otsuka Long-Evans Tokushima Fatty; PBC, primary biliary cirrhosis; PDGF-β, platelet-derived growth factor-beta; PSC, primary sclerosing cholangitis; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; sc, subcutaneous; SCID, severe combined immunodeficiency; shRNA, short hairpin RNA; siRNA, small interfering RNA; TAA, thioacetamide; TCR, T-cell receptor; TGF-β1, transforming growth factor beta 1; TRE, tetracycline responsive element; tTA, tetracycline-controlled transactivator; uPA, albumin-uroplasminogen activator; VLDL, very low density lipoprotein; YFP, yellow fluorescent protein.

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Introduction

Fibrosis is part of the general wound-healing response to liver damage of various origins and is defined by the accumulation and qualitative changes in extracellular matrix (ECM) components. The hepatic stellate cell (HSC) is the main cellular effector of this phenomenon and the major producer of scar ECM.[1]

Liver fibrosis remains a major health problem as fibrotic liver diseases have a high mortality rate and predispose to liver failure, portal hypertension, and hepatocellular carcinoma (HCC).[2] Although intense research during the last 20 years has led to considerable improvements in the understanding of liver fibrosis pathogenesis, effective antifibrotic therapies are still lacking. A better understanding of the mechanisms implicated in the initiation, progression, and resolution of fibrosis is crucially needed. Animal models are essential to study the processes underlying fibrogenesis, to identify potential therapeutic targets, and to evaluate the impact of antifibrotic therapies.[3] Larger animal (rabbit, dog, chimpanzee, etc.) may be used, but rodent (mouse and rat) models are preferred and best standardized.[4]

When using animal models, one must keep in mind some general concepts, advantages, and pitfalls. A Gold Standard Publication Checklist (GSPC) was published in 2010 to improve the quality of animal studies and related publications and the feasibility of systematic reviews.[5] GSPC directly benefits animal welfare and should be taken into account by all investigators when planning an experimental design. In studies on liver fibrosis, reproducibility, specificity, feasibility, optimal number of animals (to eliminate individual heterogeneity), and opportunity to largely sample the liver (to avoid sampling error) must be considered.[6] The rodent strain is also of importance given large variations in fibrosis susceptibility related to the genetic and immunologic background.[3]

Compared to clinical research, the use of animal models offers several advantages: (i) the possibility to collect multiple samples at different time-points and to realize sequential studies, (ii) a shorter time for disease development, (iii) the ability to control and reduce variables that cannot be closely followed in humans, and (iv) the ability to study the implication of specific genes/signaling pathways by the use of genetically modified animals. Moreover, compared to *in*

vitro systems, animal models allow for the study of the liver as a complete organ, with intact and dynamic cell-cell and cell-matrix interactions and intact crosstalk of the liver with the entire body, including immune, vascular, metabolic, and endocrine interactions.[6]

Unfortunately, animal models are not the panacea to resolve all questions. Because animals are not humans, they do not develop human diseases. Large variations in responses to noxious agents exist between humans and animals regarding pathogenicity, timing, and immuno-inflammatory reactions.[6] Differences between humans and animals reside at several levels. First, some hepatic diseases do not exist in rodents. For example, the hepatitis C virus (HCV) does not infect rodent hepatocytes; chimpanzees and Tupaia belangeri (a Northern tree shrew) are the only animals that support HCV infection, although they do not develop chronic liver disease and fibrosis.[7] Second, animals may be less or more susceptible to toxic agents than humans. Alcoholic liver disease (ALD) is particularly difficult to induce in rodents. They have a total aversion to alcohol, and rapid alcohol metabolism prevents high blood alcohol levels. Moreover, even in animals continuously and chronically fed alcohol by intragastric infusion (Tsukamoto-French model), severe liver fibrosis does not develop, arguing for a different susceptibility to alcohol toxicity between animals and humans.[8] In contrast to alcohol, common bile duct ligation (CBDL) results in secondary biliary cirrhosis after only a few weeks in rodents, whereas month-long impairment of the bile flow is needed to cause severe liver fibrosis in humans. Finally, some liver pathology occurs in a specific metabolic or immune context, like non-alcoholic steatohepatitis (NASH) and autoimmune hepatitis (AIH), or is strongly associated with particular clinical entities, like primary sclerosing cholangitis (PSC), which is preferentially observed in patients suffering from inflammatory bowel disease. To date, no animal model recapitulates complex hepatic and extra-hepatic features and succeeds in modeling intricate diseases.

To increase our understanding of human liver disorders, animal models that replicate specific disease mechanisms or the disease as a global entity, including metabolic and immune aspects, and tools able to target specific cells, components of the ECM, or signaling pathways, are valuable. In the first part of this review, we will briefly discuss the current animal models in use for liver injuries, with emphasis on fibrosis progression and translational aspects. In the second part, we will focus on the available tools that target specifically one particular element involved in fibrogenesis. These tools include the use of genetically modified animals, cell-tracking/labelling methods, and targeted delivery systems. Finally, we will discuss new models of liver disease, like the humanized mouse, and its potential applicability in the field of liver fibrosis.

Animal models of liver diseases

The use of animal models for experimental liver fibrosis research has been extensively discussed previously.[6,9] They are listed in Table 1. We will briefly highlight their main features and interesting specificities and provide relevant information for translation of experimental findings from animals to humans.

Hepatotoxin-induced liver fibrosis as a model of postnecrotic fibrosis (Table 2)

Carbon tetrachloride (CCl_4), thioacetamide (TAA), dimethylnitrosamine (DMN), and diethylnitrosamine (DEN) are the most commonly used toxic agents to induce liver fibrosis in rodents. These toxins are mainly metabolized by centrilobular hepatocytes and cause centrilobular liver damage. The resulting fibrosis first appears in the perivenular area. As fibrosis extends, bridges between central areas are formed, with portal areas being secondarily involved (portal-central septa). Importantly, in humans, fibrosis is more frequently distributed in periportal and lobular areas and central fibrosis such as caused by hepatotoxins is only seen in hemodynamic or vascular disorders, *i.e.* chronic right ventricular dysfunction or chronic Budd-Chiari syndrome.

The CCl_4-induced model of liver fibrosis is a widely used and studied, reliable animal model of hepatic fibrosis.[10] Repeated doses of CCl_4 lead to repeated rounds of wound-healing, causing HSC activation, imbalance between ECM production and degradation, and development of progressive hepatic fibrosis.[6] Multiple protocols for CCl_4 administration in mouse and rat are described in the literature, which vary in terms of route of administration (intraperitoneal (ip) injections, subcutaneous (sc) injections, oral gavage, and inhalation), dosage, adjustment of the initial dosage to daily/weekly change in body weight, frequency of dosing, duration, dilution of CCl_4, nature of the vehicle (olive oil, corn oil, paraffin oil, etc.), and the eventual use of phenobarbitone in the drinking water as enzyme inducer.[11] In addition to these parameters, the susceptibility of a given animal strain, depending on immunologic background, affects efficiency and severity of liver fibrosis development.[12–14] The impact of immune status is illustrated by variation in the severity of fibrosis following CCl_4 administration observed (i) in Balbc and C57BL/6 mice due to a different Th1/Th2 cytokines response[13] and (ii) in wild-type Balbc mice, severe combined immunodeficiency (SCID) mice (lacking B, T cells but having NK cells), and SCID beige (lacking B, T and NK cells) mice.[15]

Repeated (ip injections) or chronic (supplementation in drinking water) TAA exposure leads to severe fibrosis/cirrhosis between 12 and 16 weeks in rats and between 16 and 24 weeks in mice.[16,17] Compared to CCl_4-induced cirrhosis, TAA is associated with more prominent regenerative nodules and rapid development from periportal fibrosis to a state resembling human cirrhosis.[18] While CCl_4-induced liver fibrosis reverses in a short time,[19] fibrosis persists for more than 2 months after TAA withdrawal,[16] making these models complementary for studying processes of fibrosis reversal. Moreover, CCl_4 fibrosis develops linearly,[6] whereas with TAA, fibrogenesis initiation is slow, followed by a sudden exponential acceleration of matrix deposition to a steady state level (nonlinear fibrosis).[17] Besides causing fibrosis, TAA, but not CCl_4, has hepato- and cholangiocellular carcinogenic properties. In rats, biliary dysplasia and cholangiocellular carcinoma may be observed quickly, depending on animal strain and dose-dependent toxicity on the biliary tract.[20] HCC development is slower as hepatocellular cancers appear on a background of chronic liver fibrosis after several months of TAA administration,[21] recapitulating the multi-stage process of human carcinogenesis. Other hepatotoxins, such as DEN and DMN, are also used to induce HCC in the context of chronic fibrosis.[22–24]

Animal models of biliary fibrosis

Cholestatic liver disease encompasses a large variety of entities that may lead to biliary fibrosis, cirrhosis, and end-stage liver disease independently of etiology. Several animal models are available and attempt to reproduce cholestatic liver injuries and related fibrosis in a specific context according to the human pathology.

CBDL is the archetype model for obstructive cholestasis, since the interruption of bile flow induces an intense ductular proliferative response, portal inflammation, and rapid establishment of portal fibrosis.[25,26] The structural and functional changes due to CBDL have been extensively reported in the literature.[27] The main controversy involves the severity of induced fibrosis and the celerity of severe fibrosis development. Some works describe liver cirrhosis 15–28 days after CBDL[28,29] while others are unable to demonstrate evidence of cirrhosis after 40 days.[30–32] Interestingly, the study of fibrosis reversibility is feasible in this model by using bilioduodenal anastomosis or choledoco-jejunostomy surgical techniques to restore normal biliary outflow.[33,34] The occurrence of surgical complications (e.g. bile leakage and subsequent sepsis) is the main pitfall of the CBDL model. This may occur more frequently in mice than rats because of the more pronounced fragility of some mouse strains, especially transgenic mice, and the inevitable dilatation of the gall bladder (not present in rat) and subsequent perforation and bilioperitoneum.[35]

PSC is a chronic cholestatic liver disease characterized by strictures of the biliary tree due to an inflammatory and fibrotic process affecting the intra- and extra-hepatic bile ducts. Main complications are irregular bile duct obstruction, development of secondary biliary cirrhosis, and cholangiocarcinoma.[36] Pathogenesis of PSC is incompletely understood, but this entity has been described as an immune-mediated phenomenon triggered by environmental factors in genetically susceptible individuals.[37] The perfect animal model that summarizes all the attributes of PSC (cholangitis of the intra- and extra-hepatic bile ducts in association with gut inflammation and development of cholangiocarcinoma and/or secondary biliary cirrhosis) and exhibits a male predominance does not exist. However, several animal models may be used to study individual features.[38] From a fibrosis point of view, the most relevant one is the *Abcb4-/-* mouse model. Mice deficient in the phospholipid transporter multi-drug resistant protein 2 (MDR2), encoded by the *Abcb4* gene, rapidly develop after birth inflammatory cholangitis with portal inflammation and ductular proliferation, onionskin-type periductal fibrosis, focal obliteration of the bile ducts, extra- and intrahepatic biliary strictures, and segmental duct dilation.[39] The near total absence of phospholipid secretion into bile in *Abcb4-/-* mice results in increased concentration of free non micellar bile acids, which have toxic effects on the apical membrane of hepatocytes and cholangiocytes.[40] This animal model can also be used as a model for the human hepatic disease due to MDR3 (the human orthologue of MDR2) deficiency. In humans, this genetic defect may lead to a wide spectrum of clinical phenotypes, ranging from neonatal cholestasis to biliary cirrhosis of adulthood.[41]

Primary biliary cirrhosis (PBC) is a liver-specific autoimmune disease mostly affecting middle-aged women and is characterized by the presence of anti-mitochondrial antibodies and progressive destruction of the small bile ducts, causing liver fibrosis, portal hypertension, and potentially liver failure.[36,42] Similar to PSC, the interplay between genetic predisposition and environmental factors is a major contributor to pathogenesis of PBC and explains why it is difficult to define relevant animal models that mimic disease pathophysiology. PBC models include inducible and genetically modified animals and represent good tools to investigate the genetics and immunoregulation occurring in the earliest stage of the disease.[43,44] There are only two models in the literature that eventually lead to fibrosis: (i) C57BL/6 mice co-immunized with 2-octynoic acid coupled to bovine serum albumin and α-galactosylceramide (2OA-BSA-α-GalCer mice), an invariant natural killer T cell activator, develop fibrous septa 4 weeks postimmunization;[45] (ii) $Ae2_{a,b}^{-/-}$ mice have a widespread disruption of the Cl^-/HCO_3^- anion exchanger 2 (AE2) and develop elderly immunologic and hepatobiliary changes similar to PBC with slight liver fibrosis.[46] Increased levels of anti-mitochondrial antibodies are found in both of these models.[45,46]

Some modified diets can induce biliary damages. A diet containing 3,5-diethylcarbonyl-1,4-dihydrocollidine (DDC), a porphyrinogenic hepatotoxin, causes the formation of porphyrin crystals within the hepatocytes in the periportal region and porphyrin plugs in small bile ducts. The tissue response involves a florid ductular reaction, peri-cholangitis, periductal fibrosis, and portal-portal fibrosis after 4–8 weeks that resembles sclerosing cholangitis in humans.[47] α-naphtylisothiocyanate (ANIT) is a hepatocyte and bile duct epithelial cell toxicant. When conjugated to glutathione in hepatocytes, ANIT is secreted by the MRP2 transporter into bile, where it can exert its toxic effect on biliary cells. As the ANIT-glutathione complex is not stable in the bile, free ANIT undergoes recycling rounds of absorption and metabolism, leading to a high and toxic biliary concentration.[48,49] Animals exposed chronically to low doses of ANIT develop periportal inflammation, mild hepatocellular injury, significant bile duct proliferation, and progressive fibrosis.[50,51]

Autoimmune fibrosis

AIH consists of a progressive T cell-mediated necroinflammatory and fibrotic process in the liver, likely triggered by the combination of environmental factors, failure of immune tolerance, and genetic predisposition.[52] Considering the animal models, the difficulty lies in the breakage of immune tolerance and the long-term maintenance of immune alterations necessary for progression to chronic hepatitis and liver fibrosis. Several animal models have been proposed,[53,54] but very few reproduce chronic hepatitis and develop fibrosis. The double transgenic mouse Alb-HA/CL4-TCR spontaneously develops histologic features of AIH and hepatic fibrosis, exclusively in males. This mouse expresses the influenza virus hemagglutinin autoantigen (HA) under the control of mouse albumin regulatory elements and α-fetoprotein enhancer (Alb) (Alb-HA mouse), and a specific T-cell receptor (TCR) (CL4-TCR mouse).[55] Wild-type FVB/N mice infected with adenovirus Ad5 expressing human cytochrome P450 2D6 (Ad-2D6) develop a chronic and severe form of AIH with extensive fibrosis and generate type 1 liver kidney microsomal-like antibodies similar to type 2 AIH patients.[56]

Schistosoma infection and prolonged administration of heterologous serum, mainly porcine serum, are other ways to study hepatic fibrosis development triggered by an initial immunologic stimulus.[57–60]

Table 1. Main animal models of liver fibrosis in rodents

Liver fibrosis induced animal models		Main features	Ref.
Hepatotoxin-induced liver fibrosis as model of post-necrotic fibrosis	-CCl_4	-Peri-central fibrosis with first centro-central septa and second centro-portal septa. -Multiple protocols for administration with liver fibrosis of variable severity. -Linear development of liver fibrosis. -Variable susceptibility according to animal strain. -Fibrosis reversion in a short time after CCl_4 withdrawal. -Adverse effects depending on the route of administration: chronic peritonitis (ip) or cutaneous necrosis (sc).	6,9–15
	-TAA	-Peri-central and peri-portal fibrosis. -Regenerative nodules and periportal fibrosis resembling human cirrhosis. -Nonlinear fibrosis development. -Slow spontaneous reversal. -Hepato- and cholangiocellular carcinogenic properties.	6,16–18,20,21
	-DEN/DMN	-Rarely used to induce liver fibrosis alone. -Fibrotic septa between vascular areas. -Hepatocellular carcinogenic properties. -Auto-progression (fibrosis/HCC) after drug cessation.	6,22–24
Biliary fibrosis	-Common bile duct ligation (CBDL) model	-Obstructive cholestasis inducing portal fibrosis. -Controversies regarding fibrosis severity and celerity to induce severe fibrosis. -Study of fibrosis reversibility feasible (bilioduodenal anastomosis or choledoco-jejunostomy). -Invasive method, surgical complications (mice>rats).	26–28,30–35
	-Primary sclerosing cholangitis (PSC) models • Chemically-induced cholangitis • Knockout mouse models (*Abcb4-/-)* • Cholangitis induced by infectious agents • CBDL model • Models involving enteric bacterial cell-wall components or colitis • Models of biliary epithelial and endothelial cell injury	-No perfect animal model available summarizing all features of PSC. -Most relevant fibrosis development observed in the *Abcb4-/-* mouse model but without gut inflammation or cholangiocarcinoma. Rapid development after birth of inflammatory cholangitis, periductal fibrosis and biliary strictures.	38–40

Continued

Table 1. Continued

Liver fibrosis induced animal models		Main features	Ref.
	- Primary biliary cirrhosis (PBC) models • Inducible models • Transgenic mouse models	-No perfect animal model available summarizing all features of PBC. -Two models described leading to liver fibrosis: • 2OA-BSA-α-GalCer mice: co-immunization with 2-octynoic acid coupled to bovine serum albumin and α-galactosylceramide. Fibrous septa 4 weeks post-immunization. • $Ae2_{a,b}^{-/-}$ mice: late development of fibrosis.	43–46
	-Diet-induced cholestatic liver injury • DDC-modified diet (0.1% 3, 5-diethoxycarbonyl-1,4-dihydrocollidine) • ANIT-modified diet (α-naphtylisothiocyanate)	-DDC= porphyrinogenic hepatotoxin. Induction of peri-ductal fibrosis and porto-portal fibrotic septa after 4-8 weeks. -ANIT= hepatocytes and bile duct epithelial cells toxicant. Induction of periportal inflammation, mild hepatocellular injury, significant bile duct proliferation, and progressive fibrosis.	47–51
	-d-galactosamine–induced biliary fibrosis	-Bile duct proliferation with progressive fibrous septa. Significant fibrosis after 7–13 weeks of repeated injections (20–40 doses).	119
Auto-immune fibrosis			
	-Immunization models -Transgenic mouse models	-Very few animal models able to reproduce chronic hepatitis and to lead to fibrosis. • Wild-type FVB/N mouse infected with Ad-2D6: severe form of auto-immune hepatitis with extensive fibrosis; Type 1 liver kidney microsomal-like antibodies. • Alb-HA/CL4-TCR transgenic mouse: histologic features of AIH and hepatic fibrosis, exclusively in male.	53–56
	-Schistosoma mansoni/japonicum infection models	-Granulomas-associated fibrosis as result of a cell-mediated immunological inflammatory host-response.	57,58
	-Heterologous serum models (pig, horse, swine serum, etc.)	-Immune-mediated hepatic fibrosis with minimal hepatocellular injury.	59,60
Alcohol-induced liver disease			62,63,68
	-*Ad libitum* alcohol feeding model	-No significant fibrosis even after administration for prolonged periods.	
	-Lieber-De Carli liquid diet model	-Administration of an alcohol-containing isocalorically controlled liquid diet as the sole source of food and drink. -Mild steatosis but no significant fibrosis even after prolonged administration.	

Continued

Table 1. Continued

Liver fibrosis induced animal models		Main features	Ref.
	-Intragastric feeding model by Tsukamoto-French	-Sustained high alcohol blood level. -Fibrosis development after 6 to 8 weeks. -Main drawback: implantation and maintenance of the intragastric canula.	
Non-alcoholic fatty liver disease			
	-Dietary models		
	• Hypercaloric diets enriched in various lipid species (saturated, unsaturated, trans-fatty acids, cholesterol), carbohydrates (high fructose, high sucrose), or both (cafeteria diet, western-diet, atherogenic diets).	-Hypercaloric diets: variable degree of obesity, insulin resistance, and metabolic syndrome; variable steatosis and mild fibrosis if any. More pronounced inflammatory changes and profibrogenic response with the atherogenic diet and the trans-fatty acids enriched diet.	72–74
	• Methionine- and choline-deficient diet (MCDD)/choline-deficient, L-amino acid-defined diet (CDAA).	-MCDD: rapid development of steatohepatitis and pericellular fibrosis by week 7 to 10. Lack of metabolic features of NASH. -CDAA: steatohepatitis and pericellular fibrosis in mice but lack of metabolic features of NASH. Macrovesicular steatosis, inflammation, fibrosis (centro-portal septa), and HCC development in rats chronically fed the CDAA.	71,76,78,79 6,80,120
	-Transgenic models	-Progression to steatohepatitis and fibrosis in the nSREBP-1c transgenic mouse and in the PTEN knockout mouse.	81–85
	-Combined models: transgenic mouse fed a modified diet	e.g. Zucker fatty rats fed a HFD: lobular inflammation, ballooning degeneration and fibrosis after HFD for 8 weeks. foz/foz mice fed a HFD: steatohepatitis and pericellular fibrosis after HFD for 20–24 weeks.	86–92

Table 2. Hepatotoxin-induced liver fibrosis models

Hepatotoxin-induced liver fibrosis as model of post-necrotic fibrosis	Mechanisms	Description of hepatic damages and fibrosis	Time for fibrosis induction*	Carcinogenic properties	Ref.
-CCl4	Metabolization by cytochrome P450 CYP2E1 in centrilobular hepatocytes and transformation in highly reactive free radical metabolites.	Progressive development of fibrosis: peri-central fibrosis with centro-central septa and then porto-central septa.	Mouse -Significant fibrosis: after 2 weeks of CCl_4 administration by ip injections or inhalation; after 3–4 weeks by sc injections. -Cirrhosis: after 8 weeks of CCl_4 administration by ip injections or inhalation; after 10 weeks by sc injections. Rat -Significant fibrosis: after 2 weeks of CCl_4 administration by ip injections, inhalation or oral gavage; after 6 weeks by sc injections. -Cirrhosis: after 4 weeks CCl_4 administration by inhalation; after 8 weeks by oral gavage, after 10 weeks by ip injections, after 12 weeks by sc injections.	No carcinogenic properties when administered alone.	6,9–12,86–89,121–123
-TAA	Metabolic activation consisting in a two-phase oxidation, leading to a highly reactive product, S, S-dioxide, responsible for protein covalent binding and cellular toxicity. CYP2E1 is described as a major contributor in TAA metabolism.	Peri-central and peri-portal fibrosis (quicker emergence of portal-central and portal-portal fibrotic septa compared to CCl4).	Mouse -Severe bridging fibrosis after TAA administration in the drinking water for 16 weeks; significant fibrosis after TAA ip injections for 12 weeks. Rat -Significant fibrosis after TAA ip injections for 12 weeks. -Cirrhosis after TAA ip injections for 20 weeks.	-Dose-dependent and strain-dependent biliary carcinogenic effect. -HCC development in the context of chronic liver fibrosis after several months of TAA administration.	6,16,17,20,21,124,125
-DEN	Toxic activity mediated through DNA-adduct formation after a first activation phase mediated by CYP2E1 and other P450 isoenzymes.	-Fibrotic septa between vascular area.	-Multiple protocols of DEN administration lead to the development of fibrosis, fibrosis with HCC (long-term administration), or HCC without significant fibrosis (single administration at 2 weeks of age, short-term administration).		6,9,22,126

*Timings are indicative as they depend on several factors (dosage, adjustment of the initial dosage to daily/weekly change in body weight, frequency of dosing, animal strains).

Alcohol-induced fibrosis

ALD is a major public health burden, representing the first cause of advanced liver disease in Europe. Chronic consumption of alcohol may lead to progressive hepatic impairment, ranging from simple steatosis to alcoholic steatohepatitis (ASH), progressive fibrosis, cirrhosis, and HCC.[61] ALD pathogenesis is complex and includes changes in hepatic metabolism that lead to accumulation of lipids, depletion of essential nutrients, and enhanced hepatotoxicity. Persistent hepatic damage and sustained inflammation are associated with formation of reactive oxygen species (ROS), induction of an inflammatory immune response, HSCs activation, and collagen deposition. This complexity is enhanced when considering other factors like alcohol-induced changes in the gut microbiome, inter-individual susceptibility, and the large panel of alcohol consumption behaviors. To date, despite major efforts, no animal model has been able to recapitulate all features of alcoholic disease.[62,63] Moreover, natural aversion to alcohol, absence of an addictive behavior, spontaneous reduction in alcohol intake when acetaldehyde blood levels increase, a high rate of alcohol catabolism, and a high basal metabolic rate impair the ability to obtain and maintain over time high blood alcohol levels in rodents and explain the paucity of hepatic damage.[63]

Several animal models for ALD have been developed and have been reviewed elsewhere.[62] Briefly, the Lieber-De Carli model, the oldest model of chronic alcohol consumption, consists of administering an alcohol-containing isocalorically controlled liquid diet (with up to 36% calories from alcohol) as the sole source of food and drink.[64] It induces mild steatosis and low-grade inflammation but not significant fibrosis, even after prolonged administration.[63,65] This animal model is considered appropriate to study the early stages of ALD but not the mechanisms implicated in alcohol induced-fibrosis. Similar histological changes are observed after alcohol administration *ad libitum* in the drinking water.[66]

The intragastric feeding model was developed by Tsukamoto and French to overcome the natural aversion of animals to alcohol and to achieve a sustained high blood alcohol level.[67] Rats fed according to this method develop steatosis, inflammation, and peri-central necrosis in about 2 to 4 weeks and fibrosis after 6 to 8 weeks.[8] The implantation and maintenance of the intragastric cannula are the major technical limitations of this model.[68]

Models of NASH-associated fibrosis

Obesity, dyslipidemia, type 2 diabetes, and metabolic syndrome are major risk factors associated with non-alcoholic fatty liver disease (NAFLD). NAFLD covers a large spectrum of histological changes, including nonalcoholic fatty liver (NAFL-defined as the presence of hepatic steatosis with no evidence of hepatocellular injury), NASH (defined as the presence of steatosis, lobular or portal inflammation, and hepatocyte injury (ballooning)), pericellular fibrosis, cirrhosis, and HCC.[69]

The ideal animal model that recapitulates all aspects of the pathogenesis of human NAFLD, the typical histological features and progression, and the metabolic background (obesity, insulin resistance, hyperglycemia, hyperinsulinemia, dyslipidemia, altered adipokine profiles) does not exist. However, several models are described and may be classified in (i) dietary, (ii) genetic, and (iii) combined models.[6] Steatosis is a common feature of these models; in some, steatohepatitis does occur but progression to liver fibrosis is uncommon.[70] Obesity and metabolic syndrome are not systematically reproduced.[6]

Animals fed a large variety of modified diets have been described. The diets are hypercaloric, enriched in various lipid species (saturated, unsaturated, trans-fatty acids, cholesterol), carbohydrates (high fructose, high sucrose), or both (cafeteria diet, western-diet, atherogenic diets).[70,71] Such dietary manipulations are usually associated with varying degrees of obesity, insulin resistance, and metabolic syndrome. Steatosis may or not be seen, sustained inflammation is uncommon, and fibrosis, if any, inconspicuous. The more pronounced phenotype is observed using atherogenic diets or trans-fat enriched diets. An atherogenic diet containing cholesterol and choline induces progressive steatosis, steatohepatitis, and pericellular fibrosis in a time-dependent manner both in rats and mice.[72,73] Interestingly, mice fed this atherogenic diet remain remarkably insulin sensitive, reflecting a different metabolic status compared to the human NASH situation.[73] Trans-fatty acids are the result of the industrial hardening of the vegetable oils and are found in fast-foods. They are thought to play a major role in the development of a severe phenotype with necroinflammatory changes and profibrogenic responses in a NAFLD model in mice fed *ad libitum* high-fat chow containing trans-fats.[74]

The methionine- and choline-deficient diet (MCDD) is deficient in two essential factors for the formation of phosphatidylcholine, which is involved in very low density lipoprotein (VLDL) production and secretion from the liver.[75] Steatohepatitis occurs rapidly after starting the MCDD, and perisinusoidal fibrosis is observed by week 7 to 10.[76] The severity of MCDD-induced histological changes in rodents depends on species, strain and gender of the animals.[71,77] The MCDD is one of the most commonly used animal models of NASH, as it induces a liver pathology that recapitulates the sequence and progression of liver pathology seen in humans. However, MCDD induces hypercatabolism, significant weight loss, and hypersensitivity to insulin.[78,79] This specific metabolic profile has to be taken into account when drawing conclusions using this model.

The choline-deficient, L-amino acid-defined diet (CDAA) is a variant of the MCDD as it contains a low amount of methionine. Although it acts via a similar mechanism as the MCDD, the CDAA diet, in contrast, induces moderate pericellular fibrosis in mice. In rats, CDAA results in homogenous severe macrovesicular steatosis and unspecific inflammation. After a long time, fibrosis evolves rapidly, forming centro-portal bridges. HCC develops with a high incidence in rats chronically fed the CDAA.[6,80]

The large variety of genetic models available for NAFLD have been reviewed elsewhere.[81] Among those, only two spontaneously progress to steatohepatitis and fibrosis: nuclear sterol regulatory element-binding protein 1c (nSREBP-1c) transgenic mice and the *PTEN* knockout mice. nSREBP-1c transgenic mice overexpress nSREBP-1c in adipose tissue under the control of the adipocyte-specific aP2 enhancer/promoter. These mice are characterized by a disordered differentiation of adipose tissue, marked insulin resistance, diabetes mellitus, fatty liver with inflammatory cell infiltration, and pericellular fibrosis in mice aged 20 weeks or more.[82,83] *PTEN* is a tumor suppressor gene and a negative regulator of several signaling pathways implicated in insulin signaling, apoptosis, cell proliferation, and tumor formation.[84] A hepatocyte-specific null mutation of *PTEN*

may be generated in mice with the Cre-loxP system (AlbCrePtenflox/flox mice). Steatosis develops at 10 weeks of age, and steatohepatitis and fibrosis are present at 40 weeks of age. Moreover, two-thirds of the animals have HCC by 74–78 weeks of age.[85]

As NAFLD transgenic models rarely progress to severe stages of disease, a "second hit" is often necessary, such as the MCDD or a high fat diet (HFD), to favor NASH and fibrosis development.[86] The low-density lipoprotein receptor deficiency mouse (LDLR–/–) is a widely-used hypercholesterolemic atherosclerosis model. When fed a HFD, middle-aged *LDLR–/–* mice develop NASH associated with metabolic syndrome. In this model, aging and the LDLR deficiency status contribute to the development of a NASH severe phenotype. Middle-aged *LDLR–/–* mice develop steatosis, inflammation, and fibrosis; while young *LDLR–/–* mice and middle-age wild type mice are protected from inflammation and hepatocellular injury.[87] Fatty Zucker rats (fa/fa rats) have a natural mutation in the leptin receptor and exhibit severe obesity, insulin resistance, and hyperphagia. They have a fatty liver without signs of progression to NASH.[88,89] Administration of an HFD for 8 weeks induces lobular inflammation, ballooning degeneration, and fibrosis.[90] Another attractive "second hit" model is foz/foz mice fed a HFD. These mice have a mutation in the *Alms1* gene that encodes a ciliary protein that interferes with the central control of satiety.[91] Foz/foz mice spontaneously develop obesity, severe insulin resistance, and diabetes, whereas foz/foz mice fed a HFD progress from steatosis to steatohepatitis and pericellular fibrosis after 20-24 weeks.[92] "Second hit strategies" have been also reported using other genetically modified or selected animals with disorders in lipid/glucose homeostasis, including Abc11-/-, pparα-/-, db/db, ApoE-/-mice, and Otsuka Long-Evans Tokushima Fatty (OLETF) rats.[6]

Genetically modified models, cell tracking, and cell targeting

Gene overexpression or silencing

Targeted gene disruption or overexpression in rodents allows for the study of multiple factors associated with hepatic fibrosis and implicated in different signaling cascades related, for instance, to hepatocyte necrosis/apoptosis, growth factor-dependent fibrosis, immune response, and inflammatory cytokines. Constitutive, inducible, and/or cell specific gene manipulations are often used in combination with precited models to examine the impact of a specific pathway on the fibrotic process.[93–95] Some of these models disrupt key fibrogenic components or alter hepatocyte function and spontaneously cause liver fibrosis. These include: transgenic mice overexpressing transforming growth factor beta1 (TGF-β1) that spontaneously develop liver fibrosis;[96] liver-specific and inducible overexpression of platelet-derived growth factor-beta (PDGF-β) that induce HSCs activation and liver fibrosis;[95] MDR2 deficiency that causes biliary fibrosis;[39] and hepatospecific c-myc overexpression that is associated with HSC activation.[97] Accordingly, these transgenic mice may be used as disease models.

Single cell gene expression modulation, fate tracing, and targeting: the example of hepatic stellate cells

HSCs are key effector cells in hepatic fibrosis, and the understanding of HSC biology is crucial for the identification of novel targets for antifibrotic therapy. In the last two decades, the isolation and culture of primary HSCs, that in some respects recapitulates the activation process undergone during *in vivo* fibrogenesis, have been and remain a fantastic tool to study features of HSCs. This reductionist model, however, is artificial and not sufficient to evaluate all aspects of HSC biology. Gene expression patterns of HSCs isolated from animals with CBLD or CCl_4-induced liver fibrosis (*in vivo* activated) are significantly different from changes in genes expression during culture-activation of primary HSCs (*in vitro* activated).[98,99] Similarly, HSCs isolated from normal human liver and activated in culture express genes related to fibrogenesis and contractility while activated HSCs isolated from cirrhotic patients have a different gene expression profile related to ECM, inflammation and apoptosis.[100] The different phenotype of HSCs, whether they are activated *in vivo* or *in vitro,* suggests an important role of cell-cell and cell-ECM interactions in the control of HSC biology. Taken together, these findings demonstrate the need to develop and use tools able to experimentally target and manipulate HSCs *in vivo.*

Stellate cell specific gene silencing can be obtained using the *Cre* recombinase system under the control of the glial fibrillary acid protein (GFAP) promoter (*GFAP-Cre*) that is activated in resting HSCs. Using this approach, the role of autophagy in HSCs during fibrogenesis has been demonstrated. Deletion of the autophagy-related protein 7 (ATG7) mediated by the *Cre* recombinase under the control of the GFAP promoter reduces matrix deposition and liver fibrosis following CCl_4 or TAA injury.[101] GFAP-dependent gene overexpression or silencing in HSCs may also be combined with the tetracycline-responsive system (TRE) to reversibly control gene expression.[102] In many cell types, including HSCs, tumor suppressor p53 participates in senescence. Inducible *p53* specific silencing in HSCs can be generated by crossing mice harboring a TRE driven short hairpin RNA (shRNA) capable of efficiently suppressing *p53* expression with mice harboring a tetracycline-controlled transactivator (tTA) transgene expressed from the GFAP promoter. In the absence of tetracycline, tTA is expressed in HSCs, binds the TRE promoter, and drives shRNA transcription that suppresses *p53* expression.[103] Interestingly, this specific suppression of *p53* in HSCs leads to an increase in activated HSCs, ECM deposition, and fibrosis after CCl_4 exposition, suggesting a role for senescence in HSCs in limiting fibrosis reaction.[103] Deletion in HSCs may be reached in mice expressing the herpes simplex virus-thymidine kinase (HSV-Tk) gene driven by the GFAP promoter. In response to ganciclovir, only the proliferating HSCs are affected and depleted, allowing for the study of activated HSCs depletion on liver injury and repair. Using this system in the CCl_4 and in the CBDL model, not only was the expression of HSC activation markers decreased but liver fibrosis was significantly reduced.[104] Caution should be taken regarding these results, as GFAP expression has recently been localized in cholangiocytes as well.[105]

For several years, the nature and fate of matrix-producing cells during fibrosis induction, maintenance, and resolution has been a question of great interest. HSCs and portal fibroblasts are considered the major contributors of ECM production in the fibrotic process since they may activate into myofibroblast-like cells during chronic liver injury. Experiments using bone marrow transplantation, chimeric mice, and genetic labeling of epithelial liver cells confirmed negligible, if any, participation of extrahepatic cells or liver

epithelial cells in the production of matrix in the fibrotic liver.[106–108] Recently, Schwabe's team developed a new transgenic mouse model to perform fate mapping in HSCs and demonstrated that HSCs are the major contributor of the myofibroblast pool in CCl_4-induced liver fibrosis.[105] Mice expressing the Cre recombinase under the lecithin-retinol acyltransferase (Lrat) promoter were crossed with mice expressing ZsGreen Cre reporter. As Lrat expression is restricted to HSCs and undetectable in other liver cell types, including portal myofibroblasts, the system allow for specific tagging of 99% of HSCs.[105] In CCl_4-induced liver fibrosis, cells expressing fluorescent ZsGreen have an overlap of more than 90% with alpha-smooth muscle actin (αSMA) expressing cells, providing further evidence that HSCs are the major contributor to the myofibroblast pool. Moreover, in mice coexpressing the red-fluorescent LratCre reporter tdTomato and a green-fluorescent collagen-GFP reporter, there was a strong overlap between red and green cells, demonstrating that HSCs are a major cell source of collagen in the fibrotic liver. These results are confirmed in the TAA-induced liver fibrosis model and models of cholestatic fibrosis, such as CBDL, DDC-containing diet, and *Mdr2*$^{-/-}$ mouse models. In these cholestatic models, αSMA- and collagen-GFP positive but LratCre Tomato negative cells were described around the portal tracts and considered to be portal fibroblasts, while the population of >89% positive matrix producing activated HSCs expanded in fibrotic areas.[105] Such experiments confirm that portal myofibroblasts and activated HSCs are two distinct cell populations, with different origins and contributions to fibrogenesis.

Genetic cell tracing tools have also been used to elucidate the fate of activated HSCs/myofibroblasts during fibrosis resolution.[19] In Col-α2(I)$^{Cre\text{-}YFP}$ mice and Col-α1(1)$^{Cre\text{-}YFP}$ mice, induction of collagen expression in HSCs and myofibroblasts during fibrogenesis drives the expression of yellow fluorescent protein (YFP). The latter will remain during the entire life of the cell. Upon CCl_4-induced fibrogenesis, α-SMA positive activated HSCs and myofibroblasts coexpress YFP. After cessation of CCl_4 administration, α-SMA positive cells gradually decrease in number and are undetectable 1 month after the last CCl_4 dose, while YFP positive cells persist in liver parenchyma. This provides strong experimental evidence that a pool of previously activated HSCs reverts to an inactive phenotype during fibrosis regression.[109] In collagen-GFP mice characterized by a collagen-driven GFP expression, activated HSCs undergo apoptosis in the earliest stage of fibrosis resolution, as colocalization of caspase-3 and GFP are observed in the liver of mice 7 days after CCl_4 cessation.[109] Collagen–I degradation seems to be critical during spontaneous fibrosis recovery, since a mutation in collagen-I that confers resistance to collagenase leads to persistent HSCs activation and reduced HSCs apoptosis.[110]

Such transgenic models or other systems for genome edition such as the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system[111] will undoubtedly be used more commonly for time- and cell-type specific control of gene expression or silencing. Use of these techniques will provide deeper insight into decisive signaling pathways implicated in HSC-dependent fibrogenesis and help to identify targets for therapeutic purposes.

Drug delivery systems are designed to specifically target compounds to specific cell types. As reviewed elsewhere, several systems are available for targeting hepatocytes, Kupffer cells, sinusoidal endothelial cells, cholangiocytes, and stellate cells.[112] Vitamin A-coupled liposomes are being developed for HSC targeting. The strategy is based on the ability of HSCs to store retinol, a function shared with no other hepatic cell. Small interfering RNA (siRNA), gene vectors or drugs may be encapsulated in liposomes for specific delivery to HSCs.[113,114] *In vitro* testing confirm that Vitamin A-coupled liposomes could deliver a potential anti-fibrotic compound into HSCs and reduce the level of fibrotic factors *in vitro,* whereas most of the liposomes localize to the liver with very little spread to other organs after *in vivo* administration.[113,114] Albumin-based carriers that bind to receptors highly expressed on activated HSCs (such as the mannose 6-phosphate/insulin-like growth factor II receptor, the collagen type VI receptor, and the platelet-derived growth factor beta (PDGF-β) receptor)[112] or coupled to single cyclic peptide[115] have been designed to deliver drugs to HSCs with high efficiency.

Mice with humanized livers

Mice fail to reproduce the whole spectrum of pathological aspects observed in humans liver diseases. To overcome this problem, major efforts are devoted to generate mice with humanized livers. To do this, the murine hepatocyte population of immunodeficient mice (to avoid graft rejection) is replaced with human hepatocytes via transplantation, following constitutive or inducible hepatic injury. Four transgenic models are described: the albumin-uroplasminogen activator (uPA) transgenic mouse, the fumarylacetoacetate hydrolase (*Fah*) gene knockout mouse, the TK-NOG transgenic mouse, and the AFC8 transgenic mouse.[116] In all systems, the repopulated liver shows normal hepatocyte function and morphology and respects a normal hepatic architecture with typical zonation. A high (>70%) rate of hepatocyte repopulation is reached in the uPA transgenic mouse, the *Fah* gene KO mouse, and the TK-NOG mouse[116] but not in the AFC8 transgenic mouse (15–25%).[117] In the AFC8 transgenic mouse, not only is the liver repopulated by human hepatocytes but a functional human immune system is also reconstituted after the injection of CD34+ human hematopoietic stem cells.[117]

Several applications for mice with humanized livers have been described: infectious diseases, liver gene therapy, stem cell biology, drug metabolism, and modeling of human genetic disease.[116] The first aim of human-murine chimeric liver mouse model development was establishment of a permissive liver to viral infection. Although hepatitis B (HBV) and C (HCV) viruses infect and replicate in human hepatocytes, they do not infect rodents, since their hepatocytes do not support virus entry and replication. Several years ago, transgenic mouse models were developed that express the whole genome or individual genes of HBV or HCV. As the mouse immune system tolerates the transgenetically expressed viral proteins, infection develops without liver inflammation and without liver fibrosis.[117] The humanized uPA, the *Fah–/–*, and the TK-NOG transgenic mice support HBV/HCV infection and replication. Unfortunately, as they lack a functional human immune system, they do not allow for the study of the host immune response. The AFC8 and A2/NSG/Fas humanized mouse models have both human liver and human immune cells, and these models support HCV and HBV liver infection, respectively, leading to viral hepatitis and liver fibrosis.[117,118] These animal models provide an attractive opportunity to study virus induced liver fibrosis.

The humanized mouse models are only recently available and are currently being optimized. In addition to the study of hepatotropic viruses, the next challenge will consist of evaluating these humanized mice in several well-described liver disease models, e.g. hepatotoxin fibrosis, NALD, and ALD models. The combination of human hepatocellular metabolism and human immune repertoire will likely provide a relevant system for the study of liver damage and wound healing response in liver diseases. The results of these developments are eagerly awaited. Only the future will tell whether these new models will be instrumental in generating new information regarding disease mechanisms in humans and variation in susceptibility between animals and humans. However, immune-deficiency, immune mismatch or mosaicism, the lack of other human hepatic cell types like HSCs, the variable rate of human hepatocyte engraftment, and the inevitable interactions between the humanized liver and the non-humanized extrahepatic environment might be major limitations of such approaches.

Conclusions

The human liver is a complex organ with cell-cell and cell-matrix interactions and extrahepatic crosstalk. This complexity likely underlies the difficulty in developing animal models able to recapitulate liver diseases as global entities with relevant metabolic and immunologic backgrounds and specific hepatic features. Although the classical animal models have yielded major progress in the understanding of fibrogenesis, they are not sufficient to investigate all components implicated in the pathogenesis of human liver diseases. Today, the generation of tools that allow for the study of particular pathways, soluble factors, and cellular effectors by using either cell- and time-specific genome edition or cellular targeting is of major interest. In addition, the humanized liver mouse model represents a promising perspective, particularly when used in combination with a functional human immune system. Indeed, utilizing such experimental manipulations will likely allow for a greater understanding of human liver fibrosis pathogenesis and the identification of specific novel targets for effective antifibrotic therapies.

Conflict of interest

None

Author contributions

Writing the paper (BD, PS, IL).

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