Using CRISPR/Cas9 to model human liver disease

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Summary

CRISPR/Cas9 gene editing has revolutionised biomedical research. The ease of design has allowed many groups to apply this technology for disease modelling in animals. While the mouse remains the most commonly used organism for embryonic editing, CRISPR is now increasingly performed with high efficiency in other species. The liver is also amenable to somatic genome editing, and some delivery methods already allow for efficient editing in the whole liver. In this review, we describe CRISPR-edited animals developed for modelling a broad range of human liver disorders, such as acquired and inherited hepatic metabolic diseases and liver cancers. CRISPR has greatly expanded the repertoire of animal models available for the study of human liver disease, advancing our understanding of their pathophysiology and providing new opportunities to develop novel therapeutic approaches.

Introduction

Genetically engineered animals are powerful tools for the study of hepatic diseases. Previous approaches for creating disease models, such as recombinase-based genome engineering, were time-consuming and costly. CRISPR/Cas9, short for clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9, has become a powerful tool for modeling liver diseases. CRISPR/Cas9 systems are bacterial defence mechanisms to combat bacteriophage infection; mediated by a host RNA sequence complementary to the viral DNA, the bacterial Cas9 introduces a double-strand break into the invading genome. These "molecular scissors" have been intensively studied[1–6] and repurposed for applications in mammalian cells.[7–10] Engineered CRISPR/Cas9 systems can introduce a double-strand break into virtually any DNA sequence. These double-strand breaks are most often repaired by the error-prone non-homologous end joining (NHEJ) pathway, which will introduce frameshift mutations or deletions that inactivate genes. Hence CRISPR is very efficient for destroying or inactivating genes (introducing mutations), with current efforts focused on increasing the efficiency of homology-directed repair (template-mediated repair), which would allow for the precise insertion of new sequences and could be used to correct genes.[11]

These recent advances enable the rapid generation of embryonic and somatic modifications in animal models. Excellent reviews cover the development and application of CRISPR/Cas9,[11–15] therefore, we will focus specifically on disease modelling for the liver. We will first elaborate on some of the approaches used to generate CRISPR/Cas9-modified animals, before discussing recently published examples of human liver disease models.

CRISPR strategies to model liver disease

CRISPR/Cas9 gene editing has enabled the efficient modification of embryonic and somatic cells (Fig. 1). Liver disease models can be generated by manipulating either cell type, each having distinct advantages and drawbacks. CRISPR/Cas9 gene editing can be used to modify embryonic or somatic cells to create relevant disease models.

Embryonic genome engineering

Traditional strategies to genetically modify animals relied on the replacement or deletion of alleles via homologous recombination using embryonic stem cells (ESCs). The modified ESCs were injected into blastocytes to generate a chimera derived from modified and endogenous ESCs. In a subsequent step, the chimeric mice were bred, and if germline transmission was achieved, a genetically modified animal model was created. Due to its complexity and inefficiency, this process was arduous and expensive.[16,17] Because the first ESCs were isolated from mouse blastocysts and mouse ESCs were readily available, this work was primarily confined to mice.

CRISPR provides a technically less challenging way to genetically modify animals. Microinjection of sgRNA (single guide RNA: the bioengineered targeting RNA) and Cas9 into zygotes leads to efficient gene editing in murine embryos,[18,19] in many cases circumventing the need for prior

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ESC methods. Moreover, multiple alleles can be successfully targeted at the same time,[18,20] significantly reducing time-consuming breeding procedures. Another advantage of CRISPR editing in zygotes is that there is usually no remaining transgene, especially if Cas9 is delivered as a ribonucleoprotein complex. Traditional ESC targeting requires a selection step, commonly performed with a neomycin resistance cassette, which should be removed so that it does not interfere with hepatic gene expression[21] and trigger silencing.[22] CRISPR/Cas9 injection is now the preferred strategy for generating simple knockouts, introducing point mutations, and in some cases loxP sites for conditional models. It should be noted that insertion of larger sequences still requires targeting and selection in ESCs. Nonetheless, CRISPR has markedly accelerated the development of transgenic animal models and broadened the spectrum of species that can be targeted.[23]

These advances have greatly improved the modelling of human liver disorders and inborn errors of metabolism. Many different strains of mice, rats, rabbits and zebrafish have been generated using CRISPR technology. Moreover, additional animal models have been generated as research tools for hepatology, such as (drug) metabolism models[20,24–26] or potential transgenic sources of human albumin.[27]

Somatic genome engineering

The liver is unique compared to other organs in that it can be transfected *in vivo* via hydrodynamic tail vein injection (HTVI).[28,29] Plasmids expressing Cas9 and sgRNA can be efficiently delivered to hepatocytes with a single injection using HTVI. The only drawback is that a maximum of 30% of the liver (centrilobular) can be transfected with this method. Hence, HTVI alone is insufficient to delete a gene of interest in the whole organ in order to create a liver-specific knockout model. Pankowicz *et al.* recently addressed this limitation by combining HTVI with a growth advantage of CRISPR/Cas9 edited hepatocytes,[30] thus allowing edited hepatocytes to expand and repopulate the whole liver. This technique, called somatic liver knockout (SLiK), was used to create liver-specific knockout models of progressive familial intrahepatic cholestasis-2 (PFIC2) and argininosuccinate lyase (ASL) deficiency[30](see below). A limitation of the SLiK technique is that it is performed in fumarylacetoacetate hydrolase (FAH)-deficient mice,[20,30,31] which require special care due to the

Key Points

CRISPR/Cas9 gene editing can be used to modify embryonic or somatic cells to create relevant disease models.

Somatic genome editing is a valuable tool in liver research, allowing for efficient and practical knockout of genes of interest in the entire liver.

CRISPR/Cas9 gene editing has been used to investigate a number of liver disorders including NAFLD, wherein a number of genes have been identified that increase an individual's susceptibility to the condition

CRISPR/Cas9-mediated gene editing has been a valuable tool for the study of a number of inherited liver disorders.

CRISPR/Cas9 gene editing has been very useful in improving our understanding of the complex mutational profiles of liver cancers, with new models likely to lead to further advances.

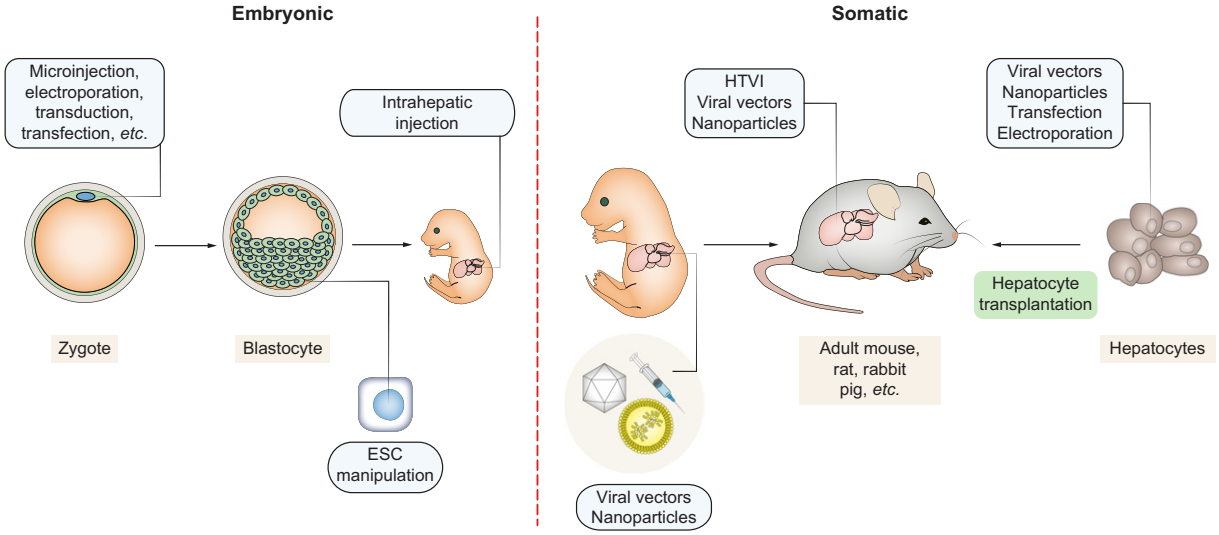

Fig. 1. Embryonic and somatic manipulations for generation of CRISPR disease models. Microinjection of CRISPR/Cas9 is the state-of the art for zygote manipulations, but other deliver methods such as electroporation have been described.[106,107] CRISPR can also be used for traditional embryonic stem cell targeting or direct embryonic editing by injection of the vitelline vein during pregnancy (E16).[108] Nanoparticles, viral vectors and HTVI can be used for somatic gene editing. Hepatocytes can also be edited using the methods depicted, establishing new disease models upon transplantation and repopulation of the liver. ESC, embryonic stem cell; HTVI, hydrodynamic tail vein injection.

hepatotoxicity caused by FAH deficiency. *Fah*$^{-/-}$ mice are administered the small-molecule drug nitisinone,[32] which inhibits the protein hydroxyphenylpyruvate dioxygenase (HPD) to rescue the wild-type phenotype. The rational of SLiK-mediated gene editing is that the simultaneous targeting of *Hpd* and a separate gene of interest will confer a selection advantage to *Hpd*-deficient hepatocytes upon withdrawal of nitisinone. A major advantage of this approach is that hepatocytes will clonally expand, propagating cells with the same genetic modifications.

CRISPR can also be combined with other delivery systems (viral or non-viral)[33] to generate organ-specific knockout models. For instance, an adenovirus vector was the first viral vector used for somatic genome editing in the liver, and succeeded in very efficient elimination of *Pcsk9*[34] and *Pten*.[35] The development of smaller orthologues of Cas9,[36,37] has made it possible to perform efficient liver-directed genome editing with adeno-associated viruses (AAV), which are currently the leading vector for liver gene therapy in humans. AAV vectors based on serotype 8 have now been used by many groups for somatic genome editing in mice.[37–41] Furthermore, lipid nanoparticles can be efficiently taken up by hepatocytes, because of their ability to interact with serum proteins,[42] and have been successfully used in combination with CRISPR/Cas9 gene editing.[43,44] A disadvantage of viral and nanoparticle delivery of CRISPR/Cas9 is the mosaicism of genetic modifications within the liver. The major advantage is the high efficiency, which allows for removal of a protein of interest across the entire liver within a week or two. Somatic genome editing is a valuable tool in liver research, allowing for efficient and practical knockout of genes of interest in the entire liver.

In summary, somatic genome editing in the liver is a valuable alternative to embryonic manipulations. Knocking out genes of interest in the liver is both efficient and practical. However, inserting precisely altering or correcting genes is very challenging, since homology-directed repair occurs only in dividing cells. Methods that promote homology-directed repair or selective expansion of gene-edited hepatocytes are in development, but much work remains. Given the multiple options for delivery of CRISPR/Cas9 to the liver, it is worth considering somatic genome editing as a faster and higher throughput alternative to embryonic manipulation.

CRISPR models of human liver disease

Non-alcoholic fatty liver disease

CRISPR/Cas9 gene editing has been used to investigate disorders of liver metabolism, such as non-alcoholic fatty liver disease (NAFLD). NAFLD is considered the hepatic manifestation of metabolic syndrome and represents the most common chronic liver disease.[45] Currently, there are no effective therapies for NAFLD apart from lifestyle changes aimed at improving fitness and promoting weight loss.[46] The first CRISPR/Cas9-mediated mouse model of NAFLD was generated by deleting the phosphatase and tensin homolog (*Pten*) gene, a negative regulator of PI3K/AKT activity.[47] Xue *et al.* used HTVI to deliver CRISPR/Cas9-machinery (Cas9 and sgRNA targeting *Pten*) to mice. In a subsequent study by the same group, more efficient liver targeting was achieved using adenoviral vectors.[48] Both delivery methods rapidly decreased PTEN expression in the liver *in vivo*: 4% and 29% of hepatocytes were *Pten*-negative following HTVI and adenoviral-mediated delivery, respectively. Whereas massive hepatic steatosis — a hallmark feature of NAFLD — was observed in both studies, steatohepatitis was only observed after adenoviral vector-delivery in a 4-month study timeframe.[47,48] Importantly, adenoviral vector-delivery led to immune responses towards the viral DNA and the SpCas9 transgene.[48]

Another group delivered CRISPR/Cas9 via HTVI to knockdown *Pten* in the livers of adult rats.[49] In this study, the authors reported that a high dosage of DNA coding for the CRISPR/Cas9 machinery was required for effective gene editing. Nonetheless, successful transfection led to a similar degree of weight gain and hepatic steatosis as observed in wild-type rats fed a high-fat diet.[49]

The transmembrane 6 superfamily member 2 (TM6SF2) variant Glu167Lys is associated with an increased incidence of NAFLD.[50] Fan *et al.* used CRISPR/Cas9 technology to generate *Tm6sf2* knockout mice in order to unravel the functional implications of this protein.[51] While plasma total cholesterol levels were reduced and hepatic expression of lipid-related genes was altered in *Tm6sf2* knockout mice, no significant changes were found in liver triglyceride accumulation either upon normal chow or high-fat diet feeding. The NAFLD phenotype of these mice turned out to be complex and more studies are needed to clarify the role of TM6SF2. CRISPR/Cas9 gene editing has been used to investigate a number of liver disorders including NAFLD, wherein a number of genes have been identified that increase an individual's susceptibility to the condition.

Hereditary tyrosinemia type I

Several groups have used CRISPR/Cas9-mediated editing to study inborn errors of hepatic metabolism (Table 1), which encompass heterogeneous and rare disorders that affect the activity of single or multiple hepatic metabolic pathways[52] and constitute a significant cause of liver transplantation in paediatric patients.[53] One example of such an inborn error is the hereditary tyrosinemia type I (HT-1). HT-1 is caused by a deficiency of FAH, the last enzyme in the catabolic pathway of tyrosine.[54–56] FAH deficiency results in the

Table 1. CRISPR-based animal models of liver disorders.

Disease	Model	Target	Tissue-specificity	Approach	Phenotype	Comments	Reference
Argininosuccinate lyase deficiency	Mouse	*Asl*, loss-of-function	Liver-targeted	SLiK	Hyperammonemia and somnolence		Pankowicz *et al.* 2018[30]
Familial dysbetalipoproteinemia	Rat	*Apoe*, loss-of-function	None	Zygote injection; CRISPR/Cas9	High level of circulating LDL-cholesterol, hypercholesterolemia, hepatosteatosis, atherosclerosis		Zhao *et al.*, 2018[73]
Familial hypercholesterolemia	Mouse, adult	*Ldlr*, loss-of-function	Liver-targeted	Intraperitoneal injection; AAV-CRISPR	High level of circulating LDL, hypercholesterolemia, atherosclerosis	AAV vector integration at CRISPR/Cas9 cut sites	Jarrett *et al.*, 2017[40] and Jarrett *et al.* 2018[71]
	Rat	*Ldlr*, loss-of-function	None	Zygote injection; CRISPR/Cas9	High level of circulating LDL-cholesterol, hypercholesterolemia, hepatosteatosis, atherosclerosis		Zhao *et al.*, 2018[73]
Hereditary tyrosinemia type I	Rat	*Fah*, loss-of-function	None	Embryo injection; CRISPR/Cas9	Hypertyrosinemia, liver fibrosis, cirrhosis		Zhang *et al.*, 2016[58]
Hypermanganesemia with dystonia, polycythemia, and cirrhosis	Zebrafish	*slc30a10*, loss-of-function	None	Embryo injection; CRISPR/Cas9	High level of circulating and hepatic Mn, hepatosteatosis, liver fibrosis		Xia *et al.*, 2017[64]
Niemann-Pick disease type C1	Zebrafish	*npc-1*, loss-of-function	None	Embryo injection; CRISPR/Cas9	Hepatic accumulation of unesterified cholesterol		Tseng *et al.*, 2018[65] Lin *et al.*, 2018[66]
Wilson's Disease	Rabbit	*Atp7b*, knock-in	None	Zygote injection; CRISPR/Cas9	Accumulation of copper in liver and kidney	High frequency of off-target editing was reported	Jiang *et al.*, 2018[60]
Non-alcoholic fatty liver disease	Mouse, adult	*Pten*, loss-of-function	Liver-targeted	Hydrodynamic injection; CRISPR/Cas9	Hepatomegaly, hepatosteatosis		Xue *et al.*, 2014[47]
	Mouse, adult	*Pten*, loss-of-function	Liver-targeted	Tail vein injection; Ad-CRISPR/Cas9	Hepatomegaly, hepatosteatosis, steatohepatitis (NASH-like)	Ad vector-associated immunotoxicity was observed in the liver	Wang *et al.*, 2015[48]
	Rat, adult	*Pten*, loss-of-function	Liver-targeted	Hydrodynamic injection; CRISPR/Cas9	Hepatosteatosis	High dosage of plasmid was required to induce NAFLD	Yu *et al.*, 2017[49]
	Mouse	*TM6SF2*, loss-of function	None	Embryo injection; CRISPR/Cas9	Decreased plasma total cholesterol and LDL	No NAFLD phenotype	Fan *et al.* 2016[51]
Progressive familial intrahepatic cholestasis type 2	Zebrafish	*abcb11b*, loss-of-function	None	Embryo injection; CRISPR/Cas9	Impaired bile excretion, hepatocellular injury, induction of autophagy in hepatocytes		Ellis *et al.*, 2018[76]
	Mouse	*Abcb11*, loss-of-function	Liver-targeted	SLiK	Impaired bile excretion with increase of bile acid in serum		Pankowicz *et al.* 2018[30]
Hepatocellular carcinoma Intrahepatic cholangiocarcinoma	Mouse, adult	Ten tumour suppressors, loss-of-function	Liver	HTVI, CRISPR-Cas9 vector flanked by SB repeats	Tumour growth	No off-target effects found by amplicon-based NGS	Weber *et al.*, 2015[84]
Hepatocellular carcinoma	Mouse, adult	*Nf1*, *Plxnb1*, *Flrt2*, *B9d1*, loss-of-function	None	Subcutaneous transplantation of CRISPR/Cas9 edited $p53^{-/-}$; Myc hepatoblasts (lentivirus)	Tumour growth		Song *et al.*, 2017[87]

(continued on next page)

Table 1 (*continued*)

Disease	Model	Target	Tissue-specificity	Approach	Phenotype	Comments	Reference
Hepatocellular carcinoma	Mouse, adult	56 known or putative tumour suppressors, loss-of-function	None	CRISPR AAV	Tumour growth		Wang *et al.*, 2018[88]
Hepatocellular carcinoma	Mouse, age uknown	*Nras* ,gain-of-function and *Pten*, loss-of-function	Liver	SB, CRISPR-Cas9, HTVI	Tumour growth, excessive lipid deposition in hepatocytes		Gao *et al.*, 2017[109]
Hepatocellular carcinoma	Mouse, adult HBV transgenic mice	*p53* and *Pten*, loss-of-function	Liver	CRISPR-Cas9, HTVI	Macroscopic tumour growth		Liu *et al.*, 2017[93]
Fibrolamellar hepatocellular carcinoma	Mouse, adult	*Dnajb1-Prkaca* gene fusion	Liver	CRISPR-Cas9, HTVI	Tumour growth		Engelholm *et al.*, 2017[97]
Fibrolamellar hepatocellular carcinoma	Mouse, adult	*Dnajb1-Prkaca* gene fusion	Liver	CRISPR-Cas9, HTVI	Tumour growth		Kastenhuber *et al.*, 2017[96]
Intrahepatic cholangiocarcinoma	Mouse, adult	*p53* and *Pten*, loss-of-function	Liver	CRISPR-Cas9, HTVI, carbon tetrachloride	Tumour growth		Xue *et al.*, 2014[47]
Intrahepatic cholangiocarcinoma	Mouse, age unknown	$HRAS^{G12V}$ gain-of-function, *p53* loss-of-fucntion	Liver	HTVI; CRISPR homology-independent target integration	Tumour growth		Mou *et al.*, 2019[91]

AAV, adeno-associated viruses; HTVI, hydrodynamic tail vein injection; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NGS, next-generation sequencing; SLiK, somatic liver knockout.

accumulation of hepatotoxic catabolites leading to cirrhosis, hepatocellular carcinoma (HCC), or metabolic decompensation in early childhood. The first *Fah*-deficient animal model, the albino lethal c14CoS mouse, was generated 40 years ago.[31] Nevertheless, there is wide interest in generating new variants of this popular disease model. CRISPR/Cas9 and microinjection technology have been used to develop $Fah^{-/-}$ mice[20,57] and rats.[58] Rats have become an increasingly popular animal for CRISPR disease models. For one, zygote injection is very efficient in rats. Additionally, rats have some distinct biological advantages over mice; for example, the ease with which liver fibrosis forms in the $Fah^{-/-}$ animals.[58] In contrast to *Fah*-deficient mouse models, $Fah^{-/-}$ rats and pigs develop hepatic fibrosis and cirrhosis, more accurately mimicking the human pathophysiology.[58,59]

Wilson disease

Jiang *et al.* created precision point mutations, using CRISPR technology in rabbits, to develop a model of Wilson disease (WD).[60] WD is an autosomal recessive genetic disorder caused by mutations in the membrane copper transporter gene, *ATP7B*.[61,62] The authors targeted the *Atp7b* gene with sgRNAs and provided a single-stranded donor oligonucleotide template for homology-directed repair, to obtain the missense mutation Arg778Leu, a common disease mutation in Asian populations.[60] When comparing zygotes obtained from donor rabbits 14 h or 19 h after human chorionic gonadotropin treatment, the authors found that injections performed in pronuclear embryos at earlier stages resulted in higher rates of point mutation (over 50%) and reduced gene knockout. *Atp7b* mutant rabbits exhibited copper accumulation in the liver and kidney, as observed in human WD, and died at an early age of 3 months. This study reported that off-target mutations were transmitted to offspring, emphasising the necessity of backcrossing mutant lines generated by zygote injection.

Hypermanganesemia with dystonia, polycythaemia, and cirrhosis

Due to their high degree of homology with humans and utility in high-throughput phenotypic screenings, zebrafish are a useful vertebrate model for human metabolic liver disorders. Autosomal recessive mutations in the human *SLC30A10* gene, which encodes a manganese transporter, are associated with hypermanganesemia with dystonia, polycythaemia, and cirrhosis (or HMDPC). This disease is characterised by increased systemic levels of manganese, which accumulates in the liver and basal ganglia, causing a wide range of hepatic

dysfunctions (*e.g.*, steatosis, fibrosis, and cirrhosis) and parkinsonian-like syndrome.[63] Zebrafish *slc30A10* mutants were generated through the injection of a sgRNA and Cas9 mRNA into single-cell embryos, leading to the knockdown of this manganese transporter.[64] Mutant animals exhibited increased systemic levels of manganese along with liver steatosis, fibrosis, and neurological defects. Like humans, *slc30A10*-mutant zebrafish were responsive to disodium calcium EDTA and ferrous fumarate therapies, which partially rescued the wild-type phenotypes.

Niemann-Pick disease type C1

Two groups independently reported zebrafish models for Niemann-Pick disease type C1 (NPC1).[65,66] NPC is a rare autosomal recessive disease caused by the accumulation of cholesterol in late endosomes/lysosomes.[67] Currently, there are no effective therapies for NPC. Mutations in either *NPC1* or *NPC2* genes cause NPC, and both genes encode lysosomal proteins involved in the transport of cholesterol from the endolysosomal lumen to other intracellular organelles. Common manifestations of NPC1 include hepatomegaly and severe cirrhosis, along with progressive neurodegeneration.[68] Tseng *et al.*[65] and Lin *et al.*[66] utilised embryo injection with sgRNA and Cas9 mRNA to generate *npc1* knockout zebrafish. Deficiency of *npc1* recapitulated both the early-onset hepatic disease and the later neurological disease observed in patients with NPC1, namely, cholesterol accumulation in the liver and symptoms of ataxia. Of note, *npc1* mutant larvae displayed a dark liver phenotype that facilitated the genotypic screening of live animals. In addition, a robust increase in *in vivo* LysoTracker Red staining was observed in *npc1* mutants as early as 3 days post fertilisation.[65] The ability to rapidly access pathophysiological readouts highlights the advantage of using CRISPR/Cas9-edited animals to accelerate large-scale phenotypic screening, which could be used to evaluate candidate drugs and compounds for new therapies.

Argininosuccinate lyase deficiency

Animal models of the urea cycle disorder ASL deficiency have also been generated using CRISPR/Cas9. The knockout mouse ($Asl^{-/-}$) is neonatally lethal and is therefore of limited use.[69] Using CRISPR and applying SLiK,[30] edited $Asl^{-/-}$ hepatocytes gradually replace the murine liver, allowing animals to be used for metabolic studies. Once $Asl^{-/-}$ hepatocytes fully replace the murine liver, animals undergo metabolic crisis and have to be euthanised. The degree of replacement can be regulated and halted at any degree (30–100%) of chimerism of $Asl^{-/-}$ and $Asl^{+/+}$ hepatocytes. Thus, SLiK allows for the study of milder phenotypes of ASL deficiency commonly observed in humans.

Familial hypercholesterolemia

Another metabolic disorder where CRISPR disease modelling has been applied is familial hypercholesterolemia (FH), an autosomal co-dominant disorder predominantly caused by mutations in the low-density lipoprotein receptor (*LDLR*) gene. Mutations in *LDLR* lead to impaired LDL uptake, hypercholesterolemia and, ultimately, severe atherosclerotic vascular disease.[70] Jarrett *et al.* generated the first inducible model of FH using AAV-mediated delivery of sgRNA targeting *Ldlr* in adult Cas9 transgenic mice.[40] *Ldlr*-edited mice exhibited increased plasma levels of LDL-cholesterol and developed atherosclerosis, thus recapitulating the human FH pathology. Follow-up work by the same group showed that delivery of CRISPR/Cas9 could be achieved with a single AAV vector,[71] and that this approach is a viable alternative to the traditional germline model used to study atherosclerosis.[72] These studies also identified integration of AAV vector sequences into CRISPR target sites, an important concern for therapeutic applications.

Fatty liver phenotypes similar to those observed in *Ldlr*-edited mice were also found in rats upon CRISPR/Cas9-mediated deletion of apolipoprotein E (*Apoe*).[73] ApoE is a component of lipoprotein remnants; its deficiency in humans leads to decreased clearance of remnants and an increased risk of atherosclerosis, a condition known as familial dysbetalipoproteinemia.[74] CRISPR/Cas9-mediated deletion of *Ldlr* and *Apoe* (either individually or in combination) was achieved by zygote microinjection of sgRNA and Cas9 mRNA, generating adult rats that exhibit hypercholesterolemia, hepatic steatosis, and atherosclerosis.[73]

Primary hyperoxaluria type I disease

Recently, Zheng *et al.* generated a rat model of primary hyperoxaluria type I disease.[75] The authors deleted alanine-glyoxylate aminotransferase in rat zygotes, resulting in severe nephrocalcinosis due to the formation of oxalate crystals, as seen in humans. Nevertheless, ethylene glycol in the drinking water was required to induce nephrocalcinosis in both rats and mice, which illustrates that animals often differ from humans when it comes to metabolism. CRISPR/Cas9-mediated gene editing has been a valuable tool for the study of a number of inherited liver disorders.

Progressive familial intrahepatic cholestasis type 2

CRISPR/Cas9-mediated gene editing has also been used to investigate PFIC2,[76] a rare autosomal recessive disorder caused by defects in bile secretion.[77] PFIC2 is caused by mutations in the ATP binding cassette family B, member 11 (*ABCB11*) gene, which encodes a bile salt export pump expressed in the apical membrane of hepatocytes involved

in the transport of monovalent bile salts across the canalicular membrane.[78] Patients with PFIC2 present with early-onset fibrosis and rapidly progress to end-stage liver disease during childhood, for which liver transplantation remains the only effective treatment.[77]

Traditional ESC targeting has been used to generate *Abcb11* deficient mice (*Abcb11*$^{-/-}$), however, offspring often die due to maternal cholestasis.[79] Producing sufficient numbers of mice to study PFIC2 is therefore challenging. SLiK is a useful alternative method to generate liver-specific *Abcb11* deletion.[30] The *Abcb11*^{slik} mice had bile acid levels comparable to the conventional *Abcb11*$^{-/-}$ mice and should be a useful new mouse model. Ellis *et al*. recently deleted *abcb11b* (the orthologue of *Abcb11*) in zebrafish using CRISPR.[76] In this work, *abcb11b* was disrupted by embryo microinjection of sgRNA and Cas9 mRNA, leading to the near absence of Abcb11b protein in the liver.

Impaired bile salt excretion and increased hepatocyte autophagy occurred early in *abcb11b* mutants and rapidly progressed to hepatocellular injury, as in patients with PFIC2. Thus, these newly generated CRISPR/Cas9-edited zebrafish provide a useful *in vivo* tool to investigate the hepatocellular mechanisms underlying the pathogenesis of PFIC2.

Hepatocellular carcinoma

Liver cancer is the second most lethal cancer worldwide.[80] The increasing mortality of both HCC[81] and intrahepatic cholangiocarcinoma (ICC)[82] reflect the lack of therapeutic alternatives, as well as our inability to model liver cancer and thus understand its molecular basis. Manipulating mouse genetics through CRISPR is now widely adopted in cancer biology[83] and has revolutionised the previously laborious undertaking of *in vivo* cancer modelling.

Using a sleeping beauty transposase vector system, another group generated a multiplex CRISPR knockout approach targeting 10 different tumour suppressor genes.[84] Again, the CRISPR/Cas9 was introduced using HTVI, however the CRISPR/Cas9 machinery was randomly integrated into the host genome. Hence, it is unclear how often the CRISPR-deleted genes(s) or the integrating transposon vector account for tumorigenesis. Insertional mutagenesis is a major limitation of forward genetic CRISPR screenings when using transposons or lentiviral vectors.[85,86] The latter was used recently in a CRISPR knockout screen targeting 20,611 genes in *p53*$^{-/-}$, *Myc* overexpressing hepatoblasts.[87] In this study, cells were transduced *ex vivo* with a lentiviral sgRNA library and then transplanted subcutaneously. After mice developed HCC, the authors sequenced tumours for sgRNA enrichment to identify cancer driver mutations and used HTVI to confirm identified genes and exclude integrational mutagenesis. Using a slightly different approach to identify cancer-driving mutations, another group designed an sgRNA library targeting 56 recurrently mutated genes but excluding known oncogenes.[88] In contrast to previous work, the library used a non-integrating, episomal AAV vector. Tumours were analysed by a targeted capture sequencing approach of sgRNA target sites. Although more elegant, this targeted approach is somewhat limited by the number of genes analysed. CRISPR/Cas9 gene editing has been very useful in improving our understanding of the complex mutational profiles of liver cancers, with new models likely to lead to further advances.

Intrahepatic cholangiocarcinoma

The first model of liver cancer that used CRISPR involved the deletion of the tumour suppressor genes *p53* and *Pten,* in combination with exposure to the carcinogen carbon tetrachloride.[47] Both tumour suppressors are frequently mutated in human ICC.[89] Xue *et al.* targeted *p53* and *Pten,* individually and in combination, by utilising HTVI of plasmids expressing Cas9 and sgRNAs. Consistent with previous studies, mice treated with either a *p53* or *Pten* sgRNA did not exhibit liver tumours 3 months post-injection, whereas all mice that received a combination plasmid developed tumours with bile duct differentiation characteristics. The resulting murine tumours also had the histological appearance of ICC and stained for the cholangiocyte marker cytokeratin 19.

In contrast to loss-of-function, generating gain-of-function alleles by CRISPR/Cas9 gene editing is more challenging, as it requires precise editing or genomic insertion of mutated oncogene cassettes. Generally, such accurate genomic alterations require homology-directed repair, which is inefficient and typically takes place during the G2/S phase of the cell cycle. Homology-independent target integration was recently developed to enable CRISPR-based targeting[90] of quiescent organs such as the liver. A similar technique was used to generate a model of ICC through integration of the oncogene *HRAS*^{G12V} in combination with deletion of *p53*.[91]

The genetic alterations driving tumorigenesis are preceded by underlying environmental or inherited factors that damage the liver. For instance, HBV infection is endemic in some areas of the world, and since there remains no curative therapy for this major pathogen, HBV accounts for 33% of worldwide liver cancer.[92] To better address its underlying aetiology, Liu *et al.* used HBV transgenic mice in combination with CRISPR/Cas9 mediated deletion of *p53* and *Pten*.[93] Although the authors deleted the same 2 tumour suppressor genes as in a previous study,[47] their tumours presented as HCC not ICC and were cytokeratin 19-negative. The reason for this discrepancy is unclear despite the

distinct settings, namely carbon tetrachloride or transgenic HBV. Nevertheless, the results established in the HBV transgenic mice imply *p53* and *Pten* deletion might act as oncogenic drivers in HBV-induced HCC, in agreement with clinical datasets.[94]

Fibrolamellar hepatocellular carcinoma

Finally, CRISPR-Cas gene editing has given rise to another model of liver cancer, fibrolamellar hepatocellular carcinoma (FL-HCC). FL-HCC is a rare form of cancer that usually occurs in young adults and is caused by a 400 kb deletion on chromosome 19, leading to a fusion protein DNAJB1-PRKACA.[95] Two groups used CRISPR to generate the *Dnajb1–Prkaca* fusion in mice.[96,97] Both groups targeted Cas9 to the first introns of *Dnajb1* and *Prkaca* to introduce double-stranded cuts in these regions, mimicking the chromosomal breaks occurring in human FL-HCC. When the genomic DNA fragment was excised in mice, the cell repair machinery utilised NHEJ to repair the DNA cuts, thereby creating the disease-associated *Dnajb1-Prkaca* gene fusion. The resulting tumours were consistent with the cytological and histological features (accumulation of mitochondria, prominent nucleoli, *etc.*) of FL-HCC.

CRISPR has facilitated the strenuous efforts of cancer gene discovery and the generation of knockout/knock-in models. For the first time, somatic gene editing in living animals is not only achievable but it is also efficient. The rapid development of liver cancer mouse models has created a new avenue to discern the mutational landscape of one of the world's deadliest cancers. CRISPR has already contributed to the discovery of a vast number of hepatic tumour suppressors and oncogenes, and it will continue to redefine our understanding of the various alterations occurring in liver tumours.

Conclusions and outlook

CRISPR/Cas9 gene editing is being rapidly integrated into biomedical research to generate new disease models for the liver. Although the mouse remains the most popular model organism, CRISPR/Cas9 editing is increasingly performed in other species such as rats, pigs and rabbits. Some experimental animals have metabolic features that closely resemble human metabolism. Hence it is worth considering studies in other species depending on the specific research question; for example, the CRISPR rat model for liver fibrosis or the rabbit model for lipoprotein metabolism. Human liver chimeric mice[98,99] could be a good alternative for modelling many metabolic liver disorders, particularly if used for validation of macromolecular therapies. The therapeutic effect of macromolecular drugs varies significantly across species, in contrast to small molecules. We have recently described the first xenograft model for metabolic liver disease and used it to validate gene therapy for FH.[100] Such xenograft models could also be generated using CRISPR/Cas9 gene editing and would have the advantage of being relevant in the human context.

The generation of CRISPR knockout animals by zygote injections is much less demanding than traditional techniques. However, it requires careful genotyping because the targeted alleles will harbour different mutations, significant deletions, and off-target editing. Backcrossing is required to eliminate mosaicism, to clearly identify the genetic alteration at the intended site, and to remove possible off-target edits elsewhere in the genome. Somatic genome editing is a very efficient approach for generating liver-specific knockouts with CRISPR/Cas9. Several gene therapy vectors efficiently transduce the liver and have been successfully used with CRISPR to edit liver genes. An alternative, particularly for multiplexing CRISPR or to investigate very severe disease phenotypes, is SliK.[30] In only a few weeks, liver-specific knockout mice can be generated without the need to produce gene therapy vectors. However, somatic gene editing will introduce many different deletions, in contrast to embryonic editing, which results in a single well-defined deletion. In addition, the introduction of patient-specific mutations into murine disease genes is inefficient, as the most widely used CRISPR/Cas9 strategies require a homology-based repair mechanism which is limited by the quiescent nature of the liver. Engineered CRISPR/Cas-associated base editors have recently emerged as an alternative to improve precision genome editing independently of homology-directed repair and double-strand DNA break formation.[101–103] For example, it has been used for somatic gene editing in the liver of adult mice to drive $Ctnnb1^{S45F}$ mutations in a model of HCC[104] and to correct phenylketonuria.[105]

In summary, many useful liver disease models have been and will be generated using CRISPR/Cas9 gene editing. The ease, accessibility, and efficiency of CRISPR have had a profound impact on animal modelling not only for the liver. In light of all the enthusiasm around CRISPR, the remaining challenges such as off-target editing are often neglected. It is conceivable that more accurate CRISPR/Cas systems will be found in bacteria or archaea, further facilitating the generation of disease models. In all, we must take advantage of this latest advance in genome engineering and build a bigger and better collection of models. This will help us to better understand liver disease and eventually develop effective therapies.

Abbreviations

AAV, adeno-associated viruses; ASL, argininosuccinate lyase; Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced short palindromic repeats; ESCs, embryonic stem cells; FH, familial hypercholesterolemia; FL-HCC, fibrolamellar HCC; HCC, hepatocellular carcinoma; HTVI, hydrodynamic tail vein injection; ICC, intrahepatic cholangiocarcinoma; LDLR, low-density lipoprotein receptor; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NGS, next-generation sequencing; NHEJ, non-homologous end joining; SLiK, somatic liver knockout; WD, Wilson disease

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

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Supplementary data

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