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Invited Mini Review

Therapeutic applications of gene editing in chronic liver diseases: an update

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Innovative genome editing techniques developed in recent decades have revolutionized the biomedical research field. Liver is the most favored target organ for genome editing owing to its ability to regenerate. The regenerative capacity of the liver enables *ex vivo* gene editing in which the mutated gene in hepatocytes isolated from the animal model of genetic disease is repaired. The edited hepatocytes are injected back into the animal to mitigate the disease. Furthermore, the liver is considered as the easiest target organ for gene editing as it absorbs almost all foreign molecules. The mRNA vaccines, which have been developed to manage the COVID-19 pandemic, have provided a novel gene editing strategy using Cas mRNA. A single injection of gene editing components with Cas mRNA is reported to be efficient in the treatment of patients with genetic liver diseases. In this review, we first discuss previously reported gene editing tools and cases managed using them, as well as liver diseases caused by genetic mutations. Next, we summarize the recent successes of *ex vivo* and *in vivo* gene editing approaches in ameliorating liver diseases in animals and humans. [BMB Reports 2022; 55(6): 251-258]

INTRODUCTION

The liver is a major organ to maintain homeostasis by promoting detoxification and lipid and protein metabolism. Liver dysfunction results in toxin accumulation, organ damage and the development of pathological conditions. Various liver diseases are caused by gene mutations. Liver transplantation was considered as the only therapeutic option for liver diseases. However, liver transplantation is associated with several limitations, including shortage of liver donors and immune rejection (1). The advent of gene editing methods has enabled the repair of pathological mutations. Thus, gene editing technology will replace liver transplantation as a therapeutic strategy for liver diseases.

The first successful gene therapy in humans was reported approximately 30 years ago. The peripheral blood T lymphocytes transduced with a retrovirus encoding *ADA* have been used in the treatment of patients with adenosine deaminase (ADA) deficiency (Fig. 1) (2). This study demonstrated the benefits of gene therapy and its therapeutic role in genetic diseases, including hereditary liver disorders (3). Over 100 types of liver-related diseases are caused by mutations of a single gene. Hence, gene therapy represents a potential alternative to liver transplantation for treating these disorders.

The first gene therapy for liver disease in humans was not successful. Jesse Gelsinger (aged 18 years) with partial ornithine transcarbamylase (OTC) deficiency, a rare liver disorder caused by a genetic mutation, participated in a clinical trial in which the wild-type OTC-encoding gene was delivered using an adenoviral vector. The patient developed pathological immune response after treatment, which led to multiple organ failure and death (Fig. 1) (4). Several efforts have since been undertaken to overcome the pathological immune response associated with gene therapy.

In the last two decades, various gene editing tools including meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system have been developed and these tools promote double-strand breaks (DSBs) in the target gene. Subsequently, DSBs activate DNA repair responses including non-homologous end joining (NHEJ), leads to the random insertions and deletions (indels) at the site of cleavage and the repair of pathological mutations. Homology-directed repair (HDR) can change the DNA at the cleavage location in the presence of a homologous DNA template. However, the efficiency of HDR is lower than that of NHEJ owing to the frequency of unwanted indels, especially in non-dividing cells. Therefore, alternative strategies are needed to repair point mutations without introducing DSBs.

Komor *et al*. fused CRISPR/Cas9 and a guide RNA and cyti-

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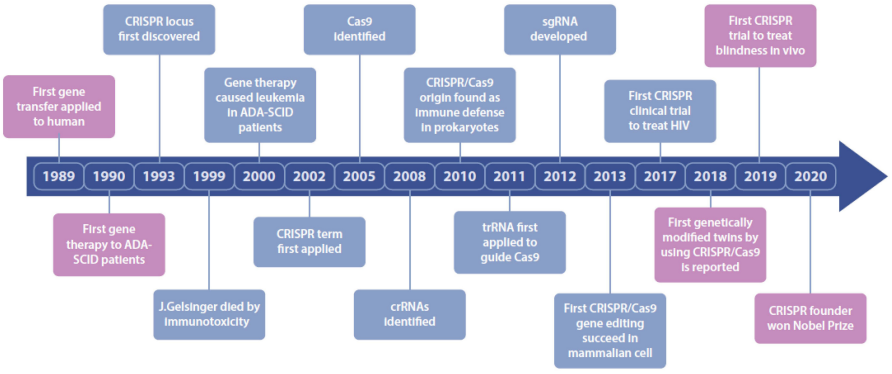

Fig. 1. Timeline of the scientific progress in gene therapy. Purple boxes indicate human trials, and important events, such as human trials and winning Novel prize. ADA-SCID: adenosine deaminase-severe combined immunodeficiency, crRNAs: CRISPR RNAs, trRNA (tracrRNA): trans-activating CRISPR RNA, sgRNA: single-guide RNA.

Table 1. Information about base and prime editors

Editor	Functions	Composition	Hurdles	Ref.
CBEs	C·G to T·A	Cytosine deaminase d/nCas9 (dead (d) or nickase (n)) UGI sgRNA	Cas9-mediated off target Unwanted ssDNA deamination	(5, 6)
ABEs	A·T to G·C	Adenine deaminase d/nCas9 (dead (d) or nickase (n)) sgRNA	Cas9-mediated off target Random deamination	(5, 6)
PEs	Insertion & deletion to any SNV	Reverse transcriptase nCas9 (nickase (n)) pegRNA	Off-target editing Immune response	(7, 8)

CBEs: cytosine base editors, ABEs: adenine base editors, PEs: prime editors, C: cytosine, G: guanine, T: thymine, A: adenine, UGI: uracil glycosylase inhibitor, sgRNA: single-guide RNA, SNV: single nucleotide variants, pegRNA: prime-editing guide RNA.

dine deaminase to convert specific cytidines to uridines, which resulted in a C>T replacement without DSBs. Additionally, there are two classes of DNA base editor: (i) cytosine base editors (CBEs) consist of a dead (d) or a nickase (n) Cas9 (d/nCas9) protein fused to cytosine deaminase and UGI (uracil glycosylase inhibitor) and sgRNA (single guide RNA) to covert C·G into T·A base pairs, (ii) adenine base editors (ABEs) consist of d/nCas9 protein fused to adenine deaminase and sgRNA to covert A·T into G·C base pairs (Table 1) (5, 6). Emmanuelle Charpentier and Jennifer Doudna, who are the pioneers of the CRISPR system, were awarded the 2020 Nobel Prize in chemistry (Fig. 1).

A new method known as "prime editing" has been developed to fix single nucleotide polymorphism (SNP) without DSBs. Prime editors (PEs) require an engineered reverse transcriptase fused to nCas9 and a prime editing guide RNA (pegRNA) that can induce both insertions and deletions in all single nucleotide variants (SNVs) of human cells (7). Prime editors exhibit enormous editing capacities, but the limitations include off-target editing, poor immune response and few delivery options (Table 1) (8).

In this review, we summarize gene mutations related to liver diseases in the descending order of their prevalence and discuss previous approaches to repair these mutations using gene editing tools (Table 2). Successful recent applications of *ex vivo* and *in vivo* gene editing strategies in the livers of mice, monkeys and human patients are discussed.

GENE EDITING IN CHRONIC LIVER DISEASES

Alpha-1 antitrypsin deficiency (AATD)

AATD is one of the most common liver disorder and an autosomal recessive metabolic disease with a high prevalence in Scandinavia and North America. The disease results from c.1096G>A mutation in *SERPINA1*, which encodes alpha-1 antitrypsin (AAT). The mutation causes Glu342Lys substitution (also called Z mutation), which decreases the levels of AAT by 70-85% (9). In addition to the most general clinical symptom of chronic obstructive pulmonary disease (COPD), AATD is associated with hepatic dysfunction that normally presents with high serum aminotransferase levels, hyperbilirubinemia and jaundice. AATD-related lung disease is managed via AAT injection, while hepatic disease often requires liver transplantation (10).

Table 2. Gene editing methods to treat liver diseases

Liver disease	Prevalence	Model	Target gene	Mutation	Method	Ref.
α-1 antitrypsin deficiency (AATD)	1:1,500-7,000	iPSCs from patients PiZ mouse C57BL/6J mouse	*SERPINA1*	*Glu342Lys*, *Ser53Phe*, *His334Asp*	ZFN-piggyBac Transposon CRISPR/Cas9 Promoterless rAAV	(9-7)
Hemophilia A (HA)	1:5,000	hiPSCs from patients Engineered hiPSCs HA/CD4 null mice	*F8*	Deletions, insertions, inversions, and point mutations	CRISPR/Cas9 TALENs; ZFNs Promoterless rAAV	(18-29)
Phenylketonuria (PKU)	1:10,000-15,000	COS-7 cells Pahenu adult mouse	*PAH*	*c.1222C>T* (*p.Arg408Trp*) *SNP*	FokI-dCas9 system CRISPR-Cas	(30-35)
Wilson's disease (WD)	1:30,000	WD mouse	*ATP7B*	*Loss- of - function*	AAV containing WT	(36-38)
Ornithine transcarbamylase (OTC) deficiency	1:70,000	spf^{ash} mouse	*OTC*	*Loss- of - function*	CRISPR/Cas9	(39-41)
Tyrosinemia type 1 (HT1)	1:100,000	fah-/- mice fah-/- rats Fahneo/PM mice fah-/- primary hepatocytes *In utero*	*FAH*/*HPD*	*Loss- of - function*	Promoterless rAAV CRISPR/Cas9 saCas9; Nme-Cas9 Cas9 nickases CRISPR-Cas	(42-53)
Arginase-1 deficiency	1:1,000,000	Induced mouse model	*ARG1*	Deletions in exon 7 and 8	TALEN	(54-56)

Smith *et al*. used the CRISPR/Cas9 system to repair the Z mutation in hiPSCs derived from patients with AATD, which demonstrated the promise of the CRISPR/Cas9 system for allele-specific genome editing (11). CRISPR/Cas9 repaired the Glu342Lys mutation in the PiZ mouse model for human AATD (Glu342Lys mutation in *SERPINA1*) back to Lys342Glu (12). In both neonatal and adult mice, *in vivo* gene editing partially recovered physiological levels of serum AAT although therapeutic levels were not achieved (13). Other therapeutic approaches for AATD involve knocking out the mutated *SERPINA1*. Bjursell *et al*. repaired human mutant *SERPINA1* in the liver of a PiZ mouse using CRISPR/Cas9 system. Disruption of the human transgene leads to a decline in hepatic and plasma hAAT levels, liver fibrosis and hepatic protein aggregation (14). Similar results were also obtained in the PiZ mouse by targeting the exon 2 of mutated human *SERPINA1* with CRISPR/Cas9, which resulted in a 98% reduction in AAT expression in hepatocytes. To alleviate lung symptoms in patients with AAT, a dual recombinant adeno-associated virus (rAAV) system was delivered to repair the E342K substitution through HDR. However, the gene collection rate was below (5%) the expected levels (15).

Some strategies involve the insertion of wild-type *SERPINA1* to increase the levels of AAT. One approach integrated wild-type human *SERPINA1* into C57BL/6J mouse liver using the CRISPR/Cas9 system targeting the ROSA26, often referred to as a "safe harbor" locus, without any side effects associated with transgene insertion; the newly inserted gene exhibits a stable expression. This knock-in approach of *SERPINA1* resulted in long-term increase of serum AAT in wild-type mice (16). Another approach integrated an AAV vector into the NSG-PiZ mouse model, which is derived from PiZ and immune-deficient (NSG) mouse strains. The AAV vector containing wild-type AAT and miRNAs targeting the pathogenic allele was inserted into the hepatocytes of NSG-PiZ mice, resulting in the upregulation of wild-type AAT levels and downregulation of mutant protein levels (17).

Hemophilia

Hemophilia caused by mutations in *F8* gene encoding coagulation factor VIII (FVIII) or *F9* gene encoding coagulation factor IX (FIX), leading to hemophilia A (HA) or hemophilia B (HB) is an X-linked bleeding disorder. Various genetic mutations including insertions, inversions, point mutations, and large deletions in the *F8* locus result in HA. Almost 50% of serious HA cases are caused by inversion breaking in intron 1 or 22 and in the upstream of correlated homologous sequences of *F8* gene (18).

Initial attempts to repair mutations via genome editing targeted these inversions using HA-derived or HB-derived iPSCs. The two inversions in hiPSCs were repaired using CRISPR/Cas9 or TALENS (19, 20). The efficiency of reversion was up to 6.7% and the wild-type F8 was expressed in the engineered cells. Furthermore, HA mice transplanted with engineered endothelial cells was significantly increased in the F8 enzymatic activity compared to non-transplanted HA mice (19).

Another approach for the repair of inversion was used TALENs to insert the exon 23-26 fragment of wild-type F8 cDNA correctly at the intersection of exon 22 and intron 22 in HA-hiPSCs via HDR. The mRNA and protein levels of F8 were rescued in the engineered hiPSC-derived endothelial cells (21). Several studies reported general strategies to repair all genetic variants

in patients with HA by nuclease-mediated insertion of wild-type F8 cDNA fragment into the F8 locus, Alb locus or H11 safe harbor (22). Interestingly, a B-domain deleted form of *F8* under the control of EF1α promoter into HA-hiPSCs using CRISPR/Cas9 were increased F8 activity in knocked-in hiPSC-derived endothelial cells (23). Also, the F8 activity increased in the F8-encoding gene inserted into the human H11 safe harbor locus in HA-hiPSCs harboring a deleted or inverted F8-encoding gene using CRISPR/Cas9 (22). This strategy may represent a potential therapeutic approach in patients with HA. The site-specific integration of *F8* into Alb locus validated the possibility of these general approaches *in vivo*. In this strategy, HA/CD4 null mice were treated with ZFN pairs, which were separately packaged into rAAV8 including intron 1 of the Alb-encoding gene and a donor rAAV encoding a docked structure of hF8. The hF8 activity was increased by 37% (24).

A similar approach was used in HB, which is an ideal disease for liver-directed genome editing strategy due to marked upregulation of hF9 activity. The cDNA of *F9* exons 2-8 flanked by a splicing a poly-A and acceptor signal were transduced with rAAV8-ZFNs into mice and the levels of hF9 were stably upregulated for up to 1 year. A similar strategy was adopted by delivering ZFNs mRNAs using lipid nanoparticles (LNPs), which are not limited by the pre-existing neutralizing antibodies (25).

In 2018, the first patient treated with SB-FIX (Clinical Trial: NCT02695160), which is ZEN-regulated genome editing transformed by rAAV inserted the wild-type of Factor IX cDNA into the Alb locus. Barzel *et al*. used the same mAlb locus via rAAV-mediated HR without the nucleases. In this strategy, the F9 plasma level was upregulated and the coagulation time reverted to physiological levels in F9 deficient mice-treated full length *F9* cDNA (26).

Alternative general knock-in strategies adopted for HB mice models entailed transgene insertion into *F9*, AAVS1, or Rosa26 loci (27). *F9* cDNA encoding exons 2-8 were inserted into intron 1 of *F9* via rAAV8-packaged ZFNs, which promoted HR, upregulated the F9 levels, and markedly shortened activated partial thromboplastin time. This approach was effective in both neonatal and adult hF9/HB mice. However, the expression of F9 in neonatal mice was 5-fold higher than in adult hF9/HB mice, which can be attributed to the decreased loss of rAAV (28). Similar strategies involved the insertion of a full-length F9 sequence into HB-derived hiPSCs with AAVS1 and Rosa26 loci in a juvenile model of HB using CRISPR/Cas9. *F9* transcript expression and hFIX activity were upregulated not only in F9-AAVS1-hiPSCs-derived hepatocytes but also in F9-Rosa26-juvenile R333Q mice with hemophilia (27, 29).

Phenylketonuria (PKU)

PKU is caused by mutations in *PAH*, which encodes phenylalanine hydroxylase (PAH). PAH regulates the catalysis of hydroxylation of phenylalanine in tyrosine. The decline of PAH activity is increased in phenylalanine in blood, which leads to neurotoxic effects. If untreated, PKU can impair cognitive development and other symptoms, including autism, eczematous rashes, motor deficits, and seizures (30). The main treatment for PKU is dietary phenylalanine restriction. However, patients often exhibit limited improvements. Other potential strategies treat with tetrahydrobiopterin to induce the enzyme activity including PAH or use the antagonists of phenylalanine uptake at the blood-brain barrier (BBB) (31). To overcome the poor outcomes of these currently used treatments, an alternative therapeutic method is needed to repair mutations in *PAH*.

Patients with PKU have mutations involving both alleles of *PAH*. The repair of only a single allele enhances PAH activity to alleviate symptoms of PKU. The missense mutations of PAH are over 50% of the PAH mutations and the c.1222C>T (p.Arg408Trp) is the most common mutation in severe PKU (32). To repair the c.1222C>T mutation used the CRISPR system with a deactivated Cas9 (dCas9) and a guided RNA for FokI nuclease to repair this mutation in a c.1222C>T mutant-expressing Cos7 cell line. The mutation was repaired in approximately 27% of treated cell clones and PAH activity was partially rescued. Furthermore, no off-target alterations were detected (33). Another recent study attempted to repair PAH pathogenic variant (c.835T>C; p.F263S) in homozygous Pah^{enu2} mouse carrying homozygous missense mutation (34). Villiger *et al*. used CRISPR with cytidine deaminase base editors packed in rAAV, and achieved an mRNA correction rate of up to 63% and recovered physiological levels of phenylalanine in the blood of adult mice. Additionally, growth retardation of Pah^{enu2} mice was mitigated in corrected heterozygous mice (35). This results suggest that base editors for gene correction are possible in adults, specifically in treating hepatic tissue of decreased proliferative ability.

Wilson's disease (WD)

WD is a rare genetic disorder caused by loss-of-function mutations in the copper-transporting P-type ATPase-encoding gene (*ATP7B*). *ATP7B* encodes a protein that transports copper out of the liver. Therefore, WD is characterized by excessive hepatic accumulation of copper, which leads to systemic toxicity. If untreated, WD progresses to liver disease, brain injury, and premature death. Patients often develop liver dysfunction, fatigue, and yellow discoloration of the skin (36). Currently, WD is treated via liver transplantation, which restores biliary copper excretion and normalizes copper level in circulation, but requires life-long immunosuppression (37).

Murillo *et al*. used AAV to repair *ATP7B* in the WD mouse model. A single injection resulted in normalized plasma holoceruloplasmin, increased biliary copper excretion, and decreased hepatic copper levels after 6 months. Interestingly, hepatocytes from AAV-transduced WD mice exhibited higher copper-excreting ability than those derived from wild-type mice, suggesting enhanced gene editing efficiency through AAV injection (38).

Ornithine transcarbamylase (OTC) deficiency

OTC deficiency is a rare X-linked genetic disorder caused by

absence of the OTC enzyme, in which plays the role in elimination of nitrogen, which results in the form of ammonia (hyperammonemia) via the urea cycle. The accumulation of ammonia caused by the lack of the OTC enzyme moves to central nervous system through the blood and leads to the symptoms with OTC deficiency. The treatment of the disease involves the treatment with ammonia scavengers, such as sodium benzoate and dietary protein restriction. The transplantation of liver is considered in cases of hyperammonemia (39).

One study repaired *OTC* mutation in an OTC deficiency mouse model using rAAV-mediated CRISPR/Cas9. Spf^{ash} mouse, which harbors a G>A point mutation at the splice site located at the end of exon 4 in *OTC*, exhibits impaired mRNA splicing and decreased OTC levels (40). The authors injected rAAV containing CRISPR/Cas9 components into newborn and adult spf^{ash} mice, which induced HDR and repaired the mutation in approximately 10% of hepatocytes in newborns. Additionally, the survival of mice was improved with a high-protein diet, which causes hyperammonemia. However, the gene correction rate in adult mice was markedly low and the toxicity was increased in unexpected large deletions of DNA. These differential results between newborn and adult mice may be attributed to the characteristics of adult hepatocytes, which are mostly non-dividing cells with NHEJ repairing mechanisms instead of HDR (41).

Tyrosinemia type 1 (HT1)

HT1 is a rare genetic disorder of tyrosine catabolism caused by a loss-of-function mutation in a gene encoding fumarylacetoacetate hydrolase (FAH). FAH deficiency promotes the accumulation of fumarylacetoacetate, maleylacetoacetate and their derivatives, and consequently causes by the damage of liver and renal tubular. Nitisinone rescues the pathological phenotype and acute liver injury by inhibiting hydroxyphenylpyruvate dioxygenase (HPD) in tyrosine catabolism upstream of FAH (42).

HT1 is amenable to gene repair therapy as the repaired hepatocytes undergo optimal proliferation in the liver (43). The two main genome editing strategies used for HT1 management include repair of *FAH* mutations and knockout of *HPD*. To repair *FAH* mutation, one study induced HDR in *FAH* using AAV containing genomic *FAH* sequence in HT1 ($Fah^{-/-}$ mice) mouse model. The engineered hepatocytes survived and repopulated the liver, resulting in improved liver function (44).

In 2014, Yin *et al*. used CRISPR/Cas9 system to repair the pathological mutations in HT1 mice. The injection of CRISPR/Cas9 components through the tail vein resulted in functional rescue of the liver damage with the downregulation of serum markers, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin (45). To insert FAH cDNA encoding exons 5-14 into intron 4 of *FAH*, Cas9 and Fah-microhomology-mediated end joining (MMEJ) constructs were injected into a $Fah^{-/-}$mouse model. The bodyweight and liver damage were significantly decreased in treated $Fah^{-/-}$mice (46). Similarly, to generate *FAH* mutant rat model, adults HT1 rats were transduced with sgRNA/donor template and nCas9 using a two-AdV system. Even though the initial correction rate was low (0.1%), the engineered hepatocytes rescued approximately 95% of the liver tissue after 9 months. The collagen deposition at 9 months post-treatment was significantly lower than that at 3 months post-treatment, which indicated amelioration of liver fibrosis (47). A different approach using the $FAH^{neo/PM}$ mouse, a heterozygous mouse model of HT1, has also been reported. Wang *et al*. used Cas9 to generate DSB-induced allelic exchange between two different mutants. The following two AAV vectors, AAV harboring Cas9 and AAV harboring a sgRNA of *FAH* intron 7, were injected in both $FAH^{neo/PM}$ newborn mice and $FAH^{neo/PM}$ young adult mice. Allelic exchange appeared to hepatocytes over the fast-proliferating, postnatal stage (48).

Ex vivo gene repair in hepatocytes obtained from animal disease models using CRISPR/Cas9 also showed therapeutic potential. Two approaches performed by Van Lith *et al*. using either AAV or lentiviral vectors were used to deliver the sgRNAs or *Staphylococcus aureus* Cas9 (saCas9) or *Streptococcus pyogenes* Cas9 (spCas9). Corrected hepatocytes were transplanted back into $Fah^{-/-}$mice via intrasplenic injection. These hepatocytes significantly repopulated the mouse liver. Additionally, the blood tyrosine levels decreased to physiological levels (49, 50). *Ex vivo* gene editing facilitates selection of accurately edited cells. Some studies have attempted to silence *Hpd* using Cas9 and base editors. One study used murine leukemia virus (MLV)-like particles loaded with Cas9-sgRNA ribonucleoproteins (Nanoblades) (51). Another study used *Neisseria meningitides* Cas9 (NmeCas9) and sgRNA packed in AAV and Rossidis *et al*. finally used base editor technique to generate a nonsense mutation in *Hpd in utero* (52). The *in utero* treatment recovered the lethal phenotype and the liver function in $Fah^{-/-}$ mice (53).

Arginase-1 deficiency (argininemia)

Arginase-1 deficiency is a genetic disorder associated with urea metabolism in which the hydrolysis of arginine to urea and ornithine is impaired due to mutations in *ARG1*. Similar to OTC deficiency management, the clinical management of arginase-1 deficiency involves dietary restrictions, treatment with nitrogen scavengers, and enzyme replacement therapies (54).

Sin *et al*. generated a mouse model of arginase-1 deficiency via Cre-loxP-regulated deletion of exons 7 and 8 of *Arg1*. Then, the authors tried to repair the genetic defect in iPSC-derived mouse hepatocytes and macrophages using the CRISPR/Cas9 system combined with the *piggyBac* technique. Gene repair was successful in iPSCs. However, differentiated hepatocytes did not restore the expression of arginase-1 markedly. Interestingly, the expression of arginase-1 significantly recovered in differentiated macrophages derived from the same iPSCs (55). And also, TALEN-regulated recombination of deleted exons was completed in iPSCs derived from the murine model. The edited cells differentiated into hepatocyte like cells, which were transferred to the liver of the arginase-1 defected mouse. However, the expression of arginase-1 was not restored owing to the poor proliferation of hepatocytes (56).

RECENT SUCCESSES IN LIVER GENE EDITING OF MOUSE, MONKEY AND HUMAN PATIENTS USING EX VIVO AND IN VIVO METHODS

Ex vivo gene editing

Recently, we demonstrated the efficacy of an *ex vivo* gene editing method targeting liver (57). The hepatocytes were extracted from the HT1 mouse model to generate chemically derived hepatic progenitors (CdHs) (58). CdHs were treated with adenine base editors (ABEs) and prime editing components to repair *FAH*. The engineered cells, which were injected back into HT1 mice through the tail vein, proliferated in the liver. HT1 mice injected with the engineered CdHs exhibited marked alleviation of hepatic dysfunction when compared with control HT1 mice (Fig. 2, right).

Our finding indicated that genetic liver disease can be treated using *ex vivo* gene editing strategies. *Ex vivo* gene editing has several advantages. First, *ex vivo* editing does not elicit pathological immune response against the edited cells, which are derived from the patient. Additionally, *in vitro* gene editing can facilitate the screening and elimination of cells with unexpected edits and only cells with the desired edited gene can be injected into patients, which can further ensure safety. However, *ex vivo* approaches require highly advanced facilities with specialized protocols and experienced researchers.

In vivo gene editing

Several successful *in vivo* gene therapies involving monkeys and humans have been reported recently. Instead of using viral vectors, lipid nanoparticles (LNPs) conjugated with apolipoprotein E can directly target the hepatocytes, which can significantly improve the safety of gene therapy. Further, the efficacy of mRNA vaccines during the COVID-19 pandemic indicated that the safety and accuracy of gene therapy can be further improved using Cas mRNA instead of *Cas* gene or Cas protein. The administration of one or two doses of LNP-packed Cas9 or base editor mRNA and CRISPR guide RNA represents successful gene therapy strategy. Two recent studies demonstrated that transiently knock-out of gene related to cardiovascular disease was efficient in non-human primates (59, 60). Highly expressed proprotein convertase subtilisin/kexin type 9 (PCSK9) in the liver plays a role in cholesterol homeostasis and the interaction between PCSK9 and low-density lipoprotein (LDL) receptor induces endocytosis and degradation of the receptor, which results in reduction of LDL cholesterol from the blood. Gain-of-function variants in *PCSK9* relate to familial hypercholesterolemia.

A recent study reported that a single administration of NLTA-2001, an *in vivo* gene-editing therapeutic agent, decreases the level of serum transthyretin (TTR) in patients, indicating potent therapeutic efficacy (61). The sustained reduction of TTR levels is supposed to alleviate disease symptoms. This finding provides strong evidence supporting the clinical application of gene editing. TTR amyloidosis caused by the accumulation of misfolded TTR proteins is formed the amyloid fibrils in cardiomyocytes and nerves, which subsequently leads to amyloid polyneuropathy and cardiomyopathy. A recent study by Gillmore *et al*. indicated that strategies for gene therapy can be upgraded from *ex vivo* to *in vivo* approaches (Fig. 2, left) (61).

CONCLUSIONS AND FUTURE PERSPECTIVES

Gene editing components can be easily delivered to the liver intravenously since liver metabolizes all foreign particles. Recent studies have demonstrated that the direct delivery of LNPs conjugated with apolipoprotein E to the hepatocytes was markedly

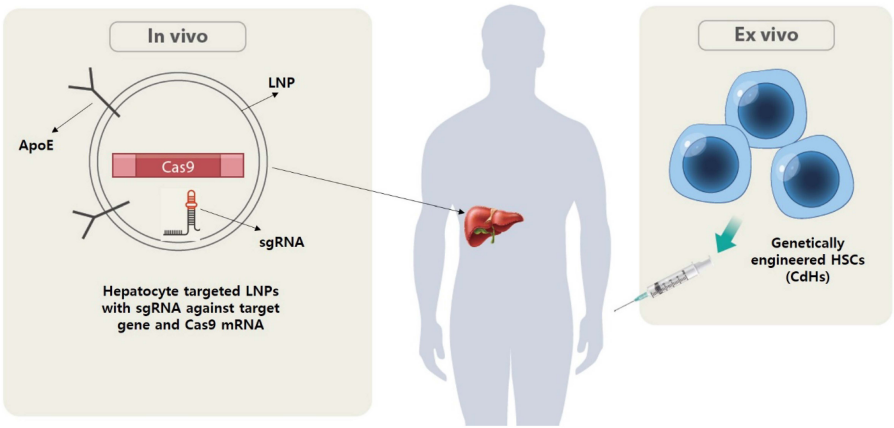

Fig. 2. *In vivo* and *ex vivo* gene editing methods targeting the liver. In *ex vivo* editing (right), genetically modified hepatic stem cells (HSCs), including chemically derived hepatic progenitors (CdHs), can be achieved from patients, genetically modified, and injected back to patients. In *in vivo* strategies (left), lipid nanoparticles (LNPs) contain apolipoprotein E (ApoE) to directly target hepatocytes. sgRNA directs to the target gene, and Cas9 mRNA is translated to perform gene editing at the specific site on the target gene.

safer than the AAV-mediated delivery. Hence, LNPs enable *in vivo* gene editing to treat various liver diseases. Additionally, *ex vivo* gene editing approaches using patient cells have yielded promising results. The application of the combination of *in vivo* and *ex vivo* gene editing approaches represents a potent therapeutic strategy against genetic liver diseases.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

REFERENCES

1. Hackl C, Schlitt HJ, Melter M, Knoppke B and Loss M (2015) Current developments in pediatric liver transplantation. World J Hepatol 7, 1509-1520
2. Blaese RM, Culver KW, Miller AD et al (1995) T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science 270, 475-480
3. Kumar SR, Markusic DM, Biswas M, High KA and Herzog RW (2016) Clinical development of gene therapy: results and lessons from recent successes. Mol Ther Methods Clin Dev 3, 16034
4. Raper SE, Chirmule N, Lee FS et al (2003) Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab 80, 148-158
5. Rees HA and Liu DR (2018) Base editing: precision chemistry on the genome and transcriptome of living cells. Nat Rev Genet 19, 770-788
6. Gaudelli NM, Komor AC, Rees HA et al (2017) Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature 551, 464-471
7. Anzalone AV, Randolph PB, Davis JR et al (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576, 149-157
8. Anzalone AV, Koblan LW and Liu DR (2020) Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. Nat Biotechnol 38, 824-844
9. Miranda E, Perez J, Ekeowa UI et al (2010) A novel monoclonal antibody to characterize pathogenic polymers in liver disease associated with alpha1-antitrypsin deficiency. Hepatology 52, 1078-1088
10. Stoller JK, Hupertz V and Aboussouan LS et al (1993) Alpha-1 Antitrypsin Deficiency. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993–2022. 2006 Oct 27 [updated 2020 May 21]
11. Smith C, Abalde-Atristain L, He C et al (2015) Efficient and allele-specific genome editing of disease loci in human iPSCs. Mol Ther 23, 570-577
12. Carlson JA, Rogers BB, Sifers RN et al (1989) Accumulation of PiZ alpha 1-antitrypsin causes liver damage in transgenic mice. J Clin Invest 83, 1183-1190
13. Song CQ, Wang D, Jiang T et al (2018) In vivo genome editing partially restores alpha1-antitrypsin in a murine model of AAT deficiency. Hum Gene Ther 29, 853-860
14. Bjursell M, Porritt MJ, Ericson E et al (2018) Therapeutic genome editing with CRISPR/Cas9 in a humanized mouse model ameliorates alpha1-antitrypsin deficiency phenotype. EBioMedicine 29, 104-111
15. Shen S, Sanchez ME, Blomenkamp K et al (2018) Amelioration of alpha-1 antitrypsin deficiency diseases with genome editing in transgenic mice. Hum Gene Ther 29, 861-873
16. Stephens CJ, Kashentseva E, Everett W, Kaliberova L and Curiel DT (2018) Targeted in vivo knock-in of human alpha-1-antitrypsin cDNA using adenoviral delivery of CRISPR/Cas9. Gene Ther 25, 139-156
17. Borel F, Tang Q, Gernoux G et al (2017) Survival advantage of both human hepatocyte xenografts and genome- edited hepatocytes for treatment of alpha-1 antitrypsin deficiency. Mol Ther 25, 2477-2489
18. Bagnall RD, Waseem N, Green PM and Giannelli F (2002) Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. Blood 99, 168-174
19. Park CY, Kim DH, Son JS et al (2015) Functional correction of large factor VIII gene chromosomal inversions in hemophilia A patient-derived iPSCs using CRISPR-Cas9. Cell Stem Cell 17, 213-220
20. Park CY, Kim J, Kweon J et al (2014) Targeted inversion and reversion of the blood coagulation factor 8 gene in human iPS cells using TALENs. Proc Natl Acad Sci U S A 111, 9253-9258
21. Wu Y, Hu Z, Li Z et al (2016) In situ genetic correction of F8 intron 22 inversion in hemophilia A patient-specific iPSCs. Sci Rep 6, 18865
22. Park CY, Sung JJ, Cho SR, Kim J and Kim DW (2019) Universal correction of blood coagulation factor viii in patient-derived induced pluripotent stem cells using CRISPR/Cas9. Stem Cell Reports 12, 1242-1249
23. Sung JJ, Park CY, Leem JW, Cho MS and Kim DW (2019) Restoration of FVIII expression by targeted gene insertion in the FVIII locus in hemophilia A patient-derived iPSCs. Exp Mol Med 51, 1-9
24. Sharma R, Anguela XM, Doyon Y et al (2015) In vivo genome editing of the albumin locus as a platform for protein replacement therapy. Blood 126, 1777-1784
25. Conway A, Mendel M, Kim K et al (2019) Non-viral delivery of zinc finger nuclease mRNA enables highly efficient in vivo genome editing of multiple therapeutic gene targets. Mol Ther 27, 866-877
26. Barzel A, Paulk NK, Shi Y et al (2015) Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. Nature 517, 360-364
27. Stephens CJ, Lauron EJ, Kashentseva E, Lu ZH, Yokoyama WM and Curiel DT (2019) Long-term correction of hemophilia B using adenoviral delivery of CRISPR/Cas9. J Control Release 298, 128-141
28. Anguela XM, Sharma R, Doyon Y et al (2013) Robust ZFN-mediated genome editing in adult hemophilic mice. Blood 122, 3283-3287
29. Lyu C, Shen J, Wang R et al (2018) Targeted genome

engineering in human induced pluripotent stem cells from patients with hemophilia B using the CRISPR-Cas9 system. Stem Cell Res Ther 9, 92
30. Blau N, van Spronsen FJ and Levy HL (2010) Phenylketonuria. Lancet 376, 1417-1427
31. Strisciuglio P and Concolino D (2014) New strategies for the treatment of phenylketonuria (PKU). Metabolites 4, 1007-1017
32. Blau N, Shen N and Carducci C (2014) Molecular genetics and diagnosis of phenylketonuria: state of the art. Expert Rev Mol Diagn 14, 655-671
33. Pan Y, Shen N, Jung-Klawitter S et al (2016) CRISPR RNA-guided FokI nucleases repair a PAH variant in a phenylketonuria model. Sci Rep 6, 35794
34. McDonald JD, Bode VC, Dove WF and Shedlovsky A (1990) The use of N-ethyl-N-nitrosourea to produce mouse models for human phenylketonuria and hyperphenylalaninemia. Prog Clin Biol Res 340C, 407-413
35. Villiger L, Grisch-Chan HM, Lindsay H et al (2018) Treatment of a metabolic liver disease by in vivo genome base editing in adult mice. Nat Med 24, 1519-1525
36. Tanzi RE, Petrukhin K, Chernov I et al (1993) The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nat Genet 5, 344-350
37. Sternlieb I, Van den Hamer CJ, Morell AG, Alpert S, Gregoriadis G and Scheinberg IH (1973) Lysosomal defect of hepatic copper excretion in Wilson's disease (hepatolenticular degeneration). Gastroenterology 64, 99-105
38. Murillo O, Luqui DM, Gazquez C et al (2016) Long-term metabolic correction of Wilson's disease in a murine model by gene therapy. J Hepatol 64, 419-426
39. Donovan K and Guzman N (2021) Ornithine Transcarbamylase Deficiency. in StatPearls, Treasure Island (FL)
40. Hodges PE and Rosenberg LE (1989) The spfash mouse: a missense mutation in the ornithine transcarbamylase gene also causes aberrant mRNA splicing. Proc Natl Acad Sci U S A 86, 4142-4146
41. Yang Y, Wang L, Bell P et al (2016) A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. Nat Biotechnol 34, 334-338
42. Lindstedt S, Holme E, Lock EA, Hjalmarson O and Strandvik B (1992) Treatment of hereditary tyrosinaemia type I by inhibition of 4-hydroxyphenylpyruvate dioxygenase. Lancet 340, 813-817
43. Azuma H, Paulk N, Ranade A et al (2007) Robust expansion of human hepatocytes in Fah-/-/Rag2-/-/Il2rg-/- mice. Nat Biotechnol 25, 903-910
44. Paulk NK, Wursthorn K, Wang Z, Finegold MJ, Kay MA and Grompe M (2010) Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. Hepatology 51, 1200-1208
45. Yin H, Xue W, Chen S et al (2014) Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nat Biotechnol 32, 551-553
46. Yao X, Wang X, Liu J et al (2017) CRISPR/Cas9 - mediated precise targeted integration in vivo using a double cut donor with short homology arms. EBioMedicine 20, 19-26
47. Shao Y, Wang L, Guo N et al (2018) Cas9-nickase-mediated genome editing corrects hereditary tyrosinemia in rats. J Biol Chem 293, 6883-6892
48. Shin JH, Jung S, Ramakrishna S, Kim HH and Lee J (2018) In vivo gene correction with targeted sequence substitution through microhomology-mediated end joining. Biochem Biophys Res Commun 502, 116-122
49. VanLith CJ, Guthman RM, Nicolas CT et al (2019) Ex vivo hepatocyte reprograming promotes homology-directed DNA repair to correct metabolic disease in mice after transplantation. Hepatol Commun 3, 558-573
50. VanLith C, Guthman R, Nicolas CT et al (2018) Curative ex vivo hepatocyte-directed gene editing in a mouse model of hereditary tyrosinemia type 1. Hum Gene Ther 29, 1315-1326
51. Mangeot PE, Risson V, Fusil F et al (2019) Genome editing in primary cells and in vivo using viral-derived Nanoblades loaded with Cas9-sgRNA ribonucleoproteins. Nat Commun 10, 45
52. Ibraheim R, Song CQ, Mir A, Amrani N, Xue W and Sontheimer EJ (2018) All-in-one adeno-associated virus delivery and genome editing by Neisseria meningitidis Cas9 in vivo. Genome Biol 19, 137
53. Rossidis AC, Stratigis JD, Chadwick AC et al (2018) In utero CRISPR-mediated therapeutic editing of metabolic genes. Nat Med 24, 1513-1518
54. Diez-Fernandez C, Rufenacht V, Gemperle C, Fingerhut R and Haberle J (2018) Mutations and common variants in the human arginase 1 (ARG1) gene: Impact on patients, diagnostics, and protein structure considerations. Hum Mutat 39, 1029-1050
55. Sin YY, Price PR, Ballantyne LL and Funk CD (2017) Proof-of-concept gene editing for the murine model of inducible arginase-1 deficiency. Sci Rep 7, 2585
56. Sin YY, Ballantyne LL, Richmond CR and Funk CD (2018) Transplantation of gene-edited hepatocyte-like cells modestly improves survival of arginase-1-deficient mice. Mol Ther Nucleic Acids 10, 122-130
57. Kim Y, Hong SA, Yu J et al (2021) Adenine base editing and prime editing of chemically derived hepatic progenitors rescue genetic liver disease. Cell Stem Cell 28, 1614-1624 e1615
58. Kim Y, Kang K, Lee SB et al (2019) Small molecule-mediated reprogramming of human hepatocytes into bipotent progenitor cells. J Hepatol 70, 97-107
59. Rothgangl T, Dennis MK, Lin PJC et al (2021) In vivo adenine base editing of PCSK9 in macaques reduces LDL cholesterol levels. Nat Biotechnol 39, 949-957
60. Musunuru K, Chadwick AC, Mizoguchi T et al (2021) In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. Nature 593, 429-434
61. Gillmore JD, Gane E, Taubel J et al (2021) CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. N Engl J Med 385, 493-502