Application of Prime Editing to Liver Hereditary Diseases

Subjects: Genetics & Heredity | Cell & Tissue Engineering | Biochemistry & Molecular Biology

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Gene therapy holds tremendous potential in the treatment of inherited diseases. Unlike traditional medicines, which only treat the symptoms, gene therapy has the potential to cure the disease by addressing the root of the problem: genetic mutations. The discovery of CRISPR/Cas9 in 2012 paved the way for the development of those therapies. Improvement of this system led to the recent development of an outstanding technology called prime editing. This system can introduce targeted insertions, deletions, and all 12 possible base-to-base conversions in the human genome. Since the first publication on prime editing in 2019, groups all around the world have worked on this promising technology to develop a treatment for genetic diseases. Liver diseases are currently the most studied field for human gene therapy by prime editing. To date, prime editing has been attempted in preclinical studies for tyrosinemia type 1, alpha-1-antitrypsin deficiency, phenylketonuria, DGAT1-deficiency, bile salt export pump deficiency, liver cancer, and for a liver disease caused by a mutation in the *DNMT1* gene.

prime editing | gene therapy | inherited diseases | genetic diseases | CRISPR/Cas9 | liver diseases | gene editing

1. Introduction

Gene therapy offers enormous potential in the treatment of genetic diseases. Its potency lies in addressing the genetic root of the problem, unlike traditional medicines, which only treat the symptoms. By correcting the mutations, gene therapy has the potential to cure hereditary diseases. Sherkow et al. [1] defined gene therapy as “the intentional, expected permanent, and specific alteration of the DNA sequence of the cellular genome, for a clinical purpose”. The first approved gene therapy occurred in 1990, when a foreign gene was inserted into a kid’s immune cells [2]. Gene therapies first took the form of DNA insertion into the host genome [3]. In the 2000s, tools allowing the introduction of modifications at specific target sites in the genome were developed, including zinc-finger nucleases (ZFNs) [4] and transcription activator-like effector nucleases (TALENs) [5].

A milestone in the development of gene therapies was the discovery of CRISPR/Cas9 in 2012. This system involves a Cas9 nuclease that induces a double-strand DNA break at a precise place in the genome. The Cas9 is directed at the right sequence in the genome by a guide RNA. This guide is a single RNA strand complementary to an 18–24 nucleotides (nt) sequence in the genome [6][7]. When the complex is fixed on the complementary DNA sequence and the Cas9 recognizes a protospacer adjacent motif (PAM), the complex is activated. This PAM sequence varies depending on the microorganism of origin of the Cas9. For example, the most widely used Cas9

is from *Streptococcus pyogenes* (SpCas9) and recognizes the PAM 5′-NGG-3′ [8]. Once this small sequence is recognized, Cas9 will cut 3 nt upstream of the PAM [9]. The cell will then repair its DNA by non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), or homology-directed repair (HDR). NHEJ is an imprecise mechanism where broken ends of DNA are joined together, which often leads to insertions or deletions of nucleotides [10]. MMEJ is also an imprecise mechanism that can lead to undesired insertions, deletions, or even translocations [11]. This mechanism works by aligning short homologous sequences that are between the broken ends [12][13]. HDR repairs the damage using a homologous donor DNA, leading to a precise repair of the cut [10].

The evolution and refinement of the CRISPR/Cas9 technology have driven the development of base editing. This system exists in three versions: cytosine base editors (CBEs) [14], adenine base editors (ABEs) [15], and C to G base editors (CGBEs) [16]. CBE can install C > T and G > A mutations, while ABE can induce A > G and T > C mutations [17], and CGBE can generate C-to-G transversions [16][18]. Those editors change all the intended base pairs in a precise window (for example, CBE switches all C•G base pairs located in the window to T•A base pairs). Compared to CRISPR/Cas9, the advantages of this technology are that base editing does not require a double-strand break (the system uses a modified D10A Cas9) and does not need an exogenous DNA template, which leads to the reduction of unwanted indels. Base editing also leads to much more precise correction of the mutation by its ability to target a particular codon. However, those systems [19] cannot be applied when the change of a base pair in the window (other than the desired one) would lead to a non-silent mutation.

In October 2019, David R. Liu's group released an outstanding discovery called prime editing [20]. This system can mediate targeted insertions, deletions, and all 12 possible base-to-base conversions. This mechanism makes DNA modifications with unprecedented precision and has substantial advantages over the traditional CRISPR/Cas9 and base editing systems (**Table 1**). Derived from the CRISPR/Cas9 system, this new technology is composed of a Cas9 nuclease fused with a reverse transcriptase (RT) at its 3′ extremity and a prime editing guide RNA (pegRNA) (**Figure 1**). The combination of Cas9 and RT forms the prime editor (PE). The pegRNA is composed of a spacer sequence, a primer binding site (PBS), a reverse transcriptase template (RTT), and a common region that binds to the Cas9 and the RT [19]. First, the complex binds to the DNA, guided by the spacer sequence in the pegRNA. The Cas9 recognizes a PAM and cuts 3 nt upstream. However, instead of creating a double-stranded cut, the modified H840A Cas9 from the prime editing induces a single-stranded nick [20]. Then, the PBS hybridizes to its complementary sequence located on the cut strand. Then, the RT will use the RTT as a template to transcribe the cut strand. At this point, one of the strands has a duplicated section, so the cell will have to remove one of the two sections to put back the DNA double-stranded again. The mismatch will be resolved by a 5′ flap or a 3′ flap. If a 3′ flap happens, the correction will be kept, but the editing will be lost if a 5′ flap occurs [20].

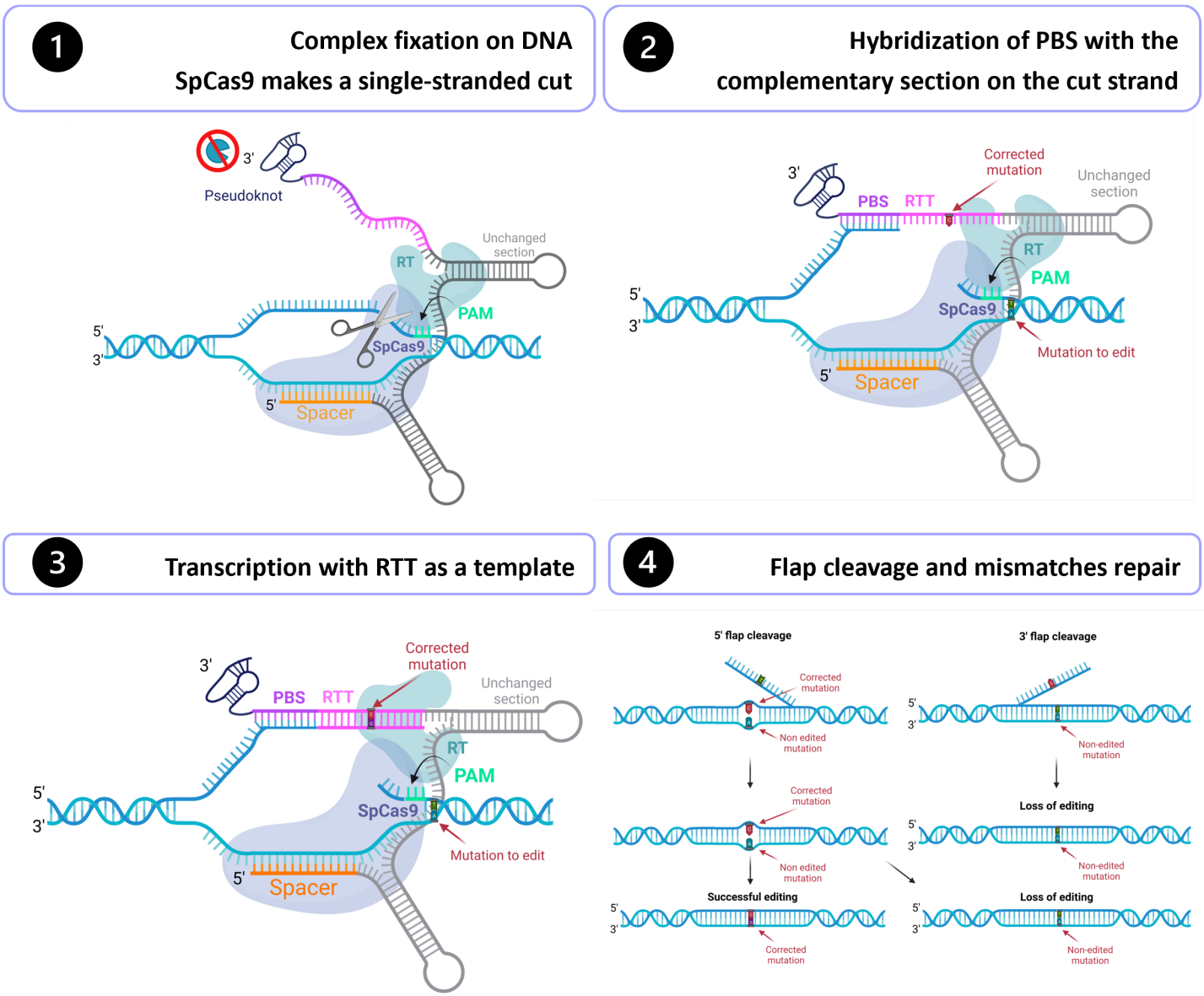

Figure 1. Prime editing mechanism. Step 1: Guided by the spacer sequence in the pegRNA, the Cas9 fused with a reverse transcriptase (RT) binds to the DNA at the desired place in the genome. The Cas9 recognizes a PAM and induce a single-stranded nick 3 nt upstream. Step 2: The PBS hybridizes to its complementary sequence on the cut strand. Step 3: The RT will use the RTT as a template to transcribe the cut strand. Step 4: Mismatches will be repaired by a 5′ flap or a 3′ flap.

To introduce any modifications, the desired correction needs to be introduced in the RTT sequence, since it serves as a template for the transcription. There are currently several versions of the prime editor, the most popular being PE2 and PE3. PE3 is similar to PE2 but has an additional guide RNA that will induce a nick on the strand not initially cut by Cas9. It will promote the replacement of the unedited strand by forcing the cell to use the edited strand as a template. This increases the chances of retaining the edit at the mismatch repair step. When a position is given, this one is defined according to the reference system based on the initial Cas9 cleavage site. Thus, nucleotides downstream of this site will have a positive position (e.g., +5 means five nucleotides after the cut site

towards 3′), and nucleotides upstream of the cut site will have a negative position (e.g., −5 means five nucleotides before the cut site towards 5′).

Table 1. Comparison of advantages and disadvantages of CRISPR/Cas9, base editing, and prime editing systems.

	CRISPR/Cas9	Base Editing	Prime Editing
Off-target effects	Significant off-target effects	Little or no off-target effects	Little or no off-target effects
	• Possibility of non-specific indels at DSB site [21]; • DNA donor template can lead to plasmid integration in the genome; • Possible genome-wide off-targets.	• No DSB; • Bystander base edits within a narrow window of 4–10 nt [22]; • Genome-wide off-targets studies need to be made.	• No DSB; • No bystander edits; • Genome-wide off-targets studies need to be made.
Flexibility	• Can introduce insertions, deletions, and all types of substitutions.	• Can introduce C > T, G > A, A > G, T > C and C > G substitutions only. • The consideration of bystander edits makes base editing more stringent on the possible sites [22].	• Can introduce insertions, deletions, and all types of substitutions. • Less stringent PAM requirements [23].
Programmability [1]	Only if a DNA donor template is given	Yes	Yes
Efficient in vivo delivery	Currently possible	Currently possible (but more difficult than CRISPR/Cas9 because of its larger size)	Need to be improved (too big for conventional vehicles)

[1] Possibility to determine the issue of editing.

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Table 1. Prime editing studies on correcting or introducing mutations causing liver diseases.

Disease	Gene	Mutation		Goal [1]	Prime Editor	% of Editing	Cells or Animal Models	Length (nt)			Edit Position from the Nick	Delivery Method	Prime Editor Form	Comments	Reference
								Spacer	PBS	RTT					
Liver cancer	*CTNNB1*	6 nt deletion				30		20	12	17	+1				
Bile salt export pump deficiency	*ABCB11*	D482G	A > G	I		20	Liver organoid	20			+7	Electroporation	Plasmid	The PAM is also mutated (+5 G > A silent mutation)	Schene 2020 [24]
DGAT1-deficiency	*DGAT1*	S210del	Del CCT		PE3	21	Patient-derived intestinal cells	20							
Bile salt export pump deficiency	*ABCB11*	R1153H	G > A	C		0	Patient-derived liver organoids								
Alpha-1-antitrypsin deficiency	*SERPINA1*	E342K	G > A	I	PE2	1.9	HEK293T cells					Lipo 2000			
					PE3	9.9									
					PE2*	6.4									
					PE3*	15.8		20	13	27			Plasmid		Liu 2021 [25]
				C	PE2	2.1	PiZ mice					Hydrodynamic TVI			
					PE2*	6.7									
					PE3	3.1						AAV8			
					PE3	0.83	hPSCs	20	9	13	+3	Electroporation	Plasmid		Habib 2022 [26]
					PE2-NGA	2.0–3.0	HEK293T cells	20	13	20		Lipo 2000	Plasmid		Lung 2021 [27]

Disease	Gene	Mutation	Goal [1]	Prime Editor	% of Editing	Cells or Animal Models	Length (nt)			Edit Position from the Nick	Delivery Method	Prime Editor Form	Comments	Reference
							Spacer	PBS	RTT					
Liver disease	dnaH1 [24]	G > C	I	PE3-NGA	3.0–5.0	Human primary fibroblasts								
				PE2-NGA	1.99									
				Intein-split PE2ΔRnH	15	C57BL/6J pups					AAV8			
				PE2ΔRnH	35.9	C57BL/6J adult mice	21				AdV	Plasmid		
					58.2	C57BL/6J pups								
Phenylketonuria	Pah^{enu2}	F263S	T > C	Intein-split PE2ΔRnH	<1%	Pah^{enu2} mice					AAV8			Böck 2022 [28]
			C	PE2ΔRnH	2.0	Adult Pah^{enu2} mice	20	13	19		AdV	Plasmid		
					6.9	Neonates Pah^{enu2} mice								
				PE3ΔRnH	11.1									
				PE3	19.6	HEK293T cells			16		Lipo 2000			
				PE3	19.7				19					
Tyrosinemia type 1	fah [25]	G > A	C	PEDAR	0.76	FahΔExon5 mice					Hydrodynamic injection	Plasmid		Jiang 2022 [29]
				PE3	2.3	HT1-mCdHs	20	11	15		Electroporation	Plasmid	sgRNA of PE3 nick in position -4	Kim 2021 [30]
					34.3	HT1 mice					Transplantation			
		c.706G > A		PE3	61	$Fah^{mut/mut}$ mice	20			+10	Hydrodynamic TVI [24]	Plasmid		Jang 2022 [31]
				PE2	33									
	FAH				18.7	HEK293T [25]					Lentiviral			

The classic PE2 requires an NGG PAM. Unfortunately, there were none near the site to be edited. Thus, they selected one that was more distant. Their pegRNA had a 20 nt spacer, a 13 nt PBS, and a 27 nt RTT. This pegRNA and PE2 achieved only 1.9% editing in HEK293T cells. However, with the same pegRNA and PE3, they achieved 9.9% editing. With this pegRNA and PE* [25] (PE that has a nuclear localization signal optimization), they obtained 6.4% of editing with PE2* and 15.8% with PE3*. They then attempted to correct in vivo a mutation in PiZ mice [25] (mouse model for the mutation E342K in the *SERPINA1* gene). To do so, they delivered prime editing's plasmid DNA by hydrodynamic tail vein injection. They obtained 2.1% editing with PE2 and 6.7% with PE2*. AAVs capacity being at most 5 kb [32], prime editing cannot be delivered using a single AAV. To address that issue, the authors tested to deliver the split-intein prime editors via AAVs by tail vein injection. By injecting a low dose of dual AAV8-PE3 (2×10^{11} viral genome), they detected 0.6% of editing after two weeks, 2.3% after six weeks, and 3.1% after ten weeks. However, the authors hypothesized that because the PAM is far from the edited site, the efficiency of prime editing is not at its best and that the utilization of a nearer PAM will lead to greater efficiency.

In the same month that Liu et al. [25] released their study, Habib et al. [26] published an article on prime editing in hiPSCs (human induced pluripotent stem cells) by generating a doxycycline-inducible prime editing platform. They also attempted to correct by prime editing the E342K mutation (1024 G > A) in the *SERPINA1* gene in hiPSCs

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Disease	Gene	Mutation	Goal [1]	Prime Editor	% of Editing	Cells or Animal Models	Length (nt)			Edit Position from the Nick	Delivery Method	Prime Editor Form	Comments	Reference
							Spacer	PBS	RTT					
						cells					vector			

[1] The goal is either the introduction (I) of the mutation or the correction (C) of the mutation. Abbreviations: AAV8 = Adeno-Associated Virus Serotype 8; AdV = human adenoviral vector 5; hiPSCs = human pluripotent stem cells; Lipo2000 = Lipofectamine 2000; TVI = tail vein injection.

... rescue for the HEK3 and RNF2 loci was possible. They obtained similar conclusions by comparing their pegRNA constructs for these loci with the results in hiPSCs of David Liu's group (the creators of prime editing) in HEK293T cells. For example, both groups concluded that, compared to the HEK3 locus, efficient prime editing at the RNF2 site required a longer PBS sequence. That conclusion suggests that the optimum PBS length depends on the sequence and, thus, might have the same optimal length even when used in different cell types. Moreover, transition substitutions (position +1 to +33), transversion substitutions (position +1 to +33), small indels (1–3 bp at +10 position), large deletions (up to 80 bp) and large insertions (up to 42 bp) are possible at the HEK3 target site in hPSCs. However, Habib et al. [26] failed to introduce small indels at the +17 and +21 positions for a mutation in the *RNF2* gene.

Another exciting aspect of Habib et al.'s [26] study is the section explaining why the PE3 system generates indel mutations. To understand what the main factor was, the authors tested five different scenarios: a single DSB induced by wild-type (wt) Cas9 and a sgRNA, a single nick induced by nCas9-RT and a sgRNA, a double DSB induced by wt Cas9 and two sgRNAs, a double nick induced by nCas9-RT and two sgRNAs, and finally the PE3 system (double nick induced by nCas9-RT, one pegRNA, and one sgRNA). From this experiment, the authors concluded that the unintended indels are not directly caused by double nicking but by the combinatory activity of the RT and the pegRNA.

In November 2021, Genesis Lung released his thesis [27] on the correction by prime editing of the E342K mutation in the *SERPINA1* gene that causes the A1AT deficiency. Lung worked on the same mutation as Schene et al. [24], Liu et al. [25], and Habib et al. [26], whose work has been described above. The last three mentioned authors failed to demonstrate a detectable correction of the mutation [25]. They used a PE that required an NGG PAM, which caused the targeted nucleotide to be far from the cut site, thus hindering good efficiency. In their original paper, Anzalone et al. [20] proposed an RTT length between 10 and 16 nt, which is much shorter than what Liu et al. [25] used (27 nt) [26]. Lung [27] attempted to correct the E342K mutation in the *SERPINA1* gene using a prime editor using another PAM. He first designed a variant of the prime editor that could use different PAMs (NGG, NGA, NGC) and conjugated them with pegRNAs that had different PBS (13 or 17 nt) and RTT lengths (between 10 and 20 nt). Liu reached up to 3% editing for the variants using an NGA PAM in HEK293T cells containing a lentivirus cassette with the E342K mutation. The best pegRNA had a spacer of 20 nt, a PBS of 13 nt, and an RTT of 20 nt. With PE3, he reached 3 to 5% editing. Those experiments were conducted by transfecting plasmids DNA with Lipofectamine 2000. He also tried to transfect the prime editing components in their RNA form, which can improve efficiency [33]. However, in this case, the efficiency was not significantly different between the use of DNA or RNA. He next tried to correct the E342K mutation in human primary fibroblasts. By using NGA-PE2, he obtained 1.99% of editing. In his thesis, Lung also described that overexpression of the three prime repair exonuclease 2 (*TREX2*) gene might decrease prime editing efficiency. It has been shown that this nuclear protein has 3′–5′ exonuclease activities [34]. Non-engineered pegs being really sensible to degradation by exonuclease, the presence of TREX2 may decrease prime editing efficiency by degrading pegs from their 3′ extremity, thus reducing the available number of pegs.

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Hence, the knockout of this gene may help prime editing. It is to note that since Lung's master's thesis is not a peer-reviewed article. Therefore, those results should be taken with caution.

Böck et al. [28]. worked on the editing of two other loci, dnmt1 and Pah^{enu2}, both of which code for proteins expressed in the liver. They first tested two variations of the prime editor, the intein-split PE2ΔRnH [28] and its unsplit version. AAV8 delivered the split version, and the unsplit version, being larger, was delivered by human adenoviral vector 5 (AdV). The latter virus can contain a larger cargo but is much more immunogenic. With the split version, they achieved 15% editing in vivo of the dnmt1 locus. Since they assumed that the unsplit version would give a better result, the authors also tested it in vivo, and they obtained much better results when the prime editor was not split. In neonatal mice, they obtained 58.2% of editing; in adult mice, they obtained 35.9%. Next, they tested the correction of a mutation at the Pah^{enu2} locus (F263S, c.835T > C), this mutation leading to phenylketonuria, a liver disease. They first tested their pegRNAs in an HEK293T cell line that had stably integrated exon 7 of the Pah^{enu2} allele. Their two best constructs had a spacer of 20 nt, a PBS of 13 nt, and an RTT of 16 or 19 nt. These two pegRNAs resulted in an editing of nearly 20%. The one with a 19 nt RTT had fewer off-targets; this pegRNA was thus chosen for the in vivo experiments. For the in vivo experiments, a mouse model of phenylketonuria (Pah^{enu2} mouse model [28]) was used. First, with the split version of the prime editor delivered by AAV8, they obtained meager results (less than 1% with PE2 and less than 2% with PE3). They then used the unsplit version of the prime editor delivered by an AdV. With the PE2 version in adult mice, they obtained only 2% editing. However, the results were better when they treated neonate mice. With PE2, they obtained 6.9% editing, and with PE3, they obtained 11.1% editing. This percentage was sufficient to lead to a therapeutic reduction of blood phenylalanine, without even inducing detectable off-target mutations and without leading to prolonged liver inflammation. Their results are encouraging. This project's major problem in pursuing clinical translation is that they deliver a massive dose of virus (7×10^{14} vector genome/kg). In addition to being very expensive, using that amount of virus results in the induction of the immune system. They however noted that the percentage of edited hepatocytes was maintained even 12 weeks after the injection.

Jiang et al. [29] optimized prime editing to delete and replace long genomic sequences. The authors combined the PE with two pegRNAs, and they called it the PE-Cas9-based deletion and repair (PEDAR) method. They tested their method to remove a 1.38 kb pathogenic insertion in the *FAH* gene in a mouse model of tyrosinemia and replace it with a 19 bp sequence. They successfully delete the fragment and repair the deletion junction to restore FAH expression in the liver. They detected FAH-expressing hepatocytes on PEDAR-treated liver sections and obtained 0.76% of correction. Even if this is low, edited hepatocytes gained a growth advantage and eventually repopulated the liver. Indeed, forty days after the treatment, widespread FAH patches were observed in PEDAR-treated mouse liver sections and edited hepatocytes showed normal morphology.

Kim et al. [30] also worked on hereditary tyrosinemia type 1. They studied the correction by prime editing of a G > A point mutation (position +10 in their pegRNA) in the *FAH* gene in chemically derived hepatic progenitors (CdHs) from a mouse model of hereditary tyrosinemia (HT1 mice) [30]. After the treatment by prime editing, they grafted those cells into the liver of HT1 mice to study the repopulation of the liver by the corrected cells. First, the authors generated CdHs from HT1 mice. Next, they electroporated the cells with PE3, a sgRNA nicking at position -4, and

a pegRNA that contained a spacer of 20 nt, a PBS of 11 nt, and an RTT of 15 nt. They obtained 2.3% editing without any off-target effects. Next, these authors tested the possibility of an ex vivo therapeutic transplantation of a corrected HT1-mCdHs-PE3b (chemically derived hepatic progenitors that are from HT1 mice and that have been treated with PE3b) cell population into the livers of HT1 mice. Because the bulk population of cells had a sufficient editing efficiency, they did not need to isolate cell clones. They thus directly grafted the bulk population of treated cells in the liver of HT1 mice. HT1-mCdHs-PE3b transplanted mice survived for more than 160 days, compared to control mice injected with PBS that died before 90 days. Liver damage of transplanted mice had also significantly decreased. After 140 days, the authors showed that the FAH-positive cell population in the HT1-mCdHs-PE3b transplanted liver repopulated the liver. Because of the repopulation of those cells, the percentage of edits in the liver increased from 2.3 to 34.3%.

Jang et al. [31] also studied the correction by prime editing of a mutation in the *FAH* gene. They worked on the G > A point mutation at the last nucleotide of exon 8. This mutation causes exon 8 skipping and results in loss of function of *FAH*, which causes hereditary tyrosinemia type 1. They first tested some pegRNAs in mutant Fah target sequence–containing HEK293T cells. The best one had a spacer of 20 nt, and the edit was at position +10 from the cut site. By using PE2, they obtained 18.7% of editing in vitro. They next tested their pegRNA in vivo in a mouse model of hereditary tyrosinemia type 1 ($Fah^{mut/mut}$). The authors tested both PE2 and PE3. The first good news was that treated mice survived till the end of the experimental period (40 days for experiments with PE3 and 60 days for experiments with PE2), unlike the sick control mice which showed substantial weight loss and died before day 30 of the experiment. With these results, they demonstrated that the treatment prevented the mice from losing weight and prolonged their survival. Quantitative analyses were conducted following the mouse sacrifice. In the PE3-treated $Fah^{mut/mut}$ mice, they observed that 12% of the Fah mRNA contained the exon 8, compared to the control $Fah^{mut/mut}$ mice that did not have any. The frequency of FAH+ cells in the liver was also quantified. At day 40, PE3-treated mice had on average 61% FAH+ cells. At day 60, PE2-treated mice had on average 33% FAH+ cells. They also determined the percentage of editing by deep sequencing. They observed 11.5% editing in PE3-treated mice and 6.9% in PE2 treated-mice. This percentage was lower than the frequency of FAH+ cells because most hepatocytes are polyploid [35] and because hepatocyte DNA was mixed with nonparenchymal cell DNA. Therefore, the frequency of FAH+ cells is more indicative of clinical improvement. The authors also investigated the presence of indels at or near the targeted nucleotide and at potential off-target sites of the pegRNA. No off-target mutations were detected.

In short, for liver diseases, prime editing has been used to correct mutations causing DGAT1-deficiency, bile salt export pump deficiency, alpha-1-antitrypsin deficiency, phenylketonuria, and tyrosinemia type 1. Prime editing has also been used to generate cell or animal models for liver cancer, bile salt export pump deficiency, alpha-1-antitrypsin deficiency, and a liver disease caused by a mutation in the *DNMT1* gene.