AAV-Mediated CRISPR/Cas9 Gene Editing in Murine Phenylketonuria

Daelyn Y. Richards, Shelley R. Winn, Sandra Dudley, Sean Nygaard, Taylor L. Mighell, Markus Grompe, and Cary O. Harding

Phenylketonuria (PKU) due to recessively inherited phenylalanine hydroxylase (PAH) deficiency results in hyperphenylalaninemia, which is toxic to the central nervous system. Restriction of dietary phenylalanine intake remains the standard of PKU care and prevents the major neurologic manifestations of the disease, yet shortcomings of dietary therapy remain, including poor adherence to a difficult and unpalatable diet, an increased incidence of neuropsychiatric illness, and imperfect neurocognitive outcomes. Gene therapy for PKU is a promising novel approach to promote lifelong neurological protection while allowing unrestricted dietary phenylalanine intake. In this study, liver-tropic recombinant AAV2/8 vectors were used to deliver CRISPR/Cas9 machinery and facilitate correction of the Pah<sup>en2</sup> allele by homologous recombination. Additionally, a non-homologous end joining (NHEJ) inhibitor, vanillin, was co-administered with the viral drug to promote homology-directed repair (HDR) with the AAV–provided repair template. This combinatorial drug administration allowed for lifelong, permanent correction of the Pah<sup>en2</sup> allele in a portion of treated hepatocytes of mice with PKU, yielding partial restoration of liver PAH activity, substantial reduction of blood phenylalanine, and prevention of maternal PKU effects during breeding. This work reveals that CRISPR/Cas9 gene editing is a promising tool for permanent PKU gene editing.

INTRODUCTION
Phenylketonuria (PKU), caused by recessively inherited phenylalanine hydroxylase (PAH) deficiency (OMIM: 261600), is one of the most common inborn errors of metabolism (IEMs) and results in hyperphenylalaninemia and neurotoxic effects of phenylalanine upon the brain. PAH is a cytosolic hepatic homo-tetramer that metabolizes L-phenylalanine (Phe) into L-tyrosine. Without PAH function, chronic untreated hyperphenylalaninemia causes severe neurological damage, leading to intellectual disability, psychological disorders, and seizures. It is important to note that human clinical PKU phenotypes form a disease continuum, ranging from mild hyperphenylalaninemia (blood Phe = 120–600 μM) to mild PKU (blood Phe = 600–1200 μM), to the most severe, classical PKU (cPKU) (blood Phe > 1200 μM), depending upon the amount of residual liver PAH activity in the patient. Due to the severity of largely preventable neurological symptoms, all infants born in medically advanced countries undergo newborn screening for hyperphenylalaninemia and are placed on dietary treatment early in life.

The current standard of care for PKU is lifelong dietary restriction of Phe intake, which requires supplementation with medical foods lacking Phe but containing critical nutrients, including amino acids other than Phe, along with vitamins and minerals that individuals with PKU are unable to retrieve from the severely restricted diet. Impaired access to specialized medical foods is prevalent in the United States due to inconsistent insurance coverage, particularly for adults. Inconsistent access to medical foods is associated with chronic hyperphenylalaninemia, cognitive and behavioral symptoms, and functional disability. Federal legislative efforts to guarantee uniform insurance coverage of medical foods across state boundaries are ongoing through the proposed Medical Nutrition Equity Act. Though the diet is successful at preventing the major manifestations of this disease, cognitive and behavioral abnormalities remain prevalent in the treated PKU population and may occur even in individuals who successfully maintain blood Phe concentrations between 120 and 360 μM, the recommended treatment range. These abnormalities can include lower IQ, executive functioning deficits, and psychiatric disorders (anxiety and depression), with the incidence of deficits increasing with increased blood Phe concentration.

A recent US Food and Drug Administration (FDA)-approved enzyme substitution therapy (pegvaliase) for adults with uncontrolled hyperphenylalaninemia on a diet has revealed dramatic improvements in blood Phe management but is associated with a significant incidence of immune-mediated hypersensitivity reactions against the foreign protein. Additionally, pegvaliase is not approved for use in individuals less than 18 years of age. Pegvaliase therapy requires daily injections, while gene therapy has the potential to correct the disease in a single treatment. In a survey performed by the National PKU Alliance (NPKUA), a national non-profit organization committed to unite, inform, and support people with PKU, over 85% of the

Received 27 November 2019; accepted 10 December 2019; https://doi.org/10.1016/j.omtm.2019.12.004.
Correspondence: Cary O. Harding, Department of Molecular and Medical Genetics, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Mail Code L103, Portland, OR 97239, USA.
E-mail: hardingc@ohsu.edu

234 Molecular Therapy: Methods & Clinical Development Vol. 17 June 2020 © 2019 The Authors. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
community were interested in gene therapy administered as a one-time infusion that would alleviate the lifelong limitations, burdens, and consequences of PKU. Preclinical studies using adeno-associated virus (AAV)-mediated gene addition therapy in the \(Pah\) mouse have shown robust efficacy, but the effectiveness is limited in neonatal mice.\(^2\)\(^3\) This loss of therapeutic efficacy is multifactorial, including the loss of AAV episomes as liver cells divide, the lack of selective advantage for PAH+ liver cells, and the high therapeutic threshold of PKU (~10% hepatocyte correction to lower blood Phe).\(^1\)\(^9\)\(^1\)\(^0\) In pursuit of a more permanent gene therapy, this study aimed to correct the mutation causing PKU in \(Pah\) mice using a targeted, integrating AAV approach. We utilized the CRISPR/Cas9 nuclease system\(^2\)\(^2\)\(^2\)\(^3\)\(^2\) to induce a double-strand break (DSB) near the \(Pah\) mutation and to enhance the opportunity for homologous recombination with the provided repair template harboring the wild-type (WT) sequence. This correction is expected to be permanent even if the targeted hepatocyte divides and its genome is replicated. The barrier impeding efficacy with this approach is the ability to achieve a sufficient number of correctly gene-edited hepatocytes to produce a physiologically relevant improvement in Phe clearance in the absence of any selective growth advantage for PAH+ cells. To address this challenge, a non-homologous end joining (NHEJ) inhibitor shown to facilitate homology-directed repair (HDR) was evaluated as a means of increasing the frequency of successful gene repair in our system.\(^2\)\(^4\)

The \(Pah\) mouse model of cPKU is ideal for this proof-of-principle study, as it contains a single missense mutation in the \(Pah\) gene associated with hyperphenylalaninemia. The pathologic c.835T > C missense mutation alters amino-acid position 263 from a phenylalanine to a serine, disrupting proper function of the catalytic domain of PAH. \(Pah\) homozygotes exhibit all of the symptoms of classical untreated human PKU, including a blood phenylalanine concentration > 2,000 \(\mu\)M while consuming normal mouse chow, hypopigmentation, and associated neuropsychology, resulting in cognitive and behavioral deficits.\(^2\)\(^5\)\(^–\)\(^2\)\(^7\)

RESULTS

Experimental Design

Gene repair occurs through homologous recombination and is greatly enhanced by DSBs, which can be mediated by the \(S.\) \textit{pyogenes} CRISPR/Cas9 system. In order to correct the \(Pah\) mutation in this fashion, three components must be delivered to hepatocytes by the liver-tropic AAV serotype 8 (AAV8): the Cas9 enzyme, a single-guide RNA (sgRNA) with homology to sequences near the target mutation, and a repair template containing homology to the target allele except for a thymine at position c.835, the WT sequence (Figure 1).\(^2\)\(^2\)\(^2\) Due to the limiting packaging capacity of AAV, this system must be manipulated into two AAV vectors in a dual-AAV approach.\(^2\)\(^2\)\(^2\) Here, one recombinant AAV genome contained the \(S.\) \textit{pyogenes} Cas9 coding sequence with expression driven by a liver-specific promoter (LSP) designated P3 (minimal transthyretin promoter [TTRmin], coupled to a \textit{de novo} designed hepatocyte-specific cis-regulatory module 8, [HS-CRM8]),\(^2\)\(^8\)\(^,\)\(^2\)\(^9\)\(^,\)\(^3\)\(^0\) and a second vector expressed a validated guide RNA 46 bp downstream of the c.835 mutation site under the expression control of the human U6 RNA polymerase III (pol III) promoter. This second vector also contained a 2-kb fragment of the WT mouse \(Pah\) genomic sequence (GRCm38: 87569274–87571296) flanking the \(Pah\) \textit{cis} promoter with purposefully introduced synonymous and intronic mutations that alter the PAM and guide sequence, thereby preventing re-cutting by Cas9 as well as facilitating downstream sequence analyses (Figure 1A). Expression of Cas9 and the guide RNA was expected to result in DNA DSBs 46 bp downstream of the \(Pah\) \textit{cis} mutation. Inmate cellular DNA repair mechanisms could alternately repair the DSBs by NHEJ, yielding either a perfectly repaired \(Pah\) \textit{cis} sequence or the introduction of small insertions or deletions (indels) through alternative end joining (Alt-EJ) or by HDR using the Pah genomic DNA fragment from the second AAV vector as a repair template (Figure 1B). The latter, desired outcome would restore the nucleotide at cDNA position 835 to a thymine and lead to the functional correction of PAH activity in the edited hepatocyte. In an effort to maximize the frequency of HDR in preference over NHEJ, vanillin, a potent NHEJ-inhibitor previously shown to increase HDR in a gene-targeting AAV system,\(^2\)\(^4\) was co-administered with the viral vectors in some mice. Animals were re-dosed with drug as adolescents due to volume restraints in neonates and to provide an opportunity for additional correction.

Three animal cohorts were tested in this study. In two cohorts, dual AAV (dAAV) and dAAV plus vanillin (dAAV+Van), all animals received both AAV vectors, and the latter also received vanillin injections. In the third cohort, repair template AAV plus vanillin (rtAAV+Van), animals received the repair template vector only, without the Cas9-expressing vector, but did receive vanillin (Figure 1C). All animals were screened for and tested positive for the presence of appropriate viral DNA according to treatment group in liver tissue via PCR post-harvest.

Therapeutic Efficacy

Single male and female mice from each treatment group were reserved for breeding experiments (Figure 2A) and allowed to survive up to 65 weeks of age. The remaining animals were euthanized at 16–24 weeks of age for characterization and tissue harvest. At the time of euthanasia, the coat color on all dAAV+Van-treated animals was indistinguishable from that of heterozygous or WT animals, while animals in the dAAV and rtAAV+Van groups had minimal, if any, coat color darkening, suggesting that substantial reduction of blood phenylalanine concentration had occurred in dAAV+Van-treated animals only (Figure 2D).

Mean serum phenylalanine concentration at euthanasia was individually variable but approximately 2-fold lower, on average, in dAAV+Van-treated mice (mean ± SEM: 685.2 ± 81.79 \(\mu\)M; range = 252–1,168 \(\mu\)M) in comparison to either dAAV-treated animals (1,518 ± 72.04 \(\mu\)M; range = 1,231–1,863 \(\mu\)M) or rtAAV+Van-treated animals (1,314 ± 119.2 \(\mu\)M; range = 827–1,850 \(\mu\)M). Statistical analysis by one-way ANOVA revealed an overall significant difference in mean serum Phe among the three treatment groups, \(F(2, 27) = 24.11, p < 0.0001\) (Figure 2B). A post hoc intergroup comparison
revealed significant differences between dAAV+Van and the other two groups but no differences between dAAV and rtAAV+Van. There appeared to be no significant additional effects from the second AAV administration in any treatment group. The decrease in serum Phe was sustained through 65 weeks in the two animals in the dAAV+Van cohort that were allowed to survive to that age (shown later in Figure 4A).

Mean liver phenylalanine hydroxylase activity at euthanasia was, again, individually variable and approximately 10-fold higher in dAAV+Van-treated mice (mean percent WT PAH activity ± SEM: 9.6% ± 1.9%; range = 3.3%–24.8%) in comparison to either dAAV-treated animals (1.2% ± 0.5%; range = 0%–5.7%) or rtAAV+Van-treated animals (0.5% ± 0.3; range = 0%–2.3%). One-way ANOVA revealed an overall significant difference in mean PAH enzyme activity among the three treatment groups, F(2, 27) = 16.02, p < 0.0001 (Figure 2B). A post hoc intergroup comparison revealed significant differences between dAAV+Van and the other two groups but no differences between dAAV and rtAAV+Van.

**On-Target Genetic Analysis**

PCR-based next-generation sequencing (NGS) was applied to on- and off-target genomic regions of treated mice (Figure 3A). In order to avoid amplifying and sequencing residual episomal or non-targeted integrations of AAV genomes containing the repair template, we used a nested PCR approach on genomic DNA isolated from treated mouse liver to amplify the on-target genomic region. To produce our first amplicon, a 1.3-kb fragment spanning *Pah*...
exon 7, we used a forward primer that was homologous to sequences in Pal intron 6 but outside the 5' end of the repair template; this fragment was subjected to a second amplification using nested primers containing convenient indexing adapters to yield a 350-bp product. The products of these PCR reactions were indexed with unique barcodes and submitted for NGS on the Illumina MiSeq platform.

We define NHEJ as DNA repair of a DSB that results in perfect restoration of the original sequence without correction of the Palenu2 mutation, an event that cannot be detected experimentally. Repair that results in a deletion of 1 bp or greater in size but retains the c.835C Palenu2 mutation is defined as Alt-EJ, and on-target HDR was defined as detecting exonic and intronic genomic sequences that were consistent with 98% identity to the repair

Figure 2. Efficacy of In Vivo CRISPR/Cas9 Gene Editing in Palenu2 Mice

(A) Experimental timeline. Palenu2/enu2 mice were born, administered the initial dose of drug in the first week of life, weaned at 4 weeks, and placed on standard mouse chow. At week 5, mice were re-dosed with drug. Between 16 and 24 weeks, mice from each cohort were harvested, and one breeding pair per treatment group was placed on high-energy mouse chow and allowed to breed. By 28 weeks, one successful litter was produced by the dAAV+Van pair, while the dAAV and rtAAV+Van breeding pairs did not successfully breed. At this point, the dAAV and rtAAV+Van breeding pairs were euthanized for tissue collection. The dAAV+Van breeder pair produced three more successful litters and were euthanized at 65 weeks. (B) Serum phenylalanine. The graph shows serum phenylalanine levels of dAAV+Van, dAAV, and rtAAV+Van animals at time of euthanasia. The left y axis indicates serum Phe in micromol/l (Mmicromol/l, while the right y axis indicates serum Phe in micrograms per deciliter. The red line indicates the mean serum phenylalanine concentration of an unrelated cohort of untreated male and female Palenu2/enu2 animals combined consuming standard mouse chow.27 The black line indicates the upper limit of serum Phe levels in wild-type mice (180 µM or 3 mg/dL). The x axis is separated by treatment group, in the order of dAAV+Van, dAAV, and rtAAV+Van from left to right, whisker plots depict mean serum phenylalanine ± 2 SD, and each dot on the graph indicates an individual animal. Males are depicted with circles, and females with triangles. The average Phe levels were 685 µM, 1,518 µM, and 1,314 µM, respectively, and ranges were 252–1,168 µM, 1,231–1,863 µM, and 827–1,850 µM, respectively. Intergroup comparison reveals significant differences between dAAV+Van and dAAV (p < 0.0001, ****) and between dAAV+Van and rtAAV+Van (p = 0.0001, ***). (C) PAH enzyme activity. The graph shows the PAH enzyme activity levels of dAAV+Van, dAAV, and rtAAV+Van animals at time of euthanasia. The y axis indicates percent wild-type PAH activity; the x axis is separated by treatment group in the order of dAAV+Van, dAAV, and rtAAV+Van from left to right, whisker plots depict mean liver PAH activity ± 2 SD, and each dot on the graph indicates an individual animal. Males are depicted with circles, and females with triangles. The average PAH activity was 9.5%, 1.2%, and 0.5%, respectively, and ranges were 3.3%–25%, 0%–5.7%, and 0%–2.3%, respectively. Intergroup comparison reveals a significant difference between dAAV+Van and dAAV (p = 0.0002, ***)

www.moleculartherapy.org
A two-way ANOVA revealed a significant difference between treatment groups, $F(2, 54) = 12.87$, $p < 0.0001$; and DNA repair, $F(1, 54) = 13.11$, $p = 0.0006$. Brackets above the treatment groups depict post hoc intergroup statistical comparisons. * - $p < 0.05$; ** - $p < 0.01$, *** - $p < 0.001$, **** - $p < 0.0001$.

Template having been appropriately incorporated into the amplicon in the expected orientation. The percentages of NGS reads with Alt-EJ (indels) or properly targeted HDR are displayed in Figure 3B.
in dAAV+Van-treated animals (mean ± SEM: 21.27% ± 2.63%; range = 12.25%–39.29%) was approximately 2- to 3-fold higher than that in either the dAAV group (12.92% ± 3.64%; range = 2.21–39.54) or the rtAAV+Van group (6.59% ± 1.81%; range = 2.52–19.34). There was no statistical difference in the percentage of indels between dAAV+Van and dAAV or between dAAV and rtAAV+Van, but there was between dAAV+Van and rtAAV+Van, with a p value of 0.0007. The percentage of reads with properly targeted HDR in dAAV+Van-treated animals (mean ± SEM: 5.58% ± 1.23%; range = 0–3.90) or the rtAAV+Van group (3.51% ± 4.32%; range = 0.01–12.57). There was no statistical difference in HDR between dAAV- and rtAAV+Van-treated animals.

NGS data were further analyzed for functional cDNA, in which NGS reads were trimmed to contain only the exon 7 sequence and evaluated for the ability to encode a functional enzyme (Figure 3C), again, revealing a similar significant difference among treatment groups, F(2, 27) = 5.805, p = 0.008 by one-way ANOVA. NGS analysis of dAAV+Van-treated animals, again, revealed an approximately 4-fold increase in the percentage of functional Pah cDNA in the dAAV+Van-treated group (mean ± SEM: 7.54% ± 2.33%; range = 1.94%-24.00%) in comparison to the dAAV group (0.70% ± 0.25%; range = 0.07%-2.54%) or the rtAAV+Van group (2.07% ± 0.73%; range = 0.08%-5.58%).

Correction of the c.835T > C mutation back to thymine by Sanger sequencing in a small portion of genomic liver DNA from treated animals could only be detected in dAAV+Van-treated animals (Figure 3D).

Off-Target Genetic Analysis
Possible sites of off-target Cas9-mediated genotoxicity were selected for NGS analysis using the in silico COSMID tool, which used the guide 1 sequence to determine the top 45 off-target cutting sites in the mouse genome, scored for likelihood of cutting where 0 = highest confidence cutting and 40.7 = lowest confidence cutting (Figure S2). The top three sites selected for further investigation were chromosome chr 10:116187078, chr 12:60323904, and chr X:140587182, with scores of 0.17, 1.33, and 2.93, respectively (Figure 3A). PCR-based NGS of liver genomic DNA from treated animals revealed minimal off-target SpCas9 cutting in all three chromosomal regions, with none accumulating to more than 2% of reads (Figure 3E). Surprisingly, a one-way ANOVA of each chromosomal region between treatment groups revealed a significant difference in only the chr 12 region, F(2, 27) = 5.71, p = 0.0086; with an approximately 2-fold higher percentage of indels in the rtAAV+Van-treated group (mean percentage of indels ± SEM: 0.89% ± 0.19%; range = 0.19%–1.66%) than either treatment group containing the SpCas9 enzyme, dAAV+Van (0.33% ± 0.09%; range = 0%–0.91%) or dAAV (0.38% ± 0.09%; range = 0%–0.80%). Despite this significant increase in indels on chr 12 in the rtAAV+Van treatment group—which cannot be easily explained, as this cohort did not receive any Cas9 enzyme—these data show minimal off-target cutting by SpCas9 at the three locations investigated in these animals. While this was not an exhaustive investigation of all off-target indels in the whole genome, this therapy seems safe for genomic integrity in the three highest predicted off-target cutting sites.

Correlations between Functional Pah Genomes, Liver PAH Activity, and Mean Serum Phe Concentrations
The amount of PAH activity measured in liver homogenates from dAAV+Van mice is linearly related to the percentage of functional PAH reads restored by CRISPR/Cas9-mediated exon 7-directed DSB and successful HDR (Figure 4C). The relationships between serum Phe and the percentage of functional PAH reads (Figure 4D) or between serum Phe and liver PAH activity (Figure 4E) fit best to a one-phase exponential decay model, which is consistent with the known first-order Michaelis-Menten kinetics of the PAH reaction.

Adverse Effects
No adverse effects of treatment were seen in any cohort. The overall health and development of the animals in the dAAV and rtAAV+Van treatment groups were consistent with that expected for untreated Pahenu2/enu2 mice, including hypopigmentation and impaired growth. The animals in the dAAV+Van treatment group showed improved health in comparison to the expected Pahenu2/enu2 development with dark coat pigmentation and improved growth (Figure 4B). Mice were euthanized at ages ranging from 16 to 65 weeks of age; the gross macroscopic appearance of all internal organs was normal at necropsy in all mice. There was no gross evidence of liver neoplasia in any animal, though molecular markers of hepatocellular carcinoma were not evaluated for low-grade carcinogenic transformation in this study.

Successful Breeding of Gene-Corrected Pahenu2 Mice
One pair (a single male and a single female) of gene-corrected Pahenu2/enu2 mice from the dAAV+Van treatment group were allowed to survive past 24 weeks to test both the duration of response and evaluate the breeding potential of gene-corrected mice. Although hyperphenylalaninemic mice are fertile, female Pahenu2/enu2 mice cananibalize all their offspring soon after birth due to a powerful maternal PKU effect, as well as congenital defects in the pups.30 Typically, the Pahenu2 colony is maintained through Pahenu2/enu2 male × Pahenu2/+ female crosses to avoid the maternal PKU effect. The efficacy of dAAV+Van upon serum Phe in both the male and female Pahenu2/enu2 mice was durable through 65 weeks of life (Figure 4A). This breeding pair utilized provided nesting materials to build well-constructed nests, a behavior not typically seen in hyperphenylalaninemic mice. While germline cells of breeders were not directly tested, these results support that there was no germline transmission of the CRISPR/Cas9-mediated gene correction from dAAV+Van-treated mice to their offspring.
In contrast, the breeding pairs from the dAAV and rtAAV+Van treatment groups that were allowed to survive past 24 weeks to evaluate the breeding potential in control treatment groups were both unsuccessful. All animals, two males and two females, that remained a breeding pair until euthanasia. Improved growth in dAAV+Van-treated animals. Three untreated age-matched males and three untreated age matched females were compared to dAAV+Van-treated animals. A three-way ANOVA comparing age, treatment group (control versus treated), and sex showed statistically significant differences between these animals, albeit low sample sizes, F(1, 10) = 10.44, p = 0.0090. Post hoc intergroup comparisons revealed consistent significant differences between each treated and untreated group of either sex (p < 0.0001) as depicted by the brackets with asterisks (**). (C) Correlation between liver PAH activity and functional Pah exon 7 reads in dAAV+Van-treated mice. Plot of percent wild-type liver PAH enzyme activity on the x axis versus the percent functional Pah exon 7 reads on the y axis with linear regression analysis. Each point is a single dAAV+Van mouse at euthanasia. Graph shows percent functional Pah exon 7 reads on the x axis and percent wild-type PAH enzyme activity on the y axis. Each point represents a dAAV+Van animal at time of euthanasia. (D) Correlation between serum Phe and percent functional Pah exon 7 reads in dAAV+Van-treated animals. Plot of serum Phe on the x axis versus percent functional Pah exon 7 reads on the y axis. Each point represents a dAAV+Van animal at time of euthanasia. The data were fit with a non-weighted, non-linear exponential one-phase decay using least-squares regression. (E) Correlation between serum Phe and liver PAH enzyme activity in dAAV+Van-treated animals. Plot of serum Phe on the y axis versus percent wild-type liver PAH enzyme activity on the x axis. Each point represents a dAAV+Van animal at time of euthanasia. The data were fit with a non-weighted, non-linear exponential one-phase decay using least-squares regression.

**DISCUSSION**

This study reveals promise for the utilization of the CRISPR/Cas9 system followed by HDR of the AAV-supplied donor DNA to achieve in vivo liver-directed gene correction for PKU. The permanent nature of editing mutations within the genome removes the concern for temporary effectiveness as seen in traditional AAV-mediated gene addition therapy for PKU. Our prior attempts with AAV-mediated, liver-directed gene addition in neonatal mice resulted in the rapid clearance of AAV episomes and only brief periods of liver PAH expression (data not shown). This result has been reported by other investigators following administration of liver-directed AAV vectors to juvenile animals in the absence of any selective growth advantage for random AAV integration events. For the first time, we have achieved liver-directed gene therapy in neonatal mice that persisted beyond adolescence into adult mice that was associated with substantial, albeit partial, correction of hyperphenylalaninemia for up to at least 6 months of age (at the time when most animals were euthanized for tissue harvest) and impressively up to 65 weeks in the case of a...
single breeder pair. Importantly, all animals in the dAAV+Van treatment group were improved in classification from severe, classical PKU to either mild PKU or mild hyperphenylalaninemia. If this dAAV+Van regimen were applied to humans suffering from classical PKU, it would greatly improve the overall quality of life by allowing increased dietary phenylalanine intake, improved maternal PKU outcomes, and possibly improved behavior and cognition, even in the case with the lowest correction (4.1% HDR, 3.3% PAH enzyme activity, 1,168 μM blood Phe on an unrestricted diet). This treatment approach was not associated with any notable acute toxicity or any long-term adverse effects. In fact, the treatment was associated with complete reversal of the abnormal phenotypes associated with chronic hyperphenylalaninemia in Pahtm2/enu2 mice, including impaired growth and hypopigmentation, despite the fact that serum phenylalanine concentrations were not fully corrected to levels typical of WT mice. Although most animals did not achieve phenylalanine levels in the recommended therapeutic range of 120–360 μM, this is the first gene therapy approach for PKU that has demonstrated lifelong effectiveness from early infancy in a PKU mouse model.

Furthermore, AAV-delivered, CRISPR/Cas9-mediated, liver-directed gene correction was associated with the prevention of maternal PKU syndrome in Pahtm2/enu2 mice. Elevated maternal blood phenylalanine in humans during pregnancy causes microcephaly, intrauterine growth retardation, congenital heart disease, and postnatal developmental disability, the so-called maternal PKU syndrome.33 Hyperphenylalaninemic Pahtm2/enu2 dams gestate to term but invariably cannibalize their progeny.30 The single breeder pair from the dAAV+Van group successfully produced four litters (Figure 2A) without any observed pregnancy losses, an outcome that is unprecedented in our laboratory, although we and others have had prior limited success with breeding Pahtm2/enu2 dams following treatment of hyperphenylalaninemia applied to adult mice.34

CRISPR/Cas9-mediated genome editing is widely used to introduce DSBs in DNA and to facilitate the deletion of functional genes in cultured cells and in whole organisms, but correction of mutant genes following Cas9-mediated DSB is entirely dependent upon the innate DNA repair activity of the host cell.35 NHEJ, in most cases, is the default repair mechanism used to repair the DSB; this blunt end joining can result in full restoration of the original sequence (an event that cannot be detected experimentally), leaving the mutant gene intact. Alternatively, some 3’ clipping of the free ends may occur prior to annealing of the break (so-called Alt-EJ) or healing of the break with incorporation of a repair template through HDR.35,36 The former, less well-understood repair mechanism, Alt-EJ, yields a small deletion at the site of the DSB. If NHEJ or Alt-EJ occurs following Cas9-mediated cleavage of a mutant gene target, a functional open reading frame will not be restored. Only HDR with the incorporation of a repair template harboring the normal functional sequence will yield a functional open reading frame, but under typical conditions, the desired HDR is a rare event. In our experiment, following administration of both AAV vectors carrying the repair template and expressing the Cas9 reagents (but without the NHEJ inhibitor vanillin), the mean percentage of functional Paht open reading frames recovered following HDR was fewer than 1%. This repair frequency is insufficient to cause any appreciable effect upon the PKU phenotype, although it may be sufficient for treatment for some disorders such as hemophilia, where secreted protein from even a small population of hepatocytes can be therapeutic.27 In diseases and model systems where corrected cells enjoy a selective growth advantage, a small number of gene-edited hepatocytes can expand under selective pressure to a therapeutically relevant population. Such results have been reported for the treatment of murine fumarylacetoacetate hydrolase deficiency, a model of human tyrosinemia type 1.38 Unfortunately, PAH-expressing hepatocytes do not enjoy a selective growth advantage over PAH-negative cells.20 Only when we treated the mice with vanillin, a potent NHEJ inhibitor, did the frequency of HDR increase to a level that became physiologically relevant in this model.

Our results were somewhat less robust than those of the Wilson laboratory, who used a similar dAAV system in the murine ornithine transcarbamylase (OTC) model, OTC sfl36, and achieved approximately 10% gene correction in a disease that does not enjoy a selective growth advantage for corrected cells without use of NHEJ inhibitors. These conflicting findings could be due to differences in either the initial efficiency of Cas9-mediated double-strand breakage or the efficiency of on-target HDR using the repair template. In our study, only 14% of Paht alleles in dAAV-treated mice demonstrated molecular evidence of genomic alterations (HDR or indels due to Alt-EJ combined) in comparison to 41% overall genomic alteration in the OTC study. This suggests that the efficiency of Cas9-mediated double-strand breakage was much lower in our experiment. This could be attributed to differences in the total dose of AAV vectors delivered or the ratio of Cas9-expressing to repair template vectors administered between the two experiments, both of which could have affected the efficiency of Cas9-mediated DSB formation. Alternatively, it is also possible that perfect NHEJ following Cas9 administration resulted in the restoration of the original pathogenic sequence in a majority of cleaved alleles, a result that cannot be detected experimentally. When we added the NHEJ inhibitor vanillin to dAAV, the mean frequency of total genomic alteration increased to 34% (21% indels and 13% HDR). This result supports our hypothesis that the initial frequency of Cas9-mediated DSBs was likely similar to that of the previously published OTC experiment but that the majority of Cas9-mediated DSBs were perfectly repaired by NHEJ in dAAV-treated mice rather than by Alt-EJ or HDR. The precise reason for the difference in the efficiency of on-target HDR is yet unknown. Both the repair template in our experiment and the OTC template contained multiple mismatches in comparison to their genomic targets; these differences could impair HDR efficiency. It is possible that nuanced design differences between the two templates or innate differences in the genomic target regions could have contributed to increased heteroduplex rejection, D-loop instability during HDR, or mismatch-repair-mediated antirecombination in our study.59 While the dAAV on-target HDR remained low, the addition of vanillin improved the on-target HDR efficiency to a level similar to that achieved in the OTC study.
The therapeutic effectiveness of this gene-editing approach was highly variable between individual mice. One source of variability could be the technically challenging facial vein injection. While we were able to measure correlations between the percentage of functional open reading frames, liver PAH activity, and final blood Phe concentration, some mice still exhibited higher than expected final blood Phe concentrations for the percentage of functional open reading frames and corresponding liver PAH activity achieved in those animals. Typically, in mice treated with AAV-mediated gene addition, restoring liver PAH activity to greater than 10% of WT activity is sufficient to fully correct blood Phe levels to normal. These discordant results could be attributed to the number of PAH-expressing cells, which is also a critical factor in total body Phe flux. PAH activity must be expressed in at least 10% of hepatocytes for complete normalization of blood Phe concentrations; restriction of PAH activity to a smaller population of hepatocytes (i.e., lower transduction frequency) significantly limits Phe clearance. We hypothesize that, in this experiment, HDR occurred in a small population of evenly dispersed hepatocytes (likely fewer than 10% of hepatocytes). Given that hepatocytes are polyploid (the genetic complement can range from diploid [2n] to octoploid [8n] and even higher [128n]), a small number of cells with many edited genomes per cell could be responsible for the relatively high number of functional genomic reads and PAH activity that we have measured. The therapeutic effect may be limited by total Phe transport capacity into the restricted cell population rather than functional enzyme availability. Alternatively, it is possible that gene correction occurred predominantly within peri-central venous hepatocytes, a zone within the hepatic acinus that is not typically responsible for substantial phenylalanine hydroxylation; this is a function normally accomplished by perportal hepatocytes. In this study, we performed systemic injections of AAV8 vectors, which, in adult mice, leads to primarily pericentral venous transduction but may lead to mixed perportal and pericentral venous transduction in newborn mice. Unfortunately, we are unable to directly evaluate the percentage or location of gene-corrected hepatocytes in treated liver by immunohistology, because the Pah<sup>enu2/enu2</sup> model produces abundant, albeit inactive, PAH protein; no available antibody is able to distinguish between active and inactive PAH protein. Further studies using single-cell omics would be informative for understanding the effects of this therapy at the level of individual cells.

While the Pah<sup>enu2/enu2</sup> mouse recapitulates human cPKU phenotypically, this isogenic model does not capture the wide range of almost 1,000 known pathogenic variants in the PAH gene. One limitation of CRISPR/Cas9-mediated gene editing as we have used it here or as others have used a CRISPR/Cas9 base-editor fusion is that a different guide RNA—and, therefore, one of the two AAV vectors—would have to be redesigned for each patient with a novel mutation, or at least there would need to be unique reagents for each exon of the Pah gene. Future development of CRISPR/Cas9 gene editing should be designed to be broadly applicable to all human PKU genotypes and should aim to drive Phe levels down to WT levels. While this study was a beginning for CRISPR/Cas9 gene editing for PKU, further studies are needed before in vivo gene-editing translation into human medicine.

**MATERIALS AND METHODS**

**Animal Husbandry**

Animal care and experimentation were performed in accordance with the guidelines of the Department of Comparative Medicine, Oregon Health & Science University, and the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6-Pah<sup>enu2/enu2</sup> mice, which are homozygous for a missense c.835T > C (p.F263S) mutation in exon 7 of the murine Pah gene, are completely deficient in liver PAH activity; are, consequently, hyperphenylalaninemic on an unrestricted diet; and are a representative animal model of human PKU. Neonatal Pah<sup>enu2/enu2</sup> mice for these experiments were generated through breeding of Pah<sup>enu2/enu2</sup> sires to Pah<sup>+/+</sup> dams. Genotyping for the presence of the Pah<sup>enu2</sup> mutation was performed by a TaqMan qPCR assay. All mice were fed tap water and standard mouse chow (LabDiet Picolab Rodent Diet 5L0D, St. Louis, MO, USA) ad libitum, providing approximately 24% protein and 1.04% L-Phe by weight, except the breeders that received high-energy chow (LabDiet Rodent High Energy Diet 5058, St. Louis, MO, USA), providing approximately 22% protein and 0.99% L-Phe. Given that adult mice consume approximately 5 g chow per day, daily L-Phe intake was estimated to be approximately 50 mg/day. The animals were housed under a standard 12-h:12-h on-off light cycle. All surgical procedures were carried out with inhaled isoflurane general anesthesia to minimize pain and discomfort.

**Guide Design and Validation**

The sequence flanking c.835T > C was entered into the MIT Guide Design tool. Three guides were chosen for validation using the cell-culture-based Surveyor system and revalidated with the in vitro Takara Guide-It Kit (Figure S1).

**Viral Production**

Plasmid AAV2 LSP-Cas9 was graciously supplied by the Grompe laboratory, and the AAV2 repair template with guide plasmid was synthesized by GeneScript. Plasmids were confirmed for overall integrity, and intact Inverted Terminal Repeats (ITRs) via RE screen using Taq1 and Ahd1, respectively. The OHSU Molecular Virology Support Core produced large-scale preparations of recombinant rAAV2/8 viruses, referenced as rAAV2/8_LSP_SpCas9 and AAV2/8_8_Ex7RepairTemp_U6G1, using standard triple plasmid transfection procedures into cultured HEK293 cells and purified by iodixanol gradient ultracentrifugation. AAV titers were determined using ITR-based qPCR analysis.

**Vector Administration and Vanillin Treatment**

Post-natal day 3 Pah<sup>enu2/enu2</sup> mice were injected through the facial vein with 2.1 × 10<sup>11</sup> vg AAV2/8_LSP_SpCas9 and 3.3 × 10<sup>11</sup> vg AAV2/8_8_Ex7RepairTemp_U6G1 in a single injection, with a total volume of 10 µL and administrated daily intraperitoneal injections of 100 mg/kg vanillin for 7 days, according to treatment designation. Mice were weaned at 4 weeks of age. Between 5 and 8 weeks of age, mice underwent a retro-orbital bleed to collect plasma.
using heparinized capillary tubes followed by a tail vein injection of 
$2.1 \times 10^{12}$ vg AAV2/8_LSP_SpCas9 and $3.3 \times 10^{12}$ vg AAV2/8_Ex7-
RepairTemp_U6G1 in a single injection, with a total volume of 
100 µL and administered daily intraperitoneal injections of 100 mg/ 
kg vanillina for 7 days, according to treatment designation.

**Euthanasia and Tissue Harvest**

Animals were sedated using inhaled isoflurane anesthesia. Whole 
blood was collected by cardiac puncture and allowed to clot in an 
Eppendorf tube, and serum was separated by centrifugation. The 
mice were then euthanized by exsanguination and perfused with 
20 mL normal saline via the left cardiac ventricle to clear blood from 
the liver.

**Serum Phenylalanine**

Serum phenylalanine was determined using an established fluoro-
metric protocol.50

**Liver PAH Enzyme Activity**

PAH enzyme activity was determined using an established radioactive 
chromatography assay,51 with modifications.52

**Liver DNA Extraction**

Separate liver biopsies, approximately 7 mg each, were collected from 
four different lobes for each animal and collectively put through the 
QIAGEN DNeasy Blood & Tissue Kit according to the manufac-
turer’s protocol.

**Screening for Viral DNA**

Liver DNA was screened for the presence of viral DNA by standard 
bench-top PCR using primers specific to each transgene. A portion of 
Cas9 sequence was amplified from the Cas9 vector (forward primer: 
5’-CAGCCAGAAAGATCTACAAAG-3’, reverse primer: 5’-CATT 
CCCTGCGTACAGTATTT-3’), and the human U6 sequence was 
amplified from the repair template vector (forward primer: 5’-GAG 
GGCCTATTTCCCATGATT-3’; reverse primer: 5’-TGTTTCTGCTCT 
TTCCACAGATA-3’).

**On-Target PCR Amplicon Preparation**

The QIAGEN LongRange PCR Kit was used according to the manufac-
turer’s protocol on genomic liver DNA using a forward primer 
targeting genomic DNA 58 bp upstream of the 5’ end of the repair tem-
plate sequence, 5’-AGTTACTGTCGTTTGCAATGCCGC-3’, and a 
reverse primer, 5’-GCACAGTAGCCTAATTTCTCTTCCTTAG-3’, 
located 426 bp downstream of the C836T mutation within the repair 
template region. A secondary nested PCR was performed using the 
5’-GGGTTGTAGTCTCTGTGGATTACCA-3’ forward primer and 
5’-GCACAGTAGCCATATTCTCTTCCTTAG-3’ reverse primer 
with Invitrogen Platinum Taq DNA Polymerase according to the manu-
facturer’s recommendations.

**On- and Off-Target NGS**

Off-target sites were selected using two in silico tools: Cas-OFFinder 
and COSMID.53,54 Three guides present in both algorithms scored 
with the highest probability of off-target cutting were selected: 
Chr10:116187094 (COSMID score, 0.17), Chr12:60323919 (COSMID 
score, 1.33), and ChrX:140587188 (COSMID score, 2.93), as well as 
the target site Chr10: 87570342 (COSMID score, 0.00). COSMID-
designed Illumina primers were ordered with adapter sequences 
developed by the O’Roak lab. The four regions were PCR amplified 
for each animal with the Invitrogen Platinum Taq DNA Polymerase 
PCR Kit using genomic liver DNA according to the manufacturer’s 
protocol. Products were run on a 1.2% agar gel and purified with the 
QIAGEN PCR Purification Kit according to the manufacturer’s 
protocol. A secondary PCR was performed to index each animal 
with a unique barcode using primers developed by the O’Roak lab 
using the Invitrogen Platinum Taq DNA Polymerase PCR Kit for 
6 cycles. Products were run on a 1.2% gel and purified with the 
QIAGEN QIAquick PCR Purification Kit. Samples were quantified 
using the Epoch Microplate Spectrophotometer and pooled in 1:1 
ratios for each region. Pooled samples were then quantified with 
the Qubit 4 Fluorometer and diluted to 2-nM concentrations. Each 
region was then combined in a 1:1 ratio in 20 µL and sent to the 
Molecular Technologies Core at Oregon Health and Science Univer-
sity. Samples were prepared with the V2 500 kit and run on the 
Illumina MiSeq instrument that downloaded raw data in FASTQ 
format to Illumina Base Space.

**MiSeq Sequencing Data Analysis**

FASTQ files generated by NGS were split by index using Python, 
paired-end reads were merged with PEAR v0.9.6,55 and common 
primer sequences were trimmed using Cutadapt v1.1856 as previously 
described.57 Reads were split into each region for each animal by 
match to the first 25 bp in each amplicon. Data were analyzed in 
CRISPResso using the Pah$^{+}\text{mut}^2$ allele as the reference sequence for 
the target region and mouse reference for each off-target region; in-
dels were called with >1-bp indel mismatch to the reference, and 
HDR was called with a 98% identity to the repair template sequence.58 
In addition, on-target data were run for percent match to the func-
tional coding sequence of the PAH exon 7 region.

**Sanger Sequencing**

The purified on-target PCR product described earlier was quantified 
using the Epoch Microplate Spectrophotometer and sent for standard 
Sanger sequencing with the 5’-GGGTTGTAGTCTCTCTGGATT 
TACCA-3’ forward primer on an ABI 3130XL sequencer at the 
Vollum DNA Services Core at Oregon Health & Science University.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10. 
1016/j.omtm.2019.12.004.

**AUTHOR CONTRIBUTIONS**

D.Y.R. performed the experiments, analyzed data, and wrote the 
manuscript. S.R.W. performed the surgical procedures and assisted 
with mouse breeding, molecular techniques, and experimental design. 
S.D. assisted with mouse breeding and molecular techniques. S.N. 
assisted with experimental design and reagents. T.L.M. assisted with
NGS preparation and bioinformatic analyses. M.G. assisted with reagents, experimental design, analyses, data interpretation, and manuscript editing and oversaw D.Y.R. as a dissertation advisory committee member. C.O.H. assisted with funding, experimental design, analyses, data interpretation, and manuscript editing and was primary mentor to D.Y.R. during graduate school.

ACKNOWLEDGMENTS

D.Y.R. and the experiments were funded by the National PKU Alliance, USA. S.R.W., S.D., and C.O.H. were funded by National Institutes of Health (USA) grant RO1 NS080866. We would like to acknowledge the O’Roak laboratory for supplying us with NGS expertise and reagents. We would also like to express our gratitude to the National PKU Alliance for supporting this research.

REFERENCES

1. Blau, N. (2016). Genetics of phenylketonuria: then and now. Hum. Mutat. 37, 508–515.
2. Flydal, M.I., and Martinez, A. (2013). Phenylalanine hydroxylase: function, structure, and regulation. IUBMB Life 65, 341–349.
3. Cazzorla, C., Bensi, G., Biasucci, G., Leuzzi, V., Manti, F., Musumeci, A., Papadla, F., Stoppioni, V., Tummolo, A., Vendemiale, M., et al. (2018). Living with phenylketonuria in adulthood: the PKU ATTITUDE study. Mol. Genet. Metab. Rep. 16, 39–45.
4. 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.
5. Lichter-Konecki, U., and Vockley, J. (2019). Phenylketonuria: current treatments and future developments. Drugs 79, 495–506.
6. Vockley, J., Anderson, H.C., Antshel, K.M., Braverman, N.E., Burton, B.K., Frazier, D.M., Mitchell, J., Smith, W.E., Thompson, B.H., and Berry, S.A.; American College of Medical Genetics and Genomics Therapeutics Committee (2014). Phenylalanine hydroxylation deficiency: diagnosis and management guideline. Genet. Med. 16, 188–200.
7. Singh, R.H., Cunningham, A.C., Medidi, S., Douglas, T.D., Frazier, D.M., Jeffers, I., McCune, H., Moseley, K.D., Ogata, B., et al. (2016). Updated, web-based nutrition management guideline for PKU: An evidence and consensus based approach. Mol. Genet. Metab. 118, 72–83.
8. Greene, C.L., and Longo, N. (2014). National Institutes of Health (NIH) review of evidence in phenylalanine hydroxylase deficiency (phenylketonuria) and recommendations/guidelines for therapy from the American College of Medical Genetics (ACMG) and Genetics Metabolic Dietitians International (GMDI). Mol. Genet. Metab. 112, 85–86.
9. Brown, C.S., and Lichter-Konecki, U. (2015). Phenylketonuria (PKU): a problem solved? Mol. Genet. Metab. Rep. 6, 8–12.
10. Ding, X.Q., Fiehler, J., Kohlschütter, B., Wittkugel, O., Zeumer, H., and Ulrich, K. (2008). MRI abnormalities in normal-appearing brain tissue of treated adult PKU patients. J. Magn. Reson. Imaging 27, 998–1004.
11. Feldmann, R., Osterloh, J., Onon, S., Fromm, J., Rutsch, F., and Weglage, J. (2008). Neurocognitive functioning in adults with phenylketonuria: report of a 10-year follow-up. Mol. Genet. Metab. 126, 246–249.
12. Stone, W.L., Basit, H., and Los, E. (2019). Phenylketonuria. StatPearls (StatPearls Publishing), https://www.ncbi.nlm.nih.gov/books/NBK53578/.
13. Gupta, S., Lau, K., Harding, C.O., Shepherd, G., Boyer, R., Atkinson, J.P., Knight, V., Olbertz, J., Larimore, K., Gu, Z., et al. (2018). Association of immune response with efficacy and safety outcomes in adults with phenylketonuria administered pegvaliase in phase 3 clinical trials. EbioMedicine 37, 366–373.
14. Harding, C.O., Amato, R.S., Stuy, M., Longo, N., Burton, B.K., Posner, J., Weng, H.H., Merlaiinen, M., Gu, Z., Jiang, I., and Vockley, J.; PRISM-2 Investigators (2018). Pegvaliase for the treatment of phenylketonuria: a pivotal, double-blind randomized discontinuation phase 3 clinical trial. Mol. Genet. Metab. 124, 20–26.
15. Longo, N., Dimmock, D., Levy, H., Viau, K., Bausell, H., Bilder, D.A., Burton, B., Gross, C., Northrup, H., Rohr, F., et al. (2019). Evidence- and consensus-based recommendations for the use of pegvaliase in adults with phenylketonuria. Genet. Med. 21, 1851–1867.
16. Thomas, J., Levy, H., Amato, S., Vockley, J., Zori, R., Dimmock, D., Harding, C.O., Bilder, D.A., Weng, H.H., Olbertz, J.; et al.; PRISM investigators (2018). Pegvaliase for the treatment of phenylketonuria: results of a long-term phase 3 clinical trial program (PRISM). Mol. Genet. Metab. 124, 27–38.
17. Harding, C.O., Gillingham, M.B., Hamman, K., Clark, H., Goebel-Daghghi, E., Bird, A., and Koebel, D.D. (2006). Complete correction of hyperphenylalaninemia following liver-directed, recombinant AAV2/8 vector-mediated gene therapy in murine phenylketonuria. Gene Ther. 13, 457–462.
18. Ding, Z., Harding, C.O., Rebuffat, A., Elzaouk, L., Wolf, J.A., and Thony, B. (2008). Correction of murine PKU following AAV-mediated intramuscular expression of a complete phenylalanine hydroxylating system. Mol. Ther. 16, 673–681.
19. Mingozzi, F., and High, K.A. (2011). Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. Nat. Rev. Genet. 12, 341–355.
20. Hamman, K., Clark, H., Montini, E., Al-Dhalimi, M., Grompe, M., Finegold, M., and Harding, C.O. (2005). Low therapeutic threshold for hepatocyte replacement in murine phenylketonuria. Mol. Ther. 12, 337–344.
21. Embury, J.E., Charron, C.E., Martinay, A., Zori, A.G., Liu, B., Ali, S.F., Rowland, N.E., and Lapin, P.J. (2007). PKU is a reversible neurodegenerative process within the nigrostriatum that begins as early as 4 weeks of age in Pah(enu2) mice. Brain Res. 1127, 136–150.
22. Vockley, J., Lemay, P.J., and Zori, R.; PRISM investigators (2014). Pegvaliase for the treatment of phenylketonuria: results of a long-term phase 3 clinical trial program (PRISM). Mol. Genet. Metab. 124, 20–26.
23. fitness and reagents. We would also like to express our gratitude to the National PKU Alliance for supporting this research.
34. Zeile, W.L., McCune, H.C., Musson, D.G., O’Donnell, B., O’Neill, C.A., Tsuruda, L.S., Zori, R.T., and Laipis, P.J. (2018). Maternal phenylketonuria syndrome: studies in mice suggest a potential approach to a continuing problem. Pediatr. Res. 83, 889–896.
35. Ceccaldi, R., Rondinelli, B., and D’Andrea, A.D. (2016). Repair pathway choices and consequences at the double-strand break. Trends Cell Biol. 26, 52–64.
36. Chang, H.H.Y., Pannunzio, N.R., Adachi, N., and Lieber, M.R. (2017). Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat. Rev. Mol. Cell Biol. 18, 495–506.
37. Ohmori, T., Nagao, Y., Mizukami, H., Sakata, A., Muramatsu, S.I., Ozawa, K., Tominaga, S.I., Hanazono, Y., Nishimura, S., Nureki, O., and Sakata, Y. (2017). CRISPR/Cas9-mediated genome editing via postnatal administration of AAV vector cures haemophilia B mice. Sci. Rep. 7, 4159.
38. Yin, H., Xue, W., Chen, S., Bogorad, R.L., Grompe, M., Koteliansky, V., Sharp, P.A., Jia, T., and Anderson, D.G. (2014). Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. Nat. Biotechnol. 32, 551–553.
39. Tham, K.C., Kanaar, R., and Lebbink, J.H.G. (2016). Mismatch repair and homologous recombination. DNA Repair (Amst.) 38, 75–83.
40. Hamman, K.J., Winn, S.R., and Harding, C.O. (2011). Hepatocytes from wild-type or heterozygous donors are equally effective in achieving successful therapeutic liver re-population in murine phenylketonuria (PKU). Mol. Genet. Metab. 104, 235–240.
41. Duncan, A.W., Taylor, M.H., Hickey, R.D., Hanlon Newell, A.E., Lenzi, M.L., Olson, S.B., Finegold, M.J., and Grompe, M. (2010). The ploydovector of mature hepatocytes as a source of genetic variation. Nature 467, 707–710.
42. Duncan, A.W. (2013). Aneuploidy, polyploidy and ploidy reversal in the liver. Semin. Cell Dev. Biol. 24, 347–356.
43. Kurrinna, S., Stratton, A.A., Coban, Z., Schumacher, J.M., Grompe, M., Duncan, A.W., and Barton, M.C. (2013). p53 regulates a mitotic transcription program and determines ploidy in normal mouse liver. Hepatology 57, 2004–2013.
44. Trefts, E., Gannon, M., and Wasserman, D.H. (2017). The liver. Curr. Biol. 27, R1147–R1151.
45. Bell, P., Wang, L., Gao, G., Haskins, M.E., Tarantal, A.F., McCarter, R.J., Zhu, Y., Yu, H., and Wilson, J.M. (2011). Inverse zonation of hepatocyte transduction with AAV vectors between mice and non-human primates. Mol. Genet. Metab. 104, 395–403.
46. Himmelreich, N., Shen, N., Okun, J.G., Thiel, C., Hoffmann, G.F., and Blau, N. (2018). Relationship between genotype, phenylalanine hydroxylase expression and in vitro activity and metabolic phenotype in phenylketonuria. Mol. Genet. Metab. 125, 86–95.
47. Villiger, L., Grisch-Chan, H.M., Lindsay, H., Ringnalda, F., Pogliano, C.B., Allegri, G., Fingeruth, R., Habele, J., Matos, J., Robinson, M.D., et al. (2018). Treatment of a metabolic liver disease by in vivo genome base editing in adult mice. Nat. Med. 24, 1519–1525.
48. McDonald, J.D., Bode, V.C., Dove, W.F., and Shedlovsky, A. (1990). Pahhph-5: a mouse mutant deficient in phenylalanine hydroxylase. Proc. Natl. Acad. Sci. USA 87, 1965–1967.
49. Kleven, M.D., Gomes, M.M., Wortham, A.M., Enns, C.A., and Kahl, C.A. (2018). Ultrathin recombinant AAV8 vector can be safely administered in vivo and efficiently transduces liver. PLoS ONE 13, e0194728.
50. McCaman, M.W., and Robins, E. (1962). Fluorimetric method for the determination of phenylalanine in serum. J. Lab. Clin. Med. 59, 885–890.
51. Ledley, F.D., Hahn, T., and Woo, S.L. (1987). Selection for phenylalanine hydroxylase activity in cells transformed with recombinant retroviruses. Somat. Cell Mol. Genet. 13, 145–154.
52. Harding, C.O., Wild, K., Chang, D., Messing, A., and Wolff, J.A. (1998). Metabolic engineering as therapy for inborn errors of metabolism—development of mice with phenylalanine hydroxylase expression in muscle. Gene Ther. 5, 677–683.
53. Bae, S., Park, J., and Kim, J.S. (2014). Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 30, 1473–1475.
54. Cradick, T.J., Qiu, P., Lee, C.M., Fine, E.J., and Bao, G. (2014). COSMID: a web-based tool for identifying and validating CRISPR/Cas off-target sites. Mol. Ther. Nucleic Acids 3, e214.
55. Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620.
56. Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J 17, 10–12.
57. Mighell, T.L., Evans-Dutton, S., and O’Roak, B.J. (2018). A saturation mutagenesis approach to understanding PTEN lipid phosphatase activity and genotype-phenotype relationships. Am. J. Hum. Genet. 102, 943–955.
58. Pinello, L., Canver, M.C., Hoban, M.D., Orkin, S.H., Kohn, D.B., Bauer, D.E., and Yuan, G.C. (2016). Analyzing CRISPR genome-editing experiments with CRISPResso. Nat. Biotechnol. 34, 695–697.