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(71) Applicant: **THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA** [US/US]; 3160 Chestnut Street, Suite 200, Philadelphia, PA 19104 (US).

(72) Inventors: **WILSON, James, M.**; 1831 Delancey Street, Philadelphia, PA 19103 (US). **SIDRANE, Jenny, Agnes**; 1280 W Evergreen Drive, Phoenixville, PA 19460 (US). **ASHLEY, Scott**; 4045 Baltimore Ave., Apt C4, Philadelphia, PA 19104 (US).

(74) Agent: **SCHALLER, Colleen, M.** et al.; Howson & Howson LLP, 350 Sentry Parkway, Building 620, Suite 210, Blue Bell, PA 19422 (US).

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(54) Title: GENE THERAPY FOR TREATING PHENYLKETONURIA

(57) Abstract: Compositions and regimens useful in treating phenylketonuria are provided. The compositions include recombinant adeno-associated virus (rAAV) with a transthyretin enhancer and promoter driving expression of a human phenylalanine hydroxylase.

WO 2018/126112 A1

GENE THERAPY FOR TREATING PHENYLKETONURIA

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN ELECTRONIC FORM

Applicant hereby incorporates by reference the Sequence Listing material filed in electronic form herewith. This file is labeled "UPN-16-7939PCT_ST25.txt".

1. INTRODUCTION

The application relates to embodiments useful for a gene therapy for treating phenylketonuria.

2. BACKGROUND

As one of the most common inborn errors of metabolism, Phenylketonuria (PKU) occurs in 1 in 10,000 to 15,000 newborns in the United States. The current treatment approaches require the affected individual to adhere consistently to an unpalatable and expensive dietary restriction and/or take enzyme substitution with phenylalanine ammonia lyase from birth for their whole life.

The most common cause of PKU is deficiency of phenylalanine hydroxylase (PAH) due to a recessively inherited mutation in the *PAH* gene. PAH is expressed primarily in the liver that catalyzes the irreversible hydroxylation of phenylalanine to tyrosine. Thus, deficiency in PAH affects the catabolic pathway of phenylalanine, resulting in accumulation of phenylalanine. High plasma phenylalanine levels results in build-up of phenylalanine in the brain and can affect brain development and function, resulting in intellectual disability and seizures. Furthermore, reduction of plasma phenylalanine via dietary restriction and enzyme substitution is expensive, inconvenient and has been linked with various adverse complications, such as persistent mild cognitive deficits.

An alternative approach to achieve sustained therapeutic levels of PAH is through continuous *in vivo* production of the native enzyme in the hepatocytes using gene transfer mediated by a cell-directed adeno-associated virus (AAV) or other viral or non-viral vector. Several attempts of vector-mediated PAH expression have been tested preliminary on mouse studies. *See, e.g.*, Harding et al, Complete correction of

hyperphenylalaninemia following liver-directed, recombinant AAV2/8 vector mediated gene therapy in murine phenylketonuria Gene Ther. 2006 Mar; 13(5):457-6 and Viecelli et al, Treatment of Phenylketonuria Using Minicircle-Based Naked-DNA Gene Transfer to Murine Liver Hepatology. 2014 Sep; 60(3): 1035–1043, which are incorporated herein by reference. However, the evaluations of delivery efficiency, immune stimulation, long-term expression stability and safety are either lacking or not optimal. Thus, more efficient AAV.hPAH vectors are needed for PKU treatment.

SUMMARY

The embodiments described herein relate to an AAV gene therapy vector for delivering normal human phenylalanine hydroxylase (PAH) to a subject in need thereof, following intravenous administration of the vector resulting in long-term, perhaps 10 years or more, of clinically meaningful correction of hyperphenylalaninemia. The subject patient population is patients with moderate to severe hyperphenylalaninemia, including those with PKU, variant PKU or non-PKU hyperphenylalaninemia. The intended vector dose is intended to deliver PAH blood levels of approximately 15% or greater as compared to wild type, which is the level which has been reported for "moderate" PKU patients. See, Kaufman, S., PNAS, 96:3160-4 (1999), which is incorporated herein by reference. In another embodiment, the intended vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 25% or greater. In one embodiment, the goal for the AAV vector treatment is conversion of severe PKU patients to either moderate or mild PKU thus lessening the burden associated with a severely limited phenylalanine diet.

In one aspect, this application provides the use of a replication deficient adeno-associated virus (AAV) to deliver a human phenylalanine hydroxylase (PAH) gene to liver cells of patients (human subjects) diagnosed with PKU. The recombinant AAV vector (rAAV) used for delivering the hPAH gene ("rAAV.hPAH") should have a tropism for the liver (*e.g.*, a rAAV bearing an AAV8 capsid), and the hPAH transgene should be controlled by liver-specific expression control elements. In one embodiment, the expression control elements include one or more of the following: an enhancer; a

promoter; an intron; a WPRE; and a polyA signal. Such elements are further described herein.

In one embodiment, the hPAH coding sequence is shown in SEQ ID NO: 1. In one embodiment, the PAH protein sequence is shown in SEQ ID NO: 2. The coding sequence for hPAH is, in one embodiment, codon optimized for expression in humans. Such sequence may share less than 80% identity to the native hPAH coding sequence (SEQ ID NO: 3). In one embodiment, the hPAH coding sequence is that shown in SEQ ID NO: 1.

In another aspect, provided herein is an aqueous suspension suitable for administration to a PKU patient which includes the rAAV described herein. In some embodiments, the suspension includes an aqueous suspending liquid and about 1 x 10^{12} to about 1x10^{14} genome copies (GC) of the rAAV/mL. The suspension is, in one embodiment, suitable for intravenous injection. In other embodiment, the suspension further includes a surfactant, preservative, and/or buffer dissolved in the aqueous suspending liquid.

In another embodiment, provided herein is a method of treating a patient having PKU with an rAAV as described herein. In one embodiment, about 1 x 10^{11} to about 3 x 10^{13} genome copies (GC) of the rAAV/kg patient body weight are delivered the patient in an aqueous suspension.

3. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of pAAV.TBG.PI.hPAHco.WPRE.bGH cis plasmid.

FIG. 2A is a bar graph of plasma phenylalanine (Phe) levels in PAH_KO_A mouse model (shown in white) and wild-type (shown in black) or heterozygous (shown in grey) littermates, as described in Example 1. These results are summarized in FIG. 2D.

FIG. 2B is a line graph of plasma phenylalanine (Phe) levels in PAH_KO_A mice (shown in white) with heterozygous (shown in grey) and wild-type (shown in black) littermates provided as controls. Mice were injected with 1 x 10^{13} GC/kg or 1 x 10^{12} GC/kg of AAV8.TBG.PI.hPAHco.WPRE.bGH on day 56 of natural history study as

described in Example 3. Experiment was performed on 7 male and 3 female PAH_KO_A mice from the natural history study.

FIG. 2C is a line graph of plasma phenylalanine (Phe) levels in PAH_KO_A mouse model injected with 1 x 10^{13} GC/kg or 1 x 10^{12} GC/kg of AAV8.TBG.PI.hPAHco.WPRE.bGH as described in Example 3. The day of injection was Day 0.

FIG. 2D is a line graph showing the mean Phe levels for the mice studied in FIG. 2A. Values expressed as mean +/- SEM.

FIG. 3A is a bar graph of plasma phenylalanine (Phe) levels in PAH_KO_B mouse model (shown in white) and wild-type (shown in black) or heterozygous (shown in grey) littermates, as described in Example 1. These results are summarized in FIG. 3D.

FIG. 3B is a line graph of plasma phenylalanine (Phe) levels in PAH_KO_B mice (shown in white) with heterozygous (shown in grey) and wild-type (shown in black) littermates provided as controls. Experiment was performed on 3 female PAH_KO_B mice from the natural history study.

FIG. 3C is a line graph of plasma phenylalanine (Phe) levels in PAH_KO_B mouse model injected with 1 x 10^{12} GC/kg of AAV8.TBG.PI.hPAHco.WPRE.bGH as described in Example 3. Mice Identification Numbers 1691, 1695 and 1696 were females injected with 1 x 10^{12} GC/kg on Day 0.

FIG. 3D is a line graph showing the mean Phe levels for the mice studied in FIG. 3A. Values expressed as mean +/- SEM.

FIG. 4A is a line graph of plasma phenylalanine (Phe) levels in PAH_KO_C mouse model (shown in white) and wild-type (shown in black) or heterozygous (shown in grey) littermates, as described in Example 1. These results are summarized in FIG. 4C.

FIG. 4B is a line graph of plasma phenylalanine (Phe) levels in PAH_KO_C mice (shown in white) with heterozygous (shown in grey) and wild-type (shown in black) littermates provided as controls. Experiment was performed on 2 male PAH_KO_C mice from the natural history study.

FIG. 4C is a line graph showing the mean Phe levels for the mice studied in FIG. 4A. Values expressed as mean +/- SEM.

FIG. 5 is a bar graph summarizing the results of FIGs. 2A, 3A and 4A. Plasma phenylalanine (Phe) levels were detected via LC/MS/MS and the data from PAH_KO_A, PAH_KO_B and PAH_KO_C mice bled from 6-8 weeks of age. Plasma was isolated and analyzed for Phe concentration. Wild-type littermates were provided as negative controls.

FIG. 6A-6C demonstrate that AAV8.TBG.hPAHco rescues phenylalanine levels in PKU_KO_B mice. PKU B mice ages 17-22 weeks were given either 10^{12} GC/kg of either AAV8.TBG.hPAHco.bGH (circles) or AAV8.TBG.hPAHco.WPRE.bGH (squares) after pretreatment phenylalanine levels were established. PBS treatment shown with triangles. Mice were then bled weekly, and plasma was isolated and phenylalanine concentration was determined (A). At the termination of the study, liver was collected and genome copy analysis (B) and immunohistochemistry (C) was performed. Values expressed as mean ± SEM.

FIG. 7 is a line graph demonstrating that AAV gene therapy lowers plasma Phe concentration in PKU_KO_A mice. WT (triangle), heterozygous (circle) and PKU_KO_A (KO) mice were injected intravenously with 10^{11} GC/kg of AAV8.TBG.hPAHco after baseline phenylalanine levels were established. Mice were then bled weekly, and plasma was isolated and analyzed for Phe concentration. Values expressed as mean +/- SEM. Phe levels in plasma decreased by 71% following intravenous administration of AAV8.TBG.hPAHco.

FIGs. 8A-8C demonstrate that high does AAV9.TBG.hPAHco rescues phenylalanine levels in PKU_KO_B mice. PKU_KO_B mice were given either 10^{12} GC/kg, 3x 10^{11} GC/kg, or 10^{11} GC/kg, of AAV8.TBG.hPAHco after baseline phenylalanine levels were established. Mice were then bled weekly, and plasma was isolated and analyzed for Phe concentration (A). Values expressed as mean +/- SEM. At termination of the study, liver was collected and genome copy analysis (B) and immunohistochemistry (C) was performed. Protein expression and reduction in Phe levels seen at a dose of 10^{12} GC/kg.

FIG. 9 shows an alignment of a portion of the PAH sequence for wild type (WT) (SEQ ID NO: 26), PAH_KO_A (Strain A) (SEQ ID NO: 27), PAH_KO_B (Strain B) (SEQ ID NO: 28), PAH_KO_C (Strain C) (SEQ ID NO: 29), PAH_KO_D (Strain D) (SEQ ID NO: 30) and consensus (SEQ ID NO: 31).

4. DETAILED DESCRIPTION

The embodiments described in the application relate to the use of a replication deficient adeno-associated virus (AAV) to deliver a human phenylalanine hydroxylase (PAH) gene to liver cells of patients (human subjects) diagnosed with phenylketonuria (PKU). The recombinant AAV vector (rAAV) used for delivering the hPAH gene ("rAAV.hPAH") should have a tropism for the liver (*e.g.*, a rAAV bearing an AAV8 capsid), and the hPAH transgene should be controlled by liver-specific expression control elements. In one embodiment, the expression control elements include one or more of the following: an enhancer; a promoter; an intron; a WPRE; and a polyA signal. Such elements are further described herein.

As used herein, "AAV8 capsid" refers to the AAV8 capsid having the amino acid sequence of GenBank, accession: YP_077180.1, SEQ ID NO: 19, which is incorporated by reference herein. Some variation from this encoded sequence is permitted, which may include sequences having about 99% identity to the referenced amino acid sequence in YP_077180.1 and WO 2003/052051 (which is incorporated herein by reference) (i.e., less than about 1% variation from the referenced sequence). Methods of generating the capsid, coding sequences therefore, and methods for production of rAAV viral vectors have been described. See, e.g., Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2015/0315612.

As used herein, the term "NAb titer" a measurement of how much neutralizing antibody (e.g., anti-AAV Nab) is produced which neutralizes the physiologic effect of its targeted epitope (e.g., an AAV). Anti-AAV NAb titers may be measured as described in, e.g., Calcedo, R., et al., Worldwide Epidemiology of Neutralizing Antibodies to Adeno-Associated Viruses. Journal of Infectious Diseases, 2009. 199(3): p. 381-390, which is incorporated by reference herein.

The terms "percent (%) identity", "sequence identity", "percent sequence identity", or "percent identical" in the context of amino acid sequences refers to the residues in the two sequences which are the same when aligned for correspondence. Percent identity may be readily determined for amino acid sequences over the full-length of a protein, polypeptide, about 32 amino acids, about 330 amino acids, or a peptide fragment thereof or the corresponding nucleic acid sequence coding sequencers. A suitable amino acid fragment may be at least about 8 amino acids in length, and may be up to about 700 amino acids. Generally, when referring to "identity", "homology", or "similarity" between two different sequences, "identity", "homology" or "similarity" is determined in reference to "aligned" sequences. "Aligned" sequences or "alignments" refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, *e.g.,* the "Clustal X", "MAP", "PIMA", "MSA", "BLOCKMAKER", "MEME", and "Match-Box" programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, *e.g.,* J. D. Thomson et al, Nucl. Acids. Res., "A comprehensive comparison of multiple sequence alignments", 27(13):2682-2690 (1999).

As used herein, the term "operably linked" refers to both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest.

A "replication-defective virus" or "viral vector" refers to a synthetic or artificial viral particle in which an expression cassette containing a gene of interest is packaged in a viral capsid or envelope, where any viral genomic sequences also packaged within the viral capsid or envelope are replication-deficient; *i.e.,* they cannot generate progeny virions but retain the ability to infect target cells. In one embodiment, the genome of the viral vector does not include genes encoding the enzymes required to replicate (the

genome can be engineered to be "gutless" - containing only the transgene of interest flanked by the signals required for amplification and packaging of the artificial genome), but these genes may be supplied during production. Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication.

It is to be noted that the term "a" or "an" refers to one or more. As such, the terms "a" (or "an"), "one or more," and "at least one" are used interchangeably herein.

The words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively. The words "consist", "consisting", and its variants, are to be interpreted exclusively, rather than inclusively. While various embodiments in the specification are presented using "comprising" language, under other circumstances, a related embodiment is also intended to be interpreted and described using "consisting of" or "consisting essentially of" language.

As used herein, the term "about" means a variability of 10% from the reference given, unless otherwise specified.

Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

5.1 Gene Therapy Vectors

In one aspect, a recombinant adeno-associated virus (rAAV) vector carrying the human PAH gene is provided for use in gene therapy. The rAAV.hPAH vector should have a tropism for the liver (*e.g.*, a rAAV bearing an AAV8 capsid) and the hPAH transgene should be controlled by liver-specific expression control elements. The vector is formulated in a buffer/carrier suitable for infusion in human subjects. The buffer/carrier should include a component that prevents the rAAV from sticking to the infusion tubing but does not interfere with the rAAV binding activity *in vivo*.

5.1.1. The rAAV.hPAH Vector

5.1.1.1. The hPAH Sequence

Phenylketonuria is an inherited error of metabolism caused predominantly by mutations in the phenylalanine hydroxylase (PAH) gene. Mutations in the PAH gene

result in decreased catalytic activity affecting the catabolic pathway of phenylalanine (Phe). PAH is a hepatic enzyme that requires the cofactor tetrahydrobiopterin (BH_4) to convert Phe to tyrosine (Tyr). A deficiency in PAH or its cofactor BH_4 results in the accumulation of excess phenylalanine, whose toxic effects can cause severe and irreversible intellectual disability and other disorders, if untreated. See, Havid and Cristodoulou, Transl Pediatr, 2015 Oct, 4(4):304-17, which is incorporated herein by reference.

Over 550 mutations of the PAH gene have been described, the majority of which result in deficient enzyme activity. See, Phenylalanine Hydroxylase Locus Knowledgebase, accessed at http://www.pahdb.mcgill.ca/, which is incorporated herein by reference. Due to the large number of known PKU mutations, and the autosomal recessive nature of the disease, a wide range of disease severity is seen. The severity of the disease is generally classified by blood phenylalanine levels, which are sometimes classified as classic PKU, moderate or variant PKU, mild PKU, or hyperphenylalaninemia. Based on blood Phe levels at diagnosis, there are 4 levels of PKU severity.

- Hyperphenylalaninemia, with Phe levels that are slightly above normal range: 120-600 µmol/L (2-10 mg/dL)
- Mild, with the lowest blood Phe levels: 600-900 µmol/L (10-15 mg/dL)
- Moderate or variant, with blood Phe levels somewhere in the middle: 900-1200 µmol/L (15-20 mg/dL)
- Severe or "classic" PKU, with extremely high blood Phe levels: >1200 µmol/L (20 mg/dL)

The goal of therapies described herein would provide functional PAH enzyme resulting in Phe levels in the 120-600 µmol/L range, e.g., a 25% or greater reduction in plasma Phe levels. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 25% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 30% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine

levels by 35% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 40% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 45% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 50% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 60% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 70% or greater. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 75% or greater.

In one embodiment, the "subject" or "patient" is a mammalian subject having PKU as described above. It is intended that a patient having PKU of any severity is the intended subject.

In one embodiment, the hPAH gene encodes the hPAH protein shown in SEQ ID NO: 2. Thus, in one embodiment, the hPAH transgene can include, but is not limited to, the sequence provided by SEQ ID NO:1 or SEQ ID NO: 3 which are provided in the attached Sequence Listing, which is incorporated by reference herein. SEQ ID NO: 3 provides the cDNA for native human PAH. SEQ ID NO: 1 provides an engineered cDNA for human PAH, which has been codon optimized for expression in humans (sometimes referred to herein as hPAHco). It is to be understood that reference to hPAH herein may, in some embodiments, refer to the hPAH native or codon optimized sequence. Alternatively or additionally, web-based or commercially available computer programs, as well as service based companies may be used to back translate the amino acid sequences to nucleic acid coding sequences, including both RNA and/or cDNA. See, e.g., backtranseq by EMBOSS, http://www.ebi.ac.uk/Tools/st/; Gene Infinity (http://www.geneinfinity.org/sms-/sms_backtranslation.html); ExPasy (http://www.expasy.org/tools/). It is intended that all nucleic acids encoding the described hPAH polypeptide sequences are encompassed, including nucleic acid sequences which have been optimized for expression in the desired target subject (e.g., by codon optimization).

In one embodiment, the nucleic acid sequence encoding hPAH shares at least 95% identity with the native hPAH coding sequence of SEQ ID NO: 3. In another embodiment, the nucleic acid sequence encoding hPAH shares at least 90, 85, 80, 75, 70, or 65% identity with the native hPAH coding sequence of SEQ ID NO: 3. In one embodiment, the nucleic acid sequence encoding hPAH shares about 78% identity with the native hPAH coding sequence of SEQ ID NO: 3. In one embodiment, the nucleic acid sequence encoding hPAH is SEQ ID NO: 1.

In one embodiment, the PAH coding sequence is optimized for expression in the target subject. Codon-optimized coding regions can be designed by various different methods. This optimization may be performed using methods which are available on-line (e.g., GeneArt,), published methods, or a company which provides codon optimizing services, e.g., as DNA2.0 (Menlo Park, CA). One codon optimizing approach is described, e.g., in International Patent Publication No. WO 2015/012924, which is incorporated by reference herein. See also, e.g., US Patent Publication No. 2014/0032186 and US Patent Publication No. 2006/0136184. Suitably, the entire length of the open reading frame (ORF) for the product is modified. However, in some embodiments, only a fragment of the ORF may be altered. By using one of these methods, one can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide.

A number of options are available for performing the actual changes to the codons or for synthesizing the codon-optimized coding regions designed as described herein. Such modifications or synthesis can be performed using standard and routine molecular biological manipulations well known to those of ordinary skill in the art. In one approach, a series of complementary oligonucleotide pairs of 80-90 nucleotides each in length and spanning the length of the desired sequence are synthesized by standard methods. These oligonucleotide pairs are synthesized such that upon annealing, they form double stranded fragments of 80-90 base pairs, containing cohesive ends, e.g., each oligonucleotide in the pair is synthesized to extend 3, 4, 5, 6, 7, 8, 9, 10, or more bases beyond the region that is complementary to the other oligonucleotide in the pair. The single-stranded ends of each pair of oligonucleotides are designed to anneal with the

single-stranded end of another pair of oligonucleotides. The oligonucleotide pairs are allowed to anneal, and approximately five to six of these double-stranded fragments are then allowed to anneal together via the cohesive single stranded ends, and then they ligated together and cloned into a standard bacterial cloning vector, for example, a TOPO® vector available from Thermo Fisher Scientific Inc. The construct is then sequenced by standard methods. Several of these constructs consisting of 5 to 6 fragments of 80 to 90 base pair fragments ligated together, i.e., fragments of about 500 base pairs, are prepared, such that the entire desired sequence is represented in a series of plasmid constructs. The inserts of these plasmids are then cut with appropriate restriction enzymes and ligated together to form the final construct. The final construct is then cloned into a standard bacterial cloning vector, and sequenced. Additional methods would be immediately apparent to the skilled artisan. In addition, gene synthesis is readily available commercially.

5.1.1.2. The rAAV vector

Because PAH is natively expressed in the liver, it is desirable to use an AAV which shows tropism for liver. In one embodiment, the AAV supplying the capsid is AAV8. In another embodiment, the AAV supplying the capsid is AAVrh.10. In yet another embodiment, the AAV supplying the capsid is a Clade E AAV. Such AAV include rh.2; rh.10; rh. 25; bb.1, bb.2, pi.1, pi.2, pi.3, rh.38, rh.40, rh.43, rh.49, rh.50, rh.51, rh.52, rh.53, rh.57, rh.58, rh.61, rh.64, hu.6, hu.17, hu.37, hu.39, hu.40, hu.41, hu.42, hu.66, and hu.67. This clade further includes modified rh. 2; modified rh. 58; and modified rh.64. See, WO 2005/033321, which is incorporated herein by reference. However, any of a number of rAAV vectors with liver tropism can be used.

In a specific embodiment described in the Examples, *infra*, the gene therapy vector is an AAV8 vector expressing an hPAH transgene under control of a thyroxine binding globulin (TBG) promoter referred to as AAV8.TBG.PI.hPAHco.WPRE.bGH. The vector genome for such a vector is shown in SEQ ID NO: 20. In another embodiment, the WPRE is omitted, i.e., AAV8.TBG.PI.hPAHco.bGH. The vector genome for such a vector is shown in SEQ ID NO: 21. The external AAV vector component is a serotype 8, $T = 1$ icosahedral capsid

consisting of 60 copies of three AAV viral proteins, VP1, VP2, and VP3, at a ratio of 1:1:10. The capsid contains a single-stranded DNA rAAV vector genome.

In one embodiment, the rAAV.hPAH genome contains an hPAH transgene flanked by two AAV inverted terminal repeats (ITRs). In one embodiment, the hPAH transgene includes one or more of an enhancer, promoter, an intron, a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) (e.g., SEQ ID NO: 15), an hPAH coding sequence, and polyadenylation (polyA) signal. In another embodiment, the hPAH transgene includes one or more of an enhancer, promoter, an intron, an hPAH coding sequence, and polyadenylation (polyA) signal. These control sequences are "operably linked" to the hPAH gene sequences. The expression cassette containing these sequences may be engineered onto a plasmid which is used for production of a viral vector.

The ITRs are the genetic elements responsible for the replication and packaging of the genome during vector production and are the only viral *cis* elements required to generate rAAV. The minimal sequences required to package the expression cassette into an AAV viral particle are the AAV 5' and 3' ITRs, which may be of the same AAV origin as the capsid, or which of a different AAV origin (to produce an AAV pseudotype). In one embodiment, the ITR sequences from AAV2, or the deleted version thereof (ΔITR), are used. However, ITRs from other AAV sources may be selected. Where the source of the ITRs is from AAV2 and the AAV capsid is from another AAV source, the resulting vector may be termed pseudotyped. Typically, an expression cassette for an AAV vector comprises an AAV 5' ITR, the hPAH coding sequences and any regulatory sequences, and an AAV 3' ITR. However, other configurations of these elements may be suitable. A shortened version of the 5' ITR, termed ΔITR, has been described in which the D-sequence and terminal resolution site (trs) are deleted. In other embodiments, the full-length AAV 5' and 3' ITRs are used. In one embodiment, the 5' ITR is that shown in SEQ ID NO: 16. In one embodiment, the 3' ITR is that shown in SEQ ID NO: 17.

In one embodiment, the expression control sequences include one or more enhancer. In one embodiment, the En34 enhancer is included (34 bp core enhancer from the human apolipoprotein hepatic control region), which is shown in SEQ ID NO: 4. In

another embodiment, the EnTTR (100 bp enhancer sequence from transthyretin) is included. Such sequence is shown in SEQ ID NO: 5. See, Wu et al, Molecular Therapy, 16(2):280–289, Feb. 2008, which is incorporated herein by reference. In yet another embodiment, the α1-microglogulin/bikunin precursor enhancer is included. In yet another embodiment, the ABPS (shortened version of the 100 bp distal enhancer from the α1-microglogulin/bikunin precursor [ABP] to 42 bp) enhancer is included. Such sequence is shown in SEQ ID NO: 6. In yet another embodiment, the ApoE enhancer is included. Such sequence is shown in SEQ ID NO: 7. In another embodiment, more than one enhancer is present. Such combination may include more than one copy of any of the enhancers described herein, and/or more than one type of enhancer.

Expression of the hPAH coding sequence is driven from a liver-specific promoter. An illustrative plasmid and vector described herein uses the thyroxine binding globulin (TBG) promoter (SEQ ID NO: 9), or a modified version thereof. One modified version of the TBG promoter is a shortened version, termed TBG-S1. A modified thyroxine binding globulin (TBG-S1) promoter sequence is shown in SEQ ID NO: 8. Alternatively, other liver-specific promoters may be used such as the transthyretin promoter. Another suitable promoter is the alpha 1 anti-trypsin (A1AT) promoter, or a modified version thereof (which sequence is shown in SEQ ID NO: 10). Another suitable promoter is the TTR promoter (SEQ ID NO: 11). Other suitable promoters include human albumin (Miyatake et al., J. Virol., 71:5124 32 (1997)), humAlb; the Liver Specific promoter (LSP), and hepatitis B virus core promoter, (Sandig *et al.*, Gene Ther., 3:1002 9 (1996). See, *e.g.*, The Liver Specific Gene Promoter Database, Cold Spring Harbor, http://rulai.schl.edu/LSPD, which is incorporated by reference. Although less desired, other promoters, such as viral promoters, constitutive promoters, regulatable promoters [*see, e.g.*, WO 2011/126808 and WO 2013/04943], or a promoter responsive to physiologic cues may be used may be utilized in the vectors described herein.

In addition to a promoter, an expression cassette and/or a vector may contain other appropriate transcription initiation, termination, enhancer sequences, and efficient RNA processing signals. Such sequences include splicing and polyadenylation (polyA) signals; regulatory elements that enhance expression (i.e., WPRE (SEQ ID NO: 15)); sequences that stabilize cytoplasmic mRNA; sequences that enhance translation

efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. In one embodiment, a polyadenylation (polyA) signal is included to mediate termination of hPAH mRNA transcripts. Examples of other suitable polyA sequences include, e.g., bovine growth hormone (SEQ ID NO: 12), SV40, rabbit beta globin, and TK polyA, amongst others.

In one embodiment, the regulatory sequences are selected such that the total rAAV vector genome is about 2.0 to about 5.5 kilobases in size. In one embodiment, the regulatory sequences are selected such that the total rAAV vector genome is about 3.4 kb, about 2.9 kb, about 3.3 kb, about 2.2 kb or about 2.5 kb in size. In one embodiment, it is desirable that the rAAV vector genome approximate the size of the native AAV genome. Thus, in one embodiment, the regulatory sequences are selected such that the total rAAV vector genome is about 4.7 kb in size. In another embodiment, the total rAAV vector genome is less about 5.2kb in size. The size of the vector genome may be manipulated based on the size of the regulatory sequences including the promoter, enhancer, intron, poly A, etc. See, Wu et al, Mol Ther, Jan 2010 18(1):80-6, which is incorporated herein by reference.

Thus, in one embodiment, an intron is included in the vector. Suitable introns include the human beta globin IVS2 (SEQ ID NO: 13). See, Kelly et al, Nucleic Acids Research, 43(9):4721-32 (2015), which is incorporated herein by reference. Another suitable promoter includes the Promega chimeric intron (SEQ ID NO: 14), sometimes referred to as "PI"). See, Almond, B. and Schenborn, E. T. A Comparison of pCI-neo Vector and pcDNA4/HisMax Vector. [Internet] 2000, which is incorporated herein by reference. Available from: www.promega.com/resources/pubhub/enotes/a-comparison-of-pcineo-vector-and-pcdna4hismax-vector/). Another suitable intron includes the hFIX intron (SEQ ID NO: 18). Various introns suitable herein are known in the art and include, without limitation, those found at http://bpg.utoledo.edu/~afedorov/lab/eid.html, which is incorporated herein by reference. See also, Shepelev V., Fedorov A. Advances in the Exon-Intron Database. Briefings in Bioinformatics 2006, 7: 178-185, which is incorporated herein by reference.

In one embodiment, the rAAV vector genome comprises SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO: 24 or SEQ ID NO:25.

5.1.2. Compositions

In one embodiment, the rAAV.hPAH virus is provided in a pharmaceutical composition which comprises an aqueous carrier, excipient, diluent or buffer. In one embodiment, the buffer is PBS. In a specific embodiment, the rAAV.hPAH formulation is a suspension containing an effective amount of rAAV.hPAH vector suspended in an aqueous solution containing 0.001% Pluronic F-68 in TMN200 (200 mM sodium chloride, 1 mM magnesium chloride, 20 mM Tris, pH 8.0). However, various suitable solutions are known including those which include one or more of: buffering saline, a surfactant, and a physiologically compatible salt or mixture of salts adjusted to an ionic strength equivalent to about 100 mM sodium chloride (NaCl) to about 250 mM sodium chloride, or a physiologically compatible salt adjusted to an equivalent ionic concentration.

For example, a suspension as provided herein may contain both NaCl and KCl. The pH may be in the range of 6.5 to 8.5, or 7 to 8.5, or 7.5 to 8. A suitable surfactant, or combination of surfactants, may be selected from among Poloxamers, i.e., nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), SOLUTOL HS 15 (Macrogol-15 Hydroxystearate), LABRASOL (Polyoxy capryllic glyceride), polyoxy 10 oleyl ether, TWEEN (polyoxyethylene sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. The surfactant may be present in an amount up to about 0.0005 % to about 0.001% of the suspension. In another embodiment, the vector is suspended in an aqueous solution containing 180 mM sodium chloride, 10 mM sodium phosphate, 0.001% Poloxamer 188, pH 7.3.

In one embodiment, the formulation is suitable for use in human subjects and is administered intravenously. In one embodiment, the formulation is delivered via a peripheral vein by bolus injection. In one embodiment, the formulation is delivered via a peripheral vein by infusion over about 10 minutes (±5 minutes). In one embodiment, the formulation is delivered via a peripheral vein by infusion over about 20 minutes (±5 minutes). In one embodiment, the formulation is delivered via a peripheral vein by infusion over about 30 minutes (±5 minutes). In one embodiment, the formulation is delivered via a peripheral vein by infusion over about 60 minutes (±5 minutes). In one embodiment, the formulation is delivered via a peripheral vein by infusion over about 90 minutes (±10 minutes). However, this time may be adjusted as needed or desired. Any suitable method or route can be used to administer an AAV-containing composition as described herein, and optionally, to co-administer other active drugs or therapies in conjunction with the AAV-mediated delivery of hPAH described herein. Routes of administration include, for example, systemic, oral, inhalation, intranasal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, and other parental routes of administration.

In one embodiment, the formulation may contain, e.g., about 1.0×10^{11} genome copies per kilogram of patient body weight (GC/kg) to about 1×10^{15} GC/kg, about 5×10^{11} genome copies per kilogram of patient body weight (GC/kg) to about 3×10^{13} GC/kg, or about 1×10^{12} to about 1×10^{14} GC/kg, as measured by oqPCR or digital droplet PCR (ddPCR) as described in, e.g., M. Lock et al, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14, which is incorporated herein by reference. In one embodiment, the rAAV.hPAH formulation is a suspension containing at least $1x10^{13}$ genome copies (GC)/mL, or greater, as measured by oqPCR or digital droplet PCR (ddPCR) as described in, e.g., M. Lock et al, supra.

In order to ensure that empty capsids are removed from the dose of AAV.hPAH that is administered to patients, empty capsids are separated from vector particles during the vector purification process, e.g., using the method discussed herein. In one embodiment, the vector particles containing packaged genomes are purified from empty capsids using the process described in US Patent Appln No. 62/322,098, filed on April 13, 2016, and entitled "Scalable Purification Method for AAV8", which is incorporated

by reference herein. Briefly, a two-step purification scheme is described which selectively captures and isolates the genome-containing rAAV vector particles from the clarified, concentrated supernatant of a rAAV production cell culture. The process utilizes an affinity capture method performed at a high salt concentration followed by an anion exchange resin method performed at high pH to provide rAAV vector particles which are substantially free of rAAV intermediates. Similar purification methods can be used for vectors having other capsids.

While any conventional manufacturing process can be utilized, the process described herein (and in US Patent Appln No. 62/322,098) yields vector preparations wherein between 50 and 70% of the particles have a vector genome, i.e., 50 to 70% full particles. Thus for an exemplary dose of $1.6x10^{12}$ GC/kg, and the total particle dose will be between $2.3x10^{12}$ and $3x10^{12}$ particles. In another embodiment, the proposed dose is one half log higher, or $5x10^{12}$ GC/kg, and the total particle dose will be between $7.6x10^{12}$ and $1.1x10^{13}$ particles. In one embodiment, the formulation is be characterized by an rAAV stock having a ratio of "empty" to “full” of 1 or less, preferably less than 0.75, more preferably, 0.5, preferably less than 0.3.

A stock or preparation of rAAV8 particles (packaged genomes) is "substantially free" of AAV empty capsids (and other intermediates) when the rAAV8 particles in the stock are at least about 75% to about 100%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least 99% of the rAAV8 in the stock and "empty capsids" are less than about 1%, less than about 5%, less than about 10%, less than about 15% of the rAAV8 in the stock or preparation.

Generally, methods for assaying for empty capsids and AAV vector particles with packaged genomes have been known in the art. See, e.g., Grimm et al., *Gene Therapy* (1999) 6:1322-1330; Sommer et al., Molec. Ther. (2003) 7:122-128. To test for denatured capsid, the methods include subjecting the treated AAV stock to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel containing 3-8% Tris-acetate in the buffer, then running the gel until sample material is separated, and blotting the gel onto nylon or nitrocellulose membranes, preferably nylon. Anti-AAV capsid antibodies are then used as the primary antibodies that bind to denatured capsid proteins, preferably an anti-AAV

capsid monoclonal antibody, most preferably the B1 anti-AAV-2 monoclonal antibody (Wobus et al., J. Virol. (2000) 74:9281-9293). A secondary antibody is then used, one that binds to the primary antibody and contains a means for detecting binding with the primary antibody, more preferably an anti-IgG antibody containing a detection molecule covalently bound to it, most preferably a sheep anti-mouse IgG antibody covalently linked to horseradish peroxidase. A method for detecting binding is used to semi-quantitatively determine binding between the primary and secondary antibodies, preferably a detection method capable of detecting radioactive isotope emissions, electromagnetic radiation, or colorimetric changes, most preferably a chemiluminescence detection kit. For example, for SDS-PAGE, samples from column fractions can be taken and heated in SDS-PAGE loading buffer containing reducing agent (*e.g.*, DTT), and capsid proteins were resolved on pre-cast gradient polyacylamide gels (*e.g.*, Novex). Silver staining may be performed using SilverXpress (Invitrogen, CA) according to the manufacturer's instructions. In one embodiment, the concentration of AAV vector genomes (vg) in column fractions can be measured by quantitative real time PCR (Q-PCR). Samples are diluted and digested with DNase I (or another suitable nuclease) to remove exogenous DNA. After inactivation of the nuclease, the samples are further diluted and amplified using primers and a TaqMan™ fluorogenic probe specific for the DNA sequence between the primers. The number of cycles required to reach a defined level of fluorescence (threshold cycle, Ct) is measured for each sample on an Applied Biosystems Prism 7700 Sequence Detection System. Plasmid DNA containing identical sequences to that contained in the AAV vector is employed to generate a standard curve in the Q-PCR reaction. The cycle threshold (Ct) values obtained from the samples are used to determine vector genome titer by normalizing it to the Ct value of the plasmid standard curve. End-point assays based on the digital PCR can also be used.

In one aspect, an optimized q-PCR method is provided herein which utilizes a broad spectrum serine protease, e.g., proteinase K (such as is commercially available from Qiagen). More particularly, the optimized qPCR genome titer assay is similar to a standard assay, except that after the DNase I digestion, samples are diluted with proteinase K buffer and treated with proteinase K followed by heat inactivation. Suitably samples are diluted with proteinase K buffer in an amount equal to

the sample size. The proteinase K buffer may be concentrated to 2 fold or higher. Typically, proteinase K treatment is about 0.2 mg/mL, but may be varied from 0.1 mg/mL to about 1 mg/mL. The treatment step is generally conducted at about 55 °C for about 15 minutes, but may be performed at a lower temperature (e.g., about 37 °C to about 50 °C) over a longer time period (e.g., about 20 minutes to about 30 minutes), or a higher temperature (e.g., up to about 60 °C) for a shorter time period (e.g., about 5 to 10 minutes). Similarly, heat inactivation is generally at about 95 °C for about 15 minutes, but the temperature may be lowered (e.g., about 70 to about 90 °C) and the time extended (e.g., about 20 minutes to about 30 minutes). Samples are then diluted (e.g., 1000 fold) and subjected to TaqMan analysis as described in the standard assay.

Additionally, or alternatively, droplet digital PCR (ddPCR) may be used. For example, methods for determining single-stranded and self-complementary AAV vector genome titers by ddPCR have been described. See, e.g., M. Lock et al, Hu Gene Therapy Methods, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14.

5.2 Patient Population

As discussed above, a subject having PKU of any severity is the intended recipient of the compositions and methods described herein.

Subjects may be permitted to continue their standard of care treatment(s) (e.g., diet low in Phe; treatment with sapropterin dihydrochloride) prior to and concurrently with the gene therapy treatment at the discretion of their caring physician. In the alternative, the physician may prefer to stop standard of care therapies prior to administering the gene therapy treatment and, optionally, resume standard of care treatments as a co-therapy after administration of the gene therapy.

Desirable endpoints of the gene therapy regimen are an increase in PAH activity resulting in Phe levels between 120-360 μmol/L. In another embodiment, the vector dose is intended to deliver PAH to result in a reduction of plasma phenylalanine levels by 25% or greater. In another embodiment, the desirable endpoint is reducing plasma Phe levels to take subject to a "moderate" phenotype from a "severe" phenotype. Methods for measurement of phenylalanine levels are known in the art e.g, as described in Gregory et al, Blood phenylalanine monitoring for dietary compliance among patients

with phenylketonuria: comparison of methods, Genetics in Medicine (November 2007) 9, 761–765, which is incorporated herein by reference. In one embodiment, patients achieve desired circulating PAH levels after treatment with rAAV.hPAH, alone and/or combined with the use of adjunctive treatments.

5.3. Dosing & Route of Administration

In one embodiment, the rAAV.hPAH vector is delivered as a single dose per patient. In one embodiment, the subject is delivered the minimal effective dose (MED) (as determined by preclinical study described in the Examples herein). As used herein, MED refers to the rAAV.hPAH dose required to achieve PAH activity resulting in Phe levels between 120-360 μmol/L.

As is conventional, the vector titer is determined on the basis of the DNA content of the vector preparation. In one embodiment, quantitative PCR or optimized quantitative PCR as described in the Examples is used to determine the DNA content of the rAAV.hPAH vector preparations. In one embodiment, digital droplet PCR as described in the Examples is used to determine the DNA content of the rAAV.hPAH vector preparations. In one embodiment, the dosage is about $1x10^{11}$ genome copies (GC)/kg body weight to about $1x10^{13}$ GC/kg, inclusive of endpoints. In one embodiment, the dosage is $5x10^{11}$ GC/kg. In another embodiment, the dosage is $5x10^{12}$ GC/kg. In specific embodiments, the dose of rAAV.hPAH administered to a patient is at least 5×10^{11} GC/kg, 1×10^{12} GC/kg, 1.5×10^{12} GC/kg, 2.0×10^{12} GC/kg, 2.5×10^{12} GC/kg, 3.0×10^{12} GC/kg, 3.5×10^{12} GC/kg, 4.0×10^{12} GC/kg, 4.5×10^{12} GC/kg, 5.0×10^{12} GC/kg, 5.5×10^{12} GC/kg, 6.0×10^{12} GC/kg, 6.5×10^{12} GC/kg, 7.0×10^{12} GC/kg, or 7.5×10^{12} GC/kg. Also, the replication-defective virus compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0×10^{9} GC to about 1.0×10^{15} GC. As used herein, the term "dosage" can refer to the total dosage delivered to the subject in the course of treatment, or the amount delivered in a single (of multiple) administration.

In one embodiment, the dosage is sufficient to decrease plasma Phe levels in the patient by 25% or more.

In some embodiments, rAAV.hPAH is administered in combination with one or more therapies for the treatment of PKU, such as a low Phe diet or administration of sapropterin dihydrochloride.

5.4. Measuring Clinical Objectives

Measurements of efficacy of treatment can be measured by transgene expression and activity as determined by plasma Phe levels and/or PAH activity. Further assessment of efficacy can be determined by clinical assessment of dietary Phe tolerance.

As used herein, the rAAV.hPAH vector herein "functionally replaces" or "functionally supplements" the patients defective PAH with active PAH when the patient expresses a sufficient level of PAH to achieve PAH activity resulting in Phe levels between 120-360 μmol/L.

The following examples are illustrative only and are not intended to limit the present invention.

EXAMPLES

The following examples are illustrative only and are not intended to limit the present invention.

EXAMPLE 1: Mouse Models of Phenylketonuria (PKU)

$PAH^{-/-}$ mice were generated by CRISPR/Cas9 technology at Jackson Labs. Wild-type C57BL/6 mice were injected with Cas9 mRNA and two guide RNAs (sgRNA) targeting the second coding exon of *PAH* gene directly into mouse zygotes. Mice that developed from these embryos were sequenced to determine the mutation(s) and then bred with C57BL/6J mice to transmit the allele and confirm germline transmission.

Four different mutations were generated and the mice strains were designated as PAH_KO_A, PAH_KO_B, PAH_KO_C, and PAH_KO_D. PAH_KO_A mice demonstrated a 3-bp substitution followed by a 64-bp deletion from the 6534 nt to the 6600 nt of *Mus musculus PAH* gene [NC_000076.6]. PAH_KO_B and PAH_KO_C mice showed a single base pair insertion after the 6589 nt and the 6539 nt respectively. PAH_KO_D showed a 6 bp deletion from the 6535 nt to the 6540 nt. FIG. 9 shows an alignment of a portion of the PAH sequence for wild type, PAH_KO_A, PAH_KO_B, PAH_KO_C, PAH_KO_D and consensus. A natural history study of these mice was

performed. Blood samples were collected via retro orbital or submandibular bleeding. Plasma phenylalanine (Phe) levels were detected via LC/MS/MS and the data from PAH_KO_A, PAH_KO_B and PAH_KO_C mice was acquired and presented in FIG. 2A, 3A and 4A respectively, and summarized in FIG. 5A. Heterozygous and wild-type littermates were provided as negative controls. Compared to the controls, the plasma phenylalanine levels in PAH_KO_A, PAH_KO_B and PAH_KO_C mice were significantly higher, indicating a functionally deficient PAH in these mice. It also suggested that PAH_KO_A, PAH_KO_B, and PAH_KO_C mice could serve as mouse models for Phenylketonuria (PKU) in human.

However, the fourth knock-out mice, PAH_KO_D, did not exhibit an elevated plasma phenylalanine level and thus excluded from further analysis.

EXAMPLE 2: AAV Vectors Containing hPAH - AAV8.TBG.PI.hPAHco.WPRE.bGH

The gene therapy vector AAV8.TBG.PI.hPAHco.WPRE.bGH was constructed by an AAV8 vector bearing a codon-optimized human PAH cDNA under the control of TBG, a hybrid promoter based on the human thyroid hormone-binding globulin promoter and microglobin/bikunin enhancer (FIG. 1). The PAH expression cassette was flanked by AAV2 derived inverted terminal repeats (ITRs) and the expression was driven by a hybrid of the TBG enhancer/promoter and the Woodchuck Hepatitis Virus (WHP) posttranscriptional regulatory element (WPRE) as an enhancer. The transgene also included the Promega SV40 misc intron (PI) and a bovine growth hormone polyadenylation signal (bGH). The vector genome sequence is shown in SEQ ID NO: 20.

The vector was prepared using conventional triple transfection techniques in 293 cells as described *e.g.*, by Mizukami, Hiroaki, et al. *A Protocol for AAV vector production and purification.* Diss. Di-vision of Genetic Therapeutics, Center for MolecularMedicine, 1998., which is incorporated herein by reference. All vectors were produced by the Vector Core at the University of Pennsylvania as previously described [Lock, M., et al, Hum Gene Ther, 21: 1259-1271 (2010)].

EXAMPLE 3: AAV8.hPAH vectors in the Model of PKU

All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.

Twenty PAH_KO_A mice were generated. Seven males and three females were assessed in natural history study. On Day 56 of natural history study, three male mice with Identification Number 1531, 1532 and 1533 were injected intravenously via the tail vein with 1 x 10^{13} GC/kg of the AAV8.TBG.PI.hPAHco.WPRE.bGH vector. Four male mice with Identification Number 1538, 1539, 1554 and 1564 were injected with 1 x 10^{12} GC/kg of the vector. Three female mice with Identification Number 1507, 1536 and 1537 were injected with 1 x 10^{12} GC/kg of the vector. The blood samples were collected weekly to evaluate plasma phenylalanine concentration (FIG. 2B). A higher level of phenylalanine was detected in the PAH_KO_A mice before injection compared to the littermate controls, indicating a deficient PAH in PAH_KO_A mice. 7 days after the vector injection, the plasma phenylalanine level of PAH_KO_A mice decreased while the controls remained stable. This result demonstrated that a single injection of AAV8.TBG.PI.hPAHco.WPRE.bGH into PAH_KO_A mice could rescue the deficient PAH and reduce the pathological accumulation of phenylalanine in the blood.

The effects of gender differences and the two doses of the AAV8.TBG.PI.hPAHco.WPRE.bGH vectors were further evaluated in PAH_KO_A mice (FIG. 2C). Plasma phenylalanine levels were observed every week for 11 weeks, e.g, as described in Gregory et al, Blood phenylalanine monitoring for dietary compliance among patients with phenylketonuria: comparison of methods, Genetics in Medicine (November 2007) 9, 761–765, which is incorporated herein by reference. Two of three female mice received 1 x 10^{12} GC/kg of the vectors and all seven male ones with both doses at 1 x 10^{12} GC/kg and 1 x 10^{13} GC/kg displayed a reduced phenylalanine concentration in the plasma. The phenylalanine of the seven male mice maintained at comparably low levels for the 11-week observation period while all three female ones demonstrated a slow increase in plasma phenylalanine level.

Three female PAH_KO_B mice were generated and exanimated in a natural history study. Weekly bleeds for phenylalanine levels were performed and the result confirmed an abnormal accumulation of phenylalanine in the blood compared to the

healthy littermate controls. Upon intravenous injection of 1 x 10^{12} GC/kg of AAV8.TBG.PI.hPAHco.WPRE.bGH, a decreased phenylalanine level was observed in all three females and the low level maintained during the 8-week observation period after the injection.

Two male PAH_KO_C mice were generated for this study and utilized in natural history study. A weekly collection of blood samples were performed and the phenylalanine concentration was assessed. The data showed that during the 9-week observation, both PAH_KO_C mice and the heterozygous/wild-type littermates maintained a comparably stable concentration of plasma phenylalanine while the knock-out mice demonstrated a significantly higher level.

A further study of expression and enzyme activity of PAH in the injected $PAH^{-/-}$ mice was performed. Livers are collected from PAH_KO_A, PAH_KO_B and PAH_KO_C mice injected with vectors or PBS only as well as the healthy littermate controls. mRNA is extracted from the liver and the expression of human PAH is evaluated via RT-PCR. To determine the protein expression of PAH, liver lysates are prepared for detection by western blot while liver sections are prepared for immunohistochemistry. Experiments are also performed to assess the PAH enzyme activity of the $PAH^{-/-}$ mice treated with the vector as well as controls.

To fully evaluate gender difference in all three $PAH^{-/-}$ mice, PAH_KO_A, PAH_KO_B and PAH_KO_C mice were bred to assess the plasma phenylalanine concentration, the expression of PAH on both mRNA and protein level and the enzyme activity of PAH before and after the injection of AAV8.TBG.PI.hPAHco.WPRE.bGH.

To determine the dose-dependent expression of AAV8.TBG.PI.hPAHco.WPRE.bGH and the potential toxicity of the highest dose, various doses of AAV8.TBG.PI.hPAHco.WPRE.bGH are injected into the $PAH^{-/-}$ mice and further assessment of phenylalanine accumulation and PAH expression/activity are performed.

Similar experiments were performed with AAV8.TBG.hPAHco.bGH. PKU B mice ages 17-22 weeks were given either 10^{12} GC/kg of either AAV8.TBG.hPAHco.bGH or AAV8.TBG.hPAHco.WPRE.bGH (or PBS for control) after pretreatment phenylalanine levels were established. Mice were then bled weekly,

and plasma was isolated and phenylalanine concentration was determined (FIG. 6A). At the termination of the study, liver was collected and genome copy analysis (FIG. 6B) and immunohistochemistry (FIG. 6C) was performed. Phenylalanine levels were reduced in both AAV8.TBG.hPAHco.bGH and AAV8.TBG.hPAHco.WPRE.bGH treated mice.

Further studies were performed with AAV8.TBG.hPAHco.bGH vector. Wildtype, heterozygous (circle) and PKU_KO_A (KO) mice were injected intravenously with 10^{11} GC/kg of AAV8.TBG.hPAHco after baseline phenylalanine levels were established. Mice were then bled weekly, and plasma was isolated and analyzed for Phe concentration. Phe levels in plasma decreased by 71% following intravenous administration of AAV8.TBG.hPAHco. FIG. 7.

PKU_KO_B mice were given either 10^{12} GC/kg, 3x 10^{11} GC/kg, or 10^{11} GC/kg, of AAV8.TBG.hPAHco after baseline phenylalanine levels were established. Mice were then bled weekly, and plasma was isolated and analyzed for Phe concentration. At termination of the study, liver was collected and genome copy analysis and immunohistochemistry was performed. Protein expression and reduction in Phe levels were seen at a dose of 10^{12} GC/kg.

Meanwhile, administration of 10^{12} GC/kg of each of the following vectors, AAV8.TBG.PI.hPAHco.bGH, AAV8.LSP.IVS2.hPAHco.bGH, AAV8.A1AT.hPAHco.BGH, AAV8.TTR.hPAHco.BGH, AAV8.TBG.PI.hPAHnativesequence.bGH, AAV8.ABPS.TBG.hFIXintron.hPAHco.BGH, AAV8.ABPS.TBG-S1.hFIXintron.hPAHco.BGH, AAV8.ApoE.A1AT.hFIXintron.hPAHco.BGH, is also performed and served as a comparison.

In conclusion, a single injection of AAV8.TBG.PI.hPAHco.WPRE.bGH vector resulted in substantial plasma phenylalanine reduction and concomitant functional correction when administered intravenously in three PAH-deficient mice.

EXAMPLE 4: AAV Gene Therapy for Phenylketonuria

Phenylketonuria (PKU) is an autosomal recessive genetic disorder caused by the attenuation of phenylalanine-4-hydroxylase (PAH) activity, resulting in the buildup of phenylalanine in the tissues and blood. High levels of phenylalanine in the bloodstream

are thought to inhibit the transport of other large neutral amino acids across the blood brain barrier, affecting brain development and resulting in intellectual disability and seizures. Treatment for PKU is currently limited to maintenance of a strict phenylalanine-restricted diet and products directed at stabilizing residual PAH. A liver-targeted AAV gene therapy approach described herein is to improve upon the current standard of care.

To investigate the development of gene therapy for PKU, four unique mouse strains were created by inducing different mutations in exon 1 of the *PAH* gene by CRISPR/Cas9 technology as described herein. A natural history study was perforemed on each of these strains to determine the progression of the disease and identify the strain that best replicated the human PKU phenotype. PKU colonies, designated B and C, both contained a single base pair (bp) deletion at different locations in exon 1 and maintained average phenylalanine levels of 2049 μM and 1705 μM, respectively, compared to normal levels of 70 μM. PKU colony A, despite having a 64 bp deletion and a 3 bp insertion in exon 1 of the *PAH* gene, had a modestly higher average phenylalanine level of 477 μM. PKU colony D, which had a 6 bp deletion, had phenylalanine levels equivalent to wild type littermates. Following AAV8 vector administration at a dose of 1 x 10^{12} GC/kg for expression of a human codon optimized version of *PAH* to the PKU B mouse colony, plasma phenylalanine levels were reduced by 87% to 222 μM. This reduction in plasma phenylalanine levels restored the ability of the males to produce offspring. These results represent development of an AAV-based therapeutic for PKU.

All publications cited in this specification, as well as US Provisional Patent Application Nos. 62/440,651, 62/469,898, and 62/505,373, are incorporated herein by reference. Similarly, the SEQ ID NOs which are referenced herein and which appear in the appended Sequence Listing are incorporated by reference. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

Sequence Listing Free Text:

Seq ID NO	Free Text

4-18	<213> Artificial Sequence <223> constructed sequence
19	<213> Unknown <223> AAV8
20-25	<213> Artificial Sequence <223> constructed sequence
27-31	<213> Artificial Sequence <223> Engineered sequence

CLAIMS:

1. A recombinant adeno-associated virus (rAAV) useful as a liver-directed therapeutic for phenylketonuria (PKU), said rAAV comprising an AAV capsid, and a vector genome packaged therein, said vector genome comprising:

(a) an AAV 5' inverted terminal repeat (ITR) sequence;

(b) a promoter;

(c) a codon optimized sequence encoding a human phenylalanine hydroxylase (PAH);

(d) an AAV 3' ITR.

2. The rAAV according to claim 1, wherein the coding sequence of (c) is SEQ ID NO: 1.

3. The rAAV according to claim 1, wherein the rAAV capsid is an AAV8 capsid.

4. The rAAV according to claim 1, wherein the promoter is TBG promoter or TBG-S1 promoter.

5. The rAAV according to claim 1, wherein the promoter is A1AT promoter.

6. The rAAV according to claim 1, wherein the promoter is LSP promoter.

7. The rAAV according to claim 1, wherein the promoter is TTR promoter.

8. The rAAV according to claim 1, wherein the AAV 5' ITR and/or AAV3' ITR is from AAV2.

9. The rAAV according to claim 1, wherein the vector genome further comprises a polyA.

10. The rAAV according to claim 1, wherein the polyA is from bGH.

11. The rAAV according to claim 1, further comprising a WPRE.

12. The rAAV according to claim 1, further comprising an intron.

13. The rAAV according to claim 12, wherein the intron is from human beta globin IVS2 or SV40.

14. The rAAV according to claim 1, further comprising an enhancer.

15. The rAAV according to claim 14, wherein the enhancer is an APB enhancer, ABPS enhancer, an alpha mic/bik enhancer, TTR enhancer, en34, or ApoE enhancer.

16. The rAAV according to claim 1, wherein the vector genome is about 3 kilobases to about 5.5 kilobases in size.

17. An aqueous suspension suitable for administration to a phenylketonuria patient, said suspension comprising an aqueous suspending liquid and about 1 $x10^{12}$ GC/mL to about 1 $x10^{14}$ GC/mL of a recombinant adeno-associated virus (rAAV) useful as a liver-directed therapeutic for phenylketonuria, said rAAV having an AAV capsid, and having packaged therein a vector genome comprising:

(a) an AAV 5' inverted terminal repeat (ITR) sequence;

(b) a promoter;

(c) a coding sequence encoding a human phenylalanine hydroxylase (PAH); and

(d) an AAV 3' ITR.

18. The suspension according to claim 17, wherein the suspension is suitable for intravenous injection.

19. The suspension according to claim 17, wherein the suspension further comprises a surfactant, preservative, and/or buffer dissolved in the aqueous suspending liquid.

20. A method of treating a patient having phenylketonuria with an rAAV according to claim 1, wherein the rAAV is delivered about 1 x 10^{10} to about 1 x 10^{15} genome copies (GC)/kg in an aqueous suspension, wherein the GC are calculated as determined based on oqPCR or ddPCR.

21. The rAAV according to claim 1, wherein the vector genome comprises SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO:22, SEQ ID NO: 23, SEQ ID NO: 24 or SEQ ID NO:25.

22. The suspension according to claim 17, wherein the rAAV capsid is an AAV8 capsid.

23. Use of an rAAV according to any of claims 1 to 16 for treatment of PKU in a subject in need thereof.

FIG. 1

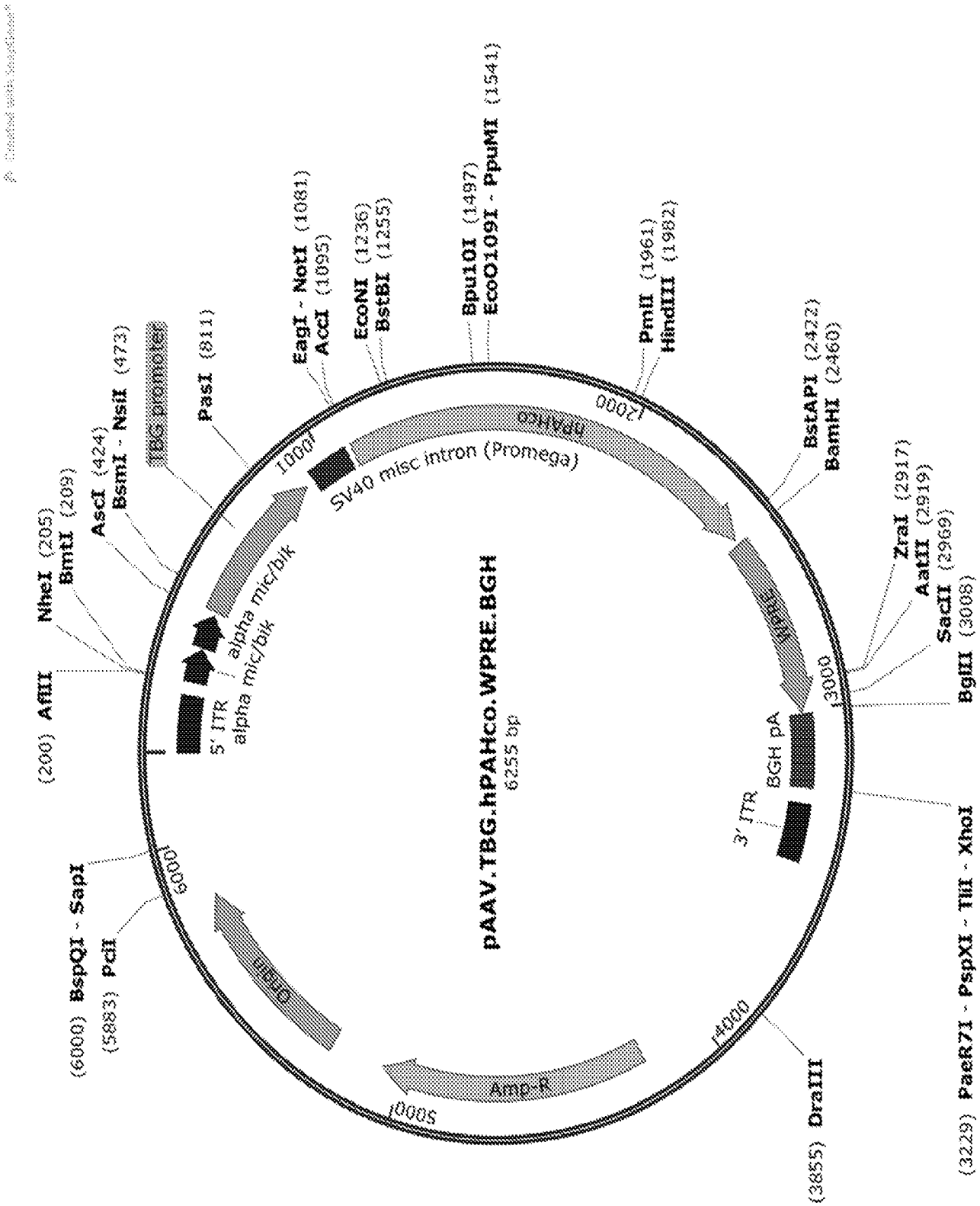

pAAV.TBG.hPAHco.WPRE.BGH
6255 bp
TBG promoter
5' ITR
alpha mic/bik
hPAHco
SV40 misc intron (Promega)
WPRE
BGH pA
3' ITR
Ampᴿ
ORIGIN
1000
2000
3000
4000
5000
6000
(200) AflII
NheI (205)
BmtI (209)
AscI (424)
BsmI - NsiI (473)
PasI (811)
EagI - NotI (1081)
AccI (1095)
EcoNI (1236)
BstBI (1255)
Bpu10I (1497)
EcoO109I - PpuMI (1541)
PmlI (1961)
HindIII (1982)
BstAPI (2422)
BamHI (2460)
ZraI (2917)
AatII (2919)
SacII (2969)
BglII (3008)
(3229) PaeR7I - PspXI - XhoI
(3855) DraIII
(5883) PciI
(6000) BspQI - SapI

FIG. 2A

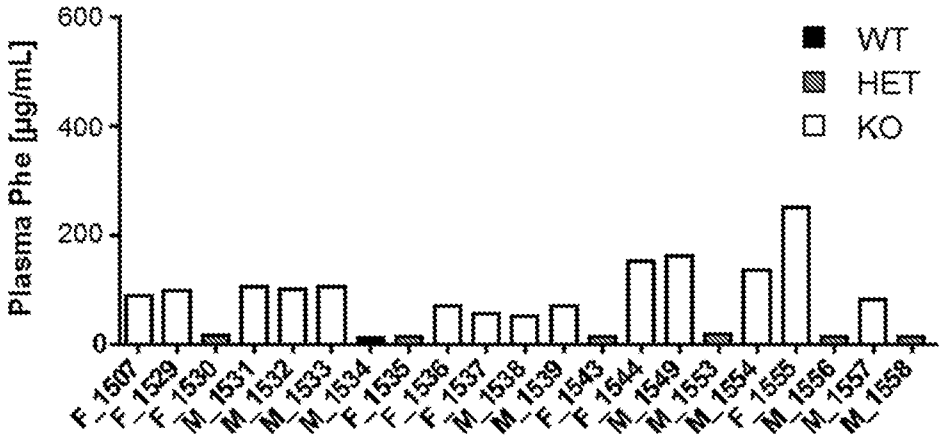

Plasma Phe [µg/mL]
600
400
200
0
WT
HET
KO
F_1507
F_1529
F_1530
M_1531
M_1532
M_1533
M_1534
F_1535
F_1536
F_1537
M_1538
M_1539
F_1543
F_1544
M_1549
M_1553
M_1554
F_1555
M_1556
M_1557
M_1558

FIG. 2B

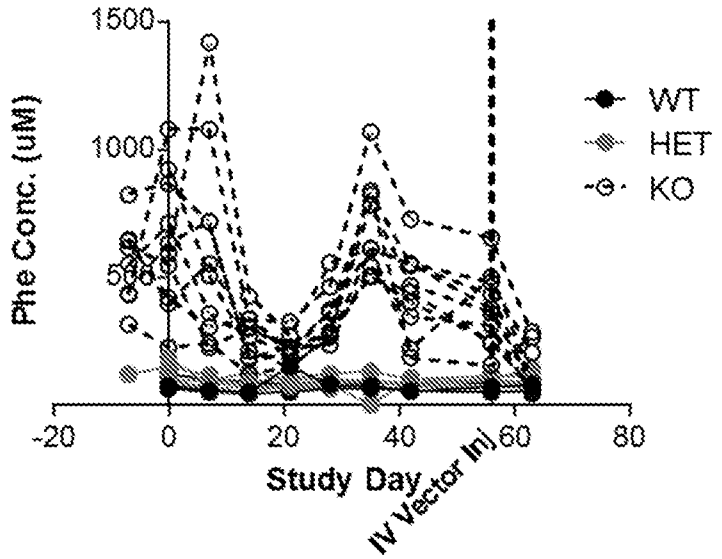

Phe Conc. (uM)
1500
1000
500
WT
HET
KO
-20
0
20
40
60
80
Study Day
IV Vector Inj

FIG. 2C

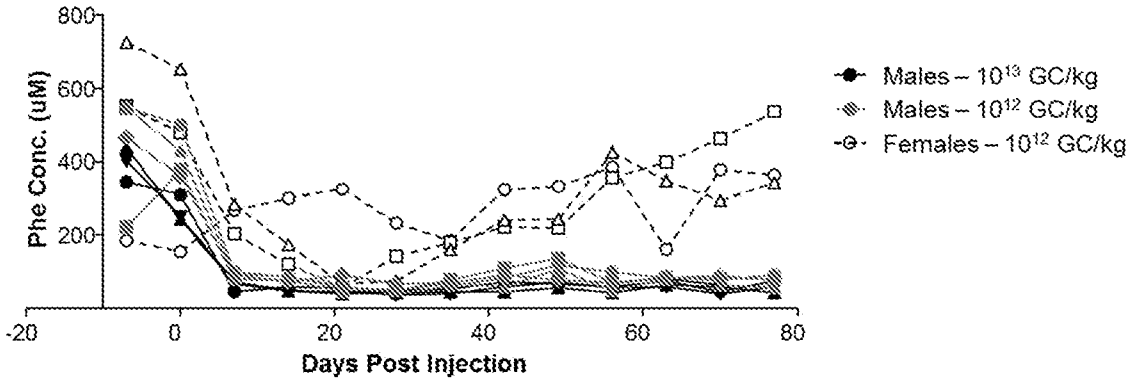

800
600
400
200
Phe Conc. (uM)
-20
0
20
40
60
80
Days Post Injection
Males – 10^13 GC/kg
Males – 10^12 GC/kg
Females – 10^12 GC/kg

FIG. 2D

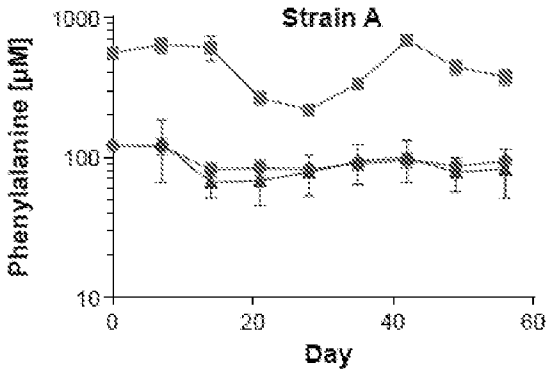

Strain A
1000
100
10
Phenylalanine [µM]
0
20
40
60
Day

FIG. 3A

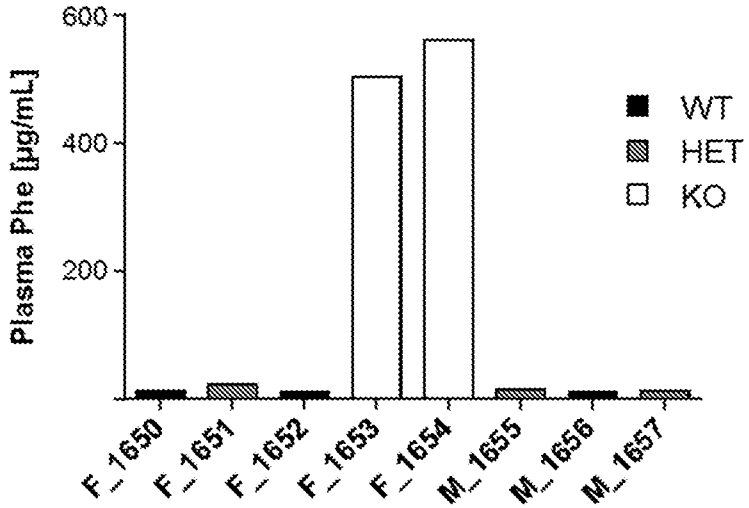

600
400
200
Plasma Phe [µg/mL]
WT
HET
KO
F_1650
F_1651
F_1652
F_1653
F_1654
M_1655
M_1656
M_1657

FIG. 3B

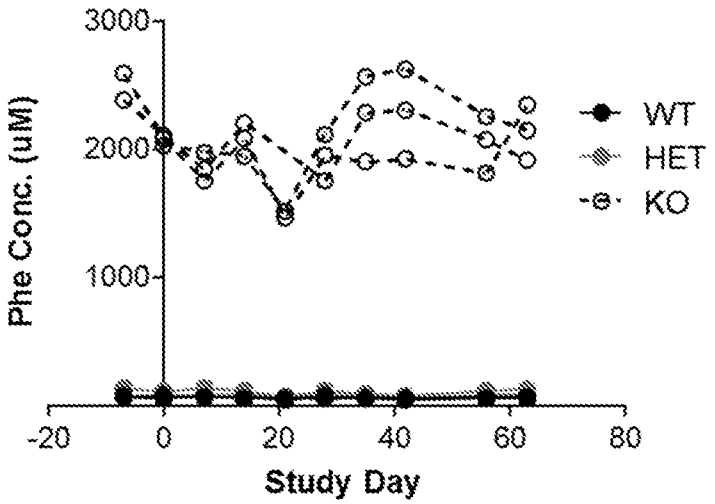

3000
2000
1000
Phe Conc. (uM)
WT
HET
KO
-20
0
20
40
60
80
Study Day

FIG. 3C

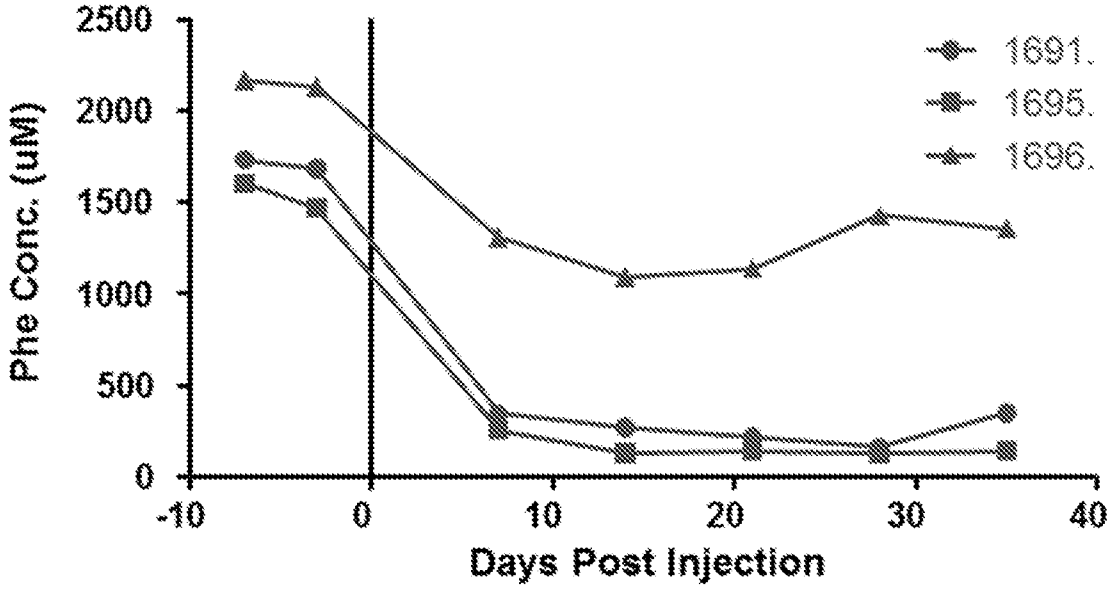

2500
2000
1500
1000
500
0
Phe Conc. (uM)
-10
0
10
20
30
40
Days Post Injection
1691.
1695.
1696.

FIG. 3D

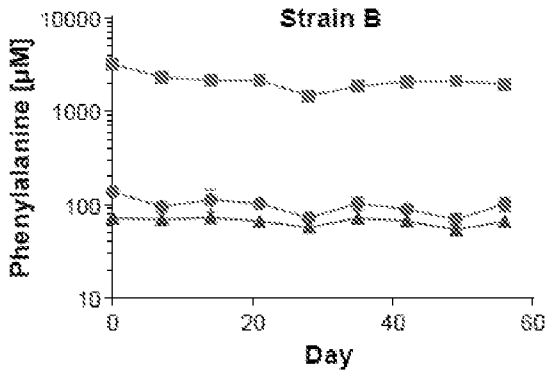

Strain B
10000
1000
100
10
Phenylalanine [µM]
0
20
40
60
Day

FIG. 4A

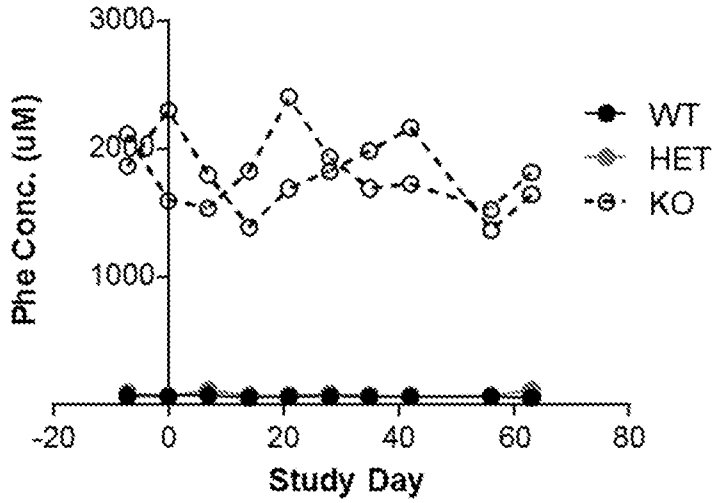


FIG. 4B

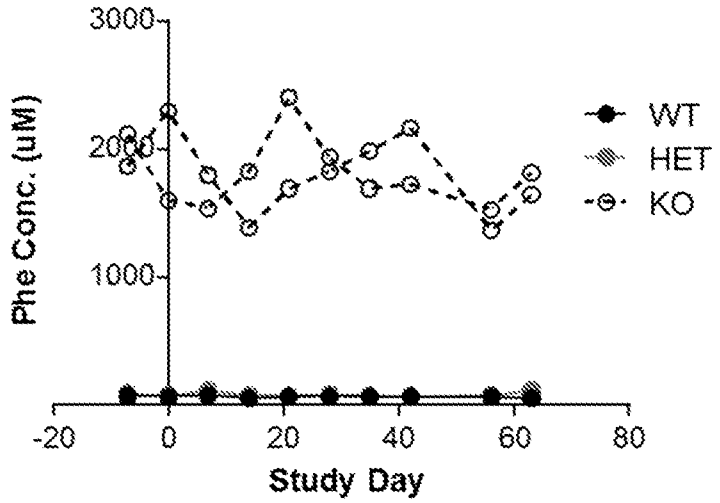


FIG. 4C

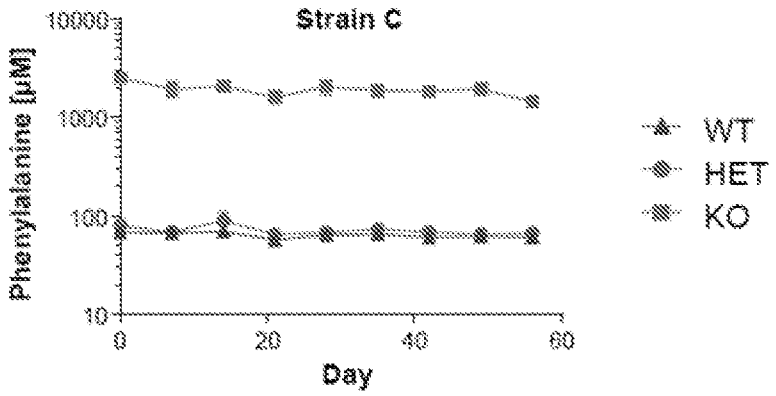


FIG. 5

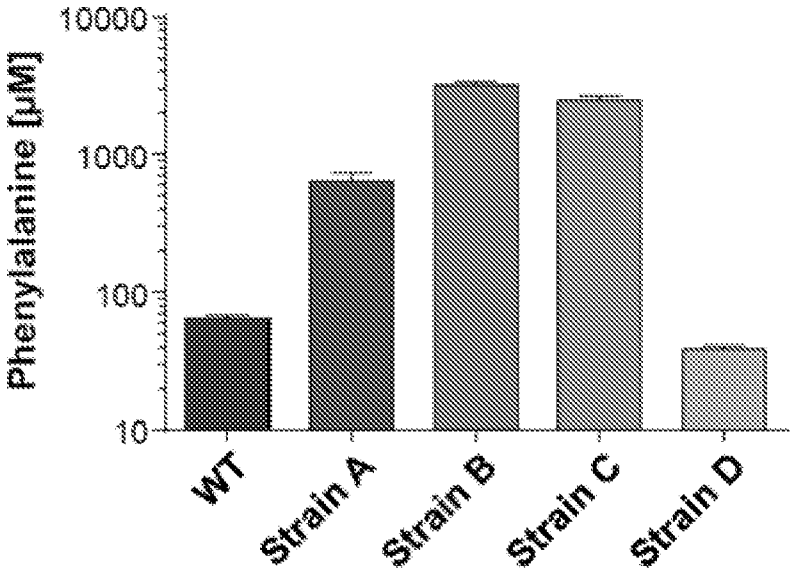

Phenylalanine [µM]
10000
1000
100
10
WT
Strain A
Strain B
Strain C
Strain D

FIG. 6A

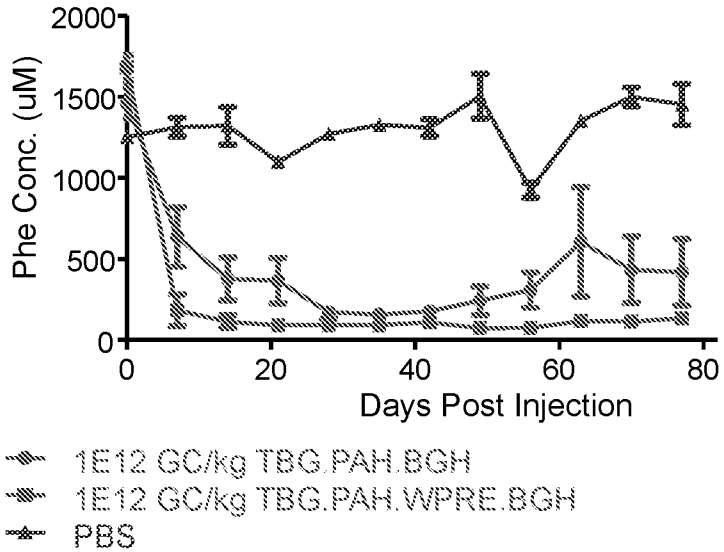
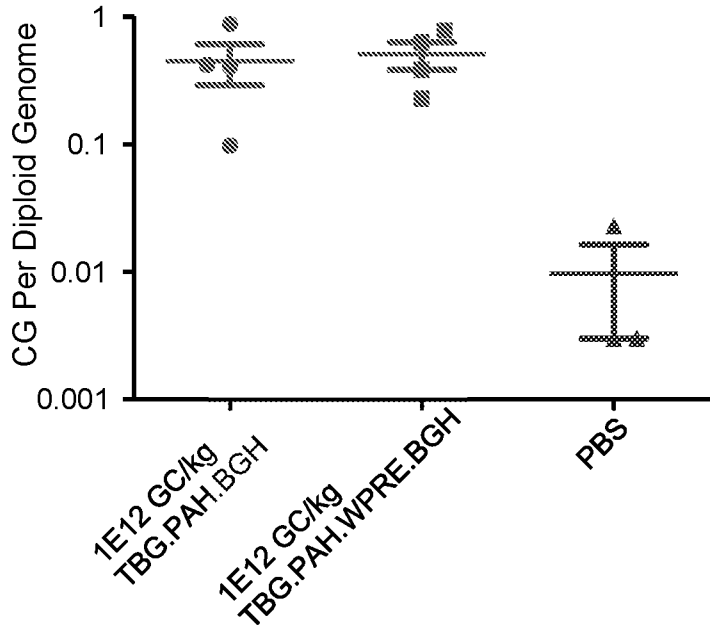

2000
1500
1000
500
0
Phe Conc. (uM)
0
20
40
60
80
Days Post Injection
1E12 GC/kg TBG.PAH.BGH
1E12 GC/kg TBG.PAH.WPRE.BGH
PBS

FIG. 6B


1
0.1
0.01
0.001
CG Per Diploid Genome
1E12 GC/kg TBG.PAH.BGH
1E12 GC/kg TBG.PAH.WPRE.BGH
PBS

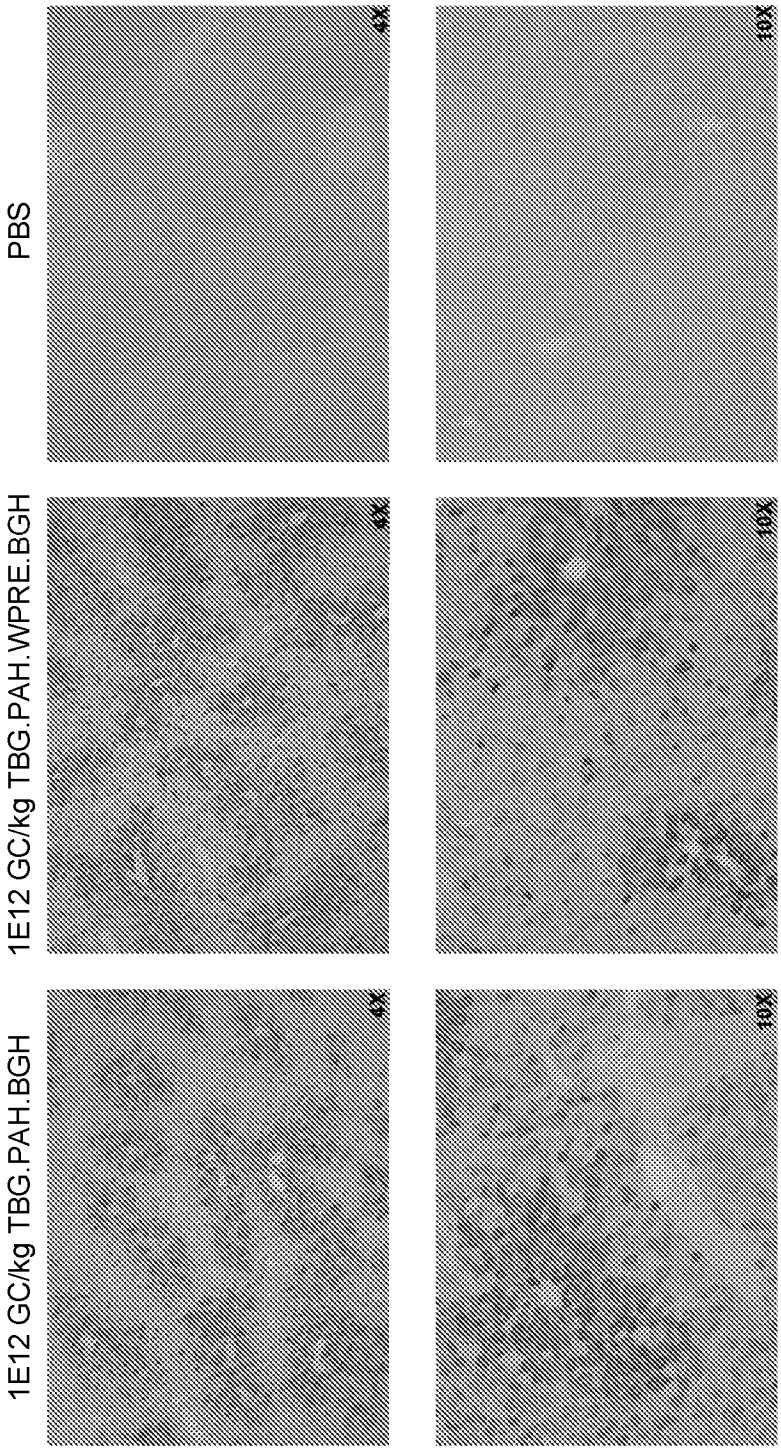

PBS
1E12 GC/kg TBG.PAH.WPRE.BGH
1E12 GC/kg TBG.PAH.BGH
4X
10X
4X
10X
4X
10X

FIG. 6C

FIG. 7

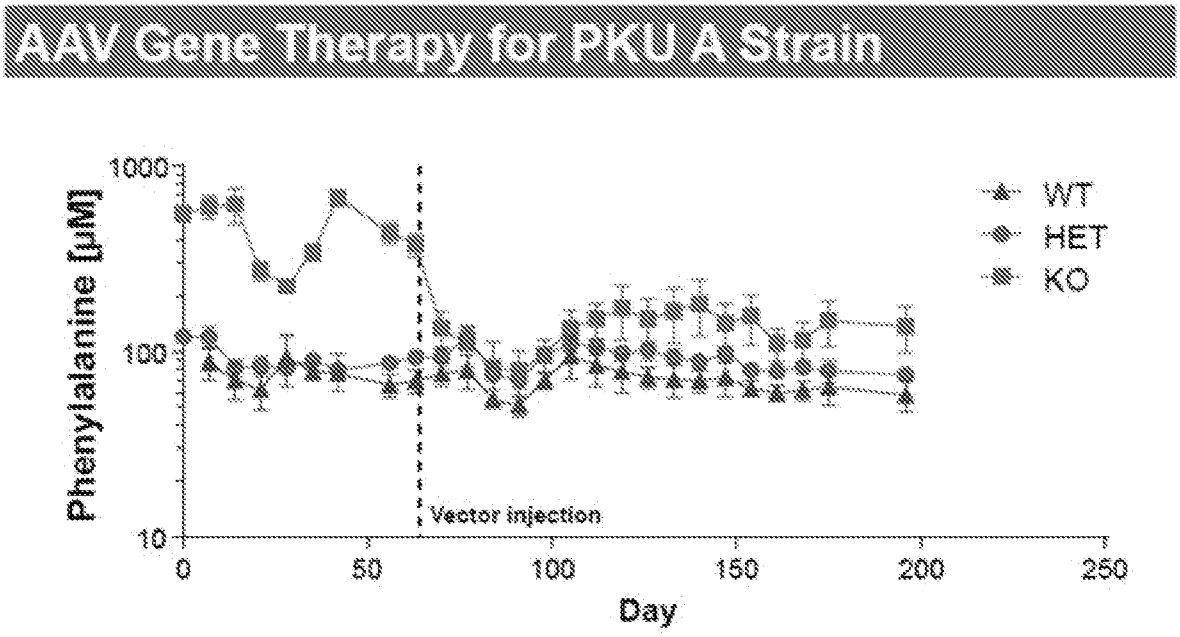
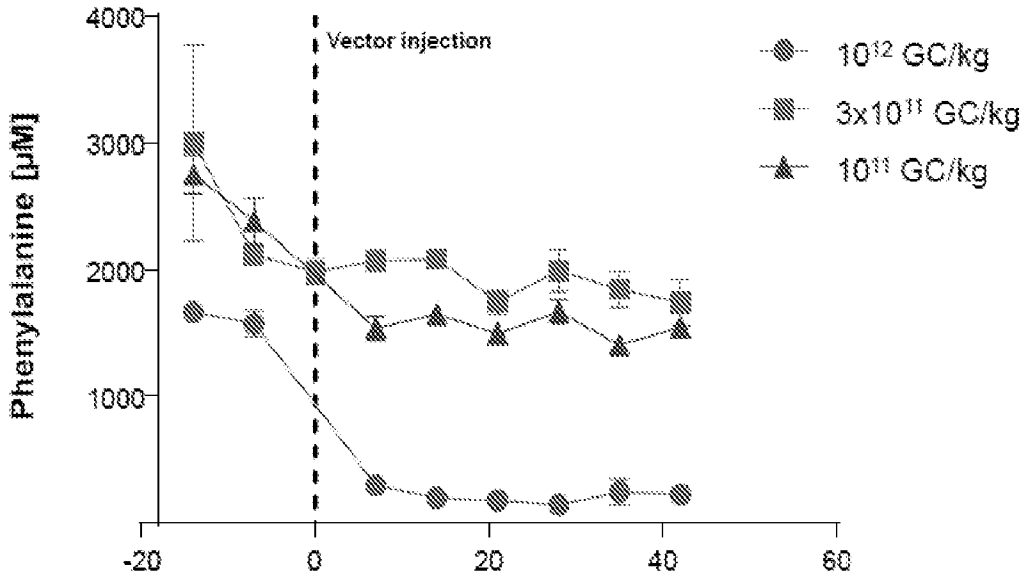

AAV Gene Therapy for PKU A Strain
Phenylalanine [µM]
1000
100
10
0
50
100
150
200
250
Day
Vector injection
WT
HET
KO

FIG 8A



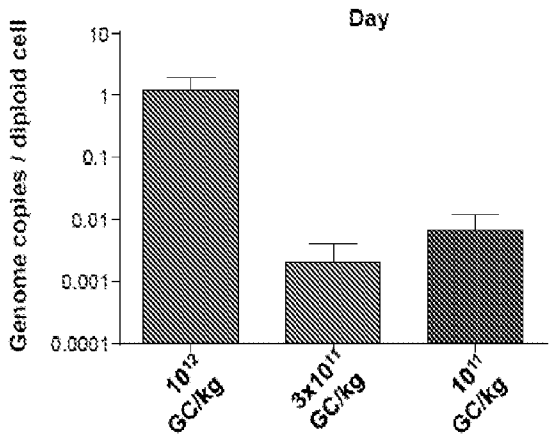


FIG 8B

FIG 8C

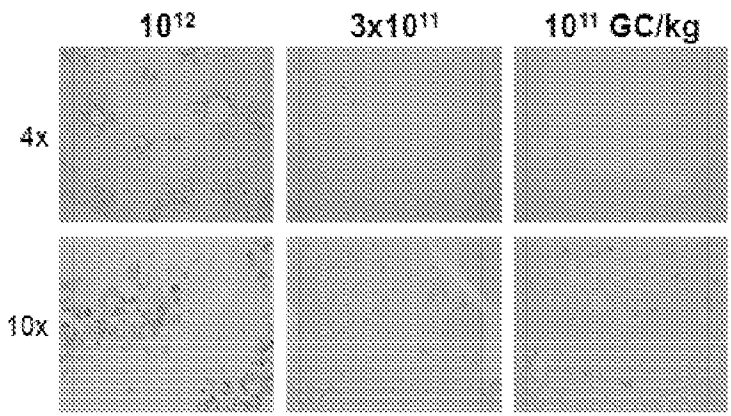
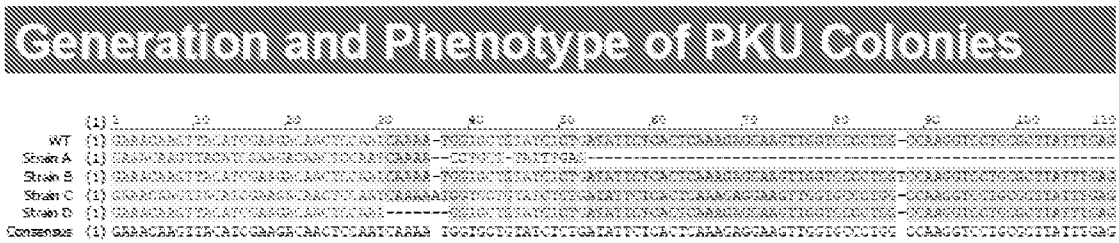


FIG. 9



Strain	Mutation in exon 1
A	3 bp insertion, 64 bp deletion
B	1 bp insertion
C	1 bp insertion
D	6 bp deletion

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/68897

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 35/76, A61K 48/00, A61P 3/00 (2018.01)
CPC - A61K 48/0058, A61K 48/0066, C12N 2800/22, C12N 2800/107, C12N 15/85, A61K 48/00, C12N 15/63

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y ---- A	US 2004/0142416 A1 (LAIPIS ET AL.) 22 July 2004 (22.07.2004) abstract; FIG. 1; para [0006]-[0009], [0019], [0023], [0039], [0047], [0060], [0070], [0084].	17-19, 22 ---------- 1, 3, 4, 8-16 ---------- 2, 21
Y ---- A	US 2015/0110858 A1 (Shire Human Genetics Therapies, Inc.) 23 April 2015 (23.04.2015) abstract; SEQ ID NO:7; para [0005], [0027].	1, 3, 4, 8-16 ---------- 21
Y	WO 2015/138348 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 17 September 2015 (17.09.2015) abstract; para [00010], [00045]-[00046].	4, 10, 15
A	US 8,999,380 B2 (BANCEL ET AL.) 07 April 2015 (07.04.2015) Table 6; SEQ ID NO:2097.	2

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 May 2018	11 MAY 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/68897

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

-----please see continuation on next supplemental sheet-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-4, 8-19, 21, 22, limited to the TBG or TBG-S1 promoter and SEQ ID NO: 20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/68897

Continued from: Box No. III Observations where unity of invention is lacking

Group I+: Claims 1-19, 21-22, drawn to an rAAV and aqueous suspension composition comprising said rAAV. The rAAV compositions will be searched to the extent that the promoter encompasses a TBG promoter or TBG-S1 promoter, and to the extent that the vector genome encompasses SEQ ID NO: 20. It is believed that claims 1-4, 8-19, 21, 22 limited to the TBG or TBG-S1 promoter and SEQ ID NO: 20, encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass an rAAV composition wherein the promoter is a TBG promoter or TBG-S1 promoter, and wherein the vector genome comprises SEQ ID NO: 20. Additional promoters and vector genome sequences will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected promoter(s) and vector genome sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be an rAAV composition wherein the promoter is a A1AT promoter, and wherein the vector genome comprises SEQ ID NO: 21, i.e. claims 1-3, 5, 8-19, 21, 22, limited to the A1AT promoter and SEQ ID NO: 21.

Group II: Claims 20, 23, drawn to a method an use of an rAAV for treatment of phenylketonuria (PKU)

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I+ requires compositions comprising rAAV, not required by Group II. The technical feature of each of the inventions listed as Group I+ is the specific promoter recited therein. Each invention requires a promoter not required by any of the other inventions.

Group II requires method steps for treating a patient having phenylketonuria, not required by Group I+.

No technical features are shared between the nucleotide sequences of vector genomes of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features of including: a recombinant rAAV and an aqueous suspension comprising the same, these shared technical features are previously disclosed as discuss below.

Common Technical Features

The feature shared by Groups I+ and II is the rAAV of claim 1.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by US 2004/0142416 A1 to Laipi et al. (hereinafter 'Laipis') in view of US 2015/0110858 A1 to DeRosa et al. (hereinafter 'DeRosa').

Laipis teaches [claim 1] a recombinant adeno-associated virus (rAAV) useful as a liver-directed therapeutic for phenylketonuria (PKA) (abstract - "Phenylalanine hydroxylase deficiency in a subject is corrected by administering to the subject rAAV-based vectors that include a sequence encoding functional phenylalanine hydroxylase"; para [0006] - "In the method, recombinant adenovirus-associated virus (rAAV) vectors carrying PAH genes are delivered to the liver of an animal with PKU") said rAAV comprising an AAV capsid, and a vector genome packaged therein (para [0007] - "The nucleic acid can be a vector such as a plasmid vector. It can also be contained within an rAAV virion"; para [0023] - "A "recombinant AAV virion," or "rAAV virion" is defined herein as an infectious, replication-defective virus composed of an AAV protein shell encapsulating a heterologous nucleotide sequence flanked on both sides by AAV ITRs"; Figure 1 shows a schematic of two PAH rAV vectors), said vector genome comprising:
(a) an AAV 5' inverted terminal repeat (ITR) sequence (para [0007] - "a phenylalanine hydroxylase-modulating sequence interposed between a first AAV inverted terminal repeat and second AAV inverted terminal repeat");
(b) a promoter (para [0009] - "The nucleic acid of the invention can include various regulatory elements such as a promoter operably lined to the phenylalanine hydroxylase-modulating sequence");
(c) a sequence encoding a human phenylalanine hydroxylase (PAH) (para [0007] - "The phenylalanine hydroxylase-modulating sequence can be (a) a polynucleotide encoding a phenylalanine hydroxylase protein"; para [0008] - "The phenylalanine hydroxylase protein encoded by the polynucleotide can be capable of catalyzing the intracellular conversion of phenylalanine to tyrosine. For example, it can be a wild-type mammalian (e.g., human) phenylalanine hydroxylase protein");
(d) an AAV 3' ITR (para [0007] - "a phenylalanine hydroxylase-modulating sequence interposed between a first AAV inverted terminal repeat and second AAV inverted terminal repeat").

Laipis does not teach that the sequence encoding human PAH is a codon optimized sequence. However, DeRosa teaches a codon optimized sequence encoding a human PAH (para [0027] - "In some embodiments, the mRNA encoding PAH is codon optimized. In some embodiments, the codon-optimized mRNA comprises SEQ ID NO:3 (corresponding to codon-optimized human PAH mRNA sequence)"). Given that codon optimization ensures optimal expression of a coding sequence within a transfected host cell, and the sequence of DeRosa is effective for treatment of PKU (para [0005] - "the present invention provides methods of treating PKU including administering to a subject in need of treatment a composition comprising an mRNA encoding phenylalanine hydroxylase (PAH)"), one of ordinary skill in the art would have found it obvious that the rAAV for treating PKU of Laipis can comprise the codon optimized sequence encoding a human PAH of DeRosa.

-----please see continuation on next supplemental sheet-----

Continued from Box No. III Observations where unity of invention is lacking

As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

Another feature shared by the inventions listed as Group I+ is the composition of claim 17.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by Laipis.

Laipis teaches [claim 17] an aqueous suspension suitable for administration to a phenylketonuria patient (para [0060] - " rAAV virions (i.e., particles) may be directly introduced into an animal, including by intravenous injection, intraperitoneal injection, or in situ injection into target tissue. . . . The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles"), said suspension comprising an aqueous suspending liquid and a recombinant adeno-associated virus (rAAV) useful as a liver-directed therapeutic for phenylketonuria (abstract - "Phenylalanine hydroxylase deficiency in a subject is corrected by administering to the subject rAAV-based vectors that include a sequence encoding functional phenylalanine hydroxylase"; para [0006] - "In the method, recombinant adenovirus-associated virus (rAAV) vectors carrying PAH genes are delivered to the liver of an animal with PKU"; para [0060] - "The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents"), said rAAV having an AAV capsid (para [0007] - "The nucleic acid can be a vector such as a plasmid vector. It can also be contained within an rAAV virion"; para [0023] - "A "recombinant AAV virion," or "rAAV virion" is defined herein as an infectious, replication-defective virus composed of an AAV protein shell encapsulating a heterologous nucleotide sequence flanked on both sides by AAV ITRs"; Figure 1 shows a schematic of two PAH rAV vectors), and having packaged therein a vector genome comprising:
(a) an AAV 5' inverted terminal repeat (ITR) sequence (para [0007] - "a phenylalanine hydroxylase-modulating sequence interposed between a first AAV inverted terminal repeat and second AAV inverted terminal repeat");
(b) a promoter (para [0009] - "The nucleic acid of the invention can include various regulatory elements such as a promoter operably lined to the phenylalanine hydroxylase-modulating sequence");
(c) a coding sequence encoding a human phenylalanine hydroxylase (para [0007] - "The phenylalanine hydroxylase-modulating sequence can be (a) a polynucleotide encoding a phenylalanine hydroxylase protein"; para [0008] - "The phenylalanine hydroxylase protein encoded by the polynucleotide can be capable of catalyzing the intracellular conversion of phenylalanine to tyrosine. For example, it can be a wild-type mammalian (e.g., human) phenylalanine hydroxylase protein");
(d) an AAV 3' ITR (para [0007] - "a phenylalanine hydroxylase-modulating sequence interposed between a first AAV inverted terminal repeat and second AAV inverted terminal repeat").
Laipis does not teach that the rAAV is present in the amount of about 1x10.sup.12 GC/mL to about 1x10.sup.14 GC/mL. However, effective parameters, such as an amount is a parameter that a person of ordinary skill in the art would routinely optimize. Optimization of parameters is a routine practice that would be obvious for a person of ordinary skill in the art to employ. It would have been customary for an artisan of ordinary skill to determine the optimal amount of rAAV needed to achieve the desired results. Thus, absent some demonstration of unexpected results from the claimed parameters, the optimization of the amount of rAAV in the aqueous suspension would have been obvious at the time of applicant's invention.

As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

The inventions listed as Groups I+ and II therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.