progressive and severe neurological impairment. PKU is estimated to affect approximately 15,000 people in the U.S. and there are no treatments available that address the genetic defect in PKU.

[0014] Despite the tremendous advances in understanding the biochemistry, molecular biology, and genetics of PKU, little progress has been made in developing new treatments for the disorder. There is large unmet need for disease-modifying therapies in PKU. First, current therapies are not disease modifying and are only effective in a subset of patients, and still require strict dietary restrictions, and non-compliance can lead to neuronal damage. Second, there are no approved gene therapies for PKU, and AAV based therapies cannot be used by 25% to 40% of patients due to pre-existing antibodies. AAV can only be administered once, and the resulting PAH levels might not be high enough to be efficacious, or may be supranormal, dose levels cannot be titrated.

[0015] Accordingly, there is need in the field for a technology that permits expression of a therapeutic PAH protein in a cell, tissue or subject for the treatment of PKU.

SUMMARY

[0015a] In one aspect, the present disclosure refers to a closed-ended DNA (ceDNA) vector comprising: at least one heterologous nucleotide sequence operably positioned between flanking inverted terminal repeats (ITRs), wherein the at least one heterologous nucleotide sequence encodes at least one phenylalanine hydroxylase (PAH) protein, wherein the at least one heterologous nucleotide sequence that encodes the at least one PAH protein is selected from a sequence having at least 90% identity to any of the sequences in Table 1.

[0015b] In another aspect, the present disclosure refers to the use of the ceDNA vector as disclosed herein in the manufacture of a medicament for expressing an PAH protein in a cell *in vivo*.

[0015c] In another aspect, the present disclosure refers to an *in vitro* method of expressing a PAH protein in a cell, the method comprising contacting the cell with the ceDNA vector as disclosed herein.

[0015d] In another aspect, the present disclosure refers to the use of the ceDNA vector as disclosed herein in the manufacture of a medicament for treating a subject with phenylketonuria (PKU).

[0015e] In another aspect, the present disclosure refers to a pharmaceutical composition comprising the ceDNA vector as disclosed herein.

[0015f] In another aspect, the present disclosure refers to a cell comprising the ceDNA vector as disclosed herein.

[0015g] In another aspect, the present disclosure refers to a composition comprising the ceDNA vector as disclosed herein and a lipid.

[0015h] In yet another aspect, the present disclosure refers to a kit comprising the ceDNA vector as disclosed herein, the pharmaceutical composition as disclosed herein, the cell as disclosed herein, or the composition as disclosed herein.

BRIEF DESCRIPTION

[0016] The technology described herein relates to methods and compositions for treatment of Phenylketonuria (PKU) by expression of enzyme phenylalanine hydroxylase (PAH) from a capsid-free (*e.g.,* non-viral) DNA vector with covalently-closed ends (referred to herein as a "closed-ended DNA vector" or a "ceDNA vector"), where the ceDNA vector comprises a PAH nucleic acid sequence or codon optimized versions thereof. These ceDNA vectors can be used to produce PAH proteins for treatment, monitoring, and diagnosis. The application of ceDNA vectors expressing PAH to a subject for the treatment of PKU is useful to: (i) provide disease modifying levels of PAH enzyme, (ii) be minimally invasive in delivery, (iii) be repeatable and dosed-to-effect, (iv) have rapid onset of therapeutic effect, (v) result in sustained expression of corrective PAH enzyme in the liver, (vi) restoring urea cycle functionphenylalanine metabolism, and/or (vii) be titratable to achieve the appropriate pharmacologic levels of the defective enzyme.

[0017] Accordingly, the invention described herein relates to a capsid-free (*e.g.,* non-viral) DNA vector with covalently-closed ends (referred to herein as a "closed-ended DNA vector" or a "ceDNA vector") comprising a heterogeneous gene encoding PAH, to permit expression of the PAH therapeutic protein in a cell.

[0018] In one aspect, disclosed herein is a close-ended DNA (ceDNA) vector comprising at least one heterologous nucleotide sequence between flanking inverted terminal repeats (ITRs), wherein at least one heterologous nucleotide sequence encodes at least one PAH protein, wherein the at least one heterologous nucleotide sequence that encodes at least one PAH protein is selected from a sequence having at least 90% identity to any of the sequences in Table 1. In one embodiment, the ceDNA vector is a casid-free vector. In one embodiment, the sequences in Table 1 are selected from the group consisting of SEQ ID NO:380, SEQ ID NO:381, SEQ ID NO:382, SEQ ID NO:383, SEQ ID NO:384,

CLAIMS

1. A closed-ended DNA (ceDNA) vector comprising:

at least one heterologous nucleotide sequence operably positioned between flanking inverted terminal repeats (ITRs), wherein the at least one heterologous nucleotide sequence encodes at least one phenylalanine hydroxylase (PAH) protein, wherein the at least one heterologous nucleotide sequence that encodes the at least one PAH protein is selected from a sequence having at least 90% identity to any of the sequences in Table 1.

2. The ceDNA vector of claim 1, wherein the ceDNA vector comprises a promoter operatively linked to the at least one heterologous nucleotide sequence that encodes at least one PAH protein.

3. The ceDNA vector of claim 2, wherein the promoter comprises a nucleic acid sequence having at least 85% identity to SEQ ID NO: 191.

4. The ceDNA vector of any of claims 1 to 3, wherein the ceDNA vector comprises:

an enhancer;

a 5' UTR and/or intron sequence;

a 3' UTR;

at least one poly A sequence; and/or

at least one promoter operably linked to the at least one heterologous nucleotide sequence.

5. The ceDNA vector of any one of claims 1-4, wherein the at least one heterologous nucleotide sequence is cDNA.

6. The ceDNA vector of any one of claims 1-5, wherein at least one of the flanking ITRs comprises a functional terminal resolution site (TRS) and a Rep binding site:

one or both of the flanking ITRs are from a virus selected from a parvovirus, a dependovirus, and an adeno-associated virus (AAV);

the flanking ITRs are symmetric or asymmetric;

the flanking ITRs are symmetrical or substantially symmetrical;

the flanking ITRs are asymmetric;

one or both of the flanking ITRs are wild type, or both of the ITRs are wild-type;

the flanking ITRs are from different viral serotypes;

the flanking ITRs are from a pair of viral serotypes shown in Table 2;

one or both of the flanking ITRs comprises a sequence selected from the sequences in Table 3, Table 5A, Table 5B, or Table 6;

at least one of the flanking ITRs is altered from a wild-type AAV ITR sequence by a deletion, addition, or substitution that affects the overall three-dimensional conformation of the ITR;

one or both of the flanking ITRs are derived from an AAV serotype selected from

AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, and AAV12;

one or both of the ITRs are synthetic; and/or

one or both of the ITRs is not a wild type ITR, or both of the ITRs are not wild-type.

7. The ceDNA vector of any one of claims 1-6, wherein one or both of the flanking ITRs is modified by a deletion, an insertion, and/or a substitution in at least one of the ITR regions selected from A, A', B, B', C, C', D, and D':

one or both of the flanking ITRs is modified by a deletion, an insertion, and/or a substitution in at least one of the ITR regions selected from A, A', B, B', C, C', D, and D', wherein the deletion, the insertion, and/or the substitution results in the deletion of all or part of a stem-loop structure normally formed by the A, A', B, B' C, or C' regions;

one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of all or part of a stem-loop structure normally formed by the B and B' regions;

one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of all or part of a stem-loop structure normally formed by the C and C' regions;

one or both of the flanking ITRs are modified by a deletion, an insertion, and/or a substitution that results in the deletion of part of a stem-loop structure normally formed by the B and B' regions and/or part of a stem-loop structure normally formed by the C and C' regions;

one or both of the flanking ITRs comprise a single stem-loop structure in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions;

one or both of the flanking ITRs comprise a single stem and two loops in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions;

one or both of the flanking ITRs comprise a single stem and a single loop in the region that normally comprises a first stem-loop structure formed by the B and B' regions and a second stem-loop structure formed by the C and C' regions; and/or

both flanking ITRs are altered in a manner that results in an overall three-dimensional symmetry when the ITRs are inverted relative to each other.

8. The ceDNA vector of any one of claims 1-7, wherein the at least one heterologous nucleotide sequence comprises a sequence having at least 90% identity to SEQ ID NO:381, SEQ ID NO:382, SEQ ID NO:383, SEQ ID NO:384, SEQ ID NO:385, SEQ ID NO:386, SEQ ID NO:387, SEQ ID NO:388, SEQ ID NO:389, SEQ ID NO:390, SEQ ID NO:391, SEQ ID NO:392, SEQ ID NO:393 or SEQ ID NO:394.

9. The ceDNA vector of any one of claims 1-8, wherein the ceDNA vector comprises a nucleic acid sequence that is at least 90% identical to SEQ ID NO: 192, SEQ ID NO:193, or SEQ ID NO:194.

10. The ceDNA vector of any one of claims 1-9, wherein at least one heterologous nucleotide sequence is under the control of at least one regulatory switch.

11. Use of the ceDNA vector of any one of claims 1-10 in the manufacture of a medicament for expressing an PAH protein in a cell *in vivo*.

12. The use of claim 11, wherein the cell is a hepatic cell.

13. An *in vitro* method of expressing a PAH protein in a cell, the method comprising contacting the cell with the ceDNA vector of any one of claims 1-10.

14. The use of any one of claims 11-12 or the method of claim 13, wherein the at least one heterologous nucleotide sequence is codon optimized for expression in the cell.

15. Use of the ceDNA vector of any one of claims 1-10 in the manufacture of a medicament for treating a subject with phenylketonuria (PKU).

16. The use of claim 15, wherein the ceDNA vector is to be administered to a hepatic cell.

17. The use of any of claims 15-16, wherein the ceDNA vector expresses the PAH protein in a hepatic cell.

18. The use of any of claims 15-17, wherein the ceDNA vector is to be administered by any one or more of: subretinal injection, suprachoroidal injection or intravitreal injection.

19. The use of any one of claims 15-18, wherein the subject exhibits at least about a 50% decrease in level of serum phenylalanine as compared to a level of serum phenylalanine in the subject prior to treatment.

20. The use of any one of claims 15-19, wherein the subject has a serum phenylalanine level of less than about 1500 uM after treatment.

21. The use of any one of claims 15-20, wherein the subject exhibits at least about a 10% increase in PAH activity after treatment as compared to a level of PAH activity prior to treatment.

22. A pharmaceutical composition comprising the ceDNA vector of any one of claims 1-10.

23. A cell comprising the ceDNA vector of any of claims 1-10.

24. The cell of claim 23, wherein the cell is a hepatic cell.

25. A composition comprising the ceDNA vector of any of claims 1-10 and a lipid.

26. The composition of claim 25, wherein the lipid is a lipid nanoparticle (LNP).

27. A kit comprising the ceDNA vector of any one of claims 1-10, the pharmaceutical composition of claim 22, the cell of claim 23 or claim 24, or the composition of claim 25 or 26.