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(54) Title: COMPOSITIONS USEFUL IN TREATMENT OF OTC DEFICENCY

(57) Abstract: Non-viral delivery systems comprising engineered hOTC DNA and RNA sequences are provided which when delivered to a subject in need thereof are useful for treating hyperammonemia, ornithine transcarbamylase transcarbamylase deficiency and symptoms associated therewith. Also provided are methods of using hOTC for treatment of liver fibrosis and/or cirrhosis in OTCD patients by administering hOTC.



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## COMPOSITIONS USEFUL IN TREATMENT OF OTC DEFICIENCY

### STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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### INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN ELECTRONIC FORM

[0002] Applicant hereby incorporates by reference the Sequence Listing material filed in electronic form herewith. This file is labeled "UPN-14-7037APCT\_ST25.txt".

### BACKGROUND OF THE INVENTION

[0003] Ornithine transcarbamylase (OTC) deficiency accounts for nearly half of all cases of inborn errors of urea synthesis, with a prevalence estimated to be at least 1 in 15,000. Urea cycle defects put patients at risk of life threatening elevation of ammonia that can lead to irreversible cognitive impairment, coma and death. Newborn males with complete deficiency develop hyperammonemic coma within the first 3 days of life, which if untreated, is lethal.

[0004] Current therapies for OTC deficiency (OTCD) have numerous challenges. Patients can be managed with a low protein diet in combination with the use of medications that activate alternate nitrogen clearance pathways, but this does not prevent hyperammonemic crises. Despite the use of dialysis and alternate pathway therapy, there is almost a 50% mortality rate in neonates. Liver transplantation can cure OTCD, but donor liver is limiting, the procedure carries significant morbidity and immunosuppressive drugs are necessary for the duration of the subject's life.

[0005] Gene therapy of a metabolic disease such as OTCD presents a more challenging model for gene replacement therapy than other conditions. Because the gene acts in a

cell-autonomous manner (*i.e.*, it can only influence the cell in which it is expressed), therapeutic effects should be directly correlated with the number of target cells that are transduced, rather than with the net level of expression in liver such as with a secreted protein where high expression per cell can overcome low transduction. Furthermore, there has been at least one published report that hOTCwt mRNA is unstable. [Wang, L., et al, Molecular Genetics and Metabolism, 105 (2012) 203-211].

[0006] There have been published reports of using viral vectors to try to treat OTC deficiency. For example, several groups have tried this in murine models of OTC deficiency, using recombinant adenoviruses carrying rat, mouse, or human OTC cDNA. Some measure of successful reconstitution of liver OTC activity and correction of metabolic derangements have been reported in animal models with viruses carrying rat or mouse OTC cDNA. Previous studies using adenoviral vectors have illustrated the difficulties of expressing sufficient levels of active human OTC in OTCD mice.

[0007] Therefore, there is a need for other approaches to OTCD therapy.

## SUMMARY OF THE INVENTION

[0008] In one aspect, a composition comprising a non-viral carrier and an engineered hOTC is provided which is less than 80% identical to the wild-type hOTC sequence over the full-length hOTC of the wild-type sequence (*e.g.*, SEQ ID NO:1), or a fragment thereof which comprises the mature hOTC but lacking at least the native leader sequence, or another intermediate which comprises at least the mature hOTC) and expresses a functional hOTCase. Suitably, the engineered sequence has been preferably codon optimized and further improved such that it enhances at least one of transduction, transcription and/or translation of the enzyme. The composition comprises a nucleic acid molecule comprising an engineered sequence encoding human ornithine transcarbamylase (hOTCase) and expression control sequences which direct expression of hOTCase in a liver cell, wherein the hOTC nucleic acid sequence is less than 80% identical to the wild-type hOTC sequence over at least the mature hOTC sequence of SEQ ID NO:1, wherein said hOTC nucleic acid sequence is selected from the nucleic acid sequence comprising SEQ ID NO: 5 or a nucleic acid sequence at least about 96 to about 99 % identical thereto, a nucleic acid sequence

selected from SEQ ID NO: 9, or a nucleic acid sequence at least about 96 to about 99 % identical thereto, a nucleic acid sequence comprising SEQ ID NO: 11 or a nucleic acid sequence at least about 96 to about 99 % identical thereto, or a nucleic acid sequence comprising SEQ ID NO: 13 or a nucleic acid sequence at least about 96 to about 99 % identical thereto. In one embodiment, the hOTC is a chimeric OTC comprises a heterologous transit sequence substituted for the native transit sequence of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12 or 13.

[0009] In one aspect, the composition comprises a plasmid. In one example, the plasmid has the sequence of SEQ ID NO: 6.

[0010] In another aspect, the engineered hOTC nucleic acid molecule is formulated in a moiety selected from the group consisting of micelles, liposomes, cationic lipid - nucleic acid compositions, poly-glycan compositions, block copolymers and other polymers, lipid and/or cholesterol-based - nucleic acid conjugates. In a further aspect, a pharmaceutical composition comprises an excipient and an effective amount of the engineered hOTC nucleic acid molecule.

[0011] In still another aspect, a composition is provided which comprises the engineered hOTC nucleic acid molecule for use in a method of treatment of a disease or medical condition associated with ornithine transcarbamylase deficiency in a human patient. In a further embodiment, a composition comprises a nucleic acid molecule encoding functional human ornithine transcarbamylase for use in a method in preventing and/or treating fibrosis or cirrhosis in a subject heterozygous for ornithine transcarbamylase deficiency in a human patient. In yet another embodiment, a composition comprises a nucleic acid molecule comprising a nucleic acid sequence encoding functional human ornithine transcarbamylase for use in a method for preventing and/or treating hepatocellular carcinoma in a subject heterozygous for ornithine transcarbamylase deficiency.

[0012] In still another embodiment, a method for delivering ornithine transcarbamylase to a subject in need thereof and/or for treating ornithine transcarbamylase deficiency in a subject comprising delivering to a subject in need thereof the composition containing an engineered hOTC as described herein.

[0013] In still another aspect, a composition comprises an mRNA according to any one of SEQ ID NO: 10, 11, or 12 in a method for delivering ornithine

transcarbamylase to a human patient in acute crisis. A composition as defined herein may also be used in a method for delivering ornithine transcarbamylase to a human patient following treatment of said patient with RNA.

[00014] In another aspect, a method for preventing and/or treating fibrosis or cirrhosis in a subject heterozygous for ornithine transcarbamylase deficiency is provided, which method comprises delivering to a subject a composition comprising a nucleic acid sequence encoding functional human ornithine transcarbamylase.

[00015] A method for preventing and/or treating hepatocellular carcinoma in a subject heterozygous for ornithine transcarbamylase deficiency is provide. This method comprises delivering a composition comprising a nucleic acid sequence encoding functional human ornithine transcarbamylase.

[00016] Other aspects and advantages of the invention will be readily apparent from the following detailed description of the invention.

## BRIEF DESCRIPTION OF THE DRAWINGS

[00017] FIG. 1A provides a wild-type hOTC cDNA, which has 324 A, 223 C, 246 G, and 269 T [SEQ ID NO: 1].

[00018] FIG. 1B - 1C provides the human ornithine transcarbamylase sequence encoded by the sequence of FIG. 1A [SEQ ID NO:2].

[00019] FIG.2 provides an engineered hOTC cDNA, with an altered GC ratio. The base count in the sequence is 283 A, 285 C, 284 G, and 216 T [SEQ ID NO: 3].

[00020] FIG. 3 provides an engineered hOTC cDNA termed LW3 [SEQ ID NO:4]. The base count in this sequence is 279 A, 303 C, 288 G, and 220 T. The start codon for the hOTC open reading frame (ORF) is preceded by a Kozak sequence in this figure. The coding sequence for the leader begins at nucleotide 15 (first 96 nucleotides), followed by the coding sequence for the 322 amino acid hOTCase. In this figure the stop codon is followed by a NotI restriction site (GCGGCCGC) which is a remnant of the vector.

[00021] FIG. 4 provides an engineered hOTC cDNA termed LW4 [SEQ ID NO:5]. The base count in this sequence is 278 A, 303 C, 289 G, and 220 T. The coding

sequence for the leader begins at nucleotide 15 (first 96 nucleotides), followed by the coding sequence for the 322 amino acid hOTCase. In this figure the stop codon is followed by a NotI restriction site (GCGGCCGC) which is a remnant of the vector.

[00022] FIGs. 5A - 5C provides an alignment of the cDNA sequences of the wild-type hOTC, and five engineered sequences, GS [SEQ ID NO: 3], LW3 [SEQ ID NO:4], LW4 [SEQ ID NO: 5], LW5 [SEQ ID NO: 8] and LW6 [SEQ ID NO:9]. The aligned sequences contain a Kozak sequence (first 14 nucleotides of LW3 and LW4) and a restriction enzyme site (following termination codon for LW3 and LW4), which are not part of the open reading frame.

## DETAILED DESCRIPTION OF THE INVENTION

[00023] An engineered human (h) ornithine transcarbamylase (OTC) cDNA is provided herein, which was designed to maximize translation and improve mRNA stability as compared to the wild-type hOTC DNA and/or mRNA. Also provided herein are engineered hOTC mRNA sequences. These compositions may be used in therapeutic and/or prophylactic methods as described herein. Optionally, these compositions are used in combination other therapies consistent with the standard of care for the conditions for which the subject (*e.g.*, a human subject) has been diagnosed.

[00024] For comparison purposes, a wild-type human OTC cDNA sequence is illustrated in FIG. 1A. This sequence encodes the human ornithine transcarbamylase of the amino acid sequence of FIGS. 1A – 1C. This same amino acid sequence is encoded by the engineered hOTC genes of FIGS. 2A – FIG. 5. The hOTC enzyme, which may be referred to as hOTCase to distinguish from the gene, is expressed from this sequence in the form of a pre-protein having a 32 amino acid leader peptide at its N-terminus (encoded by nt 1-96 of FIG.1, about amino acids 1 to about 32 of SEQ ID NO: 2) which is cleaved after directing the enzyme to the cellular mitochondria, leaving the 322 amino acid residue “mature” protein (about amino acid 33 to about amino acid 354 of SEQ ID NO: 2. This “so-called mature” hOTCase is a homotrimeric protein with a 322 amino acid residue sequence in each polypeptide chain. Optionally, as an alternative to the wild-type sequence of SEQ ID NO:2, one may select a sequence which includes one or more of the naturally occurring polymorphic positions that are not involved in disease: F101, L111, W1193-194 of SEQ ID NO: 2 (*see, e.g.*, <http://www.uniprot.org/uniprot/P00480>).

[00025] Although all of the engineered cDNA sequences are about 77% to about 78% identical to the wt hOTC nucleic acid sequence of FIG. 1A [SEQ ID NO:1], there are structural differences between these sequences (see alignment in FIG 5 illustrating same). Particularly, there is about 4% difference in nucleic acid sequences between hOTCco of FIG. 2 [SEQ ID NO: 3] and the hOTCcoLW4 of FIG4 [SEQ ID NO:5]. There is only one nt difference between LW-3 [FIG. 3, SEQ ID NO: 4] and LW-4 [FIG 4, SEQ ID NO: 5], *i.e.*, 0.094% (1/1062) difference (an A in LW-3 is changed to a G in LW-4 as shown in FIG 5) .

[00026] In one embodiment, a modified hOTC coding sequence is provided which sequence has less than about 80% identity, preferably about 77% identity or less to the full-length wild-type hOTC coding sequence (FIG. 1A, SEQ ID NO:1), which encodes functional hOTCase. In one embodiment, the modified hOTC coding sequence is characterized by improved stability as compared to wt hOTC following AAV-mediated delivery (*e.g.*, rAAV). Additionally or alternatively, a modified hOTC coding sequence is provided which lacks alternative reading frames for proteins of at least about 9 amino acids in length. Additionally, or alternatively, a modified hOTC coding sequence is provided which has hOTCase expression levels at least about 25 fold, at least about 50 fold, or at least about 100-fold when measured following expression from a viral vector, as compared to the hOTCase wild-type. Additionally, or alternatively, a modified hOTC coding sequence is provided which has hOTCase liver activity which is at least about 10-fold higher, at least about 20-fold higher, or at least about 30-fold higher as compared to the hOTCase wild-type expressed from a viral vector.

[00027] In one embodiment, a modified hOTC coding sequence is 96% to 99.9% identical to the sequence encoding the mature enzyme (about nt 99 to about 1068) or full-length of FIG. 4 (hOTCco-LW4, SEQ ID NO: 5), or 96.5% to 99% identical, or about 97% , or about 98% identical to SEQ ID NO:5 (FIG. 4).

[00028] In one embodiment, a modified hOTC coding sequence is 96% to 99.9% identical to the sequence encoding the mature enzyme (about nt 99 to about 1068) of FIG. 3 (hOTCco-LW3, SEQ ID NO:4), or 96.5% to 99% identical, or about 97% , or about 98% identical to SEQ ID NO: 4 (FIG. 3).

[00029] In another embodiment, a modified hOTC coding sequence is 96% to 99.9% identical to the sequence encoding the mature enzyme (about nt 99 to about 1068)

or the full-length of FIG. 2 (hOTCco, SEQ ID NO:3), or 96.5% to 99% identical, or about 97% , or 98% identical to SEQ ID NO: 3 (FIG. 2).

[00030] In still another embodiment, a modified hOTC coding sequence has the sequence encoding the mature protein (about nt 99 to about 1068) or the full-length of hOTCco-LW5 [SEQ ID NO: 8] or hOTCco-LW6 [SEQ ID NO:9], or a sequence 96% to 99.9% identical thereto. hOTCco-LW5 and hOTCco-LW6 are about 97% identical to each other, and each is about 78% identical to the wild-type sequence [SEQ ID NO: 1].

[00031] The sequences of FIGs. 2-5 are provided as the sense strand of the cDNA sequences. The present invention also encompasses the anti-sense strands corresponding to these cDNA sequences and corresponding RNA, e.g., mRNA, sequences. For example, the engineered mRNA of SEQ ID NO: 10, corresponds to the DNA of SEQ ID NO:4; the engineered RNA of SEQ ID NO: 11, corresponds to the DNA of SEQ ID NO: 5; the engineered RNA of SEQ ID NO: 12, corresponds to the DNA of SEQ ID NO: 8; and the RNA of SEQ ID NO: 13 corresponds to the DNA of SEQ ID NO:9. These RNA sequences, and sequences which are 95% to 99%, or about 97%, or about 98% identical to one or more of these sequences are encompassed within the scope of this invention. Methods for aligning and determining RNA identity are known the art and include published and publically available web-based or commercially available databases and services. *See, e.g.*, LocARNA, CARNA, as well as other programs identified elsewhere therein.

[00032] In one embodiment of the invention, the mRNA sequence may be delivered using a selected RNA delivery system, examples of which are supplied herein.

[00033] Also encompassed herein are fragments, *e.g.*, the sequences encoding the transit peptide (amino acids 1 to about 32), about amino acids 332 to about 354, an intermediate hOTC enzyme, or the mature enzyme, or other fragments as may be desired. Reference may be made to SEQ ID NO:2. *See, e.g.*, Ye et al. 2001, Hum Gene Ther 12: 1035-1046.

[00034] In another embodiment, a chimeric OTC is provided in which the N-terminal presequence of wild-type OTC is replaced with a transit sequence from another source which is compatible with the subject's system such that it effectively transports the mature hOTCase encoded by the chimeric OTC gene to the desired organelle. *See, e.g.*, Ye et al. 2001, Hum Gene Ther 12: 1035-1046. Such transit sequences encode a transit peptide (also termed a signal peptide, targeting signal, or localization signal) which



is fused to the coding sequence for the mature hOTC of SEQ ID NO: 1, 3, 4, 5, 8 and/or 9. For example, the wild-type hOTC transit sequence corresponds to about the first 98 nucleotides of SEQ ID NO: 1. To construct a chimeric OTC, these wild-type N-terminal sequences may be removed (about nucleic acids 1 to about nt 96 - nt 98) and replaced with a heterologous transit sequence. Suitable transit peptides are preferably, although not necessarily of human origin. Suitable transit peptides may be chosen from <http://proline.bic.nus.edu.sg/spdb/zhang270.htm>, which is incorporated by reference herein, or may be determined using a variety of computational programs for determining the transit peptide in a selected protein. Although not limited, such sequences may be from about 15 to about 50 amino acids in length, or about 20 to about 28 amino acids in length, or may be larger or smaller as required.

[00035] The term “percent (%) identity”, “sequence identity”, “percent sequence identity”, or “percent identical” in the context of nucleic acid sequences refers to the residues in the two sequences which are the same when aligned for correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, is desired. However, identity among smaller fragments, *e.g.* of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired.

[00036] 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 sequences. 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.

[00037] 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).

[00038] Multiple sequence alignment programs are also available for nucleic acid sequences. Examples of such programs include, “Clustal W”, “CAP Sequence Assembly”, “BLAST”, “MAP”, and “MEME”, which are accessible through Web Servers on the internet. Other sources for such programs are known to those of skill in the art. Alternatively, Vector NTI utilities are also used. There are also a number of algorithms known in the art that can be used to measure nucleotide sequence identity, including those contained in the programs described above. As another example, polynucleotide sequences can be compared using Fasta™, a program in GCG Version 6.1. Fasta™ provides alignments and percent sequence identity of the regions of the best overlap between the query and search sequences. For instance, percent sequence identity between nucleic acid sequences can be determined using Fasta™ with its default parameters (a word size of 6 and the NOPAM factor for the scoring matrix) as provided in GCG Version 6.1, herein incorporated by reference.

[00039] In one embodiment, the modified hOTC genes described herein are engineered into a suitable genetic element (vector) useful for generating viral vectors and/or for delivery to a host cell, e.g., naked DNA, phage, transposon, cosmid, episome, etc., which transfers the hOTC sequences carried thereon. The selected vector may be delivered by any suitable method, including transfection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion. The methods used to make such constructs are known to those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY.

[00040] As used herein, an “expression cassette” refers to a nucleic acid molecule which comprises the hOTC sequences, promoter, and may include other regulatory sequences therefor, which cassette may be packaged into the capsid of a viral vector (e.g., a viral particle). Typically, such an expression cassette for generating a viral vector

contains the hOTC sequences described herein flanked by packaging signals of the viral genome and other expression control sequences such as those described herein. For example, for an AAV viral vector, the packaging signals are the 5' inverted terminal repeat (ITR) and the 3' ITR.

[00041] In one embodiment, the ITR sequences from AAV2, or the deleted version thereof ( $\Delta$ ITR), are used for convenience and to accelerate regulatory approval. 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.

[00042] Typically, an expression cassette for an AAV vector comprises an AAV 5' ITR, the hOTC 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  $\Delta$ ITR, 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.

[00043] In one embodiment, the construct is a DNA molecule (*e.g.*, a plasmid) useful for generating viral vectors. An illustrative plasmid containing desirable vector elements is illustrated by pAAVsc.TBG.hOTCco-LW4, the sequence of which is SEQ ID NO: 6 and which is incorporated by reference. This illustrative plasmid contains an expression cassette comprising: scITR (nt 5 - 109 of SEQ ID NO: 6), a TATA signal (nt 851-854 of SEQ ID NO: 6), a synthetic hOTC coding sequence (nt 976-2037 of SEQ ID NO: 6), a poly A (nt 2182-2046 on the complement of SEQ ID NO: 6), a scITR (nt 2378-2211 on the complement of SEQ ID NO: 6), and a liver specific (TBG) promoter (nt 4172-4760) of SEQ ID NO: 6). Other expression cassettes may be generated using the other synthetic hOTC coding sequences, and other expression control elements, described herein.

[00044] The abbreviation "sc" in this context refers to self-complementary. "Self-complementary AAV" refers a construct in which a coding region carried by a recombinant AAV nucleic acid sequence has been designed to form an intra-molecular double-stranded DNA template. Upon infection, rather than waiting for cell mediated synthesis of the second strand, the two complementary halves of scAAV will associate to form one double stranded DNA (dsDNA) unit that is ready for immediate replication and transcription. *See, e.g.*, D M McCarty et al, "Self-complementary recombinant adeno-

associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis”, *Gene Therapy*, (August 2001), Vol 8, Number 16, Pages 1248-1254. Self-complementary AAVs are described in, *e.g.*, U.S. Patent Nos. 6,596,535; 7,125,717; and 7,456,683, each of which is incorporated herein by reference in its entirety.

[00045] The expression cassette typically contains a promoter sequence as part of the expression control sequences, *e.g.*, located between the selected 5' ITR sequence and the hOTC coding sequence. The illustrative plasmid and vector described herein uses the liver-specific promoter thyroxin binding globulin (TBG). Alternatively, other liver-specific promoters may be used [*see, e.g.*, The Liver Specific Gene Promoter Database, Cold Spring Harbor, <http://rulai.schl.edu/LSPD/>, such as, *e.g.*, alpha 1 anti-trypsin (A1AT); human albumin Miyatake et al., *J. Virol.*, 71:5124-32 (1997), humAlb; and hepatitis B virus core promoter, Sandig *et al.*, *Gene Ther.*, 3:1002-9 (1996)]. TTR minimal enhancer/promoter, alpha-antitrypsin promoter, LSP (845 nt)<sup>25</sup>(requires intron-less scAAV); or LSP1. Although less desired, other promoters, such as 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.

[00046] In addition to a promoter, an expression cassette and/or a vector may contain one or more other appropriate transcription initiation, termination, enhancer sequences, efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; 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. Examples of suitable polyA sequences include, *e.g.*, SV40, SV50, bovine growth hormone (bGH), human growth hormone, and synthetic polyAs. Examples of suitable enhancers include, *e.g.*, the alpha fetoprotein enhancer, the TTR minimal promoter/enhancer, LSP (TH-binding globulin promoter/alpha1-microglobulin/bikunin enhancer), amongst others. In one embodiment, the expression cassette comprises one or more expression enhancers. In one embodiment, the expression cassette contains two or more expression enhancers. These enhancers may be the same or may differ from one another. For example, an enhancer may include an Alpha mic/bik enhancer. This enhancer may be present in two copies which are located adjacent to one another. Alternatively, the dual copies of the enhancer may be separated by one or more

sequences. In still another embodiment, the expression cassette further contains an intron, e.g., the Promega intron. Other suitable introns include those known in the art, e.g., such as are described in WO 2011/126808. Optionally, one or more sequences may be selected to stabilize mRNA. An example of such a sequence is a modified WPRE sequence, which may be engineered upstream of the polyA sequence and downstream of the coding sequence [*see, e.g.*, MA Zanta-Boussif, et al, Gene Therapy (2009) 16: 605-619].

[00047] These control sequences are “operably linked” to the hOTC gene sequences. 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.

[00048] Recombinant AAV viral vectors are well suited for delivery of the hOTC expression sequences described herein. Such AAV vectors may contain ITRs which are from the same AAV source as the capsid. Alternatively, the AAV ITRs may be from a different AAV source than that which supplies the capsid.

[00049] Where pseudotyped AAV is to be produced, the ITRs in the expression are selected from a source which differs from the AAV source of the capsid. For example, AAV2 ITRs may be selected for use with an AAV capsid having a particular efficiency for targeting liver (*e.g.*, hepatocytes). AAV capsids may be selected from AAV8 [US Patent 7790449; US Patent 7282199] and rh10 [WO 2003/042397] for the compositions described herein. However, other AAV, including, *e.g.*, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, and others such as, *e.g.*, those described in WO 2003/042397; WO 2005/033321, WO 2006/110689; US 7588772 B2, which are incorporated by reference herein] may be used in human subjects.

[00050] In one embodiment, a self-complementary AAV is provided. This viral vector may contain a  $\Delta$ 5' ITR and an AAV 3' ITR. In one example, the viral vector is scAAV2/8.TBG.hOTCco. In another example, the viral vector is scAAV2/rh10.TBG.hOTCco. These vectors both contain the 5'  $\Delta$ ITR from AAV2, the liver-specific TBG promoter, an engineered hOTCco coding sequence of the invention, an SV40 polyA, and the 3' AAV2 ITR in an AAV8 capsid [*see, e.g.*, US Patent No. 8,318,480 B2] or AAV rh10 capsid. The sequence may be selected from engineered hOTC one of SEQ ID NO: 3, 4, 5, 8 or 9. Optionally, the transit sequence of the

engineered hOTC may be substituted with a heterologous transit sequence to provide a chimeric hOTC, which retains the mature hOTCase.

[00051] In another embodiment, a single-stranded AAV viral vector is provided. Such a vector may contain a 5' AAV ITR and a 3' ITR. One example is AAV2/8.TBG.hOTCco, which contains the full-length AAV2 - 5' ITR, the liver-specific TBG promoter, the hOTC coding sequence, a bovine growth hormone polyA, and AAV2 - 3' ITR. Another example is AAV2/8.TBG.hOTCco-.WPRe.bGH, which contains the same vector elements, and additionally contains the woodchuck hepatitis virus post-transcriptional regulatory element (WPRe). In other embodiments, WPRe is absent from constructs to be used *in vivo*. The engineered hOTC sequence (abbreviated herein hOTCco) may be selected from engineered hOTC of one of SEQ ID NO: 3, 4, 5, 8 or 9. Optionally, the transit peptide sequence of the engineered hOTC may be substituted with a heterologous transit sequence to provide a chimeric hOTC, which retains the mature hOTCase.

[00052] Still other promoters may be selected, including tissue specific promoters. Methods for generating and isolating AAV viral vectors suitable for delivery to a subject are known in the art. *See, e.g.* US Published Patent Application No. 2007/0036760 (February 15, 2007), US Patent 7790449; US Patent 7282199; WO 2003/042397; WO 2005/033321, WO 2006/110689; and US 7588772 B2]. The sequences of AAV8 and methods of generating vectors based on the AAV8 capsid are described in US Patent 7,282,199 B2, US 7,790,449, and US 8,318,480, which are incorporated herein by reference. In a one system, a producer cell line is transiently transfected with a construct that encodes the transgene flanked by ITRs and a construct(s) that encodes rep and cap. In a second system, a packaging cell line that stably supplies rep and cap is transiently transfected with a construct encoding the transgene flanked by ITRs. In each of these systems, AAV virions are produced in response to infection with helper adenovirus or herpesvirus, requiring the separation of the rAAVs from contaminating virus. More recently, systems have been developed that do not require infection with helper virus to recover the AAV - the required helper functions (*i.e.*, adenovirus E1, E2a, VA, and E4 or herpesvirus UL5, UL8, UL52, and UL29, and herpesvirus polymerase) are also supplied, *in trans*, by the system. In these newer systems, the helper functions can be supplied by transient transfection of the cells with constructs that encode the required helper functions, or the cells can be engineered to stably contain genes encoding the helper

functions, the expression of which can be controlled at the transcriptional or posttranscriptional level. In yet another system, the transgene flanked by ITRs and rep/cap genes are introduced into insect cells by infection with baculovirus-based vectors. For reviews on these production systems, see generally, *e.g.*, Zhang et al., 2009, "Adenovirus-adenovirus-associated virus hybrid for large-scale recombinant adeno-associated virus production," Human Gene Therapy 20:922-929, the contents of each of which is incorporated herein by reference in its entirety. Methods of making and using these and other AAV production systems are also described in the following U.S. patents, the contents of each of which is incorporated herein by reference in its entirety: 5,139,941; 5,741,683; 6,057,152; 6,204,059; 6,268,213; 6,491,907; 6,660,514; 6,951,753; 7,094,604; 7,172,893; 7,201,898; 7,229,823; and 7,439,065.

[00053] The available space for packaging may be conserved by combining more than one transcription unit into a single construct, thus reducing the amount of required regulatory sequence space. For example, a single promoter may direct expression of a single RNA that encodes two or three or more genes, and translation of the downstream genes are driven by IRES sequences. In another example, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three or more genes separated from one another by sequences encoding a self-cleavage peptide (*e.g.*, T2A) or a protease recognition site (*e.g.*, furin). The ORF thus encodes a single polyprotein, which, either during (in the case of T2A) or after translation, is cleaved into the individual proteins (such as, *e.g.*, transgene and dimerizable transcription factor). It should be noted, however, that although these IRES and polyprotein systems can be used to save AAV packaging space, they can only be used for expression of components that can be driven by the same promoter. In another alternative, the transgene capacity of AAV can be increased by providing AAV ITRs of two genomes that can anneal to form head to tail concatamers.

[00054] Optionally, the hOTC genes described herein may be used to generate viral vectors other than rAAV. Such other viral vectors may include any virus suitable for gene therapy may be used, including but not limited to adenovirus; herpes virus; lentivirus; retrovirus; *etc.* Suitably, where one of these other vectors is generated, it is produced as a replication-defective viral vector.

[00055] 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. Such replication-defective viruses may be adeno-associated viruses (AAV), adenoviruses, lentiviruses (integrating or non-integrating), or another suitable virus source.

[00056] The pharmaceutical compositions described herein are designed for delivery to subjects in need thereof by any suitable route or a combination of different routes. Direct or intrahepatic delivery to the liver is desired and may optionally be performed via intravascular delivery, *e.g.*, via the portal vein, hepatic vein, bile duct, or by transplant. Alternatively, other routes of administration may be selected (*e.g.*, oral, inhalation, intranasal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, and other parental routes). The hOTC delivery constructs described herein may be delivered in a single composition or multiple compositions. Optionally, two or more different AAV may be delivered [*see, e.g.*, WO 2011/126808 and WO 2013/049493]. In another embodiment, such multiple viruses may contain different replication-defective viruses (*e.g.*, AAV, adenovirus, and/or lentivirus). Alternatively, delivery may be mediated by non-viral constructs, *e.g.*, "naked DNA", "naked plasmid DNA", RNA, and mRNA; coupled with various delivery compositions and nano particles, including, *e.g.*, micelles, liposomes, cationic lipid - nucleic acid compositions, poly-glycan compositions and other polymers, lipid and/or cholesterol-based - nucleic acid conjugates, and other constructs such as are described herein. *See, e.g.*, X. Su et al, Mol. Pharmaceutics, 2011, 8 (3), pp 774–787; web publication: March 21, 2011; WO2013/182683, WO 2010/053572 and WO 2012/170930, both of which are incorporated herein by reference, Such non-viral hOTC delivery constructs may be administered by the routes described previously.

[00057] The viral vectors, or non-viral DNA or RNA transfer moieties, can be formulated with a physiologically acceptable carrier for use in gene transfer and gene



therapy applications. In the case of AAV viral vectors, quantification of the genome copies ("GC") may be used as the measure of the dose contained in the formulation. Any method known in the art can be used to determine the genome copy (GC) number of the replication-defective virus compositions of the invention. One method for performing AAV GC number titration is as follows: Purified AAV vector samples are first treated with DNase to eliminate un-encapsidated AAV genome DNA or contaminating plasmid DNA from the production process. The DNase resistant particles are then subjected to heat treatment to release the genome from the capsid. The released genomes are then quantitated by real-time PCR using primer/probe sets targeting specific region of the viral genome (usually poly A signal). 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  $1.0 \times 10^9$  GC to about  $1.0 \times 10^{15}$  GC (to treat an average subject of 70 kg in body weight), and preferably  $1.0 \times 10^{12}$  GC to  $1.0 \times 10^{14}$  GC for a human patient. Preferably, the dose of replication-defective virus in the formulation is  $1.0 \times 10^9$  GC,  $5.0 \times 10^9$  GC,  $1.0 \times 10^{10}$  GC,  $5.0 \times 10^{10}$  GC,  $1.0 \times 10^{11}$  GC,  $5.0 \times 10^{11}$  GC,  $1.0 \times 10^{12}$  GC,  $5.0 \times 10^{12}$  GC, or  $1.0 \times 10^{13}$  GC,  $5.0 \times 10^{13}$  GC,  $1.0 \times 10^{14}$  GC,  $5.0 \times 10^{14}$  GC, or  $1.0 \times 10^{15}$  GC.

[00058] DNA and RNA is generally measured in the nanogram (ng) to microgram ( $\mu$ g) amounts of the nucleic acids. In general, for a treatment in a human preferably dosages of the RNA is the range of 1 ng to 700  $\mu$ g, 1 ng to 500  $\mu$ g, 1 ng to 300  $\mu$ g, 1 ng to 200  $\mu$ g, or 1 ng to 100  $\mu$ g are formulated and administered. Similar dosage amounts of a DNA molecule containing an expression cassette and not delivered to a subject via a viral vector may be utilized for non-viral hOTC DNA delivery constructs.

[00059] Production of lentivirus is measured as described herein and expressed as IU per volume (*e.g.*, mL). IU is infectious unit, or alternatively transduction units (TU); IU and TU can be used interchangeably as a quantitative measure of the titer of a viral vector particle preparation. The lentiviral vector is typically non-integrating. The amount of viral particles is at least about  $3 \times 10^6$  IU, and can be at least about  $1 \times 10^7$  IU, at least about  $3 \times 10^7$  IU, at least about  $1 \times 10^8$  IU, at least about  $3 \times 10^8$  IU, at least about  $1 \times 10^9$  IU, or at least about  $3 \times 10^9$  IU.

[00060] The above-described recombinant vectors may be delivered to host cells according to published methods. The rAAV, preferably suspended in a physiologically compatible carrier, may be administered to a human or non-human mammalian patient.

Suitable carriers may be readily selected by one of skill in the art in view of the indication for which the transfer virus is directed. For example, one suitable carrier includes saline, which may be formulated with a variety of buffering solutions (*e.g.*, phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water. The selection of the carrier is not a limitation of the present invention.

[00061] Optionally, the compositions of the invention may contain, in addition to the rAAV and carrier(s), other conventional pharmaceutical ingredients, such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin.

[00062] The viral vectors described herein may be used in preparing a medicament for delivering ornithine transcarbamylase to a subject (*e.g.*, a human patient) in need thereof, supplying functional hOTCase to a subject, and/or for treating ornithine transcarbamylase deficiency. A course of treatment may optionally involve repeat administration of the same viral vector (*e.g.*, an AAV8 vector) or a different viral vector (*e.g.*, an AAV8 and an AAVrh10). Still other combinations may be selected using the viral vectors and non-viral delivery systems described herein.

[00063] In another embodiment, the nucleic acid sequences described herein may be delivered via a non-viral route. For example, a hOTC sequence may be via a carrier system for expression or delivery in RNA form (*e.g.*, mRNA) using one of a number of carrier systems which are known in the art. Such carrier systems include those provided by commercial entities, such as PhaseRx' so-called "SMARTT" technology. These systems utilize block copolymers for delivery to a target host cell. *See, e.g.*, US 2011/0286957 entitled, "Multiblock Polymers", published November 24, 2011; US 2011/0281354, published November 17, 2011; EP2620161, published July 31, 2013; and WO 2015/017519, published Feb 5, 2015,. *See, also*, S. Uchida *et al.*, (Feb 2013) PLoS ONE 8(2): e56220. Still other methods involve generating and injecting synthetic dsRNAs [*see* Soutschek *et al.* Nature (2004) 432(7014): 173-8; *see also* Morrissey *et al.* Hepatol. (2005) 41(6): 1349-56], local administration to the liver has also been demonstrated by injecting double stranded RNA directly into the circulatory system surrounding the liver using renal vein catheterization. [*See* Hamar *et al.* PNAS (2004)

101(41): 14883-8.]. Still other systems involve delivery of dsRNA and particularly siRNA using cationic complexes or liposomal formulations [*see, e.g.,* Landen *et al.* Cancer Biol. Ther. (2006) 5(12); *see also* Khoury *et al.* Arthritis Rheumatol. (2006) 54(6): 1867-77. Other RNA delivery technologies are also available, *e.g.,* from Veritas Bio [*see, e.g.,* US 2013/0323001, Dec 23, 2010, "In vivo delivery of double stranded RNA to a target cell" (cytosolic content including RNAs, *e.g.,* mRNA, expressed siRNA/shRNA/miRNA, as well as injected/introduced siRNA/shRNA/miRNA, or possibly even transfected DNA present in the cytosol packaged within exovesicles and be transported to distal sites such as the liver)]. Still other systems for *in vivo* delivery of RNA sequences have been described. *See, e.g.,* US 2012/0195917 (Aug 2, 2012) (5'-cap analogs of RNA to improve stability and increase RNA expression), WO 2013/143555A1, Oct 3, 2013, and/or are commercially available (BioNTech, Germany; Valera (Cambridge, MA); Zata Pharmaceuticals).

[00064] Thus, in one embodiment, the invention provides an engineered hOTC mRNA of the mature sequence (at least about nt 99 - 1098) or the full-length of SEQ ID NO: 10, 11, 12, 13, or a sequence having at least 97% to 99% identity thereto, in a composition for delivery of double-stranded or single stranded RNA which results in expression of the mature hOTCase in a target host cell, *e.g.,* a liver cell.

[00065] The kinetics of the composition described herein which contain mRNA (delivered directly, as compared to transcribed from a DNA delivery molecule) are particularly well suited for use in subjects in acute crisis, as expression of the hOTCase from the mRNA may be seen within a period of several hours. In order to avoid rapid clearance of the RNA, it is modified as described herein (*e.g.,* using a cap or a modified base), such that its effects may be retained for over 24 hours, over 48 hours, or up to about 3 days (about 72 hours). It may be desirable to co-administer an mRNA directly as described herein and co-administer at the same or substantially the same time, a DNA or viral vector-based hOTC composition as defined herein. Thus, a subject may receive immediate treatment, and at such time as the mRNA-mediated expression begins to wane, the longer-term hOTC expression conferred by a viral vector - mediated expression begins to take effect. Alternatively, a subject may receive a second administration of an mRNA-based composition as defined herein. The mRNA compositions described herein may be used in other therapeutic regimens or methods, including those involving OTCD patients who are not in acute crisis.

[00066] The compositions according to the present invention may comprise a pharmaceutically acceptable carrier, such as defined above. In one embodiment, the composition comprises a block copolymer associated with a hOTC polynucleotide as described herein. The block copolymers may form a micelle, such that the micelle comprises a plurality of block copolymer.

[00067] Typically, such a composition contains a nucleic acid molecule comprising the mRNA sequence corresponding to the hOTC sequence encoding the mature hOTCase (at least about nt 99 to 1098) or the full-length of any of SEQ ID NO: 10, 11, 12, 13. In addition, this nucleic acid molecule may include the 5' untranslated region (UTR), also known as the leader sequence or leader RNA, and one or more of an optional intron(s), an optional exon(s), an optional a Kozak sequence, an optional WPRE, and a polyA, and the 3' UTR flanking the coding sequences. Suitable leader sequences include those discussed above in connection with the hOTC DNA sequences, which discussion is incorporated by reference herein. Examples of sources of suitable leader sequences, other than the native hOTC leader sequences, or those corresponding to FIG 2, FIG3 or FIG4, or FIG 5 are discussed above. Similarly, sources of suitable introns, polyA, and Kozak sequences are discussed above and are applicable to the delivery of the corresponding RNA sequences discussed in the present paragraph. Further, various modifications to the RNA may be generated, e.g., a modified 5' cap structure may be engineered into the construct in order to avoid rapid clearance of the mRNA in vivo, or for another desired reason. Methods of generating such 5' cap structures is known to those of skill in the art. *See, e.g.*, US 2012/0195917 and WO 2013/143555A1, Oct 3, 2013. In addition, modified nucleotides can be used to make mRNA *in vitro*, like pseudouridine. Also RNA may be dosed repetitively, or subject can be dosed first with mRNA to manage neonatal crises followed up by viral vector-mediated delivery (*e.g.*, AAV) for long term therapy and to prevent fibrosis/cirrhosis and/or hepatocellular carcinoma.

[00068] mRNA can be synthesized from the hOTC DNA sequences described herein, using techniques that are well known in the art. For example, Cazenave C, Uhlenbeck OC, RNA template-directed RNA synthesis by T7 RNA polymerase. *Proc Natl Acad Sci U S A.* 1994 Jul 19;91(15):6972-6, describe the use of the T7 RNA polymerase for generating RNA from cDNA or RNA templates. *See also*, Wichlacz A1, Legiewicz M, Ciesiolka J., Generating in vitro transcripts with homogenous 3' ends using

trans-acting antigenomic delta ribozyme., Nucleic Acids Res. 2004 Feb 18;32(3):e39; Krieg PA, Melton DA., Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs, Nucleic Acids Res. 1984 Sep 25;12(18):7057-70; and Rio, D. C., et al. RNA: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2011, 205-220. Each of these references is incorporated herein by reference. In addition, kits and protocols for generating mRNA are available commercially including, without limitation, the Riboprobe® *In Vitro* Transcription System (Promega Corp.); RiboMAX™ Large Scale RNA Production Systems (Promega Corp.); MAXIscript Kit (Ambion); MEGIscript Kit (Ambion); MessageAmp™ aRNA Kit (Ambion); mMESSAGE mMACHINE® Kits (Ambion); and HiScribe™ T7 High Yield RNA Synthesis Kit (New England Biolabs® Inc.). Custom RNA can also be generated commercially from companies including, without limitation, TriLink Biotechnologies; bioSYNTHESIS; GE Dharmacon; and IBA Lifesciences.

[00069] The hOTC DNA sequences described herein can be generated *in vitro* and synthetically, using techniques well known in the art. For example, the PCR-based accurate synthesis (PAS) of long DNA sequence method may be utilized, as described by Xiong et al, PCR-based accurate synthesis of long DNA sequences, Nature Protocols 1, 791 - 797 (2006). A method combining the dual asymmetrical PCR and overlap extension PCR methods is described by Young and Dong, Two-step total gene synthesis method, Nucleic Acids Res. 2004; 32(7): e59. See also, Gordeeva et al, J Microbiol Methods. Improved PCR-based gene synthesis method and its application to the *Citrobacter freundii* phytase gene codon modification. 2010 May;81(2):147-52. Epub 2010 Mar 10; see, also, the following patents on oligonucleotide synthesis and gene synthesis, Gene Seq. 2012 Apr;6(1):10-21; US 8008005; and US 7985565. Each of these documents is incorporated herein by reference. In addition, kits and protocols for generating DNA via PCR are available commercially. These include the use of polymerases including, without limitation, Taq polymerase; OneTaq® (New England Biolabs); Q5® High-Fidelity DNA Polymerase (New England Biolabs); and GoTaq® G2 Polymerase (Promega). DNA may also be generated from cells transfected with plasmids containing the hOTC sequences described herein. Kits and protocols are known and commercially available and include, without limitation, QIAGEN plasmid kits; Chargeswitch® Pro Filter Plasmid Kits (Invitrogen); and GenElute™ Plasmid Kits (Sigma Aldrich). Other techniques useful herein include sequence-specific isothermal

amplification methods, that eliminate the need for thermocycling. Instead of heat, these methods typically employ a strand-displacing DNA polymerase, like Bst DNA Polymerase, Large Fragment (New England Biolabs), to separate duplex DNA. DNA may also be generated from RNA molecules through amplification via the use of Reverse Transcriptases (RT), which are RNA-dependent DNA Polymerases. RTs polymerize a strand of DNA that is complimentary to the original RNA template and is referred to as cDNA. This cDNA can then be further amplified through PCR or isothermal methods as outlined above. Custom DNA can also be generated commercially from companies including, without limitation, GenScript; GENEWIZ®; GeneArt® (Life Technologies); and Integrated DNA Technologies.

[00070] The term “expression” is used herein in its broadest meaning and comprises the production of RNA or of RNA and protein. With respect to RNA, the term “expression” or “translation” relates in particular to the production of peptides or proteins. Expression may be transient or may be stable.

[00071] The term “translation” in the context of the present invention relates to a process at the ribosome, wherein an mRNA strand controls the assembly of an amino acid sequence to generate a protein or a peptide.

[00072] According to the present invention, a “therapeutically effective amount” of the hOTC is delivered as described herein to achieve a desired result, *i.e.*, treatment of OTC deficiency or one or more symptoms thereof. As described herein, a desired result includes reducing orotic acid levels, reducing hyperammonemia and/or minimizing or eliminating one or more of the neurophysical complications including developmental delay, learning disabilities, intellectual disability, attention deficit hyperactivity disorder, and executive function deficits. Treatment may include treatment of subjects having severe neonatal-onset disease (males or, more rarely, females), and late-onset (partial) disease in males and females, which may present from infancy to later childhood, adolescence, or adulthood. In certain embodiments, the invention provides a method of treating and/or preventing fibrosis and/or cirrhosis in subjects, particularly those late-onset heterozygous subjects by administering hOTC as described herein. In one embodiment, therapeutic goals for OTC deficiency are to maintain plasma ammonia at less than  $<80 \mu\text{mol/L}$ , plasma glutamine  $<1,000 \mu\text{mol/L}$ , argininemia  $80\text{-}150 \mu\text{mol/L}$  and branched chain amino acids within the normal range. However, other therapeutic endpoints may be selected by the treating physician.

[00073] In yet another embodiment, the invention provides a method of rescuing and/or treating a neonatal subject OTCD comprising the step of delivering a hOTC gene to the liver of a newborn subject (*e.g.*, a human patient). This method may utilize any nucleic acid sequence encoding a functional hOTCase, whether a synthetic hOTC as described herein or a wild-type hOTC, or a hOTC from another source, or a combination thereof. In one embodiment, neonatal treatment is defined as being administered a hOTC as described herein within 8 hours, the first 12 hours, the first 24 hours, or the first 48 hours of delivery. In another embodiment, particularly for a primate, neonatal delivery is within the period of about 12 hours to about 1 week, 2 weeks, 3 weeks, or about 1 month, or after about 24 hours to about 48 hours. To address dilution due to the rapid turnover of liver cells in a growing mammal (*e.g.*, a non-human or human primate), neonatal therapy is desirably followed by readministration at about 3 months of age, about 6 months, about 9 months, or about 12 months. Optionally, more than one readministration is permitted. Such readministration may be with the same type of vector, a different viral vector, or via non-viral delivery. In one embodiment, an RNA based delivery system for functional hOTC is used to stabilize a subject (*e.g.*, a human patient) in crisis, followed by delivery of a viral vector mediated delivery of a functional hOTC. In another embodiment, initial therapy involves co-administration of viral and non-viral - mediated hOTC delivery systems. In a further embodiment, the hOTC DNA and RNA constructs may be used alone, or in combination with the standard of care for the patient's diagnosis and condition.

[00074] As described in the working examples herein, the inventors have found that heterozygous OTCD subjects, including those with late onset OTCD, have increased fibrosis and/or microvesicular steatosis throughout the liver. Such liver fibrosis and/or microvesicular steatosis can lead to OTCD-related cirrhosis. Thus, in another embodiment, the invention provides methods of preventing liver fibrosis and/or the associated medical condition OTCD-related cirrhosis by delivering to the subject (*e.g.*, a human patient) a hOTC. This aspect of the invention may utilize a viral or non-viral delivery system. The nucleic acid expression cassette may contain a synthetic hOTC DNA or RNA as provided herein, or another suitable sequence which expresses functional hOTCase. In one embodiment, a method of treating and/or preventing liver fibrosis, microvesicular steatosis, and/or OTCD-related cirrhosis is provided which involves delivering OTCase to a subject having OTCD. The subject may be a human

patient. In one embodiment, the patient is heterozygous and has late onset OTCD. The patient may have been previously untreated for OTCD, or may have received other conventional treatments. At present, there is no existing standard of care for OTCD, but rather symptoms are managed, *e.g.*, through discontinuation of protein intake, compensatory increases in dietary carbohydrates and lipids, hemodialysis for comatose patients with extremely high blood levels; and/or intravenous administration of sodium benzoate, arginine, and/or sodium phenylacetate. The US FDA has approved glycerol phenylbutyrate (Ravicti®) for long-term management of some urea cycle disorders for patients aged 2 years and older; this drug helps rid the body of ammonia and is intended for patients who cannot be managed by a protein-restricted diet or amino acid supplements alone. In one embodiment, treatment of the patient (*e.g.*, a first injection) is initiated prior to the first year of life. In another embodiment, treatment is initiated after the first 1 year, or after the first 2 to 3 years of age, after 5 years of age, after 11 years of age, or at an older age.

[00075] In one embodiment, the method of the invention provides for treating and/or reversing liver fibrosis and/or OTCD-related cirrhosis by delivering to the subject a functional OTCase which is encoded by an engineered DNA of SEQ ID NO: 1, 3, 4, 5, 8 or 9, or a chimeric DNA as defined herein. Delivery of the DNA may be mediated by a viral vector containing the engineered in an expression cassette, or by a non-viral delivery system, either of which mediates expression of functional OTCase in the liver cells of the subject. In another embodiment, the subject is administered an engineered RNA of SEQ ID NO: 10, 11, 12 or 13, or a chimeric RNA as defined herein. Delivery of the RNA may be mediated by a viral vector containing the engineered RNA in an expression cassette, or by a non-viral delivery system, either of which mediates expression of functional OTCase in the liver cells of the subject.

[00076] Heterozygous OTCD subjects have an increased risk of developing hepatocellular carcinoma (HCC). See, *e.g.*, JM Wilson et al, Molecular Genetics and Metabolism (2012), Mol Genet Metab. 2012 Feb; 105(2): 263–265, Published online 2011 Nov 7. Thus, in another embodiment, the invention provides methods of preventing treating and/or preventing HCC by delivering to the subject (*e.g.*, a human patient) a hOTC. This aspect of the invention may utilize a viral or non-viral delivery system. The nucleic acid expression cassette may contain a synthetic hOTC DNA or RNA as provided herein, or another suitable sequence which and expresses functional hOTCase. The



patient may have been previously untreated for OTCD, or may have received other conventional treatments, i.e., standard of care. In one embodiment, treatment of the patient (e.g., a first injection) is initiated prior diagnosis with HCC. In another embodiment, treatment of the patient is initiated following HCC diagnosis. Optionally, treatment involves co-administration with sorafenib (commercially available as Nexavar®), or being used in conjunction with chemoembolization, radiation, thermal ablation, precutaneous ethanol injection, targeted therapy (e.g., anti-angiogenesis drugs), hepatic arterial infusion of anti-cancer drugs, immunotherapy, or with surgical options including, e.g., resection, cryosurgery, and liver transplant. When used for treatment of HCC, it may be desirable to select a non-integrating delivery system (e.g., direct RNA delivery, or non-integrating viruses such as adenoviruses or non-integrating lentiviruses) for delivery of a synthetic hOTC DNA or RNA as described herein.

[00077] By “functional OTC”, is meant a gene which encodes the wild-type OTCase such as characterized by SEQ ID NO: 2 or another OTCase which provides at least about 50%, at least about 75%, at least about 80%, at least about 90%, or about the same, or greater than 100% of the biological activity level of the wild-type human ornithine transcarbamylase enzyme, which may be characterized by the sequence of SEQ ID NO:2 or a natural variant or polymorph thereof which is not associated with disease. More particularly, as heterozygous patients may have as low an OTCase functional level as about 50% or lower, effective treatment may not require replacement of OTCase activity to levels within the range of “normal” or non-OTCD patients. Similarly, patients having no detectable amounts of OTCase may be rescued by delivering OTCase function to less than 100% activity levels, and may optionally be subject to further treatment subsequently. As described herein, the gene therapy described herein, whether viral or non-viral, may be used in conjunction with other treatments, i.e., the standard of care for the subject’s (patient’s) diagnosis.

[00078] In one embodiment, such a functional OTCase has a sequence which has about 95% or greater identity to the mature protein (i.e., about the last 322 amino acids) or full-length sequence of SEQ ID NO: 2, or about 97% identity or greater, or about 99% or greater to SEQ ID NO: 2 at the amino acid level. Such a functional OTCase may have also encompass natural polymorphs which are not associated with any disease (e.g., F101, L111, and/or W1193-194 of SEQ ID NO:2). Identity may be determined by preparing an alignment of the sequences and through the use of a variety of algorithms and/or

computer programs known in the art or commercially available [*e.g.*, BLAST, ExPASy; ClustalO; FASTA; using, *e.g.*, Needleman-Wunsch algorithm, Smith-Waterman algorithm].

[00079] A variety of assays exist for measuring OTC expression and activity levels *in vitro*. See, *e.g.*, X Ye, et al, 1996 Prolonged metabolic correction in adult ornithine transcarbamylase-deficient mice with adenoviral vectors. J Biol Chem 271:3639–3646) or *in vivo*. For example, OTC enzyme activity can be measured using a liquid chromatography mass spectrometry stable isotope dilution method to detect the formation of citrulline normalized to [1,2,3,4,5-<sup>13</sup>C<sub>5</sub>] citrulline (98% <sup>13</sup>C). The method is adapted from a previously developed assay for detection of N-acetylglutamate synthase activity [Morizono H, et al, Mammalian N-acetylglutamate synthase. Mol Genet Metab. 2004;81(Suppl 1):S4–11.]. Slivers of fresh frozen liver are weighed and briefly homogenized in buffer containing 10 mM HEPES, 0.5 % Triton X-100, 2.0 mM EDTA and 0.5 mM DTT. Volume of homogenization buffer is adjusted to obtain 50 mg/ml tissue. Enzyme activity is measured using 250 µg liver tissue in 50 mM Tris-acetate, 4 mM ornithine, 5 mM carbamyl phosphate, pH 8.3. Enzyme activity is initiated with the addition of freshly prepared 50 mM carbamyl phosphate dissolved in 50 mM Tris-acetate pH 8.3, allowed to proceed for 5 minutes at 25 °C and quenched by addition of an equal volume of 5 mM <sup>13</sup>C<sub>5</sub>-citrulline in 30%TCA. Debris is separated by 5 minutes of microcentrifugation, and the supernatants are transferred to vials for mass spectroscopy. Ten µL of sample are injected into an Agilent 1100 series LC-MS under isocratic conditions with a mobile phase of 93% solvent A (1 ml trifluoroacetic acid in 1 L water):7% solvent B (1ml trifluoroacetic acid in 1L of 1:9 water/acetonitrile). Peaks corresponding to citrulline [176.1 mass:charge ratio (*m/z*)] and <sup>13</sup>C<sub>5</sub>-citrulline (181.1 *m/z*) are quantitated, and their ratios are compared to ratios obtained for a standard curve of citrulline run with each assay. Samples are normalized to either total liver tissue or to protein concentration determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Other assays, which do not require liver biopsy, may also be used. One such assay is a plasma amino acid assays in which the ratio of glutamine and citrulline is assessed and if glutamine is high (>800 microliters/liter) and citrulline low (*e.g.*, single digits), a urea cycle defect is suspected. Plasma ammonia levels can be measured and a concentration of about 100 micromoles per liter is indicative of OTCD. Blood gases can be assessed if a patient is hyperventilating; respiratory alkalosis is frequent in OTCD.

Orotic acid in urine, e.g., greater than about 20 micromoles per millimole creatine is indicative of OTCD, as is elevated urinary orotate after allopurinol challenge test. Diagnostic criteria for OTCD have been set forth in Tuchman et al, 2008, Urea Cycle Disorders Consortium (UCDC) of the Rare Disease Clinical Research Network (RDCRN). Tuchman M, et al., Consortium of the Rare Diseases Clinical Research Network. Cross-sectional multicenter study of patients with urea cycle disorders in the United States. *Mol Genet Metab.* 2008;94:397–402, which is incorporated by reference herein. See, also, <http://www.ncbi.nlm.nih.gov/books/NBK154378/>, which provides a discussion of the present standard of care for OTCD.

[00080] 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.

[00081] 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.

[00082] As used herein, the term "about" means a variability of 10 % ( $\pm 10\%$ ) from the reference given, unless otherwise specified.

[00083] The term "regulation" or variations thereof as used herein refers to the ability of a compound of formula (I) to inhibit one or more components of a biological pathway.

[00084] A "subject" is a mammal, e.g., a human, mouse, rat, guinea pig, dog, cat, horse, cow, pig, or non-human primate, such as a monkey, chimpanzee, baboon or gorilla.

[00085] As used herein, "disease", "disorder" and "condition" are used interchangeably, to indicate an abnormal state in a subject.

[00086] 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.

[00087] The following examples are illustrative only and are not intended to limit the present invention.

[00088] Example 1 – scAAV Vectors Containing hOTC

[00089] pAAVsc.TBG.hOTCwt and pAAVsc.TBG.hOTCco-LW4 were constructed by replacing the mOTC coding sequencing with wild-type (WT) hOTC (hOTCwt) or hOTCcoLW cDNA, respectively, in a plasmid derived from the previously described pAAVsc.TBG.mOTC1.3 with the intron disrupted [Moscioni D, et al, “Long-term correction of ammonia metabolism and prolonged survival in ornithine transcarbamylase-deficient mice following liver-directed treatment with adeno-associated viral vectors”, Mol Ther. 2006;14:25–33; Cunningham SC, et al, “AAV2/8-mediated correction of OTC deficiency is robust in adult but not neonatal Spf(ash) mice”, Mol Ther. 2009;17:1340–1346; Wang L, et al., “Sustained correction of OTC deficiency in spf<sup>ash</sup> mice using optimized self-complementary AAV2/8 vectors”, Gene Ther. 2012 Apr; 19(4):404-10, Epub 2011 Aug 18].

[00090] The scAAV2/8.TBG.hOTCco-LW4 contains an AAV2 3' ITR and a 5' ITR with a deletion in the D-sequence and trs (terminal resolution site), a TBG promoter, the hOTCco-LW4 gene, and a 137 bp SV40 polyA. The two vector preps (AAV2/8sc.TBG.hOTCwt and AAV2/8sc.TBG.hOTCco-LW4) used in the initial comparison study were purified by two rounds of cesium chloride gradient centrifugation, as previously described [Wang L, et al, Systematic evaluation of AAV vectors for liver directed gene transfer in murine models. Mol Ther. 2010;18:118–125]. Vectors used in the rest of the study were produced by a scaled production method based on polyethylenimine (PEI) transfection and purified from supernatant or total lysate by iodixanol gradient centrifugation as described [Lock M, et al, Hum Gene Ther. 2010;21:1259–1271]. Genome titers [genome copies (GC)/ml] of AAV vectors were determined by real-time PCR using primer and probe sets targeting the TBG promoter (forward primer 5'-AACTGCCAATTCCACTGCTG-3' [SEQ ID NO: 14], reverse primer 5'-CCATAGGCAAAGCACCAAGA-3' [SEQ ID NO:15 ], probe 6FAM-TTGCCCAATAGTGAGAACTTTTCCTGC [SEQ ID NO: 16] –TAMRA), and using a linearized plasmid as the standard. The forward primer is located 400bp downstream of the 5' closed hairpin. Fagone et al [Systemic errors in quantitative polymerase chain reaction titration of self-complementary adeno-associated viral vectors and improved over

alternative methods, Hum Gene Ther Methods. 2012 Feb;23(1):1-7.] recently reported that the quantitative PCR (Q-PCR) method could significantly underestimate the titer of scAAV vectors, especially when the PCR primers were close to the closed hairpin of the scAAV vector. The titer of one lot of AAV2/8sc.TBG.hOTCco-LW4 vector using a primer and probe set targeting the polyA region (1900bp downstream of the 5' closed hairpin), and the genome titer was 1.1-fold of the original titer, which was within the intra-assay error of Q-PCR.

[00091] OTC protein expression levels and OTC activity were evaluated in the liver of  $\text{spf}^{\text{ash}}$  mice 14 days after i.v. injection of  $1 \times 10^{11}$  GC of AAV2/8sc.TBG.hOTCwt or AAV2/8sc.TBG.hOTCco-LW4 vectors. The  $\text{spf}^{\text{ash}}$  mice are a model for late onset OTC disease in humans. All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania.  $\text{Spf}^{\text{ash}}$  mice were maintained at the Animal Facility of the Translational Research Laboratories at the University of Pennsylvania as described previously. Three to six months old  $\text{spf}^{\text{ash}}$  mice and their normal littermates were used in the studies. Vectors were administered by intravenous (i.v.) injection via the tail vein. The extent of gene transfer based on resident vector genomes was not statistically different between the two groups. Urine samples were collected before and at various time points after vector treatment for orotic acid analysis as previously described [Moscioni D, *et al*, Long-term correction of ammonia metabolism and prolonged survival in ornithine transcarbamylase-deficient mice following liver-directed treatment with adeno-associated viral vectors. Mol Ther. 2006;14:25–33].

[00092] Western blot analysis to detect hOTC expression in liver lysate was performed as previously described [Wang L, *et al*, 2012, epub 2011)]. The primary antibody to detect hOTC was a custom rabbit polyclonal antibody provided by Hiroki Morizono's laboratory at the Children's National Medical Center. Liver lysates (10  $\mu\text{g}/\text{lane}$ ) were also blotted and probed by an anti-tubulin antibody (Abcam, Cambridge, MA). Western analysis demonstrated 100-fold higher expression of hOTC from the hOTCco-LW4 vector as compared to the hOTCwt vector, reaching levels in slight excess of those seen in WT mice.

[00093] OTC enzyme activity was measured using a liquid chromatography mass spectrometry stable isotope dilution method to detect the formation of citrulline normalized to [1,2,3,4,5- $^{13}\text{C}_5$ ] citrulline (98%  $^{13}\text{C}$ ). The method is adapted from a

previously developed assay for detection of N-acetylglutamate synthase activity [Morizono H, et al, Mammalian N-acetylglutamate synthase. Mol Genet Metab. 2004;81(Suppl 1):S4–11.]. Slivers of fresh frozen liver were weighed and briefly homogenized in buffer containing 10 mM HEPES, 0.5 % Triton X-100, 2.0 mM EDTA and 0.5 mM DTT. Volume of homogenization buffer was adjusted to obtain 50 mg/ml tissue. Enzyme activity was measured using 250 µg liver tissue in 50 mM Tris-acetate, 4 mM ornithine, 5 mM carbamyl phosphate, pH 8.3. Enzyme activity was initiated with the addition of freshly prepared 50 mM carbamyl phosphate dissolved in 50 mM Tris-acetate pH 8.3, allowed to proceed for 5 minutes at 25 °C and quenched by addition of an equal volume of 5 mM <sup>13</sup>C<sub>5</sub>-citrulline in 30%TCA. Debris was separated by 5 minutes of microcentrifugation, and the supernatants were transferred to vials for mass spectroscopy. Ten µL of sample was injected into an Agilent 1100 series LC-MS under isocratic conditions with a mobile phase of 93% solvent A (1 ml trifluoroacetic acid in 1 L water):7% solvent B (1ml trifluoroacetic acid in 1L of 1:9 water/acetonitrile). Peaks corresponding to citrulline [176.1 mass:charge ratio (m/z)] and <sup>13</sup>C<sub>5</sub>-citrulline (181.1 m/z) were quantitated, and their ratios were compared to ratios obtained for a standard curve of citrulline run with each assay. Samples were normalized to either total liver tissue or to protein concentration determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

[00094] The vector carrying engineered hOTC cDNA termed herein LW4 (FIG. 4) was found to improve expressed hOTC protein levels by 100-fold. An assessment of OTC enzyme activity generally correlated with the OTC Western blot experiments although OTC protein was more elevated than OTC enzyme activity when compared to endogenous OTC. When subtracting the background activity levels in the *spf*<sup>ash</sup> mice, the hOTCco-LW4 resulted in over 33-fold higher activity than the hOTCwt. Sustained and dose-correlated hOTC expression and activity levels were observed in the treated *spf*<sup>ash</sup> mice. Compared to a previously described murine OTC vector which differed mainly in the cDNA, the vector carrying the hOTCco-LW4 vector was about 10-fold more potent.

[00095] The illustrative vector carrying the modified hOTCco-LW4 (FIG. 4) provided high level of transduction, as measured by OTC histological assays, throughout a broad range of doses. Between doses  $1 \times 10^{11}$  GC and  $3 \times 10^9$  GC, transduction efficiency, as measured by histochemical staining, varied between 50-70%. At the lowest dose of  $1 \times 10^9$  GC, 40% of the liver areas were positive by OTC histochemical staining. The lack

of a clear dose effect by histochemistry and immunostaining could be due to the fact that codon optimization significantly improved hOTC expression in the transduced hepatocytes. This leads to improved sensitivity to detect transduced cells with low vector genome copies. Transduction could be saturated with high vector doses ( $1 \times 10^{11}$  -  $1 \times 10^{10}$  GC), and therefore transduction efficiency measured by *in situ* detection methods would not discriminate between low and high dose groups in contrast to OTC enzyme activity on liver lysates measured by mass spectrometry.

[00096] A further study was performed in which neonatal expression of hOTC was assessed in  $\text{spf}^{\text{ash}}$  mice, injected on day 1 of life, using the scAAV2/8.TBG.hOTCco at a dose of  $5 \times 10^{10}$  GC/pup injected via the temporal vein. Robust expression was detected at 24 and 48 hours. Additional studies were performed using doses of  $1 \times 10^{11}$ ,  $3 \times 10^{10}$ , and  $1 \times 10^{10}$ , and assessed for 12 weeks. Over the 16 week period of the study, a reduction in the initial robust expression levels was observed at each of the doses. This is believed to be due to dilution, i.e., a natural result of the proliferation of liver cells in growing animals. Thus, while initial restoration of OTC liver activity is observed following neonatal gene transfer in  $\text{spf}^{\text{ash}}$  mice, this result is temporary, with OTC activity dropping from about 1000% of wild-type (wt) levels at about 1 week, to about 50% of wt levels at 4 weeks, to about 10% of wt levels at 12 weeks ( $1 \times 10^{11}$  GC level); or about 500% of wt levels at week 1, to about 20% of wt levels, or about 10% of wt levels at week 1 ( $3 \times 10^{10}$  GC dose) or about 200% wt levels at week 1, to about 10% wt levels at week 4 ( $1 \times 10^{10}$  GC dose). In one study, using animals receiving the first injection of  $3 \times 10^{10}$  GC at day 1, animals were injected with a second AAV vector carrying the hOTCco gene (scAAVrh10.hOTCco;  $1.8 \times 10^{10}$  GC) at week 4. As a control, one group of animals received no readministration and one group received only the second vector at 4 weeks. Readministration of the AAV.hOTCco resulted in restoration of liver OTC activity.

[00097] Further studies were designed to assess the ability to rescue OTC-KO pups by neonatal gene therapy, both short-term and long-term.

[00098] Example 2 - Production of scAAV Vectors having codon optimized sequences

[00099] A. scAAV8.TBG.hOTC-co

[000100] Plasmids containing a codon optimized hOTC sequence of SEQ ID NO: 3, 4, 5, 9 or 10, respectively, are cloned as described in Example 1 by replacing the mOTC coding sequencing with hOTCco in a plasmid derived from the previously described pAAVsc.TBG.mOTC1.3 with the intron. The resulting plasmid pAAVsc.TBG.hOTCco is cloned into an AAV8 capsid [Gao et al, PNAS USA, 2002, 99:11854-11859] using conventional techniques.

[000101] B. scAAVrh10.TBG.hOTC-co

[000102] Plasmids containing a codon optimized hOTC sequence of SEQ ID NO: 3, 4, 5, 9 or 10, respectively, are cloned as described in Example 1 by replacing the mOTC coding sequencing with hOTCco in a plasmid derived from the previously described pAAVsc.TBG.mOTC1.3 with the intron. The resulting plasmids pAAVsc.TBG.hOTCco are cloned into a AAVrh10 capsid [Gao et al, PNAS USA, 2002, 99:11854-11859] using conventional techniques.

[000103] Example 3 - Production of ssAAV Vectors having codon optimized sequences

[000104] ssAAV2/8.LSP1.hOTC-co

Plasmids containing the codon optimized hOTCco sequences are cloned as described by replacing the mOTC coding sequencing of the pLSP1mOTC plasmid [Cunningham et al, Mol Ther, 2009, 17: 1340-1346] with the corresponding cDNA sequence of SEQ ID NO:3, 4, 5, 9 or 10. The resulting plasmids pAAVsc.LSP1.hOTCco are cloned into AAV8 capsids to form the corresponding ssAAV2/8.LSP1.hOTC-co vectors using techniques described in Example 1.

[000105] B. ssAAV2/rh10.LSP1.hOTC-co

Plasmids containing the codon optimized hOTCco sequences are cloned as described by replacing the mOTC coding sequencing of the pLSP1mOTC plasmid [Cunningham et al, Mol Ther, 2009, 17: 1340-1346] with the corresponding cDNA sequence of SEQ ID NO:3, 4, 5, 9 or 10. The resulting plasmids pAAVsc.LSP1.hOTCco are cloned into AAV8 capsids to form the corresponding ssAAV2/8.LSP1.hOTC-co vectors using techniques described in Example 1.

[000106] The vectors generated according to Part A or B may be purified by two rounds of cesium chloride gradient centrifugation, buffered-exchanged with PBS, and concentrated using Amicon Ultra 15 centrifugal filter devices-100K (Millipore, Bedford,



MA). Genome titer (GC/ml) of AAV vectors can be determined by real-time PCR using a primer/probe set corresponding to the TBG promoter and linearized plasmid standards. Vectors can be subject to additional quality control tests including sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis for vector purity and Limulus amebocyte lysate (LAL) for endotoxin detection (Cambrex Bio Science, Walkersville, MD, USA).

[000107]      Example 4 - ssAAV8.TBG.hOTCco in Model of Late Onset of OTCD

[000108]      An AAV8 vector was generated using the methods described herein. The vector has packaged therein a 5' AAV2 ITR, a TBG promoter, an intron, a hOTCco, a WPRE element, a bovine growth hormone polyA, and a 3' AAV2 ITR. The expression and kinetics of this vector was compared to a self-complementary AAV8 vector with or without the WPRE element. The results show that the single-stranded constructs with the WPRE element outperformed those vectors lacking the WPRE element; at comparable doses both single-stranded vectors (with and without WPRE) were less robust than the self-complementary vector lacking WPRE in the time points measured.

[000109]      However, the single-stranded vectors may have other desirable properties, *e.g.*, in terms of kinetics, depending upon the age and condition of the patient.

[000110]      Example 5 - Production of Adenovirus Vectors having codon optimized sequences

hOTCco cDNA [SEQ ID NO: 3, 4, 5, 9 and 10] with NotI linkers is cloned downstream of a rat PEPCK promoter to generate pPEPCK-hOTC as described in A. Mian et al, Molecular Therapy, 2004, 10: 492-499 (2004). This plasmid is digested with AscI, and the resultant PEPCK-hOTCco fragment is inserted into the adenoviral backbone plasmid pC4HSU31 to generate the parental plasmids pC4HSU-PEPCK-hOTCco. Plasmid pWPRE is digested with ClaI to release the WPRE, which is then inserted into the MluI site of pPEPCK-hOTC, to generate pPEPCK-hOTCco-WPRE plasmid with their respective hOTCco sequences. The remaining steps to generate the adenoviral plasmid pC4HSU-PEPCK-hOTCco-WPRE are as previously described. All cloning sites are confirmed by DNA sequence analysis. The identity of recombinant

adenoviral plasmids can be confirmed by restriction enzyme digestion with HindIII and BamHI. The adenoviral plasmids are linearized with PmeI before transfection into 293Cre4 cells. Adenoviral vectors are rescued and amplified with 293Cre4 cells and helper virus AdLC8cluc. Suspension 293N3Scre8 cells may be used in the final step of vector production. Purification, quantification by OD260 and viral DNA extraction are performed as described in detail elsewhere [Brunetti-Pierri, N., et al. (2004). Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum. Gene Ther.* 15: 35–46; Ng, P., Parks, R. J. and Graham, F. L. (2002). Preparation of helper-dependent adenoviral vectors. *Methods Mol. Med.* 69: 371–388].

[000111]      **Example 6 - Production of hOTCco Lentiviral Vectors**

[000112]      A.      Replication-defective lentiviral vectors containing the hOTCco sequences provided herein can be produced by replacing the rat OTC gene sequence insert of the plasmid pLenti-GIII-CMV-GFP-2A-Puro [commercially available from Applied Biological Materials (ABM) Inc.; Canada] with the desired hOTCco sequence [SEQ ID NO: 3, 4, 5, 9 and 10]. The viruses are generated according to manufacturer instructions. The ABM system includes an enhancer deletion in the U3 region of 3'ΔLTR to ensure self-inactivation of the lentiviral vector following transduction and integration into the target cell's genomic DNA; contains minimal lentiviral genes necessary for packaging, replication and transduction (Gag/Pol/Rev), derived from different plasmids all lacking packaging signals; further, none of the Gag, Pol, or Rev genes are incorporated into the packaged viral genome, thus making the mature virus replication-incompetent.

[000113]      B.      Replication-defective, non-integrating hOTC Lentiviral Vectors

[000114]              A DNA construct containing a liver specific promoter and the hOTCco DNA of SEQ ID NO: 3, 4, 5, 9 and 10 are engineered into lentivirus vectors which are pseudotyped into sindbis virus E2 enveloped produced as described in US2011/0064763, which is incorporated by reference herein, All vectors contain splice donor, packaging signal (psi), a Rev-responsive element (RRE), splice donor, splice acceptor, central poly-purine tract (cPPT). The WPRE element is eliminated in certain viruses.

[000115]      C.      The hOTCco DNA of SEQ ID NO: 3, 4, 5 9 and 10, is cloned into a lentivirus pseudotyped with a vesicular stomatitis virus (VSV) envelope gene, purchased from InvivoGen (SanDiego, CA) using manufacturer's instructions.

[000116]      Example 7 - Production hOTCco RNA Delivery Systems

RNA may be prepared by *in vitro* transcription from a DNA template or synthesized. The RNA expression cassette is prepared which includes a 5' UTR, an optional intron with splice donor and acceptor sites, an optional Kozak sequence, the hOTC coding sequence provided herein, a polyA, and a 3' UTR using known techniques.

[000117]      A.      A suitable amount of mRNA are incorporated into a lipid-enveloped pH-responsive polymer nanoparticles generated using published techniques.

[X. Su et al, Mol. Pharmaceutics, 2011, 8 (3), pp 774–787; web publication: March 21, 2011].

[000118]      B.      Polymeric nanoparticle formulations with 25 kDa branched polyethyleneimine (PEI) are prepared as follows. When PEI is present, it may be branched PEI of a molecular weight ranging from 10 to 40 kDa, e.g., 25 kDa branched PEI (Sigma #408727). Additional exemplary polymers suitable for the present invention include those described in PCT Publication WO2013182683, the contents of which is hereby incorporated by reference. The required amount of mRNA is diluted just before application in water for injection (Braun, Melsungen) to a total volume of 4 ml and added quickly to 4 ml of an aqueous solution of branched PEI 25 kDa using a pipette at an N/P ratio of 10. The solution is mixed by pipetting up and down.

[000119]      C.      For a lipid-based nanoparticle, a lipid formulation is created using expression cassette containing the hOTCco RNA in a formulation of cK - E12:DOPE:Chol:PEG-DMG2K (relative amounts 50:25:20:5 (mg:mg:mg:mg)) to provide a solution for delivery. The cationic lipid cK -E12 is used (see, e.g., WO 2013/063468), and is combined with dioleoylphosphatidyl-ethanolamine or "DOPE", cholesterol (chol), and polyethylene glycol (PEG) or a PEGylated lipid (PEG-DMG2K) using e formulation methods described in international patent publications WO 2010/053572 and WO 2012/170930, both of which are incorporated herein by reference.

[000120]      Example 8 - hOTCco DNA Delivery Systems

[000121]      A.      Naked Plasmid DNA - The hOTCco sequences [SEQ ID NO: 3, 4, or 5] are engineered as naked plasmid DNA constructs which are delivered to a target

liver cell (e.g., via intravascular administration) and express the human OTC protein in the target cell.

[000122] B. Cationic lipid–DNA complexes - Cationic lipid - DNA complexes are prepared using a suitable amount of an expression cassette containing at least a promoter, an optional intron, an optional Kozak sequences, an hOTCco of SEQ ID NO: 3, 4 or 5, a polyA, and other optional expression control sequences. The promoter may be a liver specific promoter. Alternatively, another non-tissue specific promoter may be selected. For example, a suitable amount of DNA is formulated with a cationic lipid of cK - E12:DOPE:Chol:PEG-DMG2K (relative amounts 50:25:20:5 (mg:mg:mg:mg)) to form a cationic lipid - DNA complex suitable for delivery to a subject. The cationic lipid cK - E12 is used (see, e.g., WO 2013/063468), and is combined with dioleoylphosphatidyl-ethanolamine or "DOPE", cholesterol (chol), and polyethylene glycol (PEG) or a PEGylated lipid (PEG-DMG2K) using e formulation methods described in international patent publications WO 2010/053572 and WO 2012/170930, both of which are incorporated herein by reference.

[000123] Example 9 Long-term Correction of a Neonatal Lethal Form of OTC Deficiency by Multiple Treatments with AAV Vectors of Different Serotypes

[000124] In the current study, the scAAV8.TBG.hOTCcoLW4 prepared as described in Example 1 was used to rescue animals in a mouse model of neonatal (early) onset OTCD. OTC KO mice were generated through the deletion of exons 2-3, and the properties of this mouse characterized in terms of similarity to human patients with null mutations of OTC. In summary, the OTC knockout (KO) model generated in our laboratory through the deletion of exons 2-3 closely mimics the severe neonatal onset form of OTCD in humans. Neonatal male OTC KO pups have elevated plasma ammonia levels due to the absence of OTC expression in the liver, and they inevitably die within 24 hours after birth. Heterozygous females breed normally, have normal plasma ammonia levels, reduced liver OTC enzyme activity, elevated urine orotic acid levels, and in some cases lower body weight compared to wild type (WT) littermates. A single injection of scAAV8-hOTCco vector prepared as described in Example 1 at a dose of  $1-3 \times 10^9$  GC/pup immediately after birth is able to rescue the OTC KO pups and extend the life to

6 weeks. To achieve long-term correction, a group of 4-week-old OTC-KO mice received a second vector administration of scAAVrh10-hOTCco vector, which had been prepared as described in Example 1.

[000125] Over 30 OTC-KO pups retrieved by Cesarean section have been successfully rescued with gene delivery. The rescued pups had lower body weight than their normal littermates and had a transient phenotype of sparse fur and abnormal skin. Most importantly, their plasma ammonia levels were in the normal range. However, the efficacy cannot be maintained beyond 6 weeks due to loss of vector genome during fast liver proliferation in neonatal stage. A second vector administration of scAAVrh10-hOTCco vector in 4-week-old OTC-KO mice is able to further extend their lives to adulthood. The oldest mice have reached 18 months of age. The long-term rescued mice show close to normal levels of plasma ammonia, although urine orotic acid levels in a subset of these mice were significantly elevated. Sirius red staining on liver samples from heterozygous mice of different ages (6, 12, and 18 months old) showed liver fibrosis in aged (18-month old) OTC-KO heterozygous female mice, similar to a liver sample from a 11-year-old OTCD patient.

[000126] Example 10 – Treatment of Late Onset OTC Deficiency (OTCD)

[000127] Two-month old OTC-KO heterozygous mice received a single tail vein injection of a self-complementary AAV8 vector encoding a codon-optimized human OTC gene (SEQ ID NO: 5) at  $1 \times 10^{10}$ ,  $3 \times 10^{10}$ , and  $1 \times 10^{11}$  vector genome copies per mouse. One week following vector treatment, mice in all three vector dose groups had normal urine orotic acid levels which were maintained throughout the study (16 months). Liver samples were harvested from 18 month old treated mice for pathology analysis and compared to age-matched untreated heterozygous mice and WT littermates. All treated mice showed normal liver histology similar to WT, in contrast to the untreated heterozygous animals which had fibrosis throughout the liver. In conclusion, a single injection of AAV8sc-hOTCco vector can prevent liver fibrosis in OTC-KO heterozygous and has great potential for correction of liver fibrosis in OTCD patients.

[000128] Gene therapy vectors described herein are capable of rapid, robust and prolonged gene expression even in mice with a complete lack of OTC. Heterozygous females are able to reproduce and deliver hemizygous male offspring, but these pups die within a day of birth if untreated. Untreated old heterozygous female mice show evidence

of increased fibrosis and microvesicular steatosis, a finding that appears similar to observations in human heterozygous patients. A regimen of gene transfer that is able to rescue affected males has been developed and treated males have survived over 72 weeks.

[000129] Thus, these data demonstrate that liver-specific gene therapy with hOTC can prevent liver fibrosis. These data correlate with in treatment of heterozygous OTC deficient humans, e.g., subjects having late onset of OTCD.

(Sequence Listing Free Text)

The following information is provided for sequences containing free text under numeric identifier <223>.

SEQ ID NO: (containing free text)	Free text under <223>
3	Engineered hOTC
4	Engineered hOTC
5	Engineered hOTC
6	Plasmid pscAAVTBGhOTCLW
8	Engineered hOTC
9	Engineered hOTC
10	Engineered hOTC RNA
11	Engineered hOTC RNA
12	Engineered hOTC RNA
13	Engineered hOTC RNA
14	PCR forward primer
15	PCR reverse primer
16	Probe

[000130] The priority US Provisional Patent Application No. 61/950,157, filed March 9, 2014, and all published documents cited in this specification are incorporated herein by reference. Similarly, the SEQ ID NO 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.

What is Claimed Is:

1. A composition comprising a non-viral carrier and a nucleic acid molecule comprising an engineered sequence encoding human ornithine transcarbamylase (hOTCase) and expression control sequences which direct expression of hOTCase in a liver cell, wherein the hOTC nucleic acid sequence is less than 80% identical to the wild-type hOTC sequence over at least the mature hOTC sequence of SEQ ID NO:1, wherein said hOTC nucleic acid sequence is selected from the nucleic acid sequence comprising SEQ ID NO: 5 or a nucleic acid sequence at least about 96 to about 99 % identical thereto, a nucleic acid sequence selected from SEQ ID NO: 9, or a nucleic acid sequence at least about 96 to about 99 % identical thereto, a nucleic acid sequence comprising SEQ ID NO: 11 or a nucleic acid sequence at least about 96 to about 99 % identical thereto, or a nucleic acid sequence comprising SEQ ID NO: 13 or a nucleic acid sequence at least about 96 to about 99 % identical thereto.

2. The composition according to claim 1, wherein the hOTC nucleic acid sequence is a DNA sequence of SEQ ID NO: 4.

3. The composition according to claim 1, wherein the hOTC nucleic acid sequence is a DNA sequence of SEQ ID NO: 3.

4. The composition according to claim 1, wherein the hOTC nucleic acid sequence is a DNA sequence of SEQ ID NO: 8.

5. The composition according to claim 1, wherein the hOTC is an RNA sequence of SEQ ID NO: 10.

6. The composition according to claim 1, wherein the hOTC is an RNA sequence of SEQ ID NO: 12.
7. The composition according to any one of claims 1 to 6, wherein the hOTC is a chimeric OTC comprises a heterologous transit sequence substituted for the native transit sequence of SEQ ID NO: 5, 6, 7, 8, 9, 10, 11, 12 or 13.
8. The composition according to any one of claims 1 to 7, wherein the expression control sequences further comprise a liver-specific promoter.
9. The composition according to claim 8, wherein the liver-specific promoter is selected from a thyroxin-binding globulin (TBG) promoter or a lymphocyte-specific protein 1 (LSP1) promoter.
10. The composition according to any one of claims 1 to 9, wherein the expression cassette further comprises one or more of an intron, a Kozak sequence, a polyA, and a post-transcriptional regulatory elements.
11. The composition according to any one of claims 1 to 7, wherein non-viral carrier is a plasmid.
12. The composition according to claim 11, wherein the plasmid has the sequence of SEQ ID NO: 6.



13. The composition according to any one of claims 1 to 10, wherein the nucleic acid molecule is formulated in a moiety selected from the group consisting of micelles, liposomes, cationic lipid - nucleic acid compositions, poly-glycan compositions, block copolymers and other polymers, lipid and/or cholesterol-based - nucleic acid conjugates.

14. The composition according to claim 12, wherein the moiety is a nanoparticle.

15. A pharmaceutical composition comprising a carrier and an effective amount of the nucleic acid molecule according to any one of claims 1 to 13.

16. A composition comprising the nucleic acid molecule according to any one of claims 1 to 13 for use in a method of treatment of a disease or medical condition associated with ornithine transcarbamylase deficiency in a human patient.

17. A composition comprising a nucleic acid molecule encoding functional human ornithine transcarbamylase for use in a method in preventing and/or treating fibrosis or cirrhosis in a subject heterozygous for ornithine transcarbamylase deficiency in a human patient.

18. A composition comprising a nucleic acid molecule comprising a nucleic acid sequence encoding functional human ornithine transcarbamylase for use in a method for preventing and/or treating hepatocellular carcinoma in a subject heterozygous for ornithine transcarbamylase deficiency.

19. A method for delivering ornithine transcarbamylase to a subject in need thereof and/or for treating ornithine transcarbamylase deficiency in a subject comprising delivering to a subject in need thereof the composition according to any one of claims 1 to 13.

20. A composition comprising an mRNA according to any one of SEQ ID NO: 10, 11, or 12 in a method for delivering ornithine transcarbamylase to a human patient in acute crisis.

21. A composition comprising a DNA according to any one of SEQ ID NO: 3, 4, or 5 in a method for delivering ornithine transcarbamylase to a human patient following treatment of said patient with RNA.

22. A method for delivering ornithine transcarbamylase to a human patient in acute crisis comprising administering to said patient a composition comprising an mRNA according to any one of SEQ ID NO: 10, 11, or 12.

23. A method for delivering treating a human patient following treatment of the patient with OTC mRNA, said method comprising administering a composition comprising a DNA according to any one of SEQ ID NO: 3, 4, or 5.

24. A method for preventing and/or treating fibrosis or cirrhosis in a subject heterozygous for ornithine transcarbamylase deficiency, said method comprising delivering to a subject a composition comprising a nucleic acid sequence encoding functional human ornithine transcarbamylase.

25. A method for preventing and/or treating hepatocellular carcinoma in a subject heterozygous for ornithine transcarbamylase deficiency, said method comprising delivering a composition comprising a nucleic acid sequence encoding functional human ornithine transcarbamylase.

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FIG. 1A

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1 atgctgttta atctgaggat cctgtttaa acatgcagct ttagaaatgg tcacaacttc
61 atggttcgaa attttcggtg tggacaacca ctacaaaata aagtgcagct gaagggccgt
121 gaccttctca ctctaaaaaa ctttaccgga gaagaaatta aatatatgct atggctatca
181 gcagatctga aatttaggat aaaacagaaa ggagagtatt tgcctttatt gcaagggaag
241 tccttaggca tgatTTTTga gaaaagaagt actcgaacaa gattgtctac agaaacaggc
301 ttTgcacttc tgggaggaca tccttgTTTT cttaccacac aagatattca tttgggtgtg
361 aatgaaagtc tcacggacac ggcccgtgta ttgtctagca tggcagatgc agtattggct
421 cgagtgtata aacaatcaga tttggacacc ctggctaaag aagcatccat cccaattatc
481 aatgggctgt cagatttgta ccctctatc cagatcctgg ctgattacct cacgctccag
541 gaacactata gctctctgaa aggtcttacc ctgagctgga tcggggatgg gaacaatatc
601 ctgcactcca tcatgatgag cgcagcga aaTtcggaatgc accttcaggc agctactcca
661 aagggttatg agccggatgc tagtgtaacc aagtTggcag agcagtatgc caaagagaat
721 ggtaccaagc tgttgctgac aaatgatcca ttggaagcag cgcattggagg caatgtatta
781 attacagaca cttggataag catgggacaa gaagaggaga agaaaaagcg gctccaggct
841 ttccaaggTt accaggTtac aatgaagact gctaaagTtg ctgcctctga ctggacatTt
901 ttacactgct tgcccagaaa gccagaagaa gtggatgatg aagtctTtta ttctcctcga
961 tcactagtgt tcccagaggc agaaaacaga aagtggacaa tcatggctgt catggtgtcc
1021 ctgctgacag attactcacc tcagctccag aagcctaaat tt
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FIG. 1B

Met	Leu	Phe	Asn	Leu	Arg	Ile	Leu	Leu	Asn	Asn	Ala	Ala	Phe	Arg	Asn	1	5	10	15
Gly	His	Asn	Phe	Met	Val	Arg	Asn	Phe	Arg	Cys	Gly	Gln	Pro	Leu	Gln	20	25	30	
Asn	Lys	Val	Gln	Leu	Lys	Gly	Arg	Asp	Leu	Leu	Thr	Leu	Lys	Asn	Phe	35	40	45	
Thr	Gly	Glu	Glu	Ile	Lys	Tyr	Met	Leu	Trp	Leu	Ser	Ala	Asp	Leu	Lys	50	55	60	
Phe	Arg	Ile	Lys	Gln	Lys	Gly	Glu	Tyr	Leu	Pro	Leu	Leu	Gln	Gly	Lys	65	70	75	80
Ser	Leu	Gly	Met	Ile	Phe	Glu	Lys	Arg	Ser	Thr	Arg	Thr	Arg	Leu	Ser	85	90	95	
Thr	Glu	Thr	Gly	Phe	Ala	Leu	Leu	Gly	Gly	His	Pro	Cys	Phe	Leu	Thr	100	105	110	
Thr	Gln	Asp	Ile	His	Leu	Gly	Val	Asn	Glu	Ser	Leu	Thr	Asp	Thr	Ala	115	120	125	
Arg	Val	Leu	Ser	Ser	Met	Ala	Asp	Ala	Val	Leu	Ala	Arg	Val	Tyr	Lys	130	135	140	
Gln	Ser	Asp	Leu	Asp	Thr	Leu	Ala	Lys	Glu	Ala	Ser	Ile	Pro	Ile	Ile	145	150	155	160
Asn	Gly	Leu	Ser	Asp	Leu	Tyr	His	Pro	Ile	Gln	Ile	Leu	Ala	Asp	Tyr	165	170	175	
Leu	Thr	Leu	Gln	Glu	His	Tyr	Ser	Ser	Leu	Lys	Gly	Leu	Thr	Leu	Ser	180	185	190	

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## FIG. 1C

Trp Ile Gly Asp Gly Asn Asn Ile Leu His Ser Ile Met Met Ser Ala  
 195 200 205

Ala Lys Phe Gly Met His Leu Gln Ala Ala Thr Pro Lys Gly Tyr Glu  
 210 215 220

Pro Asp Ala Ser Val Thr Lys Leu Ala Glu Gln Tyr Ala Lys Glu Asn  
 225 230 235 240

Gly Thr Lys Leu Leu Leu Thr Asn Asp Pro Leu Glu Ala Ala His Gly  
 245 250 255

Gly Asn Val Leu Ile Thr Asp Thr Trp Ile Ser Met Gly Gln Glu Glu  
 260 265 270

Glu Lys Lys Lys Arg Leu Gln Ala Phe Gln Gly Tyr Gln Val Thr Met  
 275 280 285

Lys Thr Ala Lys Val Ala Ala Ser Asp Trp Thr Phe Leu His Cys Leu  
 290 295 300

Pro Arg Lys Pro Glu Glu Val Asp Asp Glu Val Phe Tyr Ser Pro Arg  
 305 310 315 320

Ser Leu Val Phe Pro Glu Ala Glu Asn Arg Lys Trp Thr Ile Met Ala  
 325 330 335

Val Met Val Ser Leu Leu Thr Asp Tyr Ser Pro Gln Leu Gln Lys Pro  
 340 345 350

Lys Phe

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FIG. 2

1 atgctgttca acctgcgaat cctgctgaac aatgccgctt ttcggaacgg gcacaatttc  
61 atggtgagga actttcgctg cggacagccc ctccagaaca aggtccagct gaagggcagg  
121 gacctgctga ccctgaaaaa tttcacaggg gaggaatatca agtacatgct gtggctgtca  
181 gccgatctga agttccggat caagcagaag ggcgaatatc tgctctgct ccagggcaaa  
241 agcctgggga tgatcttoga aaagcgcagt actcggacca gactgtcaac agagactgga  
301 ttgcactgc tgggaggaca cccatgtttt ctgaccacac aggacattca tctgggagtg  
361 aacgagtccc tgaccgacac agcacgcgtc ctgagctcca tggctgatgc agtctggct  
421 cgagtctaca aacagtctga cctggatacc ctggccaagg aagcttctat cccaatcatt  
481 aatggcctga gtgacctgta tcaccccatc cagattctgg ccgattacct gaccctccag  
541 gagcattatt ctagtctgaa agggctgaca ctgagctgga ttggggacgg aaacaatatc  
601 ctgcactcca ttatgatgag cgccgccaag tttggaatgc acctccaggc tgcaacccca  
661 aaaggctacg aaccgatgc ctccgtgaca aagctggcag aacagtatgc caaagagaac  
721 ggcactaagc tgctgctgac caatgaccct ctggaggccg ctcacggagg caacgtgctg  
781 atcactgata cctggattag tatgggacag gaggaagaga agaagaagcg gctccaggcc  
841 ttccagggct accaggtgac aatgaaaact gctaaggctc cagccagcga ctggaccttt  
901 ctgcattgcc tgcccagaaa gcctgaagag gtggacgatg aggtcttcta ctcaccaga  
961 agcctggtgt ttctgaagc tgagaatagg aagtggacaa tcatggcagt gatggtcagc  
1021 ctgctgactg attattcccc tcagctccag aaaccaaagt tctgataa

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FIG. 3

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1  ctgcagccgc caccatgctg ttcaacctgc gaatcctgct gaacaacgcc gcttttcgga
61  acgggcacaa ctttatgggt aggaactttc gctgcggaca gccctccag aataaggtcc
121 agctgaaggg cagggacctg ctgacctga aaaatttcac aggggaggaa atcaagtata
181 tgctgtggct gtcagctgat ctgaagtcc ggatcaagca gaagggcgaa tatctgcctc
241 tgctccaggg caaaagcctg gggatgatct tcgaaaagcg cagtactcgg accagactgt
301 caaccgagac tggattcgct ctgctgggag gacacccttg tttctgacc actcaggaca
361 ttcacctggg agtgaacgag tccctgaccg aactgctcg cgtcctgagc tctatggccg
421 acgctgtgct agctcgagtc tacaaacagt ccgacctgga taccctggcc aaggaagctt
481 ctatcccaat tattaacggc ctgtcagacc tgtatcacc catccagatt ctggccgatt
541 acctgaccct ccaggagcac tattctagtc tgaaagggct gacactgagt tggattgggg
601 acggaacaa tatcctgcac tctattatga tgtcagccgc caagtttgga atgcacctcc
661 aggctgcaac cccaaaaggc tacgaaccgc atgcctcagt gacaaagctg gctgaacagt
721 acgccaaaga gaacggcact aagctgctgc tgaccaacga ccctctggag gccgctcacg
781 gaggcaacgt gctgatcacc gatacctgga ttagtatggg acaggaggaa gagaagaaga
841 agcggtcca ggccttcag ggctaccagg tgacaatgaa aaccgctaag gtcgagcca
901 gcgattggac ctttctgcac tgcctgcca gaaagcccga agaggtggac gacgaggtct
961 tctactctcc cagaagcctg gtgtttcccg aagctgagaa taggaagtgg acaattatgg
1021 cagtgatggt cagcctgctg actgattatt cacctcagct ccagaaacca aagttctgat
1081 aagcgccgc
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FIG. 4

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1  ctgcagccgc caccatgctg ttcaacctgc gaatcctgct gaacaacgcc gcttttcgga
61  acgggcacaa ctttatgggt aggaactttc gctgcgga gcccctccag aataaggtcc
121 agctgaaggg cagggacctg ctgacctga aaaatttcac aggggaggaa atcaagtata
181 tgctgtggct gtcagctgat ctgaagttcc ggatcaagca gaagggcgaa tatctgcctc
241 tgctccaggg caaaaagcctg gggatgatct tcgaaaagcg cagtactcgg accagactgt
301 caaccgagac tggattcgct ctgctgggag gacacccttg ttttctgacc actcaggaca
361 ttcacctggg agtgaacgag tccctgaccg aactgctcg cgtcctgagc tctatggccg
421 acgctgtgct ggctcgagtc taaaaacagt ccgacctgga taccctggcc aaggaagctt
481 ctatcccaat tattaacggc ctgtcagacc tgtatcacc ccatccagatt ctggccgatt
541 acctgaccct ccaggagcac tattctagtc tgaaagggtc gacactgagt tggattgggg
601 acggaacaa tatcctgcac tctattatga tgtcagccgc caagtttgga atgcacctcc
661 aggctgcaac ccaaaaaggc tacgaaccgg atgcctcagt gacaaagctg gctgaacagt
721 acgccaaga gaacggcact aagctgctgc tgaccaacga ccctctggag gccgctcacg
781 gaggcaacgt gctgatcacc gatacctgga ttagtatggg acaggaggaa gagaagaaga
841 agcggtcca ggccttccag ggctaccagg tgacaatgaa aaccgctaag gtcgcagcca
901 gcgattggac ctttctgcac tgctgcccga gaaagcccga agaggtggac gacgaggtct
961 tctactctcc cagaagcctg gtgtttcccg aagctgagaa taggaagtgg acaattatgg
1021 cagtgatggt cagcctgctg actgattatt cacctcagct ccagaaacca aagttctgat
1081 aagcggccgc
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FIG. 5A

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hOTCLW5 -----ATGCTGTTCAACCTGAGAATCCTGCTGAACAACGCCGCTTCAGAA 46
hOTCLW6 -----ATGCTGTTCAACCTGCGCATCCTGCTGAACAACGCCGCTTCCGCA 46
hOTCco -----ATGCTGTTCAACCTGCGAATCCTGCTGAACAATGCCGCTTTTCGGA 46
LW3 CTGCAGCCGCCACCATGCTGTTCAACCTGCGAATCCTGCTGAACAACGCCGCTTTTCGGA 60
LW4 CTGCAGCCGCCACCATGCTGTTCAACCTGCGAATCCTGCTGAACAACGCCGCTTTTCGGA 60
hOTwt -----ATGCTGTTTAAATCTGAGGATCCTGTAAACAATGCAGCTTTTAGAA 46
          ***** ** * * * ***** * ***** ** * * * * *

hOTCLW5 ACGGCCACAACCTTCATGGTGAGAACTTCAGATGCGGCCAGCCCCCTGCAGAACAAAGGTGC 106
hOTCLW6 ACGGCCACAACCTTCATGGTGCGCAACTTCCGCTGCGGCCAGCCCCCTGCAGAACAAAGGTGC 106
hOTCco ACGGCCACAATTTTCATGGTGAGGAACCTTCGCTGCGGACAGCCCCCTCCAGAACAAAGGTCC 106
LW3 ACGGCCACAACCTTTATGGTGAGGAACCTTCGCTGCGGACAGCCCCCTCCAGAACAAAGGTCC 120
LW4 ACGGCCACAACCTTTATGGTGAGGAACCTTCGCTGCGGACAGCCCCCTCCAGAACAAAGGTCC 120
hOTwt ATGGTCACAACCTTCATGGTTCGAAATTTTCGGTGTGGACAACCACTACAAATAAAGTGC 106
          * * * ***** ** ***** * * * * * * * * * * * * * * * * *

hOTCLW5 AGCTGAAGGGCAGAGACCTGCTGACCTGAAGAACTTCACCGGCGAGGAGATCAAGTACA 166
hOTCLW6 AGCTGAAGGGCCGCGACCTGCTGACCTGAAGAACTTCACCGGCGAGGAGATCAAGTACA 166
hOTCco AGCTGAAGGGCAGGGACCTGCTGACCTGAAAAATTTACAGGGGAGGAAATCAAGTACA 166
LW3 AGCTGAAGGGCAGGGACCTGCTGACCTGAAAAATTTACAGGGGAGGAAATCAAGTATA 180
LW4 AGCTGAAGGGCAGGGACCTGCTGACCTGAAAAATTTACAGGGGAGGAAATCAAGTATA 180
hOTwt AGCTGAAGGGCCGTGACCTTCTCACTTAAAAAACTTTACCGGAGAAGAAATTAATATA 166
          ***** * ***** ** * * * * * * * * * * * * * * * * *

hOTCLW5 TGCTGTGGCTGAGCGCCGACCTGAAGTTCAGAATCAAGCAGAAGGGCGAGTACCTGCCCC 226
hOTCLW6 TGCTGTGGCTGAGCGCCGACCTGAAGTTCGCGCATCAAGCAGAAGGGCGAGTACCTGCCCC 226
hOTCco TGCTGTGGCTGTCAGCCGATCTGAAGTTCGCGATCAAGCAGAAGGGCGAATATCTGCCTC 226
LW3 TGCTGTGGCTGTCAGCTGATCTGAAGTTCGCGATCAAGCAGAAGGGCGAATATCTGCCTC 240
LW4 TGCTGTGGCTGTCAGCTGATCTGAAGTTCGCGATCAAGCAGAAGGGCGAATATCTGCCTC 240
hOTwt TGCTATGGCTATCAGCAGATCTGAAATTTAGGATAAAAACAGAAAGGAGAGTATTTGCCTT 226
          **** * * * * * * * * * * * * * * * * * * * * * * * * *

hOTCLW5 TGCTGCAGGGCAAGAGCCTGGGCATGATCTTCGAGAAGAGAAGCACCAGAACCCAGACTGA 286
hOTCLW6 TGCTGCAGGGCAAGAGCCTGGGCATGATCTTCGAGAAGCGCAGCACCCGCACCCGCTGA 286
hOTCco TGCTCCAGGGCAAAAGCCTGGGGATGATCTTCGAAAAGCGCAGTACTCGGACCAGACTGT 286
LW3 TGCTCCAGGGCAAAAGCCTGGGGATGATCTTCGAAAAGCGCAGTACTCGGACCAGACTGT 300
LW4 TGCTCCAGGGCAAAAGCCTGGGGATGATCTTCGAAAAGCGCAGTACTCGGACCAGACTGT 300
hOTwt TATTGCAAGGGAAGTCCTTAGGCATGATTTTTGAGAAAAGAAGTACTCGAACAAAGATTGT 286
          * * * * * * * * * * * * * * * * * * * * * * * * * * *

hOTCLW5 GCACCGAGACCGGCCTGGCCCTGCTGGGCGGCCACCCCTGCTTCCTGACCACCCAGGACA 346
hOTCLW6 GCACCGAGACCGGCCTGGCCCTGCTGGGCGGCCACCCCTGCTTCCTGACCACCCAGGACA 346
hOTCco CAACAGAGACTGGATTGCACTGCTGGGAGGACACCCATGTTTTCTGACCACACAGGACA 346
LW3 CAACCGAGACTGGATTGCTCTGCTGGGAGGACACCCCTGTTTTCTGACCACCTCAGGACA 360
LW4 CAACCGAGACTGGATTGCTCTGCTGGGAGGACACCCCTGTTTTCTGACCACCTCAGGACA 360
hOTwt CTACAGAAACAGGCTTTGCACTTCTGGGAGGACATCCTGTGTTTTCTTACCACACAAGATA 346
          ** * * * * * * * * * * * * * * * * * * * * * * * * *

hOTCLW5 TCCACCTGGGCGTGAACGAGAGCCTGACCGACACCGCCAGAGTGCTGAGCAGCATGGCCG 406
hOTCLW6 TCCACCTGGGCGTGAACGAGAGCCTGACCGACACCGCCGCGTGCTGAGCAGCATGGCCG 406
hOTCco TTCATCTGGGAGTGAACGAGTCCCTGACCGACACAGCACGCGTCCTGAGCTCCATGGCTG 406
LW3 TTCACCTGGGAGTGAACGAGTCCCTGACCGACACTGCTCGCGTCCTGAGCTCTATGGCCG 420
LW4 TTCACCTGGGAGTGAACGAGTCCCTGACCGACACTGCTCGCGTCCTGAGCTCTATGGCCG 420
hOTwt TTCATTTGGGTGTGAATGAAAGTCTCACGGACACGGCCGCTGATTGTCTAGCATGGCAG 406
          * * * * * * * * * * * * * * * * * * * * * * * * * *

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FIG. 5B

hOTCLW5	ACGCCGTGCTGGCCAGAGTGTACAAGCAGAGCGACCTGGACACCCTGGCCAAGGAGGCCA	466
hOTCLW6	ACGCCGTGCTGGCCCCGCGTGTACAAGCAGAGCGACCTGGACACCCTGGCCAAGGAGGCCA	466
hOTCco	ATGCAGTGCTGGCTCGAGTCTACAAACAGTCTGACCTGGATACCCTGGCCAAGGAAGCTT	466
LW3	ACGCTGTGCTAGCTCGAGTCTACAAACAGTCCGACCTGGATACCCTGGCCAAGGAAGCTT	480
LW4	ACGCTGTGCTGGCTCGAGTCTACAAACAGTCCGACCTGGATACCCTGGCCAAGGAAGCTT	480
hOTwt	ATGCAGTATTGGCTCGAGTGTATAAACAATCAGATTTGGACACCCTGGCTAAAGAAGCAT	466
	* * * * *	
hOTCLW5	GCATCCCCATCATCAACGGCCTGAGCGACCTGTACCACCCCATCCAGATCCTGGCCGACT	526
hOTCLW6	GCATCCCCATCATCAACGGCCTGAGCGACCTGTACCACCCCATCCAGATCCTGGCCGACT	526
hOTCco	CTATCCCAATCATTAAATGGCCTGAGTGACCTGTATCACCCTATCCAGATTTGGCCGATT	526
LW3	CTATCCCAATTATTAACGGCCTGTGACACCTGTATCACCCTATCCAGATTTGGCCGATT	540
LW4	CTATCCCAATTATTAACGGCCTGTGACACCTGTATCACCCTATCCAGATTTGGCCGATT	540
hOTwt	CCATCCCAATTATCAATGGGCTGTGACATTTGTACCATCCTATCCAGATCCTGGCTGATT	526
	*****	
hOTCLW5	ACCTGACCCTGCAGGAGCACTACAGCAGCCTGAAGGGCCTGACCCTGAGCTGGATCGGCG	586
hOTCLW6	ACCTGACCCTGCAGGAGCACTACAGCAGCCTGAAGGGCCTGACCCTGAGCTGGATCGGCG	586
hOTCco	ACCTGACCCTCCAGGAGCATTATTCTAGTCTGAAAGGGCTGACACTGAGCTGGATTGGGG	586
LW3	ACCTGACCCTCCAGGAGCACTATTCTAGTCTGAAAGGGCTGACACTGAGTTGGATTGGGG	600
LW4	ACCTGACCCTCCAGGAGCACTATTCTAGTCTGAAAGGGCTGACACTGAGTTGGATTGGGG	600
hOTwt	ACCTCAGCTCCAGGAACACTATAGCTCTCTGAAAGGTCTTACCCTCAGCTGGATCGGGG	586
	*****	
hOTCLW5	ACGGCAACAACATCCTGCACAGCATCATGATGAGCGCCGCCAAGTTTCGGCATGCACCTGC	646
hOTCLW6	ACGGCAACAACATCCTGCACAGCATCATGATGAGCGCCGCCAAGTTTCGGCATGCACCTGC	646
hOTCco	ACGGAAACAATATCCTGCACTCCATTATGATGAGCGCCGCCAAGTTTGGAAATGCACCTCC	646
LW3	ACGGAAACAATATCCTGCACTCTATTATGATGTGAGCGCCGCCAAGTTTGGAAATGCACCTCC	660
LW4	ACGGAAACAATATCCTGCACTCTATTATGATGTGAGCGCCGCCAAGTTTGGAAATGCACCTCC	660
hOTwt	ATGGGAACAATATCCTGCACTCCATCATGATGAGCGCAGCGAAATTTCGGAAATGCACCTTC	646
	* * *	
hOTCLW5	AGGCCGCCACCCCCAAGGGCTACGAGCCCGACGCCAGCGTGACCAAGCTGGCCGAGCAGT	706
hOTCLW6	AGGCCGCCACCCCCAAGGGCTACGAGCCCGACGCCAGCGTGACCAAGCTGGCCGAGCAGT	706
hOTCco	AGGCTGCAACCCCCAAAAGGCTACGAACCCGATGCCTCCGTGACAAAGCTGGCAGAACAGT	706
LW3	AGGCTGCAACCCCCAAAAGGCTACGAACCCGATGCCTCAGTGACAAAGCTGGCTGAACAGT	720
LW4	AGGCTGCAACCCCCAAAAGGCTACGAACCCGATGCCTCAGTGACAAAGCTGGCTGAACAGT	720
hOTwt	AGGCAGCTACTCCAAAGGGTTATGAGCCGGATGCTAGTGTAACCAAGTTGGCAGAGCAGT	706
	*****	
hOTCLW5	ACGCCAAGGAGAACGGCACCAAGCTGCTGCTGACCAACGACCCCTGGAGGCCGCCACG	766
hOTCLW6	ACGCCAAGGAGAACGGCACCAAGCTGCTGCTGACCAACGACCCCTGGAGGCCGCCACG	766
hOTCco	ATGCCAAAGAGAACGGCACTAAGCTGCTGCTGACCAATGACCCCTCTGGAGGCCGCTCACG	766
LW3	ACGCCAAAGAGAACGGCACTAAGCTGCTGCTGACCAACGACCCCTCTGGAGGCCGCTCACG	780
LW4	ACGCCAAGAGAACGGCACTAAGCTGCTGCTGACCAACGACCCCTCTGGAGGCCGCTCACG	780
hOTwt	ATGCCAAAGAGAAATGTACCAAGCTGTTGCTGACAAATGATCCATTGGAAGCAGCGCATG	766
	* * *	
hOTCLW5	GCGGCAACGTGCTGATCACCGACACCTGGATCAGCATGGGCCAGGAGGAGAGAAGAAGA	826
hOTCLW6	GCGGCAACGTGCTGATCACCGACACCTGGATCAGCATGGGCCAGGAGGAGAGAAGAAGA	826
hOTCco	GAGGCAACGTGCTGATCACTGATACCTGGATTAGTATGGGACAGGAGGAAGAGAAGAAGA	826
LW3	GAGGCAACGTGCTGATCACCGATACCTGGATTAGTATGGGACAGGAGGAAGAGAAGAAGA	840
LW4	GAGGCAACGTGCTGATCACCGATACCTGGATTAGTATGGGACAGGAGGAAGAGAAGAAGA	840
hOTwt	GAGGCAATGTATTATTACAGACACTTGGATAAGCATGGGACAAGAAGGAGAGAAGAAAA	826
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FIG. 5C

hOTCLW5	AGAGACTGCAGGCCTTCCAGGGCTACCAGGTGACCATGAAGACCGCCAAGGTGGCCGCCA	886
hOTCLW6	AGCGCCTGCAGGCCTTCCAGGGCTACCAGGTGACCATGAAGACCGCCAAGGTGGCCGCCA	886
hOTCco	AGCGGCTCCAGGCCTTCCAGGGCTACCAGGTGACAATGAAAACCGCTAAGGTCGCAGCCA	886
LW3	AGCGGCTCCAGGCCTTCCAGGGCTACCAGGTGACAATGAAAACCGCTAAGGTCGCAGCCA	900
LW4	AGCGGCTCCAGGCCTTCCAGGGCTACCAGGTGACAATGAAAACCGCTAAGGTCGCAGCCA	900
hOTwt	AGCGGCTCCAGGCCTTCCAGGTTACCAGGTTACAATGAAGACTGCTAAAGTTGCTGCCT	886
	** * ** * ** * ** * ** * ** * ** * ** * ** * ** *	
hOTCLW5	GCGACTGGACCTTTCCTGCACTGCCTGCCCAGAAAGCCCGAGGAGGTGGACGACGAGGTGT	946
hOTCLW6	GCGACTGGACCTTTCCTGCACTGCCTGCCCAGCAAGCCCGAGGAGGTGGACGACGAGGTGT	946
hOTCco	GCGACTGGACCTTTCCTGCACTGCCTGCCCAGAAAGCCTGAAGAGGTGGACGATGAGGTCT	946
LW3	GCGATTGGACCTTTCCTGCACTGCCTGCCCAGAAAGCCCGAAGAGGTGGACGACGAGGTCT	960
LW4	GCGATTGGACCTTTCCTGCACTGCCTGCCCAGAAAGCCCGAAGAGGTGGACGACGAGGTCT	960
hOTwt	CTGACTGGACATTTTTTACTGCTTGCCTGCCCAGAAAGCCAGAAAGTGGATGATGAAGTCT	946
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hOTCLW5	TCTACAGCCCCAGAAAGCCTGGTGTTCCCCGAGGCCGAGAACAGAAAGTGGACCATCATGG	1006
hOTCLW6	TCTACAGCCCCCGCAGCCTGGTGTTCCCCGAGGCCGAGAACCGCAAGTGGACCATCATGG	1006
hOTCco	TCTACTACCCAGAAAGCCTGGTGTTTCCCTGAAGCTGAGAATAGGAAGTGGACAATCATGG	1006
LW3	TCTACTCTCCAGAAAGCCTGGTGTTTCCCGAAGCTGAGAATAGGAAGTGGACAATTATGG	1020
LW4	TCTACTCTCCAGAAAGCCTGGTGTTTCCCGAAGCTGAGAATAGGAAGTGGACAATTATGG	1020
hOTwt	TTTATTCTCCTCGATCACTAGTGTTCCCGAGGCCAGAAAACAGAAAGTGGACAATCATGG	1006
	* ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *	
hOTCLW5	CCGTGATGGTGAGCCTGCTGACCGACTACAGCCCCCAGCTGCAGAAGCCCCAAGTTCTGA-	1065
hOTCLW6	CCGTGATGGTGAGCCTGCTGACCGACTACAGCCCCCAGCTGCAGAAGCCCCAAGTTCTGA-	1065
hOTCco	CAGTGATGGTCAGCCTGCTGACTGATTATTCCCCTCAGCTCCAGAAACCAAAGTTCTGAT	1066
LW3	CAGTGATGGTCAGCCTGCTGACTGATTATTACCTCAGCTCCAGAAACCAAAGTTCTGAT	1080
LW4	CAGTGATGGTCAGCCTGCTGACTGATTATTACCTCAGCTCCAGAAACCAAAGTTCTGAT	1080
hOTwt	CTGTATGGTGTCCTGCTGACAGATTACTACCTCAGCTCCAGAAGCCTAAATTT----	1062
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hOTCLW5	-----	
hOTCLW6	-----	
hOTCco	AA-----	1068
LW3	AAGCGGCCGC	1090
LW4	AAGCGGCCGC	1090
hOTwt	-----	