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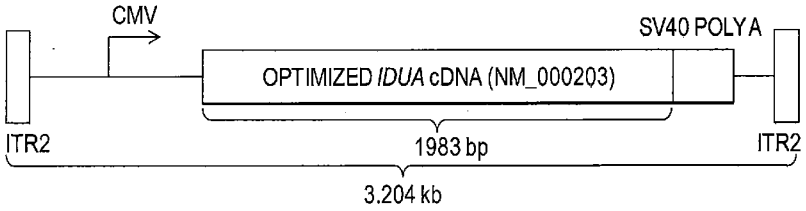



FIG. 1A

(57) Abstract: This invention relates to viral vectors for delivery of alpha-L-iduronidase to the cornea of a subject and methods of using the same for treatment and prevention of corneal clouding and blindness in a subject due to mucopolysaccharidosis I.

WO 2017/147123 A1

# AAV-IDUA Vector for Treatment of MPS I-Associated Blindness

## Statement of Priority

**[0001]** This application claims the benefit of U.S. Provisional Application Serial No. 62/298,126, filed February 22, 2016, the entire contents of which are incorporated by reference herein.

## Field of the Invention

**[0002]** This invention relates to viral vectors for delivery of alpha-L-iduronidase to the cornea of a subject and methods of using the same for treatment and prevention of corneal clouding and blindness in a subject due to mucopolysaccharidosis I.

## Background of the Invention

**[0003]** Mucopolysaccharidosis I (MPS I) is an autosomal recessive lysosomal storage disorder caused by null or nonsense mutations in the gene encoding alpha-L-iduronidase (IDUA), a ubiquitous intracellular and secreted enzyme that breaks down glycosaminoglycans (GAGs). In the absence of functional IDUA, GAGs accumulate in lysosomes and disrupt the normal intracellular trafficking of lipids, sugars, and proteins causing multisystem end organ damage. The incidence of MPS I is approximately 1 in 100,000 and the disease is characterized by hepatosplenomegaly, cardiac insufficiency, bone and joint deformities, dwarfism, mental retardation and serious nervous system problems, which commonly result in death by 10 years of age. Additional symptoms include hearing loss, joint stiffness, and clouding of the cornea which results in loss of vision (Aldenhoven *et al., Blood 125(13)*:2164 (2015)).

**[0004]** Current MPS I treatments include IDUA enzyme replacement therapy (ERT) (ALDURAZYME®) via intravenous injections, which has proven useful in reducing hepatosplenomegaly, and in improving myocardiac function, pulmonary symptoms and motility in MPS I patients with mild disease. A more promising treatment relies on allogeneic hematopoietic stem cell transplantation (HSCT) which has been used for the past 2-3 decades in MPS I patients. HSCT has proven successful at improving cognitive function, reducing hepatosplenomegaly, preventing ischemic cardiac disease and prolonging the patient's lifespan, in some cases by decades. Clinical outcomes are best when myeloablative chemotherapy and cord blood donors are

utilized and also correlates with the extent of sustained donor chimerism. A third MPS I treatment approach that is still under preclinical evaluation relies on an adeno-associated virus (AAV) IDUA gene addition strategy. Central nervous system targeted AAV-IDUA gene therapy has been explored in murine, feline, and canine MPS I models following administration via several routes including the carotid artery, intraparenchymal, intraventricular and intrathecal (Janson *et al., Neurosurgery 74(1)*:99 (2014); Wolf *et al., Neurobiol. Dis. 43(1)*:123 (2011); Hinderer *et al., Mol. Ther. 22(12)*:2018 (2014)). These gene therapy studies independently report histological, biochemical, and in particular cognitive improvements while IDUA-related toxicity was not observed. However, ERT, HSCT and AAV CNS-targeted or systemic gene therapy exhibit a common deficiency; which is the inability to correct MPS I-associated maladies in privileged compartments including the joint and eye.

**[0005]** Regarding the ocular abnormalities, approximately 90% of MPS I children lose vision due to corneal clouding, which has been attributed to the abnormal presence of vacuolated stroma cells (Huang *et al., Exp. Eye Res. 62(4)*:377 (1996)). Detailed analyses of MPS I human corneas demonstrated an accumulation of chondroitin and dermatan sulphate GAGs, which alter the uniform distribution, organization, and size of collagen fibrils (Alroy *et al., Exp. Eye Res. 68(5)*:523 (1999); Fahnehjelm *et al., Acta Ophthalmol. Scand. 84(6)*:781 (2006)). Cornea transplantation in MPS I children has been used to address the corneal blindness, however the high rejection rate has, in recent years, discouraged this treatment as standard practice. On the contrary, isolated reports using HSCT have demonstrated the capacity to stabilize, improve, and in some cases restore corneal clarity (Fahnehjelm *et al., Acta Ophthalmol. Scand. 84(6)*:781 (2006); Hobbs, *Lancet 2(8249)*:735 (1981); Hoogerbrugge *et al., Lancet, 345(8962)*:1398 (1995); Vellodi *et al., Arch. Dis. Child. 76(2)*:92 (1997); Gullingsrud *et al., Ophthalmology, 105(6)*:1099 (1998); Souillet *et al., Bone Marrow Transplant 31(12)*:1105 (2003)).

**[0006]** The present invention provides viral vectors for expression of IDUA in the cornea and methods for treating or preventing MPS I-associated corneal clouding and blindness.

## Summary of the Invention

**[0007]** This invention is based on the finding that the use of AAV vectors for delivery of IDUA to the cornea of a subject with MPS I is effective to express IDUA

throughout the cornea. Thus, one aspect of the invention relates to a recombinant nucleic acid comprising a nucleotide sequence encoding human alpha-L-iduronidase (IDUA), wherein the nucleotide sequence has been codon-optimized for expression in human cells.

**[0008]** Another aspect of the invention relates to an AAV vector genome comprising the nucleic acid of the invention, an AAV particle comprising the AAV vector genome, and a pharmaceutical composition comprising the AAV particle.

**[0009]** A further aspect of the invention relates to a method of producing a recombinant AAV particle comprising an AAV capsid, the method comprising: providing a cell *in vitro* with an AAV Cap and AAV Rep coding sequences, the AAV vector genome of the invention, and helper functions for generating a productive AAV infection; and allowing assembly of the recombinant AAV particle comprising the AAV capsid and encapsidating the AAV vector genome.

**[0010]** An additional aspect of the invention relates to a method of delivering IDUA to the cornea of a subject, comprising administering to the cornea of the subject an effective amount of an AAV particle that expresses IDUA, thereby delivering IDUA to the cornea of the subject.

**[0011]** Another aspect of the invention relates to a method of treating or delaying the onset of MPS I-associated corneal clouding in a subject in need thereof, comprising administering to the cornea of the subject a therapeutically effective amount of an AAV particle that expresses IDUA, thereby treating or delaying the onset of MPS I-associated corneal clouding in the subject.

**[0012]** These and other aspects of the invention are set forth in more detail in the description of the invention below.

## Brief Description of the Drawings

**[0013]** Figures 1A-1D show restoration of IDUA activity in MPS I patient fibroblasts by AAV gene therapy. (A) Schematic diagram of the AAV-IDUA optimized vector construct. (B) Cell lysates (Left panel) and supernatants (Right panel) from AAV2 infected and non-infected fibroblasts, normal human fibroblasts (NHF) or MPS I fibroblasts, were analyzed for IDUA protein expression. Detection of β-actin was performed as a loading control of total protein in cell lysates. (C,D) Functional activity of IDUA protein was obtained for cell lysis (C) and supernatants (D) from AAV2 infected and not-infected fibroblasts, NHF or MPS I fibroblasts. For

cell lysis, the nmoles of 4-MU were normalized to one hour reaction and mg total protein. For cell supernatants, the nmoles of 5-MU were normalized to 10 µl of the sample. 4-MU, 4-methylumbelliferone; CMV, cytomegalovirus promoter; ITR, internal terminal repeats; GFP, Green fluorescence protein.

**[0014]** Figure 2 shows no cytotoxicity following AAV2-GFP and AAV2-optIDUA administration to MPS I fibroblasts.

**[0015]** Figure 3 shows the distribution of vehicle containing india ink after injection into the cornea.

**[0016]** Figures 4A-4C show AAV capsid serotype evaluation in human cornea. (A) Human cornea stromas were injected with self-complementary CMV-GFP cassette encapsidated in AAV serotypes 8, 9, or the 8/9 chimeric 8G9. Seven days later Western blot was used to detect GFP. PBS corresponds to a human cornea injected with only PBS (vehicle control). Detection of β-actin was performed as a loading control. (B) Single-strand AAV8G9-CMV-GFP was injected into the stroma of human corneas and harvested for histology 7 days later. Left - Immunofluorescence image showing the distribution of recombinant AAV8G9-GFP viral infection across a human cornea section. Human corneas injected with PBS served as the negative control. Images were obtained with 10X objective and assembled by stitch processing. The scale bar is equal to 2000µm. Right - Different areas of the same stained human cornea taken with 20x objective. The scale bar is equal to 100µm. DAPI was used for nuclei counterstain. (C) A representative section of the corneas in (B) stained with GFP and the indicated cell marker. The scale bar is equal to 10µm. DAPI was used for nuclei counterstain.

**[0017]** Figures 5A-5C show restoration of IDUA activity in human corneas by AAV8G9-opt-IDUA. (A) A representative section of a normal non-injected cornea stained with IDUA antibody and a cell marker. DAPI was used for nuclei counterstain. The scale bar is equal to 10µm. (B) Western blot detecting IDUA amounts produced after injection of AAV8G9-IDUA. Administration of AAV8G9-GFP served as a vector infection control. B-actin serves as a loading control. (C) Functional activity of IDUA protein obtained from human corneas 7 days post-injection of AAV8G9-opt-IDUA. AAV8G9-GFP served as the negative control. The nmoles of 4-MU were normalized to one hour reaction and mg total protein. PBS corresponds to a human cornea injected with only the vehicle control.

**[0018]** Figures 6A-6B show distribution of IDUA protein following AAV-8G9-opt-

IDUA transduction. (A) Immunofluorescence image showing the distribution of IDUA protein across a human cornea section seven days post-injection of AAV8G9-opt-IDUA or PBS (vehicle). The images were obtained with 10X objective and assembled by stitch processing. The scale bar is equal to 2000μm. DAPI was used for nuclei counterstain. (B) Cross section of the same stained human corneas but taken with a 20X objective. The top figure corresponds to the negative control of staining, which was performed exactly in the same as the other samples but without the primary antibody. DAPI was used for nuclei counterstain. The scale bar is equal to 100μm.

**[0019]** Figures 7A-7B show no cytotoxicity following AAV8G9-opt-IDUA injection in human corneas. (A) Human corneas injected with PBS, AAV8G9-GFP, or AAV8G9-opt-IDUA were processed 7 days later for Tunel staining. Top, images taken at 10X. Bottom, images taken at 20X. As a positive control, human corneas injected with PBS were nuclease treated. The scale bar is equal to 100μm. (B) Quantitation of pixel area for total staining performed in (A). No statistical difference between cytotoxicity for corneas injected with AAV8G9-GFP or AAV8G9-opt-IDUA was observed ($p > 0.05$).

**[0020]** Figures 8A-8E show intrastromal injection of AAV8G9-optIDUA in MPS1 canines. The left eye of dog I-712 was injected with the AAV8G9-CMV-optIDUA (1e9 vg/μl) solution. The needle tip of an insulin syringe was inserted into the corneal stroma (A) and the solution was injected gradually (B) until the full volume (65μl) was administered (C). Immediately following the injection, an area of fluid retention was visible as clouding or edema of the axial cornea, covering 30% of the corneal surface (D). (E) Ophthalmic ultrasound biomicroscopy allowed determination of central cornea thickness at the indicated timepoints.

**[0021]** Figures 9A-9B show AAV8G9-CMV-optIDUA clears corneal cloudiness in MPS1 canines. A) MPS1 canines were administered AAV8G9-optIDUA in one eye while the contralateral eye was given AAV-GFP as a control, all by intrastromal injection. Improved reflectivity of the tapetum lucidum upon retroillumination of the fundus indicates cornea clarity over the indicated time period. B) Corneal edema and recovery. Dog I-709 developed acute corneal edema first in the left eye at 5 weeks (image on the week of onset not shown), and then in the right eye at 10 weeks. Corneal edema subsided within 2-3 weeks while receiving topical steroid (TS) with or without systemic steroid (SS), and has not recurred in either eye for up to 19 wk post-

injection. Importantly, the therapeutic effect remained evident in the IDUA-injected left eye after recovering from the transient immune reaction.

## Detailed Description of the Invention

**[0022]** The present invention will now be described with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

**[0023]** Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

**[0024]** Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right, unless specifically indicated otherwise. Nucleotides and amino acids are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission, or (for amino acids) by either the one-letter code, or the three letter code, both in accordance with 37 CFR §1.822 and established usage. *See, e.g., PatentIn User Manual*, 99-102 (Nov. 1990) (U.S. Patent and Trademark Office).

**[0025]** Except as otherwise indicated, standard methods known to those skilled in the art may be used for the construction of recombinant parvovirus and AAV (rAAV) constructs, packaging vectors expressing the parvovirus Rep and/or Cap sequences, and transiently and stably transfected packaging cells. Such techniques are known to those skilled in the art. *See, e.g.*, SAMBROOK *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL 2nd Ed. (Cold Spring Harbor, NY, 1989); AUSUBEL *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York).

**[0026]** Moreover, the present invention also contemplates that in some embodiments of the invention, any feature or combination of features set forth herein can be excluded or omitted.

**[0027]** To illustrate further, if, for example, the specification indicates that a particular amino acid can be selected from A, G, I, L and/or V, this language also indicates that the amino acid can be selected from any subset of these amino acid(s) for example A, G, I or L; A, G, I or V; A or G; only L; etc. as if each such subcombination is expressly set forth herein. Moreover, such language also indicates that one or more of the specified amino acids can be disclaimed. For example, in particular embodiments the amino acid is not A, G or I; is not A; is not G or V; etc. as if each such possible disclaimer is expressly set forth herein.

**Definitions**

**[0028]** The following terms are used in the description herein and the appended claims.

**[0029]** The singular forms "a" and "an" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

**[0030]** Furthermore, the term "about," as used herein when referring to a measurable value such as an amount of the length of a polynucleotide or polypeptide sequence, dose, time, temperature, and the like, is meant to encompass variations of 20%, 10%, 5%, 1%, 0.5%, or even 0.1% of the specified amount.

**[0031]** Also as used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative ("or").

**[0032]** As used herein, the transitional phrase "consisting essentially of" is to be interpreted as encompassing the recited materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention (*e.g.*, rAAV replication). Thus, the term "consisting essentially of" as used herein should not be interpreted as equivalent to "comprising."

**[0033]** The term "consists essentially of" (and grammatical variants), as applied to a polynucleotide or polypeptide sequence of this invention, means a polynucleotide or polypeptide that consists of both the recited sequence (*e.g.*, SEQ ID NO) and a total of ten or less (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) additional nucleotides or amino acids on the 5' and/or 3' or N-terminal and/or C-terminal ends of the recited sequence such that the function of the polynucleotide or polypeptide is not materially altered. The total of ten or less additional nucleotides or amino acids includes the total number of additional nucleotides or amino acids on both ends added together. The term

"materially altered," as applied to polynucleotides of the invention, refers to an increase or decrease in ability to express the encoded polypeptide of at least about 50% or more as compared to the expression level of a polynucleotide consisting of the recited sequence. The term "materially altered," as applied to polypeptides of the invention, refers to an increase or decrease in enzymatic activity of at least about 50% or more as compared to the activity of a polypeptide consisting of the recited sequence.

**[0034]** The term "parvovirus" as used herein encompasses the family *Parvoviridae*, including autonomously-replicating parvoviruses and dependoviruses. The autonomous parvoviruses include members of the genera *Parvovirus*, *Erythrovirus*, *Densovirus*, *Iteravirus*, and *Contravirus*. Exemplary autonomous parvoviruses include, but are not limited to, minute virus of mouse, bovine parvovirus, canine parvovirus, chicken parvovirus, feline panleukopenia virus, feline parvovirus, goose parvovirus, H1 parvovirus, muscovy duck parvovirus, snake parvovirus, and B19 virus. Other autonomous parvoviruses are known to those skilled in the art. *See, e.g.*, FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers).

**[0035]** The genus *Dependovirus* contains the adeno-associated viruses (AAV), including but not limited to, AAV type 1, AAV type 2, AAV type 3 (including types 3A and 3B), AAV type 4, AAV type 5, AAV type 6, AAV type 7, AAV type 8, AAV type 9, AAV type 10, AAV type 11, AAV type 12, AAV type 13, avian AAV, bovine AAV, canine AAV, goat AAV, snake AAV, equine AAV, and ovine AAV. *See, e.g.*, FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers); and **Table 1**.

**[0036]** As used herein, the term "adeno-associated virus" (AAV), includes but is not limited to, AAV type 1, AAV type 2, AAV type 3 (including types 3A and 3B), AAV type 4, AAV type 5, AAV type 6, AAV type 7, AAV type 8, AAV type 9, AAV type 10, AAV type 11, AAV type 12, AAV type 13, snake AAV, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV, goat AAV, shrimp AAV, and any other AAV now known or later discovered. *See, e.g.*, FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers). A number of relatively new AAV serotypes and clades have been identified (*See, e.g.*, Gao *et al.*, (2004) *J. Virol.* 78:6381; Moris *et al.*, (2004) *Virol.* 33-:375; and **Table 1**).

**[0037]** The parvovirus vectors, particles, and genomes of the present invention can

be from, but are not limited to, AAV. The genomic sequences of various serotypes of AAV and the autonomous parvoviruses, as well as the sequences of the native ITRs, Rep proteins, and capsid subunits are known in the art. Such sequences may be found in the literature or in public databases such as GenBank. *See, e.g.*, GenBank Accession Numbers NC_002077, NC_001401, NC_001729, NC_001863, NC_001829, NC_001862, NC_000883, NC_001701, NC_001510, NC_006152, NC_006261, AF063497, U89790, AF043303, AF028705, AF028704, J02275, J01901, J02275, X01457, AF288061, AH009962, AY028226, AY028223, AY631966, AX753250, EU285562, NC_001358, NC_001540, AF513851, AF513852 and AY530579; the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences. *See also, e.g.*, Bantel-Schaal *et al.*, (1999) *J. Virol.* 73: 939; Chiorini *et al.*, (1997) *J. Virol.* 71:6823; Chiorini *et al.*, (1999) *J. Virol.* 73:1309; Gao *et al.*, (2002) *Proc. Nat. Acad. Sci. USA* 99:11854; Moris *et al.*, (2004) *Virol.* 33-:375-383; Mori *et al.*, (2004) *Virol.* 330:375; Muramatsu *et al.*, (1996) *Virol.* 221:208; Ruffing *et al.*, (1994) *J. Gen. Virol.* 75:3385; Rutledge *et al.*, (1998) *J. Virol.* 72:309; Schmidt *et al.*, (2008) *J. Virol.* 82:8911; Shade *et al.*, (1986) *J. Virol.* 58:921; Srivastava *et al.*, (1983) *J. Virol.* 45:555; Xiao *et al.*, (1999) *J. Virol.* 73:3994; international patent publications WO 00/28061, WO 99/61601, WO 98/11244; and U.S. Patent No. 6,156,303; the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences. *See also* **Table 1**. An early description of the AAV1, AAV2 and AAV3 ITR sequences is provided by Xiao, X., (1996), "Characterization of Adeno-associated virus (AAV) DNA replication and integration," Ph.D. Dissertation, University of Pittsburgh, Pittsburgh, PA (incorporated herein in its entirety).

**[0038]** The term "tropism" as used herein refers to entry of the virus into the cell, optionally and preferably followed by expression (*e.g.*, transcription and, optionally, translation) of sequences carried by the viral genome in the cell, *e.g.*, for a recombinant virus, expression of the heterologous nucleotide sequences(s). Those skilled in the art will appreciate that transcription of a heterologous nucleic acid sequence from the viral genome may not be initiated in the absence of trans-acting factors, *e.g.*, for an inducible promoter or otherwise regulated nucleic acid sequence. In the case of AAV, gene expression from the viral genome may be from a stably integrated provirus, from a non-integrated episome, as well as any other form in which the virus may take within the cell.

**[0039]** As used herein, "transduction" of a cell by parvovirus or AAV refers to parvovirus/AAV-mediated transfer of genetic material into the cell. *See, e.g.*, FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (3d ed., Lippincott-Raven Publishers).

**[0040]** The terms "5' portion" and "3' portion" are relative terms to define a spatial relationship between two or more elements. Thus, for example, a "3' portion" of a polynucleotide indicates a segment of the polynucleotide that is downstream of another segment. The term "3' portion" is not intended to indicate that the segment is necessarily at the 3' end of the polynucleotide, or even that it is necessarily in the 3' half of the polynucleotide, although it may be. Likewise, a "5' portion" of a polynucleotide indicates a segment of the polynucleotide that is upstream of another segment. The term "5' portion" is not intended to indicate that the segment is necessarily at the 5' end of the polynucleotide, or even that it is necessarily in the 5' half of the polynucleotide, although it may be.

**[0041]** As used herein, the term "polypeptide" encompasses both peptides and proteins, unless indicated otherwise.

**[0042]** A "polynucleotide" is a sequence of nucleotide bases, and may be RNA, DNA or DNA-RNA hybrid sequences (including both naturally occurring and non-naturally occurring nucleotides), and can be either single or double stranded DNA sequences.

**[0043]** The term "sequence identity," as used herein, has the standard meaning in the art. As is known in the art, a number of different programs can be used to identify whether a polynucleotide or polypeptide has sequence identity or similarity to a known sequence. Sequence identity or similarity may be determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman, *Adv. Appl. Math. 2*:482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, *J. Mol. Biol. 48*:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. USA 85*:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux *et al.*, *Nucl. Acid Res. 12*:387 (1984), preferably using the default settings, or by inspection.

## Table 1

| Complete Genomes | GenBank Accession Number | | | GenBank Accession Number | | | GenBank Accession Number |
|---|---|---|---|---|---|---|---|
| **Adeno-associated virus 1** | NC_002077,AF063497 | | **Hu T88** | AY695375 | | **Clade E** | |
| **Adeno-associated virus 2** | NC_001401 | | **Hu T71** | AY695374 | | Rh38 | AY530558 |
| **Adeno-associated virus 3** | NC_001729 | | **Hu T70** | AY695373 | | Hu66 | AY530626 |
| **Adeno-associated virus 3B** | NC_001863 | | **Hu T40** | AY695372 | | Hu42 | AY530605 |
| **Adeno-associated virus 4** | NC_001829 | | **Hu T32** | AY695371 | | Hu67 | AY530627 |
| **Adeno-associated virus 5** | Y18065, AF085716 | | Hu T17 | AY695370 | | Hu40 | AY530603 |
| **Adeno-associated virus 6** | NC_001862 | | Hu LG15 | AY695377 | | Hu41 | AY530604 |
| **Avian AAV ATCC VR-865** | AY186198, AY629583, NC_004828 | | | | | Hu37 | AY530600 |
| **Avian AAV strain DA-1** | NC_006263, AY629583 | | **Clade C** | | | Rh40 | AY530559 |
| **Bovine AAV** | NC_005889, AY388617 | | Hu9 | AY530629 | | Rh2 | AY243007 |
| | | | Hu10 | AY530576 | | Bb1 | AY243023 |
| **Clade A** | | | Hu11 | AY530577 | | Bb2 | AY243022 |
| **AAV1** | NC_002077,AF063497 | | Hu53 | AY530615 | | Rh10 | AY243015 |
| **AAV6** | NC_001862 | | Hu55 | AY530617 | | Hu17 | AY530582 |
| Hu.48 | AY530611 | | Hu54 | AY530616 | | Hu6 | AY530621 |
| Hu 43 | AY530606 | | Hu7 | AY530628 | | Rh25 | AY530557 |
| Hu 44 | AY530607 | | Hu18 | AY530583 | | Pi2 | AY530554 |
| Hu 46 | AY530609 | | Hu15 | AY530580 | | Pi1 | AY530553 |
| | | | Hu16 | AY530581 | | Pi3 | AY530555 |
| **Clade B** | | | Hu25 | AY530591 | | Rh57 | AY530569 |
| Hu. 19 | AY530584 | | Hu60 | AY530622 | | Rh50 | AY530563 |
| Hu. 20 | AY530586 | | Ch5 | AY243021 | | Rh49 | AY530562 |
| Hu 23 | AY530589 | | Hu3 | AY530595 | | Hu39 | AY530601 |
| Hu22 | AY530588 | | Hu1 | AY530575 | | Rh58 | AY530570 |
| | | | Hu4 | AY530602 | | Rh61 | AY530572 |

| Hu24 | AY530590 | Hu2 | AY530585 | Rh52 | AY530565 |
|---|---|---|---|---|---|
| Hu21 | AY530587 | Hu61 | AY530623 | Rh53 | AY530566 |
| Hu27 | AY530592 | | | Rh51 | AY530564 |
| Hu28 | AY530593 | **Clade D** | | Rh64 | AY530574 |
| Hu 29 | AY530594 | Rh62 | AY530573 | Rh43 | AY530560 |
| Hu63 | AY530624 | Rh48 | AY530561 | **AAV8** | AF513852 |
| Hu64 | AY530625 | Rh54 | AY530567 | Rh8 | AY242997 |
| Hu13 | AY530578 | Rh55 | AY530568 | Rh1 | AY530556 |
| Hu56 | AY530618 | Cy2 | AY243020 | | |
| Hu57 | AY530619 | **AAV7** | AF513851 | **Clade F** | |
| Hu49 | AY530612 | Rh35 | AY243000 | Hu14 (**AAV9**) | AY530579 |
| Hu58 | AY530620 | Rh37 | AY242998 | Hu31 | AY530596 |
| Hu34 | AY530598 | Rh36 | AY242999 | Hu32 | AY530597 |
| Hu35 | AY530599 | Cy6 | AY243016 | | |
| **AAV2** | NC_001401 | Cy4 | AY243018 | **Clonal Isolate** | |
| Hu45 | AY530608 | Cy3 | AY243019 | **AAV5** | Y18065, AF085716 |
| Hu47 | AY530610 | Cy5 | AY243017 | **AAV 3** | NC_001729 |
| Hu51 | AY530613 | Rh13 | AY243013 | **AAV 3B** | NC_001863 |
| Hu52 | AY530614 | | | **AAV4** | NC_001829 |
| Hu T41 | AY695378 | | | Rh34 | AY243001 |
| **Hu S17** | AY695376 | | | Rh33 | AY243002 |
| | | | | Rh32 | AY243003 |

**[0044]** An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, *J. Mol. Evol. 35*:351 (1987); the method is similar to that described by Higgins & Sharp, *CABIOS 5*:151 (1989).

**[0045]** Another example of a useful algorithm is the BLAST algorithm, described in Altschul *et al.*, *J. Mol. Biol. 215*:403 (1990) and Karlin *et al., Proc. Natl. Acad. Sci. USA 90*:5873 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul *et al.*, *Meth. Enzymol.*, *266*:460 (1996); blast.wustl/edu/blast/README.html. WU-BLAST-2 uses several search parameters, which are preferably set to the default values. The parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

**[0046]** An additional useful algorithm is gapped BLAST as reported by Altschul *et al.*, *Nucleic Acids Res. 25*:3389 (1997).

**[0047]** A percentage amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

**[0048]** In a similar manner, percent nucleic acid sequence identity is defined as the percentage of nucleotide residues in the candidate sequence that are identical with the nucleotides in the polynucleotide specifically disclosed herein.

**[0049]** The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer nucleotides than the polynucleotides specifically disclosed herein, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical nucleotides in relation to the total number of nucleotides. Thus, for example, sequence identity of sequences shorter than a sequence specifically disclosed herein, will be determined using the number of nucleotides in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as insertions, deletions, substitutions, *etc.*

**[0050]** In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0," which obviates the need for a weighted scale or parameters as described below for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the "shorter" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

**[0051]** As used herein, an "isolated" polynucleotide (*e.g.*, an "isolated DNA" or an "isolated RNA") means a polynucleotide separated or substantially free from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polynucleotide.

**[0052]** Likewise, an "isolated" polypeptide means a polypeptide that is separated or substantially free from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polypeptide.

**[0053]** A "therapeutic polypeptide" is a polypeptide that may alleviate or reduce symptoms that result from an absence or defect in a protein in a cell or subject. Alternatively, a "therapeutic polypeptide" is one that otherwise confers a benefit to a subject, *e.g.*, anti-cancer effects or improvement in transplant survivability.

**[0054]** As used herein, the term "modified," as applied to a polynucleotide or polypeptide sequence, refers to a sequence that differs from a wild-type sequence due to one or more deletions, additions, substitutions, or any combination thereof.

**[0055]** As used herein, by "isolate" or "purify" (or grammatical equivalents) a virus vector, it is meant that the virus vector is at least partially separated from at least some of the other components in the starting material.

**[0056]** By the terms "treat," "treating," or "treatment of" (and grammatical variations thereof) it is meant that the severity of the subject's condition is reduced, at least partially improved or stabilized and/or that some alleviation, mitigation, decrease or stabilization in at least one clinical symptom is achieved and/or there is a delay in the progression of the disease or disorder.

**[0057]** The terms "prevent," "preventing," and "prevention" (and grammatical variations thereof) refer to prevention and/or delay of the onset of a disease, disorder and/or a clinical symptom(s) in a subject and/or a reduction in the severity of the onset of the disease, disorder

and/or clinical symptom(s) relative to what would occur in the absence of the methods of the invention. The prevention can be complete, *e.g.*, the total absence of the disease, disorder and/or clinical symptom(s). The prevention can also be partial, such that the occurrence of the disease, disorder and/or clinical symptom(s) in the subject and/or the severity of onset is less than what would occur in the absence of the present invention.

**[0058]** A "treatment effective" amount as used herein is an amount that is sufficient to provide some improvement or benefit to the subject. Alternatively stated, a "treatment effective" amount is an amount that will provide some alleviation, mitigation, decrease or stabilization in at least one clinical symptom in the subject. Those skilled in the art will appreciate that the therapeutic effects need not be complete or curative, as long as some benefit is provided to the subject.

**[0059]** A "prevention effective" amount as used herein is an amount that is sufficient to prevent and/or delay the onset of a disease, disorder and/or clinical symptoms in a subject and/or to reduce and/or delay the severity of the onset of a disease, disorder and/or clinical symptoms in a subject relative to what would occur in the absence of the methods of the invention. Those skilled in the art will appreciate that the level of prevention need not be complete, as long as some benefit is provided to the subject.

**[0060]** The terms "heterologous nucleotide sequence" and "heterologous nucleic acid" are used interchangeably herein and refer to a sequence that is not naturally occurring in the virus. In some embodiments, the heterologous nucleic acid comprises an open reading frame that encodes a polypeptide or nontranslated RNA of interest (*e.g.*, for delivery to a cell or subject).

**[0061]** As used herein, the terms "virus vector," "vector" or "gene delivery vector" refer to a virus (*e.g.*, AAV) particle that functions as a nucleic acid delivery vehicle, and which comprises the vector genome (*e.g.*, viral DNA [vDNA]) packaged within a virion. Alternatively, in some contexts, the term "vector" may be used to refer to the vector genome/vDNA alone or a plasmid.

**[0062]** The virus vectors of the invention can further be duplexed parvovirus particles as described in international patent publication WO 01/92551 (the disclosure of which is incorporated herein by reference in its entirety). Thus, in some embodiments, double stranded (duplex) genomes can be packaged.

**[0063]** A "rAAV vector genome" or "rAAV genome" is an AAV genome (*i.e.*, vDNA) that comprises one or more heterologous nucleic acid sequences. rAAV vectors generally require only the 145 base ITR in *cis* to generate virus. All other viral sequences are dispensable and

may be supplied in *trans* (Muzyczka (1992) *Curr. Topics Microbiol. Immunol.* 158:97). Typically, the rAAV vector genome will only retain the one or more ITR sequence so as to maximize the size of the transgene that can be efficiently packaged by the vector. The structural and non-structural protein coding sequences may be provided in *trans* (*e.g.*, from a vector, such as a plasmid, or by stably integrating the sequences into a packaging cell). In embodiments of the invention the rAAV vector genome comprises at least one ITR sequence (*e.g.*, AAV ITR sequence), optionally two ITRs (*e.g.*, two AAV ITRs), which typically will be at the 5' and 3' ends of the vector genome and flank the heterologous nucleic acid, but need not be contiguous thereto. The ITRs can be the same or different from each other.

**[0064]** The term "terminal repeat" or "TR" includes any viral terminal repeat or synthetic sequence that forms a hairpin structure and functions as an inverted terminal repeat (*i.e.*, mediates the desired functions such as replication, virus packaging, integration and/or provirus rescue, and the like). The ITR can be an AAV ITR or a non-AAV ITR. For example, a non-AAV ITR sequence such as those of other parvoviruses (*e.g.*, canine parvovirus, bovine parvovirus, mouse parvovirus, porcine parvovirus, human parvovirus B-19) or the SV40 hairpin that serves as the origin of SV40 replication can be used as an ITR, which can further be modified by truncation, substitution, deletion, insertion and/or addition. Further, the ITR can be partially or completely synthetic, such as the "double-D sequence" as described in United States Patent No. 5,478,745 to Samulski *et al.*

**[0065]** Parvovirus genomes have palindromic sequences at both their 5' and 3' ends. The palindromic nature of the sequences leads to the formation of a hairpin structure that is stabilized by the formation of hydrogen bonds between the complementary base pairs. This hairpin structure is believed to adopt a "Y" or a "T" shape. *See, e.g.*, FIELDS *et al.*, VIROLOGY, volume 2, chapters 69 & 70 (4th ed., Lippincott-Raven Publishers).

**[0066]** An "AAV inverted terminal repeat" or "AAV ITR" may be from any AAV, including but not limited to serotypes 1, 2, 3a, 3b, 4, 5, 6, 7, 8, 9, 10, 11, or 13, snake AAV, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV, goat AAV, shrimp AAV, or any other AAV now known or later discovered (*see, e.g.*, **Table 1**). An AAV ITR need not have the native terminal repeat sequence (*e.g.*, a native AAV ITR sequence may be altered by insertion, deletion, truncation and/or missense mutations), as long as the terminal repeat mediates the desired functions, *e.g.*, replication, virus packaging, persistence, and/or provirus rescue, and the like.

**[0067]** The virus vectors of the invention can further be "targeted" virus vectors (*e.g.*, having a directed tropism) and/or a "hybrid" parvovirus (*i.e.*, in which the viral ITRs and

viral capsid are from different parvoviruses) as described in international patent publication WO 00/28004 and Chao *et al.*, (2000) *Mol. Therapy* 2:619.

**[0068]** Further, the viral capsid or genomic elements can contain other modifications, including insertions, deletions and/or substitutions.

**[0069]** As used herein, the term "amino acid" encompasses any naturally occurring amino acids, modified forms thereof, and synthetic amino acids.

**[0070]** Naturally occurring, levorotatory (L-) amino acids are shown in **Table 2**.

**Table 2**

| Amino Acid Residue | Abbreviation | |
|---|---|---|
| | Three-Letter Code | One-Letter Code |
| Alanine | Ala | A |
| Arginine | Arg | R |
| Asparagine | Asn | N |
| Aspartic acid (Aspartate) | Asp | D |
| Cysteine | Cys | C |
| Glutamine | Gln | Q |
| Glutamic acid (Glutamate) | Glu | E |
| Glycine | Gly | G |
| Histidine | His | H |
| Isoleucine | Ile | I |
| Leucine | Leu | L |
| Lysine | Lys | K |
| Methionine | Met | M |
| Phenylalanine | Phe | F |
| Proline | Pro | P |
| Serine | Ser | S |
| Threonine | Thr | T |
| Tryptophan | Trp | W |
| Tyrosine | Tyr | Y |
| Valine | Val | V |

**[0071]** Alternatively, the amino acid can be a modified amino acid residue (nonlimiting examples are shown in **Table 3**) or can be an amino acid that is modified by post-translation modification (*e.g.*, acetylation, amidation, formylation, hydroxylation, methylation, phosphorylation or sulfatation).

**Table 3**: Amino Acid Residue Derivatives

| Modified Amino Acid Residue | Abbreviation |
|---|---|
| 2-Aminoadipic acid | Aad |
| 3-Aminoadipic acid | bAad |
| beta-Alanine, beta-Aminoproprionic acid | bAla |
| 2-Aminobutyric acid | Abu |
| 4-Aminobutyric acid, Piperidinic acid | 4Abu |
| 6-Aminocaproic acid | Acp |
| 2-Aminoheptanoic acid | Ahe |
| 2-Aminoisobutyric acid | Aib |
| 3-Aminoisobutyric acid | bAib |
| 2-Aminopimelic acid | Apm |
| t-butylalanine | t-BuA |
| Citrulline | Cit |
| Cyclohexylalanine | Cha |
| 2,4-Diaminobutyric acid | Dbu |
| Desmosine | Des |
| 2,2'-Diaminopimelic acid | Dpm |
| 2,3-Diaminoproprionic acid | Dpr |
| N-Ethylglycine | EtGly |
| N-Ethylasparagine | EtAsn |
| Homoarginine | hArg |
| Homocysteine | hCys |
| Homoserine | hSer |
| Hydroxylysine | Hyl |
| Allo-Hydroxylysine | aHyl |
| 3-Hydroxyproline | 3Hyp |
| 4-Hydroxyproline | 4Hyp |
| Isodesmosine | Ide |
| allo-Isoleucine | aIle |
| Methionine sulfoxide | MSO |
| N-Methylglycine, sarcosine | MeGly |
| N-Methylisoleucine | MeIle |
| 6-N-Methyllysine | MeLys |
| N-Methylvaline | MeVal |
| 2-Naphthylalanine | 2-Nal |
| Norvaline | Nva |
| Norleucine | Nle |
| Ornithine | Orn |
| 4-Chlorophenylalanine | Phe(4-Cl) |
| 2-Fluorophenylalanine | Phe(2-F) |
| 3-Fluorophenylalanine | Phe(3-F) |
| 4-Fluorophenylalanine | Phe(4-F) |
| Phenylglycine | Phg |
| Beta-2-thienylalanine | Thi |

**[0072]** Further, the non-naturally occurring amino acid can be an "unnatural" amino acid as described by Wang *et al.*, (2006) *Annu. Rev. Biophys. Biomol. Struct.* 35:225-49. These unnatural amino acids can advantageously be used to chemically link molecules of interest to the AAV capsid protein.

**[0073]** The term "template" or "substrate" is used herein to refer to a polynucleotide sequence that may be replicated to produce the parvovirus viral DNA. For the purpose of vector production, the template will typically be embedded within a larger nucleotide sequence or construct, including but not limited to a plasmid, naked DNA vector, bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC) or a viral vector (*e.g.*, adenovirus, herpesvirus, Epstein-Barr Virus, AAV, baculoviral, retroviral vectors, and the like). Alternatively, the template may be stably incorporated into the chromosome of a packaging cell.

**[0074]** As used herein, parvovirus or AAV "Rep coding sequences" indicate the nucleic acid sequences that encode the parvoviral or AAV non-structural proteins that mediate viral replication and the production of new virus particles. The parvovirus and AAV replication genes and proteins have been described in, *e.g.*, FIELDS *et al.*, VIROLOGY, volume 2, chapters 69 & 70 (4th ed., Lippincott-Raven Publishers).

**[0075]** The "Rep coding sequences" need not encode all of the parvoviral or AAV Rep proteins. For example, with respect to AAV, the Rep coding sequences do not need to encode all four AAV Rep proteins (Rep78, Rep 68, Rep52 and Rep40), in fact, it is believed that AAV5 only expresses the spliced Rep68 and Rep40 proteins. In representative embodiments, the Rep coding sequences encode at least those replication proteins that are necessary for viral genome replication and packaging into new virions. The Rep coding sequences will generally encode at least one large Rep protein (*i.e.*, Rep78/68) and one small Rep protein (*i.e.*, Rep52/40). In particular embodiments, the Rep coding sequences encode the AAV Rep78 protein and the AAV Rep52 and/or Rep40 proteins. In other embodiments, the Rep coding sequences encode the Rep68 and the Rep52 and/or Rep40 proteins. In a still further embodiment, the Rep coding sequences encode the Rep68 and Rep52 proteins, Rep68 and Rep40 proteins, Rep78 and Rep52 proteins, or Rep78 and Rep40 proteins.

**[0076]** As used herein, the term "large Rep protein" refers to Rep68 and/or Rep78. Large Rep proteins of the claimed invention may be either wild-type or synthetic. A wild-type large Rep protein may be from any parvovirus or AAV, including but not limited to serotypes 1, 2, 3a, 3b, 4, 5, 6, 7, 8, 9, 10, 11, or 13, or any other AAV now known or later discovered (*see, e.g.*, **Table 1**). A synthetic large Rep protein may be altered by insertion, deletion, truncation

and/or missense mutations.

**[0077]** Those skilled in the art will further appreciate that it is not necessary that the replication proteins be encoded by the same polynucleotide. For example, for MVM, the NS-1 and NS-2 proteins (which are splice variants) may be expressed independently of one another. Likewise, for AAV, the p19 promoter may be inactivated and the large Rep protein(s) expressed from one polynucleotide and the small Rep protein(s) expressed from a different polynucleotide. Typically, however, it will be more convenient to express the replication proteins from a single construct. In some systems, the viral promoters (*e.g.*, AAV p19 promoter) may not be recognized by the cell, and it is therefore necessary to express the large and small Rep proteins from separate expression cassettes. In other instances, it may be desirable to express the large Rep and small Rep proteins separately, *i.e.*, under the control of separate transcriptional and/or translational control elements. For example, it may be desirable to control expression of the large Rep proteins, so as to decrease the ratio of large to small Rep proteins. In the case of insect cells, it may be advantageous to down-regulate expression of the large Rep proteins (*e.g.*, Rep78/68) to avoid toxicity to the cells (*see, e.g.*, Urabe *et al.*, (2002) *Human Gene Therapy* 13:1935).

**[0078]** As used herein, the parvovirus or AAV "cap coding sequences" encode the structural proteins that form a functional parvovirus or AAV capsid (*i.e.*, can package DNA and infect target cells). Typically, the cap coding sequences will encode all of the parvovirus or AAV capsid subunits, but less than all of the capsid subunits may be encoded as long as a functional capsid is produced. Typically, but not necessarily, the cap coding sequences will be present on a single nucleic acid molecule.

**[0079]** The capsid structure of autonomous parvoviruses and AAV are described in more detail in BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapters 69 & 70 (4th ed., Lippincott-Raven Publishers).

**Parvovirus Vectors Expressing IDUA**

**[0080]** The present invention provides parvovirus vectors, *e.g.*, AAV vectors, that comprise a nucleotide sequence encoding IDUA and are capable of expressing IDUA in the cornea of a subject.

**[0081]** One aspect of the invention relates to a recombinant nucleic acid comprising, consisting essentially of, or consisting of a nucleotide sequence encoding human alpha-L-iduronidase (IDUA), wherein the nucleotide sequence has been codon-optimized for expression in human cells. In certain embodiments, the nucleic acid is a non-naturally

occurring sequence. In some embodiments, the nucleic acid comprises, consists essentially of, or consists of a nucleotide sequence that is at least 90% identical to SEQ ID NO:1, *e.g.*, at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:1. In some embodiments, the nucleic acid comprises, consists essentially of, or consists of the nucleotide sequence of SEQ ID NO:1. In some embodiments, the nucleic acid comprises at least 10 contiguous nucleotides of SEQ ID NO:1, *e.g.*, at least 10, 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800 or more.

SEQ ID NO:1

```
   1 CACCGGTCGC CACCATGCGA CCACTGAGAC CACGGGCCGC TCTGCTGGCT
  51 CTGCTGGCTT CACTGCTGGC CGCTCCCCCT GTCGCTCCTG CTGAGGCTCC
 101 CCACCTGGTG CATGTGGACG CAGCTCGCGC CCTGTGGCCA CTGAGGAGAT
 151 TCTGGAGGAG CACAGGCTTT TGCCCACCTC TGCCTCACAG CCAGGCTGAC
 201 CAGTACGTGC TGTCCTGGGA TCAGCAGCTG AACCTGGCAT ATGTGGGAGC
 251 CGTCCCCCAC AGGGGGATCA AACAGGTGAG AACTCATTGG CTGCTGGAGC
 301 TGGTCACCAC ACGAGGATCT ACTGGAAGGG GGCTGAGTTA CAACTTCACC
 351 CACCTGGACG GCTATCTGGA TCTGCTGAGA GAGAATCAGC TGCTGCCTGG
 401 ATTTGAACTG ATGGGCTCAG CCAGCGGACA TTTCACCGAC TTTGAGGATA
 451 AGCAGCAGGT GTTCGAATGG AAAGACCTGG TCAGCTCCCT GGCTCGGCGC
 501 TACATTGGGC GGTATGGCCT GGCACACGTG AGTAAGTGGA ACTTTGAGAC
 551 TTGGAATGAA CCAGACCACC ATGACTTCGA TAACGTGTCA ATGACCATGC
 601 AGGGGTTTCT GAATTACTAT GATGCCTGCT CCGAGGGCCT GCGGGCAGCC
 651 TCTCCAGCTC TGCGACTGGG AGGACCAGGC GATTCCTTCC ACACACCACC
 701 CAGAAGTCCC CTGTCATGGG GCCTGCTGCG GCACTGTCAT GACGGAACCA
 751 ACTTCTTTAC AGGAGAAGCA GGGGTGAGAC TGGATTACAT CTCCCTGCAT
 801 CGAAAGGGGG CCAGGTCTAG TATCTCTATT CTGGAGCAGG AAAAGGTGGT
 851 CGCTCAGCAG ATCCGGCAGC TGTTCCCCAA ATTTGCCGAC ACACCTATCT
 901 ACAATGACGA GGCTGATCCT CTGGTGGGAT GGTCTCTGCC TCAGCCATGG
 951 CGAGCCGATG TGACTTATGC TGCAATGGTG GTCAAAGTCA TTGCTCAGCA
1001 CCAGAACCTG CTGCTGGCAA ATACTACCAG TGCTTTCCCA TACGCACTGC
1051 TGAGTAACGA CAATGCCTTC CTGTCATATC ACCCCCATCC TTTTGCCCAG
1101 AGAACACTGA CTGCTCGGTT TCAGGTGAAC AATACCCGAC CTCCACATGT
1151 GCAGCTGCTG AGGAAGCCTG TCCTGACAGC CATGGGCCTG CTGGCTCTGC
1201 TGGACGAGGA ACAGCTGTGG GCAGAGGTGT CTCAGGCCGG GACTGTCCTG
1251 GATAGTAACC ACACCGTGGG CGTCCTGGCT TCTGCACATC GACCTCAGGG
1301 ACCAGCAGAC GCTTGGCGAG CAGCTGTGCT GATCTACGCA TCAGACGATA
1351 CACGAGCACA CCCTAACCGA AGCGTGGCAG TCACTCTGCG ACTGCGAGGA
1401 GTGCCACCTG GACCAGGACT GGTGTACGTC ACCCGCTATC TGGACAATGG
1451 CCTGTGCTCT CCCGATGGAG AGTGGCGAAG GCTGGGGAGG CCCGTGTTCC
1501 CTACAGCAGA GCAGTTTAGA CGGATGAGAG CAGCCGAAGA TCCAGTGGCT
1551 GCAGCACCAC GACCACTGCC TGCAGGAGGC AGACTGACCC TGCGGCCAGC
1601 CCTGCGCCTG CCAAGCCTGC TGCTGGTGCA CGTCTGCGCA AGGCCTGAAA
1651 AGCCACCCGG ACAGGTGACA AGGCTGAGAG CTCTGCCACT GACTCAGGGA
1701 CAGCTGGTGC TGGTCTGGTC AGACGAGCAT GTGGGGAGCA AATGTCTGTG
1751 GACTTACGAA ATTCAGTTCA GCCAGGATGG GAAGGCCTAT ACCCCTGTGA
1801 GCCGGAAACC CAGCACCTTC AACCTGTTCG TCTTTTCCCC AGACACCGGG
1851 GCCGTGTCCG GCTCTTACCG GGTCCGCGCT CTGGACTATT GGGCAAGGCC
1901 AGGCCCCTTC AGCGATCCTG TGCCATACCT GGAAGTGCCT GTGCCTCGCG
1951 GCCCACCATC TCCTGGAAAC CCTTGAGGGG ATCCGTCGAC TAG
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**[0082]** Methods of codon optimizing a nucleotide sequence to maximize expression in an organism are well known in the art and can be carried out using software available to the public. The wild-type sequence of human IDUA is known in the art and can be found in databases such as GenBank. Examples of human IDUA accession numbers include A26494, AK291816, and AH002600, incorporated by reference herein in their entirety.

**[0083]** The invention also provides a viral vector genome comprising the IDUA nucleic acid of the invention. In certain embodiments, the IDUA nucleic acid is the wild-type human IDUA sequence or the codon-optimized sequence. The viral vector genome may be a parvovirus vector genome, *e.g.*, an AAV vector genome. The viral vector may further comprise a promoter operably linked to the IDUA nucleic acid. In some embodiments, the promoter may be a constitutive promoter, *e.g.*, a CMV promoter. In other embodiments, the promoter may be a tissue-specific or preferred promoter. The invention further provides a cell *in vitro* comprising the AAV vector genome of the invention, *e.g.*, stably incorporated into the genome of the cell. The invention further provides a recombinant parvovirus particle (*e.g.*, a recombinant AAV particle) comprising the viral vector genome of the invention. Viral vectors and viral particles are discussed further below.

**[0084]** In certain embodiments, the viral vector exhibits a modified tropism due to the presence of the capsid protein of the invention. In one embodiment, the parvovirus vector exhibits systemic tropism for the cornea. In other embodiments, the parvovirus vector has reduced tropism for liver compared to a virus vector comprising a wild-type capsid protein.

## Methods of Producing Virus Vectors

**[0085]** The present invention further provides methods of producing virus vectors. In one particular embodiment, the present invention provides a method of producing a recombinant parvovirus particle, comprising providing to a cell permissive for parvovirus replication: (a) a recombinant parvovirus template comprising (i) a nucleic acid encoding IDUA, and (ii) a parvovirus ITR; (b) a polynucleotide comprising Rep and Cap coding sequences; under conditions sufficient for the replication and packaging of the recombinant parvovirus template; whereby recombinant parvovirus particles are produced in the cell. Conditions sufficient for the replication and packaging of the recombinant parvovirus template can be, *e.g.*, the presence of AAV sequences sufficient for replication of the parvovirus template and encapsidation into parvovirus capsids (*e.g.*, parvovirus *rep* sequences and parvovirus *cap*

sequences) and helper sequences from adenovirus and/or herpesvirus. In particular embodiments, the parvovirus template comprises two parvovirus ITR sequences, which are located 5' and 3' to the heterologous nucleic acid sequence, although they need not be directly contiguous thereto.

**[0086]** In some embodiments, the recombinant parvovirus template comprises an ITR that is not resolved by Rep to make duplexed AAV vectors as described in international patent publication WO 01/92551.

**[0087]** The parvovirus template and parvovirus *rep* and *cap* sequences are provided under conditions such that virus vector comprising the parvovirus template packaged within the parvovirus capsid is produced in the cell. The method can further comprise the step of collecting the virus vector from the cell. The virus vector can be collected from the medium and/or by lysing the cells.

**[0088]** The cell can be a cell that is permissive for parvoviral viral replication. Any suitable cell known in the art may be employed. In particular embodiments, the cell is a mammalian cell (*e.g.*, a primate or human cell). As another option, the cell can be a trans-complementing packaging cell line that provide functions deleted from a replication-defective helper virus, *e.g.*, 293 cells or other E1a trans-complementing cells.

**[0089]** The parvovirus replication and capsid sequences may be provided by any method known in the art. Current protocols typically express the parvovirus *rep/cap* genes on a single plasmid. The parvovirus replication and packaging sequences need not be provided together, although it may be convenient to do so. The parvovirus *rep* and/or *cap* sequences may be provided by any viral or non-viral vector. For example, the *rep/cap* sequences may be provided by a hybrid adenovirus or herpesvirus vector (*e.g.*, inserted into the E1a or E3 regions of a deleted adenovirus vector). EBV vectors may also be employed to express the parvovirus *cap* and *rep* genes. One advantage of this method is that EBV vectors are episomal, yet will maintain a high copy number throughout successive cell divisions (*i.e.*, are stably integrated into the cell as extra-chromosomal elements, designated as an "EBV based nuclear episome," *see* Margolski, (1992) *Curr. Top. Microbiol. Immun.* 158:67).

**[0090]** As a further alternative, the *rep/cap* sequences may be stably incorporated into a cell.

**[0091]** Typically the parvovirus *rep/cap* sequences will not be flanked by the TRs, to prevent rescue and/or packaging of these sequences.

**[0092]** The parvovirus template can be provided to the cell using any method known in the art. For example, the template can be supplied by a non-viral (*e.g.*, plasmid) or viral vector.

In particular embodiments, the parvovirus template is supplied by a herpesvirus or adenovirus vector (*e.g.*, inserted into the E1a or E3 regions of a deleted adenovirus). As another illustration, Palombo *et al.*, (1998) *J. Virology* 72:5025, describes a baculovirus vector carrying a reporter gene flanked by the AAV TRs. EBV vectors may also be employed to deliver the template, as described above with respect to the *rep/cap* genes.

**[0093]** In another representative embodiment, the parvovirus template is provided by a replicating rAAV virus. In still other embodiments, an AAV provirus comprising the parvovirus template is stably integrated into the chromosome of the cell.

**[0094]** To enhance virus titers, helper virus functions (*e.g.*, adenovirus or herpesvirus) that promote a productive parvovirus infection can be provided to the cell. Helper virus sequences necessary for parvovirus replication are known in the art. Typically, these sequences will be provided by a helper adenovirus or herpesvirus vector. Alternatively, the adenovirus or herpesvirus sequences can be provided by another non-viral or viral vector, *e.g.*, as a non-infectious adenovirus miniplasmid that carries all of the helper genes that promote efficient parvovirus production as described by Ferrari *et al.*, (1997) *Nature Med.* 3:1295, and U.S. Patent Nos. 6,040,183 and 6,093,570.

**[0095]** Further, the helper virus functions may be provided by a packaging cell with the helper sequences embedded in the chromosome or maintained as a stable extrachromosomal element. Generally, the helper virus sequences cannot be packaged into AAV virions, *e.g.*, are not flanked by ITRs.

**[0096]** Those skilled in the art will appreciate that it may be advantageous to provide the parvovirus replication and capsid sequences and the helper virus sequences (*e.g.*, adenovirus sequences) on a single helper construct. This helper construct may be a non-viral or viral construct. As one nonlimiting illustration, the helper construct can be a hybrid adenovirus or hybrid herpesvirus comprising the AAV *rep/cap* genes.

**[0097]** In one particular embodiment, the parvovirus *rep/cap* sequences and the adenovirus helper sequences are supplied by a single adenovirus helper vector. This vector can further comprise the parvovirus template. The parvovirus *rep/cap* sequences and/or the parvovirus template can be inserted into a deleted region (*e.g.*, the E1a or E3 regions) of the adenovirus.

**[0098]** In a further embodiment, the parvovirus *rep/cap* sequences and the adenovirus helper sequences are supplied by a single adenovirus helper vector. According to this embodiment, the parvovirus template can be provided as a plasmid template.

**[0099]** In another illustrative embodiment, the parvovirus *rep/cap* sequences and adenovirus helper sequences are provided by a single adenovirus helper vector, and the

parvovirus template is integrated into the cell as a provirus. Alternatively, the parvovirus template is provided by an EBV vector that is maintained within the cell as an extrachromosomal element (*e.g.*, as an EBV based nuclear episome).

**[0100]** In a further exemplary embodiment, the parvovirus *rep/cap* sequences and adenovirus helper sequences are provided by a single adenovirus helper. The parvovirus template can be provided as a separate replicating viral vector. For example, the parvovirus template can be provided by a parvovirus particle or a second recombinant adenovirus particle.

**[0101]** According to the foregoing methods, the hybrid adenovirus vector typically comprises the adenovirus 5' and 3' *cis* sequences sufficient for adenovirus replication and packaging (*i.e.*, the adenovirus terminal repeats and PAC sequence). The parvovirus *rep/cap* sequences and, if present, the AAV template are embedded in the adenovirus backbone and are flanked by the 5' and 3' *cis* sequences, so that these sequences may be packaged into adenovirus capsids. As described above, the adenovirus helper sequences and the parvovirus *rep/cap* sequences are generally not flanked by ITRs so that these sequences are not packaged into the parvovirus virions.

**[0102]** Zhang *et al.*, ((2001) *Gene Ther.* 18:704-12) describe a chimeric helper comprising both adenovirus and the AAV *rep* and *cap* genes.

**[0103]** Herpesvirus may also be used as a helper virus in parvovirus packaging methods. Hybrid herpesviruses encoding the parvovirus Rep protein(s) may advantageously facilitate scalable parvovirus vector production schemes. A hybrid herpes simplex virus type I (HSV-1) vector expressing the AAV-2 *rep* and *cap* genes has been described (Conway *et al.*, (1999) *Gene Ther.* 6:986 and WO 00/17377.

**[0104]** As a further alternative, the virus vectors of the invention can be produced in insect cells using baculovirus vectors to deliver the *rep/cap* genes and parvovirus template as described, for example, by Urabe *et al.*, (2002) *Human Gene Ther.* 13:1935-43.

**[0105]** Parvovirus vector stocks free of contaminating helper virus may be obtained by any method known in the art. For example, parvovirus and helper virus may be readily differentiated based on size. Parvovirus may also be separated away from helper virus based on affinity for a heparin substrate (Zolotukhin *et al.*, (1999) *Gene Therapy* 6:973). Deleted replication-defective helper viruses can be used so that any contaminating helper virus is not replication competent. As a further alternative, an adenovirus helper lacking late gene expression may be employed, as only adenovirus early gene expression is required to mediate

packaging of parvovirus. Adenovirus mutants defective for late gene expression are known in the art (*e.g.*, ts100K and ts149 adenovirus mutants).

**Recombinant Virus Vectors**

**[0106]** The virus vectors of the present invention are useful for the delivery of nucleic acids to cells *in vitro*, *ex vivo*, and *in vivo*. In particular, the virus vectors can be advantageously employed to deliver or transfer nucleic acids to animal, including mammalian, cells. In particular, the virus vectors of the present invention are useful for the delivery of a nucleic acid encoding IDUA to the cornea of a subject.

**[0107]** Cornea targeted AAV gene therapy has been investigated in animal models primarily following two routes of administration; topical applications in wound healing assays and direct injection to the corneal stroma. Regarding topical applications, AAV serotype 9 (AAV9) was reported most efficient for stromal transduction, however, this was nearly entirely localized to the epithelial/stromal boundary (Sharma *et al., Exp. Eye Res. 91(3)*:440 (2010)). Regarding AAV gene delivery following intrastromal injection into human cornea explants, it was observed that AAV8 was more efficient than AAV2 or AAV1 for stromal transduction, which encompassed multiple cell types including $CD34^+$ keratocytes and macrophages (Hippert *et al., PLoS One, 7(4)*:e35318 (2012)). Importantly, both of these routes of drug administration observed no deleterious consequences related to the AAV vector (Sharma *et al., Exp. Eye Res. 91(3)*:440 (2010); Hippert *et al., PLoS One, 7(4)*:e35318 (2012); Mohan *et al., PLoS One 6(10)*:e26432 (2011)).

**[0108]** It will be understood by those skilled in the art that the nucleic acid encoding IDUA can be operably associated with appropriate control sequences. For example, the nucleic acid can be operably associated with expression control elements, such as transcription/translation control signals, origins of replication, polyadenylation signals, internal ribosome entry sites (IRES), promoters, and/or enhancers, and the like.

**[0109]** Those skilled in the art will appreciate that a variety of promoter/enhancer elements can be used depending on the level and tissue-specific expression desired. The promoter/enhancer can be constitutive or inducible, depending on the pattern of expression desired. The promoter/enhancer can be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced.

**[0110]** In particular embodiments, the promoter/enhancer elements can be native to the target cell or subject to be treated. In representative embodiments, the promoters/enhancer

element can be native to the IDUA nucleic acid sequence. The promoter/enhancer element is generally chosen so that it functions in the target cell(s) of interest. Further, in particular embodiments the promoter/enhancer element is a mammalian promoter/enhancer element. The promoter/enhancer element may be constitutive or inducible.

**[0111]** Inducible expression control elements are typically advantageous in those applications in which it is desirable to provide regulation over expression of the nucleic acid sequence. Inducible promoters/enhancer elements for gene delivery can be tissue-specific or –preferred promoter/enhancer elements, and include eye specific or preferred (including retina-specific and cornea-specific) promoter/enhancer elements. Other inducible promoter/enhancer elements include hormone-inducible and metal-inducible elements. Exemplary inducible promoters/enhancer elements include, but are not limited to, a Tet on/off element, a RU486-inducible promoter, an ecdysone-inducible promoter, a rapamycin-inducible promoter, and a metallothionein promoter.

**[0112]** In embodiments wherein the nucleic acid sequence is transcribed and then translated in the target cells, specific initiation signals are generally included for efficient translation of inserted protein coding sequences. These exogenous translational control sequences, which may include the ATG initiation codon and adjacent sequences, can be of a variety of origins, both natural and synthetic.

**[0113]** The virus vectors of the invention can be parvovirus vectors, *e.g.*, AAV vectors. The AAV vectors may be any AAV serotype. In some embodiments, the AAV vector is an AAV2, AAV8, or AAV9 vector. In some embodiments, the AAV vector is a hybrid vector, *e.g.*, one having a capsid protein from one serotype and a genome from another serotype or one having a synthetic capsid protein. In certain embodiments, the vector comprises a hybrid capsid with an altered tropism. In one example the hybrid capsid comprising a glycan binding site (*e.g.*, a galactose binding site) from one serotype (*e.g.*, AAV9) in a capsid sequence from another serotype (*e.g.*, AAV8) (*see, e.g.*, WO 2014/144229, incorporated by reference herein in its entirety).

**[0114]** The virus vectors according to the present invention provide a means for delivering IDUA nucleic acids into a broad range of cells, including dividing and non-dividing cells. The virus vectors can be employed to deliver the nucleic acid to a cell *in vitro*, *e.g.*, to produce a polypeptide *in vitro* or for *ex vivo* gene therapy. The virus vectors are additionally useful in a method of delivering the nucleic acid to a subject in need thereof, *e.g.*, to express IDUA. In this manner, the polypeptide can be produced *in vivo* in the subject. The subject can be in need of the polypeptide because the subject has a deficiency of the polypeptide.

Further, the method can be practiced because the production of the polypeptide in the subject may impart some beneficial effect.

**[0115]** The virus vectors can also be used to produce IDUA in cultured cells or in a subject (*e.g.*, using the subject as a bioreactor to produce the polypeptide or to observe the effects of the polypeptide on the subject, for example, in connection with screening methods).

**[0116]** The virus vectors of the present invention can be employed to deliver a nucleic acid encoding IDUA to treat and/or prevent any disease state for which it is beneficial to deliver IDUA, *e.g.*, MPS I.

**[0117]** Virus vectors according to the instant invention find use in diagnostic and screening methods, whereby the IDUA nucleic acid is transiently or stably expressed in a cell culture system, in an organ or organ culture (*e.g.*, an eye),or alternatively, a transgenic animal model.

**[0118]** The virus vectors of the present invention can also be used for various non-therapeutic purposes, including but not limited to use in protocols to assess gene targeting, clearance, transcription, translation, *etc.*, as would be apparent to one skilled in the art. The virus vectors can also be used for the purpose of evaluating safety (spread, toxicity, immunogenicity, *etc.*). Such data, for example, are considered by the United States Food and Drug Administration as part of the regulatory approval process prior to evaluation of clinical efficacy.

**[0119]** Alternatively, the virus vector may be administered to a cell *ex vivo*, *e.g.*, a corneal explant, and the altered cell or explant is administered to the subject. The virus vector comprising the IDUA nucleic acid is introduced into the cell, and the cell is administered to the subject, where the nucleic acid can be expressed.

**Subjects, Pharmaceutical Formulations, and Modes of Administration**

**[0120]** Virus vectors and capsids according to the present invention find use in both veterinary and medical applications. Suitable subjects include both avians and mammals. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys, pheasant, parrots, parakeets, and the like. The term "mammal" as used herein includes, but is not limited to, humans, non-human primates, bovines, ovines, caprines, equines, felines, canines, lagomorphs, *etc.* Human subjects include neonates, infants, juveniles and adults.

**[0121]** In particular embodiments, the present invention provides a pharmaceutical composition comprising a virus vector of the invention in a pharmaceutically acceptable carrier and, optionally, other medicinal agents, pharmaceutical agents, stabilizing agents,

buffers, carriers, adjuvants, diluents, *etc.* For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and optionally can be in solid or liquid particulate form.

**[0122]** By "pharmaceutically acceptable" it is meant a material that is not toxic or otherwise undesirable, *i.e.*, the material may be administered to a subject without causing any undesirable biological effects.

**[0123]** One aspect of the present invention is a method of transferring a nucleic acid to a cell *in vitro*. The virus vector may be introduced into the cells at the appropriate multiplicity of infection according to standard transduction methods suitable for the particular target cells. Titers of virus vector to administer can vary, depending upon the target cell type and number, and the particular virus vector, and can be determined by those of skill in the art without undue experimentation. In representative embodiments, at least about $10^3$ infectious units, more preferably at least about $10^5$ infectious units are introduced to the cell.

**[0124]** The cell(s) into which the virus vector is introduced can be of any type, including but not limited to cells of the eye (including retinal cells, retinal pigment epithelium, and corneal cells (*e.g.*, keratocytes, epithelial cells, and endothelial cells). Moreover, the cell can be from any species of origin, as indicated above.

**[0125]** The virus vector can be introduced into cells *in vitro* for the purpose of administering the modified cell to a subject. In particular embodiments, the cells have been removed from a subject, the virus vector is introduced therein, and the cells are then administered back into the subject. Methods of removing cells from subject for manipulation *ex vivo*, followed by introduction back into the subject are known in the art (*see, e.g.*, U.S. Patent No. 5,399,346). Alternatively, the recombinant virus vector can be introduced into cells from a donor subject, into cultured cells, or into cells from any other suitable source, and the cells are administered to a subject in need thereof (*i.e.*, a "recipient" subject).

**[0126]** Suitable cells for *ex vivo* gene delivery are as described above. Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like. Typically, at least about $10^2$ to about $10^8$ cells or at least about $10^3$ to about $10^6$ cells will be administered per dose in a pharmaceutically acceptable carrier. In particular embodiments, the cells transduced with the virus vector are administered to the subject in a treatment effective or prevention effective amount in combination with a pharmaceutical carrier.

**[0127]** A further aspect of the invention is a method of administering the virus vector to subjects. Administration of the virus vectors according to the present invention to a human subject or an animal in need thereof can be by any means known in the art. Optionally, the virus vector is delivered in a treatment effective or prevention effective dose in a pharmaceutically acceptable carrier.

**[0128]** Dosages of the virus vector to be administered to a subject depend upon the mode of administration, the disease or condition to be treated and/or prevented, the individual subject's condition, the particular virus vector, and the nucleic acid to be delivered, and the like, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are titers of at least about $10^5$, $10^6$, $10^7$, $10^8$, $10^9$, $10^{10}$, $10^{11}$, $10^{12}$, $10^{13}$, $10^{14}$, $10^{15}$ transducing units, optionally about $10^8$ to about $10^{13}$ transducing units.

**[0129]** In particular embodiments, more than one administration (*e.g.*, two, three, four or more administrations) may be employed to achieve the desired level of gene expression over a period of various intervals, *e.g.*, daily, weekly, monthly, yearly, *etc.*

**[0130]** Exemplary modes of administration to the cornea include intrastromal, topical, intravitreal, and subretinal.

**[0131]** Delivery to a target tissue can also be achieved by delivering a depot comprising the virus vector. In representative embodiments, a depot comprising the virus vector is implanted into the cornea or other tissue of the eye or the tissue can be contacted with a film or other matrix comprising the virus vector. Such implantable matrices or substrates are described in U.S. Patent No. 7,201,898.

**[0132]** In particular embodiments, a virus vector according to the present invention is administered to the cornea to treat, delay the onset of and/or prevent corneal clouding and/or blindness associated with MPS I.

**[0133]** Thus, as one aspect, the invention further encompasses a method of delivering IDUA to the cornea of a subject, comprising administering to the cornea of the subject an effective amount of an AAV particle that expresses IDUA, thereby delivering IDUA to the cornea of the subject.

**[0134]** In another aspect, the invention further encompasses a method of treating, delaying the onset of, and/or preventing MPS I-associated corneal clouding in a subject in need thereof, comprising administering to the cornea of the subject a therapeutically effective amount of an AAV particle that expresses IDUA, thereby treating, delaying the onset of, and/or preventing MPS I-associated corneal clouding in the subject.

**[0135]** In a further aspect, the invention further encompasses a method of delivering IDUA

to a cornea *in vitro* or *ex vivo*, *e.g.*, prior to transplantation in a subject in need thereof, comprising contacting the cornea with an effective amount of an AAV particle that expresses IDUA, thereby delivering IDUA to the cornea. In some embodiments, the cornea may be incubated with the AAV particle or the AAV particle may be injected into the cornea.

**[0136]** In the methods of the invention, the subject may be one has been diagnosed with MPS I or is suspected of having MPS I. In certain embodiments, the subject is an infant or child, *e.g.*, less than 18 years old, *e.g.*, less than 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 years old. In some embodiments, the subject has not developed clouding of the cornea. In other embodiments, the subject has at least partial clouding of the cornea, *e.g.*, the subject's eyesight has been reduced by less than about 10%, *e.g.*, less than about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% relative to a subject that does not have MPS I.

**[0137]** Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus vector and/or virus capsids of the invention in a local manner, for example, in a depot or sustained-release formulation. Further, the virus vector and/or virus capsid can be delivered adhered to a surgically implantable matrix (*e.g.*, as described in U.S. Patent Publication No. 2004-0013645).

**[0138]** Having described the present invention, the same will be explained in greater detail in the following examples, which are included herein for illustration purposes only, and which are not intended to be limiting to the invention.

## Example 1

### Materials and Methods

**[0139]** **Production of AAV vectors.** For cell culture experiments, a previously described triple transfection method was used to generate the vectors used herein (Grieger *et al., Nat. Protoc. 1(3)*:1412 (2006)). This method used the pXR2 or pXR8G9 plasmids, which all contain *rep2* of AAV and individually the capsid genes of the indicated serotype. The plasmid containing the intended AAV genome was first constructed by substituting the *egfp* gene in pTR-CMV-eGFP with the codon optimized IDUA cDNA (provided by GenScript) at the AgeI and SalI sites. Following AAV production and cesium chloride gradient separation (Grieger *et al., Nat. Protoc. 1(3)*:1412 (2006)), peak fractions were dialyzed against PBS, and titered by quantitative PCR (CMV_F CAA GTA CGC CCC CTA TTG AC (**SEQ ID NO:2**), CMV_R AAG TCC CGT TGA TTT TGG TG (**SEQ ID NO:3**)) which was confirmed by

Southern dot blot (Grieger *et al., Nat. Protoc. 1(3)*:1412 (2006)). For the experiments in human explants, GMP grade vector preparations were provided by the UNC vector core.

**[0140] Cell culture and vector transduction:** Human embryonic kidney 293 cells, MPS I patient fibroblasts, and normal human fibroblasts (Simpson *et al., J. Carcinog. 4*:18 (2005)) were maintained at 37 °C in a 5% $CO_2$ atmosphere in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and penicillin–streptomycin (100 U/ml). Transduction of cultured cells was performed in a 24 well plate in a fixed volume. For these experiments, vector was added to the wells at the indicated viral genome per cell dose and was not removed for the duration of the experiment.

**[0141] Human Cornea Experiments:** All experimental protocols were approved by the University of North Carolina at Chapel Hill. Human corneas were provided by the miracles in sight tissue procurement bank. These deidentified post-mortem tissue experiments were performed in accordance with the human subjects research office of human research ethics at the University of North Carolina at Chapel Hill (IRB-14-1019). The corneas of both genders were maintained in the supplied opti-mem at 37 °C in a 5% $CO_2$ atmosphere. Injections were performed with a 31 gauge insulin syringe. India ink was used as a dye to verify the injections. Corneas were then cultured for 7 days and analyzed as described in the text.

**[0142] Functional IDUA assay:** Quantitative IDUA enzyme activities were measured as previously described (Garcia-Rivera *et al., Brain Res. Bull. 74(6)*:429 (2007)). Briefly, 10 µl of a supernatant or cell lysis solution were incubated with 50 µM 4-methylumbelliferyl alpha-L-iduronide made in 0.4M sodium formate buffer, pH 3.5, containing 0.2% Triton x-100 at 37 °C for 60 min in the dark. Reactions were stopped by adding 80µl of 0.5M NaOH/glycine buffer pH 10.3. The 96 well plates were centrifuged for 1 min 13000rpm at 4 °C and supernatants were transfered to a black 96 well plate, clear bottom with lid for measuring fluorescence at 450nm, following excitation at 365nm. The amount of cleaved substrate was calculated from a standard curve, previously established with 4-methylumbelliferone and expressed as nmol/h/mg for enzyme activity in cell lysates, or nmol/h/10 µl for analysis of supernatants. The protein amount for each sample was determined with the BCA assay.

**[0143] Tunel Assay:** Apoptotic cells generated due to the cellular cytotoxicity during AAV transduction were determined by deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick end labeling with DAB. Tunel assay was performed with TACS2 TdT-Blue Label in situ apoptosis detection kit (Trevigen, Gaithersburg, Md.). Briefly, following deparaffinization and rehydration, the human cornea sections were digested for 20 min in proteinase K. Slides were washed with 1xPBS and the endogenous peroxidase activity was

quenched by incubating the tissues with 3% $H_2O_2$ solution in methanol. After washing the slides with 1x PBS, tissue sections were incubated with 1× TdT labeling buffer for 5 min. The labeling reaction consisting of incubating the cornea tissues with TdT enzyme, dNTPs with biotinylated dUTP, and 1 x manganese chloride in 1x TdT labeling buffer for 1 h in a humidified chamber at 37C. The reaction was completed by incubating with 1x TdT stop buffer. The fragmented DNA was visualized by treating the sections with streptavidin-conjugated horseradish peroxidase and DAB solution.

**[0144] Immunofluorescence Staining:** Human cornea tissues were embedded in paraffin and sectioned at a thickness of 5 μm using a microtome. In order to stain the slide with immunofluorescence antibodies, sections were deparaffinized by incubating the slides in xylene for 5 min, two times total, followed by rehydration by immersing them sequentially in 100%, 95% and 70% ethanol solutions, 5 min each, and finally in water for 5 min. Before staining the sections, antigen retrieval procedure was performed in order to guarantee the exposure of epitopes, which were previously masked due to the paraffin embedding process. Specifically, slides were immerse into pre-heated antigen retrieval solution (Dako) at 98 °C for 7 min. Non-specific binding sites within the tissues were then blocked by incubating the slides with 10% NGS, in 1× PBS for 1 hour at RT. Slides were washed twice for 5 minutes each with 2% NGS in 1× PBS. Sections were then incubated overnight at 4 °C with primary antibody diluted appropriately in a solution consisting of 2% NGS in 1× PBS. After incubation with primary antibody, slides were washed three times, for 5 minutes each, in order to remove non-specifically bound primary antibody. The washing solution contained 2% NGS in 1× PBS. An appropriate fluorescently-labeled secondary antibody (5 μg/ml) was then added to the slides diluted in 2% NGS in 1× PBS, and slides were incubated for 1 hour at 4 °C. Finally, slides were washed twice with 2% NGS in 1× PBS three times, for five minutes each, followed by a last wash with 1× PBS at 4 °C. A couple of drops of Hoechst (Molecular probes- Life technologies, H3569 Hoechst 33258, Pentahydrate - 1 μg/mL) were added for 7 minutes to counterstain the nuclei within the section, followed by a wash with water. Slides were then coverslipped with Cytoseal 60 (Thermo Fisher Scientific (NYSE: TMO)).

**[0145] Primary antibodies used for this study were the following:** For IDUA staining, Rabbit Polyclonal IDUA antibody (Biorbyt, catalog number: orb157615, dilution 1/50); for GFP staining, Chicken anti GFP antibody (Aves, catalog number: GFP-1020, dilution 1/100); for CD34 staining, mouse monoclonal antibody (clone: B-6) (Santa Cruz, sc-74499, dilution 1/100)), for α-Smooth Muscle Actin staining, mouse monoclonal antibody (R&D, clone

#1A4, catalog number MAB1420, dilution 1/100), and for F4/80 marker staining, rat monoclonal antibody, (clone: BM8) (Santa Cruz, sc-52664, dilution 1/100). Secondary antibodies were the following: Alexa Fluor® 594 goat anti-rabbit IgG (A-11012) (Gibco – Invitrogen, Carlsbad, CA), Alexa Fluor® 594 goat anti-chicken IgG (A-11039) (Gibco – Invitrogen, Carlsbad, CA), Alexa Fluor® 594 goat anti-rat IgG (A-11006) (Gibco – Invitrogen, Carlsbad, CA) and Alexa Fluor® 488 goat anti-mouse IgG (A-11001) (Gibco – Invitrogen, Carlsbad, CA). Images from each slide were taken using a Zeiss LSM 780 Confocal Microscope with a 40× objective (Olympus, Tokyo, Japan). Images that comprised the complete human cornea were taken with 10x objective, the title function and then stitched. Images were then processed using Adobe Photoshop. Sections from each tissue were stained with secondary antibody alone as negative control staining in all experiments.

**[0146] MPS I Cell Transfection and Western Blot:** MPS I fibroblast patient cells were plated onto a 24 well plate at 20,000 cells per well. After 24 hours, nine wells were transfected for each treatment adding a mix 1μg of plasmid DNA, 3μl of PEI and 60μl of DMEM to each well. 24 hours after transfection, 40μl of supernatant were collected from each well and 3 wells were combined to form each sample. 40μl of DMEM were added to each well to maintain the same volume for later supernatant collections. 48 hours after transfection, total supernatant was collected combining 3 wells to again form triplicates. At that time total cell protein was harvested by adding 70μl of Mammalian Protein extraction Reagent (Thermo Scientific Cat: 78501), per well and following reagent protocol.

For Western blot, protein lysate was added to a solution of 5% beta-mercapto-ethanol in 4x Nupage sample buffer. The resulting solution was boiled for 10 minutes and chilled on ice for 10 minutes and then run in a 10% Bis-tris pre-cast gel. Gel was run in 1X MOPS running buffer, and transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk in ddH20, and probed with mouse host IDUA antibody (R&D Systems MAB-4119) for 2 hours at 1:500 dilution in PBS-Tween solution (0.5% Tween 20 in 1xPBS) or mouse host beta-actin antibody (Sigma) at 1:5000 dilution in PBS-Tween. This was followed by mouse horse radish peroxidase secondary antibody at 1:10000 dilution in PBS-Tween. Western-Bright Sirius chemi-luminescence reagent was used according to product protocol and blots were exposed using autoradiography film.

**[0147] 293 Cell Transfection comparison of wild-type against codon optimized IDUA:** 293 cells were plated onto a 24 well plate at 100,000 cells per well. After 24 hours, 3 wells were transfected using a mix of 1μg of plasmid DNA, 3μl of PEI and 60μl of DMEM per well. Total cell protein was harvested after 72 hours by adding 70μl of Mammalian Protein

extraction Reagent (Thermo Scientific Cat: 78501), to each well after saving the cell culture supernatant.

**[0148] MPS I Patient Cell Toxicity Assay:** Patient cells were plated onto a 24 well plate at 20,000 cells per well. AAV-2 316 was added after 24 hours in quadruplicates. After 72 hours, supernatant was saved; cells were resuspended using 0.05% trypsin and their corresponding supernatant. All samples were analyzed using a Beckman Vi-cell XR cell viability analyzer.

## Example 2

## AAV IDUA Expression Cassette in MPS I Fibroblasts

**[0149]** To develop an AAV IDUA expression cassette, the human *idua* cDNA (NM_000203) was codon optimized for human expression (opt-IDUA) and situated between the CMV promoter and the SV40 poly-adenylation sequence in an AAV inverted terminal repeat serotype 2 plasmid context (**Fig. 1A**). AAV2-opt-IDUA vectors were prepared as described (Grieger *et al., Adv. Biochem. Eng. Biotechnol. 99*:119 (2005)) and used for characterization in MPS I patient derived fibroblasts. Immortalized normal human fibroblasts (NHF) served as the control cell line (Simpson *et al., J. Carcinog. 4*:18 (2005)). In dose escalation experiments, AAV2-opt-IDUA transduction of MPS I fibroblasts resulted in increasing levels of IDUA restoration, in both cellular lysates and in the culture supernatant (**Fig. 1B**). In fact, as resting levels of IDUA in NHF are relatively low, a dose of 5,000 viral genomes/cell resulted in already supraphysiological levels in both cell lysates and in culture supernatants (**Fig. 1B**). Consistently, IDUA function was restored and elevated in transduced MPS I patient fibroblasts with a 10-fold and 30-fold increases in cellular lysate and supernatant, respectively, at the highest investigated dose (**Figs. 1C and 1D**). Despite IDUA overproduction following AAV vector transduction, no toxicity was observed in patient fibroblasts at any dose using a dye exclusion assay (**Fig. 2**). Importantly, these results demonstrate the functionality and safety of AAV2-opt-IDUA in a MPS I patient context.

## Example 3

## AAV IDUA Expression Cassette in Corneas

**[0150]** An analysis of MPS I patient corneas attributed stromal abnormalities as the cause of the corneal opacity that results in vision loss (Huang *et al., Exp. Eye Res. 62(4)*:377 (1996)). Therefore, direct administration of AAV-opt-IDUA should restore IDUA activity in the corneal stroma compartment of MPS I patients, hopefully preventing or reversing the

MPS I phenotype. Previous reports demonstrated the utility of both AAV8 (Hippert *et al., PLoS One, 7(4)*:e35318 (2012)) and AAV9 (Sharma *et al., Exp. Eye Res. 91(3)*:440 (2010)) for human keratocyte transduction, one of the most prevalent cell types present in the cornea. As such, these capsids, carrying a self-complementary (sc) AAV-CMV-GFP genome, were evaluated in normal human corneal explants, which remain viable for weeks post-mortem. Preliminary experiments demonstrated that a volume of 50µl injected into the stroma was tolerated with minimal tissue distension and a distribution across ≈50% of the adult cornea using the vector vehicle containing india ink (**Fig. 3**). Therefore, AAV serotypes 8 or 9 containing a GFP reporter gene were administered to human cornea stroma by injection ($1e^{10}$vg in 50µl) and analyzed seven days later by Western blotting. Given the reports of both 8 and 9 for cornea transduction, an AAV8/9 chimeric capsid (8G9), containing the galactose receptor of AAV9 engrafted on the AAV8 capsid was also investigated in the same manner (Sharma *et al., Exp. Eye Res. 91(3)*:440 (2010); Hippert *et al., PLoS One, 7(4)*:e35318 (2012)). Western blotting demonstrated that AAV8G9-GFP resulted in greater transduction than either parent serotype (**Fig. 4A**).

**[0151]** In order to determine the biodistribution of single-strand AAV8G9-GFP transduction (final volume 50µl), GFP immunofluorescence was performed on human corneal cross sections. The results demonstrate a lateral spread of vector transduction across the cornea with penetration into the deeper stromal layers, including some endothelial cell transduction (**Fig. 4B**). Co-staining with CD34, a marker of keratocytes, with alpha-smooth muscle actin (alpha-SM), indicative of differentiated keratocytes, and with F4/80, a macrophage marker, demonstrated the corneal cell promiscuity for the AAV8G9 capsid (**Fig. 4C**).

**[0152]** Following confirmation that the AAV8G9 capsid was the most efficient for corneal stroma transduction, it was determined if the cell types transduced by AAV8G9 naturally produce IDUA. To do this, dual staining for the cell type marker and the IDUA protein was performed in normal untreated human corneas. The results indicate that all of the confirmed corneal cell types transduced by AAV8G9 naturally produce IDUA (**Fig. 5A**).

**[0153]** Next, AAV8G9-opt-IDUA preclinical vector preparations were produced by the UNC Vector Core and evaluated in normal human corneas *ex vivo*. For these experiments, injection of single-strand AAV8G9-GFP was evaluated and PBS served as the negative control. Seven days post-injection ($1e^{10}$vg in 50µl), total protein was recovered and IDUA abundance was investigated by Western blotting. In whole corneal lysates, it was observed that the resting levels of IDUA are relatively low and that vector-derived IDUA, both the

precursor and cleaved forms, was readily detected (**Fig. 5B**). Consistently, a 10-fold elevation of IDUA activity was observed in the AAV8G9-opt-IDUA injected corneas when compared to normal levels found in human corneas (**Fig.5C**).

**[0154]** By immunofluorescence staining and detection on human cornea cross sections, the resting levels and distribution of IDUA were determined (**Fig. 6**). As MPS I patient corneas are rare, staining under the same conditions but without the IDUA primary antibody served as the negative control. The results demonstrate relatively low levels of IDUA within the corneal stroma that were also present in the corneal epithelia and to a lesser extent, in the endothelial cell layer (**Fig. 6**). IDUA staining was also performed on human cornea cross sections that were injected with AAV8G9-opt-IDUA. The results correlate to those of the IDUA Western blot and the functional assay (**Figs. 5A-5C**) in that there is an approximate 5-10 fold increase in IDUA in normal human corneas when administering AAV8G9-opt-IDUA compared to the resting level in human corneas (**Fig. 6**). Furthermore, similar to the results observed with AAV8G9-GFP, vector derived IDUA appeared well distributed (**Figs. 4B and 6**).

**[0155]** Cytotoxicity experiments were performed in normal human corneas and following ssAAV8G9-GFP or AAV8G9-opt-IDUA stromal injection (1e10vg). Tunel staining, which detects fragmented DNA indicative of cellular apoptosis, was performed on day 7 post-injection. Although the nuclease-treated positive control sample demonstrated extensive Tunel staining, corneas in which IDUA abundance/function were significantly elevated were not significantly different than non-injected or AAV8G9-GFP injected corneas (**Fig. 7**). These results are consistent with those obtained using MPS I patient fibroblasts and collectively reinforce the safety of AAV8G9-opt-IDUA gene therapy in MPS I patient corneas.

**[0156]** Hematopoietic stem cell transplantation, after myeloablative chemotherapy using allogeneic bone marrow or umbilical cord blood donors has been shown to extend life and improve its overall quality, particularly when performed in MPS I children under 2 years of age (Shapiro *et al., J. Inherit. Metab. Dis. 18(4)*:413 (1005);
Whitley *et al., Am. J. Med. Genet. 46(2)*:209 (1993); Prasad *et al., Blood 112(7)*:2979 (2008); Boelens *et al., Bone Marrow Transplant. 43(8)*:655 (2009); Summers *et al., Ophthalmology 96(7)*:977 (1989)). Engrafted donor cells provide a source of systemic IDUA and, through engraftment of donor microglial cells, enzyme replacement in the brain. Transplanted children, surviving 1-2 decades after HSCT with full donor chimerism, have normal blood IDUA levels with normal, to near normal, cognitive and cardiac functions. Later, and

ongoing, manifestations of MPS I after transplantation do occur and generally are limited to the joints, bones, and the eye, all of which are organs with lower profusion and lower delivery of donor-derived IDUA. As such, our preclinical corneal approach explored herein, was designed as a supplemental MPS I therapy to address the shortcomings of stem cell transplantation and AAV gene therapy targeting the CNS.

**[0157]** As MPS I patient corneas are rarely available, we initially investigated disease correction in MPS I patient fibroblasts. The data demonstrate restoration of IDUA function using a low vector dose. This is, in part, due to the relatively low level of IDUA in normal fibroblasts which is presumably sufficient for normal cellular physiology (**Fig. 1**). Consistently, the abundance of IDUA found in normal human corneas was also modest. In fact, in whole cornea lysates, IDUA abundance by Western blotting was not readily detected, however, tissue histology identified resting levels of IDUA in corneal epithelia, endothelia and the majority in multiple cell types found in the stroma (**Figs. 5 and 6**). This evaluation of IDUA abundance and AAV-mediated restoration highlights a very important aspect to the present corneal gene therapy approach: mainly that only a low amount of IDUA is necessary to restore WT IDUA function in the corneas of MPS I children. The chosen AAV dose in the cornea explants (1e10vg) was found to be excessive and resulted in a 10-fold supraphysiological elevation of IDUA function. However, even at this high IDUA level no toxicity was detected (**Figs. 5, 6, 7**). Given the ability of secreted IDUA to cross-correct neighboring cells, the low resting levels in normal human corneas, and the efficiency of AAV8G9-opt-IDUA for human cornea transduction, the data herein suggest that a low vector dose will result in normal levels of IDUA in MPS I patient corneas. Furthermore, reported complications associated with a neutralizing antibody response to the IDUA transgene product (IDUA) are not anticipated as there are no antibodies in the human cornea (Hinderer *et al., Mol. Ther. 23(8)*:1298 (2015)).

**[0158]** Given the collective data herein, restoring IDUA function in MPS I corneas seems quite feasible. Furthermore, it appears that AAV8G9 elicits IDUA production in stromal cells that naturally produce IDUA, as well as in the endothelial layer (**Fig. 6**). As the present AAV-opt-IDUA strategy is capable of transducing both of these compartments (**Fig. 6**), the likelihood of reversing or preventing MPS I-associated corneal blindness, as a supplemental therapy to HSCT or AAV systemic gene delivery remains a viable possibility.

## Example 4

### AAV IDUA Expression Cassette in Corneas In Vivo

**[0159]** The effect of AAV IDUA expression in vivo was tested in MPS1 canines. The left eye of dog I-712 was injected with the AAV8G9-CMV-optIDUA (1e9 vg/μl) solution as shown in **Figs. 8A-8D**. The needle tip of an insulin syringe was inserted into the corneal stroma (A) and the solution was injected gradually (B) until the full volume (65μl) was administered (C). Immediately following the injection, an area of fluid retention was visible as clouding or edema of the axial cornea, covering 30% of the corneal surface (D). Ophthalmic ultrasound biomicroscopy allowed determination of central cornea thickness at the indicated timepoints (**Fig. 8E**). The distension seen two minutes after injection was resolved within two days.

**[0160]** In the next experiment, two MPS1 canines were administered AAV8G9-optIDUA in one eye while the contralateral eye was given AAV-GFP as a control, all by intrastromal injection. Improved reflectivity of the tapetum lucidum upon retroillumination of the fundus indicates cornea clarity over the indicated time period (**Fig. 9A**). In both dogs, a substantial improvement in reflectivity was observed within 7 days and maintained for 113 days, demonstrating that IDUA expression cleared the corneal cloudiness.

**[0161]** Dog I-709 developed acute corneal edema first in the left eye at 5 weeks (image on the week of onset not shown), and then in the right eye at 10 weeks (**Fig. 9B**). Corneal edema subsided within 2-3 weeks while receiving topical steroid (TS) with or without systemic steroid (SS), and did not recurred in either eye for up to 19 wk post-injection. Importantly, the therapeutic effect remained evident in the IDUA-injected left eye after recovering from the transient immune reaction.

**[0162]** The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

**THAT WHICH IS CLAIMED IS:**

1. A recombinant nucleic acid comprising a sequence encoding human alpha-L-iduronidase (IDUA), wherein the nucleotide sequence has been codon-optimized for expression in human cells.

2. The recombinant nucleic acid of claim 1, comprising a nucleotide sequence at least 90% identical to SEQ ID NO:1.

3. The recombinant nucleic acid of claim 1, comprising the nucleotide sequence of SEQ ID NO:1.

4. An adeno-associated virus (AAV) vector genome comprising the nucleic acid of claim 1.

5. The AAV vector genome of claim 4, wherein the nucleic acid is operably linked to a constitutive promoter.

6. A cell *in vitro* comprising the AAV vector genome of claim 4 or 5.

7. The cell of claim 6, wherein the vector genome is stably incorporated into the cell genome.

8. An AAV particle comprising the AAV vector genome of claim 4 or 5.

9. A method of producing a recombinant AAV particle comprising an AAV capsid, the method comprising:

providing a cell *in vitro* with AAV Cap and AAV Rep coding sequences, the AAV vector genome of claim 4 or 5, and helper functions for generating a productive AAV infection; and

allowing assembly of the recombinant AAV particle comprising the AAV capsid and encapsidating the AAV vector genome.

10. An AAV particle produced by the method of claim 9.

11. The AAV particle of claim 8 or 10, wherein the AAV particle is an AAV2, AAV8, or AAV9 particle.

12. The AAV particle of claim 8 or 10, wherein the AAV particle is a chimeric AAV8/AAV9 particle.

13. A pharmaceutical formulation comprising the AAV particle of any one of claims 8 or 10-12 and a pharmaceutically acceptable carrier.

14. A method of delivering IDUA to the cornea of a subject, comprising administering to the cornea of the subject an effective amount of an AAV particle that expresses IDUA, thereby delivering IDUA to the cornea of the subject.

15. A method of treating or delaying the onset of mucopolysaccharidosis I (MPS I)-associated corneal clouding in a subject in need thereof, comprising administering to the cornea of the subject a therapeutically effective amount of an AAV particle that expresses IDUA, thereby treating or delaying the onset of MPS I-associated corneal clouding in the subject.

16. A method of delivering IDUA to a cornea *in vitro* or *ex vivo*, comprising contacting the cornea with an effective amount of an AAV particle that expresses IDUA, thereby delivering IDUA to the cornea.

17. The method of claim 16, further comprising transplanting the cornea to a subject in need thereof.

18. The method of any one of claims 14-17, wherein the AAV particle is the AAV particle of any one of claims 8 or 10-12 or the pharmaceutical composition of claim 13.

19. The method of any one of claims 14-18, wherein the AAV particle is administered to the cornea by intrastromal injection.

20. The method of any one of claims 14-18, wherein the AAV particle is administered to the cornea topically.

21. The method of any one of claims 14-20, wherein the subject is a human subject.

22. The method of any one of claims 14-21, wherein the subject has been diagnosed with MPS I.

23. The method of any one of claims 14-22, wherein the subject has not developed clouding of the cornea.

24. The method of any one of claims 14-22, wherein the subject has developed clouding of the cornea.

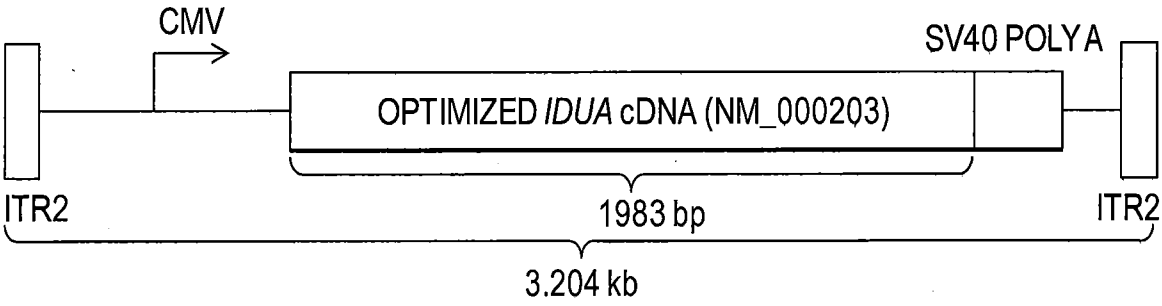



FIG. 1A

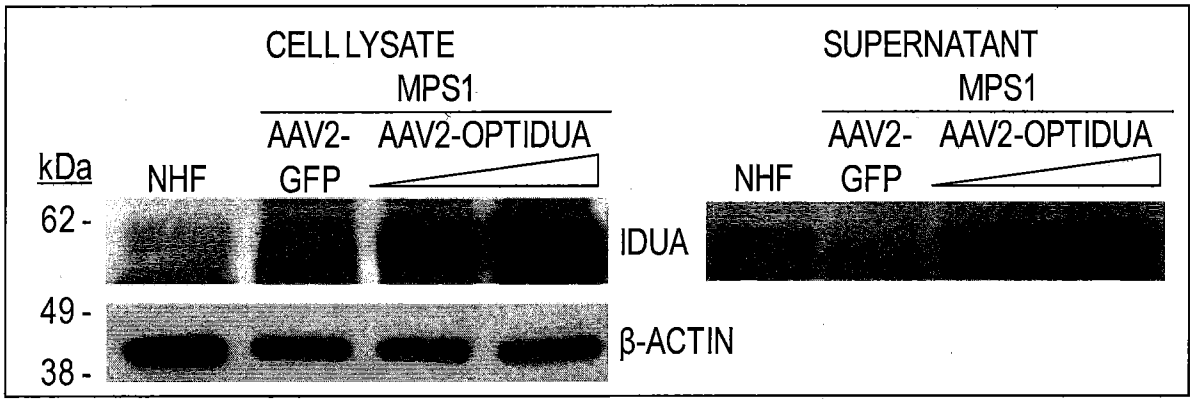



FIG. 1B

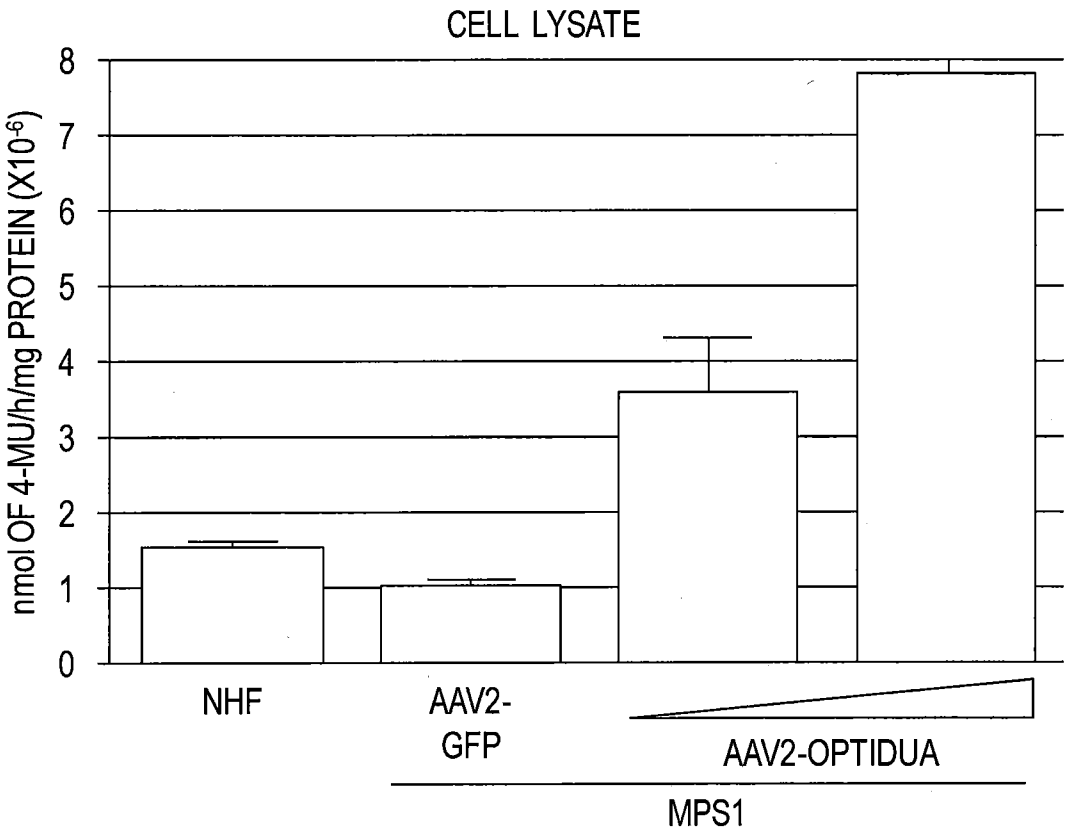



FIG. 1C

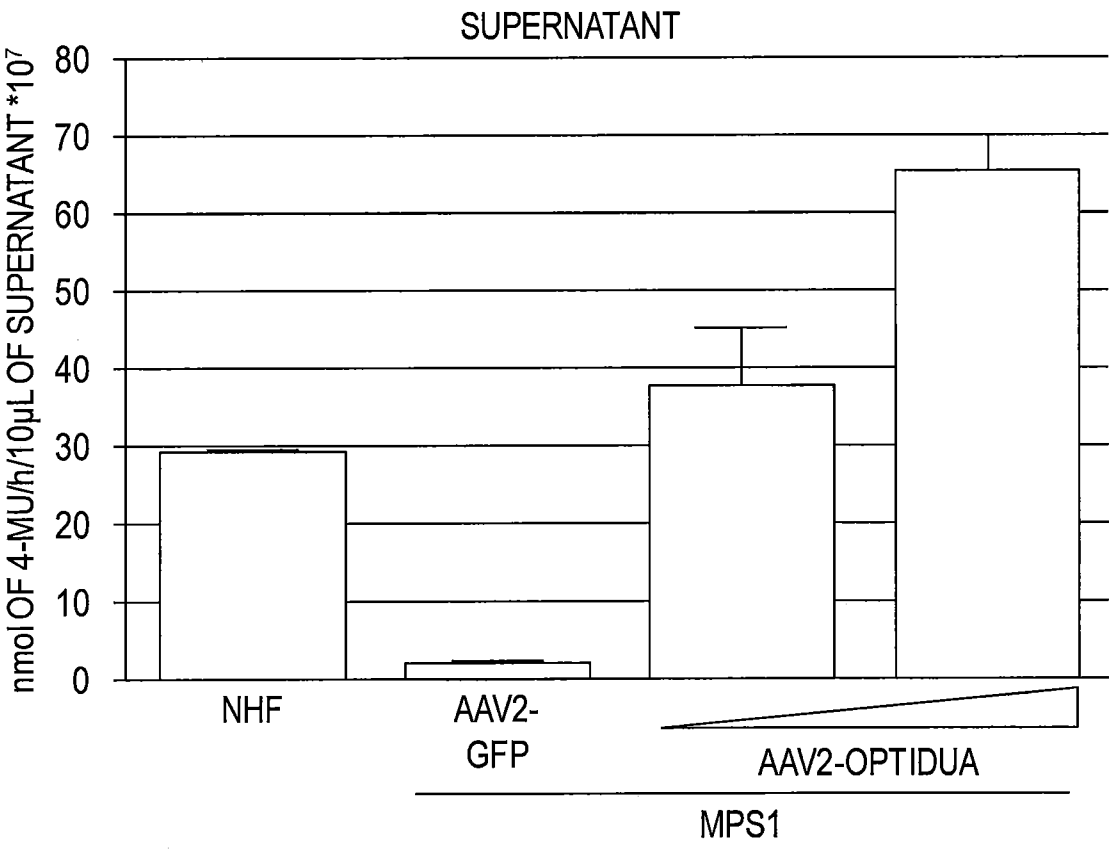

SUPERNATANT
nmol OF 4-MU/h/10µL OF SUPERNATANT *10^7
80
70
60
50
40
30
20
10
0
NHF
AAV2-GFP
AAV2-OPTIDUA
MPS1

FIG. 1D

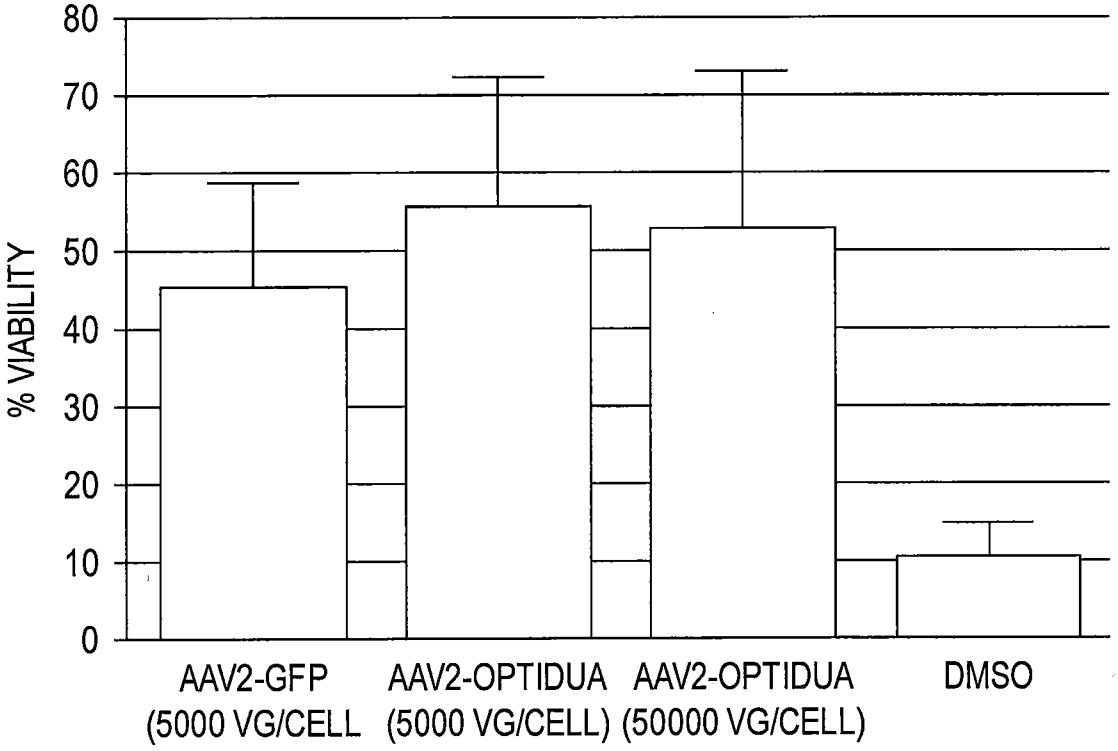

% VIABILITY
80
70
60
50
40
30
20
10
0
AAV2-GFP (5000 VG/CELL
AAV2-OPTIDUA (5000 VG/CELL)
AAV2-OPTIDUA (50000 VG/CELL)
DMSO

FIG. 2

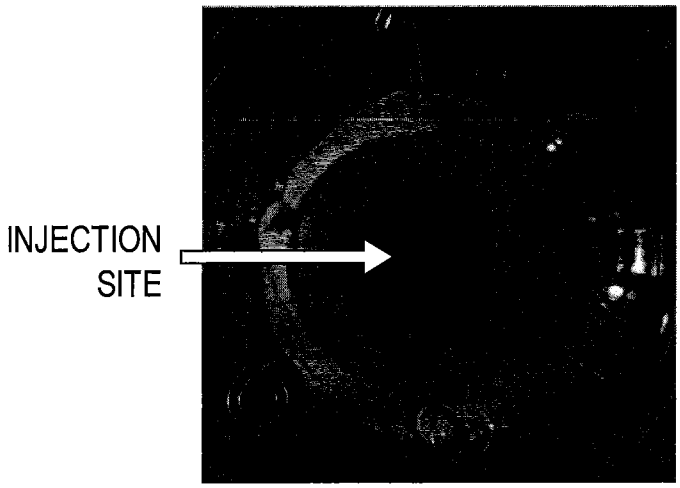



FIG. 3

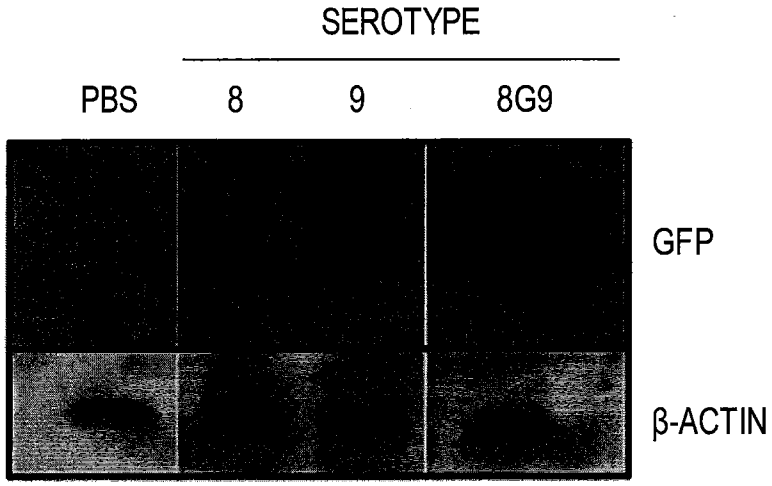



FIG. 4A

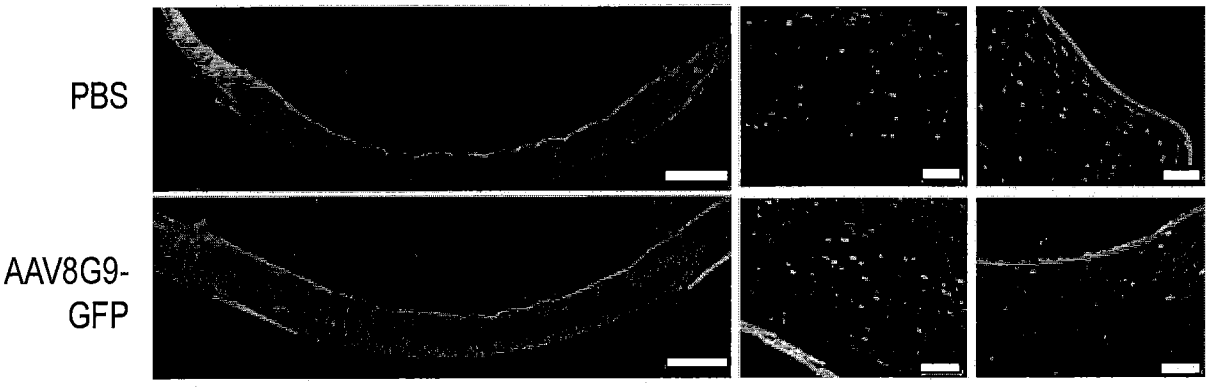



FIG. 4B

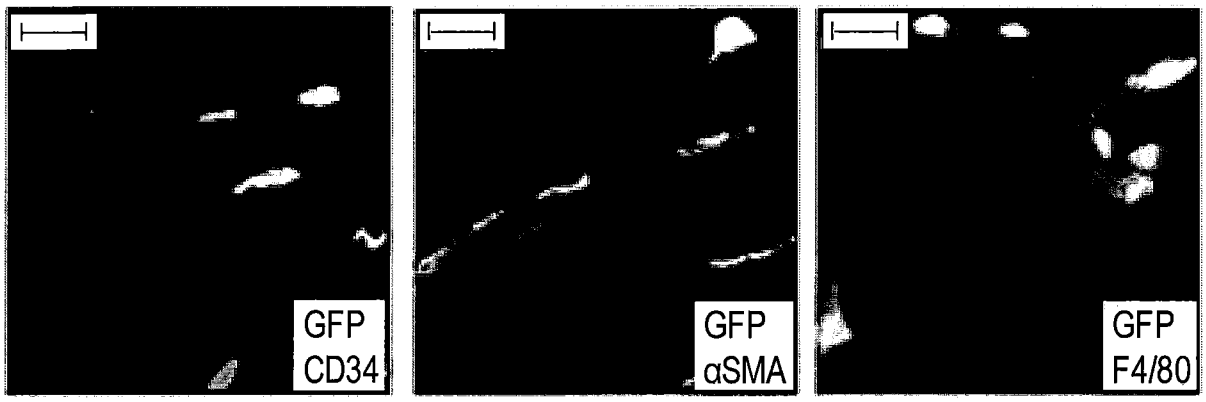



FIG. 4C

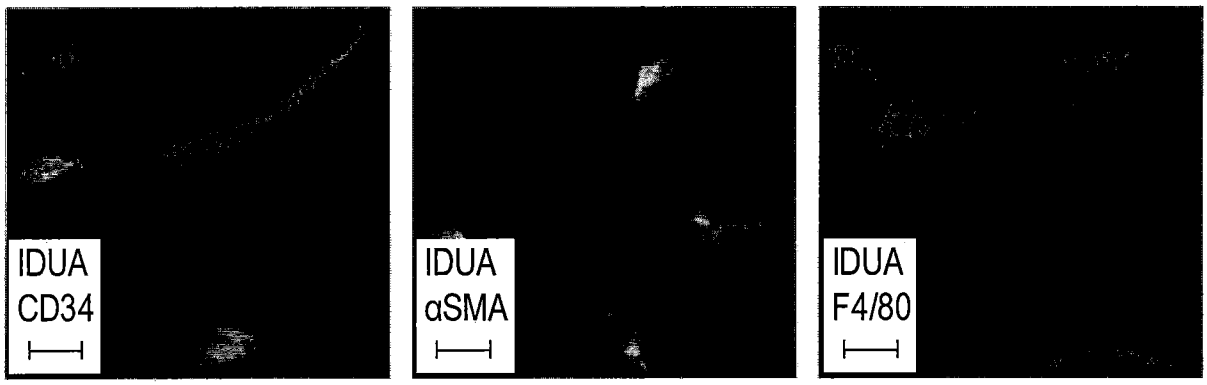



FIG. 5A

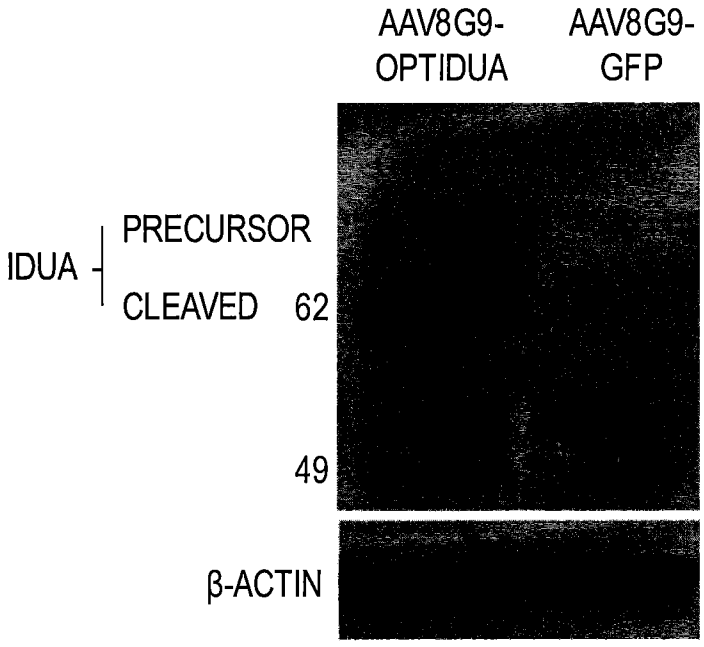



FIG. 5B

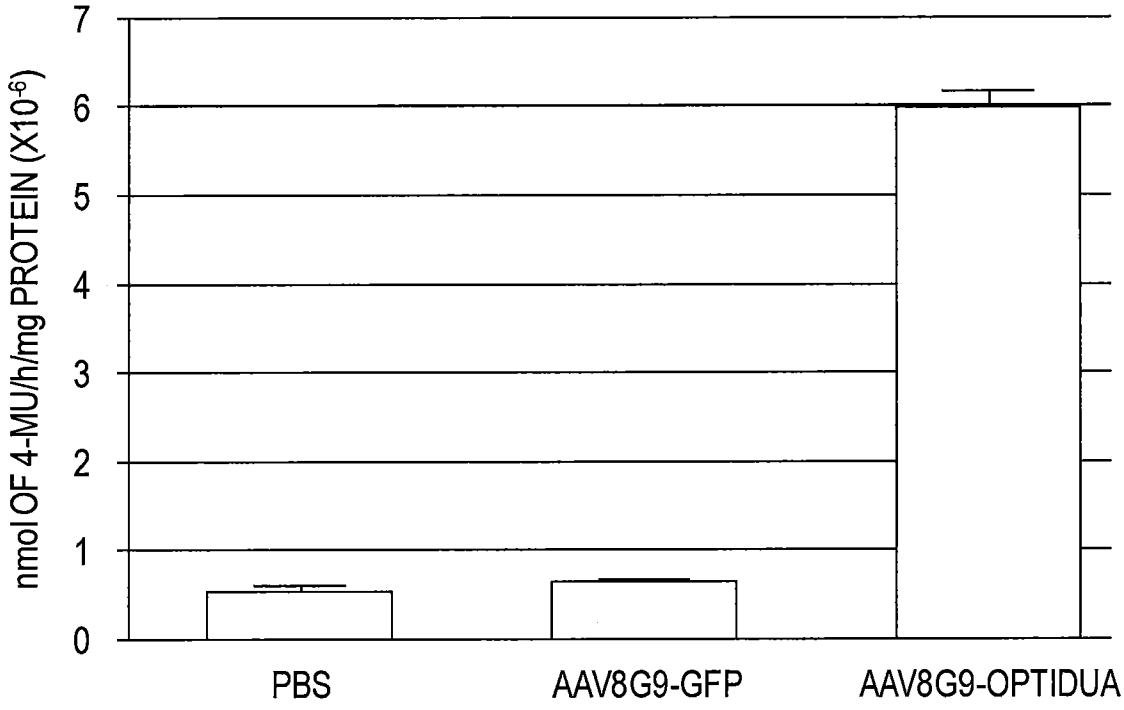

7
6
5
4
3
2
1
0
nmol OF 4-MU/h/mg PROTEIN (X10-6)
PBS
AAV8G9-GFP
AAV8G9-OPTIDUA

*FIG. 5C*

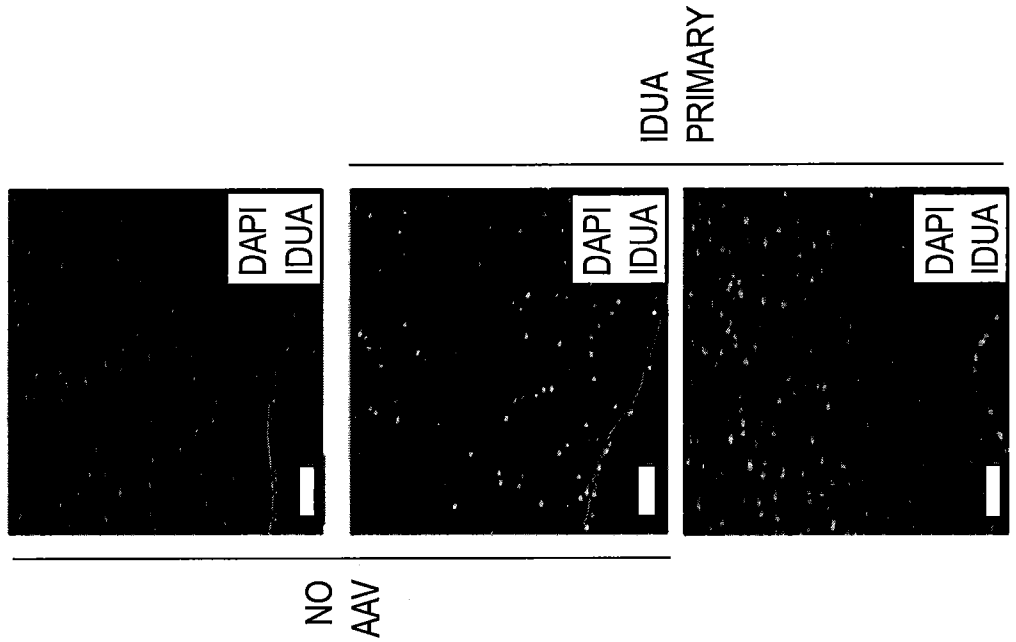

NO PRIMARY
IDUA PRIMARY
DAPI
IDUA
NO AAV

FIG. 6B

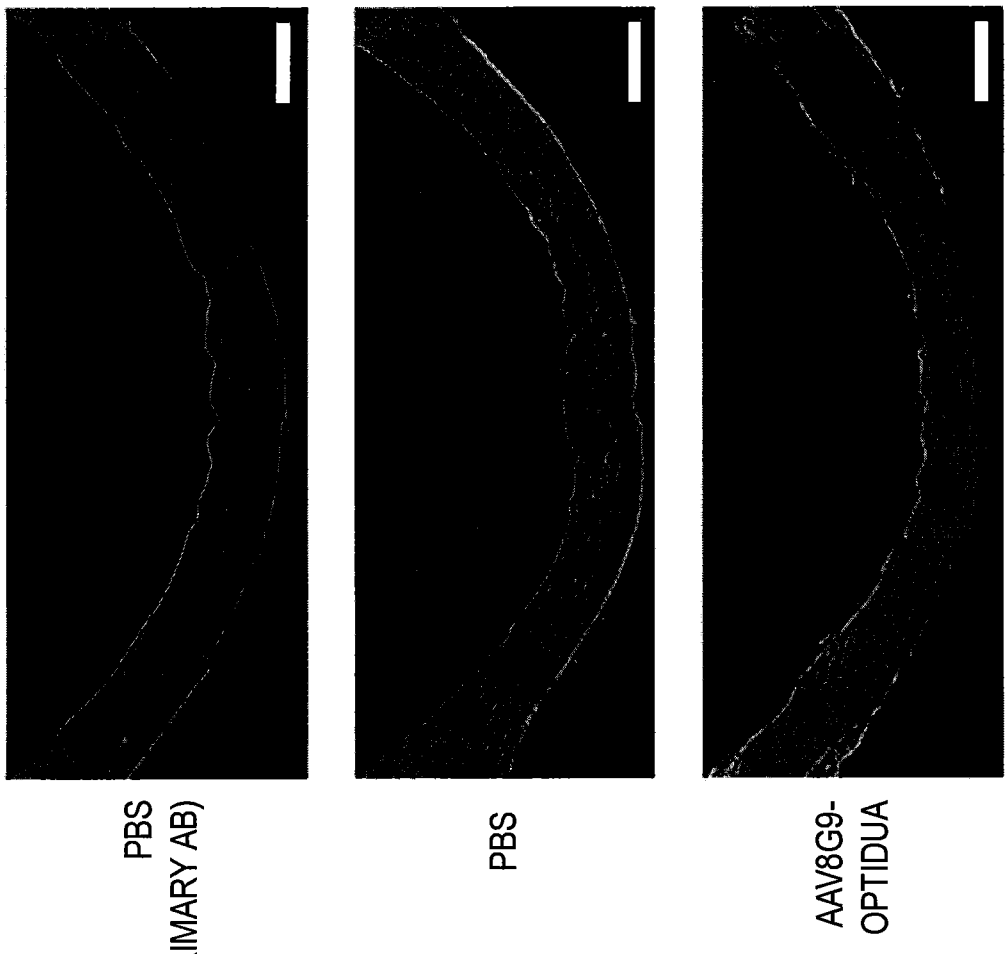

PBS (NO PRIMARY AB)
PBS
AAV8G9-OPTIDUA

FIG. 6A

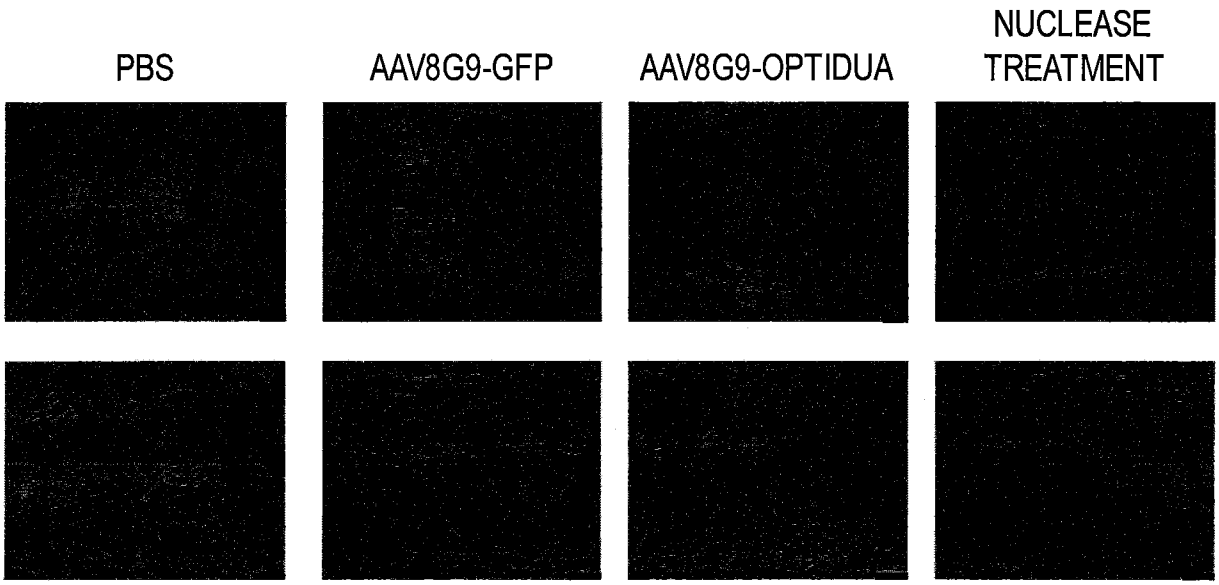

PBS
AAV8G9-GFP
AAV8G9-OPTIDUA
NUCLEASE TREATMENT

FIG. 7A

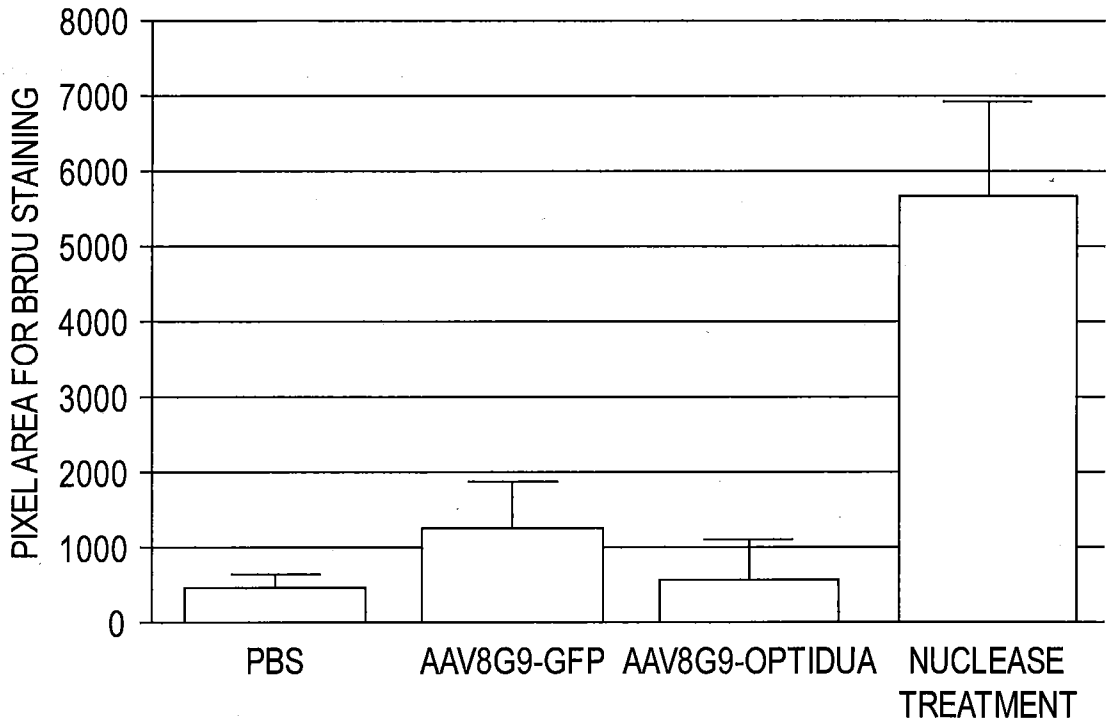

PIXEL AREA FOR BRDU STAINING
8000
7000
6000
5000
4000
3000
2000
1000
0
PBS
AAV8G9-GFP
AAV8G9-OPTIDUA
NUCLEASE TREATMENT

FIG. 7B

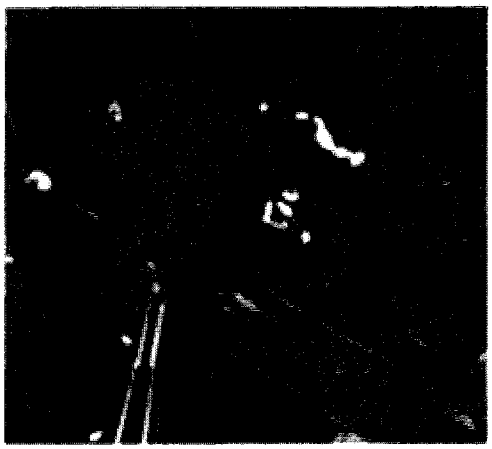


FIG. 8A

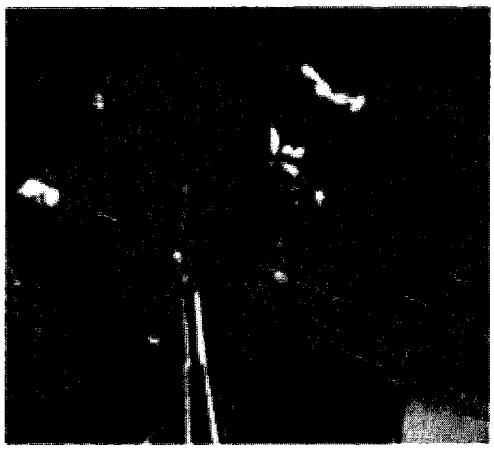


FIG. 8B

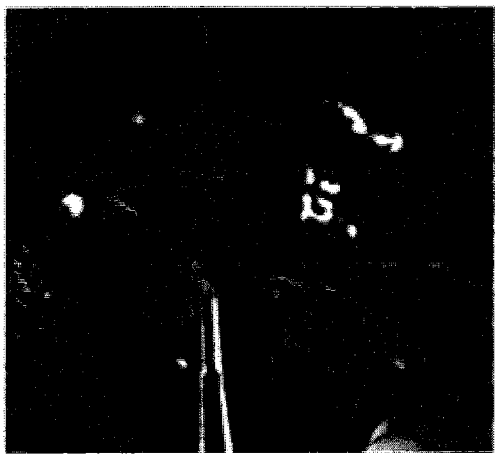


FIG. 8C

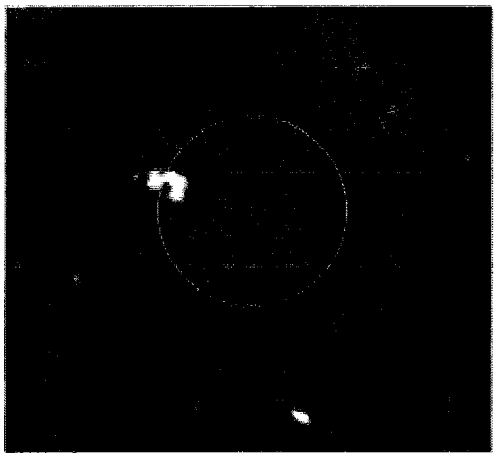


FIG. 8D

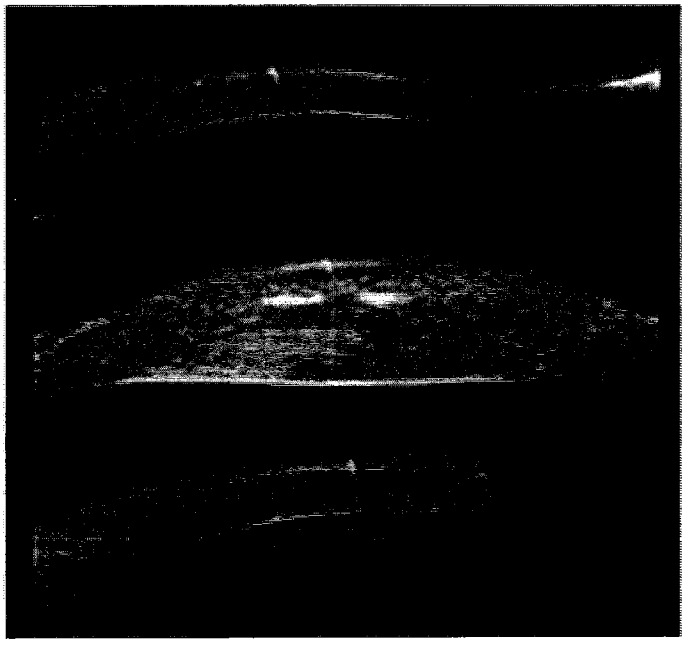



FIG. 8E

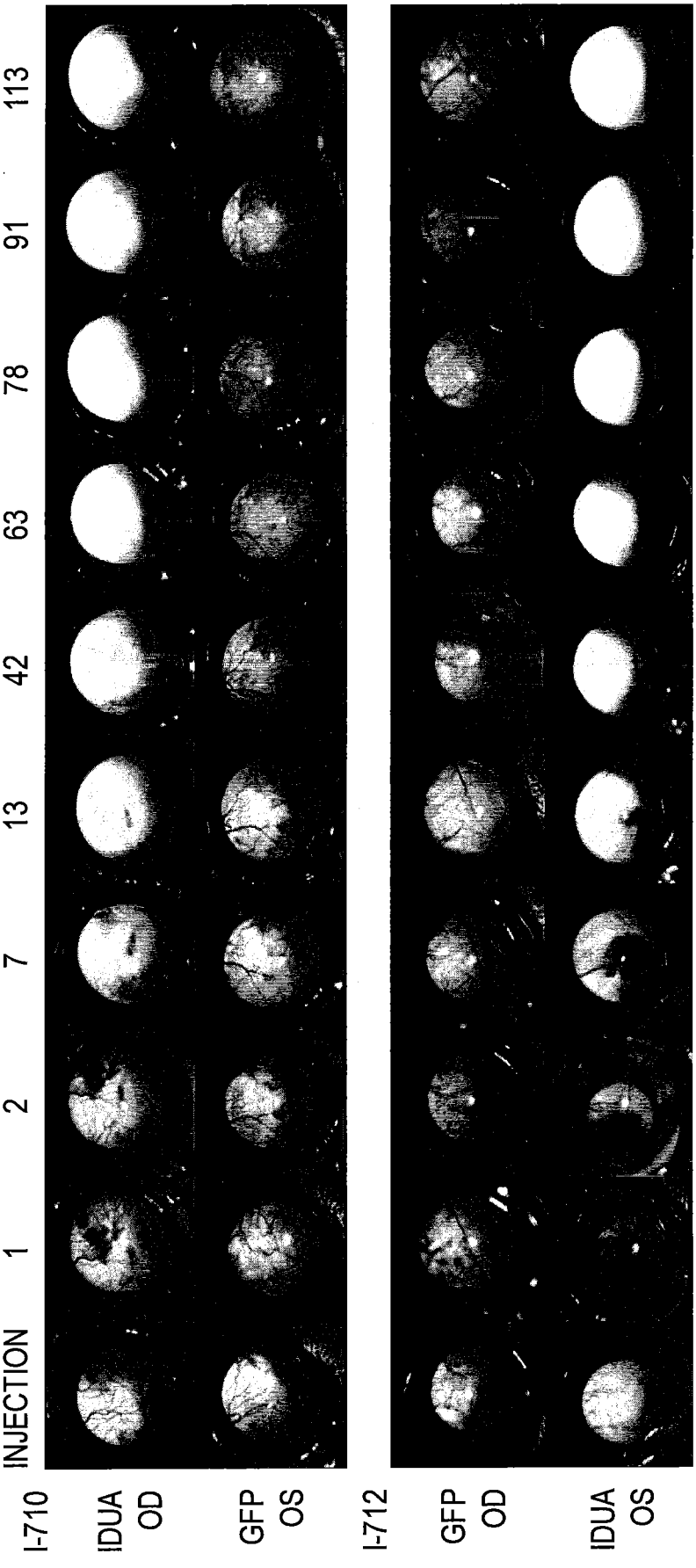

PRE-INJECTION
DAY 1
DAY 2
DAY 7
DAY 13
DAY 42
DAY 63
DAY 78
DAY 91
DAY 113
I-710
IDUA OD
GFP OS
I-712
GFP OD
IDUA OS

FIG. 6A

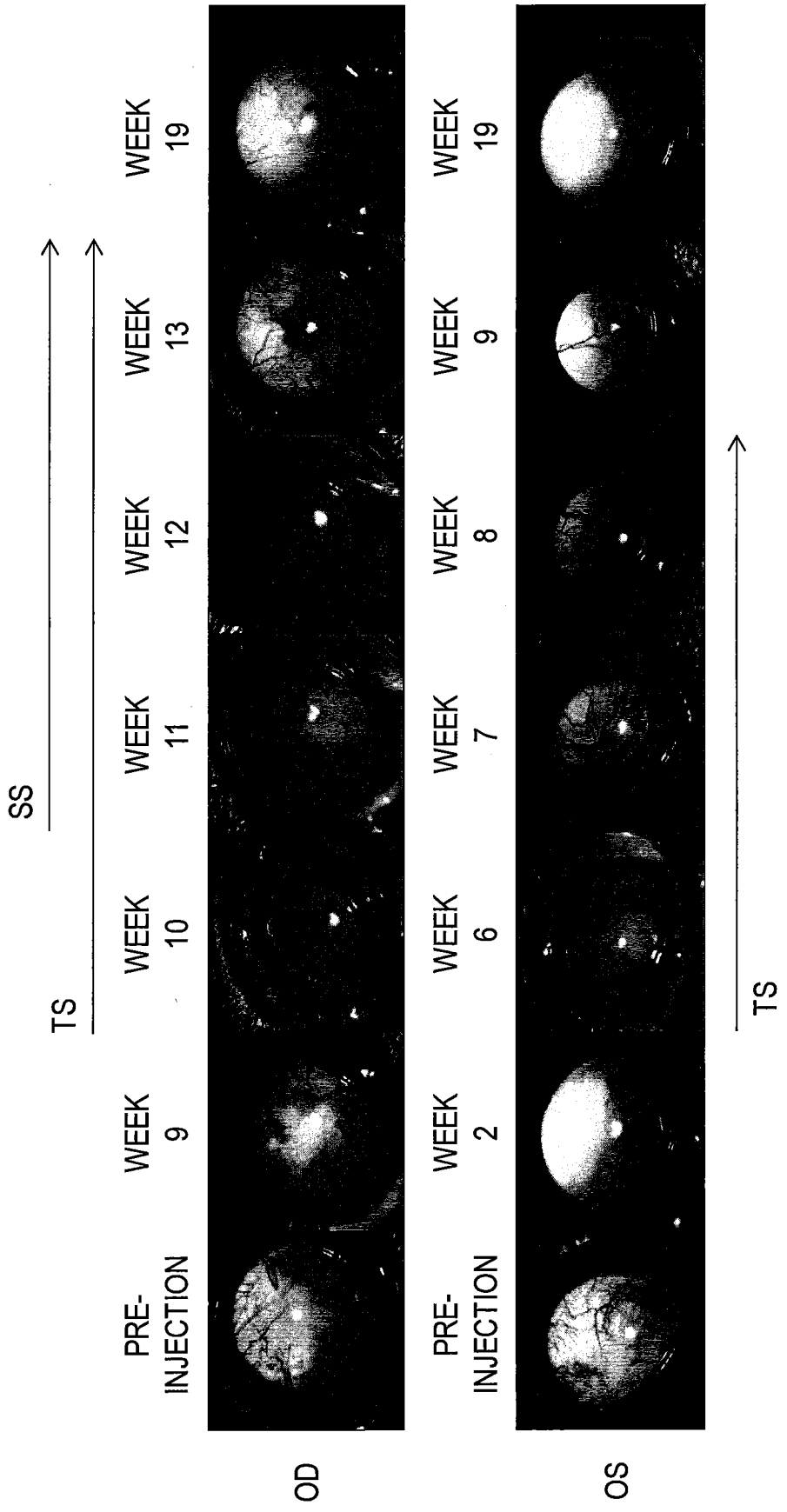

OD
OS
PRE-INJECTION
WEEK 2
WEEK 6
WEEK 7
WEEK 8
WEEK 9
WEEK 10
WEEK 11
WEEK 12
WEEK 13
WEEK 19
TS
SS

FIG. 6B

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 2017/018829

A. CLASSIFICATION OF SUBJECT MATTER

(see extra sheet)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 35/76, 48/00, C12N 7/00, 15/00, 9/24

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

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

NCBI, PubMed, RUPTO, USPTO, WIPO, PAJ, Esp@cenet, PCTonline, USPTO DB, CIPO(Canada PO), SIPO DB, CA, K-PION, KIPRIS, PabMed, (Chem.Abstr.)., GoogleScholar, PatSearch

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WALTER ACOSTA et al. RTB Lectin: a novel receptor-independent delivery system for lysosomal enzyme replacement therapies. Scientific Reports, 2015, Vol.5, no. 14144, pp.1-11, especially abstract, p.2, 3, 7, fig.1,2 | 1 |
| X<br>Y<br>A | WO 2014/151341 A1 (THE TRUSTEEES OF THE UNIVERSITY OF PENNSYLVANIA ) 25.09.2014, p.2-6, 9-12, 14-17, claims 1-4, 13, 21, 23-26, 29 | 1, 4-11, 13<br>12, 14-18<br>2, 3 |
| Y | WO 2016/005514 A1 (STICHTING KATHOLIEKE UNIVERSITEIT) 14.01.2016, p.22-23 | 12 |
| Y | WO 2015/ 191508 A1 (VOYAGER THERAPEUTICS, INC.) 17.12.2015, paragraphs [0039],[00440], claims | 14-18 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 24 April 2017 (24.04.2017) | 08 June 2017 (08.06.2017) |
| Name and mailing address of the ISA/RU:<br>Federal Institute of Industrial Property,<br>Berezhkovskaya nab., 30-1, Moscow, G-59,<br>GSP-3, Russia, 125993<br>Facsimile No: (8-495) 531-63-18, (8-499) 243-33-37 | Authorized officer<br>A.Zhogina<br>Telephone No. 495 531 65 15 |

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 2017/018829

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | BECK M. Mucopolysaccharidosis type I (MPSI). Orphanet encyclopedia, 2003, p.1-3, retrieved from the Internet: http://www.orpha.net/data/patho/GB/uk- MPS1.pdf | 17 |
| A | WO 2001/068888 A2 (NEUROLOGIX, INC.) 20.09.2001, p. 11, 20, 25-27, claims 15, 54, 76 | 1-18 |
| A | RU 2013115853 A (KURNA INK. ) 27.11.2014 , claims | 1-18 |

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 2017/018829

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

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

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

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

3. ☒ Claims Nos.: 19-24
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

According to Rule 13 of the Regulations under the PCT the claimed inventions does not meet the requirement of unity of invention , therefore independent claims 1, 4, 6, 8-10 and 13-16 form several groups of inventions, which are not so linked as to form a single general inventive concept.

The special technical feature of independent claims 1, 4, 6, 8-10, 13 is a sequence encoding human alpha-L-iduronidase (IDUA).

The special technical feature of independent claims 14-16 is an AAV particle that expresses IDUA.

Hence, claims comprise 2 groups of inventions, namely:

1 invention – claims 1-13 and 18 (partially)

2 invention – claims 14-17 and 18 (partially)

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

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

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

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

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

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

☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**
Classification of subject matter

International application No.
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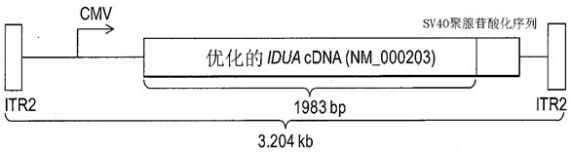
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序列表2页 附图9页

(54)发明名称

# 治疗MPS I-相关失明的AAV-IDUA载体

(57)摘要

本发明涉及向受试者角膜递送α-L-艾杜糖醛酸酶的病毒载体及采用所述病毒载体治疗和预防受试者因粘多糖贮积症I型引发的角膜浑浊和失明。




CN 108697774 A

1.一种重组核酸,包括编码人α-L-艾杜糖醛酸酶(IDUA)的核苷酸序列,其中所述核苷酸序列经密码子优化用于人类细胞表达。

2.根据权利要求1所述的重组核酸,包括至少90%与SEQ ID NO:1相同的核苷酸序列。

3.根据权利要求1所述的重组核酸,包括SEQ ID NO:1核苷酸序列。

4.一种腺相关病毒(AAV)载体基因组,包括权利要求1所述的核酸。

5.根据权利要求4所述的AAV载体基因组,其中所述核酸可与组成型启动子操作连接。

6.一种体外细胞,包括权利要求4或5所述的AAV载体基因组。

7.根据权利要求6所述的细胞,其中所述载体基因组稳定地整合到细胞基因组内。

8.一种AAV粒子,包括权利要求4或5所述的AAV载体基因组。

9.一种制备包括AAV衣壳的重组AAV粒子的方法,所述方法包括:

提供一种含AAV Cap和AAV Rep编码序列的体外细胞、权利要求4或5所述的AAV载体基因组,及产生增殖性AAV感染的辅助功能;及

组装包括所述AAV衣壳的重组AAV粒子和包裹所述AAV载体基因组。

10.一种根据权利要求9所述方法制备的AAV粒子。

11.根据权利要求8或10所述的AAV粒子,其中所述AAV粒子是AAV2、AAV8或AAV9粒子。

12.根据权利要求8或10所述的AAV粒子,其中所述AAV粒子是嵌合AAV8/AAV9粒子。

13.一种药物制剂,包括权利要求8或10-12中任一项所述的AAV粒子及药学上可接受的载体。

14.一种递送IDUA至受试者角膜的方法,包括向受试者角膜施用有效剂量的表达IDUA的AAV粒子,从而将IDUA递送至受试者的角膜。

15.一种治疗或推迟受试者MPS I-相关角膜浑浊的方法,包括向受试者角膜施用有效剂量的表达IDUA的AAV粒子,从而治疗或推迟受试者MPS I-相关角膜浑浊。

16.一种体外或间接体内递送IDUA至角膜的方法,包括将有效剂量表达IDUA的AAV粒子与角膜接触,从而将IDUA递送至角膜。

17.根据权利要求16所述的方法,进一步向需要的受试者移植角膜。

18.根据权利要求14-17任一项所述的方法,其中所述AAV粒子是权利要求8或10-12中任一项所述的AAV粒子或权利要求13所述的药物组合物。

19.根据权利要求14-18任一项所述的方法,其中所述AAV粒子经基质内注射施用于角膜。

20.根据权利要求14-18任一项所述的方法,其中所述AAV粒子局部施用于角膜。

21.根据权利要求14-20任一项所述的方法,其中所述受试者是人类受试者。

22.根据权利要求14-21任一项所述的方法,其中所述受试者经诊断患有MPS I。

23.根据权利要求14-22任一项所述的方法,其中所述受试者尚未出现角膜浑浊。

24.根据权利要求14-22任一项所述的方法,其中所述受试者已经出现角膜浑浊。

# 治疗MPS I-相关失明的AAV-IDUA载体

优先权声明

[0001] 本申请要求2016年2月22日提交的序列号为62/298,126的美国临时申请的权益，通过引用,该临时申请全文纳入本申请中。

## 技术领域

[0002] 本发明涉及向受试者角膜递送α-L-艾杜糖醛酸酶的病毒载体及采用所述病毒载体治疗和预防受试者因粘多糖贮积症I型引发的角膜浑浊和失明。

## 背景技术

[0003] 粘多糖贮积症I型(MPS I)是编码α-L-艾杜糖醛酸酶(IDUA)的基因无效突变或无义突变引发的一种常染色体隐性溶酶体贮积症,α-L-艾杜糖醛酸酶是普遍存在的分解粘多糖(GAG)的细胞内分泌酶。缺乏功能IDUA时,GAG会在溶酶体内积聚,破坏脂质、糖和蛋白质正常的细胞内转运,导致多系统终末器官损伤。MPS I的发病率是大约10万分之一,该疾病的特征有肝脾肿大、心功能不全、骨和关节畸形、侏儒症、智力迟钝及严重的神经系统问题,通常导致患者不到10岁即死亡。其它症状包括听力损失、关节僵硬,及角膜浑浊,导致视力减退(Aldenhoven等人,Blood 125(13):2164(2015))。

[0004] 目前MPS I的治疗包括静脉注射IDUA酶替代治疗(ERT)(ALDURAZYME®),已经证明,该方法可减少轻度MPSI患者的肝脾肿大,并改善其心功能、肺部症状和运动性。更具前景的治疗方法依赖于异基因造血干细胞移植(HSCT),该方法过去20-30年已经用于MPS I患者。已经证明,HSCT在改善认知功能、减少肝脾肿大、预防缺血性心脏病及延长患者寿命(某些情况下可延长几十年)方面非常成功。当采用清髓性化疗和脐带血捐献时,临床结果最佳,而且结果与持续的供体嵌合程度有关。第三种MPS I治疗方法依赖于腺-相关病毒(AAV)IDUA基因添加策略,该方法仍处于临床前评估阶段。人们通过几种途径施用,包括颈动脉、实质内、室内和鞘内施用,采用鼠、猫和犬MPS I模型探索了中枢神经系统靶向AAV-IDUA基因治疗方法(Janson等人,Neurosurgery 74(1):99(2014);Wolf等人,Neurobiol.Dis.43(1):123(2011);Hinderer等人,Mol.Ther.22(12):2018(2014))。这些基因治疗研究独立报道了组织学、生物化学方面,特别是认知方面得到了改善,同时并未观察到与IDUA-相关的毒性。但是,ERT、HSCT和AAV CNS-靶向或全身基因治疗具有共同的缺点:即其无法治疗特殊隔室,包括关节和眼睛的MPS I-相关疾病。

[0005] 至于眼睛问题,大约90％MPS I儿童患者因角膜浑浊导致视力减退,角膜浑浊是因为存在异常的空泡基质细胞(Huang等人,Exp.Eye Res.62(4):377(1996))。MPS I人类患者角膜的详细分析表明其存在软骨素和硫酸皮肤素GAG积聚,改变了胶原纤维的均匀分布、组织和尺寸(Alroy等人,Exp.Eye Res.68(5):523(1999);Fahnehjelm等人,Acta Ophthalmol.Scand.84(6):781(2006))。通过角膜移植治疗MPS I儿童的角膜盲,但是,近年来,由于这种治疗方法的排斥率非常高,因此,这种方法未能成为标准实践方法。相反,HSCT独立报告已经表明,HSCT方法能够稳定、改善及某些情况下恢复角膜透明性(Fahnehjelm等

人,Acta Ophthalmol.Scand.84(6):781(2006);Hobbs,Lancet 2(8249):735(1981);Hoogerbrugge等人,Lancet,345(8962):1398(1995);Vellodi等人,Arch.Dis.Child.76(2):92(1997);Gullingsrud等人,Ophthalmology,105(6):1099(1998);Souillet等人,Bone Marrow Transplant 31(12):1105(2003))。

[0006] 本发明提供在角膜内表达IDUA的病毒载体及治疗或预防MPS I-相关角膜浑浊和失明的方法。

## 发明内容

[0007] 本发明基于以下发现:采用AAV载体递送IDUA至MPS I受试者角膜,能够在整个角膜内有效表达IDUA。因此,本发明的一个方面涉及一种包括编码人α-L-艾杜糖醛酸酶(IDUA)的核苷酸序列的重组核酸,其中所述核苷酸序列经密码子优化用于人类细胞表达。

[0008] 本发明的另一个方面涉及一种包括本发明所述核酸的AAV载体基因组、包括所述AAV载体基因组的AAV粒子及包括所述AAV粒子的药物组合物。

[0009] 本发明的另一个方面涉及一种制备包括AAV衣壳的重组AAV粒子的方法,所述方法包括:提供一种含AAV Cap和AAV Rep编码序列的体外细胞,本发明所述AAV载体基因组,及产生增殖性AAV感染的辅助功能;组装包括所述AAV衣壳的重组AAV粒子和包裹所述AAV载体基因组。

[0010] 本发明的另一个方面涉及一种递送IDUA至受试者角膜的方法,包括向受试者角膜施用有效剂量的表达IDUA的AAV粒子,从而将IDUA递送至受试者的角膜。

[0011] 本发明的另一个方面涉及一种治疗受试者MPS I-相关角膜浑浊或推迟受试者MPS I-相关角膜浑浊发作的方法,包括向受试者角膜施用有效剂量的表达IDUA的AAV粒子,从而治疗受试者MPS I-相关角膜浑浊或推迟受试者MPS I-相关角膜浑浊发作。

[0012] 本发明的这些方面和其它方面将在下面进行更详细的说明。

## 附图说明

[0013] 图1A-1D表明,通过AAV基因治疗,MPS I患者成纤维细胞恢复了IDUA活性。(A)AAV-IDUA优化载体构建体示意图。(B)对AAV2感染和非感染成纤维细胞、正常人类成纤维细胞(NHF)或MPS I患者成纤维细胞的细胞裂解液(左边)和上清液(右边)的IDUA蛋白质表达进行了分析。检测β-肌动蛋白,作为细胞裂解液中总蛋白质的内参。(C、D)AAV2感染和非感染成纤维细胞、NHF或MPS I成纤维细胞的细胞裂解液(C)和上清液(D)的IDUA蛋白质功能活性。对细胞裂解液,4-MU的纳摩尔数归一化到1小时反应和mg总蛋白质。对细胞上清液,5-MU的纳摩尔数归一化到10μl样品。4-MU:4-伞形酮;CMV:巨细胞病毒启动子;ITR:内部末端重复序列;GFP:绿色荧光蛋白。

[0014] 图2表明,向MPS I成纤维细胞施用AAV2-GFP和AAV2-optIDUA后未出现细胞毒性。

[0015] 图3示出了注射到角膜后含墨汁溶媒的分布。

[0016] 图4A-4C示出了人角膜内的AAV衣壳血清型评估情况。(A)向人角膜基质中注射AAV血清型8、9或8/9嵌合8G9包裹的自补CMV-GFP盒。7天后,采用蛋白质印迹法检测GFP。PBS对应的是仅注射PBS(溶媒对照)的人角膜。检测β-肌动蛋白作为内参。(B)将单链AAV8G9-CMV-GFP注射到人角膜基质中,并在7天后收获开展组织学分析。左-表明重组AAV8G9-GFP病毒感

染在人角膜截面上分布情况的免疫荧光图像。采用注射PBS的人角膜作为阴性对照。图像采用10X物镜拍摄，并通过拼接处理组装。比例尺等于2000μm。右-同一染色人角膜采用20倍物镜拍摄的不同区域。比例尺等于100μm。采用DAPI用于核复染。(C)采用GFP和所示细胞标记物染色的(B)中角膜的代表性部分。比例尺等于10μm。采用DAPI用于核复染。

[0017] 图5A-5C表明，通过AAV8G9-opt-IDUA，人角膜恢复了IDUA活性。(A)采用IDUA抗体和细胞标记物染色的正常未注射角膜的代表性截面。采用DAPI用于核复染。比例尺等于10μm。(B)采用蛋白质印迹法检测得到的注射AAV8G9-IDUA后产生的IDUA数量。施用AAV8G9-GFP作为载体感染对照。β-肌动蛋白作为内参。(C)注射AAV8G9-opt-IDUA 7天后得到的人角膜IDUA蛋白质功能活性。AAV8G9-GFP作为阴性对照。4-MU的纳摩尔数归一化到1小时反应和mg总蛋白质。PBS对应的是仅注射溶媒对照的人角膜。

[0018] 图6A-6B示出了AAV-8G9-opt-IDUA转导后IDUA蛋白质的分布情况。(A)表明注射AAV8G9-opt-IDUA或PBS(溶媒)7天后人角膜截面IDUA蛋白质分布的免疫荧光图像。图像采用10X物镜拍摄，并通过拼接处理组装。比例尺等于2000μm。采用DAPI用于核复染。(B)同一染色人角膜采用20倍物镜拍摄的截面图。上图与阴性对照染色对应，阴性对照与其它样品按照完全相同的方法开展，但不含一抗。采用DAPI用于核复染。比例尺等于100μm。

[0019] 图7A-7B表明，人角膜注射AAV8G9-opt-IDUA后未出现细胞毒性。(A)将注射PBS、AAV8G9-GFP或AAV8G9-opt-IDUA的人角膜进行处理，7天后用于Tunel染色。上图是10倍物镜拍摄的图像。下图是20倍物镜拍摄的图像。作为阳性对照，将注射PBS的人角膜进行核酸酶处理。比例尺等于100μm。(B)(A)中总染色像素区定量情况。注射AAV8G9-GFP或AAV8G9-opt-IDUA的角膜细胞毒性不存在统计学差异($p>0.05$)。

[0020] 图8A-8E示出了向MPS 1犬的角膜基质内注射AAV8G9-optIDUA。向I-712狗的左眼内注射AAV8G9-CMV-optIDUA(1e9vg/μl)溶液。将胰岛素注射器针尖插入到角膜基质(A)内，缓慢注射所述溶液(B)，直到施用全部体积(65μl)(C)。注射后，可以立即看到呈浑浊或轴向角膜水肿的流体保留区，该区域覆盖30％角膜表面(D)。(E)采用眼部超声生物显微镜检测所示时间点中心角膜的厚度。

[0021] 图9A-9B表明，AAV8G9-CMV-optIDUA消除了MPS1犬的角膜浑浊。A)向MPS1犬的一只眼内施用AAV8G9-optIDUA，而对侧眼内施用AAV-GFP作为对照，施用方式都为角膜基质内注射。后部反光照射基底时，照膜反射率改善，表明所示期间角膜透明。B)角膜水肿和恢复。首先，I-709犬在5周时左眼出现急性角膜水肿(初始发作周图像未示出)，然后，右眼在10周时出现急性角膜水肿。当局部施用类固醇(TS)时，在全身施用或不施用类固醇(SS)条件下，角膜水肿在2-3周内消失，两只眼睛在注射后长达19周都未再复发。重要的是，从短暂的免疫反应恢复后，注射IDUA的左眼的治疗效果仍然非常明显地得到保留。

## 具体实施方式

[0022] 现在，将参考示出本发明优选实施例的附图对本发明进行说明。但是，本发明可以采用各种不同形式实施，并且不应认为本发明限于此处提出的实施例。相反，提供这些实施例是为了充分完整地公开本发明，本领域技术人员将全面了解本发明的范围。

[0023] 除非特别规定，本发明使用的所有术语(包括技术名词和科学术语)的意义与本发明所属领域技术人员通常理解的相同。本发明说明中使用的术语仅仅是为了描述特定实施

例，并不是用于限制本发明。此处提到的所有出版物、专利申请、专利和其它公开文献都通过引用而纳入本申请中。

[0024] 除非特别说明，此处核苷酸序列仅以单链、5'至3'方向、从左至右显示。此处核苷酸和氨基酸按照IUPAC-IUB生化命名委员会推荐的方式表示，或(氨基酸)按照37CFR§1.822和惯用法以单字母代码或三字母代码表示。参见，例如PatentIn User Manual，99-102(1990年11月)(美国专利商标局)。

[0025] 除非另外指出，可以采用本领域技术人员熟悉的标准方法构建重组细小病毒和AAV(rAAV)构建体，包装表达细小病毒Rep与/或Cap序列的载体，及瞬时稳定地转染包装细胞。所述方法是本领域技术人员熟悉的，参见，例如，SAMBROOK等人，MOLECULAR CLONING:A LABORATORY MANUAL 2nd Ed.(Cold Spring Harbor,NY,1989)；AUSUBEL等人，CURRENT PROTOCOLS IN MOLECULAR BIOLOGY(Green Publishing Associates,Inc.and John Wiley&Sons,Inc.,New York)。

[0026] 此外，本发明还设想在本发明的一些实施例中，可以排除或省略此处提出的任何特征或特征组合。

[0027] 为了进一步说明，例如，如果说明书表明某一氨基酸可以选自A、G、I、L与/或V，则该陈述亦表明该氨基酸可以选自这些氨基酸的任何子集，例如，A、G、I或L；A、G、I或V；A或G；仅L等，就好像每个所述子组合在此处明确提出一样。此外，所述陈述还表明，可以放弃一个或多个规定氨基酸。例如，在特定实施例中，氨基酸不是A、G或I；不是A；不是G或V等，犹如所述可能放弃在此处明确提出的那样。

定义

[0028] 此处发明内容和所附权利要求书中使用下述术语。

[0029] 除非上下文中清楚表明，否则，单数形式“一”也包括复数形式。

[0030] 此外，提到可测量值，如多核苷酸或多肽序列的长度、剂量、时间、温度等时使用的词语“大约”指的是包括规定数值20％、10％、5％、1％、0.5％或甚至0.1％的变化范围。

[0031] 此外，此处所述“与/或”指的是和包括一个或多个相关列出项目的任何和所有可能组合，当解释为可选择的(“或”)，是指缺少组合。

[0032] 连接词“基本上由……组成”解释为包括列出的材料或步骤及“那些并不显著影响本发明基本新特点”的材料或步骤(例如，rAAV复制)。因此，此处词语“基本上由……组成”不应解释为相当于“包括”。

[0033] 正如应用于本发明多核苷酸或多肽序列时，词语“基本上由……组成”(和语法变化形式)指的是多核苷酸或多肽由所提出序列(例如SEQ ID NO)及所提出序列5'与/或3'或N-端与/或C-端上共10个或更少数量(例如1、2、3、4、5、6、7、8、9或10个)的其它核苷酸或氨基酸组成，从而多核苷酸或多肽的功能并未显著改变。共10个或更少数量的其它核苷酸或氨基酸包括两端加和的其它核苷酸或氨基酸的总数量。正如应用于本发明多核苷酸时那样，词语“显著改变”指的是与所提出序列组成的多核苷酸的表达水平相比，表达编码多肽的能力提高或降低至少大约50％或更高。正如应用于本发明多肽时那样，词语“显著改变”指的是与所提出序列组成的多肽的活性相比，转导活性提高或降低至少大约50％或更高。

[0034] 此处所述词语“细小病毒”包括细小病毒科，包括自主复制细小病毒和依赖病毒。自主复制细小病毒包括细小病毒属、红病毒属、浓核病毒属、重复病毒属(Iteravirus)和康

特拉病毒属(Contravirus)的成员。示例性自主复制细小病毒包括但不限于小鼠微小病毒、牛细小病毒、犬细小病毒、鸡细小病毒、猫泛白细胞减少症病毒、猫细小病毒、鹅细小病毒、H1细小病毒、番鸭细小病毒、蛇细小病毒和B19病毒。其它自主复制细小病毒是本领域技术人员熟悉的。参见例如,FIELDS等所著《病毒学》第2卷第69章(4版,利平科特-雷文出版公司(Lippincott-Raven Publishers))。

[0035] 依赖病毒属包括腺相关病毒(AAV),包括但不限于AAV 1、AAV 2、AAV 3(包括3A和3B型)、AAV 4、AAV 5、AAV 6、AAV 7、AAV 8、AAV 9、AAV 10、AAV 11、AAV 12、AAV 13、鸟AAV、牛AAV、犬AAV、山羊AAV、蛇AAV、马AAV和绵羊AAV。参见,例如FIELDS等所著《病毒学》第2卷第69章(4版,利平科特-雷文出版公司)和表1。

[0036] 正如此处所述,词语"腺相关病毒"(AAV)包括但不限于AAV 1、AAV 2、AAV 3(包括3A和3B型)、AAV 4、AAV 5、AAV 6、AAV 7、AAV 8、AAV 9、AAV 10、AAV 11、AAV 12、AAV 13、蛇AAV、鸟AAV、牛AAV、犬AAV、马AAV、绵羊AAV、山羊AAV、虾AAV及目前了解或以后发现的任何其它AAV。参见,例如FIELDS等所著《病毒学》第2卷第69章(4版,利平科特-雷文出版公司)。已经鉴定了多种相对较新的AAV血清型和分支(参见,例如Gao等人,(2004)J.Virol.78:6381;;Moris等人,(2004)Virol.33-:375;和表1)。

[0037] 本发明的细小病毒载体、粒子和基因组可以来源于AAV,但不限于来源于AAV。AAV各种血清型及自主复制细小病毒的基因组序列,以及天然ITR、Rep蛋白质和衣壳亚单位的序列是本领域熟悉的。这些序列可以在文献或公开数据库,如基因库(GenBank)中找到。参见,例如基因库保藏号NC_002077、NC_001401、NC_001729、NC_001863、NC_001829、NC_001862、NC_000883、NC_001701、NC_001510、NC_006152、NC_006261、AF063497、U89790、AF043303、AF028705、AF028704、J02275、J01901、J02275、X01457、AF288061、AH009962、AY028226、AY028223、AY631966、AX753250、EU285562、NC_001358、NC_001540、AF513851、AF513852和AY530579;通过引用,有关细小病毒和AAV核酸和氨基酸序列的公开内容并入本申请中。亦参见,例如,Bantel-Schaal等人,(1999)J.Virol.73:939;Chiorini等人,(1997)J.Virol.71:6823;Chiorini等人,(1999)J.Virol.73:1309;Gao等人,(2002)Proc.Nat.Acad.Sci.USA 99:11854;Moris等人,(2004)Virol.33-:375-383;Mori等人,(2004)Virol.330:375;Muramatsu等人,(1996)Virol.221:208;Ruffing等人,(1994)J.Gen.Virol.75:3385;Rutledge等人,(1998)J.Virol.72:309;Schmidt等人,(2008)J.Virol.82:8911;Shade等人,(1986)J.Virol.58:921;Srivastava等人,(1983)J.Virol.45:555;Xiao等人,(1999)J.Virol.73:3994;国际专利公开WO 00/28061、WO 99/61601、WO 98/11244;及美国专利第6,156,303号;通过引用,有关细小病毒和AAV核酸和氨基酸序列的公开内容并入本申请中。亦参见表1。最初有关AAV1、AAV2和AAV3ITR序列的描述由Xiao,X.,(1996)在宾夕法尼亚州匹兹堡大学博士论文"Characterization of Adeno-associated virus(AAV)DNA replication and integration"中提供(该论文全文并入本申请中)。

[0038] 此处所述词语"嗜性"指的是病毒进入细胞,任选和优选然后表达(转录和任选翻译)细胞中病毒基因组携带的序列,例如,对重组病毒来说,表达异源核苷酸序列。本领域技术人员将了解的是,病毒基因组异源核酸序列的转录可以在缺乏反式作用因子时启动,例如,用于诱导型启动子或其它调节核酸序列。在AAV时,病毒基因组的基因表达可以来自稳

定整合的细小病毒、非整合附加体，以及病毒可能进入细胞内的任何其它形式。

[0039] 正如此处所述，细小病毒或AAV的细胞“转导”指的是细小病毒/AAV-介导将遗传物质传输到细胞内。参见，例如FIELDS等所著《病毒学》第2卷第69章(3版，利平科特-雷文出版公司)。

[0040] 词语“5’部分”和“3’部分”是定义两个或多个元件之间空间关系的相对词语。因此，例如，多核苷酸的“3’部分”指的是多核苷酸另一段下游的一段。词语“3’部分”并不表示所述段必定在多核苷酸3’端，或甚至不必在多核苷酸的3’半部分，虽然情况可能是这样。同样，例如，多核苷酸的“5’部分指的是多核苷酸另一段上游的一段。”词语“5’部分”并不表示所述段必定在多核苷酸5’端，或甚至不必在多核苷酸的5’半部分，虽然情况可能是这样。

[0041] 正如此处所述，除非另外说明，词语“多肽”包括肽和蛋白质。

[0042] “多核苷酸”是核苷酸碱基序列，可能是RNA、DNA或DNA-RNA混合序列(包括天然和非天然核苷酸)，可以是单链或双链DNA序列。

[0043] 词语“序列一致性”是本领域的标准意义。正如本领域熟悉的那样，可以利用许多不同的程序鉴定多核苷酸或多肽与已知序列是否具有序列一致性或类似性。序列一致性或类似性可以采用本领域熟悉的标准方法测定，包括但不限于Smith&Waterman局部序列一致性算法(Adv.Appl.Math.2:482(1981))、Needleman&Wunsch序列一致性比对算法(J.Mol.Biol.48:443(1970))、Pearson&Lipman相似性检索方法(Proc.Natl.Acad.Sci.USA 85:2444(1988))、这些算法的计算机实施(威斯康星遗传学软件包中的GAP、BESTFIT、FASTA和TFASTA，该软件包由威斯康星州麦迪逊市科学大道575号的Genetics Computer Group提供)，及Devereux等人，Nucl.Acid Res.12:387(1984)描述的最佳拟合序列程序，最好是采用默认值，或检查确定。

表1

| 、<br>全基因组 | 基因库保藏号 | | | 基因库保藏号 | | | 基因库保藏号 |
|---|---|---|---|---|---|---|---|
| | | | Hu T88 | AY695375 | | 分支 E | |
| 腺相关病毒 1 | NC_002077,AF063497 | | **Hu T71** | AY695374 | | Rh38 | AY530558 |
| 腺相关病毒 2 | NC_001401 | | **Hu T70** | AY695373 | | Hu66 | AY530626 |
| 腺相关病毒 3 | NC_001729 | | **Hu T40** | AY695372 | | Hu42 | AY530605 |
| 腺相关病毒 3B | NC_001863 | | **Hu T32** | AY695371 | | Hu67 | AY530627 |
| 腺相关病毒 4 | NC_001829 | | Hu T17 | AY695370 | | Hu40 | AY530603 |
| 腺相关病毒 5 | Y18065, AF085716 | | Hu LG15 | AY695377 | | Hu41 | AY530604 |
| 腺相关病毒 6 | NC_001862 | | | | | Hu37 | AY530600 |
| 鸟 AAV ATCC VR-865 | AY186198, AY629583, NC_004828 | | 分支 C | | | Rh40 | AY530559 |
| 鸟 AAV 菌株 DA-1 | NC_006263, AY629583 | | Hu9 | AY530629 | | Rh2 | AY243007 |
| 牛 AAV | NC_005889, AY388617 | | Hu10 | AY530576 | | Bb1 | AY243023 |
| | | | Hu11 | AY530577 | | Bb2 | AY243022 |
| 分支 A | | | Hu53 | AY530615 | | Rh10 | AY243015 |
| AAV1 | NC_002077,AF063497 | | Hu55 | AY530617 | | Hu17 | AY530582 |
| AAV6 | NC_001862 | | Hu54 | AY530616 | | Hu6 | AY530621 |
| Hu.48 | AY530611 | | Hu7 | AY530628 | | Rh25 | AY530557 |
| Hu 43 | AY530606 | | Hu18 | AY530583 | | Pi2 | AY530554 |
| Hu 44 | AY530607 | | Hu15 | AY530580 | | Pi1 | AY530553 |
| Hu 46 | AY530609 | | Hu16 | AY530581 | | Pi3 | AY530555 |
| | | | Hu25 | AY530591 | | Rh57 | AY530569 |
| 分支 B | | | Hu60 | AY530622 | | Rh50 | AY530563 |
| Hu. 19 | AY530584 | | Ch5 | AY243021 | | Rh49 | AY530562 |
| Hu. 20 | AY530586 | | Hu3 | AY530595 | | Hu39 | AY530601 |
| Hu 23 | AY530589 | | Hu1 | AY530575 | | Rh58 | AY530570 |
| Hu22 | AY530588 | | Hu4 | AY530602 | | Rh61 | AY530572 |
| Hu24 | AY530590 | | Hu2 | AY530585 | | Rh52 | AY530565 |
| Hu21 | AY530587 | | Hu61 | AY530623 | | Rh53 | AY530566 |
| Hu27 | AY530592 | | | | | Rh51 | AY530564 |
| Hu28 | AY530593 | | 分支 D | | | Rh64 | AY530574 |
| Hu 29 | AY530594 | | Rh62 | AY530573 | | Rh43 | AY530560 |
| Hu63 | AY530624 | | Rh48 | AY530561 | | AAV8 | AF513852 |
| Hu64 | AY530625 | | Rh54 | AY530567 | | Rh8 | AY242997 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hu13 | AY530578 | | Rh55 | AY530568 | | Rh1 | AY530556 |
| Hu56 | AY530618 | | Cy2 | AY243020 | | | |
| Hu57 | AY530619 | | **AAV7** | AF513851 | | **分支 F** | |
| Hu49 | AY530612 | | Rh35 | AY243000 | | Hu14 (**AAV9**) | AY530579 |
| Hu58 | AY530620 | | Rh37 | AY242998 | | Hu31 | AY530596 |
| Hu34 | AY530598 | | Rh36 | AY242999 | | Hu32 | AY530597 |
| Hu35 | AY530599 | | Cy6 | AY243016 | | | |
| **AAV2** | NC_001401 | | Cy4 | AY243018 | | **克隆分离体** | |
| Hu45 | AY530608 | | Cy3 | AY243019 | | **AAV5** | Y18065, AF085716 |
| Hu47 | AY530610 | | Cy5 | AY243017 | | **AAV 3** | NC_001729 |
| Hu51 | AY530613 | | Rh13 | AY243013 | | **AAV 3B** | NC_001863 |
| Hu52 | AY530614 | | | | | **AAV4** | NC_001829 |
| Hu T41 | AY695378 | | | | | Rh34 | AY243001 |
| **Hu S17** | AY695376 | | | | | Rh33 | AY243002 |
| | | | | | | Rh32 | AY243003 |

[0044] 有用算法的一个实例是PILEUP。PILEUP利用渐进、双序列比对，根据一组相关序列建立了多序列比对法。它还绘制了表明聚类关系的树图，用于建立比对。PILEUP采用Feng&Doolittle(J.Mol.Evol.35:351(1987))渐进比对法的简化方法；这种方法与Higgins&Sharp,CABIOS 5:151(1989)描述的方法类似。

[0045] 另一个有用算法的实例是Altschul等人,J.Mol.Biol.215:403(1990)和Karlin等人,Proc.Natl.Acad.Sci.USA 90:5873(1993)描述的BLAST算法。特别有用的BLAST程序是Altschul等人,Meth.Enzymol.,266:460(1996)；blast.wustl/edu/blast/README.html的WU-BLAST-2程序。

WU-BLAST-2采用几个搜索参数，这些参数优选设定为默认值。这些参数是动态值，是由程序本身建立的，取决于具体序列的组成和搜索感兴趣序列的具体数据库的组成；但是，可以调节这些值以提高灵敏性。

[0046] 另一种有用的算法是Altschul等人,Nucleic Acids Res.25:3389(1997)报告的间隔BLAST。

[0047] 氨基酸序列一致性百分数通过匹配相同残基的数量除以比对区“长”序列残基的总数量确定。“长”序列是比对区中实际残基数最多的序列(为了最大程度地提高比对分数而通过WU-Blast-2引入的空位被忽略)。

[0048] 按照类似方式，核酸序列一致性百分数定义为候选序列中与此处特别公开的多核苷酸中核苷酸相同的核苷酸残基百分数。

[0049] 比对可能包括在比对序列中引入空位。此外，对于核苷酸比此处特别公开多核苷

酸多或少的序列来说，应该了解的是，在一个实施例中，序列一致性百分数将根据相同核苷酸的数量相对核苷酸总数量确定。因此，例如，在一个实施例中，比此处特别公开序列短的序列的序列一致性将采用短序列中的核苷酸数确定。在一致性百分数计算中，相对权重并未分配给各种不同的序列变化表示，如插入、删除、替换等。

[0050] 在一个实施例中，仅一致性评为正分(+1)，所有序列变化形式，包括空位都分配一个数值“0”，序列相似性计算不需要下述加权尺度或参数。序列一致性百分数可以，例如，将匹配相同残基数除以比对区“短”序列残基总数，然后乘以100计算得出。“长”序列是比对区实际残基数最多的序列。

[0051] “分离”多核苷酸(例如，“分离DNA”或“分离RNA”)指的是与天然生物或病毒至少其它一些组分(例如，通常发现与多核苷酸有关的细胞或病毒结构组分或其它多肽或核酸)分离的多核苷酸或基本上不含天然生物或病毒至少其它一些组分的多核苷酸。

[0052] 同样，“分离”多肽指的是与天然生物或病毒至少其它一些组分(例如，通常发现与多肽有关的细胞或病毒结构组分或其它多肽或核酸)分离的多肽或基本上不含天然生物或病毒至少其它一些组分的多肽。

[0053] “治疗多肽”是可以缓解或减轻细胞或受试者因蛋白质缺少或缺陷所引发症状的多肽。或者，“治疗多肽”是给受试者带来好处(例如，抗癌效果或改善移植物存活性)的多肽。

[0054] 正如应用于多核苷酸或多肽序列时，词语“修饰”，指的是由于一个或多个删除、加入、替换或它们的任何组合，得到的与野生型序列不同的序列。

[0055] “分离”或“提纯”(或语法相当形式)病毒载体，指的是将病毒载体与初始材料中的至少其它一些组分至少部分分离。

[0056] 词语“治疗”(及其语法变化形式)指的是受试者疾病严重程度减轻、至少部分改善或稳定与/或至少一种临床症状减轻、缓解、减少或稳定与/或疾病或病症进程延迟。

[0057] 词语“预防”(及其语法变化形式)指的是与不采用本发明方法相比，阻止与/或延迟受试者疾病、病症与/或临床症状的发生与/或减轻疾病、病症与/或临床症状发生的严重程度。预防可以是全面的，例如，完全不出现疾病、病症与/或临床症状。预防还可以是部分的，从而受试者疾病、病症与/或临床症状的发生与/或疾病、病症与/或临床症状发生的严重程度低于不采用本发明方法时的情况。

[0058] “治疗有效”剂量是足够为受试者提供某些改善或好处的剂量。或者，“治疗有效”剂量是将减轻、缓解、减少或稳定受试者至少一种临床症状的剂量。本领域技术人员将了解的是，治疗效果并不需要全面或治愈，只要为受试者提供某些好处即可。

[0059] “预防有效”剂量是与不采用本发明方法相比，足够阻止与/或延迟受试者疾病、病症与/或临床症状的发生与/或减轻疾病、病症与/或临床症状发生的严重程度的剂量。本领域技术人员将了解的是，预防水平并不需要全面，只要为受试者提供某些好处即可。

[0060] 词语“异源核苷酸序列”和“异源核酸”在此处互换使用，指的是病毒中的非天然序列。在一些实施例中，异源核酸包括编码感兴趣多肽或非翻译RNA的开放阅读框(例如，用于输送至细胞或受试者)。

[0061] 词语“病毒载体”、“载体”或“基因递送载体”指的是作为核酸递送媒介的病毒(例如，AAV)粒子，包括包装在病毒粒子内的载体基因组(例如，病毒DNA[vDNA])。或者，在一些

背景中，词语“载体”可用于仅指载体基因组/vDNA或指质粒。

[0062] 本发明的病毒载体可以进一步是国际专利公开WO 01/92551所述的双链细小病毒粒子(通过引用，该专利公开全文纳入本申请中)。因此，在一些实施例中，可以包装双链(双)基因组。

[0063] “rAAV载体基因组”或“rAAV基因组”是包括一个或多个异源核酸序列的AAV基因组(即vDNA)。rAAV载体通常仅需顺式145碱基ITR来产生病毒。所有其它病毒序列是可有可无的，可能以反式提供(Muzyczka(1992)Curr.Topics Microbiol.Immunol.158:97)。通常，rAAV载体基因组将仅保留一个或多个ITR序列，从而最大限度增大载体有效包装转基因的尺寸。结构和非结构蛋白质编码序列可以以反式提供(例如，来自载体，如质粒，或将序列稳定整合到包装细胞内)。在本发明的实施例中，rAAV载体基因组包括至少一个ITR序列(例如，AAV ITR序列)，任选两个ITR序列(例如，两个AAV ITR序列)，所述序列通常将位于载体基因组的5’端和3’端，侧接异源核酸，但并不需要与其连接。ITR可以彼此相同或不同。

[0064] 词语“末端重复序列”或“TR”包括形成发夹结构并作为反向末端重复序列(即调节要求的功能，例如，复制、病毒包装、整合与/或前病毒补救等)的任何病毒末端重复序列或合成序列。ITR可以是AAV ITR或非-AAV ITR。例如，非-AAV ITR序列，如其它细小病毒(例如，犬细小病毒、牛细小病毒、鼠细小病毒、猪细小病毒、人细小病毒B-19)的序列，或作为SV40复制源的SV40发夹可以作为ITR使用，ITR可以通过截断、替换、删除、插入与/或加入进一步进行修饰。此外，ITR可以部分或全部合成，例如，授权给Samulski等人的美国专利第5,478,745号中描述的“双-D序列”。

[0065] 细小病毒基因组在其5’端和3’端都有回文序列。序列的回文性质导致形成发夹结构，发夹结构通过在互补碱基对之间形成氢键稳定。人们认为，这种发夹结构采用“Y”形或“T”形。参见，例如FIELDS等所著《病毒学》第2卷第69&70章(4版，利平科特-雷文出版公司)。

[0066] “AAV反向末端重复序列”或“AAV ITR”可以来自任何AAV，包括但不限于血清型1、2、3a、3b、4、5、6、7、8、9、10、11或13、蛇AAV、鸟AAV、牛AAV、犬AAV、马AAV、绵羊AAV、山羊AAV、虾AAV或现在熟知或以后发现的任何其它AAV(参见，例如表1)。AAV ITR不需要拥有天然末端重复序列(例如，天然AAV ITR序列可以通过插入、删除、截断与/或错义突变改变)，只要所述末端重复序列调节所要求的功能，例如，复制、病毒包装、持久性，与/或前病毒拯救等即可。

[0067] 本发明的病毒载体可以进一步是“靶向”病毒载体(例如，拥有定向嗜性)与/或“混合”细小病毒(即其中病毒ITR和病毒衣壳来自不同的细小病毒)，正如国际专利公开WO00/28004和Chao等人，(2000)Mol.Therapy 2:619所述。

[0068] 此外，病毒衣壳或基因组元件可以包含其它修饰，包括插入、删除与/或替换。

[0069] 正如此处所述，词语“氨基酸”包括任何天然氨基酸、其修饰形式及合成氨基酸。

[0070] 天然左旋(L-)氨基酸如表2所示。

表2

| 氨基酸残基 | 缩写 | |
|---|---|---|
| | 三字母代码 | 单字母代码 |
| 丙氨酸 | Ala | A |
| 精氨酸 | Arg | R |
| 天冬酰胺 | Asn | N |
| 天冬氨酸（天冬氨酸盐） | Asp | D |
| 半胱氨酸 | Cys | C |
| 谷氨酰胺 | Gln | Q |
| 谷氨酸（谷氨酸盐） | Glu | E |
| 甘氨酸 | Gly | G |
| 组氨酸 | His | H |
| 异亮氨酸 | Ile | I |
| 亮氨酸 | Leu | L |
| 赖氨酸 | Lys | K |
| 蛋氨酸 | Met | M |
| 苯丙氨酸 | Phe | F |
| 脯氨酸 | Pro | P |
| 丝氨酸 | Ser | S |
| 苏氨酸 | Thr | T |
| 色氨酸 | Trp | W |
| 酪氨酸 | Tyr | Y |
| 缬氨酸 | Val | V |

[0071] 或者，氨基酸可以是修饰氨基酸残基(非限制性实例在表3中示出)或可以是后-翻译修饰(例如，乙酰化、酰胺化、甲酰化、羟基化、甲基化、磷酸化或硫酸化)氨基酸。

表3：氨基酸残基衍生物

| 修饰氨基酸残基 | 缩写 |
|---|---|
| 2-氨基己二酸 | Aad |
| 3-氨基己二酸 | bAad |
| β-丙氨酸、β-氨基丙酸 | bAla |
| 2-氨基丁酸 | Abu |
| 4-氨基丁酸、哌啶酸 | 4Abu |
| 6-氨基己酸 | Acp |
| 2-氨基庚酸 | Ahe |
| 2-氨基异丁酸 | Aib |
| 3-氨基异丁酸 | bAib |
| 2-氨基庚二酸 | Apm |
| 叔丁基丙氨酸 | t-BuA |
| 瓜氨酸 | Cit |
| 环己基丙氨酸 | Cha |
| 2,4-二氨基丁酸 | Dbu |
| 锁链素 | Des |
| 2,2'-二氨基庚二酸 | Dpm |
| 2,3-二氨基丙酸 | Dpr |
| N-乙基甘氨酸 | EtGly |
| N-乙基天冬酰胺 | EtAsn |
| 高精氨酸 | hArg |
| 高半胱氨酸 | hCys |
| 高丝氨酸 | hSer |
| 羟赖氨酸 | Hyl |
| 别-羟赖氨酸 | aHyl |
| 3-羟基脯氨酸 | 3Hyp |
| 4-羟基脯氨酸 | 4Hyp |
| 异锁链素 | Ide |
| 别-异亮氨酸 | aIle |
| 蛋氨酸亚砜 | MSO |
| N-甲基甘氨酸、肌氨酸 | MeGly |
| N-甲基异亮氨酸 | MeIle |
| 6-N-甲基赖氨酸 | MeLys |
| N-甲基缬氨酸 | MeVal |
| 2-萘基丙氨酸 | 2-Nal |
| | Nva |

| | |
|---|---|
| 正缬氨酸 | Nle |
| 正亮氨酸 | Orn |
| 鸟氨酸 | Phe(4-Cl) |
| 4-氯苯基丙氨酸 | Phe(2-F) |
| 2-氟苯基丙氨酸 | Phe(3-F) |
| 3-氟苯基丙氨酸 | Phe(4-F) |
| 4-氟苯基丙氨酸 | Phg |
| 苯基甘氨酸 | Thi |
| β-2-噻吩丙氨酸 | |

[0072] 此外，非天然氨基酸可以是Wang等人，(2006)Annu.Rev.Biophys.Biomol.Struct.35:225-49描述的“非天然”氨基酸。这些非天然氨基酸可以有利地用于将感兴趣的分子与AAV衣壳蛋白化学连接。

[0073] 词语“模板”或“底物”指的是可复制产生细小病毒DNA的多核苷酸序列。为了产生载体，模板通常将嵌在更大的核苷酸序列或构建体内，包括但不限于质粒、裸DNA载体、细菌人工染色体(BAC)、酵母人工染色体(YAC)或病毒载体(例如，腺病毒、疱疹病毒、Epstein-Barr病毒、AAV、杆状病毒、逆转录病毒载体等)。或者，所述模板稳定地包含在包装细胞的染色体内。

[0074] 细小病毒或AAV“Rep编码序列”指的是编码细小病毒或编码调节病毒复制和新病毒粒子产生的AAV非结构蛋白的核酸序列。细小病毒和AAV复制基因和蛋白质在例如，FIELDS等所著《病毒学》第2卷第69&70章(4版，利平科特-雷文出版公司)中进行了描述。

[0075] “Rep编码序列”并不需要编码所有细小病毒或AAV Rep蛋白质。例如，谈到AAV时，Rep编码序列并不需要编码所有四个AAV Rep蛋白质(Rep78、Rep 68、Rep52和Rep40)，实际上，我们认为，AAV5仅表达剪接Rep68和Rep40蛋白质。在代表性实施例中，Rep编码序列至少对病毒基因组复制和包装到新病毒粒子内必须的复制蛋白质进行编码。Rep编码序列通常将对至少一种大型Rep蛋白质(即Rep78/68)和一种小型Rep蛋白质(即Rep52/40)编码。在具体实施例中，Rep编码序列编码AAV Rep78蛋白质和AAV Rep52与/或Rep40蛋白质。在其它实施例中，Rep编码序列编码Rep68蛋白质和Rep52蛋白质与/或Rep40蛋白质。在另一个实施例中，Rep编码序列编码Rep68和Rep52蛋白质、Rep68和Rep40蛋白质、Rep78和Rep52蛋白质、Rep78和Rep40蛋白质。

[0076] 词语大型“Rep蛋白质”指的是Rep68与/或Rep78。发明所述大型Rep蛋白质可以是野生型或合成型。野生型大型Rep蛋白质可以来自任何细小病毒或AAV，包括但不限于血清型1、2、3a、3b、4、5、6、7、8、9、10、11或13，或现在熟知或以后发现的任何其它AAV。合成大型Rep蛋白质可以通过插入、删除、截断与/或错义突变改变。

[0077] 本领域技术人员将进一步了解的是，复制蛋白质并不一定由同一多核苷酸编码。例如，对MVM来说，NS-1和NS-2蛋白质(剪接变体)可以由另一种多核苷酸独立表达。同样，对AAV来说，p19启动子可以失活，大型Rep蛋白质由一种多核苷酸表达，而小型Rep蛋白质由另一种不同的多核苷酸表达。但是，复制蛋白质由单一构建体表达通常更方便。在一些系统中，病毒启动子(例如，AAV p19启动子)可能无法被细胞识别，因此，必须由不同的表达盒表达大型和小型Rep蛋白质。在其它情况下，可能要求分别表达大型Rep和小型Rep蛋白质，即

在独立转录与/或翻译控制元件控制下。例如，可能要求控制大型Rep蛋白质的表达，从而降低大型Rep蛋白质和小型Rep蛋白质之比。在昆虫细胞的情况下，下调大型Rep蛋白质(例如Rep78/68)的表达，避免对细胞的毒性比较有利(参见，例如Urabe等人，(2002)Human GeneTherapy 13:1935)。

[0078] 细小病毒或AAV帽子(cap)编码序列编码构成功能细小病毒或AAV衣壳(即可以包装DNA和感染靶细胞)的结构蛋白质。通常，帽子编码序列将编码所有细小病毒或AAV衣壳亚单位，但可以并非对全部衣壳亚单位进行编码，只要产生功能衣壳即可。通常，帽子编码序列将位于单个核酸分子上，但并非必须如此。

[0079] 自主细小病毒和AAV的衣壳结构在FIELDS等所著《病毒学》第2卷第69&70章(4版，利平科特-雷文出版公司)中进行了更详细的描述。

表达IDUA的细小病毒载体

[0080] 本发明提供细小病毒载体，例如，AAV载体，所述载体包括编码IDUA的核苷酸序列，并且能够在受试者角膜内表达IDUA。

[0081] 本发明的一个方面涉及一种重组核酸，所述核酸包括编码人α-L-艾杜糖醛酸酶(IDUA)的核苷酸序列，基本上由或由所述编码人α-L-艾杜糖醛酸酶(IDUA)的核苷酸序列组成，其中所述核苷酸序列经密码子优化用于人类细胞表达。在一些实施例中，所述核酸是非天然序列。在一些实施例中，所述核酸包括至少90％与SEQ ID NO:1相同的核苷酸序列，或基本上由或由所述核苷酸序列组成，例如，至少91％、92％、93％、94％、95％、96％，、97％、98％或99％与SEQ ID NO:1相同。在一些实施例中，所述核苷酸包括SEQ ID NO:1核苷酸序列，基本上由或由SEQ ID NO:1核苷酸序列组成。在一些实施例中，所述核酸包括至少10个连续SEQ ID NO:1核苷酸，例如，至少10、25、50、100、200、300、400、500，、600、700、800、900、1000、1100、1200、1300、1400、1500、1600、1700、1800个或更多个连续SEQ ID NO:1核苷酸。

SEQ ID NO 1:

```
  1 CACCGGTCGC CACCATGCGA CCACTGAGAC CACGGGCCGC TCTGCTGGCT
 51 CTGCTGGCTT CACTGCTGGC CGCTCCCCCT GTCGCTCCTG CTGAGGCTCC
101 CCACCTGGTG CATGTGGACG CAGCTCGCGC CCTGTGGCCA CTGAGGAGAT
151 TCTGGAGGAG CACAGGCTTT TGCCCACCTC TGCCTCACAG CCAGGCTGAC
201 CAGTACGTGC TGTCCTGGGA TCAGCAGCTG AACCTGGCAT ATGTGGGAGC
251 CGTCCCCCAC AGGGGGATCA AACAGGTGAG AACTCATTGG CTGCTGGAGC
301 TGGTCACCAC ACGAGGATCT ACTGGAAGGG GGCTGAGTTA CAACTTCACC
351 CACCTGGACG GCTATCTGGA TCTGCTGAGA GAGAATCAGC TGCTGCCTGG
401 ATTTGAACTG ATGGGCTCAG CCAGCGGACA TTTCACCGAC TTTGAGGATA
451 AGCAGCAGGT GTTCGAATGG AAAGACCTGG TCAGCTCCCT GGCTCGGCGC
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 501 TACATTGGGC GGTATGGCCT GGCACACGTG AGTAAGTGGA ACTTTGAGAC
 551 TTGGAATGAA CCAGACCACC ATGACTTCGA TAACGTGTCA ATGACCATGC
 601 AGGGGTTTCT GAATTACTAT GATGCCTGCT CCGAGGGCCT GCGGGCAGCC
 651 TCTCCAGCTC TGCGACTGGG AGGACCAGGC GATTCCTTCC ACACACCACC
 701 CAGAAGTCCC CTGTCATGGG GCCTGCTGCG GCACTGTCAT GACGGAACCA
 751 ACTTCTTTAC AGGAGAAGCA GGGGTGAGAC TGGATTACAT CTCCCTGCAT
 801 CGAAAGGGGG CCAGGTCTAG TATCTCTATT CTGGAGCAGG AAAAGGTGGT
 851 CGCTCAGCAG ATCCGGCAGC TGTTCCCCAA ATTTGCCGAC ACACCTATCT
 901 ACAATGACGA GGCTGATCCT CTGGTGGGAT GGTCTCTGCC TCAGCCATGG
 951 CGAGCCGATG TGACTTATGC TGCAATGGTG GTCAAAGTCA TTGCTCAGCA
1001 CCAGAACCTG CTGCTGGCAA ATACTACCAG TGCTTTCCCA TACGCACTGC
1051 TGAGTAACGA CAATGCCTTC CTGTCATATC ACCCCCATCC TTTTGCCCAG
1101 AGAACACTGA CTGCTCGGTT TCAGGTGAAC AATACCCGAC CTCCACATGT
1151 GCAGCTGCTG AGGAAGCCTG TCCTGACAGC CATGGGCCTG CTGGCTCTGC
1201 TGGACGAGGA ACAGCTGTGG GCAGAGGTGT CTCAGGCCGG GACTGTCCTG
1251 GATAGTAACC ACACCGTGGG CGTCCTGGCT TCTGCACATC GACCTCAGGG
1301 ACCAGCAGAC GCTTGGCGAG CAGCTGTGCT GATCTACGCA TCAGACGATA
1351 CACGAGCACA CCCTAACCGA AGCGTGGCAG TCACTCTGCG ACTGCGAGGA
1401 GTGCCACCTG GACCAGGACT GGTGTACGTC ACCCGCTATC TGGACAATGG
1451 CCTGTGCTCT CCCGATGGAG AGTGGCGAAG GCTGGGGAGG CCCGTGTTCC
1501 CTACAGCAGA GCAGTTTAGA CGGATGAGAG CAGCCGAAGA TCCAGTGGCT
1551 GCAGCACCAC GACCACTGCC TGCAGGAGGC AGACTGACCC TGCGGCCAGC
1601 CCTGCGCCTG CCAAGCCTGC TGCTGGTGCA CGTCTGCGCA AGGCCTGAAA
1651 AGCCACCCGG ACAGGTGACA AGGCTGAGAG CTCTGCCACT GACTCAGGGA
1701 CAGCTGGTGC TGGTCTGGTC AGACGAGCAT GTGGGGAGCA AATGTCTGTG
1751 GACTTACGAA ATTCAGTTCA GCCAGGATGG GAAGGCCTAT ACCCCTGTGA
1801 GCCGGAAACC CAGCACCTTC AACCTGTTCG TCTTTTCCCC AGACACCGGG
1851 GCCGTGTCCG GCTCTTACCG GGTCCGCGCT CTGGACTATT GGGCAAGGCC
1901 AGGCCCCTTC AGCGATCCTG TGCCATACCT GGAAGTGCCT GTGCCTCGCG
1951 GCCCACCATC TCCTGGAAAC CCTTGAGGGG ATCCGTCGAC TAG
```

[0082] 密码子优化核苷酸序列，从而提高生物体内表达的方法是本领域熟悉的，可以采用公开的软件开展。人IDUA的野生型序列是本领域熟悉的，可以在数据库(例如基因库)中找到。人IDUA保藏号实例包括A26494、AK291816和AH002600，通过引用，它们都纳入本发明中。

[0083] 本发明还提供一种包括本发明IDUA核酸的病毒载体基因组。在一些实施例中，IDUA核酸是野生型人IDUA序列或密码子-优化序列。病毒载体基因组可以是细小病毒载体基因组，例如，AAV载体基因组。所述病毒载体可以进一步包括与IDUA核酸操作连接的启动子。在一些实施例中，所述启动子可以是组成型启动子，例如，CMV启动子。在其它实施例中，启动子可以是组织特异性或优先性启动子。本发明进一步提供一种包含本发明所述AAV载体基因组的体外细胞，例如，稳定地整合到细胞基因组内。本发明进一步提供一种包括本发明所述病毒载体基因组的重组细小病毒粒子(例如，重组AAV粒子)。下面进一步讨论病毒载体和病毒粒子。

[0084] 在一些实施例中，由于本发明衣壳蛋白的存在，病毒载体显示出修饰嗜性。在一个

实施例中，所述细小病毒载体对角膜显示出全身嗜性。在其它实施例中，与含野生型衣壳蛋白的病毒载体相比，所述细小病毒载体对肝脏的嗜性降低。

产生病毒载体的方法

[0085] 本发明进一步提供产生病毒载体的方法。在一个具体实施例中，本发明提供一种产生重组细小病毒粒子的方法，包括向允许细小病毒复制的细胞提供：(a)重组细小病毒模板，包括(i)编码IDUA的核酸，和(ii)细小病毒ITR；(b)多核苷酸，包括Rep和Cap编码序列；在足够复制和包装重组细小病毒模板的条件下；从而在细胞中产生重组细小病毒粒子。足够复制和包装重组细小病毒模板的条件可以是，例如，存在足够复制细小病毒模板和包裹到细小病毒衣壳内的AAV序列(例如，细小病毒rep序列和细小病毒cap序列)及来自腺病毒与/或疱疹病毒的辅助序列。在具体实施例中，细小病毒模板包括两个细小病毒ITR序列，这两个序列位于异源核酸序列的5’和3’端，虽然它们并不需要与它们直接连接。

[0086] 在一些实施例中，重组细小病毒模板包括非Rep分辨的ITR，从而形成国际专利公开WO 01/92551所述的双链AAV载体。

[0087] 细小病毒模板和细小病毒rep和cap序列在某种条件下提供，从而在细胞内产生病毒载体，所述病毒载体包括细小病毒衣壳包装的细小病毒模板。所述方法可以进一步包括从细胞采集病毒载体的步骤。病毒载体可以从培养基采集与/或通过裂解细胞采集。

[0088] 细胞可以是允许细小病毒复制的细胞。可以采用本领域熟悉的任何合适细胞。在具体实施例中，细胞是哺乳动物细胞(例如，灵长类动物或人类细胞)。作为另一种选择，所述细胞可以是反式-互补包装细胞系，提供复制-缺陷辅助病毒删除的功能，例如，293细胞或其它E1a反式互补细胞。

[0089] 可以采用本发明熟悉的任何方法提供细小病毒复制和衣壳序列。目前的方案通常表达单质粒上的细小病毒rep/cap基因。细小病毒复制和包装序列不需要一起提供，虽然一起提供的话可能比较方便。细小病毒rep与/或cap序列可以由任何病毒或非-病毒载体提供。例如，rep/cap序列可以由混合腺病毒或疱疹病毒载体提供(例如，插入到删除腺病毒载体的E1a或E3区内)。还可以采用EBV载体来表达细小病毒cap和rep基因。本方法的一个优点是EBV载体是附加体，但将在整个连续细胞分化期间保持高拷贝数(即作为额外-染色体元件稳定整合到细胞内，称为“EBV基核附加体”，参见Margolski，(1992) Curr.Top.Microbiol.Immun.158:67)。

[0090] 作为另一个替代，rep/cap序列可以稳定整合到细胞内。

[0091] 通常，细小病毒rep/cap序列将不侧接TR，以防止这些序列的补救与/或包装。

[0092] 可以采用本领域熟悉的任何方法向细胞提供细小病毒模板。例如，模板可由非病毒(例如质粒)或病毒载体提供。在具体实施例中，细小病毒模板由疱疹病毒或腺病毒载体提供(例如，插入到删除腺病毒的E1a或E3区内)。作为另一种阐述，Palombo等人，(1998) J.Virology72:5025描述了携带侧接AAV TR报告基因的杆状病毒载体。正如上文谈到rep/cap基因时所述，还可以采用EBV载体递送模板。

[0093] 在另一个代表实施例中，细小病毒模板通过复制rAAV病毒提供。在另一个其它实施例中，包括细小病毒模板的AAV前病毒稳定地整合到细胞的染色体内。

[0094] 为了提高病毒滴度，可以向细胞提供促进增殖性细小病毒感染的辅助病毒功能(例如，腺病毒或孢疹病毒)。细小病毒复制必须的辅助病毒序列是本领域熟悉的。通常，这

些序列将由辅助腺病毒或孢疹病毒载体提供。或者，腺病毒或孢疹病毒序列可由另一种非病毒或病毒载体提供，例如，作为携带所有促进高效细小病毒产生的辅助基因非感染性腺病毒微质粒，如Ferrari等人，(1997)Nature Med.3:1295和美国专利第6,040,183号和第6,093,570号所述。

[0095] 此外，辅助病毒功能可由包装细胞提供，其中辅助序列嵌在染色体内或作为稳定的染色体外元件维持。通常，辅助病毒序列不能包装到AAV病毒粒子内，例如，不侧接ITR。

[0096] 熟悉本领域的技术人员将了解的是，在单一辅助构建体上提供细小病毒复制和衣壳序列及辅助病毒序列(例如，腺病毒序列)比较有利。这种辅助构建体可以是非病毒或病毒构建体。作为非限制性说明，辅助构建体可以是包括AAV rep/cap基因的混合腺病毒或混合孢疹病毒。

[0097] 在一个具体实施例中，细小病毒rep/cap序列和腺病毒辅助序列由单个腺病毒辅助载体提供。这种载体可以进一步包括细小病毒模板。细小病毒rep/cap序列与/或细小病毒模板可以插入到腺病毒的删除区(例如，E1a或E3区内)。

[0098] 在另一个实施例中，细小病毒rep/cap序列和腺病毒辅助序列由单个腺病毒辅助载体提供。根据该实施例，细小病毒模板可以作为质粒模板提供。

[0099] 在另一个示范实施例中，细小病毒rep/cap序列和腺病毒辅助序列由单一腺病毒辅助载体提供，细小病毒模板作为前病毒整合到细胞内。或者，细小病毒模板由细胞内作为染色体外元件维持的EBV载体提供(例如，作为EBV基核附加体)。

[0100] 在另一个示范实施例中，细小病毒rep/cap序列和腺病毒辅助序列由单个辅助腺病毒提供。细小病毒模板可以作为独立的复制病毒载体提供。例如，细小病毒模板可以由细小病毒粒子或第二重组腺病毒粒子提供。

[0101] 根据前面的方法，混合腺病毒载体通常包括足够腺病毒复制和包装的腺病毒5'和3'顺式序列(即腺病毒末端重复序列和PAC序列)。细小病毒rep/cap序列，及AAV模板(如果存在的话)嵌在腺病毒骨架内，侧接5'和3'顺式序列，从而这些序列可以包装到腺病毒衣壳内。如上所述，腺病毒辅助序列和腺病毒rep/cap序列通常并不侧接ITR，从而这些序列不包装到细小病毒粒子内。

[0102] Zhang等人，((2001)Gene Ther.18:704-12)描述了一种包括腺病毒及AAV rep和cap基因的嵌合辅助者。

[0103] 疱疹病毒也可以作为细小病毒包装方法中的辅助病毒。编码细小病毒Rep蛋白的混合疱疹病毒可有利地促进可扩展细小病毒载体产生方案。已经有人描述(Conway等人，(1999)Gene Ther.6:986和WO 00/17377)了表达AAV-2rep和cap基因的混合I型单纯疱疹病毒(HSV-1)。

[0104] 作为另一个替代方案，正如，例如Urabe等人，(2002)Human Gene Ther.13:1935-43所述，本发明的病毒载体可以采用杆状病毒在昆虫细胞中产生，以递送rep/cap基因和细小病毒模板。

[0105] 不含污染辅助病毒的细小病毒载体库可采用本领域熟悉的任何方法获取。例如，细小病毒和辅助病毒很容易根据尺寸区分。细小病毒还可以根据对硫酸肝素的亲合性与辅助病毒区分(Zolotukhin等人，(1999)Gene Therapy 6:973)。可以采用删除复制-缺陷辅助病毒，从而任何污染辅助病毒不会复制。作为另一个替代方案，可以采用缺少晚期基因表达

的辅助腺病毒，因为细小病毒包装调节仅需腺病毒早期基因表达。晚期基因表达缺陷腺病毒突变体是本领域熟悉的(例如，ts100K和ts149腺病毒突变体)。

重组病毒载体

[0106] 本发明的病毒载体用于将核酸递送给试管内、活体外、活体内细胞。特别是，可以有利地采用病毒载体，递送或输送核酸至动物(包括哺乳动物)细胞。特别是，本发明的所述病毒载体用于递送编码IDUA的核酸至受试者的角膜。

[0107] 已经通过两种施用途径：伤口愈合分析局部施用和直接注射到角膜基质，在动物模型中对角膜靶向AAV基因治疗进行了研究。关于局部施用，据报道，AAV血清型9(AAV9)的角膜转导最有效，但是，这种血清型几乎全部位于上皮/基质边界(Sharma等人，Exp.Eye Res.91(3):440(2010))。至于在基质内注射到人角膜外植体后递送AAV基因方面，据观察，AAV8的基质转导效率比AAV2或AAV1高，包括多种细胞类型，包括$CD34^+$角膜细胞和巨噬细胞(Hippert等人，PLoS One,7(4):e35318(2012))。重要的是，据观察，这两种给药途径都未出现任何与AAV载体有关系的不良后果(Sharma等人，Exp.Eye Res.91(3):440(2010)；Hippert等人，PLoS One,7(4):e35318(2012)；Mohan等人，PLoS One 6(10):e26432(2011))。

[0108] 本领域技术人员将了解的是，编码IDUA的核酸可以与合适的控制序列操作连接。例如，所述核酸可以与表达控制元件操作连接，例如，转录/翻译控制信号、复制源、多腺苷酸化信号、内部核糖体进入位点(IRES)、启动子，与/或增强子等。

[0109] 本领域技术人员将了解的是，根据期望的水平和组织-特异性表达，可以采用各种启动子/增强子元件。根据期望的表达模式，启动子/增强子可以是组成型或诱导型。启动子/增强子可以是本身就有的或外来的，可以是天然的或合成的序列。若为外来的，表示野生型宿主中未发现转录起始区，在其中引入转录起始区。

[0110] 在具体实施例中，启动子/增强子元件可以是靶细胞或治疗受试者本身就有的。在代表性实施例中，启动子/增强子元件可以是IDUA核酸序列本身就有的。通常选择启动子/增强子元件，从而其在感兴趣的靶细胞中发挥作用。此外，在具体实施例中，启动子/增强子元件是哺乳动物启动子/增强子元件。启动子/增强子元件可以是组成型或诱导型。

[0111] 可诱导表达控制元件通常在期望提供核酸序列调节过表达的应用中比较有利。基因递送诱导型启动子/增强子元件可以是组织特异性或-优先性启动子/增强子元件，包括眼睛特异性或优先性(包括视网膜-特异性和角膜-特异性)启动子/增强子元件。其它可诱导启动子/增强子元件包括激素-可诱导和金属-可诱导元件。示例性可诱导启动子/增强子元件包括但不限于Tet开/关元件、RU486-可诱导启动子、蜕皮激素-可诱导启动子、雷帕霉素-可诱导启动子及金属硫蛋白启动子。

[0112] 在核酸序列在靶细胞中转录，然后翻译的实施例中，通常包括特异性起始信号用于高效翻译插入的蛋白编码序列。这些外源翻译控制序列，可能包括ATG起始密码子和相邻的序列，可以来自各种天然和合成来源。

[0113] 本发明的病毒载体可以是细小病毒载体，例如，AAV载体。AAV载体可以是任何AAV血清型。在一些实施例中，AAV载体是AAV2、AAV8或AAV9载体。在一些实施例中，AAV载体是混合载体，例如，混合载体拥有来自一种血清型的衣壳蛋白及来自另一种血清型的基因组或混合载体拥有合成衣壳蛋白。在一些实施例中，载体包括嗜性改变的混合衣壳。在一个实例

中，混合衣壳在一种血清型(例如，AAV8)的衣壳序列中包括另一种血清型(例如，AAV9)的聚糖结合位点(参见，例如，WO 2014/144229，该专利通过引用全文纳入本申请中)。

[0114] 本发明的病毒载体提供一种递送IDUA核酸到范围广泛的各种细胞(包括分化和未分化细胞)内的手段。可以采用所述病毒载体将核酸递送到体外细胞内，例如，从而试管内或活体外产生用于基因治疗的多肽。所述病毒载体还用于将核酸递送到受试者体内的方法，例如，从而表达IDUA。采用这种方式，可以在受试者体内产生多肽。受试者需要这种多肽，因为受试者缺乏这种多肽。此外，所述方法可以实施，因为受试者体内多肽的产生可能赋予某些有益效果。

[0115] 病毒载体还可用于在培养细胞中或受试者体内产生IDUA(例如，采用受试者作为生物反应器，产生多肽，或观察多肽对受试者的影响，例如，与筛选方法一起使用)。

[0116] 本发明的病毒载体可用于递送编码IDUA的核酸，治疗与/或预防从递送IDUA中受益的任何疾病，例如，MPS I。

[0117] 本发明的病毒载体可用于诊断和筛选方法，从而IDUA核酸在细胞培养系统、器官或器官培养基(例如，眼睛)或转基因动物模型中短暂或稳定表达。

[0118] 本发明的病毒载体还可以用于各种非治疗用途，包括但不限于评价基因打靶、清除、转录、翻译等方案中的用途，这些用途是本领域技术人员显而易见的。病毒载体还可以用于评价安全性(扩散、毒性、免疫原性等)。这些数据，例如，被美国食品药物管理局视为评价临床效果前监管批准过程的一部分。

[0119] 或者，可向体外细胞，例如，角膜外植体施用病毒载体，然后，将所述改变的细胞或外植体向受试者施用。向细胞内加入包含IDUA核酸的病毒载体，及向受试者施用所述细胞，其中所述核酸可以被表达。

受试者、药物制剂及施用方式

[0120] 本发明的病毒载体和衣壳用于兽医和医学应用。合适的受试者包括鸟类和哺乳动物。词语“鸟类”包括但不限于鸡、鸭、鹅、鹌鹑、火鸡、野鸡、鹦鹉、长尾鹦鹉等。词语“哺乳动物”包括但不限于人类、非人类灵长类动物、牛、绵羊、山羊、马、猫、犬、兔等。人类受试者包括婴儿、胎儿、少年和成人。

[0121] 在具体实施例中，本发明提供一种药物组合物，包括药学上可接受载体中的本发明病毒载体与/或衣壳及，任选其它药剂、药物、稳定剂、缓冲液、载体、佐剂、稀释剂等。注射用时，载体通常是液体。对于其它施用方法，载体可以是固体或液体。对于吸入施用，载体是可以吸入的，及任选可以是固体或液体颗粒形式。

[0122] “药学上可接受的”指的是材料无毒或者是符合要求的，即材料可以向受试者施用而不会造成任何不期望发生的生物效应。

[0123] 本发明的一个方面是一种将核酸递送到体外细胞的方法。按照适合具体靶细胞的标准转导方法，可以按合适的感染复数将病毒载体引入到细胞内。施用病毒载体的滴度可以根据靶细胞类型和数量及具体的病毒载体而变化，可以由本领域技术人员确定，无需开展过多实验。在代表性实施例中，至少大约$10^3$感染单位，更优选至少大约$10^5$感染单位被引入到细胞内。

[0124] 引入病毒载体的细胞可以是任何类型的细胞，包括但不限于眼细胞(包括视网膜细胞、视网膜色素上皮细胞和角膜细胞(例如，角膜细胞、上皮细胞和内皮细胞)。此外，所述

细胞可以源自上文所述任何物种。

[0125] 病毒载体可以引入到体外细胞中，用于向受试者施用所述修饰细胞。在具体实施例中，从受试者采集所述细胞，在细胞内引入所述病毒载体，然后，将所述细胞施用回到受试者体内。体外操作从受试者采集细胞，然后将细胞引回到受试者体内的方法是本领域熟悉的(参见，例如美国专利第5,399,346号)。或者，可以将重组病毒载体引入到供体受试者的细胞内、培养细胞内或其它任何合适来源的细胞内，并将所述细胞向需要的受试者施用(即“受体”受试者)。

[0126] 体外基因递送的合适细胞如上文所述。受试者细胞施用剂量将根据受试者年龄、状况和物种、细胞类型、细胞表达的核酸及施用方式等变化。通常，药学上可接受载体内每次剂量施用的细胞数是至少大约$10^2$至大约$10^8$个细胞或至少大约$10^3$个细胞至大约$10^6$个细胞。在具体实施例中，病毒载体转导的细胞以治疗有效或预防有效剂量与药学载体一起向受试者施用。

[0127] 本发明的另一个方面是一种向受试者施用病毒载体的方法。可以采用本领域熟悉的任何方法向需要的人类受试者或动物施用本发明病毒载体。任选病毒载体在药学上可接受载体内按治疗有效或预防有效剂量递送。

[0128] 受试者病毒载体的施用剂量取决于施用方式、治疗与/或预防的疾病或病症、受试者的状况、具体的病毒载体，及递送的核酸等，并且可以按照常规方式确定。实现治疗效果的示例剂量是至少大约$10^5$、$10^6$、$10^7$、$10^8$、$10^9$、$10^{10}$、$10^{11}$、$10^{12}$、$10^{13}$、$10^{14}$、$10^{15}$转导单位滴度，任选大约$10^8$-$10^{13}$转导单位滴度。

[0129] 在具体实施例中，要实现要求的基因表达水平，可以在不同的间隔期间，例如，每天、每周、每月、每年等多次施用(例如，施用两次、三次、四次或更多次)。

[0130] 角膜的示例施用模式包括基质内施用、局部施用、玻璃体内施用和视网膜下施用。

[0131] 向靶组织递送还可以通过递送包含病毒载体的储库实现。在代表性实施例中，包含病毒载体的储库被移植到角膜内或眼睛的其它组织内或可将组织与包含所述病毒载体的薄膜或其它基质接触。合适的可移植基质或基材在美国专利第7,201,898号中进行了描述。

[0132] 在具体实施例中，本发明的一种病毒载体施用于角膜，用于治疗、推迟与/或预防与MPS I有关的角膜浑浊与/或失明。

[0133] 因此，本发明的一个方面进一步包括一种递送IDUA至受试者角膜的方法，包括向受试者角膜施用有效剂量的表达IDUA的AAV粒子，从而将IDUA递送至受试者的角膜。

[0134] 另一方面，本发明进一步包括一种治疗、推迟与/或预防受试者MPS I-相关角膜浑浊的方法，包括向受试者角膜施用治疗有效剂量的表达IDUA的AAV粒子，从而治疗、推迟与/或预防受试者MPS I-相关角膜浑浊。

[0135] 另一方面，本发明进一步包括一种体外或间接体内递送IDUA至角膜的方法，例如，在受试者移植之前，包括将有效剂量表达IDUA的AAV粒子与角膜接触，从而将IDUA递送至角膜。在一些实施例中，角膜可以采用AAV粒子培养或将AAV粒子注射到角膜内。

[0136] 在本发明的方法中，受试者可以是诊断患有MPS I或怀疑患有MPS I的患者。在一些实施例中，受试者是婴儿或儿童，例如，小于18岁，例如，小于18、17、16、15、14、13、12、11、10、9、8、7、6或5岁。在一些实施例中，受试者未出现角膜浑浊。在其它实施例中，受试者至少

出现部分角膜浑浊，例如，与未患MPS I的受试者相比，受试者视力降低大约不到10％，例如，大约不到10％、20％、30％、40％、50％、60％、70％、80％或90％。

[0137] 注射液可以以传统形式制备，以注射前的液体溶液或悬浮液、适合配制成溶液或悬浮液的固体形式，或者以乳液形式制备。或者，可以局部施用本发明的病毒载体与/或病毒衣壳，例如，以储库或缓释制剂的形式。此外，病毒载体与/或病毒衣壳可以粘附在外科可移植基质上递送(例如，如美国专利公开第2004-0013645号所述)。

[0138] 上面已经对本发明进行了描述，将在下述实例中对这些方面进行更详细的说明，这些实例在此仅用于阐述目的，而非用于限制本发明。

实例1

材料和方法

[0139] 制备AAV载体。对于细胞培养实验，采用以前描述的三次转染法产生此处使用的载体(Grieger等人，Nat.Protoc.1(3):1412(2006))。该方法采用pXR2或pXR8G9质粒，这些质粒都包含AAV的rep2及独立包含所示血清型的衣壳基因。首先，用AgeI和SalI位点的密码子优化IDUA cDNA(由GenScript提供)替换pTR-CMV-eGFP中的egfp基因，构建包含所示AAV基因组的质粒。继AAV产生和氯化铯梯度分离(Grieger等人，Nat.Protoc.1(3):1412(2006))后，采用PBS对峰组分进行透析，并采用定量PCR滴定(CMV_F CAA GTA CGC CCC CTA TTG AC(SEQ ID NO:2)，CMV_R AAG TCC CGT TGA TTT TGG TG(SEQ ID NO:3))，该定量PCR滴定经过Southern印迹分析证明(Grieger等人，Nat.Protoc.1(3):1412(2006))。对于人类外植体实验，采用北卡罗来纳大学教堂山分校(UNC)载体中心提供的GMP级载体制剂。

[0140] 细胞培养和载体转导：将人胚胎肾293细胞、MPS I患者成纤维细胞和正常人的成纤维细胞(Simpson等人，J.Carcinog.4:18(2005))在37℃、5％$CO_2$气氛中维持在补充10％胎牛血清(FCS)、青霉素/链霉素(100U/ml)的Dulbecco改良伊格尔培养基(Sigma)中。培养细胞转导按固定体积在24孔板中进行。在这些实验中，将载体按所示病毒基因组/细胞数量加入到孔内，并且在实验期间不清除载体。

[0141] 人角膜实验：所有实验方案都经过北卡罗来纳大学教堂山分校批准。人角膜由视力组织采购库提供。这些去身份死后组织实验按照北卡罗来纳大学教堂山分校人类研究伦理学人类受试者研究办公室的规定执行(IRB-14-1019)。将两种性别的角膜都在37℃、5％$CO_2$气氛中保持在提供的opti-mem中。采用31号胰岛素注射器进行注射。采用墨水作染料来验证注射。然后，将角膜培养7天，并按照文中所述进行分析。

[0142] 功能IDUA检验：按照前面所述(Garcia-Rivera等人，Brain Res.Bull.74(6):429(2007))，测定定量IDUA酶活性。简单地说，将10μl上清液或细胞裂解液采用50μM 4-甲基伞形酮α-L-艾杜糖醛酸酶在37℃于暗处培养60分钟，其中4-甲基伞形酮α-L-艾杜糖醛酸酶采用pH 3.5、含0.2％Triton x-100的0.4M甲酸钠缓冲液配制。加入80μl 0.5M NaOH/甘氨酸缓冲液(pH 10.3)终止反应。将96孔板在4℃以13000rpm条件离心分离1分钟，上清液转移到底部透明的带盖黑色96孔板内，在365nm激发后，测定450nm处的荧光。根据之前采用4-甲基伞形酮，并以nmol/h/mg表达细胞裂解液中酶的活性，或上清液分析时以nmol/h/10μl表达酶活性建立的标准曲线，计算裂解底物的含量。每个样品的蛋白质含量采用BCA分析测定。

[0143] Tunel检测：AAV转导期间因细胞毒性导致的凋亡细胞采用脱氧核苷酸基转移酶(TdT)-介导的dUTP-生物素缺口末端标记测定，dUTP-生物素缺口末端采用DAB标记。Tunel

检测采用TACS2TdT-Blue Label原位凋亡检测试剂盒(Trevigen,Gaithersburg,Md.)进行。简单地说,在脱蜡和再水合后,将人角膜切片在蛋白酶K中消解20分钟。载波片用1xPBS洗涤,采用3%$H_2O_2$甲醇溶液培养组织,淬灭内源性过氧化物酶的活性。采用1xPBS洗涤载玻片后,将组织切片采用1×TdT标记缓冲液培养5分钟。标记反应由TdT酶培养角膜组织、生物素化dUTP培养dNTP,及37℃加湿室内1x氯化锰(1x TdT标记缓冲液中)培养1小时组成。采用1x TdT终止缓冲液培养结束反应。采用链霉素亲和素-结合辣根过氧化物酶和DAB溶液处理切片,使断裂DNA直观显化。

[0144] 免疫荧光染色:将人角膜组织嵌在石蜡中,并采用切片机切成5μm厚度。为了采用免疫荧光抗体对载玻片染色,将载玻片在二甲苯中培养5分钟,共培养两次,从而脱除切片的石蜡,然后将其顺序浸在100%、95%和70%乙醇溶液中,每次浸5分钟,最后在水中浸5分钟,开展再水合。在切片染色之前,开展抗原修复程序,确保表位暴露。由于石蜡嵌入过程,这些表位之前被掩蔽。特别是,将载玻片在预加热的98℃抗原修复液(Dako)中浸7分钟。然后,在室温用10%NGS(1×PBS中)培养载玻片1小时,阻断这些组织内的非特异性结合位点。采用2%NGS(1×PBS中)洗涤载玻片2次,每次洗5分钟。然后,采用大致在含2%NGS(1×PBS中)的溶液中稀释的一抗在4℃培养一个晚上。一抗培养后,洗涤载玻片3次,每次5分钟,从而清除非特异性结合的一抗。洗涤溶液包含2%NGS(1×PBS中)。然后,将合适的荧光-标记二抗(5μg/ml)加入到2%NGS(1×PBS中)稀释的载玻片上,并将载玻片在4℃培养1小时。最后,采用2%NGS(1×PBS中)洗涤载玻片3次,每次5分钟,最后用4℃1×PBS洗涤。加入几滴Hoechst(Molecular probes-Life technologies,H3569Hoechst 33258,五水合物-1μg/mL)7分钟,对切片核心进行复染,然后用水洗涤。然后,用Cytoseal 60盖玻片(Thermo Fisher Scientific(NYSE:TMO))盖好载玻片。

[0145] 此研究采用的一抗如下:IDUA染色时,采用兔子多克隆IDUA抗体(Biorbyt,目录号:orb157615,稀释1/50);GFP染色时,采用鸡抗GFP抗体(Aves,目录号:GFP-1020,稀释1/100);CD34染色时,采用小鼠单克隆抗体(克隆:B-6)(Santa Cruz,sc-74499,稀释1/100)),α-平滑肌肌动蛋白染色时,采用小鼠单克隆抗体(R&D,克隆#1A4,目录号MAB1420,稀释1/100),F4/80标记物染色时,采用大鼠单克隆抗体(克隆:BM8)(Santa Cruz,sc-52664,稀释1/100)。采用的二抗如下:Alexa Fluor®594羊抗兔IgG(A-11012)(Gibco-Invitrogen,Carlsbad,CA)、Alexa Fluor®594羊抗鸡IgG(A-11039)(Gibco-Invitrogen,Carlsbad,CA)、Alexa Fluor®594羊抗大鼠IgG(A-11006)(Gibco-Invitrogen,Carlsbad,CA)和Alexa Fluor®488羊抗小鼠IgG(A-11001)(Gibco-Invitrogen,Carlsbad,CA)。采用配备40×物镜的Zeiss LSM 780Confocal显微镜(日本东京奥林巴斯公司)拍摄每个载玻片的图像。包括完整的人角膜的图像采用10x物镜、标题功能拍摄,然后拼接。然后,采用AdobePhotoshop对图像进行处理。在所有实验中,都单独采用二抗对每个组织切片进行染色,作为阴性对照染色。

[0146] MPS I细胞转染和蛋白质印迹分析:将MPS I患者成纤维细胞按20000个细胞/孔接种在24孔板内。24小时后,每次处理向每个孔内加入混合1μg质粒DNA、3μl PEI和60μl DMEM,对9个孔进行转染。转染24小时后,从每个孔采集40μl上清液,3孔混合,形成样品。向每个孔内加入40μl DMEM,以使后面的上清液采集维持同样的体积。转染48小时后,采集全部上清液,再一次3孔混合,形成三个重复样品。此时,每个孔内加入70μl哺乳动物蛋白质提取试剂(Thermo Scientific Cat:78501),并按照试剂协议,收获全部细胞蛋白质。为了开

展蛋白质印迹分析，将蛋白裂解液加入到5％β-巯基-乙醇的4x Nupage样品缓冲液溶液中。将所得溶液煮沸10分钟，并在冰上冷却10分钟，然后在10％Bis-tris预制凝胶中开展电泳。凝胶在1X MOPS电泳缓冲液中进行电泳，并转移到硝基纤维素膜。该膜采用5％牛奶ddH20溶液阻断，并以PBS-Tween溶液按1:500稀释(0.5％Tween 20的1xPBS溶液)的小鼠宿主IDUA抗体(R&D Systems MAB-4119)或以PBS-Tween溶液按1:5000稀释的小鼠宿主β-肌动蛋白抗体(Sigma)检测2小时。然后，采用以PBS-Tween溶液按1:10000稀释的小鼠辣根过氧化物酶二抗检测。按照产品协议，采用Western-Bright Sirius化学-荧光试剂，并采用放射自显膜暴露印迹。

[0147] 野生型和密码子优化IDUA的293细胞转染对比：将293细胞按每孔100,000个细胞接种到24孔板内。24小时后，向每个孔内加入混合1μg质粒DNA、3μl PEI和60μl DMEM，对3个孔进行转染。保留细胞培养上清液后，每个孔内加入70μl哺乳动物蛋白质提取试剂(Thermo Scientific Cat:78501)，72小时后，收获全部细胞蛋白质。

[0148] MPS I患者细胞毒性分析：将患者细胞按每孔20,000个细胞接种到24孔板内。24小时后，加入AAV-2 316，共四个重复样品。72小时后，保留上清液；将细胞用0.05％胰蛋白酶和其相应的上清液重新悬浮。采用Beckman Vi-cell XR细胞活力分析仪分析所有样品。

实例2

MPS I成纤维细胞中的AAV IDUA表达盒

[0149] 为了制备AAV IDUA表达盒，将人idua cDNA(NM_000203)进行密码子优化用于人类表达(opt-IDUA)，并使其位于AAV反向末端重复序列血清型2质粒的CMV启动子和SV40聚腺苷酸化序列之间(图1A)。按照所述制备AAV2-opt-IDUA载体(Grieger等人，Adv.Biochem.Eng.Biotechnol.99:119(2005))，并用于表征MPS I患者成纤维细胞。采用永生化正常人成纤维细胞(NHF)作为对照细胞系(Simpson等人，J.Carcinog.4:18(2005))。在剂量递增实验中，MPS I成纤维细胞的AAV2-opt-IDUA转导导致细胞裂解液和培养上清液中IDUA恢复，其含量水平不断增加(图1B)。实际上，由于NHF中IDUA的静息水平相对较低，因此，5,000病毒基因组/细胞用量即导致细胞裂解液和培养上清液内达到超生理水平(图1B)。同样，转导MPS I患者成纤维细胞的IDUA功能得到恢复和提高，在研究的最高剂量时，细胞裂解液和上清液中分别提高10倍和30倍(图1C和1D)。虽然在AAV载体转导后，过量产生IDUA，但是，染料排除试验表明，任何剂量时患者成纤维细胞都未观察到任何毒性(图2)。重要的是，这些结果证明了AAV2-opt-IDUA对MPS I患者的功能性和安全性。

实例3

角膜中的AAV IDUA表达盒

[0150] 对MPS I患者角膜的分析表明基质异常是导致角膜不透明、从而视力减退的原因(Huang等人，Exp.Eye Res.62(4):377(1996))。因此，直接施用AAV-opt-IDUA应恢复MPS I患者角膜基质室内的IDUA活性，有望预防或逆转MPS I显型。以前的报告证明了采用AAV8(Hippert等人，PLoS One,7(4):e35318(2012))和AAV9(Sharma等人，Exp.Eye Res.91(3):440(2010))用于人类角膜细胞转导，角膜细胞是角膜中最常见的细胞类型。因此，在死后数周仍然有活力的正常人角膜外植体中对这些携带自补(sc)AAV-CMV-GFP基因组的衣壳进行评估。初步实验表明，向基质内注射50μl，可以耐受，组织扩张小，采用包含墨水的载体溶媒，发现其在成人角膜上的分布≈50％(图3)。因此，将含GFP报告基因的AAV血清型8或9注

射施用于人角膜基质(50μl,含1e$^{10}$vg),7天后采用蛋白质印迹法进行分析。鉴于血清型8和9的角膜转导报告,还按同样的方式对AAV8/9嵌合衣壳(8G9)进行了研究。AAV8/9嵌合衣壳包含连接在AAV8衣壳中的AAV9半乳糖受体(Sharma等人,Exp.Eye Res.91(3):440(2010);Hippert等人,PLoS One,7(4):e35318(2012))。蛋白质印迹分析表明,AAV8G9-GFP导致的转导比其任一种亲代血清型都要高(图4A)。

[0151] 为了确定单链AAV8G9-GFP转导的生物分布(最终体积50μl),开展了人角膜截面GFP免疫荧光分析。结果证明载体在角膜上横向扩散转导,渗透到更深的基质层,包括某些内皮细胞转导(图4B)。与CD34-角膜细胞标志物、α-平滑肌肌动蛋白(α-SM)-分化角膜细胞指标及F4/80-巨噬细胞标志物共染,证明了AAV8G9衣壳的角膜细胞混杂性(图4C)。

[0152] 继确认AAV8G9衣壳对角膜基质转导最有效后,确认了AAV8G9转导的细胞类型是否自然产生IDUA。为此,采用正常未处理的人角膜开展细胞类型标志物和IDUA蛋白质双重染色。结果表明,AAV8G9转导的所有类型的角膜细胞都自然产生IDUA(图5A)。

[0153] 接下来,北卡罗来纳大学教堂山分校(UNC)载体中心制备了AAV8G9-opt-IDUA临床前载体制剂,并在正常人的角膜内开展体外评估。在这些实验中,对注射单链AAV8G9-GFP的情况进行评价,并以PBS作为阴性对照。注射7天后(1e$^{10}$vg,50μl),蛋白质印迹法分析表明,总蛋白质恢复,且IDUA丰富。在整个角膜裂解液中,观察到IDUA的静息水平相对较低,而载体衍生IDUA,不管其是前体形式还是断裂形式,都很容易检测出来(图5B)。同样,与人角膜的正常水平相比,注射AAV8G9-opt-IDUA的角膜内IDUA的活性提高10倍(图5C)。

[0154] 通过对人角膜截面进行免疫荧光染色和检测,测定了IDUA的静息水平和分布(图6)。由于MPS I患者角膜稀少,在相同条件下染色,但没有采用IDUA一抗作为阴性对照。结果表明,角膜基质内的IDUA含量相对较低,IDUA亦存在于角膜上皮,还存在于内皮细胞层中,但含量更低(图6)。还对注射AAV8G9-opt-IDUA的人角膜截面进行IDUA染色。这些结果与IDUA蛋白质印迹分析及功能分析(图5A-5C)的结果密切相关,其中当施用AAV8G9-opt-IDUA时,与人角膜静息水平相比,正常人角膜内的IDUA增加大约5-10倍(图6)。此外,与采用AAV8G9-GFP观察到的结果类似,载体衍生IDUA的分布良好(图4B和图6)。

[0155] 基质注射ssAAV8G9-GFP或AAV8G9-opt-IDUA(1e10vg)后,开展正常人的角膜细胞毒性实验。在注射7天后,开展Tunel染色-检测细胞凋亡指标-断裂DNA。虽然核酸酶-处理阳性对照样品证明了广泛的Tunel染色,但是,其中IDUA丰度/功能显著提高的角膜与未注射或注射AAV8G9-GFP的角膜相比并没有明显差异(图7)。这些结果与采用MPS I患者成纤维细胞得到的结果一致,总体提高了AAV8G9-opt-IDUA基因治疗在MPS I患者角膜中使用的安全性。

[0156] 已经证明,利用异基因骨髓或脐带血捐献开展清髓性化疗之后,采用造血干细胞移植可以延长寿命,改善其总体质量,特别是在2岁以下MPS I儿童的情况下(Shapiro等人,J.Inherit.Metab.Dis.18(4):413(1005);

Whitley等人,Am.J.Med.Genet.46(2):209(1993);Prasad等人,Blood 112(7):2979(2008);Boelens等人,Bone Marrow Transplant.43(8):655(2009);Summers等人,Ophthalmology 96(7):977(1989))。移植供体细胞提供全身IDUA来源,通过移植供体小胶质细胞,实现脑内酶替换。移植儿童在完全供体嵌合的HSCT后存活10-20年,具有正常的血液IDUA水平,正常、接近正常的认知和心脏功能。后来,及后续,移植后MPS I确实有所表现,

通常限于关节、骨骼和眼睛，这些都是供体衍生IDUA丰度更低和递送更少的器官。因此，我们此处探索的临床前角膜方法设计为补充MPS I治疗，用于克服干细胞移植和AAV基因治疗靶向CNS的缺点。

[0157] 由于MPS I患者角膜稀少，因此，我们首先研究了MPS I患者成纤维细胞的治疗情况。数据表明，采用较低剂量的载体即恢复了IDUA功能。这部分是由于正常成纤维细胞IDUA水平相对较低，预计足以应对正常的细胞生理需求(图1)。同样，正常人角膜中IDUA的丰度也适中。实际上，在整个角膜裂解液中，采用蛋白质印迹法测定IDUA丰度并不容易，但是，采用组织学研究测定了角膜上皮、内皮和基质内大多数细胞类型中的IDUA静息水平(图5和图6)。IDUA丰度和AAV-介导恢复的这种评估表明了本发明角膜基因治疗一个非常重要的方面：仅需少量IDUA即可恢复MPS I儿童角膜的WT IDUA功能。我们发现，角膜外植体中的选择AAV剂量(1e10vg)过量，导致IDUA功能超出生理需求10-倍。但是，即使在如此高的IDUA剂量条件下，也未检测出任何毒性(图5、6、7)。鉴于分泌IDUA交叉治疗附近细胞的能力、正常人角膜中较低的静息水平及AAV8G9-opt-IDUA对人角膜转导的效率，此处的数据表明，较低的载体剂量将导致MPS I患者角膜内的IDUA达到正常水平。此外，由于人角膜中没有任何抗体，因此，预期不会出现因响应IDUA转基因产品(IDUA)而出现与中和抗体有关的并发症(Hinderer等人,Mol.Ther.23(8):1298(2015))。

[0158] 鉴于此处的数据，恢复MPS I角膜的IDUA功能似乎相当可行。此外，似乎AAV8G9导致在基质细胞内，以及在内皮层中(天然)产生IDUA(图6)。由于本发明AAV-opt-IDUA策略能够转导这些隔室(图6)，因此，可以作为HSCT或AAV全身性基因递送的补充治疗，逆转或预防与MPS I-相关的角膜失明。

实例4

体内角膜中的AAV IDUA表达盒

[0159] 对MPS1犬中AAV IDUA的体内表达影响进行了试验。如图8A-8D所示，在I-712狗左眼内注射AAV8G9-CMV-optIDUA(1e9vg/μl)溶液。将胰岛素注射器针尖插入到角膜基质(A)内，缓慢注射所述溶液(B)，直到施用全部体积(65μl)(C)。注射后，可以立即看到呈浑浊或轴向角膜水肿的流体保留区，该区域覆盖30％角膜表面(D)。采用眼部超声生物显微镜检测所示时间点中心角膜的厚度(图8E)。注射2分钟后出现的扩张在两天内消退。

[0160] 在接下来的实验中，向两只MPS1犬的一只眼内施用AAV8G9-optIDUA，而对侧眼内施用AAV-GFP作为对照，施用方式都为角膜基质内注射。后部反光照射基底时，照膜反射率改善，表明所示期间角膜透明(图9A)。在两只狗中，7天内都观察到反射大幅改善，并且维持了113天，表明IDUA表达消除了角膜浑浊。

[0161] 首先，I-709犬在5周时左眼出现急性角膜水肿(初始发作周图像未示出)，然后，右眼在10周时出现水肿(图9B)。当局部施用类固醇(TS)时，在全身施用或不施用类固醇(SS)条件下，角膜水肿在2-3周内消失，两只眼睛在注射后长达19周都未再复发。重要的是，从短暂的免疫反应恢复后，注射IDUA的左眼的治疗效果仍然非常明显地得到保留。

[0162] 上述说明是为了阐明本发明，不应视为限制本发明。本发明由下述权利要求定义，与所述权利要求相当的内容也包括在本发明中。

序列表

<110> 北卡罗来纳大学教堂山分校

<120> 治疗MPS I-相关失明的AAV-IDUA载体

<130> 5470-772WO

<150> US 62/298,126

<151> 2016-02-22

<160> 3

<170> SIPOSequenceListing 1.0

<210> 1

<211> 1993

<212> DNA

<213> 智人(Homo sapiens)

<400> 1

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cagctcgcgc cctgtggcca ctgaggagat tctggaggag cacaggcttt tgcccacctc 180
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gctcttaccg ggtccgcgct ctggactatt gggcaaggcc aggccccttc agcgatcctg 1920
tgccatacct ggaagtgcct gtgcctcgcg gcccaccatc tcctggaaac ccttgagggg 1980
atccgtcgac tag 1993

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caagtacgcc ccctattgac 20

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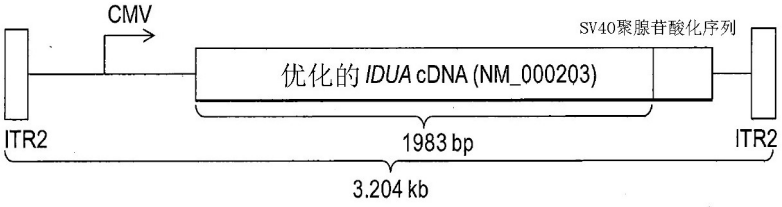

CMV
SV40聚腺苷酸化序列
优化的 IDUA cDNA (NM_000203)
ITR2
1983 bp
ITR2
3.204 kb

图1A

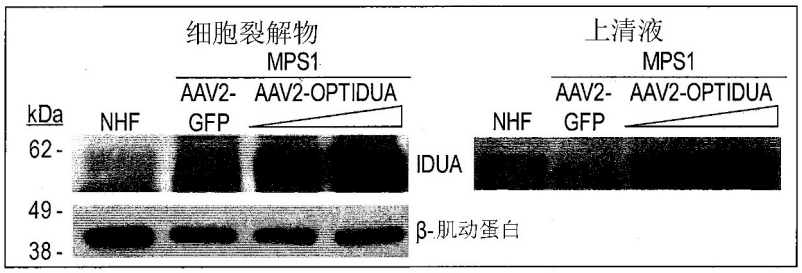

细胞裂解物
MPS1
AAV2-GFP
AAV2-OPTIDUA
NHF
kDa
62-
49-
38-
IDUA
β-肌动蛋白
上清液
MPS1
AAV2-GFP
AAV2-OPTIDUA
NHF

图1B

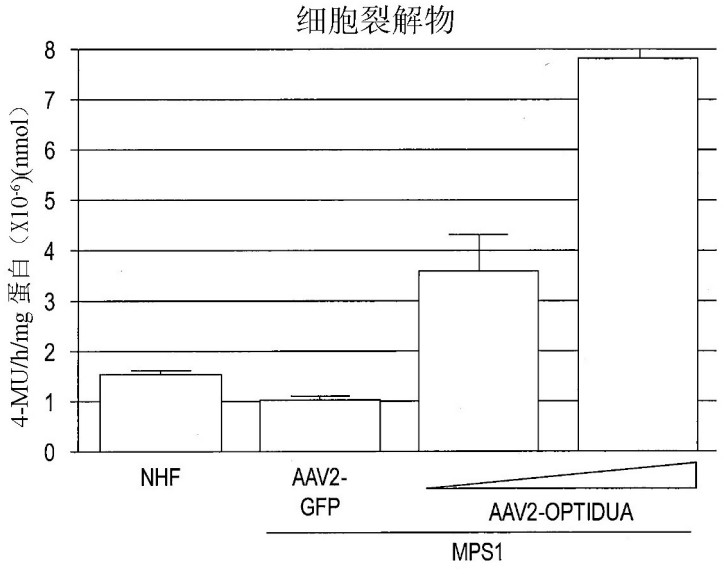

细胞裂解物
4-MU/h/mg 蛋白（X10⁻⁶)(nmol)
0
1
2
3
4
5
6
7
8
NHF
AAV2-GFP
AAV2-OPTIDUA
MPS1

图1C

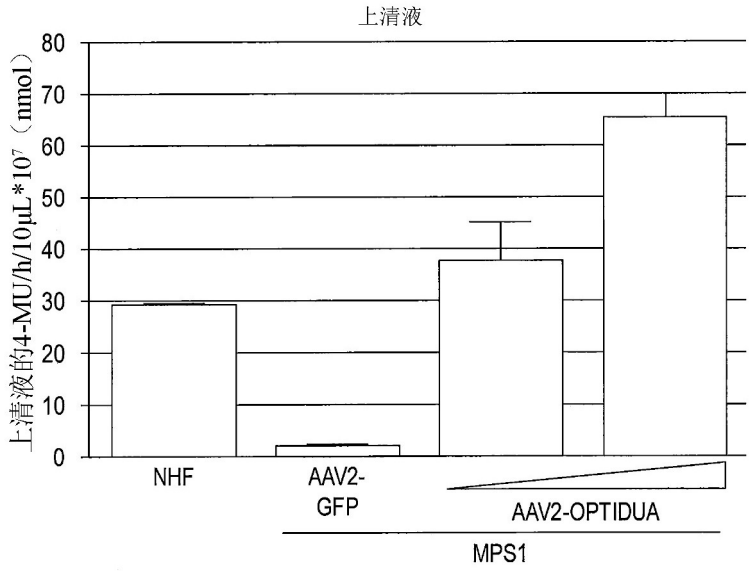

上清液
上清液的4-MU/h/10μL*10^7（nmol）
80
70
60
50
40
30
20
10
0
NHF
AAV2-GFP
AAV2-OPTIDUA
MPS1

图1D

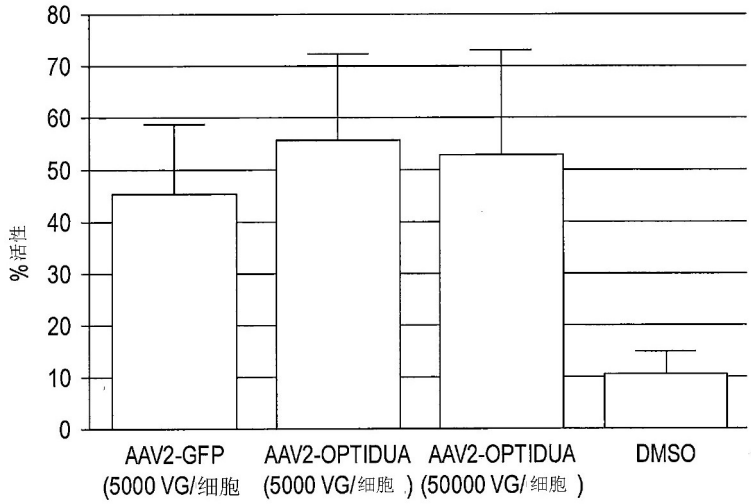

%活性
80
70
60
50
40
30
20
10
0
AAV2-GFP (5000 VG/细胞
AAV2-OPTIDUA (5000 VG/细胞 )
AAV2-OPTIDUA (50000 VG/细胞 )
DMSO

图2

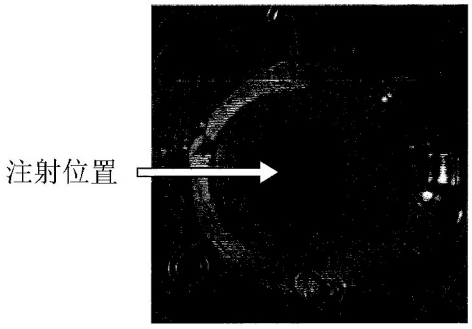



图3

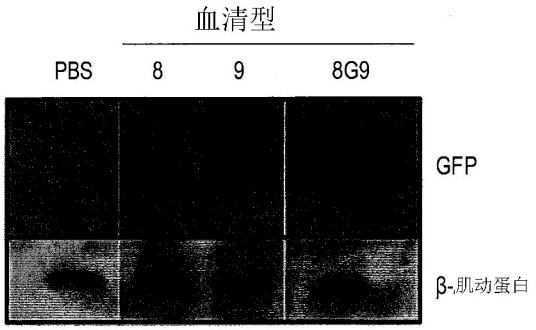



图4A

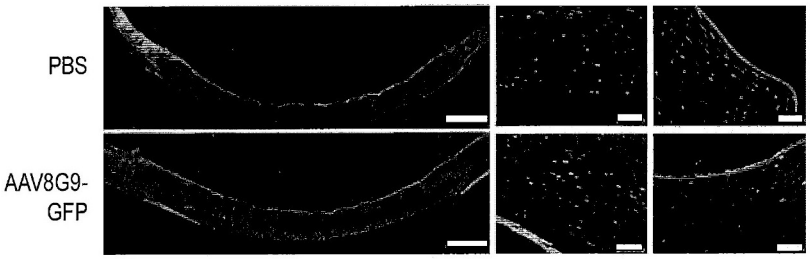



图4B

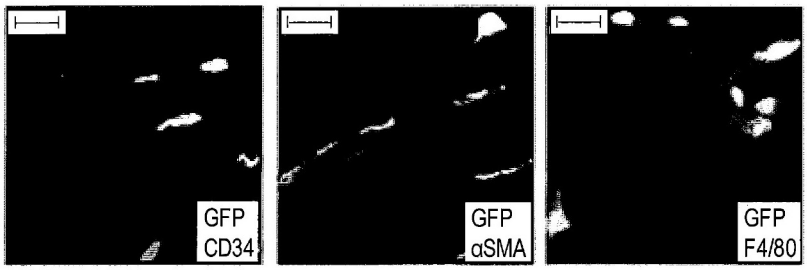

GFP
CD34
GFP
αSMA
GFP
F4/80

图4C

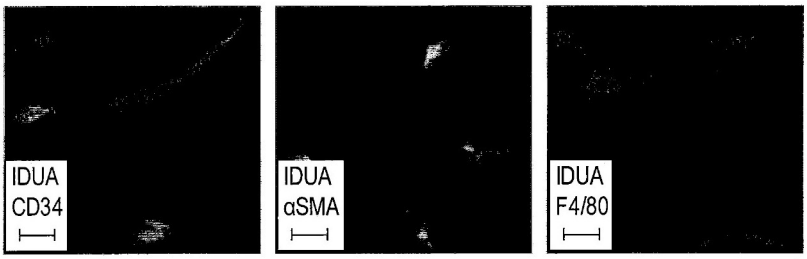

IDUA
CD34
IDUA
αSMA
IDUA
F4/80

图5A

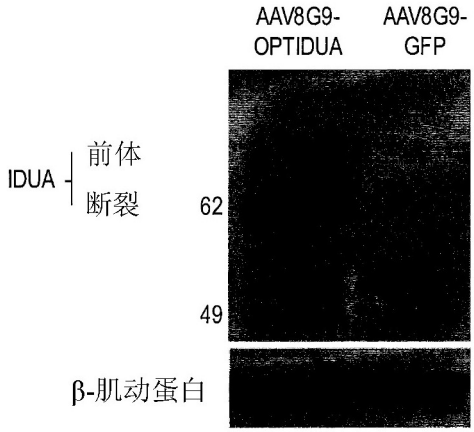

AAV8G9-
OPTIDUA
AAV8G9-
GFP
IDUA
前体
断裂
62
49
β-肌动蛋白

图5B

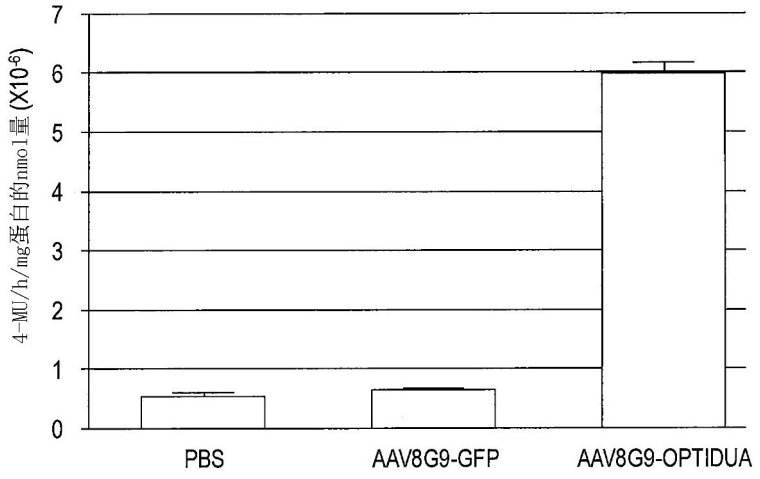

4-MU/h/mg蛋白的nmol量 (X10-6)
7
6
5
4
3
2
1
0
PBS
AAV8G9-GFP
AAV8G9-OPTIDUA

图5C

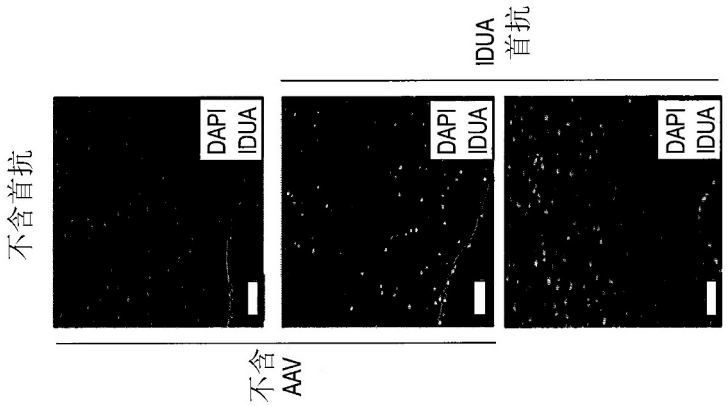

IDUA
首抗
不含首抗
DAPI
IDUA
DAPI
IDUA
DAPI
IDUA
不含
AAV

图6B

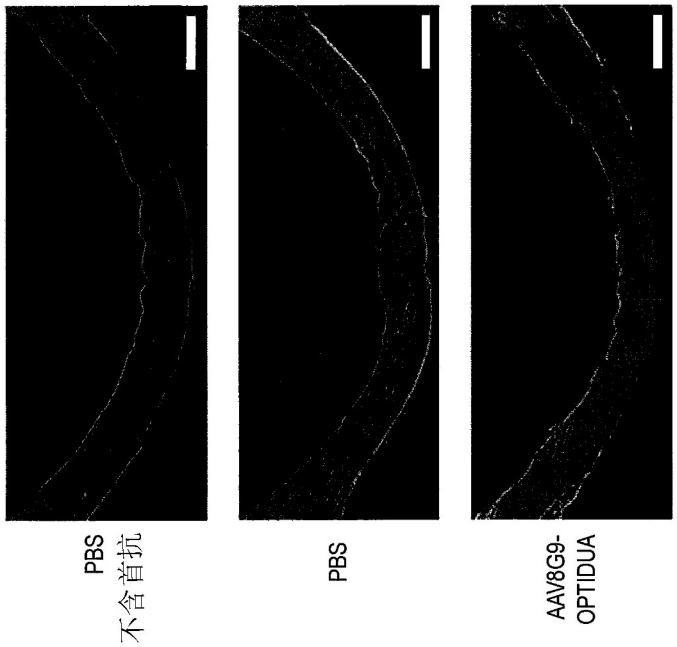

PBS
不含首抗
PBS
AAV8G9-
OPTIDUA

图6A

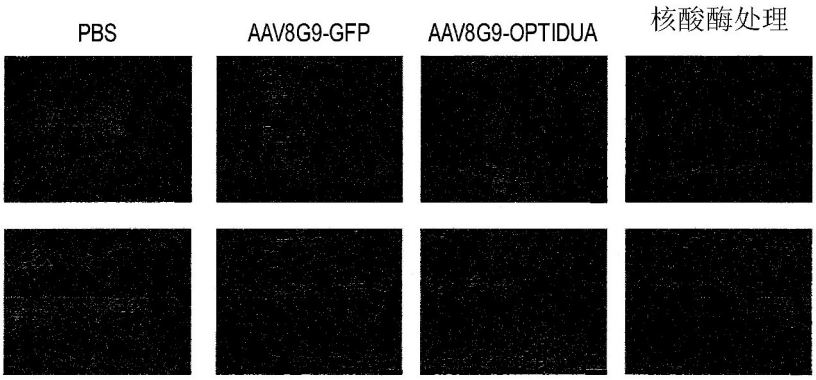

PBS
AAV8G9-GFP
AAV8G9-OPTIDUA
核酸酶处理

图7A

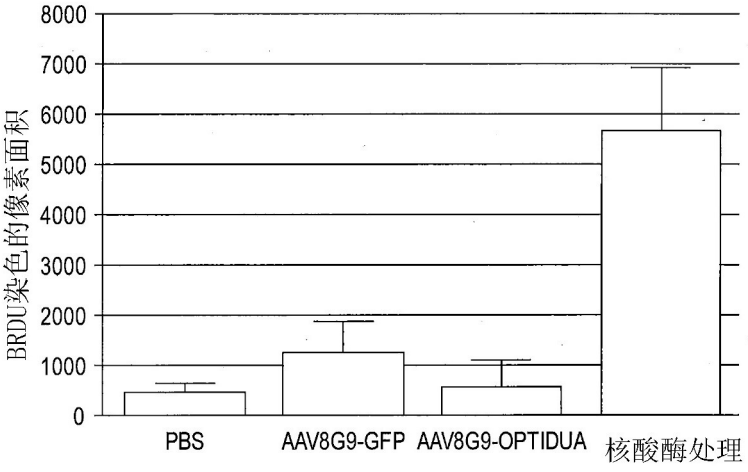

BRDU染色的像素面积
8000
7000
6000
5000
4000
3000
2000
1000
0
PBS
AAV8G9-GFP
AAV8G9-OPTIDUA
核酸酶处理

图7B

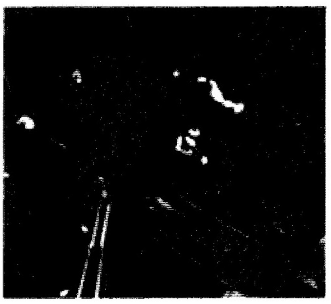


图8A

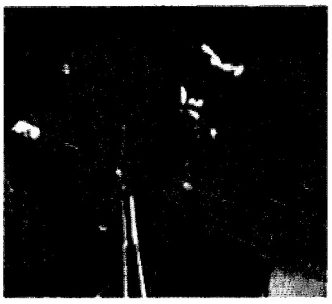


图8B

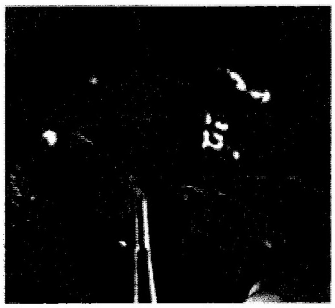


图8C

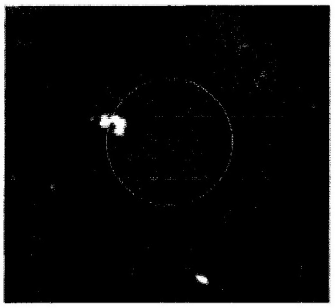


图8D

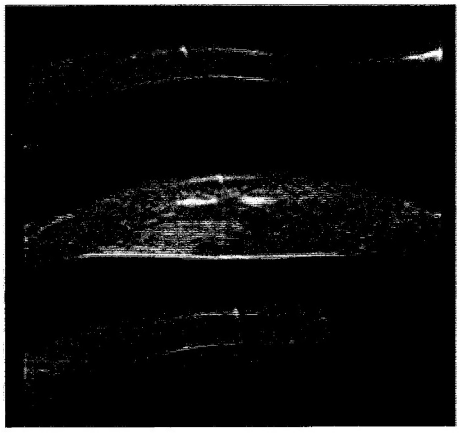



图8E

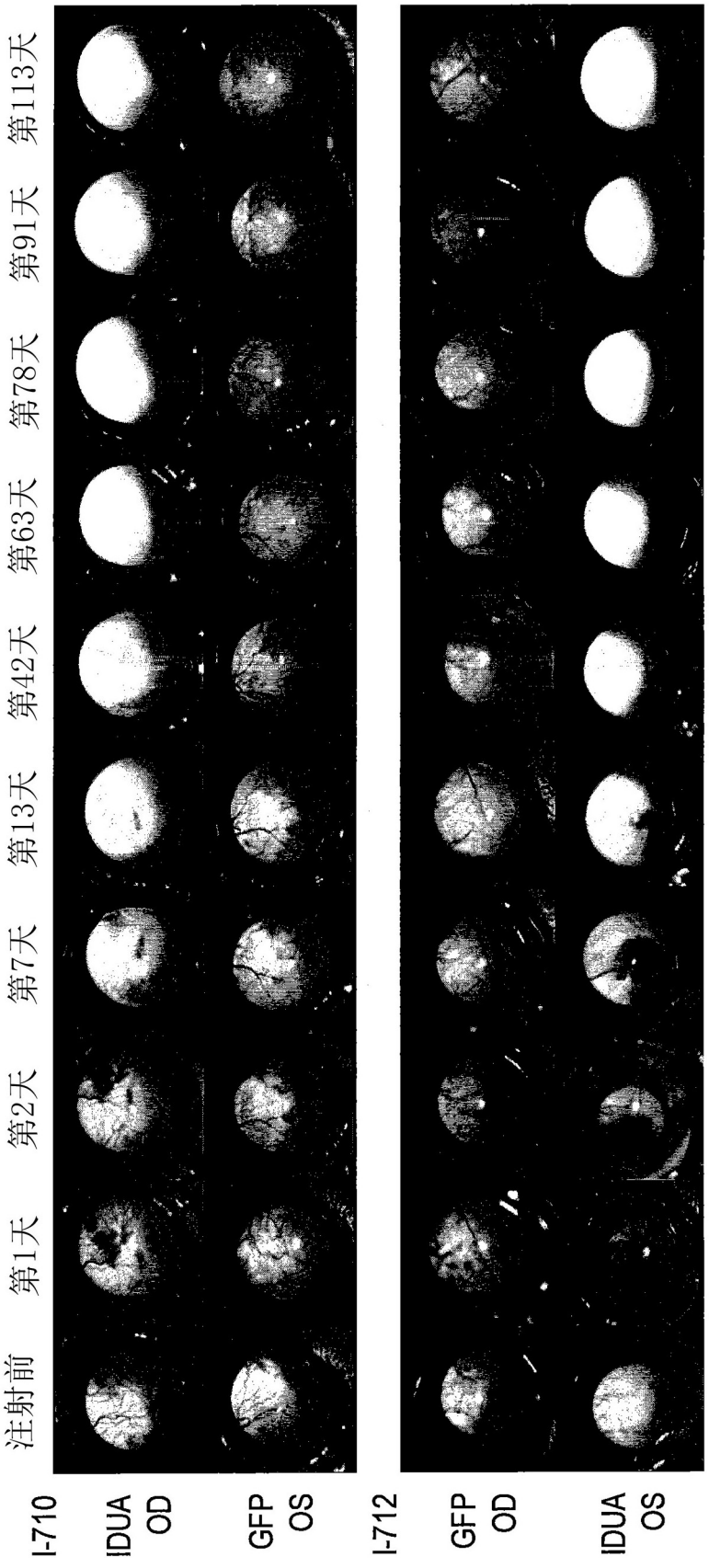

注射前
第1天
第2天
第7天
第13天
第42天
第63天
第78天
第91天
第113天
I-710
IDUA OD
GFP OS
I-712
GFP OD
IDUA OS

图6A

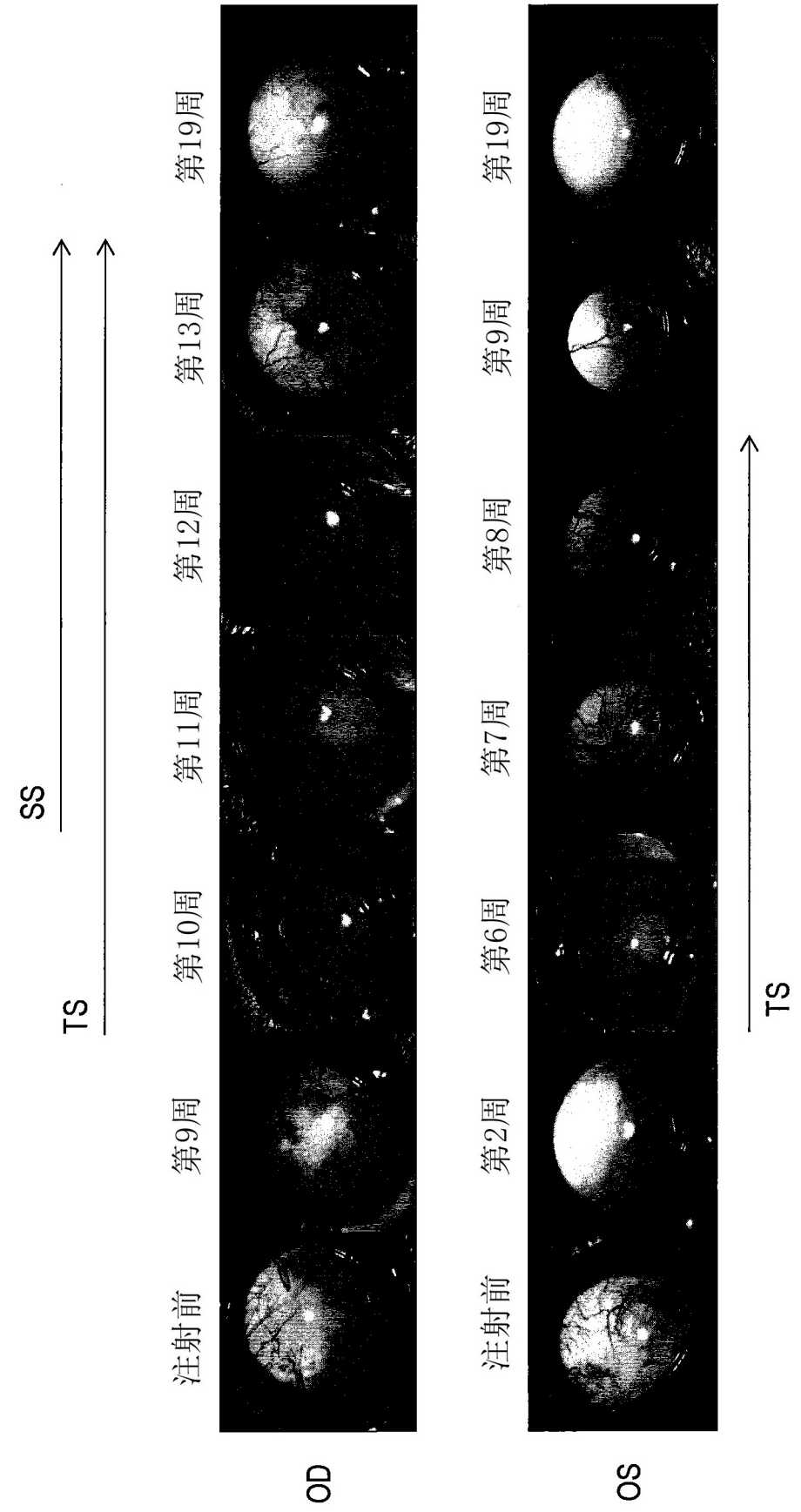

OD
注射前
第9周
第10周
第11周
第12周
第13周
第19周
SS
TS
OS
注射前
第2周
第6周
第7周
第8周
第9周
第19周
TS

图6B