COMPOSITIONS AND METHODS FOR INTRAVITREAL DELIVERY OF POLYNUCLEOTIDES TO RETINAL CONES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/127,194, filed on March 2, 2015, and U.S. Provisional Application No. 62/134,466, filed on March 17, 2015; each of which is incorporated by reference herein in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is AVBI_006_02WO_ST25.txt. The text file is 74 KB, was created on March 1, 2016, and is being submitted electronically via EFS-Web.

FIELD OF THE INVENTION

This invention pertains to viral-based gene therapy of retinal disorders.

BACKGROUND OF THE INVENTION

Photoreceptors are a specialized type of neuron found in the retina that are capable detecting light and converting that light signal into electrical signals. There are two types of photoreceptors in the retina: rod photoreceptors, which are more sensitive to light and hence support vision in dim lighting; and cone photoreceptors, which are sensitive to specific wavelengths of light and hence support the perception of color, and which respond faster to stimuli than rods so perceive finer detail and more rapid changes in images than rods and hence support high acuity vision.

A number of vision disorders are associated with a loss of viability or function of the cone photoreceptors, including, for example, those associated with defects within cones, i.e. cone-intrinsic defects, such as Stargardt's macular dystrophy, cone dystrophy, cone-rod dystrophy, Spinocerebellar ataxia type 7, and Bardet-Biedl syndrome-1, as well as color vision disorders, including achromotopsia, blue cone monochromacy, and protan, deutan, and tritan defects; and those that are associated with retinal disorders that affect the central macula, such as age-

related macular degeneration, macular telangiectasia, retinitis pigmentosa, diabetic retinopathy, retinal vein occlusions, glaucoma, Sorsby's fundus dystrophy, adult vitelliform macular dystrophy, Best's disease, and X-linked retinoschisis. It is expected that these cone cell disorders may be treated by delivering to cone photoreceptors a therapeutic gene that, when expressed by the cone photoreceptors, complements the deficiency and "rescues" the cone cell viability and/or function.

The highest density of cone photoreceptors exist at the 1.5mm depression located in the center of the macula of the retina. This region, called the "fovea centralis" or "foveal pit", is responsible for sharp central vision (also called foveal vision), which is necessary in humans for activities where visual detail is of primary importance, such as reading and driving. The fovea centralis consists of two sub-regions: the foveola, a 0.35mm diameter rod-free region of retina at the center of the pit; and the fovea, a 1.5mm-diameter cone-enriched region of retina that surrounds the foveola and forms the slopes of the pit. Surrounding the fovea centralis is the parafovea, which forms the lip of the depression and is comprised of all cells of the retina, cone photoreceptors being represented in reduced numbers relative to in the fovea centralis. Beyond the parafovea is the perifovea, a region of retina which contains an even more diminished density of cones. Because cone cells of the fovea constitute the vast majority of cone photoreceptors in the retina, these cells are ideal target recipients of therapeutic genes delivered for the treatment of cone-associated disorders (Oster 1935).

Some success at delivering genes to cells of the retina has been achieved by employing viral vectors such as adeno-associated virus (AAV) or lentivirus. However, these vectors must be administered by subretinal injection, a procedure that disrupts the structure of the retina and carries with it a risk of creating additional damage to retinal tissue that is often already damaged by the disorder being treated. One alternative is to deliver the viral vector to the retina intravitreally, i.e., by injecting the vector into the vitreous of the eye and hoping that the vector permeates the retina and transduces the retinal cells. However, as demonstrated by the art, foveal cone cells are notoriously resistant to transduction by viral vectors delivered intravitreally to the retina.

Thus, there is a need in the art for viral vectors that transduce cone cells with high efficiency when delivered from the vitreous of the eye. The present invention addresses these issues.

SUMMARY

Methods and compositions are provided for intravitreally delivering a polynucleotide to cone photoreceptors. Examples of the methods include injecting a recombinant adeno-associated virus comprising a polynucleotide of interest into the vitreous of the eye. These methods and compositions find particular use in treating ocular disorders associated with cone dysfunction and/or death. In a first aspect, the present disclosure refers to use of a recombinant adeno-associated virus serotype 2 (rAAV2) variant comprising a therapeutic polynucleotide in the manufacture of a medicament for treating or preventing a cone-associated retinal disorder in a primate subject having or at risk for developing the cone-associated retinal disorder, wherein:

I) said rAAV2 variant comprises a variant AAV2 capsid protein comprising the amino acid sequence LGETTRP (SEQ ID NO:11) inserted into the GH loop of the VP1 capsid protein; and

II) the therapeutic polynucleotide comprises a MNTC regulatory cassette operably linked to a polynucleotide encoding a therapeutic protein,

wherein the MNTC regulatory cassette comprises a human L/M opsin Locus Control Region ("LCR") enhancer and a truncated M opsin promoter comprising about 140 nucleotides upstream of the transcriptional start site;

and wherein the medicament is to be administered intravitreally in an amount effective to treat or prevent the cone-associated retinal disorder.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not to-scale. On the contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Included in the drawings are the following figures.

Figure 1 illustrates how intravitreally-delivered AAV2 variant AAV2-7m8 transduces retinal cells in the fovea centralis and parafovea of primates more efficiently than intravitreally-delivered AAV2. 5 x 10^{11} vector genomes of AAV2.CMV.GFP (upper left); AAV-2.5T.CMV.GFP (upper right) (Excoffon K. J., et al. 2009. Proc. Natl. Acad. Sci. U. S. A. 106:3865–3870); (lower left) AAV2-7.8.CMV.GFP (Dalkara D, et al. Sci Transl Med. 2013 Jun 12;5(189):189ra76); or AAV-ShH10.CMV.GFP (lower right) (Klimczak RR et al. PLoS One. 2009 Oct 14;4(10):e7467) was injected into the vitreous of an African green monkey in a volume of 50 uL, and GFP expression was observed 8 weeks later by OCT fluorescence imaging in vivo.

Figure 2 illustrates how the robustly the AAV2-7m8 capsid transduces foveal cones of primates. (a-b) AAV2-7m8.MNTC.GFP was injected into the central vitreous of a baboon and expression was observed (a) 5 weeks and (b) 8 weeks later by fundus fluorescence. (c and d) Natural GFP fluorescence within a 15 micron section of the fovea at approximately 6 months after injection with AAV2-7m8.MNTC.GFP at low magnification (c) and high magnification (d).

Figure 3 illustrates robust and cone-specific gene expression in the cones of a mouse retina following intravitreal injection of AAV-7m8 delivered MNTC.GFP. (a-b) Examples of GFP fluorescence 11 weeks after mice received intravitreal injections of 5.04×10^{10} vector genomes via intravitreal injection. (c-e) retinas were harvested for histology 14 weeks after injection and cone outer segments were labeled with an antibody to L/M opsin (red). In (c) the red channel is turned off so only the native GFP is visible, (d) is the same image with the red channel on to allow visualization of cone outer segments. Comparison of (c) and (d) shows that most if not all cones were transduced by the virus. (e) Image from the same retina as in c and d from different angle showing profiles of cone photoreceptors.

Figure 4A-4B illustrates gene expression directed by the pMNTC regulatory cassette in the cones of the Mongolian gerbil retina. 1×10^{10} - 2×10^{10} vector genomes of virus carrying GFP under the control of the CMV, pR2.1, or MNTC promoter were injected in a volume of 5 uL into the vitreous of a Mongolian gerbil, and GFP expression visualized at the designated time points by fundus fluorescence imaging. (a) Expression of GFP directed by AAV2-7m8.CMV.GFP and AAV2-7m8.MNTC.GFP, visualized 4 weeks after intravitreal administration. Gerbils 12-10, 12-11, and 12-12 were injected with AAV2-7m8.CMV.GFP, while gerbils 12-13, 12-14, and 12-15 were injected with AAV2-7m8.MNTC.GFP. OD, oculus dexter (right eye). OS, oculus sinister (left eye). (b) Expression of GFP directed by AAV2-7m8.pR2.1.GFP and AAV2-7m8.MNTC.GFP, 4 and 8 weeks later as detected by fundus fluorescence imaging.

Figure 5A-5D demonstrate that the pMNTC regulatory cassette provides for more robust gene expression in foveal cones of primates than the cone promoter pR2.1. 5×10^{11}vector genomes of AAV2-7m8.MNTC.GFP or AAV2-7m8.pR2.1.GFP were injected in a volume of 50 uL into the vitreous of African Green Monkeys as indicated (AAV2-7m8.MNTC.GFP into animals 271 and 472; AAV2-7m8.pR2.1.GFP into animals 500 and 509). Retinas were visualized in vivo at (a) 2 weeks, (b) 4 weeks, (c) 8 weeks, and (d) 12 weeks for GFP using a fundus fluorescence camera (a, b, c, d) or autofluorescence on Heidelberg Spectralis OCT (a, b; data not shown for weeks 8 and 12). OD, oculus dexter (right eye). OS, oculus sinister (left eye).

Figure 6A-6D demonstrate the contribution of each of the optimized pMNTC elements to the more robust expression observed. (a) The pMNTC and pR2.1 expression cassettes. (b) The experimental expression cassettes, in which each element in pMNTC is replaced one-by-one by the corresponding element in pR2.1. (c,d) Expression of the luciferase transgene in the retinas of gerbils intravitreally injected with each of the test articles (n=6-8 eyes per construct) as detected (c) 4 weeks and (d) 8 weeks after injection by IVIS imaging. "7m8.CMV" served as the positive control.

Figure 7 illustrates cone-specific gene expression directed by the pR2.1 regulatory cassette in a non-human primate (NHP). $5x10^{11}$ vector genomes of AAV2-7m8.pR2.1.GFP were injected in a volume of 50 uL into the vitreous of an African Green Monkey. GFP transgene expression was observed by stereo fluorescence microscopy of an 8 μm cross-section of the retina. GFP was stained with an anti-GFP antibody (green; chicken polyclonal; Abcam Cat #13970); opsin cone cells were stained with an anti-L/M Opsin antibody specific for opsin cones (red; rabbit polyclonal; Abcam Cat #5405); rod cells were stained with an anti-rhodopsin antibody (1D4 pink; mouse monoclonal; Abcam Cat #5417); and nuclei were stained with Dapi (blue/all nuclei; Invitrogen REF #D21490. GFP staining co-localized with L/M opsin staining but not rhodopsin staining. GFP transgene expression was present in L/M-opsin cones across the photoreceptor layer, but GFP transgene expression was not observed in rods. Arrows indicate illustrative cone cells double-stained for both GFP and opsin.

DETAILED DESCRIPTION OF THE INVENTION

Methods and compositions are provided for intravitreally delivering a polynucleotide to cone photoreceptors. Aspects of the methods include injecting a recombinant adeno-associated virus comprising the polynucleotide of interest into the vitreous of the eye. These methods and compositions find particular use in treating ocular disorders associated with cone dysfunction and/or death. These and other objects, advantages, and features of the invention will become apparent to those persons skilled in the art upon reading the details of the compositions and methods as more fully described below.

Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular method or composition described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the

present invention will be limited only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

Unless defined otherwise, 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. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described.

All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supersedes any disclosure of an incorporated publication to the extent there is a contradiction.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely", "only" and the like in connection with the recitation of claim elements, or the use of a "negative" limitation.

It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the

polynucleotide" includes reference to one or more polynucleotides and equivalents thereof, e.g. nucleic acid sequences, known to those skilled in the art, and so forth.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

DEFINITIONS

A "vector" as used herein refers to a macromolecule or association of macromolecules that comprises or associates with a polynucleotide and which can be used to mediate delivery of the polynucleotide to a cell. Illustrative vectors include, for example, plasmids, viral vectors (virus or the viral genome thereof), liposomes, and other gene delivery vehicles.

By a “virus” it is meant a viral particle comprising a viral capsid and a viral genome. For example, an adeno-associated virus refers to a viral particle comprising at least one adeno-associated virus capsid protein or variant thereof and an encapsidated adeno-associated virus vector genome or variant thereof.

By a viral “capsid” it is meant the protein shell of a virus. Viral capsids typically comprise several oligomeric structural subunits made of protein called protomers. The capsid encloses, or “encapsidates”, the genetic material, or “genome”, of the virus. In some viruses, the capsid is enveloped, meaning that the capsid is coated with a lipid membrane known as a viral envelope.

By a viral “genome” (referred to interchangeably herein as “viral genome”, “viral vector DNA” and “viral DNA”), it is meant a polynucleotide sequence comprising at least one, and generally two, viral terminal repeats (e.g. inverted terminal repeats (ITRs), long terminal repeats (LTR)) at its ends.

By a “recombinant viral genome” it is meant a viral genome comprising a heterologous nucleic acid sequence and at least one, and generally two, viral terminal repeats at its ends. By a “recombinant virus” it is meant a viral particle comprising a recombinant viral genome.

As used herein, the term “heterologous” means derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared. For example, a polynucleotide introduced by genetic engineering techniques into a plasmid or vector derived from a different species, e.g. a viral genome, is a heterologous polynucleotide. As another example, a promoter removed from its native coding sequence and operatively linked to a

coding sequence with which it is not naturally found linked is a heterologous promoter. As a third example, a heterologous gene product, e.g. RNA, protein, is a gene product not normally encoded by a cell in which it is being expressed.

The term "replication defective" as used herein relative to the viruses of the disclosure refers to a virus that cannot independently replicate and package its genome. For example, when a cell of a subject is infected with recombinant virions, the heterologous gene is expressed in the infected cells; however, due to the fact that the infected cells lack AAV rep and cap genes and accessory function genes, the recombinant virus is not able to replicate further.

The term "AAV" is an abbreviation for adeno-associated virus. When used herein, the term AAV may be used to refer to the virus itself or derivatives thereof, e.g. the viral capsid, the viral genome, and the like. The term “AAV” encompasses all subtypes, both naturally occurring and recombinant forms, and variants thereof except where required otherwise.

By “naturally occurring” or “wild-type” AAV it is meant any adeno-associated virus or derivative thereof comprising a viral capsid that consists of viral capsid proteins that occur in nature. Non-limiting examples of naturally occurring AAV include AAV type 1 (AAV-1), AAV type 2 (AAV-2), AAV type 3 (AAV-3), AAV type 4 (AAV-4), AAV type 5 (AAV-5), AAV type 6 (AAV-6), AAV type 7 (AAV-7), AAV type 8 (AAV-8), AAV9, AAV10, AAV11, AAV12, rh10, avian AAV, bovine AAV, canine AAV, equine AAV, primate AAV, non-primate AAV, and ovine AAV. "Primate AAV" refers to AAV that infect primates, "non-primate AAV" refers to AAV that infect non-primate mammals, "bovine AAV" refers to AAV that infect bovine mammals, etc.

By an “AAV variant” or a “variant AAV” it is meant to include an AAV viral particle comprising a variant, or mutant, AAV capsid protein. Examples of variant AAV capsid proteins include AAV capsid proteins comprising at least one amino acid difference (e.g., amino acid substitution, amino acid insertion, amino acid deletion) relative to a corresponding parental AAV capsid protein, i.e. an AAV capsid protein from which it was derived, a wild type AAV capsid protein, etc., where the variant AAV capsid protein does not consist of an amino acid sequence present in a naturally occurring AAV capsid protein. In addition to differing structurally, i.e. at the sequence level, from the corresponding parental AAV, the AAV variant may differ functionally from the corresponding parental AAV. Put another way, the variant capsid protein comprising the at least one amino acid difference relative to a corresponding parental AAV capsid protein may confer functional characteristics on the AAV variant that are not possessed by the corresponding parental AAV. For example, the AAV variant may have a different cellular tropism, i.e. a different affinity for and/or ability to infect a particular type of cell, e.g. the AAV

variant may bind to a cell, e.g. a retinal cell, with an increased (or decreased) affinity than the parental AAV, and/or infect/transduce a cell, e.g. a retinal cell, with an increased (or decreased) efficiency than the parental AAV such that more (or less) cells of a cell population is transduced/infected with the same titer of viral particles. As a second example, the AAV variant may have a greater (or lesser) affinity for antibodies produced by the host animal, e.g. the AAV variant may bind with greater (or lesser) affinity to neutralizing antibodies and be cleared from the host tissue to a greater (or lesser) extent.

By “recombinant AAV”, or “rAAV” it is meant to include any AAV that comprises a heterologous polynucleotide sequence in its viral genome. In general, the heterologous polynucleotide is flanked by at least one, and generally by two naturally occurring or variant AAV inverted terminal repeat sequences (ITRs). The term rAAV vector encompasses both rAAV vector particles and rAAV vector plasmids. Thus, for example, an rAAV that comprises a heterologous polynucleotide sequence would be an rAAV that includes a nucleic acid sequence not normally included in a naturally-occurring, wild-type AAV, for example, a transgene (e.g. a non-AAV RNA-coding polynucleotide sequence, non-AAV protein-coding polynucleotide sequence), a non-AAV promoter sequence, a non-AAV poly-adenylation sequence, etc.

As used herein, the term "expression vector" refers to a vector comprising a region which encodes a gene product of interest, and is used for effecting the expression of a gene product in an intended target cell. An expression vector also comprises control elements operatively linked to the encoding region to facilitate expression of the protein in the target. The combination of control elements and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.

As used herein, the term "expression" refers to the transcription and/or translation of a coding sequence, e.g. an endogenous gene, a heterologous gene, in a cell.

As used herein, the terms "gene" or "coding sequence" refer to a polynucleotide sequence that encodes a gene product, and encompasses both naturally occurring polynucleotide sequences and cDNA. A gene may or may not include regions preceding and following the coding region, e.g. 5' untranslated (5' UTR) or "leader" sequences and 3' UTR or "trailer" sequences, or intervening sequences (introns) between individual coding segments (exons).

As used herein, the term “gene product” refers the desired expression product of a

polynucleotide sequence such as a polypeptide, peptide, protein or RNA including, for example, a ribozyme, short interfering RNA (siRNA), miRNA or small hairpin RNA (shRNA). The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component.

As used herein, the terms "operatively linked" or "operably linked" refers to a juxtaposition of genetic elements on a single polynucleotide, wherein the elements are in a relationship permitting them to operate in the expected manner. For instance, a promoter is operatively linked to a coding region if the promoter helps initiate transcription of the coding sequence. There may be intervening residues between the promoter and coding region so long as this functional relationship is maintained. The combination of control elements, e.g. promoter, enhancer(s), etc. and a gene or genes to which they are operably linked for expression is sometimes referred to as an "expression cassette," a large number of which are known and available in the art or can be readily constructed from components that are available in the art.

By a "promoter" it is generally meant a DNA sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis, i.e., a minimal sequence sufficient to direct transcription. Promoters and corresponding protein or polypeptide expression may be ubiquitous, meaning strongly active in a wide range of cells, tissues and species or cell-type specific, tissue-specific, or species-specific. Promoters may be “constitutive,” meaning continually active, or “inducible,” meaning the promoter can be activated or deactivated by the presence or absence of biotic or abiotic factors.

By an "enhancer" it is generally meant a cis-acting regulatory element that stimulates, i.e. promotes or enhances, transcription of an adjacent genes. By a “silencer” it is meant a cis-acting regulatory element that inhibits, i.e. reduces or suppresses, transcription of an adjacent gene, e.g. by actively interfering with general transcription factor assembly or by inhibiting other regulatory elements, e.g. enhancers, associated with the gene. Enhancers can function (i.e., can be associated with a coding sequence) in either orientation, over distances of up to several kilobase pairs (kb) from the coding sequence and from a position downstream of a transcribed region. Enhancer sequences influence promoter-dependent gene expression and may be located in the 5' or 3' regions of the native gene. Enhancer sequences may or may not be contiguous with the promoter sequence. Likewise, enhancer sequences may or may not be

immediately adjacent to the gene sequence. For example, an enhancer sequence may be several thousand basepairs from the promoter and/or gene sequence.

A "termination signal sequence" within the meaning of the invention may be any genetic element that causes RNA polymerase to terminate transcription, such as for example a polyadenylation signal sequence. A polyadenylation signal sequence is a recognition region necessary for endonuclease cleavage of an RNA transcript that is followed by the polyadenylation consensus sequence AATAAA. A polyadenylation signal sequence provides a "polyA site", i.e. a site on a RNA transcript to which adenine residues will be added by post-transcriptional polyadenylation.

The terms "identical" or percent "identity" in the context of two or more nucleotide sequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described herein, e.g. the Smith-Waterman algorithm, or by visual inspection.

As used herein, the term "sequence identity" refers to the degree of identify between nucleotides in two or more aligned sequences, when aligned using a sequence alignment program. The term "% homology" is used interchangeably herein with the term "% identity" herein and refers to the level of nucleic acid or amino acid sequence identity between two or more aligned sequences, when aligned using a sequence alignment program. For example, as used herein, 80% homology means the same thing as 80% sequence identity determined by a defined algorithm, and accordingly a homologue of a given sequence has greater than 80% sequence identity over a length of the given sequence. Sequence identity may be determined by aligning sequences using any of a number of publicly available alignment algorithm tools, e.g., the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2: 482 (1981), the homology alignment algorithm of Needleman & Wunsch, J Mol. Biol. 48: 443 (1970), the search for similarity method of Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85: 2444 (1988), computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), by the BLAST algorithm, Altschul et al., J Mol. Biol. 215: 403-410 (1990), with software that is publicly available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/), or by visual inspection (see generally, Ausubel et al., infra).

The terms "complement" and "complementary" refer to two antiparallel nucleotide sequences capable of pairing with one another upon formation of hydrogen bonds between the

complementary base residues in the antiparallel nucleotide sequences.

The term "native", when used in the context of a polynucleotide or polypeptide herein, refers to a polynucleotide or polypeptide sequence that is found in nature; i.e., that is present in the genome of a wild-type virus or cell.

The term “variant’, when used in the context of a polynucleotide or polypeptide herein, refers to a mutants of a native polynucleotide or polypeptide having less than 100% sequence identity with the native sequence or any other native sequence. Such variants may comprise one or more substitutions, deletions, or insertions in the corresponding native gene or gene product sequence. The term “variant” also includes fragments of the native gene or gene product, and mutants thereof, e.g. fragments comprising one or more substitutions, deletions, or insertions in the corresponding native gene or gene product fragment. In some embodiments, the variant retains a functional activity of the native gene product, e.g. ligand binding, receptor binding, protein signaling, etc., as known in the art.

The term "fragment," when referring to a recombinant protein or polypeptide of the invention means a polypeptide having an amino acid sequence which is the same as part of, but not all of, the amino acid sequence of the corresponding full length protein or polypeptide, which retains at least one of the functions or activities of the corresponding full length protein or polypeptide. The fragment preferably includes at least 20-100 contiguous amino acid residues of the full length protein or polypeptide.

As used herein, the terms "biological activity" and "biologically active" refer to the activity attributed to a particular gene product, e.g. RNA or protein, in a cell line in culture or in vivo. For example, the "biological activity" of an RNAi molecule refers to the ability of the molecule to inhibit the production of a polypeptide from a target polynucleotide sequence.

As used herein, the term “antagonist” refers a molecule that acts to inhibit the activity of a target molecule. Antagonists include both structural antagonists that inhibit the activity of the target molecule by, for example, binding directly to the target or inactivating its receptor and functional antagonists, which, for example, decrease production of the target in a biological system or increase production of inhibitors of the target in a biological system.

The terms "administering" or "introducing", as used herein refer to contacting a cell, tissue, or subject with a vector for the purposes of delivering a polynucleotide to the cell or to cells and or organs of the subject. Such administering or introducing may take place in vivo, in vitro or ex vivo. A vector for expression of a gene product may be introduced into a cell by transfection, which typically means insertion of heterologous DNA into a cell by physical means

(e.g., calcium phosphate transfection, electroporation, microinjection or lipofection); infection, which typically refers to introduction by way of an infectious agent, i.e. a virus; or transduction, which typically means stable infection of a cell with a virus or the transfer of genetic material from one microorganism to another by way of a viral agent (e.g., a bacteriophage).

"Transformation" or "transfection" as used herein refers to the delivery of a heterologous DNA to the interior of a cell, e.g. a mammalian cell, an insect cell, a bacterial cell, etc. by a vector. A vector used to "transform" a cell may be a plasmid, minicircle DNA, or other vehicle. Typically, a cell is referred to as "transduced", "infected"; "transfected" or "transformed" dependent on the means used for administration, introduction or insertion of heterologous DNA (i.e., the vector) into the cell. The terms "transfected" and "transformed" are used interchangeably herein to refer to the introduction of heterologous DNA by non-viral methods, e.g. electroporation, calcium chloride transfection, lipofection, etc., e.g. as when preparing the subject viral vectors for use in the subject methods. The terms "transduced" and "infected" are used interchangeably herein to refer to introduction of the heterologous DNA to the cell in the context of a viral particle.

The term "host cell", as used herein refers to a cell which has been transduced, infected, transfected or transformed with a vector. The vector may be a plasmid, a viral particle, a phage, etc. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to those skilled in the art. It will be appreciated that the term "host cell" refers to the original transduced, infected, transfected or transformed cell and progeny thereof.

As used herein, a "therapeutic" gene refers to a gene that, when expressed, confers a beneficial effect on the cell or tissue in which it is present, or on a mammal in which the gene is expressed. Examples of beneficial effects include amelioration of a sign or symptom of a condition or disease, prevention or inhibition of a condition or disease, or conferral of a desired characteristic. Therapeutic genes include genes that correct a genetic deficiency in a cell or mammal.

The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof, e.g. reducing the likelihood that the disease or symptom thereof occurs in the subject, and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, and

includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; or (c) relieving the disease, i.e., causing regression of the disease. The therapeutic agent may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest. Such treatment is desirably performed prior to complete loss of function in the affected tissues. The subject therapy will desirably be administered during the symptomatic stage of the disease, and in some cases after the symptomatic stage of the disease.

The terms "individual," "subject," "host," and "patient," are used interchangeably herein and refer to any mammalian subject for whom diagnosis, treatment, or therapy is desired, including, but not limited to, human and non-human primates, including simians and humans; mammalian sport animals (e.g., horses); mammalian farm animals (e.g., sheep, goats, etc.); mammalian pets (dogs, cats, etc.); and rodents (e.g., mice, rats, etc.); particularly humans.

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms “a”, “an” and “the” are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms “including”, “includes”, “having”, “has”, “with”, or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term “comprising”.

By “comprising” it is meant that the recited elements are required in, for example, the composition, method, kit, etc., but other elements may be included to form the, for example, composition, method, kit etc. within the scope of the claim. For example, an expression cassette “comprising” a gene encoding a therapeutic polypeptide operably linked to a promoter is an expression cassette that may include other elements in addition to the gene and promoter, e.g. poly-adenylation sequence, enhancer elements, other genes, linker domains, etc.

By “consisting essentially of”, it is meant a limitation of the scope of the, for example, composition, method, kit, etc., described to the specified materials or steps that do not materially affect the basic and novel characteristic(s) of the, for example, composition, method, kit, etc. For example, an expression cassette “consisting essentially of” a gene encoding a therapeutic polypeptide operably linked to a promoter and a polyadenylation sequence may include additional sequences, e.g. linker sequences, so long as they do not materially affect the transcription or translation of the gene. As another example, a variant polypeptide fragment

"consisting essentially of" a recited sequence has the amino acid sequence of the recited sequence plus or minus about 10 amino acid residues at the boundaries of the sequence based upon the full length naïve polypeptide from which it was derived, e.g. 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 residue less than the recited bounding amino acid residue, or 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues more than the recited bounding amino acid residue.

By "consisting of", it is meant the exclusion from the composition, method, or kit of any element, step, or ingredient not specified in the claim. For example, an expression cassette "consisting of" a gene encoding a therapeutic polypeptide operably linked to a promoter and a polyadenylation sequence consists only of the promoter, polynucleotide sequence encoding the therapeutic polypeptide, and polyadenlyation sequence. As another example, a polypeptide "consisting of" a recited sequence contains only the recited amino acid sequence.

The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term "about" meaning within an acceptable error range for the particular value should be assumed.

METHODS AND COMPOSITIONS

In some aspects of the invention, methods and compositions are provided for delivering a polynucleotide to cone photoreceptors. As discussed above, cone photoreceptors, referred to interchangeably herein as "cone cells", "retinal cones", and most simply, "cones," are one of two subtypes of photoreceptor cells in the retina of the eye, the other being rod photoreceptors. Cone photoreceptors may be readily distinguished from rod photoreceptors by a number of physical, biochemical, and functional characteristics. For example, cone photoreceptors comprise an outer segment region that is shaped like a cone, whereas rod photoreceptors comprise an outer segment that is shaped like a rod. Cone photoreceptors express a number of proteins that are not expressed by rod photoreceptors, including, e.g., L-opsin (OPN1LW, the nucleic acid and amino acid sequences for which may be found at GenBank Accession No:

NM_020061.5), M-opsin (OPN1MW, the nucleic acid and amino acid sequences for which may be found at GenBank Accession No: NM_000513.2), or S-opsin (OPN1SW, the nucleic acid and amino acid sequences for which may be found at GenBank Accession No: NM_001708.2); whereas rod photoreceptors express a number of proteins that are not expressed by cone photoreceptors, e.g. rhodopsin (RHO, the nucleic acid and amino acid sequences for which may be found at GenBank Accession No: NM_000539.3) and rod-derived cone viability factor (RDCVF, also known as NXNL1, the nucleic acid and amino acid sequences for which may be found at GenBank Accession No:NM_138454.1). Functionally, cone photoreceptors differ from rod photoreceptors in that cone photoreceptors are responsible for color vision and function best in relatively bright light, whereas rod photoreceptors support vision at low light levels and function best in dim light; cones and rods can be distinguished based on this difference using an electroretinogram (ERG) or color ERG (cERG). Finally, cone photoreceptors may be distinguished from rod photoreceptors by their location in the retina. As discussed above, the vast majority of cone photoreceptors – all of them L- and M-cone photoreceptors -- are densely packed in a 1.5mm depression located in the center of the macula of the retina, called the fovea centralis, with the remaining L- and M-cone photoreceptors and the S-cone photoreceptors scattered in the parafovea, the perifovea, and the peripheral retina. In contrast, rod photoreceptors are excluded from the foveola and are poorly represented in the fovea, instead being primarily found in the parafovea, the perifovea, and the peripheral retina.

As discussed above, prior to the present disclosure, it was common understanding in the art that cone photoreceptors -- and more particularly, the L- and M-cone photoreceptors in the fovea -- were resistant to transduction by AAV delivered from the vitreous. However, as demonstrated by the working examples herein, foveal cones can, in fact, be transduced by intravitreally delivery using the methods and compositions of the present disclosure. In some embodiments, the cone photoreceptors that are transduced by the subject methods and compositions reside anywhere in the retina, i.e. the macula (the foveal centralis, the parafovea, the perifovea), or the periphery. In some embodiments, the cone photoreceptors reside in the fovea centralis. In certain embodiments, the cone photoreceptors are foveal cones, that is, they are L- or M-cones that reside within the fovea, this being the region of the fovea centralis spanning from about 0.175mm from the center of the fovea centralis to about 0.75mm from the center of the fovea centralis.

rAAV virions

In practicing the subject methods, the polynucleotide of interest is delivered to cone photoreceptors by injecting into the vitreous of the eye a recombinant viral particle comprising the polynucleotide of interest as a heterologous sequence within its genome. In some instances, the recombinant viral particles are recombinant adeno-associated virus (rAAV) particles. In some embodiments, the rAAV are of a wild-type serotype; that is, they comprise a viral capsid that consists of viral capsid proteins that occur in nature. In other embodiments, the rAAV are an AAV serotype variant, i.e., they comprise a variant AAV capsid protein, that is, an AAV capsid protein that comprises at least one amino acid difference relative to a corresponding parental AAV capsid protein, e.g. a wild type AAV capsid protein, and does not consist of an amino acid sequence present in a naturally occurring AAV capsid protein.

As demonstrated in the working examples of the present application, rAAV virions comprising a variant AAV capsid protein comprising at least one amino acid difference in the GH loop, or more particularly, in subloop IV of the GH loop, demonstrate an increased infectivity of cone photoreceptors relative to rAAV virions comprising wild type AAV capsid protein when delivered intravitreally. By "increased infectivity," it is meant that the variant rAAV virion is better able to transduce the target cell than the wild type AAV capsid protein. Improvements in the ability of an AAV to transduce a cell can be observed by observing more polynucleotide being delivered to each cell and more cells being transduced in a tissue, resulting in an increase in the amount of polynucleotide delivered to each cell and to the tissue. Accordingly, in some aspects of the invention, methods are provided for the improved delivery of a polynucleotide of interest to cone photoreceptors, the improvement comprising delivering to the vitreous of the eye an effective amount of a rAAV variant, the rAAV variant comprising i) a variant AAV capsid protein that comprises at least one amino acid difference relative to a corresponding parental AAV capsid protein, e.g. a wild type AAV capsid protein, and does not consist of an amino acid sequence present in a naturally occurring AAV capsid protein, and ii) the polynucleotide of interest as a heterologous sequence within the viral genome.

Of particular interest in the subject disclosure are rAAV variants that comprise at least one amino acid difference in the GH loop, or "loop IV", of an AAV capsid protein relative to a corresponding parental AAV capsid protein. By the GH loop, or loop IV, it is meant the loop created between the G and H strands of the jelly-roll β-barrel of the AAV capsid protein VP1, as described in, e.g., Xie et al. (2002) PNAS 99(16):10405-10410, van Vliet et al. (2006) Mol. Ther. 14:809; Padron et al. (2005) J. Virol. 79:5047; and Shen et al. (2007) Mol. Ther. 15:1955. In some instances, the at least one amino acid difference is within subloop 4 of the GH loop, i.e.,

the solvent-accessible portion of the GH loop, consisting essentially of about amino acids 571-612 of AAV1 VP1 (SEQ ID NO:1), about amino acids 570-611 of AAV2 VP1 (SEQ ID NO:2), about amino acids 571-612 of AAV3 VP1 (SEQ ID NO:3), about amino acids 569-610 of AAV4 VP1 (SEQ ID NO:4), about amino acids 560-601 of AAV5 VP1 (SEQ ID NO:5), about amino acids 571 to 612 of AAV6 VP1 (SEQ ID NO:6), about amino acids 572 to 613 of AAV7 VP1 (SEQ ID NO:7), about amino acids 573 to 614 of AAV8 VP1 (SEQ ID NO:8), about amino acids 571 to 612 of AAV9 VP1 (SEQ ID NO:9), about amino acids 573 to 614 of AAV10 VP1 (SEQ ID NO:10); or about the corresponding amino acid range of a variant thereof. In certain instances, the at least one amino acid difference is within the range of amino acids consisting essentially of amino acids 581-596 of AAV1 VP1, 580-595 of AAV2 VP1, 581-596 of AAV3 VP1, 579-594 of AAV4, 570-585 of AAV5 VP1, 581-596 of AAV6 VP1, 582-597 of AAV7 VP1, 583-598 of AAV8 VP1, 581-596 of AAV9 VP1, 583-598 of AAV10 VP1, or within the corresponding amino acid range of a variant thereof. Those skilled in the art would know, based on a comparison of the amino acid sequences of capsid proteins of various AAV serotypes, where the amino acids "corresponding to amino acids 570-611 of VP1 from AAV2", for example, would be in a capsid protein of any given AAV serotype.

In some embodiments, the at least one amino acid difference is an insertion of a peptide between two amino acids in the GH loop of the AAV capsid protein, e.g. between about amino acids 571-612 of AAV1 VP1 (SEQ ID NO:1), about amino acids 570-611 of AAV2 VP1 (SEQ ID NO:2), about amino acids 571-612 of AAV3 VP1 (SEQ ID NO:3), about amino acids 569-610 of AAV4 VP1 (SEQ ID NO:4), about amino acids 560-601 of AAV5 VP1 (SEQ ID NO:5), about amino acids 571 to 612 of AAV6 VP1 (SEQ ID NO:6), about amino acids 572 to 613 of AAV7 VP1 (SEQ ID NO:7), about amino acids 573 to 614 of AAV8 VP1 (SEQ ID NO:8), about amino acids 571 to 612 of AAV9 VP1 (SEQ ID NO:9), about amino acids 573 to 614 of AAV10 VP1 (SEQ ID NO:10); or about the corresponding amino acid range of a variant thereof; for example, between two amino acids within amino acids 581-596 of AAV1 VP1, 580-595 of AAV2 VP1, 581-596 of AAV3 VP1, 579-594 of AAV4, 570-585 of AAV5 VP1, 581-596 of AAV6 VP1, 582-597 of AAV7 VP1, 583-598 of AAV8 VP1, 581-596 of AAV9 VP1, 583-598 of AAV10 VP1, or within the corresponding amino acid range of a variant thereof. For example, the insertion site can be between amino acids 580 and 581, amino acids 581 and 582, amino acids 582 and 583, amino acids 583 and 584, amino acids 584 and 585, amino acids 585 and 586, amino acids 586 and 587, amino acids 587 and 588, amino acids 588 and 589, amino acids 589 and 590, amino acids 590 and 591, amino acids 591 and 592, amino acids 592 and 593, amino acids 593 and

594, or amino acids 594 and 595 of AAV2 VP1, or the corresponding amino acids in another AAV VP1 or variant thereof.

Of particular interest in some embodiments of the present disclosure are the rAAV variants comprising a peptide insertion as disclosed in PCT Publication No. WO 2012/145601, the full disclosure of which is incorporated herein by reference. These rAAV variants comprise a peptide insert having 5 to 11 amino acids in length, that is, the inserted peptide comprises 5 amino acids, 6 amino acids, 7 amino acids, 8 amino acids, 9 amino acids, 10 amino acids, or 11 amino acids.

One exemplary peptide of particular interest is a peptide of Formula I:

$Y_1Y_2X_1X_2X_3X_4X_5X_6X_7Y_3Y_4$ (SEQ ID NO:20)

where:

each of Y1 –Y4, if present, is independently selected from Ala, Leu, Gly, Ser, and Thr;

X1, if present, is selected from Leu, Asn, and Lys;

X2 is selected from Gly, Glu, Ala, and Asp;

X3 is selected from Glu, Thr, Gly, and Pro;

X4 is selected from Thr, Ile, Gln, and Lys;

X5 is selected from Thr and Ala;

X6 is selected from Arg, Asn, and Thr;

X7, if present, is selected from Pro and Asn.

In certain embodiments, X1 and/or X7 is absent.

A second exemplary peptide of particular interest is a peptide of Formula II:

$Y_1Y_2X_1X_2X_3X_4X_5X_6X_7Y_3Y_4$ (SEQ DI NO:21)

where:

each of Y1 –Y4, if present, is independently selected from Ala, Leu, Gly, Ser, and Thr;

each of X1 – X4 is any amino acid;

X5 is Thr

X6 is Arg; and

X7 is Pro.

In certain embodiments, any one or more of Y1-Y4 are absent.

A third exemplary peptide of particular interest is a peptide of Formula III:

$Y_1Y_2X_1X_2X_3X_4X_5X_6X_7Y_3Y_4$ (SEQ ID NO:22)

where:

each of Y1 –Y4, if present, is independently selected from Ala, Leu, Gly, Ser, and Thr;

X1, if present, is selected from Leu and Asn;
X2, if present, is selected from Gly and Glu;
X3 is selected from Glu and Thr;
X4 is selected from Thr and Ile;
X5 is Thr;
X6 is Arg; and
X7 is Pro.
In certain embodiments, any one or more of Y1-Y4, X1 and X2 are absent.

A fourth exemplary peptide of particular interest is a peptide of Formula IV:

Y1Y2X1X2X3X4X5X6X7Y3Y4 (SEQ ID NO:23)
where:
each of Y1 –Y4, if present, is independently selected from Ala, Leu, Gly, Ser, and Thr;
X1, if present, is selected from Leu, Asn, Arg, Ala, Ser, and Lys;
X2 is selected from Gly, Glu, Ala, Val, Thr, and Asp;
X3 is selected from Glu, Thr, Gly, Asp, or Pro;
X4 is selected from Thr, Ile, Gly, Lys, Asp, and Gln;
X5 is selected from Thr, Ser, Val, and Ala;
X6 is selected from Arg, Val, Lys, Pro, Thr, and Asn; and
X7 is selected from Pro, Gly, Phe, Asn, and Arg.
In certain embodiments, any one or more of Y1-Y4 and X1 are absent.

Exemplary insertion peptides of particular interest having these formulas include peptides comprising the sequence LGETTRP (SEQ ID NO:11) and NETITRP (SEQ ID NO:12), or variants thereof. In some cases, the insertion peptide has from 1 to 4 spacer amino acids (Y1-Y4) at the amino terminus and/or at the carboxyl terminus. Suitable spacer amino acids include, but are not limited to, leucine, alanine, glycine, and serine. For example, in some cases, an insertion peptide has the amino acid sequence: LALGETTRPA (SEQ ID NO:13); LANETITRPA (SEQ ID NO:14), As another example, in some cases, the insertion peptide has the amino acid sequence AALGETTRPA (SEQ ID NO:15) or AANETITRPA (SEQ ID NO:16), As yet another example, in some cases, an insertion peptide has the amino acid sequence GLGETTRPA (SEQ ID NO:17) or GNETITRPA (SEQ ID NO:18).

In some embodiments, a subject rAAV virion capsid does not include any amino acid substitutions, insertions, or deletions, other than an insertion of from about 5 to 11 amino acids

in the GH loop or subregion thereof relative to a corresponding parental AAV capsid protein. In other embodiments, a subject rAAV virion capsid may include from 1 to about 25 amino acid insertions, deletions, or substitutions, compared to the parental AAV capsid protein, in addition to an insertion of from about 5 to 11 amino acids in the GH loop or subregion thereof as described above. For example, a number of amino acid sequence alterations have been disclosed in the art, any of which may be included in the subject rAAV. In some embodiments, a subject rAAV virion capsid is a chimeric capsid, e.g., the capsid comprises a portion of an AAV capsid of a first AAV serotype and a portion of an AAV capsid of a second serotype; and comprises an insertion of from about 5 amino acids to about 11 amino acids in the GH loop or subregion thereof relative to a corresponding parental AAV capsid protein.

In some embodiments, a subject rAAV virion comprises a capsid protein comprising an amino acid sequence having a sequence identity of 80% or more to the VP1 capsid protein of the corresponding parental capsid protein, e.g. 85% or more, 90% or more, 95% or more or 97C% identity or more to the corresponding parental capsid protein and an insertion of from about 5 to 11 amino acids in the GH loop or subregion thereof relative to a corresponding parental AAV capsid protein. For example a sequence identity of 80% or more to the 7m8 VP1 sequence described in SEQ ID NO:19, e.g. 85% identity or more, 90% identity or more, or 95% identity or more to the 7m8 VP1 sequence, in some instances 97% identity or more, 98% identity or more, or at least about 99% sequence identity to the amino acid sequence provided in SEQ ID NO:19.

rAAV variants that are encompassed by the subject compositions and that find use in the subject methods may be readily validated as such by determining the efficacy by which they transduce cone photoreceptors, e.g. foveal cone photoreceptors. For example, viral particles may be created comprising an AAV viral genome comprising an expression cassette comprising GFP operably linked to a cone promoter as known in the art, packaged into the subject rAAV, and the viral particles injected into the vitreous of a mammalian eye, e.g. the eye of a mouse, rat, rabbit, gerbil, hamster, squirrel, or primate, e.g. non-human primate. rAAV virions encompassed by the present disclosure will typically exhibit at least a 2-fold, at least a 5-fold, at least a 10-fold, at least a 15-fold, at least a 20-fold, at least a 25-fold, at least a 50-fold, in some instances, more than 50-fold, e.g. at least a 60-fold, at least a 70-fold, at least an 80-fold, at least a 90-fold, for example, a 100-fold increased infectivity of cone photoreceptors or more when administered via intravitreal injection as compared to the infectivity of cone photoreceptors by an AAV virion comprising the corresponding parental AAV capsid protein. Put another way,

rAAV virions suitable for use in the subject methods will infect at least 10-fold more, at least 15-fold more, at least 20-fold more, at least 50-fold more, in some instances more than 50-fold more cone photoreceptors, e.g. at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, for example, a 100-fold more cone photoreceptors than AAV virions comprising the corresponding parental AAV capsid protein.

In some embodiments, the method may further comprise the step of detecting the presence of the delivered polynucleotide in the cone photoreceptor. Any convenient method may be employed for detecting the presence of the polynucleotide. For example, the polynucleotide may be detecting using, e.g., PCR, Next Gen sequencing, and the like, or the expression of a gene product encoded by the polynucleotide may be detected by, e.g., RT-PCR, Northern blot, RNAse protection, Western blot, ELISA, immunohistochemistry, and the like. These methods are particularly suited to preclinical studies. In clinical studies, in may be preferably to detect the presence of the polynucleotide by detecting the presence of a functional gene product, that is, by detecting the impact of the gene product on the viability or function of the cone photoreceptor in the subject. For example, if the gene product encoded by the polynucleotide improves the viability of the cone photoreceptor, an improvement in viability of the cone photoreceptor may be detected by, e.g., fundus photography, Optical coherence tomography (OCT), Adaptive Optics (AO), and the like, as a way of detecting the presence of the polynucleotide. If the gene product encoded by the polynucleotide alters the activity of the cone photoreceptor, the modified activity of the cone photoreceptor may be detected by, e.g., electroretinogram (ERG) and color ERG (cERG); color vision tests such as pseudoisochromatic plates (Ishihara plates, Hardy-Rand-Ritter polychromatic plates), the Farnsworth-Munsell 100 hue test, the Farnsworth's panel D-15, the City university test, Kollner's rule, and the like; and visual acuity tests such as the ETDRS letters test, Snellen visual acuity test, and the like, as a way of detecting the presence of the delivered polynucleotide.

As discussed above, in some embodiments, the polynucleotide that is delivered by the subject compositions and methods is expressed by the cone photoreceptor to which it is delivered. In other words, in some aspects of the invention, methods are provided for expressing a gene product in a cone photoreceptor, the methods comprising delivering to the cone photoreceptor a polynucleotide that encodes the gene product of interest. As will be well understood by the ordinarily skilled artisan, expression by a cone cell of a polynucleotide of interest typically requires that the polynucleotide of interest be operably linked to a promoter. As will also be appreciated by the ordinarily skilled artisan, there are a number of ways in which

this can be achieved. For example, the polynucleotide may be delivered to the host cell, i.e. the cone photoreceptor, operatively linked to a promoter. In other words, the viral genome comprising the polynucleotide of interest also comprises a promoter, wherein the promoter is operably linked to the polynucleotide to form an expression cassette. As another example, the polynucleotide may be delivered to the host cell i.e. the cone photoreceptor, flanked by sequences that promoter the integration of the polynucleotide into the host genome. In other words, the viral genome comprising the polynucleotide of interest comprises sequences flanking the polynucleotide of interest that are homologous to sequences flanking the 3' end of a host cell promoter and promote the recombination of the polynucleotide of interest into the host genome such that it is operably linked to the host cell promoter. Other arrangements of the recombinant viral genome that may be employed to ensure the expression of the polynucleotide of interest will be readily envisioned by the ordinarily skilled artisan; see, for example, US Application Publication No. 2013/0280222, the full disclosure of which is incorporated herein by reference.

Accordingly, in some instances, the viral genome comprised by the rAAV comprises a promoter operably linked to the polynucleotide of interest. In some instances, the promoter is a ubiquitous promoter, i.e., it is a promoter that is active in a wide range of cells, tissues and species. In other instances, the promoter is a cone promoter. By a cone promoter it is meant a promoter that is active in cone photoreceptors, i.e., that promotes the expression in cone photoreceptors of a polynucleotide to which it is operably linked. Non-limiting examples of cone promoters that find use in the subject compositions include the pMNTC promoter as disclosed in US Provisional Application Nos. 61/954,330 and 62/127,185; the pR2.1 promoter or variants thereof (e.g. pR1.7, pR1.5, pR1.1, etc.) as disclosed in, e.g., US Application No. 2013/0317091; or the synthetic IRBP/GNAT2 promoter as disclosed in US Application No. 2014/0275231; the full disclosures of which are incorporated herein by reference. In other instances, the viral genome comprised by the rAAV comprises two sequences having homology to a target integration site in the host genome, a first sequence that is homologous to the region 5' of the integration site and located 5' to the polynucleotide on the viral genome, and a second sequence that is homologous to the region 3' of the integration site and located 3' to the polynucleotide on the viral genome, wherein the target integration site is 3' to and operably linked to a host promoter, e.g. a cone promoter, e.g. an L-opsin promoter, an M-opsin promoter.

In some embodiments, transduction is enhanced relative to expression as observed when a wild type or other parental capsid is employed. By enhanced, it is meant transduction

that is elevated, increased, or augmented for example, at least 2-fold, at least 5-fold, at least 10-fold, at least 15-fold, at least 20-fold, at least 25-fold, at least 50-fold, in some instances, more than 50-fold, e.g. at least 60-fold, at least 70-fold, at least 80-fold, at least 90-fold, for example, 100-fold in a subject's cone photoreceptors over levels that would be observed using a wild type or other parental capsid protein, and usually to an amount to have an impact on cone viability and/or function, e.g. to provide a therapeutic benefit to the subject.

Enhanced transduction of cone cells by the subject variant rAAVs is expected to result in enhanced expression of polynucleotides, e.g., expression cassettes, being delivered to those cells by the variant rAAV. Enhanced expression of a polynucleotide by the rAAVs of the subject disclosure may be observed in a number of ways. For example, enhanced expression may be observed by detecting the expression of the polynucleotide following contact of the variant rAAV to the cone cells sooner, e.g. 7 days sooner, 2 weeks sooner, 3 weeks sooner, 4 weeks sooner, 8 weeks sooner, 12 weeks sooner, or more, than expression would be detected if the polynucleotide were delivered by the parental rAAV. Enhanced expression may also be observed as an increase in the amount of gene product per cell. For example, there may be a 2-fold increase or more, e.g. a 3-fold increase or more, a 4-fold increase or more, a 5-fold increase or more, or a 10-fold increase or more in the amount of gene product per cone cell. Enhanced expression may also be observed as an increase in the number of cone cells that express detectable levels of the polynucleotide carried by the variant rAAV. For example, there may be a 2-fold increase or more, e.g. a 3-fold increase or more, a 4-fold increase or more, a 5-fold increase or more, or a 10-fold increase or more in the number of cone cells that express detectable levels of the polynucleotide. As another example, the polynucleotide of the present invention may promote detectable levels of the polynucleotide in a greater percentage of cells as compared to a parental rAAV; for example, where a parental rAAV may promote detectable levels of polynucleotide expression in, for example, less than 5% of the cone cells in a certain region, the rAAV of the present invention promotes detectable levels of expression in 5% or more of the cone cells in that region; e.g. 10% or more, 15% or more, 20% or more, 25% or more, 30% or more, 35% or more, 40% or more, or 45% or more, in some instances 50% or more, 55% or more; 60% or more, 65% or more, 70% or more, or 75% or more, for example 80% or more, 85% or more, 90% or more, or 95% or more of the cone cells that are contacted, will express detectable levels of gene product. Enhanced expression may also be observed as an alteration in the viability and/or function of the cone cells, e.g. as measured using assessment tools such as fundus photography, OCT, adaptive optics, cERG, color vision tests,

visual acuity tests, and the like, as known in the art and as described herein.

In some embodiments, the method may further comprise the step of detecting the expression of the polynucleotide in the cone photoreceptor. In such embodiments, any convenient method as known in the art or described herein may be employed for detecting the expression of the polynucleotide, including, for example, detecting the gene product, i.e., the encoded RNA or protein, e.g., by RT-PCR, Northern blot, RNAse protection, Western blot, ELISA, immunohistochemistry, and the like; detecting the impact of the gene product on the viability of the cone photoreceptor, e.g., by fundus photography, Optical coherence tomography (OCT), Adaptive Optics (AO); or detecting the impact of the gene product on cone function, e.g. electroretinography (ERG), color vision tests, visual acuity tests, etc., any of which may be employed in the subject methods.

rAAV virions comprising the polynucleotide of interest of the present disclosure may be produced using any convenient methodologies, AAV packaging cells, and packaging technology as known to those of skill in the art. For example, an AAV expression vector (that is, a plasmid comprising the rAAV genome as well as elements useful for the cloning of the genomic elements in, e.g. bacteria, e.g. origin of replication, selectable marker, etc.) may be transfected into mammalian producer cells. Also transfected into the mammalian producer cells is an AAV helper construct, i.e. a plasmid comprising AAV REP and CAP coding regions that can be expressed in the producer cell, which complement AAV helper functions absent from the AAV expression vector. The dually-transfected producer cells are then infected by a helper virus, e.g. adenovirus, or transfected with a plasmid comprising helper virus accessory genes that promote AAV vector replication, e.g., regions VA, E2A, E4, so as to promote efficient rAAV virus production. The producer cells are then cultured to produce rAAV, and AAV vectors are purified and formulated using standard techniques known in the art.

As another example, an AAV expression vector may be packaged as a baculovirus and introduced into insect producer cells, e.g. Sf9 cells. Also introduced into the insect cells by another baculovirus are the AAV REP and CAP genes. Baculovirus-being a virus--comprises the genes encoding the accessory functions necessary for efficient rAAV virus production. Accordingly, upon infection of the insect cells by the two baculoviruses, the producer cells can be cultured to produce rAAV, and AAV vectors purified and formulated using standard techniques known in the art.

Examples of these and other methods may be found in, for example, U.S. Pat. Nos. 5,436,146; 5,753,500, 6,040,183, 6,093,570 and 6,548,286, expressly incorporated by

reference herein in their entirety. Further compositions and methods for packaging are described in Wang et al. (US 2002/0168342), also incorporated by reference herein in its entirety.

Any convenient host cells used in the art for producing rAAV virions may be employed in the production of the subject vectors, including, for example, mammalian cells, insect cells, microorganisms and yeast, e.g. SF-9, 293, A549, HeLa cells, etc. In some instances, the host cells are packaging cells in which the AAV rep and cap genes are stably maintained in the host cell. In some instances, the host cells are producer cells in which the AAV vector genome is stably maintained and packaged.

Pharmaceutical compositions and unit dosages

In some embodiments, e.g. gene therapy uses, it will be desirable to formulate the subject rAAV as a pharmaceutical composition. In certain embodiments, a pharmaceutical composition comprises a vector or virion (e.g., rAAV) described herein and one or more pharmaceutically acceptable carriers, diluents or excipients. Pharmaceutical compositions suitable for use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the internal compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile solutions can be prepared by incorporating the active compound in the required

amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof

In one embodiment, active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

The pharmaceutical compositions of the subject disclosure encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal comprising a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bio-equivalents.

The term “prodrug” indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions.

The term “pharmaceutically acceptable salt” refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Metals used as cations comprise sodium, potassium, magnesium, calcium, and the like. Amines comprise N-N’-dibenzylethylenediamine,

chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," J. Pharma Sci., 1977, 66, 119). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

As used herein, a "pharmaceutical addition salt" comprises a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These comprise organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and comprise basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in Nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2-or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and comprise alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible. For oligonucleotides, preferred examples of pharmaceutically acceptable salts comprise but are not limited to: (l) salts formed with cations such as sodium, potassium,

ammonium, magnesium, calcium, polyamides such as spermine and spermidine, and the like; (II) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (III) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, napthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (IV) salts formed from elemental anions such as chlorine, bromine, and iodine.

Pharmaceutical compositions of the present invention comprise, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that comprise, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, “carrier compound” or “carrier” can refer to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by in vivo processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The co-administration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extra circulatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is co-administered with polyinosinic acid, dextran sulphate, polycytidic acid or 4-acetamido-4’isothiocyano-stilbene-2,2’disulfonic acid (Miyao et al., Antisense Res. Dev., 1995, 5, 115-121; Takakura et al., Antisense & Nucl. Acid Drug Dev., 1996, 6, 177-183).

The subject recombinant AAV can be incorporated into pharmaceutical compositions for administration to mammalian patients, particularly humans. The virions can be formulated in nontoxic, inert, pharmaceutically acceptable aqueous carriers, preferably at a pH ranging from 3 to 8, more preferably ranging from 6 to 8. Such sterile compositions will comprise the vector or virion containing the nucleic acid encoding the therapeutic molecule dissolved in an aqueous buffer having an acceptable pH upon reconstitution.

In some embodiments, the pharmaceutical composition provided herein comprise a

therapeutically effective amount of a vector or virion in admixture with a pharmaceutically acceptable carrier and/or excipient, for example saline, phosphate buffered saline, phosphate and amino acids, polymers, polyols, sugar, buffers, preservatives and other proteins. Exemplary amino acids, polymers and sugars and the like are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds, polyoxyethylene sorbitan fatty acid esters, sucrose, fructose, dextrose, maltose, glucose, mannitol, dextran, sorbitol, inositol, galactitol, xylitol, lactose, trehalose, bovine or human serum albumin, citrate, acetate, Ringer's and Hank's solutions, cysteine, arginine, carnitine, alanine, glycine, lysine, valine, leucine, polyvinylpyrrolidone, polyethylene and glycol. Preferably, this formulation is stable for at least six months at 4º C.

In some embodiments, the pharmaceutical composition provided herein comprises a buffer, such as phosphate buffered saline (PBS) or sodium phosphate/sodium sulfate, tris buffer, glycine buffer, sterile water and other buffers known to the ordinarily skilled artisan such as those described by Good et al. (1966) Biochemistry 5:467. The pH of the buffer in which the pharmaceutical composition comprising the tumor suppressor gene contained in the adenoviral vector delivery system, may be in the range of 6.5 to 7.75, preferably 7 to 7.5, and most preferably 7.2 to 7.4.

In some embodiments, the pharmaceutical composition provided herein comprises substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran, in the amount about 1-10 percent, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 percent.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

In some instances, e.g. for administration intraocularly, orally, or parentally, it may be especially advantageous to formulate the pharmaceutical composition in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

In some cases, the unit dose of the pharmaceutical composition of the disclosure may be measured as pfu (plaque forming units). In some cases, the pfu of the unit dose of the pharmaceutical composition of the disclosure may be about $1x10^8$ to about $5x10^{10}$ pfu. In some cases, the pfu of the unit dose of the pharmaceutical composition of the disclosure is at least about $1x10^8$, $2x10^8$, $3x10^8$, $4x10^8$, $5x10^8$, $6x10^8$, $7x10^8$, $8x10^8$, $9x10^8$, $1x10^9$, $2x10^9$, $3x10^9$, $4x10^9$, $5x10^9$, $6x10^9$, $7x10^9$, $8x10^9$, $9x10^9$, $1x10^{10}$, $2x10^{10}$, $3x10^{10}$, $4x10^{10}$, and $5x10^{10}$ pfu. In some cases, the pfu of the unit dose of the pharmaceutical composition of the disclosure is at most about $1x10^8$, $2x10^8$, $3x10^8$, $4x10^8$, $5x10^8$, $6x10^8$, $7x10^8$, $8x10^8$, $9x10^8$, $1x10^9$, $2x10^9$, $3x10^9$, $4x10^9$, $5x10^9$, $6x10^9$, $7x10^9$, $8x10^9$, $9x10^9$, $1x10^{10}$, $2x10^{10}$, $3x10^{10}$, $4x10^{10}$, and $5x10^{10}$ pfu.

In some cases, the viral vector of the disclosure may be measured as vector genomes. In some cases, the unit dose of the pharmaceutical composition of the disclosure is $1x10^8$ vector genomes or more, e.g.$1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, or $1x10^{13}$ vector genomes or more, in certain instances, $1x10^{14}$ vector genomes or more, and usually no more than $1x10^{15}$ vector genomes. In some embodiments, the unit dose of the pharmaceutical composition of the disclosure is at most about $1x10^{15}$ vector genomes, e.g. $1x10^{14}$ vector genomes or less, for example $1x10^{13}$, $1x10^{12}$, $1x10^{11}$, $1x10^{10}$, or $1x10^9$ vector genomes or less, in certain instances $1x10^8$vector genomes, and typically no less than $1x10^8$ vector genomes. In some cases, the unit dose of the pharmaceutical composition of the disclosure is $1x10^{10}$ to $1x10^{11}$ vector genomes. In some cases, the unit dose of the pharmaceutical composition of the disclosure is $1x10^{10}$ to $3x10^{12}$ vector genomes. In some cases, the unit dose of the pharmaceutical composition of the disclosure is $1x10^9$ to $3x10^{13}$ vector genomes. In some cases, the unit dose of the pharmaceutical composition of the disclosure is $1x10^8$ to $3x10^{14}$ vector genomes. .

In some cases, the unit dose of the pharmaceutical composition of the disclosure may be measured using multiplicity of infection (MOI). In some cases, MOI may refer to the ratio, or multiple of vector or viral genomes to the cells to which the nucleic may be delivered. In some cases, the MOI may be $1x10^6$. In some cases, the MOI may be $1x10^5$ -$1x10^7$. In some cases, the MOI may be $1x10^4$ -$1x10^8$. In some cases, recombinant viruses of the disclosure are at least about $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, $1x10^{13}$, $1x10^{14}$, $1x10^{15}$, $1x10^{16}$, $1x10^{17}$, and $1x10^{18}$ MOI. In some cases, recombinant viruses of this disclosure are $1x10^8$ to $3x10^{14}$ MOI. In some cases, recombinant viruses of the disclosure are at most about $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, $1x10^{13}$, $1x10^{14}$, $1x10^{15}$, $1x10^{16}$, $1x10^{17}$, and $1x10^{18}$ MOI.

In some aspects, the pharmaceutical composition comprises about 1 x 10^8 to about 1 x

10^{15} recombinant viruses, about 1 x 10^{9} to about 1 x 10^{14} recombinant viruses, about 1 x 10^{10} to about 1 x 10^{13} recombinant viruses, about 1 x 10^{109} to about 3 x 10^{12} recombinant viruses, or about 1 x 10^{11} to about 3 x 10^{12} recombinant viruses.

Methods of administration

The pharmaceutical composition of the present invention may be administered to the eye of the subject by any convenient method, e.g. intraocularly, intravenously, intraperitoneally, etc. In some instances, the administration is intraocular, e.g. by intravitreal injection or subretinal injection. The general methods for delivering a vector via intravitreal injection or via subretinal injection may be illustrated by the following brief outlines. These examples are merely meant to illustrate certain features of the methods, and are in no way meant to be limiting.

In preferred embodiments, the subject rAAV is delivered intravitreally. For intravitreal administration, the vector can be delivered in the form of a suspension. Initially, topical anesthetic is applied to the surface of the eye followed by a topical antiseptic solution. The eye is held open, with or without instrumentation, and the vector is injected through the sclera with a short, narrow, for example a 30 gauge needle, into the vitreous cavity of the eye of a subject under direct observation. Intravitreal administration is generally well tolerated. At the conclusion of the procedure, there is sometimes mild redness at the injection site. There is occasional tenderness, but most patients do not report any pain. No eye patch or eye shield is necessary after this procedure, and activities are not restricted. Sometimes, an antibiotic eye drop is prescribed for several days to help prevent infection.

In some embodiments, the subject rAAV is delivered subretinally. For subretinal administration, the vector can be delivered in the form of a suspension injected subretinally under direct observation using an operating microscope. This procedure may involve vitrectomy followed by injection of vector suspension using a fine cannula through one or more small retinotomies into the subretinal space.

Briefly, an infusion cannula can be sutured in place to maintain a normal globe volume by infusion (of e.g. saline) throughout the operation. A vitrectomy is performed using a cannula of appropriate bore size (for example 20 to 27 gauge), wherein the volume of vitreous gel that is removed is replaced by infusion of saline or other isotonic solution from the infusion cannula. The vitrectomy is advantageously performed because (1) the removal of its cortex (the posterior hyaloid membrane) facilitates penetration of the retina by the cannula; (2) its removal and replacement with fluid (e.g. saline) creates space to accommodate the intraocular injection of

vector, and (3) its controlled removal reduces the possibility of retinal tears and unplanned retinal detachment.

In practicing the subject methods, the subject rAAV virion is delivered to the eye in an amount effective to deliver the polynucleotide of interest to 5% or more of the subject's cone photoreceptors, for example, 10% or more, 20% or more, 30% or more, 40% or more, or 50% or more of the subject's cone photoreceptors, e.g. 60% or more, 70% or more, 80% or more, or 90% or more of the subject's cone photoreceptors, in some instance, 95% or more, 98% or more, or 100% of the subject's cone photoreceptors to provide therapeutic benefit to the subject individual. Put another way, following the administering, 5% or more of the subject's cone photoreceptors, e.g. 10% or more, 20% or more, 30% or more, 40% or more, or 50% or more, in some instance 60% or more, 70% or more, 80% or more, or 90% or more, e.g. 95%, 98%, or 100% of the cones, will comprise a sufficient amount of the polynucleotide of interest to have an impact on cone viability and/or function, e.g. to treat or prevent a disorder. In some embodiments, the transduced cones photoreceptors will be located throughout the retina. In some embodiments, the transduced cone photoreceptors will be cones in the fovea and foveola. In some embodiments, the transduced cone photoreceptors will be foveal cones, i.e. L- or M-cones located in the fovea.

Typically, an effective amount will be about $1x10^8$ vector genomes or more of the subject rAAV, e.g. $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, or $1x10^{13}$ vector genomes or more, in certain instances, $1x10^{14}$ vector genomes or more, and usually no more than $1x10^{15}$ vector genomes. In some cases, the amount of vector genomes that is delivered is at most about $1x10^{15}$ vector genomes, e.g. $1x10^{14}$ vector genomes or less, for example $1x10^{13}$, $1x10^{12}$, $1x10^{11}$, $1x10^{10}$, or $1x10^9$ vector genomes or less, in certain instances $1x10^8$vector genomes, and typically no less than $1x10^8$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^{10}$ to $1x10^{11}$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^{10}$ to $3x10^{12}$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^9$ to $3x10^{13}$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^8$ to $3x10^{14}$ vector genomes.

In some cases, the amount of pharmaceutical composition to be administered may be measured using multiplicity of infection (MOI). In some cases, MOI may refer to the ratio, or multiple of vector or viral genomes to the cells to which the nucleic may be delivered. In some cases, the MOI may be $1x10^6$. In some cases, the MOI may be $1x10^5$ -$1x10^7$. In some cases, the MOI may be $1x10^4$ -$1x10^8$. In some cases, recombinant viruses of the disclosure are at

least about $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, $1x10^{13}$, $1x10^{14}$, $1x10^{15}$, $1x10^{16}$, $1x10^{17}$, and $1x10^{18}$ MOI. In some cases, recombinant viruses of this disclosure are $1x10^8$ to $3x10^{14}$ MOI. In some cases, recombinant viruses of the disclosure are at most about $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, $1x10^{13}$, $1x10^{14}$, $1x10^{15}$, $1x10^{16}$, $1x10^{17}$, and $1x10^{18}$ MOI.

In some aspects, the amount of pharmaceutical composition comprises about 1×10^8 to about 1×10^{15} recombinant viruses, about 1×10^9 to about 1×10^{14} recombinant viruses, about 1×10^{10} to about 1×10^{13} recombinant viruses, about 1×10^{10} to about 3×10^{12} recombinant viruses, or about 1×10^{11} to about 3×10^{12} recombinant viruses.

UTILITY

Methods and compositions for the intravitreal delivery of polynucleotides to cone photoreceptors, and more particular foveal cones, find many uses in research and in medicine.

For example, such methods and compositions may be used in research to test the function of the gene product encode by the polynucleotide in vivo, e.g. to better understand the function of the cone photoreceptor and/or whether the gene product will impact the viability and/or function of the cone photoreceptor.

As alluded to above, the subject rAAVs, referred to collectively herein as “subject compositions”, find use in expressing a transgene in cone cells of an animal, for example, in foveal cones of an animal. For example, the subject compositions may be used in research, e.g. to determine the effect that the gene has on cone cell viability and/or function. As another example, the subject compositions may be used in medicine, e.g. to treat a cone cell disorder. Thus, in some aspects of the invention, methods are provided for the expression of a gene in cone cells, the method comprising contacting cone cells with a composition of the present disclosure. In some embodiments, contacting occurs in vitro. In some embodiments, contacting occurs in vivo, i.e., the subject composition is administered to a subject.

For instances in which cone cells are to be contacted in vitro with a subject rAAV, the cells may be from any mammalian species, e.g. rodent (e.g. mice, rats, gerbils, squirrels), rabbit, feline, canine, goat, ovine, pig, equine, bovine, primate, human. Cells may be from established cell lines, e.g. WERI cells, 661W cells, or they may be primary cells, where “primary cells”, “primary cell lines”, and “primary cultures” are used interchangeably herein to refer to cells and cells cultures that have been derived from a subject and allowed to grow in vitro for a limited number of passages, i.e. splittings, of the culture. For example, primary cultures are

cultures that may have been passaged 0 times, 1 time, 2 times, 4 times, 5 times, 10 times, or 15 times, but not enough times go through the crisis stage. Typically, the primary cell lines of the present invention are maintained for fewer than 10 passages in vitro.

If the cells are primary cells, they may be harvested from a mammal by any convenient method, e.g. whole explant, biopsy, etc. An appropriate solution may be used for dispersion or suspension of the harvested cells. Such solution will generally be a balanced salt solution, e.g. normal saline, PBS, Hank's balanced salt solution, etc., conveniently supplemented with fetal calf serum or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from 5-25 mM. Convenient buffers include HEPES, phosphate buffers, lactate buffers, etc. The cells may be used immediately, or they may be stored, frozen, for long periods of time, being thawed and capable of being reused. In such cases, the cells will usually be frozen in 10% DMSO, 50% serum, 40% buffered medium, or some other such solution as is commonly used in the art to preserve cells at such freezing temperatures, and thawed in a manner as commonly known in the art for thawing frozen cultured cells.

To promote expression of the transgene, the subject rAAV will be contacted with the cells for about 30 minutes to 24 hours or more, e.g., 1 hour, 1.5 hours, 2 hours, 2.5 hours, 3 hours, 3.5 hours 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 12 hours, 16 hours, 18 hours, 20 hours, 24 hours, etc. The subject rAAV may be provided to the subject cells one or more times, e.g. one time, twice, three times, or more than three times, and the cells allowed to incubate with the agent(s) for some amount of time following each contacting event e.g. 16-24 hours, after which time the media is replaced with fresh media and the cells are cultured further. Contacting the cells may occur in any culture media and under any culture conditions that promote the survival of the cells. For example, cells may be suspended in any appropriate nutrient medium that is convenient, such as Iscove's modified DMEM or RPMI 1640, supplemented with fetal calf serum or heat inactivated goat serum (about 5-10%), L-glutamine, a thiol, particularly 2-mercaptoethanol, and antibiotics, e.g. penicillin and streptomycin. The culture may contain growth factors to which the cells are responsive. Growth factors, as defined herein, are molecules capable of promoting survival, growth and/or differentiation of cells, either in culture or in the intact tissue, through specific effects on a transmembrane receptor. Growth factors include polypeptides and non-polypeptide factors.

Typically, an effective amount of subject rAAV is provided to produce the expression of the transgene in cells. As discussed elsewhere herein, the effective amount may be readily determined empirically, e.g. by detecting the presence or levels of transgene gene product, by

detecting an effect on the viability or function of the cone cells, etc. Typically, an effect amount of subject rAAV will promote greater expression of the transgene in cone cells than the same amount of parental rAAV from which its capsid was derived. Typically, expression will be enhanced 2-fold or more relative to the expression from parental rAAV, for example 3-fold, 4-fold, or 5-fold or more, in some instances 10-fold, 20-fold or 50-fold or more, e.g. 100-fold.

In some embodiments, as when the transgene is a selectable marker, the population of cells may be enriched for those comprising the transgene by separating the modified cells from the remaining population. Separation may be by any convenient separation technique appropriate for the selectable marker used. For example, if the transgene is a fluorescent marker, cells may be separated by fluorescence activated cell sorting, whereas if the transgene is a cell surface marker, cells may be separated from the heterogeneous population by affinity separation techniques, e.g. magnetic separation, affinity chromatography, "panning" with an affinity reagent attached to a solid matrix, or other convenient technique. Techniques providing accurate separation include fluorescence activated cell sorters, which can have varying degrees of sophistication, such as multiple color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc. The cells may be selected against dead cells by employing dyes associated with dead cells (e.g. propidium iodide). Any technique may be employed which is not unduly detrimental to the viability of the cells. Cell compositions that are highly enriched for cells comprising the transgene are achieved in this manner. By “highly enriched”, it is meant that the genetically modified cells will be 70% or more, 75% or more, 80% or more, 85% or more, 90% or more of the cell composition, for example, about 95% or more, or 98% or more of the cell composition. In other words, the composition may be a substantially pure composition of genetically modified cells.

For instances in which cone cells are to be contacted in vivo with the subject rAAV, the subject may be any mammal, e.g. rodent (e.g. mice, rats, gerbils), rabbit, feline, canine, goat, ovine, pig, equine, bovine, or primate. In certain embodiments, the subject is a primate of the Parvorder Catarrhini. As is known in the art, Catarrhini is one of the two subdivisions of the higher primates (the other being the New World monkeys), and includes Old World monkeys and the apes, which in turn are further divided into the lesser apes or gibbons and the great apes, consisting of the orangutans, gorillas, chimpanzees, bonobos, and humans. In a further preferred embodiment, the primate is a human.

The subject rAAV may be administered to the retina of the subject by any suitable method. For example, the subject composition may be administered intraocularly via intravitreal

injection or subretinal injection. The general methods for delivering a vector via intravitreal injection or via subretinal injection may be illustrated by the following brief outlines. These examples are merely meant to illustrate certain features of the methods, and are in no way meant to be limiting.

For subretinal administration, the subject rAAV can be delivered in the form of a suspension injected subretinally under direct observation using an operating microscope. Typically, a volume of 1 to 200 uL, e.g. 50 uL, 100 uL, 150 ul, or 200 uL, but usually no more than 200 uL, of the subject composition will be administered by such methods. This procedure may involve vitrectomy followed by injection of vector suspension using a fine cannula through one or more small retinotomies into the subretinal space. Briefly, an infusion cannula can be sutured in place to maintain a normal globe volume by infusion (of e.g. saline) throughout the operation. A vitrectomy is performed using a cannula of appropriate bore size (for example 20 to 27 gauge), wherein the volume of vitreous gel that is removed is replaced by infusion of saline or other isotonic solution from the infusion cannula. The vitrectomy is advantageously performed because (1) the removal of its cortex (the posterior hyaloid membrane) facilitates penetration of the retina by the cannula; (2) its removal and replacement with fluid (e.g. saline) creates space to accommodate the intraocular injection of vector, and (3) its controlled removal reduces the possibility of retinal tears and unplanned retinal detachment.

For intravitreal administration, the subject rAAV can be delivered in the form of a suspension. Initially, topical anesthetic is applied to the surface of the eye followed by a topical antiseptic solution. The eye is held open, with or without instrumentation, and the rAAV is injected through the sclera with a short, narrow, for example a 30 gauge needle, into the vitreous cavity of the eye of a subject under direct observation. Typically, a volume of 1 to 100 uL, e.g. 25 uL, 50 uL, or 100 uL, and usually no more than 100uL, of the subject composition may be delivered to the eye by intravitreal injection without removing the vitreous. Alternatively, a vitrectomy may be performed, and the entire volume of vitreous gel is replaced by an infusion of the subject composition. In such cases, up to about 4 mL of the subject composition may be delivered, e.g. to a human eye. Intravitreal administration is generally well tolerated. At the conclusion of the procedure, there is sometimes mild redness at the injection site. There is occasional tenderness, but most patients do not report any pain. No eye patch or eye shield is necessary after this procedure, and activities are not restricted. Sometimes, an antibiotic eye drop is prescribed for several days to help prevent infection.

The subject methods and/or compositions may be used in medicine to express a

therapeutic polynucleotide in cone photoreceptors as a therapy to treat or prevent a retinal disorder. The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof, e.g. reducing the likelihood that the disease or symptom thereof occurs in the subject, and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; or (c) relieving the disease, i.e., causing regression of the disease. The therapeutic agent may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest. Such treatment is desirably performed prior to complete loss of function in the affected tissues. The subject therapy will desirably be administered during the symptomatic stage of the disease, and in some cases after the symptomatic stage of the disease.

There are a number of retinal disorders that may be treated or prevented using the subject methods and/or compositions. Of particular interest are cone-associated disorders; that is, disorders that are associated with a loss of cone viability and/or a reduction in cone function. As discussed above, cone photoreceptors are responsible for color vision and high acuity foveal vision, and are densely packed in a 1.5mm depression located in the center of the macula of the retina, called the fovea centralis. Consistent with this, disorders associated with cone dysfunction and viability typically manifest in the macula and impact color vision and high acuity vision. Non-limiting examples of cone-associated disorders include rod-cone dystrophy; cone-rod dystrophy; progressive cone dystrophy; retinitis pigmentosa (RP); Stargardt Disease; macular telangiectasia, Leber hereditary optic neuropathy, Best's disease; adult vitelliform macular dystrophy; X-linked retinoschisis; color vision disorders such as blue cone monochromacy, achromatopsia, incomplete achromatopsia, protan defects, deutan defects, and tritan defects; and retinal disorders that affect the central macula, such as, for example, age-related macular degeneration, wet age-related macular degeneration, geographic atrophy, macular telangiectasia, retinitis pigmentosa, diabetic retinopathy, retinal vein occlusions, glaucoma, Sorsby's fundus dystrophy, adult vitelliform macular dystrophy, Best's disease, and X-linked retinoschisis.

Stargardt's macular dystrophy. Stargardt's macular dystrophy, also known as Stargardt Disease and fundus flavimaculatus, is an inherited form of juvenile macular degeneration that causes progressive vision loss usually to the point of legal blindness. The onset of symptoms usually appears between the ages of six and thirty years old (average of about 16-18 years). Mutations in several genes, including ABCA4, CNGB3, ELOVL4, PROM1, are associated with the disorder. Symptoms typically develop by twenty years of age, and include wavy vision, blind spots, blurriness, impaired color vision, and difficulty adapting to dim lighting. The main symptom of Stargardt disease is loss of visual acuity, which ranges from 20/50 to 20/200. In addition, those with Stargardt disease are sensitive to glare; overcast days offer some relief. Vision is most noticeably impaired when the macula is damaged, which can be observed by fundus exam.

Cone dystrophy. Cone dystrophy (COD) is an inherited ocular disorder characterized by the loss of cone cells. The most common symptoms of cone dystrophy are vision loss (age of onset ranging from the late teens to the sixties), sensitivity to bright lights, and poor color vision. Visual acuity usually deteriorates gradually, but it can deteriorate rapidly to 20/200; later, in more severe cases, it drops to "counting fingers" vision. Color vision testing using color test plates (HRR series) reveals many errors on both red-green and blue-yellow plates. It is believed that the dystrophy is primary, since subjective and objective abnormalities of cone function are found before ophthalmoscopic changes can be seen. However, the retinal pigment epithelium (RPE) rapidly becomes involved, leading to a retinal dystrophy primarily involving the macula. The fundus exam via ophthalmoscope is essentially normal early on in cone dystrophy, and definite macular changes usually occur well after visual loss. The most common type of macular lesion seen during ophthalmoscopic examination has a bull's-eye appearance and consists of a doughnut-like zone of atrophic pigment epithelium surrounding a central darker area. In another, less frequent form of cone dystrophy there is rather diffuse atrophy of the posterior pole with spotty pigment clumping in the macular area. Rarely, atrophy of the choriocapillaris and larger choroidal vessels is seen in patients at an early stage. Fluorescein angiography (FA) is a useful adjunct in the workup of someone suspected to have cone dystrophy, as it may detect early changes in the retina that are too subtle to be seen by ophthalmoscope. Because of the wide spectrum of fundus changes and the difficulty in making the diagnosis in the early stages, electroretinography (ERG) remains the best test for making the diagnosis. Abnormal cone function on the ERG is indicated by a reduced single-flash and flicker response when the test is carried out in a well-lit room (photopic ERG). Mutations in

several genes, including GUCA1A, PDE6C, PDE6H, and RPGR, are associated with the disorder.

Cone-rod dystrophy. Cone-rod dystrophy (CRD, or CORD) is an inherited retinal dystrophy that belongs to the group of pigmentary retinopathies. CRD is characterized by retinal pigment deposits visible on fundus examination, predominantly localized to the macular region and the loss of both cone and rod cells. In contrast to rod-cone dystrophy (RCD) resulting from the primary loss in rod photoreceptors and later followed by the secondary loss in cone photoreceptors, CRD reflects the opposite sequence of events: primary cone involvement, or, sometimes, by concomitant loss of both cones and rods. Symptoms include decreased visual acuity, color vision defects, photoaversion and decreased sensitivity in the central visual field, later followed by progressive loss in peripheral vision and night blindness. Mutations in several genes, including ADAM9, PCDH21, CRX, GUCY2D, PITPNM3, PROM1, PRPH2, RAX2, RIMS1, RPGR, and RPGRIP1, are associated with the disorder.

Spinocerebellar ataxia type 7. Spinocerebellar ataxia is a progressive, degenerative, inherited disease characterized by slowly progressive incoordination of gait and is often associated with poor coordination of hands, speech, and eye movements. There are multiple types of SCA, with Spinocerebellar ataxia type 7 (SCA-7) differing from most other SCAs in that visual problems can occur in addition to poor coordination. SCA-7 is associated with automosmal dominant mutations in the ATXN7/SCA7 gene. When the disease manifests itself before age 40, visual problems rather than poor coordination are typically the earliest signs of disease. Early symptoms include difficulty distinguishing colors and decreased central vison. In addition, symptoms of ataxia (incoordination, slow eye movements, and mild changes in sensation or reflexes) may be detectable. Loss of motor control, unclear speech, and difficulty swallowing become prominent as the disease progresses.

Bardet-Biedl syndrome-1. Bardet-Biedl syndrome-1 (BBS-1) is a pleiotropic disorder with variable expressivity and a wide range of clinical variability observed both within and between families. The main clinical features are rod–cone dystrophy, with childhood-onset visual loss preceded by night blindness; postaxial polydactyly; truncal obesity that manifests during infancy and remains problematic throughout adulthood; specific learning difficulties in some but not all individuals; male hypogenitalism and complex female genitourinary malformations; and renal dysfunction, a major cause of morbidity and mortality. Vision loss is one of the major features of Bardet-Biedl syndrome. Problems with night vision become apparent by mid-childhood, followed by blind spots that develop in the peripheral vision. Over time, these blind spots enlarge and

merge to produce tunnel vision. Most people with Bardet-Biedl syndrome also develop blurred central vision (poor visual acuity) and become legally blind by adolescence or early adulthood. Bardet-Biedl syndrome can result from mutations in at least 14 different genes (often called BBS genes) known or suspected to play critical roles in cilia function, with mutations in BBS1 and BBS10 being the most common.

Achromatopsia. Achromatopsia, or Rod monochromatism, is a disorder in which subjects experience a complete lack of the perception of color, such that the subject sees only in black, white, and shades of grey. Other symptoms include reduced visual acuity, photophobia, nystagmus, small central scotoma, and eccentric fixation. The disorder is frequently noticed first in children around six months of age by their photophobic activity and/or their nystagmus. Visual acuity and stability of the eye motions generally improve during the first 6–7 years of life (but remain near 20/200). Mutations in CNGB3, CNGA3, GNAT2, PDE6C, and PDE6HI have been associated with the disorder.

Incomplete achromatopsia. Incomplete achromatopsia is similar to Achromatopsia but with less penetrance. In incomplete achromatopsia, the symptoms are similar to those of complete achromatopsia except in a diminished form. Individuals with incomplete achromatopsia have reduced visual acuity with or without nystagmus or photophobia. Furthermore, these individuals show only partial impairment of cone cell function but again have retained rod cell function.

Blue cone monochromacy. Blue cone (S cone) monochromatism (BCM) is a rare X-linked congenital stationary cone dysfunction syndrome, affecting approximately 1 in 100,000 individuals. Affected males with BCM have no functional long wavelength sensitive (L) or medium wavelength sensitive (M) cones in the retina, due to mutations at the genetic locus for the L and M-opsin genes. Color discrimination is severely impaired from birth, and vision is derived from the remaining preserved S cones and rod photoreceptors. BCM typically presents with reduced visual acuity (6/24 to 6/60), pendular nystagmus, photophobia, and patients often have myopia. The rod-specific and maximal electroretinogram (ERG) usually show no definite abnormality, whereas the 30Hz cone ERG cannot be detected. Single flash photopic ERG is often recordable, albeit small and late, and the S cone ERG is well preserved.

Color vision deficiency. Color vision deficiency (CVD), or color blindness, is the inability or decreased ability to see color, or perceive color differences, under normal lighting conditions. Individuals suffering from color blindness may be identified as such using any of a number of color vision tests, e.g., color ERG (cERG), pseudoisochromatic plates (Ishihara plates, Hardy-

Rand-Ritter polychromatic plates), the Farnsworth-Munsell 100 hue test, the Farnsworth's panel D-15, the City University test, Kollner's rule, etc. Examples of color vision deficiencies include protan defects, deutan defects, and tritan defects. Protan defects include protanopia (an insensitivity to red light) and protanomaly (a reduced sensitivity to red light), and are associated with mutations in the L-Opsin gene (OPN1LW). Deutan defects include deuteranopia (an insensitivity to green light) and deutanomaly (a reduced sensitivity to green light), and are associated with mutations in the M-Opsin gene (OPN1MW). Tritan defects include tritanopia (an insensitivity to blue light) and tritanomaly (a reduced sensitivity to blue light), and are associated with mutations in the S-Opsin gene (OPN1SW).

Age-related macular degeneration. Age-related macular degeneration (AMD) is one of the leading causes of vision loss in people over the age of 50 years. AMD mainly affects central vision, which is needed for detailed tasks such as reading, driving, and recognizing faces. The vision loss in this condition results from a gradual deterioration of photoreceptors in the macula. Side (peripheral) vision and night vision are generally not affected.

Researchers have described two major types of age-related macular degeneration, known as the dry, or "nonexudative" form, and the wet, or "exudative" or "neovascular", form, both of which may be treated by delivering transgenes packaged in the subject rAAV.

Dry AMD is characterized by a buildup of yellow deposits called drusen between the retinal pigment epithelium and the underlying choroid of the macula, which may be observed by Fundus photography. This results in a slowly progressive loss of vision. The condition typically affects vision in both eyes, although vision loss often occurs in one eye before the other. Other changes may include pigment changes and RPE atrophy. For example, in certain cases called central geographic atrophy, or "GA", atrophy of the retinal pigment epithelial and subsequent loss of photoreceptors in the central part of the eye is observed. Dry AMD has been associated with mutations in CD59 and genes in the complement cascade.

Wet AMD is a progressed state of dry AMD, and occurs in abut 10% of dry AMD patients. Pathological changes include retinal pigment epithelial cells (RPE) dysfunction, fluid collecting under the RPE, and choroidal neovascularization (CNV) in the macular area. Fluid leakage, RPE or neural retinal detachment and bleeding from ruptured blood vessels can occur in severe cases. Symptoms of wet AMD may include visual distortions, such as straight lines appearing wavy or crooked, a doorway or street sign looking lopsided, or objects appearing smaller or farther away than they really are; decreased central vision; decreased intensity or brightness of colors; and well-defined blurry spot or blind spot in the field of vision. Onset may

be abrupt and worsen rapidly. Diagnosis may include the use of an Amsler grid to test for defects in the subject's central vision (macular degeneration may cause the straight lines in the grid to appear faded, broken or distorted), fluorescein angiogram to observe blood vessel or retinal abnormalities, and optical coherence tomography to detect retina swelling or leaking blood vessels. A number of cellular factors have been implicated in the generation of CNV, among which are vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), pigment epithelium-derived factor (PEDF), hypoxia inducible factor (HIF), angiopoietin (Ang), and other cytokines, mitogen-activated protein kinases (MAPK) and others.

Macular telangiectasia. Macular telangiectasia (MacTel) is a form of pathologically dilated blood vessels (telangiectasia) in the parafoveal region of the macula. The tissue deteriorates and the retinal structure becomes scarred due to the development of liquid-filled cysts, which impairs nutrition of the photoreceptor cells and destroys vision permanently. There are two types of MacTel, type 1 and type 2. Macular telangiectasia type 2 is a bilateral disease, whose prevalence has recently been shown to be as high as 0.1% in persons 40 years and older. Biomicroscopy may show reduced retinal transparency, crystalline deposits, mildly ectatic capillaries, blunted venules, retinal pigment plaques, foveal atrophy, and neovascular complexes. Fluorescein angiography shows telangiectatic capillaries predominantly temporal to the foveola in the early phase and a diffuse hyperfluorescence in the late phase. High-resolution optical coherence tomography (OCT) may reveal disruption of the photoreceptor inner segment-outer segment border, hyporeflective cavities at the level of the inner or outer retina, and atrophy of the retina in later stages. In Type 1 macular telangiectasia, the disease almost always occurs in one eye, which differentiates it from Type 2. While MacTel does not usually cause total blindness, it commonly causes loss of the central vision, which is required for reading and driving vision, over a period of 10–20 years.

Retinitis pigmentosa. Retinitis Pigmentosa (RP) is a group of inherited disorders characterized by progressive peripheral vision loss and night vision difficulties (nyctalopia) that can lead to central vision loss. Presenting signs and symptoms of RP vary, but the classic ones include nyctalopia (night blindness, most commonly the earliest symptom in RP); visual loss (usually peripheral, but in advanced cases, central visual loss); and photopsia (seeing flashes of light). Because RP is a collection of many inherited diseases, significant variability exists in the physical findings. Ocular examination involves assessment of visual acuity and pupillary reaction, as well as anterior segment, retinal, and funduscopic evaluation. In some instances, the RP is one aspect of a syndrome, e.g. syndromes that are also associated with hearing loss

(Usher syndrome, Waardenburg syndrome, Alport syndrome, Refsum disease); Kearns-Sayre syndrome (external ophthalmoplegia, lid ptosis, heart block, and pigmentary retinopathy); Abetalipoproteinemia (Fat malabsorption, fat-soluble vitamin deficiencies, spinocerebellar degeneration, and pigmentary retinal degeneration); mucopolysaccharidoses (eg, Hurler syndrome, Scheie syndrome, Sanfilippo syndrome); Bardet-Biedl syndrome (Polydactyly, truncal obesity, kidney dysfunction, short stature, and pigmentary retinopathy); and neuronal ceroid lipofuscinosis (Dementia, seizures, and pigmentary retinopathy; infantile form is known as Jansky-Bielschowsky disease, juvenile form is Vogt-Spielmeyer-Batten disease, and adult form is Kufs syndrome). Retinitis pigmentosa is most commonly associated with mutations in the RHO, RP2, RPGR, RPGRIP1, PDE6A, PDE6B, MERTK, PRPH2, CNGB1, USH2A, ABCA4, BBS genes.

Diabetic retinopathy. Diabetic retinopathy (DR) is damage to the retina caused by complications of diabetes, which can eventually lead to blindness. Without wishing to be bound by theory, it is believed that hyperglycemia-induced intramural pericyte death and thickening of the basement membrane lead to incompetence of the vascular walls. These damages change the formation of the blood-retinal barrier and also make the retinal blood vessels become more permeable.

There are two stages of diabetic retinopathy: non-proliferative diabetic retinopathy (NPDR), and proliferative diabetic retinopathy (PDR). Nonproliferative diabetic retinopathy is the first stage of diabetic retinopathy, and is diagnosed by fundoscopic exam and coexistent diabetes. In cases of reduced vision, fluorescein angiography may be done to visualize the vessles in the back of the eye to and any retinal ischemia that may be present. All people with diabetes are at risk for developing NPDR, and as such, would be candidates for prophylactic treatment with the subject vectors. Proliferative diabetic retinopathy is the second stage of diabetic retinopathy, characterized by neovascularization of the retina, vitreous hemorrhage, and blurred vision. In some instances, fibrovascular proliferation causes tractional retinal detachment. In some instances, the vessels can also grow into the angle of the anterior chamber of the eye and cause neovascular glaucoma. Individuals with NPDR are at increased risk for developing PDR, and as such, would be candidates for prophylactic treatment with the subject vectors.

Diabetic macular edema. Diabetic macular edema (DME) is an advanced, vision-limiting complication of diabetic retinopathy that affects nearly 30% of patients who have had diabetes for at least 20 years, and is responsible for much of the vision loss due to DR. It results from

retinal microvascular changes that compromise the blood-retinal barrier, causing leakage of plasma constituents into the surrounding retina and, consequently, retinal edema. Without wishing to be bound by theory, it is believed that hyperglycemia, sustained alterations in cell signaling pathways, and chronic microvascular inflammation with leukocyte-mediated injury leads to chronic retinal microvascular damage, which triggers an increase in intraocular levels of VEGF, which in turn increases the permeability of the vasculature.

Patients at risk for developing DME include those who have had diabetes for an extended amount of time and who experience one or more of severe hypertension (high blood pressure), fluid retention, hypoalbuminemia, or hyperlipidemia. Common symptoms of DME are blurry vision, floaters, double vision, and eventually blindness if the condition is allowed to progress untreated. DME is diagnosed by funduscopic examination as retinal thickening within 2 disc diameters of the center of the macula. Other methods that may be employed include Optical coherence tomography (OCT) to detect retinal swelling, cystoid edema, and serous retinal detachment; fluorescein angiography, which distinguishes and localizes areas of focal versus diffuse leakage, thereby guiding the placement of laser photocoagulation if laser photocoagulation is to be used to treat the edema; and color stereo fundus photographs, which can be used to evaluate long-term changes in the retina. Visual acuity may also be measured, especially to follow the progression of macular edema and observe its treatment following administration of the subject pharmaceutical compositions.

Retinal vein occlusions. A retinal vein occlusion (RVO) is a blockage of the portion of the circulation that drains the retina of blood. The blockage can cause back-up pressure in the capillaries, which can lead to hemorrhages and also to leakage of fluid and other constituents of blood.

Glaucoma. Glaucoma is a term describing a group of ocular (eye) disorders that result in optic nerve damage, often associated with increased fluid pressure in the eye (intraocular pressure)(IOP). The disorders can be roughly divided into two main categories, "open-angle" and "closed-angle" (or "angle closure") glaucoma. Open-angle glaucoma accounts for 90% of glaucoma cases in the United States. It is painless and does not have acute attacks. The only signs are gradually progressive visual field loss, and optic nerve changes (increased cup-to-disc ratio on fundoscopic examination). Closed-angle glaucoma accounts for less than 10% of glaucoma cases in the United States, but as many as half of glaucoma cases in other nations (particularly Asian countries). About 10% of patients with closed angles present with acute angle closure crises characterized by sudden ocular pain, seeing halos around lights, red eye, very

high intraocular pressure (>30 mmHg), nausea and vomiting, suddenly decreased vision, and a fixed, mid-dilated pupil. It is also associated with an oval pupil in some cases. Modulating the activity of proteins encoded by DLK, NMDA, INOS, CASP-3, Bcl-2, or Bcl-xl may treat the condition.

Sorsby's fundus dystrophy. Sorsby's fundus dystrophy is an autosomal dominant, retinal disease associated with mutations in the TIMP3 gene. Clinically, early, mid-peripheral, drusen and colour vision deficits are found. Some patients complain of night blindness. Most commonly, the presenting symptom is sudden acuity loss, manifest in the third to fourth decades of life, due to untreatable submacular neovascularisation. Histologically, there is accumulation of a confluent lipid containing material 30 μm thick at the level of Bruch's membrane.

Vitelliform macular dystrophy. Vitelliform macular dystrophy is a genetic eye disorder that can cause progressive vision loss. Vitelliform macular dystrophy is associated with the buildup of fatty yellow pigment (lipofuscin) in cells underlying the macula. Over time, the abnormal accumulation of this substance can damage cells that are critical for clear central vision. As a result, people with this disorder often lose their central vision, and their eyesight may become blurry or distorted. Vitelliform macular dystrophy typically does not affect side (peripheral) vision or the ability to see at night.

Researchers have described two forms of vitelliform macular dystrophy with similar features. The early-onset form (known as Best disease) usually appears in childhood; the onset of symptoms and the severity of vision loss vary widely. It is associated with mutations in the VMD2/BEST1 gene. The adult-onset form (Adult vitelliform macular dystrophy) begins later, usually in mid-adulthood, and tends to cause vision loss that worsens slowly over time. It has been associated with mutations in the PRPH2 gene. The two forms of vitelliform macular dystrophy each have characteristic changes in the macula that can be detected during an eye examination.

Rod-cone dystrophy. Rod-cone dystrophies are a family of progressive diseases in which rod dysfunction, which leads to night blindness and loss of peripheral visual field expanses, is either the prevailing problem or occurring at least as severely as cone dysfunction. A scallop-bordered lacunar atrophy may be seen in the midperiphery of the retina. The macula is only mildly involved by clinical examination although central retinal thinning is seen in all cases. Dyschromatopsia is mild early and usually becomes more severe. The visual fields are moderately to severely constricted although in younger individuals a typical ring scotoma is

present. The peripheral retina contains 'white dots' and often resembles the retinal changes seen in retinitis punctate albescens. Retinitis pigmentosa is the main group of diseases included under this definition and, as a whole, is estimated to affect approximately one in every 3,500 people. Depending on the classification criteria used, about 60-80% of all retinitis pigmentosa patients have a clear-cut rod-cone dystrophy pattern of retinal disease and once other syndromic forms are taken into account, about 50-60% of all retinitis pigmentosas fall in the rod-cone dystrophy nonsyndromic category.

Leber's congenital amaurosis. Leber's congenital amaurosis (LCA) is a severe dystrophy of the retina that typically becomes evident in the first year of life. Visual function is usually poor and often accompanied by nystagmus, sluggish or near-absent pupillary responses, photophobia, high hyperopia, and keratoconus. Visual acuity is rarely better than 20/400. A characteristic finding is Franceschetti's oculo-digital sign, comprising eye poking, pressing, and rubbing. The appearance of the fundus is extremely variable. While the retina may initially appear normal, a pigmentary retinopathy reminiscent of retinitis pigmentosa is frequently observed later in childhood. The electroretinogram (ERG) is characteristically "nondetectable" or severely subnormal. Mutations in 17 genes are known to cause LCA: GUCY2D (locus name: LCA1), RPE65 (LCA2), SPATA7 (LCA3), AIPL1 (LCA4), LCA5 (LCA5), RPGRIP1 (LCA6), CRX (LCA7), CRB1 (LCA8), NMNAT1 (LCA9), CEP290 (LCA10), IMPDH1 (LCA11), RD3 (LCA12), RDH12 (LCA13), LRAT (LCA14), TULP1 (LCA15), KCNJ13 (LCA16), and IQCB1. Together, mutations in these genes are estimated to account for over half of all LCA diagnoses. At least one other disease locus for LCA has been reported, but the gene is not known.

X-linked retinoschisis. X-linked retinoschisis (XLRS) is characterized by symmetric bilateral macular involvement with onset in the first decade of life, in some cases as early as age three months. Fundus examination shows areas of schisis (splitting of the nerve fiber layer of the retina) in the macula, sometimes giving the impression of a spoke wheel pattern. Schisis of the peripheral retina, predominantly inferotemporally, occurs in approximately 50% of individuals. Affected males typically have vision of 20/60 to 20/120. Visual acuity often deteriorates during the first and second decades of life but then remains relatively stable until the fifth or sixth decade. The diagnosis of X-linked juvenile retinoschisis is based on fundus findings, results of electrophysiologic testing, and molecular genetic testing. RS1 is the only gene known to be associated with X-linked juvenile retinoschisis.

An individual affected by a cone cell disorder or at risk for developing a cone cell disorder can be readily identified using techniques to detect the symptoms of the disorder as

known in the art, including, without limitation, fundus photography; Optical coherence tomography (OCT); adaptive optics (AO); electroretinography, e.g. ERG, color ERG (cERG); color vision tests such as pseudoisochromatic plates (Ishihara plates, Hardy-Rand-Ritter polychromatic plates), the Farnsworth-Munsell 100 hue test, the Farnsworth's panel D-15, the City university test, Kollner's rule, and the like; and visual acuity tests such as the ETDRS letters test, Snellen visual acuity test, visual field test, contrast sensitivity test, and the like; as will be known by the ordinarily skilled artisan. Additionally or alternatively, the individual affected by a cone cell disorder or at risk for developing a cone cell disorder can be readily identified using techniques to detect gene mutations that are associated with the cone cell disorder as known in the art, including, without limitation, PCR, DNA sequence analysis, restriction digestion, Southern blot hybridization, mass spectrometry, etc. In some embodiments, the method comprises the step of identifying the individual in need of a cone cell therapy. In such instances, any convenient method for determining if the individual has the symptom(s) of a cone cell disorder or is at risk for developing a cone cell disorder, for example by detecting the symptoms described herein or known in the art, by detecting a mutation in a gene as herein or as known in the art, etc. may be utilized to identify the individual in need of a cone cell therapy.

In practicing the subject methods, the subject composition is typically delivered to the retina of the subject in an amount that is effective to result in the expression of the transgene in the cone cells. In some embodiments, the method comprises the step of detecting the expression of the transgene in the cone cells.

There are a number of ways to detect the expression of a transgene, any of which may be used in the subject embodiments. For example, expression may be detected directly, i.e. by measuring the amount of gene product, for example, at the RNA level, e.g. by RT-PCR, Northern blot, RNAse protection; or at the protein level, e.g. by Western blot, ELISA, immunohistochemistry, and the like. As another example, expression may be detected indirectly, i.e. by detecting the impact of the gene product on the viability or function of the cone photoreceptor in the subject. For example, if the gene product encoded by the transgene improves the viability of the cone cell, the expression of the transgene may be detected by detecting an improvement in viability of the cone cell, e.g. by fundus photography, Optical coherence tomography (OCT), Adaptive Optics (AO), and the like. If the gene product encoded by the transgene alters the activity of the cone cell, the expression of the transgene may be detected by detecting a change in the activity of the cone cell, e.g. by electroretinogram (ERG) and color ERG (cERG); functional adaptive optics; color vision tests such as

pseudoisochromatic plates (Ishihara plates, Hardy-Rand-Ritter polychromatic plates), the Farnsworth-Munsell 100 hue test, the Farnsworth's panel D-15, the City university test, Kollner's rule, and the like; and visual acuity tests such as the ETDRS letters test, Snellen visual acuity test, visual field test, contrast sensitivity test, and the like, as a way of detecting the presence of the delivered polynucleotide. In some instances, both an improvement in viability and a modification in cone cell function may be detected.

In some embodiments, the subject method results in a therapeutic benefit, e.g. preventing the development of a disorder, halting the progression of a disorder, reversing the progression of a disorder, etc. In some embodiments, the subject method comprises the step of detecting that a therapeutic benefit has been achieved. The ordinarily skilled artisan will appreciate that such measures of therapeutic efficacy will be applicable to the particular disease being modified, and will recognize the appropriate detection methods to use to measure therapeutic efficacy. For example, therapeutic efficacy in treating macular degeneration may be observed as a reduction in the rate of macular degeneration or a cessation of the progression of macular degeneration, effects which may be observed by, e.g., fundus photography, OCT, or AO, by comparing test results after administration of the subject composition to test results before administration of the subject composition. As another example, therapeutic efficacy in treating a progressive cone dysfunction may be observed as a reduction in the rate of progression of cone dysfunction, as a cessation in the progression of cone dysfunction, or as an improvement in cone function, effects which may be observed by, e.g., ERG and/or cERG; color vision tests; functional adaptive optics; and/or visual acuity tests, for example, by comparing test results after administration of the subject composition to test results before administration of the subject composition and detecting a change in cone viability and/or function. As a third example, therapeutic efficacy in treating a color vision deficiency may be observed as an alteration in the individual's perception of color, e.g. in the perception of red wavelengths, in the perception of green wavelengths, in the perception of blue wavelengths, effects which may be observed by, e.g., cERG and color vision tests, for example, by comparing test results after administration of the subject composition to test results before administration of the subject composition and detecting a change in cone viability and/or function.

Expression of a transgene delivered by the subject rAAV is expected to be robust. Accordingly, in some instances, the expression of the transgene, e.g. as detected by measuring levels of gene product, by measuring therapeutic efficacy, etc, may be observed two months or less after administration, e.g. 4, 3 or 2 weeks or less after administration, for example, 1 week

after administration of the subject composition. Expression of the transgene is also expected to persist over time. Accordingly, in some instances, the expression of the transgene, e.g. as detected by measuring levels of gene product, by measuring therapeutic efficacy, etc., may be observed 2 months or more after administration of the subject composition, e.g., 4, 6, 8, or 10 months or more, in some instances 1 year or more, for example 2, 3, 4, or 5 years, in certain instances, more than 5 years.

In certain embodiments, the method comprises the step of detecting expression of the polynucleotide delivered by the subject rAAV in the cone cells, wherein expression is enhanced relative to expression from an AAV not comprising a 7-10 amino acid insert in the GH loop. i.e. a reference control, e.g. a parental rAAV into which the peptide has been inserted. Typically, expression will be enhanced 2-fold or more relative to the expression from a reference, e.g.a parental rAAV, for example 3-fold, 4-fold, or 5-fold or more, in some instances 10-fold, 20-fold or 50-fold or more, e.g. 100-fold, as evidenced by, e.g. earlier detection, higher levels of gene product, a stronger functional impact on the cells, etc.

Typically, an effective amount to achieve a change in will be about $1x10^8$ vector genomes or more, in some cases $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, or $1x10^{13}$ vector genomes or more, in certain instances, $1x10^{14}$ vector genomes or more, and usually no more than $1x10^{15}$ vector genomes. In some cases, the amount of vector genomes that is delivered is at most about 1x1015 vector genomes, e.g. $1x10^{14}$ vector genomes or less, for example $1x10^{13}$, $1x10^{12}$, $1x10^{11}$, $1x10^{10}$, or $1x10^9$ vector genomes or less, in certain instances $1x10^8$ vector genomes, and typically no less than $1x10^8$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^{10}$ to $1x10^{11}$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^{10}$ to $3x10^{12}$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^9$ to $3x10^{13}$ vector genomes. In some cases, the amount of vector genomes that is delivered is $1x10^8$ to $3x10^{14}$ vector genomes.

In some cases, the amount of pharmaceutical composition to be administered may be measured using multiplicity of infection (MOI). In some cases, MOI may refer to the ratio, or multiple of vector or viral genomes to the cells to which the nucleic may be delivered. In some cases, the MOI may be $1x10^6$. In some cases, the MOI may be $1x10^5$ -$1x10^7$. In some cases, the MOI may be $1x10^4$ -$1x10^8$. In some cases, recombinant viruses of the disclosure are at least about $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, $1x10^{13}$, $1x10^{14}$, $1x10^{15}$, $1x10^{16}$, $1x10^{17}$, and $1x10^{18}$ MOI. In some cases, recombinant viruses of this disclosure are $1x10^8$ to $3x10^{14}$ MOI. In some cases, recombinant viruses of the

disclosure are at most about $1x10^1$, $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$, $1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$, $1x10^{12}$, $1x10^{13}$, $1x10^{14}$, $1x10^{15}$, $1x10^{16}$, $1x10^{17}$, and $1x10^{18}$ MOI.

In some aspects, the amount of pharmaceutical composition comprises about 1×10^8 to about 1×10^{15} particles of recombinant viruses, about 1×10^9 to about 1×10^{14} particles of recombinant viruses, about 1×10^{10} to about 1×10^{13} particles of recombinant viruses, or about 1×10^{11} to about 3×10^{12} particles of recombinant viruses.

Individual doses are typically not less than an amount required to produce a measurable effect on the subject, and may be determined based on the pharmacokinetics and pharmacology for absorption, distribution, metabolism, and excretion ("ADME") of the subject composition or its by-products, and thus based on the disposition of the composition within the subject. This includes consideration of the route of administration as well as dosage amount, which can be adjusted for subretinal (applied directly to where action is desired for mainly a local effect), intravitreal (applied to the vitreaous for a pan-retinal effect), or parenteral (applied by systemic routes, e.g. intravenous, intramuscular, etc.) applications. Effective amounts of dose and/or dose regimen can readily be determined empirically from preclinical assays, from safety and escalation and dose range trials, individual clinician-patient relationships, as well as in vitro and in vivo assays such as those described herein and illustrated in the Experimental section, below.

All of the above U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, are incorporated herein by reference, in their entirety.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts,

temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

General methods in molecular and cellular biochemistry can be found in such standard textbooks as Molecular Cloning: A Laboratory Manual, 3rd Ed. (Sambrook et al., HaRBor Laboratory Press 2001); Short Protocols in Molecular Biology, 4th Ed. (Ausubel et al. eds., John Wiley & Sons 1999); Protein Methods (Bollag et al., John Wiley & Sons 1996); Nonviral Vectors for Gene Therapy (Wagner et al. eds., Academic Press 1999); Viral Vectors (Kaplift & Loewy eds., Academic Press 1995); Immunology Methods Manual (I. Lefkovits ed., Academic Press 1997); and Cell and Tissue Culture: Laboratory Procedures in Biotechnology (Doyle & Griffiths, John Wiley & Sons 1998), the disclosures of which are incorporated herein by reference. Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, Sigma-Aldrich, and ClonTech.

EXAMPLE 1

BACKGROUND

New therapies are needed for the treatment of many cone photoreceptor associated disorders, including macular dystrophies such as cone-rod dystrophy, cone dystrophy, Stargardt macular dystrophy, and achromatopsia; color vision disorders such as protan, deutan, and tritan defects; and vision disorders of the central macula such as age-related macular degeneration, macular telangiectasia, retinitis pigmentosa, diabetic retinopathy, retinal vein occlusions, glaucoma, Sorsby's fundus dystrophy, adult vitelliform macular dystrophy, Best's disease, and X-linked retinoschisis. As these vision disorders are associated with a loss of function and/or viability of the cone photoreceptors, it is hypothesized that these disorders may be treatable by delivering a therapeutic gene to cone photoreceptors to rescue cone viability and function.

To that end, the polynucleotide cassette "pMNTC" was designed in which enhancer, promoter, 5'UTR, intron, Kozak, and polyadenylation sequences were designed for cone-specific expression (Fig. 6a). The cassette included an LCR enhancer sequence from the L- and M-opsin genomic locus and a truncated promoter sequence from the M-Opsin gene, comprising about 140 nucleotides upstream of the transcriptional start site. In addition, the cassette included a 5' untranslated region (5' UTR) based on the M-opsin 5'UTR but modified to have minimal secondary structure and to include additional sequence at its 3' end into which an

intron was inserted. The intronic sequence used was a pSI chimeric intron having the 5´-donor site from the first intron of the human β-globin gene and the branch and 3´-acceptor site from the intron that lies between the leader and the body of an immunoglobulin gene heavy chain variable region (Bothwell, A.L. et al. (1981) Heavy chain variable region contribution to the NPb family of antibodies: Somatic mutation evident in a gamma 2a variable region. Cell 24, 625–37). The sequences of the donor and acceptor sites, along with the branchpoint site, were changed to match the consensus sequences for splicing (Senapathy, P., Shapiro, M.B. and Harris, N.L. (1990) Meth. Enzymol. 183, 252–78). Also included in the pMNTC polynucleotide cassette was a strong Kozak sequence and an SV40 polyadenylation sequence.

Experiments were also performed to identify the best AAV with which to deliver transgenes to cone cells. Successful delivery of polynucleotides to cells of the retina for the purposes of gene therapy has been achieved using viral vectors such as AAV and lentivirus. However, these viruses must be injected subretinally to reach the cells of the non-human primate (NHP) retina, a procedure that carries with it the risk of retinal damage. A less disruptive approach is administration by intravitreal injection. However, efficient transduction of cone photoreceptors following intravitreal delivery of AAV or lentivirus has never been demonstrated: while reports exist of AAVs with the ability to transduce retinal cone cells with high efficiency (Merigan et al. IOVS 2008,49 E-abstract 4514), later reports have questioned the efficacy of these vectors (Yin et al. IOVS 2011, 52(5):2775-2783).

RESULTS

Directed evolution of AAV2 has led to the identification of the viral variant “7m8” that is able to transduce photoreceptors better than wild type AAV2 (Dalkara et al. Sci Transl Med 2013). However, the retina contains two types of photoreceptors – rods and cones – and no reports exist demonstrated whether AAV2-7m8 can transduce cone photoreceptors, per se, and more particularly, cone photoreceptors in the highly cone-enriched area of the fovea. To test this possibility, we delivered AAV2-7m8 carrying an expression cassette of the ubiquitous promoter CMV operably linked to GFP to the retina of African Green monkey by intravitreal injection. Intravitreally delivered AAV2-7m8.CMV.GFP appeared to transduce retinal cells in the fovea centralis (the 0.35mm diameter rod-free region of retina at the center of the foveal pit) and parafovea (the lip of the depression) of primates more efficiently than intravitreally-delivered AAV2 or other AAV variants previously shown in the art to transduce retinal cells. Neither AAV2-7m8 nor the other AAVs tested tested appeared to be able to transduce the cones of the primate fovea, the 1.5mm-diameter cone-enriched region of retina that surrounds the foveola

and forms the slopes of the pit (Fig. 1).

We next packaged a genome comprising pMNTC operably linked to GFP within the AAV2-7m8 capsid, and assessed the ability of this vector composition to express the GFP transgene in cone cells in vivo when injected intravitreally. Expression was evaluated in a number of species with varying numbers of retinal cones cells among total photoreceptors, including mouse (3% cones), rat (1% cones), gerbil (13% cones), and nonhuman primate (5% cones). Contrary to our results in Fig. 1, strong gene expression could be detected throughout the nonhuman primate fovea (Fig. 2). These data indicate that intravitreally delivered AAV2-7m8 can, in fact, transduce retinal cones, and that pMNTC acts as a robust expression cassette in cone cells. Robust reporter gene expression was also seen in the intravireally injected retina of the rat (data not shown) and gerbil (Fig. 4A), with expression levels and anatomic location correlating with cone abundance and location in all species.

To determine the cell-specificity of pMNTC-directed expression, whole mounts of transduced mouse retina were analyzed by immunohistochemistry using an antibody that is specific for cone L and M opsins. The expression of L/M opsin, which labels the outer segments of cone photoreceptors only, was observed in virtually all of the cones of the mouse retina that expressed GFP from the AAV2-7m8.MNTC.GFP vector (Fig. 3), indicating that MNTC-directed expression of transgenes is highly cone-specific. Moreover 80% or more of the cone outer segments that were labelled by the L/M opsin-specific antibody also expressed the GFP transgene, indicating that AAV2-7m8 transduces cones highly efficiently (Fig. 3).

We also determined the cell-specificity of pR2.1-directed expression by packaging a genome comprising pR2.1 operably linked to GFP within the AAV2-7m8 capsid (AAV2-7m8pR2.1.GFP vector). pR2.1 comprises the human L/M opsin enhancer ("LCR") and the promoter region from the human L-Opsin gene. In addition, pR2.1 comprises the L-Opsin 5'UTR fused to additional 5'UTR sequence at its 3' end, into which modified SV40 late 16s intronic sequence has been inserted. This is followed by the L-Opsin Kozak sequence, which is then typically linked in-frame to a transgene. At the end of the cassette is an SV40 polyA tail. The ability of this vector composition to express the GFP transgene in cone cells in vivo was assessed 12 weeks after intravitreal injection in an African green monkey (non-human primate; NHP). Briefly, the NHP received bilateral intravitreal administrations of 50 uL of $1.0x10^{13}$ vg/mL AAV2-7m8pR2.1.GFP to yield a final dose of 5×10^{11} vg per eye. Retinal examination, including fundus color and fluorescence photography, was performed by using a Topcon TRC-50EX retinal camera with Canon 6D digital imaging hardware and a Spectralis OCT Plus at baseline

and at weeks 4, 8, and 12 post-intravitreal vector injection. The animal was terminated at 12 weeks and eyes processed. A cross-section of a treated retina from the NHP was stained with a chicken polyclonal anti-GFP antibody (Abcam Cat #13970; Cambridge, UK); a rabbit polyclonal anti-L/M Opsin antibody specific for opsin cones (Abcam Cat #5405); a 1D4 mouse monoclonal anti-rhodopsin antibody (Abcam Cat #5417); and Dapi to stain all nuclei (Invitrogen Ref # D21490). GFP-tagged transgene containing cells were imaged by multispectral analysis along with the antibody probes and DIC (differential interference contract for topology). As flattened stacks of optical planes through the entire section. Cell analysis for transgene was optimized using morphology and colocalization with probes. GFP (transgene) staining co-localized with L/M opsin staining and not with rhodopsin staining, indicating that pR2.1 promotes expression in cone cells specifically (Fig. 7). GFP transgene signal was observed at fovea, mid and far periphery; GFP transgene signal colocalized with L/M-opsin, calbindin and PNA probe; clear exclusion of 1D4-containing cells in fovea was observed; and there was no GFP transgene positive cell association with rods or other probe-containing cells. (Figure 7). In summary, cells double-stained for GFP (transgene expression) and L/M opsin were observed, but there was a lack of cells double-staining for both GFP and rhodopsin, indicating that the AAV2-7m8pR2.1.GFP vector specifically directed expression in cone cells and not rod cells.

We next compared the ability of pMNTC to promote expression in cone cells to that of pR2.1. Viral preparations of AAV2-7m8.MNTC.GFP and AAV2-7m8.pR2.1.GFP were delivered intravitreally to the retinas of gerbils and nonhuman primates in vivo, and the retinas imaged in vivo 2 weeks, 4 weeks, 8 weeks, and 12 weeks later by fundus autofluorescence and OCT. GFP reporter expression was detected sooner, more strongly, and in more cones in gerbil retina transduced with rAAV carrying the pMNTC.GFP expression cassette than in gerbil retinas carrying the pR2.1.GFP expression cassette (Fig. 4B). Likewise, GFP reporter expression was detected sooner and in more cones in nonhuman primate retinas transduced with rAAV carrying the pMNTC.GFP expression cassette as compared to NHP retinas transduced with the pR2.1 expression cassette (Fig. 5, n = 4 eyes). In both gerbils and NHP, GFP was consistently observed to be stronger from pMNTC than from pR2.1 throughout the duration of the study.

To determine the contribution of each of the elements in the pMNTC expression cassette to the overall improvement in expression, a series of expression constructs were cloned in which each of the elements in pMNTC was substituted one-by-one with the corresponding element from the pR2.1 expression cassette. These constructs were then packaged into AAV2-7m8 and delivered by intravitreal injection to the gerbil retina. Gerbil retinas were assessed 4

and 8 weeks later in vivo by in vivo bioluminescence (IVIS imaging system, PerkinElmer), which provides a quantitative readout of reporter expression across the entire eye.

As expected, expression of the luciferase reporter under the control of pMNTC was higher than expression of the luciferase reporter under the control of pR2.1 (Fig. 6). Replacement of the pMNTC promoter sequence with the pR2.1 promoter sequence having the most sequence homology to it reduced expression (construct pMNTC_pR2.1 L3'P), as did the inclusion of pR2.1 promoter sequence that lies more distal to the 5'UTR of pR2.1 (construct pMNTC_pR2.1-L5'P). Expression was also reduced by the introduction into the pMNTC 5'UTR of two false start sequences ("AUG1" and "AUG2") that were observed in the pR2.1 5'UTR (construct pMNTC_2.1-AUG1/2). Interestingly, expression was not reduced when the pMNTC 5'UTR was replaced with a modified pR2.1 5'UTR sequence in which these false starts had been removed (nucleotide 17 changed to C, nt 61and 62 changed to CA) (pMNTC_pR2.1-5'UTR), suggesting that the pR2.1 5'UTR would promote strong expression in cone cells but for the false AUGs in the pR2.1 5'UTR element. Also interestingly, the pR2.1 intron appeared to provide more robust expression than the pSI chimeric intron of pMNTC, suggesting that inclusion of the pR2.1 intron in the polynucleotide cassettes of the present disclosure may be used to further improve expression in cone cells. Lastly, removal of the L/M enhancer (found in both pR2.1 and pMNTC) reduced expression as well. While the polyA tailed seemed at first to also have a significant impact on expression, re-sequencing of the pMNTC construct comprising this pR2.1 element revealed that the polyA tail was not operably linked to the transgene, thereby explaining why only background levels of expression were observed from this construct. Thus, the L/M opsin LCR, the inclusion of the M opsin core promoter rather than the L opsin promoter, and the exclusion of false starts in the 5'UTR all contribute to the enhancement in gene expression achieved using the pMNTC promoter.

In conclusion, we have identified an AAV variant, the AAV variant comprising a 7m8 peptide in the GH loop, which may be used for the intravitreal delivery of polynucleotides to retinal cones. Likewise, we have identified a number of polynucleotide cassette elements that may be used to promote strong expression in cone photoreceptors. Together, these discoveries represent improvements that may facilitate the development of therapeutic agents for cone-associated disorders.

MATERIALS AND METHODS

Transgene expression in vitro in WERI-RB-1 cells. WERI-Rb-1 retinoblastoma cells expressing cone photoreceptor pigments cells are transfected with a polynucleotide cassette of

the present disclosure according to the method described by Shaaban and Deeb, 1998; IOVS 39(6)885-896. The polynucleotide cassettes are transfected as plasmid DNA using well established techniques of molecular biology, such as cloning (Maniatis et al.) or via de novo DNA synthesis. All regulatory elements are placed in the cassette and used to drive the enhanced GFP protein. Plasmid DNA is then introduced into cells using established techniques for non-viral transfection, for example using a lipid-based transfection reagent (Altogen Biosystems, NV) or Lipofectamine LTX (Life Technologies). Cells are then cultured for 72 hours and eGFP expression is measured using flow cytometry and fluorescence microscopy. Transgene expression in cells transfected with the polynucleotide cassette of the present invention (i.e., constructs designed for cone photoreceptor expression) is compared to the un-optimized counterparts (i.e., those based on pR2.1) and is found to be stronger from cassettes carrying improved elements

In vitro expression is also evaluated using other mammalian cell lines that express cone opsins, such as 661W cells (Tan et al., IOVS 2004; 45(3) 764-768).

Similarly, in vitro expression is evaluated using non-photoreceptor cell lines that have been engineered to express cone photoreceptor-specific proteins. Such a system has been described with HEK293 cells that have been genetically engineered to express CRX/Sp1 (Khani et al., IOVS 2007; 48: 3954). Marker genes are also used (eGFP, dsRed, mCherry, luciferase) as well as physiologic genes (opsin, ACHR genes). Physiologic genes are tested by examining mRNA levels (e.g., by RT-PCR) or protein levels (e.g., by ELISA or Western blot).

Animal care. All experiments conformed to the principles regarding the care and use of animals adopted by the American Physiological Society and the Society for Neuroscience, and were approved by the Institutional Animal Care and Use Committee (IACUC).

Small animal studies. The expression of the gene products encoded by the coding sequence of the expression cassettes was evaluated in vivo in mice, rats, and gerbils. This was accomplished by intravitreal injection in vivo of an rAAV preparation comprising the expression cassette (Li et al., 2008; Mol Vis 48: 332-338). Note that electroporation of plasmid DNA may be performed instead (Matsuda/Cepko).

Mouse studies. Mice used in this study were C57BL/6. Animals were anesthetized with ketamine/xylazine (110 mg/kg intraperitoneal). A beveled 34 gauge disposable needle loaded with test article was inserted into the vitreous of the eye, and 5.04 x 1010 vector genomes of rAAV in a volume of 1.5µl was injected into the vitreous.

Gerbil and rat studies. Mongolian gerbils (Meriones unguiculatus) and brown Norway

rats were used in this study. Pupils were dilated with 10% phenylephrine and 0.5% tropicamide. Animals were anesthetized with an intraperitoneal or intramuscular injection of 0.1-0.2 mL of a ketamine/xylazine solution (70 mg/mL ketamine and 10 mg/mL xylazine for rats; 25 mg/mL ketamine and 0.3 mg/mL xylazine for gerbils). A beveled 34 gauge disposable needle loaded with test article in a 100 µL Hamilton syringe was inserted into the vitreous of the eye through the sclera at an optimized superior-temporal point about 1 mm from Limbus. 1 x 1010 - 2 x 1010 vector genomes of test article (2 x 1010 vg of rAAV.GFP, or 1.15 x 1010 vg of rAAV.luciferase) in a 5 uL volume was injected slowly with a micro-injection pump into the vitreous, after which the needle tip was held in the injected eye at the injected position for 10 seconds so as to ensure adequate test article dispensing. The needle was then withdrawn.

Non-human primate (NHP) studies. The polynucleotide cassettes and expression vectors were also tested in large animals. This was done by using AAV, for example using the techniques of Mancuso et al. Briefly, an AAV cassette was made, the AAV encapsidating the expression cassette was manufactured, and the viral prep was injected intravitreally (up to 170 uL in the vitreous) or subretinally (up to 3, 100 uL injections at different locations; vitrectomy may be performed prior to injection) in nonhuman primates. Expression was evaluated by reporter (GFP), color ERG, and/or behavioral testing using the Cambridge Color Test or on animals trained to make a saccade (eye movement) when a target enters the field of view. The saccades are monitored using an eye tracker. Prior to treatment animals are trained to perform a color vision test or to make a saccade when it sees a colored target. An ERG is performed to estimate the spectral sensitivity of the cones present. Data from the color vision test performance and the ERG provide evidence that the animal is dichromatic (colorblind). For animals that receive a vector carrying the GFP gene, expression is monitored using fundus imaging with RetCam II or similar device under light that produces excitation of the GFP. For animals receiving a photopigment gene that differs in spectral sensitivity compared to the animal's endogenous pigments, expression is monitored using the multifocal color ERG to measure spectral sensitivity at up to 106 different retinal locations, and by behavioral testing.

Baboons were sedated with 10-15 mg/kg ketamine following by sevofluorane. African Green monkeys were sedated with an intramuscular injection of 5:1 ketamine:xylazine mix (0.2 ml/kg of 100 mg/ml ketamine and 20 mg/ml xylazine). Mydriasis was achieved with topical 10% phenylephrine. An eye speculum was placed in the eye to facilitate injections. A drop of proparacaine hydrochloride 0.5% and then 5% betadine solution was applied, followed by a rinse with sterile saline. Baboons (Fig. 2) received 60 µl of a 3.4×10^{13} vg preparation of rAAV

by intravitreal (ITV) injection to yield a final dose of 2.02 x 10^{12} vg per eye. African Green monkeys received 50 uL of a 1 x 10^{13} preparation of rAAV vector by ITV injection to yield a final dose of 5 x 10^{11} vg per eye. ITV injections to the central vitreous were administered using a 31-gauge 0.375 inch needle (Terumo) inserted inferotemporally at the level of the ora serrata ~2.5 mm poster to the limbus under a surgical magnification to allow full visualization of extraocular and intraocular needle placement. Central vitreous placement was confirmed by direct observation of the needle tip at the time of the injection. Following ITV injections a topical triple antibiotic ointment was administered.

Slit-lamp biomicroscopy. The anterior segment of each monkey eye was examined by slit-lamp biomicroscopy during baseline screening and at week 4 (day 28), week 8 (day 56) and week 12 (day 84) post-injection to monitor inflammation. No abnormalities were observed.

NHP Necropsy and Eye Processing. Animals were euthanized with pentobarbital 12 weeks post intravitreal injection. Eyes were tagged with a suture at the 12 o'clock position before enucleating and trimming of extraocular tissues. Posterior cups were isolated by removing tissues anterior to the limbus and fixed by immersion in 4% paraformaldehyde and stored in 70% ethanol.

Immunolabeling. Eyes were rehydrated into water then PBS buffer before flattening and delaminating retina as whole mounts. Preparations were imaged by stereo fluorescence microscopy (Discovery FI V20, Carl Zeiss Microscopy, LLC, Thornwood, NY) for GFP. Quadrant with fovea was detached from flat-mount, mounted under coverslip and imaged as full montage (5x tiling and stitching, Axio Observer Z1, Zeiss). Strip of retina was isolated central at fovea out to periphery, cryoprotected in sucrose and frozen in OCT. 8µm sections were immunostained with antibodies to proteins enriched in specific retinal cell populations, including, L/M- and S-opsins, glutamine synthetase (GS), calbindin, rhodopsin (1D4), β-III Tubulin, Laminin, peanut agglutinin (PNA) and/or others. GFP-tagged transgene containing cells were imaged by multispectral analysis along with antibody probes and DIC (differential interference contract for topology) as flattened stacks of optical plains through entire section (Axio Observer Z1, with Apotome, Zeiss). Cell analysis for transgene was optimized using morphology and colocalization with probes.

Fundus examination and photography. Eye examination and fundus photography of rat and gerbil retinas was performed using a Phoenix Micron IV fundus microscope. All animals received a baseline screening/photographing to confirm ocular health, and then photographed at the designated timepoints to monitor the expression of the GFP transgene. Any change to the

optic nerves and retina or appearance of gross lesions were recorded by a color fundus photography and expression of GFP was visualized using fluorescence fundus imaging with a fluorescein filter.

Retinal examination, fundus color and fluorescence photography, and autofluorescence OCT of NHP were performed by using a Topcon TRC-50EX retinal camera with Canon 6D digital imaging hardware and New Vision Fundus Image Analysis System software and Spectralis OCT Plus. All animals received a baseline imaging. GFP expression was also documented at week 2, 4, 8, and 12 post-intravitreal vector injection.

IVIS Imaging System. Expression of luciferase in the retina following delivery of rAAV.luciferase was quantified in vivo 2, 4 and 8 weeks post-intravitreal injection using an IVIS Imaging System. Gerbils were injected subcutaneously with 150mg/kg luciferin (PerkinElmer) (15mg/ml luciferin at a dose of 15ml/kg). Approximately 22 minutes later, animals were sedated by inhalation of 4% isoflurane for 3-5 minutes. Immediately thereafter, animals were placed on the imaging platform in pairs, and the luminescence of the one eye of each animal quantified followed immediately by imaging of the contralateral eye. A naïve gerbil was used as a negative standard, with background levels of luminescence typically registering a luminescence of 1×10^4 photons/second. Bioluminescence verification using a phantom mouse (XPM-2 Perkin Elmer phantom mouse for bioluminescence imaging) was performed prior to imaging to ensure calibration of the imaging system.

Immunohistochemistry. Mice were euthanized with a lethal dose of sodium pentobarbital and tissues fixed via cardiac perfusion first with 0.13M phosphate buffered saline (PBS) pH 7.2–7.4 containing 2 units of heparin per mL, followed by 4% paraformaldehyde (PFA) in PBS, followed by 4% paraformaldehyde plus 1% glutaraldehyde in PBS. Glutaraldehyde served to keep the neural retina attached to the RPE so that the cone outer segments would remain intact. Each solution was warmed to ~37°C just prior to administration and ~35–40mL of perfusate was delivered at each stage. Once the perfusion was stopped, the mouse was wrapped in a moist paper towel and left to further fix for 2–3 hours before enucleation and dissection.

Permanent ink was used to mark the orientation of the eye, the anterior segment was removed, and the eye-cup was fixed in 4% PFA overnight at 4°C and then stored in PBS at 4°C. Retinal whole-mounts were made by flattening the dissected retina between tissues soaked in 4% PFA for two hours and then transferring them to a culture plate for 6 more hours of fixation. Afterward, the PFA was replaced with PBS containing 0.03% sodium azide (Sigma).

Antibody labeling was carried out on a rotating table shaker. To block non-specific labeling, whole mounts were incubated overnight at 4°C with a solution containing 5% donkey serum (Jackson ImmunoResearch, Cat #004-000-120), 1mg/ml BSA (Jackson ImmunoResearch, Cat #001-000-161), and 0.03% Triton X-100 in PBS (pH 7.4). The primary antibody used in this study was rabbit anti red-green (L/M) opsin diluted 1:200 (Millipore, Cat # AB5405. Specimens were washed in PBS 3 times for 30 minutes each, then incubated at 4°C overnight with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride 1:10,000; Invitrogen, Cat # D-21490) plus secondary antibodies. The secondary antibody for the L/M-opsin antibody was Alexa Fluor 488 labeled donkey anti-rabbit IgG(H+L) diluted 1:200 in antibody dilution buffer (Invitrogen, Cat # A21206). The incubation with secondary antibody was followed by three 30 minute PBS washes, 30 minutes of post-fixation with 4% paraformaldehyde, and three more 30 minute PBS washes. Finally, the retinal slices were placed on slides with 2% DABCO in glycerol and covered with cover slips.

Microscopy. Widefield images of mouse retina whole mounts were acquired using a Nikon Eclipse E1000 with a 20x (open-air) objective and camera set with a 1.5x optical zoom. For each specimen, 50 optical sections were taken 0.5 µm apart and the M-opsin Z-stack was reconstructed in ImageJ. The Z-stack was oriented so that the lengths of the outer segments were in plane, and the distance between where antibody staining began and ended was measured as an estimate of the length of the outer segments. Further, a 3D projection of the Z-stack was generated and the number of cones with visible M-opsin in the outer segment could be quantified.

Confocal image slices were acquired using an Olympus FluoViewTM FV1000. Sections were imaged using a 20x oil immersion lens (40 images taken 0.5 µm apart) and the Z-stacks were reconstructed in ImageJ. Channel exposure levels were balanced within and across images using Adobe Photoshop. For the retinal whole mounts, images were taken using a 10x open-air lens and mosaics were constructed with Adobe Photoshop's native mosaic construction software.

Experiments testing the tissue specificity of the polynucleotide cassettes. In this instance, a construct encoding GFP is injected via one or more routes of administration, such as intravitreal, subretinal, or intravenously. The animal is then sacrificed and tissues are analyzed by qPCR – to detect DNA sequences indicating presence of the construct – and GFP expression – to detect areas where the construct is actively expressed. Whereas absence of DNA sequence indicates lack of biodistribution to a given tissue, the presence of DNA sequence

together with the lack of transgene expression (mRNA or protein level) indicates presence of vector but lack of expression in that tissue. In this way, the level of specificity for cone photoreceptors can be established, and used to determine the utility of this invention in terms of restricting expression to target cone photoreceptor cells without expression in non-targeted tissues such as optic nerve, liver, spleen, or brain tissue. Intravitreal AAV is known to biodistribute to the brain (Provost et al) so highly expressed, improved constructs for targeting cone photoreceptors would be useful to limit expression to target cells of the retina and limit potential adverse events associated with off-target transgene expression.

The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.

All publications and patent applications described herein are hereby incorporated by reference in their entireties.

CLAIMS

That which is claimed is:

1. Use of a recombinant adeno-associated virus serotype 2 (rAAV2) variant comprising a therapeutic polynucleotide in the manufacture of a medicament for treating or preventing a cone-associated retinal disorder in a primate subject having or at risk for developing the cone-associated retinal disorder, wherein:

I) said rAAV2 variant comprises a variant AAV2 capsid protein comprising the amino acid sequence LGETTRP (SEQ ID NO:11) inserted into the GH loop of the VP1 capsid protein; and

II) the therapeutic polynucleotide comprises a MNTC regulatory cassette operably linked to a polynucleotide encoding a therapeutic protein,

wherein the MNTC regulatory cassette comprises a human L/M opsin Locus Control Region ("LCR") enhancer and a truncated M opsin promoter comprising about 140 nucleotides upstream of the transcriptional start site;

and wherein the medicament is to be administered intravitreally in an amount effective to treat or prevent the cone-associated retinal disorder.

2. The use according to claim 1, wherein the variant AAV2 capsid protein comprises a VP1 protein having a sequence identity of at least 90% to the polypeptide of SEQ ID NO: 19, and wherein the variant AAV2 capsid protein comprises the amino acid sequence LGETTRP (SEQ ID NO:11) inserted into the GH loop of the VP1 capsid protein.

3. The use according to claim 2, wherein the variant AAV2 capsid protein comprises a VP1 protein having a sequence identity of at least 95% to the polypeptide of SEQ ID NO: 19, and wherein the variant AAV2 capsid protein comprises the amino acid sequence LGETTRP (SEQ ID NO:11) inserted into the GH loop of the VP1 capsid protein.

4. The use according to claim 2, wherein the variant AAV2 capsid protein comprises a VP1 protein of SEQ ID NO: 19.

5. The use according to claim 1, wherein the human L/M opsin LCR enhancer comprises: GACTTGATCTTCTGTTAGCCCTAATCATCAATTAGC.

6. The use according to claim 1, wherein the human L/M opsin LCR enhancer comprises:
CCTACAGCAGCCAGGGTGAGATTATGAGGCTGAGCTGAGAATATCAAGACTGTACCGAGT
AGGGGGCCTTGGCAAGTGTGGAGAGCCCGGCAGCTGGGGCAGAGGGCGGAGTACGGTG
TGCGTTTACGGACCTCTTCAAACGAGGTAGGAAGGTCAGAAGTCAAAAAGGGAACAAATGA
TGTTTAACCACACAAAAATGAAAATCCAATGGTTGGATATCCATTCCAAATACACAAAGGCA
ACGGATAAGTGATCCGGGCCAGGCACAGAAGGCCATGCACCCGTAGGATTGCACTCAGA
GCTCCCAAATGCATAGGAATAGAAGGGTGGGTGCAGGAGGCTGAGGGGTGGGGAAAGGG
CATGGGTGTTTCATGAGGACAGAGCTTCCGTTTCATGCAATGAAAAGAGTTTGGAGACGGA
TGGTGGTGACTGGACTATACACTTACACACGGTAGCGATGGTACACTTTGTATTATGTATAT
TTTACCACGATCTTTTTAAAGTGTCAAAGGCAAATGGCCAAATGGTTCCTTGTCCTATAGCT
GTAGCAGCCATCGGCTGTTAGTGACAAAGCCCCTGAGTCAAGATGACAGCAGCCCCCATA
ACTCCTAATCGGCTCTCCCGCGTGGAGTCATTTAGGAGTAGTCGCATTAGAGACAAGTCCA
ACATCTAATCTTCCACCCTGGCCAGGGCCCCAGCTGGCAGCGAGGGTGGGAGACTCCGG
GCAGAGCAGAGGGCGCTGACATTGGGGCCCGGCCTGGCTTGGGTCCCTCTGGCCTTTCC
CCAGGGGCCCTCTTTCCTTGGGGCTTTCTTGGGCCGCCACTGCTCCCGCTCCTCTCCCCC
CATCCCACCCCCTCACCCCCTCGTTCTTCATATCCTTCTCTAGTGCTCCCTCCACTTTCATC
CACCCTTCTGCAAGAGTGTGGGACCACAAATGAGTTTTCACCTGGCCTGGGGACACACGT
GCCCCCACAGGTGCTGAGTGACTTTCTAGGACAGTAATCTGCTTTAGGCTAAAATGGGACT
TGATCTTCTGTTAGCCCTAATCATCAATTAGCAGAGCCGGTGAAGGTGCAGAACCTACCGC
CTTTCCAGGCCTCCTCCCACCTCTGCCACCTCCACTCTCCTTCCTGGGATGTGGGGGCTG
GCACACGTGTGGCCCAGGGCATTGGTGGGATTGCACTGAGCTGGGTCATTAGCGTAATCC
TGGACAAGGGCAGACAGGGCGAGCGGAGGGCCAGCTCCGGGGCTCAGGCAAGGCTGGG
GGCTTCCCCCAGACACCCCACTCCTCCTCTGCTGGACCCCCACTTCATAGGGCACTTCGT
GTTCTCAAAGGGCTTCCAAATAGCATGGTGGCCTTGGATGCCCAGGGAAGCCTCAGAGTT
GCTTATCTCCCTCTAGACAGAAGGGGAATCTCGGTCAAGAGGGAGAGGTCGCCCTGTTCA
AGGCCACCCAGCCAGCTCATGGCGGTAATGGGACAAGGCTGGCCAGCCATCCCACCCTC
AGAAGGGACCCGGTGGGGCAGGTGATCTCAGAGGAGGCTCACTTCTGGGTCTCACATTCT
T.

7. The use according to claim 1, wherein the truncated M opsin promoter comprises:
CCAGCAAATCCCTCTGAGCCGCCCCCGGGGGCTCGCCTCAGGAGCAAGGAAGCAAGGGG
TGGGAGGAGGAGGTCTAAGTCCCAGGCCCAATTAAGAGATCAGATGGTGTAGGATTTGGG
AGCTTTTAAGGTGAAGAGGCCCGGGCTGAT.

8. The use according to claim 1, wherein the MNTC regulatory cassette comprises:
CCTACAGCAGCCAGGGTGAGATTATGAGGCTGAGCTGAGAATATCAAGACTGTACCGAGT
AGGGGGCCTTGGCAAGTGTGGAGAGCCCGGCAGCTGGGGCAGAGGGCGGAGTACGGTG
TGCGTTTACGGACCTCTTCAAACGAGGTAGGAAGGTCAGAAGTCAAAAAGGGAACAAATGA
TGTTTAACCACACAAAAATGAAAATCCAATGGTTGGATATCCATTCCAAATACACAAAGGCA
ACGGATAAGTGATCCGGGCCAGGCACAGAAGGCCATGCACCCGTAGGATTGCACTCAGA
GCTCCCAAATGCATAGGAATAGAAGGGTGGGTGCAGGAGGCTGAGGGGTGGGGAAAGGG
CATGGGTGTTTCATGAGGACAGAGCTTCCGTTTCATGCAATGAAAAGAGTTTGGAGACGGA
TGGTGGTGACTGGACTATACACTTACACACGGTAGCGATGGTACACTTTGTATTATGTATAT
TTTACCACGATCTTTTTAAAGTGTCAAAGGCAAATGGCCAAATGGTTCCTTGTCCTATAGCT
GTAGCAGCCATCGGCTGTTAGTGACAAAGCCCCTGAGTCAAGATGACAGCAGCCCCCATA
ACTCCTAATCGGCTCTCCCGCGTGGAGTCATTTAGGAGTAGTCGCATTAGAGACAAGTCCA
ACATCTAATCTTCCACCCTGGCCAGGGCCCCAGCTGGCAGCGAGGGTGGGAGACTCCGG
GCAGAGCAGAGGGCGCTGACATTGGGGCCCGGCCTGGCTTGGGTCCCTCTGGCCTTTCC
CCAGGGGCCCTCTTTCCTTGGGGCTTTCTTGGGCCGCCACTGCTCCCGCTCCTCTCCCCC
CATCCCACCCCCTCACCCCCTCGTTCTTCATATCCTTCTCTAGTGCTCCCTCCACTTTCATC
CACCCTTCTGCAAGAGTGTGGGACCACAAATGAGTTTTCACCTGGCCTGGGGACACACGT
GCCCCCACAGGTGCTGAGTGACTTTCTAGGACAGTAATCTGCTTTAGGCTAAAATGGGACT
TGATCTTCTGTTAGCCCTAATCATCAATTAGCAGAGCCGGTGAAGGTGCAGAACCTACCGC
CTTTCCAGGCCTCCTCCCACCTCTGCCACCTCCACTCTCCTTCCTGGGATGTGGGGGCTG
GCACACGTGTGGCCCAGGGCATTGGTGGGATTGCACTGAGCTGGGTCATTAGCGTAATCC
TGGACAAGGGCAGACAGGGCGAGCGGAGGGCCAGCTCCGGGGCTCAGGCAAGGCTGGG
GGCTTCCCCCAGACACCCCACTCCTCCTCTGCTGGACCCCCACTTCATAGGGCACTTCGT
GTTCTCAAAGGGCTTCCAAATAGCATGGTGGCCTTGGATGCCCAGGGAAGCCTCAGAGTT
GCTTATCTCCCTCTAGACAGAAGGGGAATCTCGGTCAAGAGGGAGAGGTCGCCCTGTTCA
AGGCCACCCAGCCAGCTCATGGCGGTAATGGGACAAGGCTGGCCAGCCATCCCACCCTC
AGAAGGGACCCGGTGGGGCAGGTGATCTCAGAGGAGGCTCACTTCTGGGTCTCACATTCT

TCCAGCAAATCCCTCTGAGCCGCCCCCGGGGGCTCGCCTCAGGAGCAAGGAAGCAAGGG
GTGGGAGGAGGAGGTCTAAGTCCCAGGCCCAATTAAGAGATCAGATGGTGTAGGATTTGG
GAGCTTTTAAGGTGAAGAGGCCCGGGCTGATCCCACTGGCCGGTATAAAGCACCGTGACC
CTCAGGTGACGCACCAGGGCCGGCTGCCGTCGGGGACAGGGCTTTCCATAGCCCAGGTA
AGTATCAAGGTTACAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCTTGTCGAGACAGA
GAAGACTCTTGCGTTTCTGATAGGCACCTATTGGTCTTACTGACATCCACTTTGCCTTTCTC
TCCACAGGCCCAGAGAGGAGACAGGCCGCCACC.

9. The use according to claim 1, wherein the cone-associated retinal disorder is selected from the group consisting of: rod-cone dystrophy; cone-rod dystrophy; progressive cone dystrophy; retinitis pigmentosa (RP); Stargardt Disease; macular telangiectasia, Leber hereditary optic neuropathy, Best's disease; adult vitelliform macular dystrophy; X-linked retinoschisis; a color vision disorder; age-related macular degeneration; wet age-related macular degeneration; geographic atrophy; diabetic retinopathy; a retinal vein occlusion; retinal ischemia; Familial Exudative Vitreoretinopathy (FEVR); COATs disease; and Sorsby's fundus dystrophy.

10. The use according to claim 1, wherein the cone-associated retinal disorder is a color vision disorder.

11. The use according to claim 10, wherein the color vision disorder is blue cone monochromacy.

12. The use according to claim 1, wherein the color vision disorder is color vision deficiency.