METHODS AND COMPOSITIONS FOR TREATING GENETIC EYE DISEASES

RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 62/118,934, filed February 20, 2015, the entirety of which is incorporated herein by reference.

INTRODUCTION

Gene transfer is now widely recognized as a powerful tool for analysis of biological events and disease processes at both the cellular and molecular levels. Recently, the application of gene therapy for the treatment of human diseases, either inherited (*e.g.,* adenosine deaminase (ADA) deficiency) or acquired (*e.g.,* cancer or infectious disease), has received considerable attention. With the advent of improved gene transfer techniques and the identification of an ever expanding library of defective gene-related diseases, gene therapy has rapidly evolved from a treatment theory to a practical reality.

Traditionally, gene therapy has been defined as a procedure in which an exogenous gene is introduced into the cells of a patient in order to correct an inborn genetic error. Although more than 4500 human diseases are currently classified as genetic, specific mutations in the human genome have been identified for relatively few of these diseases. Until recently, these rare genetic diseases represented the exclusive targets of gene therapy efforts. Accordingly, most of the NIH approved gene therapy protocols to date have been directed toward the introduction of a functional copy of a defective gene into the somatic cells of an individual having a known inborn genetic error. Only recently, have researchers and clinicians begun to appreciate that most human cancers, certain forms of cardiovascular disease, and many degenerative diseases also have important genetic components, and for the purposes of designing novel gene therapies, should be considered "genetic disorders." Therefore, gene therapy has more recently been broadly defined as the correction of a disease phenotype through the introduction of new genetic information into the affected organism.

In *in vivo* gene therapy, a transferred gene is introduced into cells of the recipient organism *in situ* that is, within the recipient. *In vivo* gene therapy has been examined in several animal models. Several recent publications have reported the feasibility of direct gene transfer *in situ* into organs and tissues such as muscle, hematopoietic stem cells, the arterial wall, the nervous system, and lung. Direct injection of DNA into skeletal muscle, heart muscle and injection of DNA-lipid complexes into the vasculature also has been reported to yield a detectable expression level of the inserted gene product(s) *in vivo.*

Treatment of genetic diseases of the eye, *e.g.,* inherited genetic diseases of the eye, such as diseases that cause blindness, remains a problem. Examples of such are retinitis pigmentosa, maculopathies, Leber's congenital amaurosis, Leber's hereditary optic neuropathy, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related macular degeneration, Spinocerebellar Ataxia Type 7 (SCAT), color blindness, and lysosomal storage diseases that affect the cornea, such as Mucopolysaccharidosis (MPS) IV and MPS VII. Thus, therapies for genetic diseases of the eye need to be developed.

SUMMARY

The present inventors have discovered peptides that function to target agents, such as viral vectors, to ocular cells. The present disclosure describes a method to utilize these novel peptides to direct, for example, viral capsids to the cell type of interest. In this instance, ocular cells (such as retinal cells) are targeted by the identified peptides. Vectors harboring capsid proteins modified to include such peptides can be used to provide therapeutic agents to the eye.

As used herein, the term "targets" means that the capsid protein of a virus, such as an adeno-associated virus (AAV), preferentially binds to one type of tissue (e.g., retinal cells) over another type of tissue (e.g., brain tissue) In certain embodiments, the genetically modified capsid protein may "target" ocular cells by binding at level of 10% to 1000% higher than a comparable, unmodified capsid protein. For example, an AAV having a genetically-modified capsid protein may bind to ocular cells at a level 50% to 100% greater than an unmodified AAV virus.

The present invention provides a modified adeno-associated virus (AAV) capsid protein containing a targeting peptide, wherein the targeting peptide is from 3 to 10 amino acids (i.e., 3, 4, 5, 6, 7, 8, 9, or 10 amino acids) in length and wherein the targeting peptide targets an AAV to an ocular cell. In certain embodiments, the targeting peptide is 3, 4, 5, 6, 7, 8, 9 or 10 amino acids in length.

The present invention provides an AAV virus containing the capsid protein modified genetically to encode the peptides described hereinabove.

The present invention provides a method of treating disease (e.g., an ocular disease) in a mammal by administering the viral vector described hereinabove or the cell described hereinabove to the mammal. In certain embodiments, the mammal is human. In certain embodiments, the disease is retinitis pigmentosa, maculopathies, Leber's congenital

amaurosis, Leber's hereditary optic neuropathy, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related macular degeneration, SCAT, color blindness, and lysosomal storage diseases that affect the cornea, such as MPS IV and MPS VII.

In certain embodiments, the disease is a lysosomal storage disease or disorder. In certain embodiments, the disease is a deficiency or defect in TPP1 (tripeptidyl peptidase I), CLN3 (Battenin), PPT1 (palmitoyl protein thioesterase I), CLN6 (neuronal ceroid lipofuscinosis protein 6) or CLN8 expression or activity. In certain embodiments, the disease is a neurodegenerative disease such as neuronal ceroid lipofuscinosis (NCL), such as infantile NCL, late infantile NCL, juvenile NCL (Batten disease) and adult NCL.

The present invention provides a method to deliver an agent to the eye of a subject, by transducing ocular cells with a viral vector described hereinabove so that the transduced ocular cells express the therapeutic agent and deliver the agent to the eye of the subject. In certain embodiments, the agent is Ataxin 7 mirRNA (for example to treat SCA 7). In certain embodiments, the agent is Retinal Pigment Epithelium-specific 65 kDa protein (RPE 65) (for example to treat Leber's congenital amaurosis). In certain embodiments, the agent is Vascular Endothelial Growth Factor (VEGF) inhibitor or soluble VEGF receptor 1 (sFifl) (for example to treat age related macular degeneration). In certain embodiments, the agent is REP1 (for example to treat Choroideremia). In certain embodiments, the agent is L-opsin (for example to treat color blindness). Table 1 sets forth exemplary diseases, target genes and corresponding encoded proteins.

Table 1

Condition to be Treated	**Target Gene for gene therapy**	**Protein Encoded**
Retinitis Pigmentosa	*Rho*	Rhodopsin
	PDE6ß	Phosphodiesterase 6ß
	ABCA4	ATP-binding cassette, sub-family A, member 4
	RPE65	Retinal pigment epithelium-specific 65 kDa protein
	LRAT	Lecithin Retinol Acyltransferase
	RDS/Peripherin	Retinal degeneration, slow/Peripherin
	MERTK	Tyrosine-protein kinase Mer
	IMPDHI	Inosine-5-prime-monophosphate dehydrogenase, type I
Maculopathies	GUCY2D	Guanylate Cyclase 2D
	RDS/Peripherin	Retinal degeneration, slow/Peripherin
	AIPL1	Aryl-hydrocarbon interacting protein-like 1
	ABCA4	ATP-binding cassette, sub-family A, member 4
	RPGRIP1	Retinitis pigmentosa GTPase regulator interacting protein 1

Condition to be Treated	Target Gene for gene therapy	Protein Encoded
Leber's congenital amaurosis and early onset severe retinal dystrophy	IMPDH1	Inosine-5-prime-monophosphate dehydrogenase, type I
	AIPL1	Aryl-hydrocarbon interacting protein-like 1
	GUCY2D	Guanylate Cyclase 2D
	LRAT	Lecithin Retinol Acyltransferase
	MERTK	Tyrosine-protein kinase Mer
	RPGRIP1	Retinitis pigmentosa GTPase regulator interacting protein 1
	RPE65	Retinal pigment epithelium-specific 65 kDa protein
Leber's hereditary optic neuropathy	Mitochondria Complex I genes, ND1, ND4, ND6, etc.	NADH Dehydrogenase, Subunit 1, NADH Dehydrogenase, Subunit 4, NADH Dehydrogenase, Subunit 6
Stargardt disease	ABCA4	ATP-binding cassette, sub-family A, member 4
Achromatopsia	GNAT2	Guanine nucleotide binding protein, alpha transducing activity polypeptide 2
	CNGB3	Cyclic nucleotide gated channel beta 3
X-linked retinoschisis	Rsl	Retinoschisin 1
Ocular albinism	OA1	Ocular albinism type 1
Oculocutaneous albinism	(OCA1) tyrosinase	Oculocutaneous albinism type 1 tyrosinase
Glaucoma	p21 WAF-1/0Cip 1	Cyclin-dependent kinase inhibitor interacting protein 1
Choroideremia	REP-1	Rab escort protein-1
Age related macular degeneration	PDGF	Platelet-derived growth factor
	Endostatin	
	Angiostatin	
	VEGF inhibitor	Vascular endothelial growth factor inhibitor
SCA 7	Ataxin 7 mirRNA	
Color blindness	Opsin	Opsin
Lysosomal storage disease IV	arylsulfatase B	Arylsulfatase B
Lysosomal storage disease VII	ß - glucuronidase	ß -Glucuronidase

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Sequence of peptide modified AAV (PMAAV)-GST. AAV2/2 (AAV2 ITR/AAV2 capsid) capsid sequence (Italicized) and glutathione S-transferase (GST) retina target peptide (Bold and underlined).

Figure 2. Fluorescent imaging in live animals showed relative infection efficiencies. Speckled regions of green fluorescent protein (GFP) positivity were observed in AAV2/2 injection eyes, whereas there were broad and uniform GFP signals in PMAAV-GST eyes.

Figure 3. PMAAV-GST.CMV.eGFP targeted the retinal pigment epithelium (RPE) and Outer Nuclear Layer (ONL) layers, and some cells in the ganglion cell layer (GCL). AAV2/2 targeted the OPL primarily. CMV - Cytomegalovirus; eGFP - enhanced GFP.

Figure 4. Subretinal injection.

Figure 5. Unmodified AAV2 - subretinal injection.

Figure 6. Unmodified AAV2 - subretinal injection.

Figure 7. 20X images PM modified AAV2 - subretinal injection.

Figure 8. Intravitreal injection.

Figure 9. AAV2 Intravitreal injection.

Figure 10. AAV2 Intravitreal injection.

Figure 11. AAV2 PM GST intravitreal injection.

Figure 12. AAV2 PM GST intravitreal injection.

DETAILED DESCRIPTION

Certain embodiments of the present disclosure provide a viral vector comprising a modified capsid, wherein the modified capsid comprises at least one amino acid sequence that targets the viral vector to ocular cells. In certain embodiments, the ocular cell is a retinal cell.

In certain embodiments, the viral vector is an adeno associated viral vector (AAV). In certain embodiments, the AAV is AAV2, although the tropism is modified so it would follow that such modifications would change the tropism of any AAV.

In certain embodiments, the targeting peptide comprises a sequence up to 10 amino acids in length having therein (or consisting of) of GSTPPPM (SEQ ID NO: 1) or amino acid sequence GETRAPL (SEQ ID NO: 4), as expressed in an amino to carboxy orientation or in a carboxy to amino orientation. It should be noted that the orientation of the sequence is not important. For example, the peptide may be oriented from the amino-terminal end to carboxy terminal end of the peptide to be GSTPPPM (SEQ ID NO:1) or may be from the amino-terminal end to carboxy-terminal end of the peptide to be MPPPTSG (SEQ ID NO:5).

In certain embodiments, the targeting peptide is inserted between the AAV2 capsid residues at positions P34-A35, T138-A139, A139-P140, G453-T454, N587-R588, and/or

R588-Q589. An exemplary wildtype reference AAV2 capsid protein sequence is provided in SEQ ID NO:10.

In certain embodiments, the targeting peptide is inserted after the AAV9 capsid residues at positions D384, G385, I560, T561, N562, E563, E564, E565, N704, and/or Y705. An exemplary wildtype reference AAV9 capsid protein sequence is provided in SEQ ID NO:11.

In certain embodiments, the capsid protein comprises or consists of SEQ ID NO:8 or SEQ ID NO:9.

The present invention provides a viral vector comprising a nucleic acid encoding the capsid protein as described hereinabove. In certain embodiments, the viral vector further contains a nucleic acid sequence encoding a nucleic acid of interest. In certain embodiments, the nucleic acid of interest is a therapeutic agent. In certain embodiments, the therapeutic agent is an enzyme or an RNAi molecule (*e.g.,* siRNA, shRNA or miRNA molecule). In certain embodiments, the therapeutic agent is Ataxin 7 mirRNA, RPE65, VEGF inhibitor or soluble VEGF receptor 1 (sFifl), REP1, L-opsin, Rho, PDE6ß, ABCA4, LRAT, RDS/Peripherin, MERTK, IMPDH1, GUCY2D, RDS/Peripherin, AIPL1, ABCA4, RPGRIP1, IMPDH1, AIPL1, GUCY2D, LRAT, MERTK, RPGRIP1, RPE65, ABCA4, GNAT2, CNGB3, Rsl, OA1, (OCAI) tyrosinase, P21 WAF-1/Cipl, PDGF, Endostatin, Angiostatin, arylsulfatase B, or ß - glucuronidase.

Certain embodiments of the present disclosure provide a nucleic acid sequence encoding a viral vector as described herein.

Certain embodiments of the present disclosure provide a nucleic acid sequence encoding a modified capsid as described herein. Certain embodiments of the present disclosure provide a nucleic acid sequence comprising or consisting of SEQ ID NO:2 (GGGTCGACGCCGCCTCCTATG). Certain embodiments of the present disclosure provide a modified capsid encoded by a nucleic acid sequence described herein.

Certain embodiments of the present disclosure provide a cell comprising a viral vector as described herein.

Certain embodiments of the present disclosure provide a cell transduced by a viral vector as described herein.

In certain embodiments, the cell is a mammalian cell. In certain embodiments, the cell is a human cell. In certain embodiments, the cell is a non-human cell. In certain

embodiments, the cell is in vitro. In certain embodiments, the cell is *in vivo*. In certain embodiments, the cell is an ocular cell, such as a retinal cell.

Certain embodiments of the present disclosure provide a method of treating a disease in a mammal comprising administering a viral vector or the cell as described herein to the mammal. In certain embodiments, the targeting peptide targets a diseased ocular cell. In certain embodiments, the targeting peptide targets an ocular cell in a subject that has retinitis pigmentosa, maculopathies, Leber's congenital amaurosis, Leber's hereditary optic neuropathy, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related macular degeneration, SCAT, color blindness, or lysosomal storage diseases that affect the cornea, such as MPS IV and MPS VII.

In certain embodiments, the subject has a lysosomal storage disease or disorder. In certain embodiments, the disease is a deficiency or defect in TPP1 (tripeptidyl peptidase I), CLN3 (Battenin), PPT1 (palmitoyl protein thioesterase I), CLN6 (neuronal ceroid lipofuscinosis protein 6) or CLN8 expression or activity. Diseases include neurodegenerative diseases such as neuronal ceroid lipofuscinosis (NCL), such as infantile NCL, late infantile NCL, juvenile NCL (Batten disease) and adult NCL.

In certain embodiments, the mammal is human.

Certain embodiments of the present disclosure provide a method to deliver an agent to the eye of a subject, comprising transducing ocular cells with a viral vector described herein so that the transduced ocular cells express the therapeutic agent and deliver the agent to the eye of the subject.

In certain embodiments, the viral vector transduces ocular cells.

In certain embodiments, the transduced ocular cells are within or comprise retina outer plexiform layer (OPL).

Certain embodiments of the present disclosure provide a viral vector or cell as described herein for use in medical treatment or diagnosis.

Certain embodiments of the present disclosure provide a use of a viral vector or cell as described herein to prepare a medicament useful for treating an ocular disease, *e.g.*, a genetic eye disease, in a mammal.

Certain embodiments of the present disclosure provide a method for identifying peptides that target ocular tissue comprising using phage display biopanning so as to identify such peptides.

The vector may further comprise an enzyme, a secreted protein, a nuclear protein, or a cytoplasmic protein. As used herein, the term "secreted protein" includes any secreted protein, whether naturally secreted or modified to contain a signal sequence so that it can be secreted. For example, the secreted protein could be beta-glucuronidase.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. Generally, "operably linked" means that the DNA sequences being linked are contiguous. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. Additionally, multiple copies of the nucleic acid encoding enzymes may be linked together in the expression vector. Such multiple nucleic acids may be separated by linkers.

The vector may be an adeno-associated virus (AAV) vector, an adenoviral vector, a retrovirus, or a lentivirus vector based on human immunodeficiency virus or feline immunodeficiency virus. Examples of such AAVs are found in Davidson et al., PNAS (2000) 97:3428-3432. The AAV and lentiviruses can confer lasting expression while the adenovirus can provide transient expression.

The present disclosure also provides a mammalian cell containing a vector described herein. The cell may be human, and may be from an eye. The cell type may be a stem or progenitor cell population.

The present disclosure provides a method of treating a disease such as a genetic eye disease or cancer in a mammal by administering a polynucleotide, polypeptide, expression vector, or cell described herein. The genetic eye disease or cancer may be retinitis pigmentosa, maculopathies, Leber's congenital amaurosis, Leber's hereditary optic neuropathy, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related macular degeneration, SCAT , color blindness, and lysosomal storage diseases that affect the cornea, such as MPS IV and MPS VII.

The genetic disease may be a lysosomal storage disease or disorder. In certain embodiments, the disease is a deficiency or defect in TPP1 (tripeptidyl peptidase I), CLN3 (Battenin), PPT1 (palmitoyl protein thioesterase I), CLN6 (neuronal ceroid lipofuscinosis protein 6) or CLN8 expression or activity. The genetic disease may be a neurodegenerative disease, for example, a neuronal ceroid lipofuscinosis (NCL), such as infantile NCL, late infantile NCL, juvenile NCL (Batten disease) and adult NCL.

Certain aspects of the disclosure relate to polynucleotides, polypeptides, vectors, and genetically engineered cells (modified *in vivo*), and the use of them. In particular, the disclosure relates to a method for gene or protein therapy that is capable of local delivery of a therapeutically effective dose of the therapeutic agent.

According to one aspect, a cell expression system for expressing a therapeutic agent in a mammalian recipient is provided. The expression system (also referred to herein as a "genetically modified cell") comprises a cell and an expression vector for expressing the therapeutic agent. Expression vectors include, but are not limited to, viruses, plasmids, and other vehicles for delivering heterologous genetic material to cells. Accordingly, the term "expression vector" as used herein refers to a vehicle for delivering heterologous genetic material to a cell. In particular, the expression vector is a recombinant adenoviral, adeno-associated virus, or lentivirus or retrovirus vector.

The expression vector further includes a promoter for controlling transcription of the heterologous gene. The promoter may be an inducible promoter (described below). The expression system is suitable for administration to the mammalian recipient. The expression system may comprise a plurality of non-immortalized genetically modified cells, each cell containing at least one recombinant gene encoding at least one therapeutic agent.

The cell expression system can be formed *in vivo*. According to yet another aspect, a method for treating a mammalian recipient *in vivo* is provided. The method includes introducing an expression vector for expressing a heterologous gene product into a cell of the patient *in situ*, such as via intraocular administration. To form the expression system *in vivo*, an expression vector for expressing the therapeutic agent is introduced *in vivo* into the mammalian recipient.

According to yet another aspect, a method for treating a mammalian recipient *in vivo* is provided. The method includes introducing the target protein into the patient *in vivo*.

The expression vector for expressing the heterologous gene may include an inducible promoter for controlling transcription of the heterologous gene product. Accordingly, delivery of the therapeutic agent *in situ* is controlled by exposing the cell *in situ* to conditions, which induce transcription of the heterologous gene.

The mammalian recipient may have a condition that is amenable to gene replacement therapy. As used herein, "gene replacement therapy" refers to administration to the recipient of exogenous genetic material encoding a therapeutic agent and subsequent expression of the administered genetic material *in situ*. Thus, the phrase "condition amenable to gene

replacement therapy" embraces conditions such as genetic diseases (*i.e.*, a disease condition that is attributable to one or more gene defects), acquired pathologies (*i.e.*, a pathological condition which is not attributable to an inborn defect), cancers and prophylactic processes (*i.e.*, prevention of a disease or of an undesired medical condition). Accordingly, as used herein, the term "therapeutic agent" refers to any agent or material, which has a beneficial effect on the mammalian recipient. Thus, "therapeutic agent" embraces both therapeutic and prophylactic molecules having nucleic acid or protein components.

According to one embodiment, the mammalian recipient has a genetic disease and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the disease. In yet another embodiment, the mammalian recipient has an acquired pathology and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the pathology. According to another embodiment, the patient has a cancer and the exogenous genetic material comprises a heterologous gene encoding an anti-neoplastic agent. In yet another embodiment the patient has an undesired medical condition and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the condition.

According to yet another embodiment, a pharmaceutical composition is disclosed. The pharmaceutical composition comprises a plurality of the above-described genetically modified cells or polypeptides and a pharmaceutically acceptable carrier. The pharmaceutical composition may be for treating a condition amenable to gene replacement therapy and the exogenous genetic material comprises a heterologous gene encoding a therapeutic agent for treating the condition. The pharmaceutical composition may contain an amount of genetically modified cells or polypeptides sufficient to deliver a therapeutically effective dose of the therapeutic agent to the patient. Exemplary conditions amenable to gene replacement therapy are described below.

According to another aspect, a method for forming the above-described pharmaceutical composition is provided. The method includes introducing an expression vector for expressing a heterologous gene product into a cell to form a genetically modified cell and placing the genetically modified cell in a pharmaceutically acceptable carrier.

These and other aspects, as well as various advantages and utilities will be more apparent with reference to the detailed description and to the accompanying Figures.

As used herein, the term "enzyme," a "secreted protein," a "nuclear protein," or a "cytoplasmic protein" include variants or biologically active or inactive fragments of these

polypeptides. A "variant" of one of the polypeptides is a polypeptide that is not completely identical to a native protein. Such variant protein can be obtained by altering the amino acid sequence by insertion, deletion or substitution of one or more amino acid. The amino acid sequence of the protein is modified, for example by substitution, to create a polypeptide having substantially the same or improved qualities as compared to the native polypeptide. The substitution may be a conserved substitution. A "conserved substitution" is a substitution of an amino acid with another amino acid having a similar side chain. A conserved substitution would be a substitution with an amino acid that makes the smallest change possible in the charge of the amino acid or size of the side chain of the amino acid (alternatively, in the size, charge or kind of chemical group within the side chain) such that the overall peptide retains its spacial conformation but has altered biological activity. For example, common conserved changes might be Asp to Glu, Asn or Gln; His to Lys, Arg or Phe; Asn to Gln, Asp or Glu and Ser to Cys, Thr or Gly. Aline is commonly used to substitute for other amino acids. The 20 essential amino acids can be grouped as follows: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan and methionine having nonpolar side chains; glycine, serine, threonine, cystine, tyrosine, asparagine and glutamine having uncharged polar side chains; aspartate and glutamate having acidic side chains; and lysine, arginine, and histidine having basic side chains.

The amino acid changes are achieved by changing the codons of the corresponding nucleic acid sequence. It is known that such polypeptides can be obtained based on substituting certain amino acids for other amino acids in the polypeptide structure in order to modify or improve biological activity. For example, through substitution of alternative amino acids, small conformational changes may be conferred upon a polypeptide that results in increased activity. Alternatively, amino acid substitutions in certain polypeptides may be used to provide residues, which may then be linked to other molecules to provide peptide-molecule conjugates which retain sufficient properties of the starting polypeptide to be useful for other purposes.

One can use the hydropathic index of amino acids in conferring interactive biological function on a polypeptide, wherein it is found that certain amino acids may be substituted for other amino acids having similar hydropathic indices and still retain a similar biological activity. Alternatively, substitution of like amino acids may be made on the basis of hydrophilicity, particularly where the biological function desired in the polypeptide to be generated is intended for use in immunological embodiments. The greatest local average

hydrophilicity of a "protein", as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity. Accordingly, it is noted that substitutions can be made based on the hydrophilicity assigned to each amino acid.

In using either the hydrophilicity index or hydropathic index, which assigns values to each amino acid, it is preferred to conduct substitutions of amino acids where these values are ±2, with ±1 being particularly preferred, and those with in ±0.5 being the most preferred substitutions.

The variant protein has at least 50%, at least about 80%, or even at least about 90% but less than 100%, contiguous amino acid sequence homology or identity to the amino acid sequence of a corresponding native protein.

The amino acid sequence of the variant polypeptide corresponds essentially to the native polypeptide's amino acid sequence. As used herein "correspond essentially to" refers to a polypeptide sequence that will elicit a biological response substantially the same as the response generated by the native protein. Such a response may be at least 60% of the level generated by the native protein, and may even be at least 80% of the level generated by native protein.

A variant may include amino acid residues not present in the corresponding native protein or deletions relative to the corresponding native protein. A variant may also be a truncated "fragment" as compared to the corresponding native protein, i.e., only a portion of a full-length protein. Protein variants also include peptides having at least one D-amino acid.

The variant protein may be expressed from an isolated DNA sequence encoding the variant protein. "Recombinant" is defined as a peptide or nucleic acid produced by the processes of genetic engineering. It should be noted that it is well-known in the art that, due to the redundancy in the genetic code, individual nucleotides can be readily exchanged in a codon, and still result in an identical amino acid sequence. The terms "protein," "peptide" and "polypeptide" are used interchangeably herein.

The present disclosure provides methods of treating a disease in a mammal by administering an expression vector to a cell or patient. For the gene therapy methods, a person having ordinary skill in the art of molecular biology and gene therapy would be able to determine, without undue experimentation, the appropriate dosages and routes of administration of the expression vector used in the novel methods of the present disclosure.

According to one embodiment, the cells are transformed or otherwise genetically modified *in vivo*. The cells from the mammalian recipient are transformed (i.e., transduced or

transfected) *in vivo* with a vector containing exogenous genetic material for expressing a heterologous (e.g., recombinant) gene encoding a therapeutic agent and the therapeutic agent is delivered *in situ*.

As used herein, "exogenous genetic material" refers to a nucleic acid or an oligonucleotide, either natural or synthetic, that is not naturally found in the cells; or if it is naturally found in the cells, it is not transcribed or expressed at biologically significant levels by the cells. Thus, "exogenous genetic material" includes, for example, a non-naturally occurring nucleic acid that can be transcribed into anti-sense RNA, as well as a "heterologous gene" (*i.e.*, a gene encoding a protein which is not expressed or is expressed at biologically insignificant levels in a naturally-occurring cell of the same type).

In the certain embodiments, the mammalian recipient has a condition that is amenable to gene replacement therapy. As used herein, "gene replacement therapy" refers to administration to the recipient of exogenous genetic material encoding a therapeutic agent and subsequent expression of the administered genetic material *in situ*. Thus, the phrase "condition amenable to gene replacement therapy" embraces conditions such as genetic diseases (i.e., a disease condition that is attributable to one or more gene defects), acquired pathologies (i.e., a pathological condition which is not attributable to an inborn defect), cancers and prophylactic processes (i.e., prevention of a disease or of an undesired medical condition). Accordingly, as used herein, the term "therapeutic agent" refers to any agent or material, which has a beneficial effect on the mammalian recipient. Thus, "therapeutic agent" embraces both therapeutic and prophylactic molecules having nucleic acid (e.g., antisense RNA) and/or protein components.

A number of genetic eye diseases are known. Therapeutic agents effective against these diseases are also known, since it is the protein/enzyme known to be deficient in these disorders. In certain embodiments, the disease or condition is retinitis pigmentosa, maculopathies, Leber's congenital amaurosis, Leber's hereditary optic neuropathy, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related macular degeneration, SCAT , color blindness, and lysosomal storage diseases that affect the cornea, such as MPS IV and MPS VII.

In certain embodiments, the disease or condition may be a lysosomal storage disease or disorder. In certain embodiments, the disease is a deficiency or defect in TPP1 (tripeptidyl peptidase I), CLN3 (Battenin), PPT1 (palmitoyl protein thioesterase I), CLN6 (neuronal

ceroid lipofuscinosis protein 6) or CLN8 expression or activity. The disease may be a neurodegenerative disease such as neuronal ceroid lipofuscinosis (NCL), such as infantile NCL, late infantile NCL, juvenile NCL (Batten disease) and adult NCL.

As used herein, "acquired pathology" refers to a disease or syndrome manifested by an abnormal physiological, biochemical, cellular, structural, or molecular biological state. Exemplary acquired pathologies are provided in Table 2. Therapeutic agents effective against these diseases are also given.

Table 2. Potential Gene Therapies for Acquired Pathologies. Corresponding Proteins Encoded by the Genes are set forth in Table 1.

Diseases	Target Gene for gene therapy
Retinitis Pigmentosa	Rho, PDE6ß, ABCA4, RPE65, LRAT, RDS/Peripherin, MERTK, IMPDH1
Maculopathies	GUCY2D, RDS/Peripherin, AIPL1, ABCA4, RPGRIP 1
Leber's congenital amaurosis and early onset severe retinal dystrophy	IMPDH1, AIPL1, GUCY2D, LRAT, MERTK, RPGRIP1, RPE65
Leber's hereditary optic neuropathy	Mitochondria Complex I genes, ND1, ND4, ND6, etc.
Stargardt disease,	ABCA4
Achromatopsia	GNAT2, CNGB3
X-linked retinoschisis	Rs1
Ocular albinism	OA1
Oculocutaneous albinism	(OCA1) tyrosinase
Glaucoma	P21 WAF-1 /Cip1
Choroideremia	REP-1
Age related macular degeneration	PDGF, Endostatin Angiostatin, VEGF inhibitor
SCA 7	Ataxin 7 mirRNA
Color blindness	Opsin
Lysosomal storage disease IV	arylsulfatase B
Lysosomal storage disease VII	ß -glucuronidase

Alternatively, the condition amenable to gene replacement therapy is a prophylactic process, *i.e.*, a process for preventing disease or an undesired medical condition. Thus, the instant disclosure embraces a cell expression system for delivering a therapeutic agent that has a prophylactic function (*i.e.*, a prophylactic agent) to the mammalian recipient.

In summary, the term "therapeutic agent" includes, but is not limited to, the agents listed in the Tables above, as well as their functional equivalents. As used herein, the term "functional equivalent" refers to a molecule (*e.g.*, a peptide or protein) that has the same or an

improved beneficial effect on the mammalian recipient as the therapeutic agent of which is it deemed a functional equivalent. As will be appreciated by one of ordinary skill in the art, a functionally equivalent proteins can be produced by recombinant techniques, *e.g.,* by expressing a "functionally equivalent DNA." As used herein, the term "functionally equivalent DNA" refers to a non-naturally occurring DNA, which encodes a therapeutic agent. For example, many, if not all, of the agents disclosed in Tables 1-2 have known amino acid sequences, which are encoded by naturally occurring nucleic acids. However, due to the degeneracy of the genetic code, more than one nucleic acid can encode the same therapeutic agent. Accordingly, the instant disclosure embraces therapeutic agents encoded by naturally-occurring DNAs, as well as by non-naturally-occurring DNAs, which encode the same protein as, encoded by the naturally-occurring DNA.

The above-disclosed therapeutic agents and conditions amenable to gene replacement therapy are merely illustrative and are not intended to limit the scope of the instant disclosure. The selection of a suitable therapeutic agent for treating a known condition is deemed to be within the scope of one of ordinary skill of the art without undue experimentation.

Screening Methods

The present disclosure provides methods to screen for and identify amino acid sequences that target, e.g., specifically target, a specific area, such as the vasculature of the central nervous system. This method can be used to identify targeting sequences that are specific for specific disease states. In other words, targeting sequences may be identified and used in the treatment of specific diseases.

AAV Vectors

Adeno associated virus (AAV) is a small (20 nm), nonpathogenic virus that is useful in treating human diseases, such as Parkinson's disease and recessive genetic diseases. A construct is generated that surrounds a promoter linked to a target gene with AAV inverted terminal repeat (ITR) sequences.

In one embodiment, a viral vector of the disclosure is an AAV vector. An "AAV" vector refers to an adeno-associated virus, and may be used to refer to the naturally occurring wild-type virus itself or derivatives thereof. The term covers all subtypes, serotypes and pseudotypes, and both naturally occurring and recombinant forms, except where required otherwise. As used herein, the term "serotype" refers to an AAV which is identified by and distinguished from other AAVs based on capsid protein reactivity with defined antisera, *e.g.,* there are many known serotypes of primate AAVs (*e.g.,* AAV1, AAV2, AAV3, AAV4,

AAV5, AAV6, AAV7, AAV8, AAV9, AAV 10, AAV-Rh74, and AAVRh10, and modified capsids of these serotypes). For example, serotype AAV-2 is used to refer to an AAV which contains capsid proteins encoded from the cap gene of AAV-2 and a genome containing 5' and 3' ITR sequences from the same AAV-2 serotype. Pseudotyped AAV refers to an AAV that contains capsid proteins from one serotype and a viral genome including 5'-3' ITRs of a second serotype. Pseudotyped rAAV would be expected to have cell surface binding properties of the capsid serotype and genetic properties consistent with the ITR serotype. Pseudotyped rAAV are produced using standard techniques described in the art. As used herein, for example, rAAV1 may be used to refer an AAV having both capsid proteins and 5'-3' ITRs from the same serotype or it may refer to an AAV having capsid proteins from serotype 1 and 5'-3' ITRs from a different AAV serotype, e.g., AAV serotype 2. For each example illustrated herein the description of the vector design and production describes the serotype of the capsid and 5'-3' ITR sequences. The abbreviation "rAAV" refers to recombinant adeno-associated virus, also referred to as a recombinant AAV vector (or "rAAV vector").

An "AAV virus" or "AAV viral particle" refers to a viral particle composed of at least one AAV capsid protein (preferably by all of the capsid proteins of a wild-type AAV) and an encapsidated polynucleotide. If the particle comprises heterologous polynucleotide (i.e., a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as "rAAV".

In one embodiment, the AAV expression vectors are constructed using known techniques to at least provide as operatively linked components in the direction of transcription, control elements including a transcriptional initiation region, the DNA of interest and a transcriptional termination region. The control elements are selected to be functional in a mammalian cell. The resulting construct which contains the operatively linked components is flanked (5' and 3') with functional AAV ITR sequences.

By "adeno-associated virus inverted terminal repeats" or "AAV ITRs" is meant the art recognized regions found at each end of the AAV genome which function together in cis as origins of DNA replication and as packaging signals for the virus. AAV ITRs, together with the AAV rep coding region, provide for the efficient excision and rescue from, and integration of a nucleotide sequence interposed between two flanking ITRs into a mammalian cell genome.

The nucleotide sequences of AAV ITR regions are known. See for example Kotin, R.M. (1994) Human Gene Therapy 5:793-801; Berns, K.I. "Parvoviridae and their Replication" in Fundamental Virology, 2nd Edition, (B.N. Fields and D.M. Knipe, eds.). As used herein, an "AAV ITR" need not have the wild-type nucleotide sequence depicted, but may be altered, *e.g.,* by the insertion, deletion or substitution of nucleotides. Additionally, the AAV ITR may be derived from any of several AAV serotypes, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV-Rh74, and AAVRh10, and modified capsids of these serotypes. Furthermore, 5' and 3' ITRs which flank a selected nucleotide sequence in an AAV vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, *i.e.*, to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the heterologous sequence into the recipient cell genome when AAV Rep gene products are present in the cell.

In one embodiment, AAV ITRs can be derived from any of several AAV serotypes, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV-Rh74 (Rhesus macaque-derived AAV), and AAVRh10. Furthermore, 5' and 3' ITRs which flank a selected nucleotide sequence in an AAV expression vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, *i.e.*, to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the DNA molecule into the recipient cell genome when AAV Rep gene products are present in the cell.

In one embodiment, AAV capsids can be derived from any of several AAV serotypes, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV-Rh74 (Rhesus macaque-derived AAV), and AAVRh10, and the AAV ITRs are derived from AAV serotype 2. In certain embodiments, the AAV capsid is AAV2 capsid. Suitable DNA molecules for use in AAV vectors will be less than about 5 kilobases (kb), less than about 4.5 kb, less than about 4 kb, less than about 3.5 kb, less than about 3 kb, less than about 2.5 kb in size and are known in the art.

In some embodiments of the disclosure the DNA molecules for use in the AAV vectors will contain one or more copies of a single siRNA sequence. As used herein the term multiple copies of an siRNA sequences means at least 2 copies, at least 3 copies, at least 4 copies, at least 5 copies, at least 6 copies, at least 7 copies, at least 8 copies, at least 9 copies, and at least 10 copies. In some embodiments the DNA molecules for use in the AAV vectors

will contain multiple siRNA sequences. As used herein the term multiple siRNA sequences means at least 2 siRNA sequences, at least 3 siRNA sequences, at least 4 siRNA sequences, at least 5 siRNA sequences, at least 6 siRNA sequences, at least 7 siRNA sequences, at least 8 siRNA sequences, at least 9 siRNA sequences, and at least 10 siRNA sequences. In some embodiments suitable DNA vectors of the disclosure will contain a sequence encoding the siRNA molecule of the disclosure and a stuffer fragment. Suitable stuffer fragments of the disclosure include sequences known in the art including without limitation sequences which do not encode an expressed protein molecule; sequences which encode a normal cellular protein which would not have deleterious effect on the cell types in which it was expressed; and sequences which would not themselves encode a functional siRNA duplex molecule.

In one embodiment, suitable DNA molecules for use in AAV vectors will be less than about 5 kilobases (kb) in size and will include, for example, a stuffer sequence and a sequence encoding a siRNA molecule of the disclosure. For example, in order to prevent any packaging of AAV genomic sequences containing the rep and cap genes, a plasmid containing the rep and cap DNA fragment may be modified by the inclusion of a stuffer fragment as is known in the art into the AAV genome which causes the DNA to exceed the length for optimal packaging. Thus, the helper fragment is not packaged into AAV virions. This is a safety feature, ensuring that only a recombinant AAV vector genome that does not exceed optimal packaging size is packaged into virions. An AAV helper fragment that incorporates a stuffer sequence can exceed the wild-type genome length of 4.6 kb, and lengths above 105% of the wild-type will generally not be packaged. The stuffer fragment can be derived from, for example, such non-viral sources as the Lac-Z or beta-galactosidase gene.

In one embodiment, the selected nucleotide sequence is operably linked to control elements that direct the transcription or expression thereof in the subject *in vivo*. Such control elements can comprise control sequences normally associated with the selected gene. Alternatively, heterologous control sequences can be employed. Useful heterologous control sequences generally include those derived from sequences encoding mammalian or viral genes. Examples include, but are not limited to, the Simian virus 40 (SV40) early promoter, mouse mammary tumor virus long terminal repeat (LTR) promoter; adenovirus major late promoter (Ad MLP); a herpes simplex virus (HSV) promoter, a cytomegalovirus (CMV) promoter such as the CMV immediate early promoter region (CMVIE), a rous sarcoma virus (RSV) promoter, pol II promoters, pol III promoters, synthetic promoters, hybrid promoters,

and the like. In addition, sequences derived from nonviral genes, such as the murine metallothionein gene, will also find use herein. Such promoter sequences are commercially available from, *e.g.,* Stratagene (San Diego, Calif.).

In one embodiment, both heterologous promoters and other control elements, such as retina-specific and inducible promoters, enhancers and the like, will be of particular use. Examples of heterologous promoters include the CMV promoter. Examples of retina-specific promoters include IRBP (interphotoreceptor retinoid-binding protein) promoter, hGRK1 (human rhodopsin kinase) promoter, IRBPe/GNAT2 (an enhancer element of inter-photoreceptor retinoid-binding protein promoter) and a minimal sequence of the human transducin alpha-subunit promoter are examples of retina-specific promoter.

In one embodiment, the AAV expression vector which harbors the DNA molecule of interest bounded by AAV ITRs, can be constructed by directly inserting the selected sequence(s) into an AAV genome which has had the major AAV open reading frames ("ORFs") excised therefrom. Other portions of the AAV genome can also be deleted, so long as a sufficient portion of the ITRs remain to allow for replication and packaging functions. Such constructs can be designed using techniques well known in the art.

Alternatively, AAV ITRs can be excised from the viral genome or from an AAV vector containing the same and fused 5' and 3' of a selected nucleic acid construct that is present in another vector using standard ligation techniques. For example, ligations can be accomplished in 20 mM Tris-Cl pH 7.5, 10 mM MgC12, 10 mM DTT, 33 gg/ml BSA, 10 mM-50 mM NaCl, and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 30-100 μg/ml total DNA concentrations (5-100 nM total end concentration). AAV vectors which contain ITRs. In particular, several AAV vectors are described therein which are available from the American Type Culture Collection ("ATCC") under Accession Numbers 53222, 53223, 53224, 53225 and 53226.

Additionally, chimeric genes can be produced synthetically to include AAV ITR sequences arranged 5' and 3' of one or more selected nucleic acid sequences. The complete chimeric sequence is assembled from overlapping oligonucleotides prepared by standard methods.

In order to produce rAAV virions, an AAV expression vector is introduced into a suitable host cell using known techniques, such as by transfection. A number of transfection

techniques are generally known in the art. *See, e.g.,* Sambrook *et al.* (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York. Particularly suitable transfection methods include calcium phosphate co-precipitation, direct micro-injection into cultured cells, electroporation, liposome mediated gene transfer, lipid-mediated transduction, and nucleic acid delivery using high-velocity microprojectiles.

In one embodiment, suitable host cells for producing rAAV virions include microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of a heterologous DNA molecule. The term includes the progeny of the original cell which has been transfected. Thus, a "host cell" as used herein generally refers to a cell which has been transfected with an exogenous DNA sequence. Cells from the stable human cell line, 293 (readily available through, *e.g.,* the American Type Culture Collection under Accession Number ATCC CRL1573) can be used in the practice of the present disclosure. Particularly, the human cell line 293 is a human embryonic kidney cell line that has been transformed with adenovirus type-5 DNA fragments, and expresses the adenoviral Ela and Elb genes. The 293 cell line is readily transfected, and provides a particularly convenient platform in which to produce rAAV virions.

In one embodiment, host cells containing the above-described AAV expression vectors are rendered capable of providing AAV helper functions in order to replicate and encapsidate the nucleotide sequences flanked by the AAV ITRs to produce rAAV virions. AAV helper functions are generally AAV-derived coding sequences which can be expressed to provide AAV gene products that, in turn, function in trans for productive AAV replication. AAV helper functions are used herein to complement necessary AAV functions that are missing from the AAV expression vectors. Thus, AAV helper functions include one, or both of the major AAV ORFs (open reading frames), namely the rep and cap coding regions, or functional homologues thereof.

The Rep expression products have been shown to possess many functions, including, among others: recognition, binding and nicking of the AAV origin of DNA replication; DNA helicase activity; and modulation of transcription from AAV (or other heterologous) promoters. The Cap expression products supply necessary packaging functions. AAV helper functions are used herein to complement AAV functions in trans that are missing from AAV vectors.

In one embodiment, AAV helper functions are introduced into the host cell by transfecting the host cell with an AAV helper construct either prior to, or concurrently with,

the transfection of the AAV expression vector. The term "AAV helper construct" refers generally to a nucleic acid molecule that includes nucleotide sequences providing AAV functions deleted from an AAV vector which is to be used to produce a transducing vector for delivery of a nucleotide sequence of interest. AAV helper constructs are commonly used to provide transient expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for lytic AAV replication; however, helper constructs lack AAV ITRs and can neither replicate nor package themselves. AAV helper constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45 which encode both Rep and Cap expression products. A number of other vectors have been described which encode Rep and/or Cap expression products.

By "AAV rep coding region" is meant the art-recognized region of the AAV genome which encodes the replication proteins Rep 78, Rep 68, Rep 52 and Rep 40. These Rep expression products have been shown to possess many functions, including recognition, binding and nicking of the AAV origin of DNA replication, DNA helicase activity and modulation of transcription from AAV (or other heterologous) promoters. The Rep expression products are collectively required for replicating the AAV genome. Suitable homologues of the AAV rep coding region include the human herpesvirus 6 (HHV-6) rep gene which is also known to mediate AAV-2 DNA replication.

By "AAV cap coding region" is meant the art-recognized region of the AAV genome which encodes the capsid proteins VP1, VP2, and VP3, or functional homologues thereof. These Cap expression products supply the packaging functions which are collectively required for packaging the viral genome. In certain embodiments, modifications, such as insertions are made in AAV2 capsid proteins at P34-A35, T138-A139, A139-P140, G453-T454, N587-R588, and/or R588-Q589. In certain embodiments, insertions are made at D384, G385, I560, T561, N562, E563, E564, E565, N704, and/or Y705 of AAV9.

In one embodiment, both AAV expression vectors and AAV helper constructs can be constructed to contain one or more optional selectable markers. Suitable markers include genes which confer antibiotic resistance or sensitivity to, impart color to, or change the antigenic characteristics of those cells which have been transfected with a nucleic acid construct containing the selectable marker when the cells are grown in an appropriate selective medium. Several selectable marker genes that are useful in the practice of the disclosure include the hygromycin B resistance gene (encoding Aminoglycoside

phosphotranferase (APH)) that allows selection in mammalian cells by conferring resistance to G418 (available from Sigma, St. Louis, Mo.). Other suitable markers are known to those of skill in the art.

In one embodiment, the host cell (or packaging cell) is rendered capable of providing non AAV derived functions, or "accessory functions," in order to produce rAAV virions. Accessory functions are non AAV derived viral and/or cellular functions upon which AAV is dependent for its replication. Thus, accessory functions include at least those non AAV proteins and RNAs that are required in AAV replication, including those involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of Cap expression products and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses.

In one embodiment, accessory functions can be introduced into and then expressed in host cells using methods known to those of skill in the art. Commonly, accessory functions are provided by infection of the host cells with an unrelated helper virus. A number of suitable helper viruses are known, including adenoviruses; herpesviruses such as herpes simplex virus types 1 and 2; and vaccinia viruses. Nonviral accessory functions will also find use herein, such as those provided by cell synchronization using any of various known agents.

In one embodiment, accessory functions are provided using an accessory function vector. Accessory function vectors include nucleotide sequences that provide one or more accessory functions. An accessory function vector is capable of being introduced into a suitable host cell in order to support efficient AAV virion production in the host cell. Accessory function vectors can be in the form of a plasmid, phage, transposon or cosmid. Accessory vectors can also be in the form of one or more linearized DNA or RNA fragments which, when associated with the appropriate control elements and enzymes, can be transcribed or expressed in a host cell to provide accessory functions.

In one embodiment, nucleic acid sequences providing the accessory functions can be obtained from natural sources, such as from the genome of an adenovirus particle, or constructed using recombinant or synthetic methods known in the art. In this regard, adenovirus-derived accessory functions have been widely studied, and a number of adenovirus genes involved in accessory functions have been identified and partially characterized. Specifically, early adenoviral gene regions E1a, E2a, E4, VAI RNA and, possibly, E1b are thought to participate in the accessory process. Herpesvirus-derived

accessory functions have been described. Vaccinia virus-derived accessory functions have also been described.

In one embodiment, as a consequence of the infection of the host cell with a helper virus, or transfection of the host cell with an accessory function vector, accessory functions are expressed which transactivate the AAV helper construct to produce AAV Rep and/or Cap proteins. The Rep expression products excise the recombinant DNA (including the DNA of interest) from the AAV expression vector. The Rep proteins also serve to duplicate the AAV genome. The expressed Cap proteins assemble into capsids, and the recombinant AAV genome is packaged into the capsids. Thus, productive AAV replication ensues, and the DNA is packaged into rAAV virions.

In one embodiment, following recombinant AAV replication, rAAV virions can be purified from the host cell using a variety of conventional purification methods, such as CsCl gradients. Further, if infection is employed to express the accessory functions, residual helper virus can be inactivated, using known methods. For example, adenovirus can be inactivated by heating to temperatures of approximately 60°C for, *e.g.*, 20 minutes or more. This treatment effectively inactivates only the helper virus since AAV is extremely heat stable while the helper adenovirus is heat labile. The resulting rAAV virions are then ready for use for DNA delivery to the eye of the subject.

Methods of delivery of viral vectors include, but are not limited to, intra-ocular, sub retinal, and intra-vitreal routes. Generally, rAAV virions may be introduced into cells of the eye using either *in vivo* or *in vitro* transduction techniques. If transduced *in vitro*, the desired recipient cell will be removed from the subject, transduced with rAAV virions and reintroduced into the subject. Alternatively, syngeneic or xenogeneic cells can be used where those cells will not generate an inappropriate immune response in the subject.

Suitable methods for the delivery and introduction of transduced cells into a subject have been described. For example, cells can be transduced *in vitro* by combining recombinant AAV virions with ocular cells *e.g.*, in appropriate media, and screening for those cells harboring the DNA of interest can be screened using conventional techniques such as Southern blots and/or PCR, or by using selectable markers. Transduced cells can then be formulated into pharmaceutical compositions, described more fully below, and the composition introduced into the subject by various techniques, such as by grafting, intramuscular, intravenous, subcutaneous and intraperitoneal injection.

In one embodiment, for *in vivo* delivery, the rAAV virions are formulated into pharmaceutical compositions and will generally be administered intra-ocularly, *e.g.*, by injection sub-retinally.

In one embodiment, pharmaceutical compositions will comprise sufficient genetic material to produce a therapeutically effective amount of the nucleic acid of interest, *i.e.*, an amount sufficient to reduce or ameliorate symptoms of the disease state in question or an amount sufficient to confer the desired benefit. The pharmaceutical compositions will also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, Tween80, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

As is apparent to those skilled in the art in view of the teachings of this specification, an effective amount of viral vector which must be added can be empirically determined. Administration can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosages of administration are well known to those of skill in the art and will vary with the viral vector, the composition of the therapy, the target cells, and the subject being treated. Single and multiple administrations can be carried out with the dose level and pattern being selected by the treating physician.

It should be understood that more than one transgene could be expressed by the delivered viral vector. Alternatively, separate vectors, each expressing one or more different transgenes, can also be delivered to the eye as described herein. Furthermore, it is also intended that the viral vectors delivered by the methods of the present disclosure be combined with other suitable compositions and therapies.

Methods for Introducing Genetic Material into Cells

The exogenous genetic material (*e.g.*, a cDNA encoding one or more therapeutic proteins) is introduced into the cell *ex vivo* or *in vivo* by genetic transfer methods, such as transfection or transduction, to provide a genetically modified cell. Various expression vectors (*i.e.*, vehicles for facilitating delivery of exogenous genetic material into a target cell) are known to one of ordinary skill in the art.

As used herein, "transfection of cells" refers to the acquisition by a cell of new genetic material by incorporation of added DNA. Thus, transfection refers to the insertion of nucleic acid into a cell using physical or chemical methods. Several transfection techniques are known to those of ordinary skill in the art including: calcium phosphate DNA co-precipitation; DEAE-dextran; electroporation; cationic liposome-mediated transfection; and tungsten particle-facilitated microparticle bombardment. Strontium phosphate DNA co-precipitation is another possible transfection method.

In contrast, "transduction of cells" refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus. A RNA virus (*i.e.*, a retrovirus) for transferring a nucleic acid into a cell is referred to herein as a transducing chimeric retrovirus. Exogenous genetic material contained within the retrovirus is incorporated into the genome of the transduced cell. A cell that has been transduced with a chimeric DNA virus (*e.g.*, an adenovirus carrying a cDNA encoding a therapeutic agent), will not have the exogenous genetic material incorporated into its genome but will be capable of expressing the exogenous genetic material that is retained extrachromosomally within the cell.

Typically, the exogenous genetic material includes the heterologous gene (usually in the form of a cDNA comprising the exons coding for the therapeutic protein) together with a promoter to control transcription of the new gene. The promoter characteristically has a specific nucleotide sequence necessary to initiate transcription. Optionally, the exogenous genetic material further includes additional sequences (*i.e.*, enhancers) required to obtain the desired gene transcription activity. For the purpose of this discussion an "enhancer" is simply any non-translated DNA sequence which works contiguous with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The exogenous genetic material may introduced into the cell genome immediately downstream from the promoter so that the promoter and coding sequence are operatively linked so as to permit transcription of the coding sequence. A retroviral expression vector may include an exogenous promoter element to control transcription of the inserted exogenous gene. Such exogenous promoters include both constitutive and inducible promoters.

Naturally-occurring constitutive promoters control the expression of essential cell functions. As a result, a gene under the control of a constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes which encode certain constitutive or "housekeeping" functions: hypoxanthine phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR), adenosine deaminase, phosphoglycerol kinase (PGK), pyruvate kinase, phosphoglycerol mutase, the actin promoter, and other constitutive promoters known to those of skill in the art. In addition, many viral promoters function constitutively in eucaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRs) of Moloney Leukemia Virus and other retroviruses; and the thymidine kinase promoter of Herpes Simplex Virus, among many others. Accordingly, any of the above-referenced constitutive promoters can be used to control transcription of a heterologous gene insert.

Genes that are under the control of inducible promoters are expressed only or to a greater degree, in the presence of an inducing agent, (*e.g.*, transcription under control of the metallothionein promoter is greatly increased in presence of certain metal ions). Inducible promoters include responsive elements (REs) which stimulate transcription when their inducing factors are bound. For example, there are REs for serum factors, steroid hormones, retinoic acid and cyclic AMP. Promoters containing a particular RE can be chosen in order to obtain an inducible response and in some cases, the RE itself may be attached to a different promoter, thereby conferring inducibility to the recombinant gene. Thus, by selecting the appropriate promoter (constitutive versus inducible; strong versus weak), it is possible to control both the existence and level of expression of a therapeutic agent in the genetically modified cell. If the gene encoding the therapeutic agent is under the control of an inducible promoter, delivery of the therapeutic agent *in situ* is triggered by exposing the genetically modified cell *in situ* to conditions for permitting transcription of the therapeutic agent, *e.g.*, by intraperitoneal injection of specific inducers of the inducible promoters which control transcription of the agent. For example, *in situ* expression by genetically modified cells of a therapeutic agent encoded by a gene under the control of the metallothionein promoter, is enhanced by contacting the genetically modified cells with a solution containing the appropriate (*i.e.*, inducing) metal ions *in situ*.

Accordingly, the amount of therapeutic agent that is delivered *in situ* is regulated by controlling such factors as: (1) the nature of the promoter used to direct transcription of the inserted gene, (*i.e.*, whether the promoter is constitutive or inducible, strong or weak); (2) the

number of copies of the exogenous gene that are inserted into the cell; (3) the number of transduced/transfected cells that are administered (*e.g.*, implanted) to the patient; (4) the size of the implant (*e.g.*, graft or encapsulated expression system); (5) the number of implants; (6) the length of time the transduced/transfected cells or implants are left in place; and (7) the production rate of the therapeutic agent by the genetically modified cell. Selection and optimization of these factors for delivery of a therapeutically effective dose of a particular therapeutic agent is deemed to be within the scope of one of ordinary skill in the art without undue experimentation, taking into account the above-disclosed factors and the clinical profile of the patient.

In addition to at least one promoter and at least one heterologous nucleic acid encoding the therapeutic agent, the expression vector may include a selection gene, for example, a neomycin resistance gene, for facilitating selection of cells that have been transfected or transduced with the expression vector. Alternatively, the cells are transfected with two or more expression vectors, at least one vector containing the gene(s) encoding the therapeutic agent(s), the other vector containing a selection gene. The selection of a suitable promoter, enhancer, selection gene and/or signal sequence (described below) is deemed to be within the scope of one of ordinary skill in the art without undue experimentation.

The therapeutic agent can be targeted for delivery to an extracellular, intracellular or membrane location. If it is desirable for the gene product to be secreted from the cells, the expression vector is designed to include an appropriate secretion "signal" sequence for secreting the therapeutic gene product from the cell to the extracellular milieu. If it is desirable for the gene product to be retained within the cell, this secretion signal sequence is omitted. In a similar manner, the expression vector can be constructed to include "retention" signal sequences for anchoring the therapeutic agent within the cell plasma membrane. For example, all membrane proteins have hydrophobic transmembrane regions, which stop translocation of the protein in the membrane and do not allow the protein to be secreted. The construction of an expression vector including signal sequences for targeting a gene product to a particular location is deemed to be within the scope of one of ordinary skill in the art without the need for undue experimentation.

The following discussion is directed to various utilities of the instant disclosure. For example, the instant disclosure has utility as an expression system suitable for detoxifying intra- and/or extracellular toxins *in situ*. By attaching or omitting the appropriate signal sequence to a gene encoding a therapeutic agent capable of detoxifying a toxin, the

therapeutic agent can be targeted for delivery to the extracellular milieu, to the cell plasma membrane or to an intracellular location. In one embodiment, the exogenous genetic material containing a gene encoding an intracellular detoxifying therapeutic agent, further includes sequences encoding surface receptors for facilitating transport of extracellular toxins into the cell where they can be detoxified intracellularly by the therapeutic agent. Alternatively, the cells can be genetically modified to express the detoxifying therapeutic agent anchored within the cell plasma membrane such that the active portion extends into the extracellular milieu. The active portion of the membrane-bound therapeutic agent detoxifies toxins, which are present in the extracellular milieu.

In addition to the above-described therapeutic agents, some of which are targeted for intracellular retention, the instant disclosure also embraces agents intended for delivery to the extracellular milieu and/or agents intended to be anchored in the cell plasma membrane.

The selection and optimization of a particular expression vector for expressing a specific gene product in an isolated cell is accomplished by obtaining the gene, potentially with one or more appropriate control regions (*e.g.*, promoter, insertion sequence); preparing a vector construct comprising the vector into which is inserted the gene; transfecting or transducing cultured cells *in vitro* with the vector construct; and determining whether the gene product is present in the cultured cells. In certain embodiments, a virus from the adeno-associated virus family is used. In certain embodiments, an expression vector for gene therapy based on AAV2, AAV4 and/or AAV5 is used.

Thus, as will be apparent to one of ordinary skill in the art, a variety of suitable viral expression vectors are available for transferring exogenous genetic material into cells. The selection of an appropriate expression vector to express a therapeutic agent for a particular condition amenable to gene replacement therapy and the optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation.

In an alternative embodiment, the expression vector is in the form of a plasmid, which is transferred into the target cells by one of a variety of methods: physical (*e.g.*, microinjection, electroporation, scrape loading, microparticle bombardment) or by cellular uptake as a chemical complex (*e.g.*, calcium or strontium co-precipitation, complexation with lipid, complexation with ligand). Several commercial products are available for cationic liposome complexation including Lipofectin™ (Gibco-BRL, Gaithersburg, Md.) and Transfectam™ (ProMega, Madison, Wis.). However, the efficiency of transfection by these

methods is highly dependent on the nature of the target cell and accordingly, the conditions for optimal transfection of nucleic acids into cells using the above-mentioned procedures must be optimized. Such optimization is within the scope of one of ordinary skill in the art without the need for undue experimentation.

The instant disclosure also provides various methods for making and using the above-described genetically-modified cells. As used herein, the term "isolated" means a cell or a plurality of cells that have been removed from their naturally-occurring *in vivo* location. Methods for removing cells from a patient, as well as methods for maintaining the isolated cells in culture are known to those of ordinary skill in the art.

The instant disclosure also provides methods for genetically modifying cells of a mammalian recipient *in vivo*. According to one embodiment, the method comprises introducing an expression vector for expressing a heterologous gene product into cells of the mammalian recipient *in situ* by, for example, injecting the vector into the recipient.

In one embodiment, the preparation of genetically modified cells contains an amount of cells sufficient to deliver a therapeutically effective dose of the therapeutic agent to the recipient *in situ*. The determination of a therapeutically effective dose of a specific therapeutic agent for a known condition is within the scope of one of ordinary skill in the art without the need for undue experimentation. Thus, in determining the effective dose, one of ordinary skill would consider the condition of the patient, the severity of the condition, as well as the results of clinical studies of the specific therapeutic agent being administered.

If the genetically modified cells are not already present in a pharmaceutically acceptable carrier they are placed in such a carrier prior to administration to the recipient. Such pharmaceutically acceptable carriers include, for example, isotonic saline and other buffers as appropriate to the patient and therapy.

More than one recombinant gene can be introduced into each genetically modified cell on the same or different vectors, thereby allowing the expression of multiple therapeutic agents by a single cell.

EXAMPLE 1

Preparation of Modified AAVs That Target Ocular Cells

The present inventors designed AAVs that are modified to target ocular cells after local delivery. To generate an adeno-associated virus (AAV) that targets the ocular cells, the inventors first used *in vivo* phage display panning to identify peptide motifs that bind preferentially to ocular cells. A phage-display library was injected into the eyes of mice, and

eye cells were subsequently isolated along with the bound phage. The isolated phage was then amplified and reinjected, and after five rounds of such *in vivo* panning, DNA sequencing of the recovered phage revealed an enrichment of distinct peptide motifs from the initial phage library.

In the mice, the peptide motifs GSTPPPM (SEQ ID NO: 1) and GETRAPL (SEQ ID NO: 4) were identified. To confirm the affinity of these phage for the ocular cells, each phage was individually re-injected intravenously into mice, and visualized. Consistent with the panning results, each of the selected phage accumulated in eye beyond the background levels observed for controls.

Peptide-modified AAVs were generated by inserting the peptides identified from phage display panning into the AAV2 capsid. Peptides were inserted between positions 587 and 588 of the VP3 capsid protein to yield clones. AAV-WT (Wild Type, no insert) served as a control virus. The 587/588 site is located in a domain of the VP3 capsid protein involved in the binding of AAV2 with its major receptor, heparin sulfate proteoglycan (HSPG), and insertion of peptides in this site can alter the tropism of AAV without compromising virus viability. The modified capsid proteins packaged AAV vector genomes with genomic titers comparable to those of wildtype virus.

Sequence of AAV2 wild-type capsid

```
              587 588
5'-AGA GGC AAC AGA CAA GCA-3'      (SEQ ID NO: 6)
   R   G   N   R   Q   A           (SEQ ID NO: 7)
```

The sequence of AAV2 wild type capsid is depicted below in SEQ ID NO:8.

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYL
GPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKE
DTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTG
KAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMAD
NNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGA
SNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQV
KEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQ
YGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQS
LDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYR
QQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQS
GVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRG**NR**QAAT

ADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQ
ILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNY
NKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL* (SEQ IDNO:8)

In the example below, the sequence of AAV2 wild type capsid with inserted motif (SEQ ID NO:1) that targets ocular cells is italicized, and the underlined amino acids are spacers.

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYL
GPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKE
DTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTG
KAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMAD
NNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGA
SNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQV
KEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQ
YGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQS
LDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYR
QQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQS
GVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNAAA*GS*
*TPPPM*AA**R**QAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPL
MGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKE
NSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL* (SEQ ID NO:9)

The sequence of AAV2 wild type capsid with SEQ ID NO:4 inserted motif is depicted below in SEQ ID NO:10. The amino acid motif that targets ocular cells is italicized, and the underlined amino acids are spacers.

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYL
GPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKE
DTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTG
KAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMAD
NNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGA
SNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQV
KEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQ
YGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQS
LDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYR

QQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQS
GVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNAAA*GET*
*RAPL*AA**R**QAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPL
MGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKE
NSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL* (SEQ ID
NO:10)

The sequence of AAV9 wild type capsid is depicted below in SEQ ID NO:11.
MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLG
PGNGLDKGEPVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTS
FGGNLGRAVFQAKKRLLEPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQP
AKKRLNFGQTGDTESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVADNNEGADGVG
SSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISNSTSGGSSNDNAYFGYST
PWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTDNNGVKTIA
NNLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYGYLTLNDGSQAVGRS
SFYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLIDQYLYYLSKT
INGSGQNQQTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQNNNSEFAWPGA
SSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTGRDNVDADKVMITN
EEEIKTTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMVWQDRDVYLQGP
IWAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYST
GQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLT
RNL* (SEQ ID NO:11)

Materials and Methods:

***In vivo* biopanning**: 1µl of Ph.D.™ 7 phage library (titer 2.0x 10^{13} pfu/ml from NEB) was injected into the subretinal space of mice eyes. After 30 minutes, the retina and RPE from injected eye were separated, washed with 5 ml cold PBS 3-5 times. The retina and RPE were homogenized in 500 u1 PBS. The recovered phage were titered, amplified, purified, and re-injected into additional eyes for the next round of *in vivo* panning. The whole procedure was repeated for four rounds. After this, 50 random selected clones were sequenced to determine enriched motifs.

Construction of peptide modified AAV2 capsids: The plasmid for cloning of modified capsids was developed from pXX2, containing the wild-type AAV2 Rep and Cap. A plasmid with a DNA fragment encoding amino acids AAAstopA and the restriction sites NotI and AscI inserted between AAV2 Cap amino acid position 587 and 588 was constructed

as the backbone plasmid. dsDNA inserts encoding selected peptides were cloned into NotI and AscI site as peptide modified pXX2.

AAV2 production and titer: Plates of 293T cells were cotransfected with three plasmids: pXX2 or peptide modified pXX2, which supplied the Rep and Cap proteins of AAV2; pHelper, which contained the adenovirus helper functions; and a vector plasmid, which contained the AAV2 ITRs and the transgene of interest. Twenty 150mm-diameter plates were cotransfected 90 μg DNA of plasmids pXX2, pHelper, and vector at a molar ratio of 1:1:1. After incubating for 60 hours, the virus was purified with iodixanol gradients and further purification through a mustang Q membrane. Titers of recombinant AAV were determined by real-time PCR.

EXAMPLE 2

Treating Genetic Eye Disease by Means of Gene Therapy

Two methods of administering viral vectors into the eye were tested in order to test the delivery of gene therapy agents *in vivo*. The modes of administration were by injecting the viral vectors via subretinally (Fig. A-D) and intravitreally (Fig. E-I).

The virus used in this study was AAV2/2. As a control, Wild type AAV2/2 .CMV. eGFP was used, and the test agent was PMAAV-GST. CMV. eGFP. The virus was injected into five week old C57BL/6 female mice. The mice were subretinal injected with virus (2.26 x 109 vg/eye). Three weeks later, the mice were anesthetized and the pupils were dilated. They were placed on a stereotactic platform, and dynamic fluorescent fundus photographs were taken. Four weeks later, the mice were sacrificed and the eyes were enucleated and fixated in 4% of paraformaldehyde. The tissue sections were cut and mounted. The expression of eGFP was observed under fluorescent microscope.

Three weeks after subretinal injection of wild type AAV2/2.CMV.eGFP and PMAAV-GST.CMV.eGFP, fluorescent imaging in live animals showed relative infection efficiencies. The targeting peptides used in this experiment was GSTPPPM (SEQ ID NO: 1). Speckled regions of GFP positivity were observed in AAV2/2 injection eyes, whereas there were broad and uniform GFP signals in PMAAV-GST eyes (Fig. 2). In other experiments, the targeting peptide was GETRAPL (SEQ ID NO: 4).

Four weeks after subretinal injection, PMAAV-GST.CMV.eGFP targeted the retinal pigment epithelium (RPE) and Outer Nuclear Layer (ONL) layers, and some cells in the ganglion cell layer (GCL). AAV2/2 targeted the outer plexiform layer (OPL) primarily (Fig. 3).

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (*i.e.*, meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A modified adeno-associated virus (AAV) capsid protein comprising an ocular cell targeting peptide, wherein the targeting peptide comprises a sequence from 7 to 10 amino acids in length that comprises GSTPPPM (SEQ ID NO: 1), in an amino to carboxy orientation or in a carboxy to amino orientation, and wherein the targeting peptide targets an AAV to an ocular cell.

2. The capsid protein of claim 1, wherein the targeting peptide is 7 amino acids in length.

3. The capsid protein of claim 1, wherein the targeting peptide comprises a sequence up to 10 amino acids in length having therein amino acid sequence GSTPPPM (SEQ ID NO: 1) in an amino to carboxy orientation or in a carboxy to amino orientation.

4. The capsid protein of claim 1, wherein the targeting peptide consists of amino acid sequence GSTPPPM (SEQ ID NO: 1) in an amino to carboxy orientation or in a carboxy to amino orientation.

5. The capsid protein of claim 1, wherein the targeting peptide is inserted between the AAV2 capsid residues at positions P34-A35, T138-A139, A139-P140, G453-T454, N587-R588, and/or R588-Q589 of SEQ ID NO:8.

6. The capsid protein of claim 1, wherein the targeting peptide is inserted after the AAV9 capsid residues at positions D384, G385, I560, T561, N562, E563, E564, E565, N704, and/or Y705 of SEQ ID NO:11.

7. The capsid protein of claim 1, wherein the capsid protein comprises or consists of SEQ ID NO:9.

8. The capsid protein of claim 1 or 2, wherein the targeting peptide targets a diseased ocular cell.

9. The capsid protein of claim 8, wherein the target peptide targets an ocular cell in a subject that has retinitis pigmentosa, maculopathies, Leber’s congenital amaurosis, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related

macular degeneration, Spinocerebellar Ataxia type 7 (SCAT), color blindness, and lysosomal storage diseases that affect the cornea, such as Mucopolysaccharidosis (MPS) IV and MPS VII.

10. The capsid protein of any one of claims 1-4 or 7-9, wherein the AAV capsid is AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV-Rh74 (Rhesus macaque-derived AAV), or AAVRh10, or modified capsids of serotypes AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV-Rh74 (Rhesus macaque-derived AAV), or AAVRh10.

11. The capsid protein of claim 10, wherein the AAV capsid is AAV2 capsid.

12. A nucleic acid sequence encoding the modified capsid as described in any one of claims 1-11.

13. The nucleic acid sequence of claim 12, comprising the nucleic acid sequence GGGTCGACGCCGCCTCCTATG (SEQ ID NO:2).

14. An AAV virus containing the capsid protein of any one of claims 1-11.

15. A viral vector comprising the capsid protein of any one of claims 1-11.

16. The viral vector of claim 15, wherein the viral vector further comprises a nucleic acid sequence encoding a nucleic acid or protein of interest.

17. The viral vector of claim 16, wherein the nucleic acid or protein of interest is a therapeutic agent.

18. The viral vector of claim 17, wherein the therapeutic agent is an enzyme or an RNAi molecule.

19. The viral vector of claim 17, wherein the therapeutic agent is Ataxin 7 miRNA, Retinal pigment epithelium-specific 65 kDa protein (RPE 65), vascular endothelial growth factor (VEGF) inhibitor or soluble VEGF receptor 1 (sFifl), (Rab escort protein-1) REP1, L-opsin, rhodopsin (Rho), phosphodiesterase 6(3 (PDE6I3), ATP-binding cassette, sub-family A, member 4 (ABCA4), lecithin retinol acyltransferase (LRAT), Retinal degeneration, slow/Peripherin (RDS/Peripherin), Tyrosine-protein kinase Mer (MERTK), Inosine-5 prime-monophosphate dehydrogenase, type I

(IMPDHI), guanylate cyclase 2D (GUCY2D), aryl-hydrocarbon interacting protein-like 1 (AIPL 1), retinitis pigmentosa GTPase regulator interacting protein 1 (RPGRIPI), Inosine-5-prime-monophosphate dehydrogenase, type I (IMPDH1 guanine nucleotide binding protein, alpha transducing activity polypeptide 2 (GNAT2), cyclic nucleotide gated channel beta 3 (CNGB3), retinoschisin 1 (Rsl), ocular albinism type 1 (OA1), oculocutaneous albinism type 1 (OCA1) tyrosinase, P21 WAF-1/Cipl, platelet-derived growth factor (PDGF), Endostatin Angiostatin, arylsulfatase B, or 13 -glucuronidase.

20. A composition comprising the AAV virus of claim 14 or viral vector of any of claims 15-19 and a carrier.

21. A pharmaceutical composition comprising the AAV virus of claim 14 or viral vector of any of claims 15-19 and a pharmaceutically acceptable carrier.

22. An *in vitro* cell comprising the viral vector of any one of claims 15-19.

23. The cell of claim 22, wherein the cell is a mammalian cell.

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

25. The cell of claim 23, wherein the cell is a non-human cell.

26. The cell of any one of claims 22-25, wherein the cell is an ocular cell.

27. A viral vector as described by any one of claims 15-19 for use as a medicament or in diagnosis, wherein the viral vector is an AAV vector.

28. Use of the viral vector of any one of claims 15-19 in the manufacture of a medicament for the prophylactic or therapeutic treatment of an ocular disease in a mammal wherein the disease is retinitis pigmentosa, maculopathies, Leber's congenital amaurosis, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related macular degeneration, SCAT, color blindness, or lysosomal storage diseases that affect the cornea, such as MPS IV and MPS VII, wherein the viral vector is an AAV vector.

29. A cell as described by any one of claims 22-25 for use as a medicament or in diagnosis, wherein the cell is an ocular cell.

30. Use of the cell of any one of claims 22-25 in the manufacture of a medicament for the treatment of an ocular disease in a mammal wherein the disease is retinitis pigmentosa, maculopathies, Leber's congenital amaurosis, early onset severe retinal dystrophy, achromatopsia, retinoschisis, ocular albinism, oculocutaneous albinism, glaucoma, Stargardt disease, choroideremia, age-related macular degeneration, SCAT, color blindness, or lysosomal storage diseases that affect the cornea, such as MPS IV and MPS VII, wherein the cell is an ocular cell.

31. The use of claim 30, wherein the cells used in the manufacture of the medicament comprise retina outer plexiform layer (OPL) cells.