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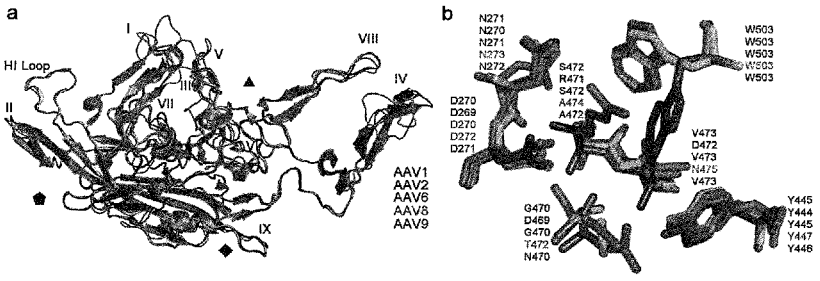

FIG. 1

(57) Abstract: The present invention provides methods and compositions comprising an adeno-associated virus (AAV) capsid protein, comprising one or more amino acids substitutions, wherein the substitutions introduce a new glycan binding site into the AAV capsid protein.

WO 2014/144229 A1

METHODS AND COMPOSITIONS FOR DUAL GLYCAN BINDING AAV VECTORS

STATEMENT OF PRIORITY

This application claims the benefit, under 35 U.S.C. § 119(e), of U.S. Provisional Application Serial No. 61/802,111, filed March 15, 2013, the entire contents of which are incorporated by reference herein.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under Grant Nos. R01HL089221, P01HL112761 and R01AI072176 awarded by the National Institutes of Health. The United States government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to modified capsid proteins from adeno-associated virus (AAV), virus capsids and virus vectors comprising the same, as well as methods of their use.

BACKGROUND OF THE INVENTION

Virus-glycan interactions are critical determinants of host cell invasion. Cell surface carbohydrates such as sialic acids, gangliosides or heparan sulfate are exploited by a vast number of viruses such as influenza, herpesvirus, SV40, polyomavirus, papillomavirus and other pathogens[1,2]. In most cases, a single class of glycans primarily serves as the cell surface attachment factor for viruses, leading to sequential or parallel engagement of other receptors/co-receptors for cell entry. Adeno-associated viruses (AAV) are helper-dependent parvoviruses that exploit heparan sulfate (HS), galactose (Gal) or sialic acids (Sia) as primary receptors for cell surface binding[3,4]. For instance, AAV serotypes 2 and 3b utilize HS; AAV1, 4 and 5 bind Sia with different linkage specificities; while AAV9 exploits Gal for host cell attachment. Different AAV strains also require subsequent interaction with co-receptors such as integrin αVβ5 or α5β1, fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR) or the laminin receptor for cellular uptake[3,4].

A notable exception to the monogamous relationship between a specific AAV strain and a single class of carbohydrates is AAV serotype 6, which recognizes both Sia and HS[5]. However, only Sia has been shown essential for viral transduction. Structural studies have

now established that the K531 residue in conjunction with R488, K528 and K533 in the VP3 subunit of the AAV6 capsid form a continuous basic patch for electrostatic recognition of HS glycosaminoglycans[6-8]. Similarly, the structural basis for HS recognition by AAV2 and AAV3b is well known and attributed to similar clusters of basic amino acid residues located at the three-fold axis of symmetry[9-12]. The Sia binding footprints for AAV1, AAV4, AAV5 and AAV6 remain to be determined. More recently, key amino acid residues involved in Gal recognition by AAV9 capsids were identified by using a combination of molecular docking and site-directed mutagenesis[13]. What is needed are virus vectors that have multiple glycan binding capability to exploit alternative pathways for cell entry and transduction.

The present invention overcomes previous shortcomings in the art by providing modified capsid proteins with multiple glycan binding sites, AAV vectors comprising these capsid proteins and methods for their use as therapeutic vectors.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides an adeno-associated virus (AAV) capsid protein, comprising one or more amino acids substitutions, wherein the substitutions introduce a new glycan binding site into the AAV capsid protein. In some embodiments, the amino acid substitutions are in amino acid 266, amino acids 463-475 and amino acids 499-502 in AAV2 or the corresponding amino acid positions in AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV10.

In some embodiments, new glycan binding site can be a hexose binding site, wherein the hexose is a galactose (Gal), a mannose (Man), a glucose (Glu) or a fucose (fuc).

In some embodiments, the new glycan binding site can be a sialic acid (Sia) binding site, wherein the Sia residue is N-acetylneuraminic acid (Neu5Ac) or N-Glycolylneuraminic acid (Neu5Gc).

In some embodiments, the new glycan binding site can be a disaccharide binding site, wherein the disaccharide is a sialic acid linked to galactose in the form Sia(alpha2,3)Gal or Sia(alpha2,6)Gal.

In some embodiments, the substitutions introduce a new glycan binding site from a first AAV serotype into the capsid protein of a second AAV serotype that is different from said first AAV serotype.

The present invention also provides an AAV capsid comprising the AAV capsid protein of this invention.

Further provided herein is a virus vector comprising the AAV capsid of this invention as well as a composition comprising the AAV capsid protein, AAV capsid and/or virus vector of this invention in a pharmaceutically acceptable carrier.

The present invention additionally provides a method of introducing a nucleic acid into a cell, comprising contacting the cell with the virus vector of this invention. The cell can be in a subject and in some embodiments, the subject can be a human subject.

These and other aspects of the invention are addressed in more detail in the description of the invention set forth below.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Structural alignment of AAV1, AAV2, AAV6, AAV8, and AAV9 VP3 monomers. (a) Superposition of the VP3 monomers of AAV1 (purple blue), AAV2 (deep blue), AAV6 (light magenta), AAV8 (green), and AAV9 (brown) with loops I-IX labeled and axes of symmetry indicated. **(b)** Close-up views of overlay of the galactose binding site on AAV9 and equivalent residues on AAV1, AAV2, AAV6, and AAV8. Amino acid residues are marked by the color code in **(a)**. Coordinates were obtained from X-ray crystallography structure of VP monomers (PDB accession#: AAV1-3NG9, AAV2-1LP3, AAV6-3OAH, AAV8-2QA0, AAV9-3UX1). Structure alignment was performed and visualized using PyMOL.

FIG. 2. G-mutants utilize Gal as a novel glycan receptor to transduce cells *in vitro*. (a) Transduction efficiency of AAV1, 1G9, and AAV9 on Chinese Hamster Ovary (CHO) cell lines. Pro5 and Lec2 cells were pre-chilled to 4°C for 30 minutes prior to AAV-CBA-Luciferase infection at an MOI of 1000 vg/cell at 4°C for 60 minutes. After removing unbound virions by three washes with ice-cold PBS, infected cells were cultured in 37°C incubator for 24 hours. Luminometric analysis was performed to quantify the luciferase transgene expression efficiencies from cell lysates. **(b)** Transduction efficiency of AAV2i8, 2i8G9, and AAV9 on Pro5 and Lec2 cells. **(c)** Transduction efficiency of AAV6, 6G9, and AAV9 on Pro5 and Lec2 cells. **(d)** Transduction efficiency of AAV8, 8G9, and AAV9 on Pro5 and Lec2 cells. Results are presented as mean ± s.e.m. (n=4). Statistical significance was assessed using the one-tailed Student's *t*-test (n.s., not significant; * $p < 0.05$; ** $p < 0.01$).

FIG. 3. Three-dimensional models of the dual glycan binding AAV2G9 chimera and its parental strains AAV2 and AAV9. (A) Three-dimensional structural model of an intact AAV2G9 capsid with existing HS and "grafted" Gal binding sites colored in purple and

orange, respectively. **(B-D)** Illustrations of the three-dimensional surface model of VP3 trimers at the three-fold symmetry axes of AAV2 **(B)**, AAV9 **(C)**, and AAV2G9 **(D)** capsids. Residues involved in HS binding (AAV2 VP1 numbering: R487, K527, K532, R585, R588) and Gal binding (AAV9 VP1 numbering: D271, N272, Y446, N470, A472, V473, W503) are highlighted as in **(A)**. Black triangles indicate the three-fold symmetry axes.

FIG. 4. *In vitro* characterization of the dual glycan binding AAV2G9 chimera. ***(A-C)*** Inhibition of AAV2 ***(A)***, AAV9 ***(B)***, and AAV2G9 ***(C)*** transduction on CHO Lec2 cells with FITC-ECL and soluble heparin. CHO Lec2 cells were pre-chilled at 4°C and incubated with FITC-ECL, soluble heparin or both prior to infection with AAV2, AAV9 or AAV2G9 packaging a CBA-luciferase reporter transgene cassette. Transduction efficiency was measured 24 hours post infection as luciferase activity in relative light units (RLU). Percentage of transgene expression was calculated by normalizing transduction efficiency to RLU from controls. Results are presented as mean ± s.e.m. (n=4). ***(D-F)*** Competitive inhibition of cell surface binding of AAV2 ***(D)***, AAV9 ***(E)***, and AAV2G9 ***(F)*** on CHO Lec2 cells with FITC-ECL and soluble heparin. Different AAV particles were bound to cells pre-chilled at 4°C and unbound virions removed by washing with cold PBS. Bound virions were quantified using qPCR after viral genome extraction. Percentage of bound virions was determined by normalizing number of bound virions to that of corresponding controls. Results are presented as mean ± s.e.m. (n=5). Statistical significance was analyzed using the one-tailed Student's *t*-test (* $p < 0.05$; ** $p < 0.01$).

FIG. 5. Immunofluorescence of bound virions on Lec2 cell surface at 0 hours post-infection (hpi). CHO Lec2 cells were plated on 12 mm coverslips at density of $5x10^4$ cells/coverslip overnight. After being pre-chilled at 4°C for 30 minutes, Lec2 cells were infected with AAV2, AAV 2G9, and AAV9 at an MOI of 1000 vg/cell at 4°C for 30 minutes. After removal of unbound virions, cells were fixed with 2% paraformaldehyde in 1xPBS. Intact virions were detected using the monoclonal antibodies (A20 for AAV2/AAV2G9 and ADK9 for AAV9) obtained as media supernatant from corresponding hybridoma cultures with 1:10 dilution in immunofluorescence wash buffer (IFWB). Alexa Fluor 594® goat anti-mouse IgG was utilized at a dilution of 1:1000 in IFWB as the secondary antibody for immunofluorescence detection. Coverslips were then mounted onto glass slides in Prolong® Gold anti-fade reagent with DAPI. Fluorescence micrographs were acquired using a Zeiss® 710 confocal laser scanning microscope equipped with a 63x oil immersion objective and a spectral detection system. Image processing was carried out using LSM® viewer and Image J® software. The white scale bar indicates 10 μm.

FIG. 6. Competitive inhibition of AAV2G9 transduction on Lec2 cells by AAV2 capsids or AAV9 capsids. Lec2 cells were preincubated with (*A*) AAV2 or (*B*) AAV9-CBA-tdTomato at multiplicity of infection (MOI) ranging from 500 to 100,000 vg/cell for 2 hours prior to infection with AAV2G9-CBA-Luc particles (MOI 1000 vg/cell). Percentage inhibition of AAV2G9 transduction was calculated by normalizing luciferase transgene expression levels to that of untreated control. Results are presented as mean ± s.e.m. (n=4). Statistical significance was analyzed using the one-tailed Student's *t*-test (* $p < 0.05$; ** $p < 0.01$).

FIG. 7. Kinetics of transduction efficiency profiles of AAV 2G9 compared to parental AAV2 and AAV9 on Lec2 cells at indicated time points post infection. Pre-chilled Lec2 cells were infected with AAV2, AAV2G9, or AAV9-CBA-luciferase vectors at an MOI of 1000 vg/cell as described. At indicated time points (18, 24, 28, 42 and 54 hours) post-infection, cells were lysed prior to luminometric analysis. Luciferase transgene expression was measured by luciferase activities of cell lysates in relative light units (RLU) (n=5). Statistical significance was assessed using the one-tailed Student's *t*-test (* $p < 0.05$; ** $p < 0.01$).

FIG. 8. AAV2G9 mediates rapid onset and enhanced transgene expression *in vivo*. (*A*) *In vivo* transgene expression kinetics of AAV2, AAV 2G9, and AAV9 vectors packaging CBA-luciferase transgene cassette. BALB/c mice (n=4) were administered AAV vectors at a dose of $1x10^{11}$ vg/animal through the tail vein and bioluminescent images collected at 3, 7, and 18 days post-injection using an Xenogen® Lumina imaging system. Representative live animal images are shown with bioluminescence on a rainbow colored scale ($1x10^5$-$1x10^6$ photons/second/cm^2/steradian). AAV2G9 maintains the hepatic tropism of AAV2, but demonstrates a more rapid and robust luciferase signal than both parental AAV strains. (***B and C***) Quantitation of the kinetics of light signal output (expressed as photons/second/cm^2/steradian) was performed by marking regions of interest (ROIs) around images of the (*B*) liver region and (*C*) entire animals obtained at different time intervals (n=4). Statistical significance was assessed using the one-tailed Student's *t*-test (n.s., not significant; * $p < 0.05$; ** $p < 0.01$).

FIG. 9. Quantification of transgene expression and biodistribution profiles of AAV2G9 in mice. (*A*) Quantitation of luciferase transgene expression from heart and liver tissue lysates of AAV2 (black), AAV2G9 (grey), or AAV9 (white) treated animals at days 3 and 18 (n=4). (*B*) Biodistribution of vector genomes in liver and heart lysates obtained from BALB/c mice administered with AAV2 (black), AAV2G9 (grey), or AAV9 (white) at days 3

and 18 (n=4). At indicated time points, host genomic DNA and viral genomes were isolated from tissue lysates and quantified using qPCR with primer sets specific to mouse lamin gene and luciferase transgene. Results are presented as mean ± s.e.m. (n=4). Statistical significance was assessed using the one-tailed Student's *t*-test (n.s., not significant; * $p < 0.05$; ** $p < 0.01$).

FIG. 10. *In vivo* transgene expression kinetics of AAV2i8, 2i8G9, and AAV9 vectors packaging CBA-luciferase transgene cassette. BALB/c mice (n=4) were administered AAV vectors at a dose of $1x10^{11}$ vg/animal through the tail vein and bioluminescence images collected at 3, 7, and 18 days post-injection using a Xenogen® Lumina imaging system. Representative live animal images are shown with bioluminescence expressed on a rainbow colored scale (10^5-10^6 photons/second/cm^2/steradian).

FIG. 11. CNS tropism profiles of representative AAV "G9" strains in neonatal mice. Postnatal 0 (P0) pups (n=3) were unilaterally injected into the left cerebral ventricle with 3.5x10e9 AAV vector genomes containing a GFP transgene driven by a hybrid chicken beta actin (CBh) promoter. At 2 wks post injection, GFP immunohistochemistry revealed differential spread, regional and cellular tropisms for each AAV "G9" strain within the murine brain.

DETAILED DESCRIPTION OF THE INVENTION

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

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

The present invention is based on the discovery of a "pocket" on the AAV capsid protein that defines a glycan recognition footprint. Specific amino acids that define this pocket have been identified and are described herein, for example for the galactose binding

site of AAV9. In the present invention, this AAV9 galactose binding footprint was grafted into an AAV2 capsid protein template, resulting in the introduction of a new glycan binding site in the engrafted AAV2 capsid protein template. This AAV galactose binding footprint can be introduced into any other AAV serotype, by substituting the corresponding amino acids, which are shown, for example, in **Table 3** herein.

Thus the present invention is directed to molecular grafting of a glycan recognition footprint from one AAV strain onto another, which is guided by structural modeling studies and achieved by site-directed mutagenesis. Recombinant vectors (derived from these new strains) packaging reporter cassettes display rapid onset and enhanced transgene expression in cell culture and animal models. Using naturally occurring serotypes/isolates as templates, this universal strategy can be applied to generate a panel of synthetic dual glycan binding AAV strains that could address challenges such as dose-dependent immunotixicity observed in human gene therapy clinical trials.

Thus, in one aspect, the present invention provides an adeno-associated virus (AAV) capsid protein, comprising one or more amino acids substitutions, wherein the substitutions introduce a new glycan binding site into the AAV capsid protein. In some embodiments, the amino acid substitutions are in amino acid 266, amino acids 463-475 and amino acids 499-502 in AAV2 or the corresponding amino acid positions in AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV10 or any other AAV serotype as identified in **Table 3**.

In some embodiments, the new glycan binding site can be a hexose binding site, wherein the hexose is a galactose (Gal), a mannose (Man), a glucose (Glu) or a fucose (fuc).

In some embodiments, the new glycan binding site can be a sialic acid (Sia) binding site, wherein the Sia residue is N-acetylneuraminic acid (Neu5Ac) or N-Glycolylneuraminic acid (Neu5Gc).

In some embodiments, the new glycan binding site can be a disaccharide binding site, wherein the disaccharide is a sialic acid linked to galactose in the form Sia(alpha2,3)Gal or Sia(alpha2,6)Gal.

In some embodiments, the substitutions introduce a new glycan binding site from a capsid protein of a first AAV serotype ("donor") into the capsid protein of a second AAV serotype ("template) that is different from said first AAV serotype.

The present invention also provides an AAV capsid comprising the AAV capsid protein of this invention.

Further provided herein is a virus vector comprising the AAV capsid of this invention as well as a composition comprising the AAV capsid protein, AAV capsid and/or virus vector of this invention in a pharmaceutically acceptable carrier.

The present invention additionally provides a method of introducing a nucleic acid into a cell, comprising contacting the cell with the virus vector of this invention. The cell can be in a subject and in some embodiments, the subject can be a human subject.

In some exemplary embodiments, the AAV capsid protein donor can be AAV serotype 9 and the AAV capsid protein template can be AAV serotype 1 (AAV1), AAV serotype 2 (AAV2), AAV serotype 3a (AAV3a), AAV serotype 3b (AAV3b), AAV serotype 4 (AAV4), AAV serotype 5 (AAV5), AAV serotype 6 (AAV6), AAV serotype 7 (AAV7), AAV serotype 8 (AAV8), or AAV serotype 10 (AAV10).

In some exemplary embodiments, the AAV capsid protein template can be from AAV2, and a) the substitution at amino acid 266 is A266S; b) the substitutions at amino acids 463-475 are SQAGASDIRDQSR463-475SX$_1$AGX$_2$SX$_3$X$_4$X$_5$X$_6$QX$_7$R, wherein X$_{1-7}$ can be any amino acid; and c) the substitutions at amino acids 499-502 are EYSW499-502EX$_8$X$_9$W, wherein X$_{8-9}$ can be any amino acid. In some embodiments, X$_1$ can be V; X$_2$ can be P; X$_{3-6}$ can be NMAV; and X$_7$ can be G, resulting in the sequence SVAGPSNMAVQGR. In some embodiments, X$_8$ can be F and X$_9$ can be W, resulting in the sequence EFAW.

The example above is provided to demonstrate the substitutions possible for introducing a galactose binding site from an AAV9 donor into an AAV2 template. **Table 3** lists several AAV serotypes for which these corresponding amino acids are identified and exemplary substitutions that can be made in each of these serotypes to introduce the galactose binding site of AAV9. What is shown in **Table 3** and described in detail herein is that specific amino acid positions are conserved and others are substituted. Where a substitution is shown, the substitution set forth in **Table 3** is exemplary of various substitutions that can be made at these residue positions. It is contemplated that the embodiments of this invention encompass other donor AAV serotypes besides AAV9 and other glycan binding sites besides the galactose binding site.

Table 2 lists non-limiting exemplary serotypes of AAV and accession numbers of the genome and capsid sequences that may be encompassed by the invention. The AAV serotype of the donor and the template is not limited to human AAV, but may include non-human AAV, for example, Avian or Bovine AAV, as well as non-human primate AAV, examples of which are shown in **Table 1**.

The example above shows the possible amino acid substitutions in an AAV2 template for introduction of a galactose binding site from an AAV9 donor. In another example, the template can be AAV1 or AAV6 and the substitutions at the amino acid positions corresponding to positions 463-475 of AAV2 can be $SX_1X_2X_3PX_4X_5MX_6VQX_7X_8$, wherein X_{1-8} can be any amino acid. In a particular embodiment, X_{1-3} is VAG; X_4 is S; X_5 is N; X_6 is A; X_7 is G and X_8 is R, resulting in the sequence SVAGPSNMAVQGR. In further embodiments, substitution at the amino acid positions corresponding to positions 499-502 in AAV2 can be $X_9FX_{10}W$, wherein X_9 andX_{10} may be any amino acid. In a particular embodiment, X_9 is E and X_{10} is W, resulting in the sequence EFAW.

In another example, the template can be AAV3a or AAV3b and the substitutions at the amino acid positions corresponding to positions 463-475 of AAV2 can be $SX_1AGPX_2X_3MX_4X_5QX_6R$ wherein X_{1-6} can be any amino acid. In a particular embodiment, X_1 is V; X_2 is S;X_3 is N; X_4 is A; X_5 is N; and X_6 is G, resulting in the sequence SVAGPSNMAVQGR. In further embodiments, substitution at the amino acid positions corresponding to positions 499-502 in AAV2 can be X_7FX_8W, wherein X_7 andX_8 may be any amino acid. In a particular embodiment, X_7 is E and X_8 is W, resulting in the sequence EFAW.

In another example, the template can be AAV4 and the substitutions at the amino acid positions corresponding to positions 463-475 of AAV2 can be $X_1X_2X_3X_4PX_5NX_6X_7X_8X_9X_{10}X_{11}$ wherein X_{1-11} can be any amino acid. In a particular embodiment, X_1 is S; X_2 is V;X_3 is A; X_4 is G; X_5 is S; X_6 is M; X_7 is A; X_8 is V; X_9 is Q; X_{10} is G; and X_{11} is R, resulting in the sequence SVAGPSNMAVQGR. In further embodiments, substitution at the amino acid positions corresponding to positions 499-502 in AAV2 can be $X_{12}X_{13}X_{14}X_{15}$, wherein $X_{12\text{-}15}$ can be any amino acid. In a particular embodiment, X_{12} is E; X_{13} is F; X_{14} is A; and X_{15} is W, resulting in the sequence EFAW.

In another example, the template can be AAV5and the substitutions at the amino acid positions corresponding to positions 463-475 of AAV2 can be $X_1X_2X_3X_4X_5X_6X_7X_8AX_9X_{10}X_{11}X_{12}$, wherein X_{1-12} can be any amino acid. In a particular embodiment, X_1 is S; X_2 is V;X_3 is A; X_4 is G; X_5 is P; X_6 is S; X_7 is N; X_8 is M; X_9 is V; X_{10} is Q; X_{12} is G; and X_{12} is R, resulting in the sequence SVAGPSNMAVQGR. In further embodiments, substitution at the amino acid positions corresponding to positions 499-502 in AAV2 can be $X_{13}X_{14}AX_{15}$, wherein X_{13-15} can be any amino acid. In a particular embodiment, X_{13} is E; X_{14} is F; X_{15} is A; and X_{16} is W, resulting in the sequence EFAW.

In another example, the template can be AAV7 and the substitutions at the amino acid positions corresponding to positions 463-475 of AAV2 can be $X_1X_2X_3GPSX_4MAX_5QX_6X_7$, wherein X_{1-7} can be any amino acid. In a particular embodiment, X_1 is S; X_2 is V;X_3 is A; X_4 is N; X_5 is V; X_6 is G; and X_7 is R, resulting in the sequence SVAGPSNMAVQGR. In further embodiments, substitution at the amino acid positions corresponding to positions 499-502 in AAV2 can be X_8FAW, wherein X_8 can be any amino acid. In a particular embodiment, wherein X_8 is E, resulting in the sequence EFAW.

In another example, the template can be AAV8 and the substitutions at the amino acid positions corresponding to positions 463-475 of AAV2 can be $SX_1X_2GPX_3X_4MAX_5QX_6X_7$, wherein X_{1-7} can be any amino acid. In a particular embodiment, X_1 is V; X_2 is A; X_3 is S; X_4 is N; X_5 is V; X_6 is G; and X_7 is R, resulting in the sequence SVAGPSNMAVQGR. In further embodiments, substitution at the amino acid positions corresponding to positions 499-502 in AAV2 can be X_8FAW, wherein X_8 can be any amino acid. In a particular embodiment, X_8 can be E, resulting in the sequence EFAW.

In another example, the template can be AAV10 and the substitutions at the amino acid positions corresponding to positions 463-475 of AAV2 can be $X_1X_2AGPX_3NMX_4X_5QX_6X_7$, wherein X_{1-7} can be any amino acid. In a particular embodiment, X_1 is S; X_2 is V;X_3 is S; X_4 is A; X_5 is V; X_6 is G; and X_7 is R, resulting in the sequence SVAGPSNMAVQGR. In further embodiments, substitution at the amino acid positions corresponding to positions 499-502 in AAV2 can be X_8FAW, wherein X_8 can be any amino acid. In a particular embodiment, X_8 can be E, resulting in the sequence EFAW.

The examples above describe introduction of a galactose binding site from AAV9 into a capsid protein template that can be AAV2, AAV3a, AAV3b, AAV4, AAV5, AAV7, AAV8 or AAV10. These examples, which are not intended to be limiting, demonstrate this universal principle that a glycan binding site from a donor AAV serotype can be introduced into a capsid protein template of a different AAV serotype (e.g., as listed in **Table 3**) by modifying residues the define the "pocket" described herein. Such modified or chimeric capsid proteins comprising a new glycan binding site can be assembled into capsids that make up virus particles that can be used as virus vectors that have the beneficial phenotype of increased cell surface binding and more rapid and enhanced transgene expression *in vivo*.

As used herein, the term "adeno-associated virus" (AAV), includes but is not limited to, AAV type 1, AAV type 2, AAV type 3 (including types 3A and 3B), AAV type 4, AAV type 5, AAV type 6, AAV type 7, AAV type 8, AAV type 9, AAV type 10, AAV type 11, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV, Clade F AAV and any

other AAV now known or later discovered. *See, e.g.*, BERNARD N. FIELDS *et al.*, VIROLOGY, volume 2, chapter 69 (4th ed., Lippincott-Raven Publishers). A number of relatively new AAV serotypes and clades have been identified (*see, e.g.*, Gao et al. (2004) *J. Virology* 78:6381-6388 and **Table 1**).

The genomic sequences of various serotypes of AAV, as well as the sequences of the native terminal repeats (TRs), Rep proteins, and capsid subunits are known in the art. Exemplary but non-limiting examples of such sequences may be found in the literature or in public databases such as GenBank® Database. *See, e.g.*, GenBank® Database Accession Numbers NC_002077.1, NC_001401.2, NC_001729.1, NC_001863.1, NC_001829.1, NC_006152.1, NC_001862.1, AF513851.1, AF513852.1, the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences. *See also, e.g.*, Srivistava et al. (1983) *J. Virology* 45:555; Chiorini et al. (1998) *J. Virology* 71:6823; Chiorini *et al.* (1999) *J. Virology* 73:1309; Bantel-Schaal et al. (1999) *J. Virology* 73:939; Xiao et al. (1999) *J. Virology* 73:3994; Muramatsu et al. (1996) *Virology* 221:208; Shade et al. (1986) *J. Virol.* 58:921; Gao et al. (2002) *Proc. Nat. Acad. Sci. USA* 99:11854; international patent publications WO 00/28061, WO 99/6160 and WO 98/11244; and U.S. Patent No. 6,156,303; the disclosures of which are incorporated by reference herein for teaching parvovirus and AAV nucleic acid and amino acid sequences.

The capsid structures of autonomous parvoviruses and AAV are described in more detail in BERNARD N. FIELDS *et al.*, Virology, Volume 2, Chapters 69 & 70 (4th ed., Lippincott-Raven Publishers). *See also*, description of the crystal structure of AAV2 (Xie et al. (2002) *Proc. Nat. Acad. Sci.* 99:10405-10), AAV4 (Padron et al. (2005) *J. Virol.* 79: 5047-58), AAV5 (Walters et al. (2004) *J. Virol.* 78: 3361-71) and CPV (Xie et al. (1996) *J. Mol. Biol.* 6:497-520 and Tsao et al. (1991) *Science* 251: 1456-64).

Definitions

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

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

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

Unless the context indicates otherwise, it is specifically intended that the various features of the invention described herein can be used in any combination.

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

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

As used herein, the terms "reduce," "reduces," "reduction" and similar terms mean a decrease of at least about 5%, 10%, 15%, 20%, 25%, 35%, 50%, 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, 100% or more.

As used herein, the terms "enhance," "enhances," "enhancement" and similar terms indicate an increase of at least about 10%, 20%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400%, 500% or more.

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

A "polynucleotide" is a sequence of nucleotide bases, and may be RNA, DNA or DNA-RNA hybrid sequences (including both naturally occurring and non-naturally occurring nucleotide), but in representative embodiments are either single or double stranded DNA sequences.

As used herein, an "isolated" polynucleotide (e.g., an "isolated DNA" or an "isolated RNA") means a polynucleotide at least partially separated from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polynucleotide. In representative embodiments an "isolated" nucleotide is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

Likewise, an "isolated" polypeptide means a polypeptide that is at least partially separated from at least some of the other components of the naturally occurring organism or virus, for example, the cell or viral structural components or other polypeptides or nucleic acids commonly found associated with the polypeptide. In representative embodiments an "isolated" polypeptide is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

As used herein, by "isolate" or "purify" (or grammatical equivalents) a virus vector, it is meant that the virus vector is at least partially separated from at least some of the other components in the starting material. In representative embodiments an "isolated" or "purified" virus vector is enriched by at least about 10-fold, 100-fold, 1000-fold, 10,000-fold or more as compared with the starting material.

A "therapeutic polypeptide" is a polypeptide that can alleviate, reduce, prevent, delay and/or stabilize symptoms that result from an absence or defect in a protein in a cell or subject and/or is a polypeptide that otherwise confers a benefit to a subject, *e.g.*, anti-cancer effects or improvement in transplant survivability.

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

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

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

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

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

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

The virus vectors of the invention can further be "targeted" virus vectors (*e.g.*, having a directed tropism) and/or a "hybrid" parvovirus (*i.e.*, in which the viral TRs and viral capsid are from different parvoviruses) as described in international patent publication WO 00/28004 and Chao et al. (2000) *Molecular Therapy* 2:619.

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

Methods of Producing Virus Vectors.

The invention also encompasses virus vectors comprising the modified capsid proteins and capsids of the invention. In particular embodiments, the virus vector is a parvovirus vector (*e.g.*, comprising a parvovirus capsid and/or vector genome), for example, an AAV vector (*e.g.*, comprising an AAV capsid and/or vector genome). In representative embodiments, the virus vector comprises a modified AAV capsid comprising a modified capsid subunit of the invention and a vector genome.

For example, in representative embodiments, the virus vector comprises: (a) a modified virus capsid (*e.g.*, a modified AAV capsid) comprising a modified capsid protein of the invention; and (b) a nucleic acid comprising a terminal repeat sequence (*e.g.*, an AAV TR), wherein the nucleic acid comprising the terminal repeat sequence is encapsidated by the

modified virus capsid. The nucleic acid can optionally comprise two terminal repeats (*e.g.*, two AAV TRs).

In representative embodiments, the virus vector is a recombinant virus vector comprising a heterologous nucleic acid encoding a polypeptide or functional RNA of interest. Recombinant virus vectors are described in more detail below.

In particular embodiments, the virus vectors of the invention have reduced transduction of liver as compared with the level of transduction by a virus vector without the modified capsid protein. In particular embodiments, the virus vector has systemic transduction toward muscle, *e.g.*, the vector transduces multiple skeletal muscle groups throughout the body and optionally transduces cardiac muscle and/or diaphragm muscle.

It will be understood by those skilled in the art that the modified capsid proteins, virus capsids and virus vectors of the invention exclude those capsid proteins, capsids and virus vectors that have the indicated amino acids at the specified positions in their native state (i.e., are not mutants).

The present invention further provides methods of producing the inventive virus vectors. In one representative embodiment, the present invention provides a method of producing a virus vector, the method comprising providing to a cell: (a) a nucleic acid template comprising at least one TR sequence (*e.g.*, AAV TR sequence), and (b) AAV sequences sufficient for replication of the nucleic acid template and encapsidation into AAV capsids (*e.g.*, AAV *rep* sequences and AAV *cap* sequences encoding the AAV capsids of the invention). Optionally, the nucleic acid template further comprises at least one heterologous nucleic acid sequence. In particular embodiments, the nucleic acid template comprises two AAV ITR sequences, which are located 5' and 3' to the heterologous nucleic acid sequence (if present), although they need not be directly contiguous thereto.

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

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

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

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

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

The nucleic acid template can be provided to the cell using any method known in the art. For example, the template can be supplied by a non-viral (*e.g.*, plasmid) or viral vector. In particular embodiments, the nucleic acid template is supplied by a herpesvirus or adenovirus vector (*e.g.*, inserted into the E1a or E3 regions of a deleted adenovirus). As another illustration, Palombo et al. (1998) *J. Virology* 72:5025, describes a baculovirus vector carrying a reporter gene flanked by the AAV TRs. EBV vectors may also be employed to deliver the template, as described above with respect to the *rep/cap* genes.

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

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

Further, the helper virus functions may be provided by a packaging cell with the helper sequences embedded in the chromosome or maintained as a stable extrachromosomal

element. Generally, the helper virus sequences cannot be packaged into AAV virions, *e.g.*, are not flanked by TRs.

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

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

In a further embodiment, the AAV *rep/cap* sequences and the adenovirus helper sequences are supplied by a single adenovirus helper vector. According to this embodiment, the rAAV template can be provided as a plasmid template.
In another illustrative embodiment, the AAV *rep/cap* sequences and adenovirus helper sequences are provided by a single adenovirus helper vector, and the rAAV template is integrated into the cell as a provirus. Alternatively, the rAAV template is provided by an EBV vector that is maintained within the cell as an extrachromosomal element (*e.g.*, as an EBV based nuclear episome).

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

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

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

Herpesvirus may also be used as a helper virus in AAV packaging methods. Hybrid herpesviruses encoding the AAV Rep protein(s) may advantageously facilitate scalable AAV vector production schemes. A hybrid herpes simplex virus type I (HSV-1) vector expressing the AAV-2 *rep* and *cap* genes has been described (Conway et al. (1999) *Gene Therapy* 6:986 and PCT Publication No. WO 00/17377.

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

AAV vector stocks free of contaminating helper virus may be obtained by any method known in the art. For example, AAV and helper virus may be readily differentiated based on size. AAV may also be separated away from helper virus based on affinity for a heparin substrate (Zolotukhin et al. (1999) *Gene Therapy* 6:973). Deleted replication-defective helper viruses can be used so that any contaminating helper virus is not replication competent. As a further alternative, an adenovirus helper lacking late gene expression may be employed, as only adenovirus early gene expression is required to mediate packaging of AAV virus. Adenovirus mutants defective for late gene expression are known in the art (*e.g.*, ts100K and ts149 adenovirus mutants).

Recombinant Virus Vectors.

The virus vectors of the present invention are useful for the delivery of nucleic acids to cells *in vitro*, *ex vivo*, and *in vivo*. In particular, the virus vectors can be advantageously employed to deliver or transfer nucleic acids to animal cells, including e.g., mammalian cells.

Any heterologous nucleic acid sequence(s) of interest may be delivered in the virus vectors of the present invention. Nucleic acids of interest include nucleic acids encoding polypeptides, including therapeutic (*e.g.*, for medical or veterinary uses) or immunogenic (*e.g.*, for vaccines) polypeptides.

Therapeutic polypeptides include, but are not limited to, cystic fibrosis transmembrane regulator protein (CFTR), dystrophin (including mini- and micro-dystrophins, *see, e.g.*, Vincent *et al.* (1993) *Nature Genetics* 5:130; U.S. Patent Publication No. 2003017131; PCT Publication No. WO/2008/088895, Wang et al. *Proc. Natl. Acad. Sci. USA* 97:13714-13719 (2000); and Gregorevic et al. *Mol. Ther.* 16:657-64 (2008)), myostatin propeptide, follistatin, activin type II soluble receptor, IGF-1, anti-inflammatory polypeptides such as the I kappa B dominant mutant, sarcospan, utrophin (Tinsley *et al.* (1996) *Nature* 384:349), mini-utrophin, clotting factors (*e.g.*, Factor VIII, Factor IX, Factor X, *etc.),* erythropoietin, angiostatin, endostatin, catalase, tyrosine hydroxylase, superoxide dismutase,

leptin, the LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α_1-antitrypsin, adenosine deaminase, hypoxanthine guanine phosphoribosyl transferase, β-glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase A, branched-chain keto acid dehydrogenase, RP65 protein, cytokines (*e.g.*, α-interferon, β-interferon, interferon-γ, interleukin-2, interleukin-4, granulocyte-macrophage colony stimulating factor, lymphotoxin, and the like), peptide growth factors, neurotrophic factors and hormones (*e.g.*, somatotropin, insulin, insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, fibroblast growth factor, nerve growth factor, neurotrophic factor –3 and –4, brain-derived neurotrophic factor, bone morphogenic proteins [including RANKL and VEGF], glial derived growth factor, transforming growth factor –α and –β, and the like), lysosomal acid α-glucosidase, α-galactosidase A, receptors (*e.g.*, the tumor necrosis growth factorα soluble receptor), S100A1, parvalbumin, adenylyl cyclase type 6, a molecule that modulates calcium handling (*e.g.*, $SERCA_{2A}$, Inhibitor 1 of PP1 and fragments thereof [*e.g.*, PCT Publication Nos. WO 2006/029319 and WO 2007/100465]), a molecule that effects G-protein coupled receptor kinase type 2 knockdown such as a truncated constitutively active bARKct, anti-inflammatory factors such as IRAP, anti-myostatin proteins, aspartoacylase, monoclonal antibodies (including single chain monoclonal antibodies; an exemplary Mab being the Herceptin® Mab), neuropeptides and fragments thereof (*e.g.*, galanin, Neuropeptide Y (*see* U.S. Patent No. 7,071,172), angiogenesis inhibitors such as Vasohibins and other VEGF inhibitors (*e.g.*, Vasohibin 2 [*see* PCT Publication WO JP2006/073052]). Other illustrative heterologous nucleic acid sequences encode suicide gene products (*e.g.*, thymidine kinase, cytosine deaminase, diphtheria toxin, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, tumor suppressor gene products (*e.g.*, p53, Rb, Wt-1), TRAIL, FAS-ligand, and any other polypeptide that has a therapeutic effect in a subject in need thereof. AAV vectors can also be used to deliver monoclonal antibodies and antibody fragments, for example, an antibody or antibody fragment directed against myostatin (*see, e.g.*, Fang et al. *Nature Biotechnology* 23:584-590 (2005)).

Heterologous nucleic acid sequences encoding polypeptides include those encoding reporter polypeptides (*e.g.*, an enzyme). Reporter polypeptides are known in the art and include, but are not limited to, Green Fluorescent Protein, β-galactosidase, alkaline phosphatase, luciferase, and chloramphenicol acetyltransferase gene.

Optionally, the heterologous nucleic acid encodes a secreted polypeptide (*e.g.*, a polypeptide that is a secreted polypeptide in its native state or that has been engineered to be secreted, for example, by operable association with a secretory signal sequence as is known in the art).

Alternatively, in particular embodiments of this invention, the heterologous nucleic acid may encode an antisense nucleic acid, a ribozyme (*e.g.*, as described in U.S. Patent No. 5,877,022), RNAs that effect spliceosome-mediated *trans*-splicing (*see*, Puttaraju *et al.* (1999) *Nature Biotech.* 17:246; U.S. Patent No. 6,013,487; U.S. Patent No. 6,083,702), interfering RNAs (RNAi) including siRNA, shRNA or miRNA that mediate gene silencing (*see*, Sharp et al. (2000) *Science* 287:2431), and other non-translated RNAs, such as "guide" RNAs (Gorman *et al.* (1998) *Proc. Nat. Acad. Sci. USA* 95:4929; U.S. Patent No. 5,869,248 to Yuan *et al.*), and the like. Exemplary untranslated RNAs include RNAi against a multiple drug resistance (MDR) gene product (*e.g.*, to treat and/or prevent tumors and/or for administration to the heart to prevent damage by chemotherapy), RNAi against myostatin (e.g., for Duchenne muscular dystrophy), RNAi against VEGF (*e.g.*, to treat and/or prevent tumors), RNAi against phospholamban (*e.g.*, to treat cardiovascular disease, see e.g., Andino et al. *J. Gene Med.* 10:132-142 (2008) and Li et al. *Acta Pharmacol Sin.* 26:51-55 (2005)); phospholamban inhibitory or dominant-negative molecules such as phospholamban S16E (*e.g.*, to treat cardiovascular disease, *see e.g.*, Hoshijima et al. *Nat. Med.* 8:864-871 (2002)), RNAi to adenosine kinase (*e.g.*, for epilepsy), and RNAi directed against pathogenic organisms and viruses (*e.g.*, hepatitis B and/or C virus, human immunodeficiency virus, CMV, herpes simplex virus, human papilloma virus, *etc.*).

Further, a nucleic acid sequence that directs alternative splicing can be delivered. To illustrate, an antisense sequence (or other inhibitory sequence) complementary to the 5' and/or 3' splice site of dystrophin exon 51 can be delivered in conjunction with a U1 or U7 small nuclear (sn) RNA promoter to induce skipping of this exon. For example, a DNA sequence comprising a U1 or U7 snRNA promoter located 5' to the antisense/inhibitory sequence(s) can be packaged and delivered in a modified capsid of the invention.

The virus vector may also comprise a heterologous nucleic acid that shares homology with and recombines with a locus on a host chromosome. This approach can be utilized, for example, to correct a genetic defect in the host cell.

The present invention also provides virus vectors that express an immunogenic polypeptide, *e.g.*, for vaccination. The nucleic acid may encode any immunogen of interest known in the art including, but not limited to, immunogens from human immunodeficiency

virus (HIV), simian immunodeficiency virus (SIV), influenza virus, HIV or SIV gag proteins, tumor antigens, cancer antigens, bacterial antigens, viral antigens, and the like.

The use of parvoviruses as vaccine vectors is known in the art (*see, e.g.*, Miyamura *et al.*, (1994) *Proc. Nat. Acad. Sci USA* 91:8507; U.S. Patent No. 5,916,563 to Young *et al.*, U.S. Patent No. 5,905,040 to Mazzara *et al.*, U.S. Patent No. 5,882,652, U.S. Patent No. 5,863,541 to Samulski *et al.*). The antigen may be presented in the parvovirus capsid. Alternatively, the antigen may be expressed from a heterologous nucleic acid introduced into a recombinant vector genome. Any immunogen of interest as described herein and/or as is known in the art can be provided by the virus vector of the present invention.

An immunogenic polypeptide can be any polypeptide suitable for eliciting an immune response and/or protecting the subject against an infection and/or disease, including, but not limited to, microbial, bacterial, protozoal, parasitic, fungal and/or viral infections and diseases. For example, the immunogenic polypeptide can be an orthomyxovirus immunogen (*e.g.*, an influenza virus immunogen, such as the influenza virus hemagglutinin (HA) surface protein or the influenza virus nucleoprotein, or an equine influenza virus immunogen) or a lentivirus immunogen (*e.g.*, an equine infectious anemia virus immunogen, a Simian Immunodeficiency Virus (SIV) immunogen, or a Human Immunodeficiency Virus (HIV) immunogen, such as the HIV or SIV envelope GP160 protein, the HIV or SIV matrix/capsid proteins, and the HIV or SIV *gag*, *pol* and *env* gene products). The immunogenic polypeptide can also be an arenavirus immunogen (*e.g.*, Lassa fever virus immunogen, such as the Lassa fever virus nucleocapsid protein and/or the Lassa fever envelope glycoprotein), a poxvirus immunogen (*e.g.*, a vaccinia virus immunogen, such as the vaccinia L1 or L8 gene product), a flavivirus immunogen (*e.g.*, a yellow fever virus immunogen or a Japanese encephalitis virus immunogen), a filovirus immunogen (*e.g.*, an Ebola virus immunogen, or a Marburg virus immunogen, such as NP and GP gene products), a bunyavirus immunogen (*e.g.*, RVFV, CCHF, and/or SFS virus immunogens), or a coronavirus immunogen (*e.g.*, an infectious human coronavirus immunogen, such as the human coronavirus envelope glycoprotein, or a porcine transmissible gastroenteritis virus immunogen, or an avian infectious bronchitis virus immunogen). The immunogenic polypeptide can further be a polio immunogen, a herpesvirus immunogen (*e.g.*, CMV, EBV, HSV immunogens) a mumps virus immunogen, a measles virus immunogen, a rubella virus immunogen, a diphtheria toxin or other diphtheria immunogen, a pertussis antigen, a hepatitis (*e.g.*, hepatitis A, hepatitis B, hepatitis C, etc.) immunogen, and/or any other vaccine immunogen now known in the art or later identified as an immunogen.

Alternatively, the immunogenic polypeptide can be any tumor or cancer cell antigen. Optionally, the tumor or cancer antigen is expressed on the surface of the cancer cell. Exemplary cancer and tumor cell antigens are described in S.A. Rosenberg (*Immunity* 10:281 (1991)). Other illustrative cancer and tumor antigens include, but are not limited to: BRCA1 gene product, BRCA2 gene product, gp100, tyrosinase, GAGE-1/2, BAGE, RAGE, LAGE, NY-ESO-1, CDK-4, β-catenin, MUM-1, Caspase-8, KIAA0205, HPVE, SART-1, PRAME, p15, melanoma tumor antigens (Kawakami et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:3515; Kawakami et al. (1994) *J. Exp. Med.*, 180:347; Kawakami et al. (1994) *Cancer Res.* 54:3124), MART-1, gp100 MAGE-1, MAGE-2, MAGE-3, CEA, TRP-1, TRP-2, P-15, tyrosinase (Brichard et al. (1993) *J. Exp. Med.* 178:489); HER-2/neu gene product (U.S. Pat. No. 4,968,603), CA125, LK26, FB5 (endosialin), TAG 72, AFP, CA19-9, NSE, DU-PAN-2, CA50, SPan-1, CA72-4, HCG, STN (sialyl Tn antigen), c-erbB-2 proteins, PSA, L-CanAg, estrogen receptor, milk fat globulin, p53 tumor suppressor protein (Levine, (1993) *Ann. Rev. Biochem.* 62:623); mucin antigens (PCT Publication No. WO 90/05142); telomerases; nuclear matrix proteins; prostatic acid phosphatase; papilloma virus antigens; and/or antigens now known or later discovered to be associated with the following cancers: melanoma, adenocarcinoma, thymoma, lymphoma (*e.g.*, non-Hodgkin's lymphoma, Hodgkin's lymphoma), sarcoma, lung cancer, liver cancer, colon cancer, leukemia, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer, brain cancer and any other cancer or malignant condition now known or later identified (*see, e.g.*, Rosenberg, (1996) *Ann. Rev. Med.* 47:481-91).

As a further alternative, the heterologous nucleic acid can encode any polypeptide that is desirably produced in a cell *in vitro*, *ex vivo*, or *in vivo*. For example, the virus vectors may be introduced into cultured cells and the expressed gene product isolated therefrom.

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

Further, regulated expression of the heterologous nucleic acid(s) of interest can be achieved at the post-transcriptional level, *e.g.*, by regulating selective splicing of different introns by the presence or absence of an oligonucleotide, small molecule and/or other

compound that selectively blocks splicing activity at specific sites (*e.g.*, as described in PCT Publication No. WO 2006/119137).

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

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

Inducible expression control elements are typically advantageous in those applications in which it is desirable to provide regulation over expression of the heterologous nucleic acid sequence(s). Inducible promoters/enhancer elements for gene delivery can be tissue-specific or preferred promoter/enhancer elements, and include muscle specific or preferred (including cardiac, skeletal and/or smooth muscle specific or preferred), neural tissue specific or preferred (including brain-specific or preferred), eye specific or preferred (including retina-specific and cornea-specific), liver specific or preferred, bone marrow specific or preferred, pancreatic specific or preferred, spleen specific or preferred, and/or lung specific or preferred promoter/enhancer elements. Other inducible promoter/enhancer elements include hormone-inducible and metal-inducible elements. Exemplary inducible promoters/enhancer elements include, but are not limited to, a Tet on/off element, a RU486-inducible promoter, an ecdysone-inducible promoter, a rapamycin-inducible promoter, and a metallothionein promoter.

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

The virus vectors according to the present invention provide a means for delivering heterologous nucleic acids into a broad range of cells, including dividing and non-dividing

cells. The virus vectors can be employed to deliver a nucleic acid of interest to a cell *in vitro*, *e.g.*, to produce a polypeptide *in vitro* or for *ex vivo* gene therapy. The virus vectors are additionally useful in a method of delivering a nucleic acid to a subject in need thereof, *e.g.*, to express an immunogenic or therapeutic polypeptide or a functional RNA. In this manner, the polypeptide or functional RNA can be produced *in vivo* in the subject. The subject can be in need of the polypeptide because the subject has a deficiency of the polypeptide. Further, the method can be practiced because the production of the polypeptide or functional RNA in the subject may impart some beneficial effect.

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

In general, the virus vectors of the present invention can be employed to deliver a heterologous nucleic acid encoding a polypeptide or functional RNA to treat and/or prevent any disease state for which it is beneficial to deliver a therapeutic polypeptide or functional RNA. Illustrative disease states include, but are not limited to: cystic fibrosis (cystic fibrosis transmembrane regulator protein) and other diseases of the lung, hemophilia A (Factor VIII), hemophilia B (Factor IX), thalassemia (ß-globin), anemia (erythropoietin) and other blood disorders, Alzheimer's disease (GDF; neprilysin), multiple sclerosis (ß-interferon), Parkinson's disease (glial-cell line derived neurotrophic factor [GDNF]), Huntington's disease (RNAi to remove repeats), amyotrophic lateral sclerosis, epilepsy (galanin, neurotrophic factors), and other neurological disorders, cancer (endostatin, angiostatin, TRAIL, FAS-ligand, cytokines including interferons; RNAi including RNAi against VEGF or the multiple drug resistance gene product, mir-26a [*e.g.*, for hepatocellular carcinoma]), diabetes mellitus (insulin), muscular dystrophies including Duchenne (dystrophin, mini-dystrophin, insulin-like growth factor I, a sarcoglycan [*e.g.*, α, β, γ], RNAi against myostatin, myostatin propeptide, follistatin, activin type II soluble receptor, anti-inflammatory polypeptides such as the Ikappa B dominant mutant, sarcospan, utrophin, mini-utrophin, antisense or RNAi against splice junctions in the dystrophin gene to induce exon skipping [*see e.g.*, PCT Publication No. WO/2003/095647], antisense against U7 snRNAs to induce exon skipping [*see e.g.*, PCT Publication No. WO/2006/021724], and antibodies or antibody fragments against myostatin or myostatin propeptide) and Becker, Gaucher disease (glucocerebrosidase), Hurler's disease (α-L-iduronidase), adenosine deaminase deficiency (adenosine deaminase), glycogen storage diseases (*e.g.*, Fabry disease [α-galactosidase] and

Pompe disease [lysosomal acid α-glucosidase]) and other metabolic disorders, congenital emphysema (α1-antitrypsin), Lesch-Nyhan Syndrome (hypoxanthine guanine phosphoribosyl transferase), Niemann-Pick disease (sphingomyelinase), Tays Sachs disease (lysosomal hexosaminidase A), Maple Syrup Urine Disease (branched-chain keto acid dehydrogenase), retinal degenerative diseases (and other diseases of the eye and retina; *e.g.*, PDGF for macular degeneration and/or vasohibin or other inhibitors of VEGF or other angiogenesis inhibitors to treat/prevent retinal disorders, *e.g.*, in Type I diabetes), diseases of solid organs such as brain (including Parkinson's Disease [GDNF], astrocytomas [endostatin, angiostatin and/or RNAi against VEGF], glioblastomas [endostatin, angiostatin and/or RNAi against VEGF]), liver, kidney, heart including congestive heart failure or peripheral artery disease (PAD) (*e.g.*, by delivering protein phosphatase inhibitor I (I-1) and fragments thereof (*e.g.*, I1C), serca2a, zinc finger proteins that regulate the phospholamban gene, Barkct, β2-adrenergic receptor, β2-adrenergic receptor kinase (BARK), phosphoinositide-3 kinase (PI3 kinase), S100A1, parvalbumin, adenylyl cyclase type 6, a molecule that effects G-protein coupled receptor kinase type 2 knockdown such as a truncated constitutively active bARKct; calsarcin, RNAi against phospholamban; phospholamban inhibitory or dominant-negative molecules such as phospholamban S16E, *etc.*), arthritis (insulin-like growth factors), joint disorders (insulin-like growth factor 1 and/or 2), intimal hyperplasia (*e.g.*, by delivering enos, inos), improve survival of heart transplants (superoxide dismutase), AIDS (soluble CD4), muscle wasting (insulin-like growth factor I), kidney deficiency (erythropoietin), anemia (erythropoietin), arthritis (anti-inflammatory factors such as IRAP and TNFα soluble receptor), hepatitis (α-interferon), LDL receptor deficiency (LDL receptor), hyperammonemia (ornithine transcarbamylase), Krabbe's disease (galactocerebrosidase), Batten's disease, spinal cerebral ataxias including SCA1, SCA2 and SCA3, phenylketonuria (phenylalanine hydroxylase), autoimmune diseases, and the like. The invention can further be used following organ transplantation to increase the success of the transplant and/or to reduce the negative side effects of organ transplantation or adjunct therapies (*e.g.*, by administering immunosuppressant agents or inhibitory nucleic acids to block cytokine production). As another example, bone morphogenic proteins (including BNP 2, 7, *etc.*, RANKL and/or VEGF) can be administered with a bone allograft, for example, following a break or surgical removal in a cancer patient.

The invention can also be used to produce induced pluripotent stem cells (iPS). For example, a virus vector of the invention can be used to deliver stem cell associated nucleic

acid(s) into a non-pluripotent cell, such as adult fibroblasts, skin cells, liver cells, renal cells, adipose cells, cardiac cells, neural cells, epithelial cells, endothelial cells, and the like. Nucleic acids encoding factors associated with stem cells are known in the art. Nonlimiting examples of such factors associated with stem cells and pluripotency include Oct-3/4, the SOX family (*e.g.*, SOX1, SOX2, SOX3 and/or SOX15), the Klf family (*e.g.*, Klf1, Klf2, Klf4 and/or Klf5), the Myc family (*e.g.*, C-myc, L-myc and/or N-myc), NANOG and/or LIN28.

The invention can also be practiced to treat and/or prevent a metabolic disorder such as diabetes (*e.g.*, insulin), hemophilia (*e.g.*, Factor IX or Factor VIII), a lysosomal storage disorder such as a mucopolysaccharidosis disorder (*e.g.*, Sly syndrome [β-glucuronidase], Hurler Syndrome [α-L-iduronidase], Scheie Syndrome [α-L-iduronidase], Hurler-Scheie Syndrome [α-L-iduronidase], Hunter's Syndrome [iduronate sulfatase], Sanfilippo Syndrome A [heparan sulfamidase], B [N-acetylglucosaminidase], C [acetyl-CoA:α-glucosaminide acetyltransferase], D [N-acetylglucosamine 6-sulfatase], Morquio Syndrome A [galactose-6-sulfate sulfatase], B [β-galactosidase], Maroteaux-Lamy Syndrome [N-acetylgalactosamine-4-sulfatase], *etc.*), Fabry disease (α-galactosidase), Gaucher's disease (glucocerebrosidase), or a glycogen storage disorder (*e.g.*, Pompe disease; lysosomal acid α-glucosidase).

Gene transfer has substantial potential use for understanding and providing therapy for disease states. There are a number of inherited diseases in which defective genes are known and have been cloned. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, which may involve regulatory or structural proteins, and which are typically inherited in a dominant manner. For deficiency state diseases, gene transfer can be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer can be used to create a disease state in a model system, which can then be used in efforts to counteract the disease state. Thus, virus vectors according to the present invention permit the treatment and/or prevention of genetic diseases.

The virus vectors according to the present invention may also be employed to provide a functional RNA to a cell *in vitro* or *in vivo*. Expression of the functional RNA in the cell, for example, can diminish expression of a particular target protein by the cell. Accordingly, functional RNA can be administered to decrease expression of a particular protein in a subject in need thereof. Functional RNA can also be administered to cells *in vitro* to regulate gene expression and/or cell physiology, *e.g.*, to optimize cell or tissue culture systems or in screening methods.

In addition, virus vectors according to the instant invention find use in diagnostic and screening methods, whereby a nucleic acid of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

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

As a further aspect, the virus vectors of the present invention may be used to produce an immune response in a subject. According to this embodiment, a virus vector comprising a heterologous nucleic acid sequence encoding an immunogenic polypeptide can be administered to a subject, and an active immune response is mounted by the subject against the immunogenic polypeptide. Immunogenic polypeptides are as described hereinabove. In some embodiments, a protective immune response is elicited.

Alternatively, the virus vector may be administered to a cell *ex vivo* and the altered cell is administered to the subject. The virus vector comprising the heterologous nucleic acid is introduced into the cell, and the cell is administered to the subject, where the heterologous nucleic acid encoding the immunogen can be expressed and induce an immune response in the subject against the immunogen. In particular embodiments, the cell is an antigen-presenting cell (*e.g.*, a dendritic cell).

An "active immune response" or "active immunity" is characterized by "participation of host tissues and cells after an encounter with the immunogen. It involves differentiation and proliferation of immunocompetent cells in lymphoreticular tissues, which lead to synthesis of antibody or the development of cell-mediated reactivity, or both." Herbert B. Herscowitz, *Immunophysiology: Cell Function and Cellular Interactions in Antibody Formation, in* IMMUNOLOGY: BASIC PROCESSES 117 (Joseph A. Bellanti ed., 1985). Alternatively stated, an active immune response is mounted by the host after exposure to an immunogen by infection or by vaccination. Active immunity can be contrasted with passive immunity, which is acquired through the "transfer of preformed substances (antibody, transfer factor, thymic graft, interleukin-2) from an actively immunized host to a non-immune host." *Id.*

A "protective" immune response or "protective" immunity as used herein indicates that the immune response confers some benefit to the subject in that it prevents or reduces the

incidence of disease. Alternatively, a protective immune response or protective immunity may be useful in the treatment and/or prevention of disease, in particular cancer or tumors (*e.g.*, by preventing cancer or tumor formation, by causing regression of a cancer or tumor and/or by preventing metastasis and/or by preventing growth of metastatic nodules). The protective effects may be complete or partial, as long as the benefits of the treatment outweigh any disadvantages thereof.

In particular embodiments, the virus vector or cell comprising the heterologous nucleic acid can be administered in an immunogenically effective amount, as described herein.

The virus vectors of the present invention can also be administered for cancer immunotherapy by administration of a virus vector expressing one or more cancer cell antigens (or an immunologically similar molecule) or any other immunogen that produces an immune response against a cancer cell. To illustrate, an immune response can be produced against a cancer cell antigen in a subject by administering a virus vector comprising a heterologous nucleic acid encoding the cancer cell antigen, for example to treat a patient with cancer and/or to prevent cancer from developing in the subject. The virus vector may be administered to a subject *in vivo* or by using *ex vivo* methods, as described herein. Alternatively, the cancer antigen can be expressed as part of the virus capsid or be otherwise associated with the virus capsid (*e.g.*, as described above).

As another alternative, any other therapeutic nucleic acid (*e.g.*, RNAi) or polypeptide (*e.g.*, cytokine) known in the art can be administered to treat and/or prevent cancer.

As used herein, the term "cancer" encompasses tumor-forming cancers. Likewise, the term "cancerous tissue" encompasses tumors. A "cancer cell antigen" encompasses tumor antigens.

The term "cancer" has its understood meaning in the art, for example, an uncontrolled growth of tissue that has the potential to spread to distant sites of the body (*i.e.*, metastasize). Exemplary cancers include, but are not limited to melanoma, adenocarcinoma, thymoma, lymphoma (*e.g.*, non-Hodgkin's lymphoma, Hodgkin's lymphoma), sarcoma, lung cancer, liver cancer, colon cancer, leukemia, uterine cancer, breast cancer, prostate cancer, ovarian cancer, cervical cancer, bladder cancer, kidney cancer, pancreatic cancer, brain cancer and any other cancer or malignant condition now known or later identified. In representative embodiments, the invention provides a method of treating and/or preventing tumor-forming cancers.

The term "tumor" is also understood in the art, for example, as an abnormal mass of undifferentiated cells within a multicellular organism. Tumors can be malignant or benign. In representative embodiments, the methods disclosed herein are used to prevent and treat malignant tumors.

By the terms "treating cancer," "treatment of cancer" and equivalent terms it is intended that the severity of the cancer is reduced or at least partially eliminated and/or the progression of the disease is slowed and/or controlled and/or the disease is stabilized. In particular embodiments, these terms indicate that metastasis of the cancer is prevented or reduced or at least partially eliminated and/or that growth of metastatic nodules is prevented or reduced or at least partially eliminated.

By the terms "prevention of cancer" or "preventing cancer" and equivalent terms it is intended that the methods at least partially eliminate or reduce and/or delay the incidence and/or severity of the onset of cancer. Alternatively stated, the onset of cancer in the subject may be reduced in likelihood or probability and/or delayed.

In particular embodiments, cells may be removed from a subject with cancer and contacted with a virus vector expressing a cancer cell antigen according to the instant invention. The modified cell is then administered to the subject, whereby an immune response against the cancer cell antigen is elicited. This method can be advantageously employed with immunocompromised subjects that cannot mount a sufficient immune response *in vivo* (*i.e.*, cannot produce enhancing antibodies in sufficient quantities).

It is known in the art that immune responses may be enhanced by immunomodulatory cytokines (*e.g.*, α-interferon, β-interferon, γ-interferon, ω-interferon, τ-interferon, interleukin-1α, interleukin-1β, interleukin-2, interleukin-3, interleukin-4, interleukin 5, interleukin-6, interleukin-7, interleukin-8, interleukin-9, interleukin-10, interleukin-11, interleukin 12, interleukin-13, interleukin-14, interleukin-18, B cell Growth factor, CD40 Ligand, tumor necrosis factor-α, tumor necrosis factor-β, monocyte chemoattractant protein-1, granulocyte-macrophage colony stimulating factor, and lymphotoxin). Accordingly, immunomodulatory cytokines (preferably, CTL inductive cytokines) may be administered to a subject in conjunction with the virus vector.

Cytokines may be administered by any method known in the art. Exogenous cytokines may be administered to the subject, or alternatively, a nucleic acid encoding a cytokine may be delivered to the subject using a suitable vector, and the cytokine produced *in vivo*.

Subjects, Pharmaceutical Formulations, and Modes of Administration.

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

In representative embodiments, the subject is "in need of" the methods of the invention and thus in some embodiments can be a "subject in need thereof."

In particular embodiments, the present invention provides a pharmaceutical composition comprising a virus vector and/or capsid of the invention in a pharmaceutically acceptable carrier and, optionally, other medicinal agents, pharmaceutical agents, stabilizing agents, buffers, carriers, adjuvants, diluents, *etc.* For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid. For inhalation administration, the carrier will be respirable, and optionally can be in solid or liquid particulate form.

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

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

The cell(s) into which the virus vector is introduced can be of any type, including but not limited to neural cells (including cells of the peripheral and central nervous systems, in particular, brain cells such as neurons and oligodendricytes), lung cells, cells of the eye (including retinal cells, retinal pigment epithelium, and corneal cells), epithelial cells (*e.g.*, gut and respiratory epithelial cells), muscle cells (*e.g.*, skeletal muscle cells, cardiac muscle cells, smooth muscle cells and/or diaphragm muscle cells), dendritic cells, pancreatic cells (including islet cells), hepatic cells, myocardial cells, bone cells (*e.g.*, bone marrow stem

cells), hematopoietic stem cells, spleen cells, keratinocytes, fibroblasts, endothelial cells, prostate cells, germ cells, and the like. In representative embodiments, the cell can be any progenitor cell. As a further embodiment, the cell can be a stem cell (*e.g.*, neural stem cell, liver stem cell). As still a further embodiment, the cell can be a cancer or tumor cell. Moreover, the cell can be from any species of origin, as indicated above.

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

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

In some embodiments, the virus vector is introduced into a cell and the cell can be administered to a subject to elicit an immunogenic response against the delivered polypeptide (*e.g.*, expressed as a transgene or in the capsid). Typically, a quantity of cells expressing an immunogenically effective amount of the polypeptide in combination with a pharmaceutically acceptable carrier is administered. An "immunogenically effective amount" is an amount of the expressed polypeptide that is sufficient to evoke an active immune response against the polypeptide in the subject to which the pharmaceutical formulation is administered. In particular embodiments, the dosage is sufficient to produce a protective immune response (as defined above). The degree of protection conferred need not be complete or permanent, as long as the benefits of administering the immunogenic polypeptide outweigh any disadvantages thereof.

A further aspect of the invention is a method of administering the virus vector and/or virus capsid to a subject. Administration of the virus vectors and/or capsids according to the

present invention to a human subject or an animal in need thereof can be by any means known in the art. Optionally, the virus vector and/or capsid can be delivered in a treatment effective or prevention effective dose in a pharmaceutically acceptable carrier.

The virus vectors and/or capsids of the invention can further be administered to elicit an immunogenic response (*e.g.*, as a vaccine). Typically, immunogenic compositions of the present invention comprise an immunogenically effective amount of virus vector and/or capsid in combination with a pharmaceutically acceptable carrier. Optionally, the dosage is sufficient to produce a protective immune response (as defined above).

Dosages of the virus vector and/or capsid to be administered to a subject depend upon the mode of administration, the disease or condition to be treated and/or prevented, the individual subject's condition, the particular virus vector or capsid, the nucleic acid to be delivered, and the like, and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^3, 10^{14}, 10^{15} transducing units, optionally about $10^8 - 10^{13}$ transducing units.

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

Exemplary modes of administration include oral, rectal, transmucosal, intranasal, inhalation (*e.g.*, via an aerosol), buccal (*e.g.*, sublingual), vaginal, intrathecal, intraocular, transdermal, *in utero* (or *in ovo*), parenteral (*e.g.*, intravenous, subcutaneous, intradermal, intramuscular [including administration to skeletal, diaphragm and/or cardiac muscle], intradermal, intrapleural, intracerebral, and intraarticular), topical (*e.g.*, to both skin and mucosal surfaces, including airway surfaces, and transdermal administration), intralymphatic, and the like, as well as direct tissue or organ injection (*e.g.*, to liver, skeletal muscle, cardiac muscle, diaphragm muscle or brain). Administration can also be to a tumor (*e.g.*, in or near a tumor or a lymph node). The most suitable route in any given case will depend on the nature and severity of the condition being treated and/or prevented and on the nature of the particular vector that is being used.

Administration to skeletal muscle according to the present invention includes but is not limited to administration to skeletal muscle in the limbs (*e.g.*, upper arm, lower arm, upper leg, and/or lower leg), back, neck, head (*e.g.*, tongue), thorax, abdomen, pelvis/perineum, and/or digits. Suitable skeletal muscles include but are not limited to abductor digiti minimi (in the hand), abductor digiti minimi (in the foot), abductor hallucis, abductor ossis metatarsi quinti, abductor pollicis brevis, abductor pollicis longus, adductor

brevis, adductor hallucis, adductor longus, adductor magnus, adductor pollicis, anconeus, anterior scalene, articularis genus, biceps brachii, biceps femoris, brachialis, brachioradialis, buccinator, coracobrachialis, corrugator supercilii, deltoid, depressor anguli oris, depressor labii inferioris, digastric, dorsal interossei (in the hand), dorsal interossei (in the foot), extensor carpi radialis brevis, extensor carpi radialis longus, extensor carpi ulnaris, extensor digiti minimi, extensor digitorum, extensor digitorum brevis, extensor digitorum longus, extensor hallucis brevis, extensor hallucis longus, extensor indicis, extensor pollicis brevis, extensor pollicis longus, flexor carpi radialis, flexor carpi ulnaris, flexor digiti minimi brevis (in the hand), flexor digiti minimi brevis (in the foot), flexor digitorum brevis, flexor digitorum longus, flexor digitorum profundus, flexor digitorum superficialis, flexor hallucis brevis, flexor hallucis longus, flexor pollicis brevis, flexor pollicis longus, frontalis, gastrocnemius, geniohyoid, gluteus maximus, gluteus medius, gluteus minimus, gracilis, iliocostalis cervicis, iliocostalis lumborum, iliocostalis thoracis, illiacus, inferior gemellus, inferior oblique, inferior rectus, infraspinatus, interspinalis, intertransversi, lateral pterygoid, lateral rectus, latissimus dorsi, levator anguli oris, levator labii superioris, levator labii superioris alaeque nasi, levator palpebrae superioris, levator scapulae, long rotators, longissimus capitis, longissimus cervicis, longissimus thoracis, longus capitis, longus colli, lumbricals (in the hand), lumbricals (in the foot), masseter, medial pterygoid, medial rectus, middle scalene, multifidus, mylohyoid, obliquus capitis inferior, obliquus capitis superior, obturator externus, obturator internus, occipitalis, omohyoid, opponens digiti minimi, opponens pollicis, orbicularis oculi, orbicularis oris, palmar interossei, palmaris brevis, palmaris longus, pectineus, pectoralis major, pectoralis minor, peroneus brevis, peroneus longus, peroneus tertius, piriformis, plantar interossei, plantaris, platysma, popliteus, posterior scalene, pronator quadratus, pronator teres, psoas major, quadratus femoris, quadratus plantae, rectus capitis anterior, rectus capitis lateralis, rectus capitis posterior major, rectus capitis posterior minor, rectus femoris, rhomboid major, rhomboid minor, risorius, sartorius, scalenus minimus, semimembranosus, semispinalis capitis, semispinalis cervicis, semispinalis thoracis, semitendinosus, serratus anterior, short rotators, soleus, spinalis capitis, spinalis cervicis, spinalis thoracis, splenius capitis, splenius cervicis, sternocleidomastoid, sternohyoid, sternothyroid, stylohyoid, subclavius, subscapularis, superior gemellus, superior oblique, superior rectus, supinator, supraspinatus, temporalis, tensor fascia lata, teres major, teres minor, thoracis, thyrohyoid, tibialis anterior, tibialis posterior, trapezius, triceps brachii, vastus intermedius, vastus lateralis, vastus medialis,

zygomaticus major, and zygomaticus minor, and any other suitable skeletal muscle as known in the art.

The virus vector and/or capsid can be delivered to skeletal muscle by intravenous administration, intra-arterial administration, intraperitoneal administration, limb perfusion, (optionally, isolated limb perfusion of a leg and/or arm; *see e.g.* Arruda et al. (2005) *Blood* 105:3458-3464), and/or direct intramuscular injection. In particular embodiments, the virus vector and/or capsid is administered to a limb (arm and/or leg) of a subject (*e.g.*, a subject with muscular dystrophy such as DMD) by limb perfusion, optionally isolated limb perfusion (*e.g.*, by intravenous or intra-articular administration). In embodiments of the invention, the virus vectors and/or capsids of the invention can advantageously be administered without employing "hydrodynamic" techniques. Tissue delivery (*e.g.*, to muscle) of vectors is often enhanced by hydrodynamic techniques (*e.g.*, intravenous/intravenous administration in a large volume), which increase pressure in the vasculature and facilitate the ability of the vector to cross the endothelial cell barrier. In particular embodiments, the viral vectors and/or capsids of the invention can be administered in the absence of hydrodynamic techniques such as high volume infusions and/or elevated intravascular pressure (*e.g.*, greater than normal systolic pressure, for example, less than or equal to a 5%, 10%, 15%, 20%, 25% increase in intravascular pressure over normal systolic pressure). Such methods may reduce or avoid the side effects associated with hydrodynamic techniques such as edema, nerve damage and/or compartment syndrome.

Administration to cardiac muscle includes administration to the left atrium, right atrium, left ventricle, right ventricle and/or septum. The virus vector and/or capsid can be delivered to cardiac muscle by intravenous administration, intra-arterial administration such as intra-aortic administration, direct cardiac injection (*e.g.*, into left atrium, right atrium, left ventricle, right ventricle), and/or coronary artery perfusion.

Administration to diaphragm muscle can be by any suitable method including intravenous administration, intra-arterial administration, and/or intra-peritoneal administration.

Delivery to a target tissue can also be achieved by delivering a depot comprising the virus vector and/or capsid. In representative embodiments, a depot comprising the virus vector and/or capsid is implanted into skeletal, cardiac and/or diaphragm muscle tissue or the tissue can be contacted with a film or other matrix comprising the virus vector and/or capsid. Such implantable matrices or substrates are described, e.g., in U.S. Patent No. 7,201,898.

In particular embodiments, a virus vector and/or virus capsid according to the present invention is administered to skeletal muscle, diaphragm muscle and/or cardiac muscle (*e.g.*, to treat and/or prevent muscular dystrophy, heart disease [for example, PAD or congestive heart failure]).

In representative embodiments, the invention is used to treat and/or prevent disorders of skeletal, cardiac and/or diaphragm muscle.

In a representative embodiment, the invention provides a method of treating and/or preventing muscular dystrophy in a subject in need thereof, the method comprising: administering a treatment or prevention effective amount of a virus vector of the invention to a mammalian subject, wherein the virus vector comprises a heterologous nucleic acid encoding dystrophin, a mini-dystrophin, a micro-dystrophin, myostatin propeptide, follistatin, activin type II soluble receptor, IGF-1, anti-inflammatory polypeptides such as the Ikappa B dominant mutant, sarcospan, utrophin, a micro-dystrophin, laminin-α2, α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, δ-sarcoglycan, IGF-1, an antibody or antibody fragment against myostatin or myostatin propeptide, and/or RNAi against myostatin. In particular embodiments, the virus vector can be administered to skeletal, diaphragm and/or cardiac muscle as described elsewhere herein.

Alternatively, the invention can be practiced to deliver a nucleic acid to skeletal, cardiac or diaphragm muscle, which is used as a platform for production of a polypeptide (*e.g.*, an enzyme) or functional RNA (*e.g.*, RNAi, microRNA, antisense RNA) that normally circulates in the blood or for systemic delivery to other tissues to treat and/or prevent a disorder (*e.g.*, a metabolic disorder, such as diabetes [*e.g.*, insulin], hemophilia [*e.g.*, Factor IX or Factor VIII], a mucopolysaccharide disorder [*e.g.*, Sly syndrome, Hurler Syndrome, Scheie Syndrome, Hurler-Scheie Syndrome, Hunter's Syndrome, Sanfilippo Syndrome A, B, C, D, Morquio Syndrome, Maroteaux-Lamy Syndrome, *etc.*] or a lysosomal storage disorder such as Gaucher's disease [glucocerebrosidase] or Fabry disease [α-galactosidase A] or a glycogen storage disorder such as Pompe disease [lysosomal acid α glucosidase]). Other suitable proteins for treating and/or preventing metabolic disorders are described herein. The use of muscle as a platform to express a nucleic acid of interest is described in U.S. Patent Publication No. 20020192189.

Thus, as one aspect, the invention further encompasses a method of treating and/or preventing a metabolic disorder in a subject in need thereof, the method comprising: administering a treatment or prevention effective amount of a virus vector of the invention to

skeletal muscle of a subject, wherein the virus vector comprises a heterologous nucleic acid encoding a polypeptide, wherein the metabolic disorder is a result of a deficiency and/or defect in the polypeptide. Illustrative metabolic disorders and heterologous nucleic acids encoding polypeptides are described herein. Optionally, the polypeptide is secreted (*e.g.*, a polypeptide that is a secreted polypeptide in its native state or that has been engineered to be secreted, for example, by operable association with a secretory signal sequence as is known in the art). Without being limited by any particular theory of the invention, according to this embodiment, administration to the skeletal muscle can result in secretion of the polypeptide into the systemic circulation and delivery to target tissue(s). Methods of delivering virus vectors to skeletal muscle are described in more detail herein.

The invention can also be practiced to produce antisense RNA, RNAi or other functional RNA (*e.g.*, a ribozyme) for systemic delivery.

The invention also provides a method of treating and/or preventing congenital heart failure or PAD in a subject in need thereof, the method comprising administering a treatment or prevention effective amount of a virus vector of the invention to a mammalian subject, wherein the virus vector comprises a heterologous nucleic acid encoding, for example, a sarcoplasmic endoreticulum Ca^{2+}-ATPase (SERCA2a), an angiogenic factor, phosphatase inhibitor I (I-1) and fragments thereof (*e.g.*, I1C), RNAi against phospholamban; a phospholamban inhibitory or dominant-negative molecule such as phospholamban S16E, a zinc finger protein that regulates the phospholamban gene, β2-adrenergic receptor, β2-adrenergic receptor kinase (BARK), PI3 kinase, calsarcan, a β-adrenergic receptor kinase inhibitor (βARKct), inhibitor 1 of protein phosphatase 1 and fragments thereof (*e.g.*, I1C), S100A1, parvalbumin, adenylyl cyclase type 6, a molecule that effects G-protein coupled receptor kinase type 2 knockdown such as a truncated constitutively active bARKct, Pim-1, PGC-1α, SOD-1, SOD-2, EC-SOD, kallikrein, HIF, thymosin-β4, mir-1, mir-133, mir-206, mir-208 and/or mir-26a.

Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus vector and/or virus capsids of the invention in a local rather than systemic manner, for example, in a depot or sustained-release formulation. Further, the virus vector and/or virus capsid can be delivered adhered to a surgically implantable matrix (*e.g.*, as described in U.S. Patent Publication No. 20040013645).

The virus vectors and/or virus capsids disclosed herein can be administered to the lungs of a subject by any suitable means, optionally by administering an aerosol suspension of respirable particles comprised of the virus vectors and/or virus capsids, which the subject inhales. The respirable particles can be liquid or solid. Aerosols of liquid particles comprising the virus vectors and/or virus capsids may be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in the art. *See e.g.*, U.S. Patent No. 4,501,729. Aerosols of solid particles comprising the virus vectors and/or capsids may likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

The virus vectors and virus capsids can be administered to tissues of the central nervous system (CNS) (*e.g.*, brain, eye) and may advantageously result in broader distribution of the virus vector or capsid than would be observed in the absence of the present invention.

In particular embodiments, the delivery vectors of the invention may be administered to treat diseases of the CNS, including genetic disorders, neurodegenerative disorders, psychiatric disorders and tumors. Illustrative diseases of the CNS include, but are not limited to Alzheimer's disease, Parkinson's disease, Huntington's disease, Canavan disease, Leigh's disease, Refsum disease, Tourette syndrome, primary lateral sclerosis, amyotrophic lateral sclerosis, progressive muscular atrophy, Pick's disease, muscular dystrophy, multiple sclerosis, myasthenia gravis, Binswanger's disease, trauma due to spinal cord or head injury, Tay Sachs disease, Lesch-Nyan disease, epilepsy, cerebral infarcts, psychiatric disorders including mood disorders (*e.g.*, depression, bipolar affective disorder, persistent affective disorder, secondary mood disorder), schizophrenia, drug dependency (*e.g.*, alcoholism and other substance dependencies), neuroses (*e.g.*, anxiety, obsessional disorder, somatoform disorder, dissociative disorder, grief, post-partum depression), psychosis (*e.g.*, hallucinations and delusions), dementia, paranoia, attention deficit disorder, psychosexual disorders, sleeping disorders, pain disorders, eating or weight disorders (*e.g.*, obesity, cachexia, anorexia nervosa, and bulemia) and cancers and tumors (*e.g.*, pituitary tumors) of the CNS.

Disorders of the CNS include ophthalmic disorders involving the retina, posterior tract, and optic nerve (*e.g.*, retinitis pigmentosa, diabetic retinopathy and other retinal degenerative diseases, uveitis, age-related macular degeneration, glaucoma).

Most, if not all, ophthalmic diseases and disorders are associated with one or more of three types of indications: (1) angiogenesis, (2) inflammation, and (3) degeneration. The delivery vectors of the present invention can be employed to deliver anti-angiogenic factors;

anti-inflammatory factors; factors that retard cell degeneration, promote cell sparing, or promote cell growth and combinations of the foregoing.

Diabetic retinopathy, for example, is characterized by angiogenesis. Diabetic retinopathy can be treated by delivering one or more anti-angiogenic factors either intraocularly (*e.g.*, in the vitreous) or periocularly (*e.g.*, in the sub-Tenon's region). One or more neurotrophic factors may also be co-delivered, either intraocularly (*e.g.*, intravitreally) or periocularly.

Uveitis involves inflammation. One or more anti-inflammatory factors can be administered by intraocular (*e.g.*, vitreous or anterior chamber) administration of a delivery vector of the invention.

Retinitis pigmentosa, by comparison, is characterized by retinal degeneration. In representative embodiments, retinitis pigmentosa can be treated by intraocular (*e.g.*, vitreal administration) of a delivery vector encoding one or more neurotrophic factors.

Age-related macular degeneration involves both angiogenesis and retinal degeneration. This disorder can be treated by administering the inventive delivery vectors encoding one or more neurotrophic factors intraocularly (*e.g.*, vitreous) and/or one or more anti-angiogenic factors intraocularly or periocularly (*e.g.*, in the sub-Tenon's region).

Glaucoma is characterized by increased ocular pressure and loss of retinal ganglion cells. Treatments for glaucoma include administration of one or more neuroprotective agents that protect cells from excitotoxic damage using the inventive delivery vectors. Such agents include N-methyl-D-aspartate (NMDA) antagonists, cytokines, and neurotrophic factors, delivered intraocularly, optionally intravitreally.

In other embodiments, the present invention may be used to treat seizures, *e.g.*, to reduce the onset, incidence and/or severity of seizures. The efficacy of a therapeutic treatment for seizures can be assessed by behavioral (*e.g.*, shaking, ticks of the eye or mouth) and/or electrographic means (most seizures have signature electrographic abnormalities). Thus, the invention can also be used to treat epilepsy, which is marked by multiple seizures over time.

In one representative embodiment, somatostatin (or an active fragment thereof) is administered to the brain using a delivery vector of the invention to treat a pituitary tumor. According to this embodiment, the delivery vector encoding somatostatin (or an active fragment thereof) is administered by microinfusion into the pituitary. Likewise, such treatment can be used to treat acromegaly (abnormal growth hormone secretion from the pituitary). The nucleic acid sequences (*e.g.*, GenBank Accession No. J00306) and amino acid

sequences (*e.g.*, GenBank Accession No. P01166; contains processed active peptides somatostatin-28 and somatostatin-14) of somatostatins are known in the art.

In particular embodiments, the vector can comprise a secretory signal as described, e.g., in U.S. Patent No. 7,071,172.

In representative embodiments of the invention, the virus vector and/or virus capsid is administered to the CNS (*e.g.*, to the brain or to the eye). The virus vector and/or capsid may be introduced into the spinal cord, brainstem (medulla oblongata, pons), midbrain (hypothalamus, thalamus, epithalamus, pituitary gland, substantia nigra, pineal gland), cerebellum, telencephalon (corpus striatum, cerebrum including the occipital, temporal, parietal and frontal lobes. cortex, basal ganglia, hippocampus and portaamygdala), limbic system, neocortex, corpus striatum, cerebrum, and/or inferior colliculus. The virus vector and/or capsid may also be administered to different regions of the eye such as the retina, cornea and/or optic nerve.

The virus vector and/or capsid may be delivered into the cerebrospinal fluid (*e.g.*, by lumbar puncture) for more disperse administration of the delivery vector. The virus vector and/or capsid may further be administered intravascularly to the CNS in situations in which the blood-brain barrier has been perturbed (*e.g.*, brain tumor or cerebral infarct).

The virus vector and/or capsid can be administered to the desired region(s) of the CNS by any route known in the art, including but not limited to, intrathecal, intracerebral, intraventricular, intravenous (*e.g.*, in the presence of a sugar such as mannitol), intranasal, intra-aural, intra-ocular (*e.g.*, intra-vitreous, sub-retinal, anterior chamber) and peri-ocular (*e.g.*, sub-Tenon's region) delivery as well as intramuscular delivery with retrograde delivery to motor neurons.

In particular embodiments, the virus vector and/or capsid is administered in a liquid formulation by direct injection (*e.g.*, stereotactic injection) to the desired region or compartment in the CNS. In other embodiments, the virus vector and/or capsid may be provided by topical application to the desired region or by intra-nasal administration of an aerosol formulation. Administration to the eye may be by topical application of liquid droplets. As a further alternative, the virus vector and/or capsid may be administered as a solid, slow-release formulation (*see, e.g.,* U.S. Patent No. 7,201,898).

In yet additional embodiments, the virus vector can used for retrograde transport to treat and/or prevent diseases and disorders involving motor neurons (e.g., amyotrophic lateral sclerosis (ALS); spinal muscular atrophy (SMA), etc.). For example, the virus vector can be delivered to muscle tissue from which it can migrate into neurons.

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

EXAMPLES: Engineering of dual glycan binding AAV

Structural modeling. Coordinates for the AAV2 and AAV9 viral protein (VP) crystal structures were obtained from RCSB Protein Databank (PDB accession# 1LP3 and 3UX1, respectively)[30,31]. Using the SWISS-MODEL protein structure modeling server (http://swissmodel.expasy.org/)[32], homology models of the 2G9 VP3 monomer were generated with crystal structures of AAV2 VP3 as template. A three-dimensional icosahedral model of an intact 2G9 capsid was created using the Oligomer Generator utility in VIPERdb-Virus Particle ExploreR2[33]. Similarly, illustration of the AAV2 VP3 trimer, 2G9 trimer, and AAV9 trimer were obtained using the Oligomer Generator utility. All structural models were visualized using PyMOL with residues forming the galactose binding site (AAV9 VP1 numbering: D271, N272, Y446, N470, A472, V473, W503)[13] and heparan sulfate binding site (AAV2 VP1 numbering: R487, K527, K532, R585, R588)[10-12,34] highlighted in orange and purple, respectively. Different monomers were colored in pale green, light blue and light pink.

Generation of dual glycan binding AAV strains. Helper plasmids pXR1, 2, 6, 8 and 9 were obtained from UNC vector core. The prototypical pXR2G9 chimera plasmid construct was generated by substituting amino acid residues directly involved or flanking the Gal recognition site on the AAV9 capsid protein subunit onto corresponding residues on the capsid subunit of AAV2 (AAV2 VP1 numbering: A266S, Q464V, A467P, D469N, I470M, R471A, D472V, S474G, Y500F, S501A). Substitutions were generated using the QuikChange® Lightning site-directed mutagenesis kit (Agilent) using the following primers (IDT): 5'- GGAACCACCA CGCAGTCAAG GCTTCAGTTT TCTGTGGCCG GACCCAGTAA CATGGCTGTC CAGGGAAGGA ACTGGCTTCCT GGACCCTGTT ACCGC-3' and 5'- GACATCTGCG GATAACAACA ACAGTGAATTT GCTTGGACTG GAGCTACCAA GTACCACCT-3'. Recombinant AAV vectors packaging the CBA-Luc transgene cassettes were generated as described previously[14]. Viral titers were obtained by quantitative PCR.

***In vitro* binding, transduction and competitive inhibition assays.** CHO Lec2 cells were cultured in αMEM (Thermo Scientific) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin (Cellgro), 100 μg/ml of streptomycin (Cellgro), and 2.5 μg/ml

of amphotericin B (Sigma). Cells were seeded at a density of $1x10^5$ cells/well in 24 well plates. For competitive inhibition assays, cells were pre-chilled at 4°C for 30 minutes and incubated with 100 μg/ml of FITC-labeled *Erythrina Cristagalli* Lectin (FITC-ECL, Vector Labs) in αMEM at 4°C for 1 hour. Alternatively, different viral capsids were incubated with 100μg/ml of soluble heparin (Sigma) or 1xPBS (control) at room temperature for 1 hour. Mock-treated or FITC-ECL treated cells were then infected with HS-bound or mock-treated AAV2, AAV2G9, or AAV9 capsids packaging a CBA-Luc transgene cassette at an MOI of 1000 vector genome (vg) copies/cell. Following incubation in the cold room for 1 hour, unbound virions were removed by three washes with ice cold 1xPBS. For cell surface binding assays, the number of bound virions was measured by quantifying vector genome copy numbers/cell in each well using quantitative PCR. For transduction assays, infected Lec2 cells were moved to 37°C and incubated for 24 hours prior to quantitation of luciferase transgene expression from cell lysates.

For competitive inhibition with parental AAV2 or AAV9 capsids, vectors packaging CBA promoter-driven tdTomato transgene cassette were utilized. Briefly, Lec2 cells were seeded in 24 well plates overnight at a density of $1x10^5$ cells/well. After being pre-chilled at 4°C for 30 minutes, Lec2 cells were pre-incubated with either AAV2-tdTomato or AAV9-tdTomato vectors at multiplicities of infection (MOI) ranging from 500 to 100,000 vg/cell at 4°C for another 30 minutes. Cells were then super-infected with AAV2G9-CBA-Luc at an MOI of 1000 vg/cell for 45 minutes at 4°C, followed by removal of unbound virions using ice cold PBS. Infected cells were then incubated at 37°C for 24 hours prior to luciferase expression analysis. Controls included AAV2-CBA-Luc or AAV9-CBA-Luc vectors.

Kinetics of transgene expression *in vivo*. Female BALB/c mice (6-8 weeks old) were purchased from Jackson Laboratories and handled in accordance with NIH guidelines using IACUC approved protocols at UNC Chapel Hill. Different AAV vectors packaging the CBA-Luc cassette were injected intravenously into the tail vein at a dose of $1x10^{11}$ vg/mouse. At indicated time intervals post-administration (3, 7, and 18 days), mice were intraperitoneally injected with luciferin (120mg/kg; Nanolight) and bioluminescent images obtained using a Xenogen IVIS® Lumina system (Caliper Lifesciences). Quantitation of light output from liver and whole animal images was carried out using WAVEMETRICS® software. Further quantitation of luciferase transgene expression and vector genome biodistribution in different tissues was carried out in two different groups of mice that were sacrificed at days 3 and 18 post-vector administration. Luciferase transgene expression was monitored in different tissue lysates as described earlier. Vector genome biodistribution was

determined by first extracting genomic DNA from tissue lysates using a DNeasy® Kit (Qiagen). Luciferase transgene copy number was determined using qPCR and normalized to the number of copies of the mouse lamin gene to determine vg/cell in each tissue. Specific primer sets were 5'-AGGGCACCTC CATCTCGGAA AC-3' / 5'-GGACCCAAGG ACTACCTCAA GGG-3' (for mouse lamin) and 5'-AAAAGCACTC TGATTGACAA ATAC-3'/ 5'-CCTTCGCTTC AAAAAATGGA AC-3'(for CBA-Luc) , respectively.

Statistical Analysis. All data is expressed as mean ± standard error mean and the number of replicates for each experiment is provided in the corresponding figure legends. Statistical significance was determined using the unpaired one-tail student's *t*-test and p-values less than 0.05 considered statistically significant for different experiments unless indicated otherwise.

Results

To explore the feasibility of "grafting" the Gal footprint of AAV9 onto several AAV strains, we first compared the three-dimensional structures of VP3 subunit trimers of AAV serotypes 1, 2, 6 and 8 in alignment with that of AAV9 (**Figure 1**). Amino acid residues on the template capsids that overlapped with corresponding AAV9 VP3 residues directly involved in binding or immediately flanking the Gal receptor footprint were modified by multiple rounds of site-directed mutagenesis. All of the chimeric AAV strains generated were prepared as recombinant vectors packaging a chicken beta-actin promoter driven firefly luciferase (CBA-Luc) reporter transgene cassette using previously established protocols[14]. Amino acid residues involved in Gal recognition and other flanking residues from AAV9 were remarkably well tolerated on different AAV serotype capsids as the packaging efficiencies of these AAV chimeras are comparable with parental strains. Multiple AAV chimeras based on AAV serotypes 1, 2, 6, 8 and the previously engineered AAV2i8 mutant[15] were obtained (at titers ranging from 5×10^{11} to 5×10^{12} viral genome copies/mL) and observed to exploit Gal as a novel primary receptor in transducing CHO Lec2 cells *in vitro* (**Figure 2**). We then carried out a detailed characterization of a prototypical dual glycan binding AAV chimera, dubbed AAV2G9 (where G stands for the Gal footprint and the numbers identify the recipient and donor capsid serotypes, respectively).

Three-dimensional models of synthetically engineered AAV2G9 (full capsid in **Figure 3A** and VP3 trimer in **Figure 3D**) with the putative dual glycan receptor binding sites (HS and Gal) highlighted were generated by homology modeling using Swiss Model®. The molecular model of AAV2G9 full capsids demonstrates the geometrical distribution and orthogonality of HS and Gal binding sites located around the three-fold symmetry axis on the

icosahedral capsid. Close-up views of HS and Gal receptor footprints from the three-fold axes further support the observation that grafting orthogonal Gal binding sites on the backbone of AAV2 capsid can be tolerated with regard to capsid assembly. Three-dimensional structures of the AAV2 VP3 subunit trimer with side chains of positively charged residues involved in HS recognition (**Figure 3A**) as well as the side chains of amino acid residues comprising the Gal recognition site on the AAV9 VP3 subunit trimer (**Figure 3C**) are also shown.

AAV2G9 exploits HS and Gal receptors interchangeably *in vitro*. The first line of evidence supporting the usage of dual glycan receptors by AAV2G9 was obtained from competitive inhibition assays of virus binding on cell surface involving soluble heparin and *Erythrina Cristagalli* lectin (ECL), which selectively binds terminally galactosylated glycans. As seen in **Figures 4A-B**, HS, but not ECL significantly inhibits AAV2 transduction in CHO Lec2 cells (dark grey bars), while ECL selectively blocks AAV9 transduction by nearly two log units (white bars). These results are consistent with the expected transduction profiles for AAV2 and AAV9[16-18]. In contrast, AAV2G9 can only be effectively neutralized by pre-treatment with a combination of both ECL and HS (light grey bars, **Figure 4C**). A small, yet significant inhibitory effect is observed for ECL.

Transduction profiles for AAV2 and AAV9 were further corroborated by inhibition of cell surface binding by each strain using ECL or HS (**Figures 4D-E**). The unique cell surface attachment of the chimeric AAV strain is further supported by competitive inhibition of cell surface attachment of AAV2G9 exclusively by a combination of ECL and HS, but neither reagent alone (**Figure 4F**). In addition, confocal immunofluorescence micrographs (**Figure 5**) obtained using monoclonal antibodies against different AAV capsids suggest that AAV2G9 binds more robustly to the surface of CHO Lec2 cells than AAV2 or AAV9. Such a scenario can be expected based on the apparent ability of AAV2G9 to bind two different glycans interchangeably.

In order to further interrogate the exploitation of alternate transduction pathways by AAV2G9, we conducted competition assays with the parental serotypes, AAV2 and AAV9. As shown in **Figures 6A-B**, pre-incubation with AAV2-CBA-tdTom or AAV9-CBA-tdTom competing vectors at MOIs ranging from 500 to 100,000 vg/cell efficiently blocks transduction by AAV2-CBA-Luc or AAV9-CBA-Luc, respectively as measured by luciferase transgene expression. However, both AAV2 and AAV9 are unable to effectively block AAV2G9 transduction at 10-fold excess multiplicities of infection (MOI). At higher MOI (100-fold excess), AAV2 appears to compete less effectively than AAV9 in neutralizing AAV2G9 transduction. Taken together, these results support the notion that AAV2G9 is

indeed a novel, dual glycan binding strain with the unique ability to exploit both HS and Gal as primary receptors for transduction.

AAV2G9 mediates rapid onset of transgene expression. We then investigated whether dual glycan binding confers specific advantages to viral transduction *in vitro* and *in vivo*. Monitoring the time course of luciferase reporter expression in CHO Lec2 cells revealed that AAV2G9 mediates rapid onset and improved gene transfer *in vitro* (**Figure 7**). Live animal imaging studies were then carried out to monitor luciferase transgene expression following systemic administration of different AAV strains in BALB/c mice (**Figure 8A**). Bioluminescent images and quantitative assessment of light output within the liver and the whole animal obtained at days 3, 7 and 18 post-injection correlate with *in vitro* data and support the notion that AAV2G9 can mediate rapid onset and enhanced gene expression (**Figures 8B-C**). Interestingly, the kinetic profile displayed by AAV2G9 mirrors that of AAV9 but not AAV2. In contrast, the transduction profile/tissue tropism of AAV2G9 appears to be primarily hepatotropic, similar to AAV2 and unlike the systemic tropism displayed by AAV9 as established previously[4,19-21]. Thus, dual glycan receptor engagement appears to improve the transduction efficiency of AAV strains, but does not alter tissue tropism.

Transduction and biodistribution profile of AAV2G9 vectors *in vivo*. To further evaluate the *in vivo* transduction and biodistribution profiles of AAV2G9, quantitative analysis of tissue lysates from BALB/c mice were carried out at days 3 and 18 post-administration. Specifically, AAV2G9 displays markedly higher luciferase transgene expression in liver compared to AAV2 (nearly two log units) and AAV9 (~1 log unit) at 3 days post-administration (**Figure 9A**). While AAV9 displays more than 10-fold higher transduction efficiency in heart than AAV2G9, a modest increase in cardiac transduction by AAV2G9 compared to AAV2 is also observed. At day 18, cardiac and liver tissues harvested from mice treated with AAV2G9 continue to demonstrate higher transgene expression, although AAV9 emerges as the most efficient strain at this stage. Specifically, transduction efficiencies in cardiac tissue by AAV2, AAV2G9, and AAV9 maintain a similar trend as observed 3 days post-administration. In the liver, the differences between luciferase transgene expression by AAV2, AAV2G9, and AAV9 diminish upon progressing to 18 days post-injection. Specifically, AAV9 demonstrates between 5 to 10-fold higher transgene expression when compared to AAV2G9 and AAV2, respectively.

Quantitative analysis of vector genome copy numbers in liver and heart by AAV2G9 and the parental AAV strains at 3 days post-administration (**Figure 9B**) is consistent with the trends observed for transduction efficiencies shown in **Figure 5A**. Specifically, AAV2G9

accumulated to a higher extent in cardiac tissue compared to AAV2, but was still ~2 log units lower than AAV9. In liver, AAV2G9 copy number is comparable to that of AAV9, but over one log unit higher than AAV2. At day 18, copy numbers for all serotypes were decreased presumably due to continuous cell turnover and degradation of single-stranded AAV genomes as reported previously[22,23].

Figure 10 shows *in vivo* transgene expression kinetics of AAV2i8, 2i8G9, and AAV9 vectors packaging CBA-luciferase transgene cassette. BALB/c mice (n=4) were administered AAV vectors at a dose of $1x10^{11}$ vg/animal through the tail vein and bioluminescence images were collected at 3, 7, and 18 days post-injection using a Xenogen® Lumina imaging system. Representative live animal images are shown with bioluminescence expressed on a rainbow colored scale (10^5-10^6 photons/second/cm^2/steradian).

Figure 11 shows central nervous system (CNS) tropism profiles of representative AAV G9 strains in neonatal mice. Postnatal 0 (P0) pups (n=3) were unilaterally injected into the left cerebral ventricle with 3.5x10e9 AAV vector genomes containing a GFP transgene driven by a hybrid chicken beta actin (CBh) promoter. At 2 wks post injection, GFP immunohistochemistry revealed differential spread, regional and cellular tropisms for each AAV "G9" strain within the murine brain.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof.

Table 1. AAV Genomes

Table 1 Complete Genomes	GenBank® Accession Number		GenBank® Accession Number			GenBank® Accession Number
		Hu T88	AY695375		**Clade E**	
AAV1	NC_002077,AF063497	**Hu T71**	AY695374		Rh38	AY530558
AAV2	NC_001401	**Hu T70**	AY695373		Hu66	AY530626
AAV 3	NC_001729	**Hu T40**	AY695372		Hu42	AY530605
AAV3B	NC_001863	**Hu T32**	AY695371		Hu67	AY530627
AAV4	NC_001829	Hu T17	AY695370		Hu40	AY530603
AAV5	Y18065, AF085716	Hu LG15	AY695377		Hu41	AY530604
AAV6	NC_001862				Hu37	AY530600
AAV	AY186198, AY629583, NC_004828	**Clade C**			Rh40	AY530559
Avian AAV strain DA-1	NC_006263, AY629583	Hu9	AY530629		Rh2	AY243007
Bovine AAV	NC_005889, AY388617	Hu10	AY530576		Bb1	AY243023
		Hu11	AY530577		Bb2	AY243022
Clade A		Hu53	AY530615		Rh10	AY243015
AAV1	NC_002077,AF063497	Hu55	AY530617		Hu17	AY530582
AAV6	NC_001862	Hu54	AY530616		Hu6	AY530621
Hu.48	AY530611	Hu7	AY530628		Rh25	AY530557
Hu 43	AY530606	Hu18	AY530583		Pi2	AY530554
Hu 44	AY530607	Hu15	AY530580		Pi1	AY530553
Hu 46	AY530609	Hu16	AY530581		Pi3	AY530555
		Hu25	AY530591		Rh57	AY530569
Clade B		Hu60	AY530622		Rh50	AY530563
Hu. 19	AY530584	Ch5	AY243021		Rh49	AY530562
Hu. 20	AY530586	Hu3	AY530595		Hu39	AY530601
Hu 23	AY530589	Hu1	AY530575		Rh58	AY530570
Hu22	AY530588	Hu4	AY530602		Rh61	AY530572
Hu24	AY530590	Hu2	AY530585		Rh52	AY530565
Hu21	AY530587	Hu61	AY530623		Rh53	AY530566
Hu27	AY530592				Rh51	AY530564
Hu28	AY530593	**Clade D**			Rh64	AY530574
Hu 29	AY530594	Rh62	AY530573		Rh43	AY530560
Hu63	AY530624	Rh48	AY530561		**AAV8**	AF513852
Hu64	AY530625	Rh54	AY530567		Rh8	AY242997
Hu13	AY530578	Rh55	AY530568		Rh1	AY530556
Hu56	AY530618	Cy2	AY243020			
Hu57	AY530619	**AAV7**	AF513851		**Clade F**	
Hu49	AY530612	Rh35	AY243000		Hu14 **(AAV9)**	AY530579
Hu58	AY530620	Rh37	AY242998		Hu31	AY530596
Hu34	AY530598	Rh36	AY242999		Hu32	AY530597
Hu35	AY530599	Cy6	AY243016			
AAV2	NC_001401	Cy4	AY243018		**Clonal Isolate**	
Hu45	AY530608	Cy3	AY243019		**AAV5**	Y18065, AF085716
Hu47	AY530610	Cy5	AY243017		**AAV 3**	NC_001729
Hu51	AY530613	Rh13	AY243013		**AAV 3B**	NC_001863
Hu52	AY530614				**AAV4**	NC_001829
Hu T41	AY695378				Rh34	AY243001
Hu S17	AY695376				Rh33	AY243002
					Rh32	AY243003

Table 2. Exemplary AAV Genome and Capsid Accession Nos.

Virus and Serotype	Genome Accession No.	Capsid/VP1 Accession No.
AAV1	NC_002077.1	NP_049542.1
AAV2	NC_001401.2	YP_680426.1
AAV3A	NC_001729.1	NP_043941.1
AAV3B	NC_001863.1	NP_045760.1
AAV4	NC_001829.1	NP_044927.1
AAV5	NC_006152.1	YP_068409.1
AAV6	NC_001862.1	NP_045758.1
AAV7	AF513851.1	AAN03855.1
AAV8	AF513852.1	AAN03857.1
AAV9	AY530579.1	AAS99264.1
AAV10	AY631965.1*	AAT46337.1
AAV11	AY631966.1*	AAT46339.1
AAV13	EU285562.1	ABZ10812.1

* Incomplete sequence

Table 3. Amino acid positions of mutations to graft the Gal binding footprint of AAV9 into different AAV strains

AAV Strain	Accession No.	Mutations to Graft Galactose Binding Footprint
AAV1	NP_049542.1	A267S, SRGSPAGMSVQPK464-476SVAGPSNMAVQGR, NFTW500-503EFAW
AAV2	YP_680426.1	A266S, SQAGASDIRDQSR463-475SVAGPSNMAVQGR, EYSW499-502EFAW
AAV3a	NP_043941.1	A266S, SQAGPQSMSLQAR464-476SVAGPSNMAVQGR, NFPW500-503EFAW
AAV3b	NP_045760.1	A266S, SQAGPQSMSLQAR464-476SVAGPSNMAVQGR, NFPW500-503EFAW
AAV4	NP_044927.1	insert SSND before N261, TKLRPTNFSNFKK458-470SVAGPSNMAVQGR, DSLI499-502EFAW
AAV5	YP_068409.1	G257S, NKNLAGRYANTYK450-463SVAGPSNMAVQGR, VSAF486-489EFAW
AAV6	NP_045758.1	A267S, SRGSPAGMSVQPK464-476SVAGPSNMAVQGR, NFTW500-503EFAW
AAV7	AAN03855.1	VQGGPSTMAEQAK466-478SVAGPSNMAVQGR, NFAW502-505EFAW
AAV8	AAN0857.1	A269S,SQGGPNTNANQAK466-478SVAGPSNMAVQGR, NFAW502-505EFAW
AAV9	**AAS99264.1**	**DONOR STRAIN**
AAV10	AAT46337.1	T270S, SQAGPANMSAQAK466-478SVAGPSNMAVQGR, N502E
Avian AAV	NP_852781.1	D273S, N275D, insert ANS before D273, SRATKTNMAAQYR467-479SVAGPSNMAVQGR, FSVW505-508EFAW
BB1	AAO88209.1	T270S, SQAGPNNMSAQAR466-478SVAGPSNMAVQGR, N502E
BB2	AAO88208.1	T270S, SQAGPNNMSAQAR466-478SVAGPSNMAVQGR, N502E
CH5	AAO88207.1	AT266-267SS, SQAGPSSMAQQAK463-475SVAGPSNMAVQGR
CY2	AAO88206.1	T269S, YQGGPSTMAEQAK466-478SVAGPSNMAVQGR, N502E
CY3	AAO88205.1	AT262-263SS, HQAGPNTMAEQSK457-469SVAGPSNMAVQGR, N493E
CY4	AAO88204.1	AT262-263SS, HQAGPNTVAEQSK457-469SVAGPSNMAVQGR, N493E
CY5	AAO88203.1	AT262-263SS, HQAGPNTMAEQSK457-469SVAGPSNMAVQGR, N493E
CY6	AAO88202.1	AT262-263SS, HQAGPNTMAEQSK457-469SVAGPSNMAVQGR, N493E
Hu LG15	AAU05371.1	A266S, QAGASDIRDQWR464-475VAGPSNMAVGQR, DYS499-501EFA
Hu S17	AAU05370.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu T17	AAU05358.1	A266S, QAGASDIRDQWR464-475VAGPSNMAVGQR, DYS499-501EFA
Hu T41	AAU05372.1	A266S, QAGASDIRDQWR464-475VAGPSNMAVGQR, DYS499-501EFA
Hu T70	AAU05364.1	A266S, QAGASDIRDQWR464-475VAGPSNMAVGQR, EYS499-501EFA
Hu T71	AAU05366.1	A266S, QAGASDIRDQWR464-475VAGPSNMAVGQR, DYS499-501EFA
Hu T88	AAU05368.1	A266S, QAGASDIRDQWR464-475VAGPSNMAVGQR, DYS499-501EFA
Hu1	AAS99260.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu2	AAS99270.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu3	AAS99280.1	AC267-268SS, QAGPTNMSLQAK465-476VAGPSNMAVQGR, NFP500-502EFA
Hu4	AAS99287.1	A266S, QAGPTNMSLQAK464-475VAGPSNMAVQGR, NFP499-501EFA
Hu6	AAS99306.1	T270S, SQAGPNNMSAQAK466-478SVAGPSNMAVQGR, N502E
Hu7	AAS99313.1	A266S, SQAGPTSMSLQAK463-475SVAGPSNMAVQGR, NFP499-501EFA
Hu9	AAS99314.1	A266S, SQAGPTSMSLQAK463-475SVAGPSNMAVQGR, NFP499-501EFA
Hu10	AAS99261.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu11	AAS99262.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu13	AAS99263.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, EYS499-501EFA
Hu15	AAS99265.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu16	AAS99266.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu17	AAS99267.1	T270S, QAGPNNMSAQAK467-478VAGPSNMAVGQR, NFA502-504EFA
Hu18	AAS99268.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu19	AAS99269.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, DYS499-501EFA

AAV Strain	Accession No.	Mutations to Graft Galactose Binding Footprint
Hu20	AAS99271.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, DYS499-501EFA
Hu21	AAS99272.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, DYS499-501EFA
Hu22	AAS99273.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, DYS499-501EFA
Hu23	AAS99274.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, DYS499-501EFA
Hu24	AAS99275.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, DYS499-501EFA
Hu25	AAS99276.1	A266S, QAGPTSMSLQAK464-475VAGPSNMAVGQR, NFP499-501EFA
Hu27	AAS99277.1	A266S, QAGASDVRDQSR464-475VAGPSNMAVGQR, DYS499-501EFA
Hu28	AAS99278.1	A266S, QAGASDIQDQSR464-475VAGPSNMAVGQR, EYS499-501EFA
Hu29	AAS99279.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVGQRN, EYS499-501EFA
Hu34	AAS99283.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu35	AAS99284.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu37	AAS99285.1	T270S, QAGPANMSAQAK467-478VAGPSNMAVQGR, N502E
Hu39	AAS99286.1	T270S, RAGPSNMSAQAR467-478VAGPSNMAVQGR, N502E
Hu40	AAS99288.1	T270S, QAGPANMSAQAK467-478VAGPSNMAVQGR, N502E
Hu41	AAS99289.1	T270S, QAGPANMSAQAK467-478VAGPSNMAVQGR, N502E
Hu42	AAS99290.1	T270S, QAGPANMSAQAK467-478VAGPSNMAVQGR, N502E
Hu43	AAS99291.1	A268S, RGSPAGMSVQPK466-477VAGPSNMAVQGR, NFT502-503EFA
Hu44	AAS99292.1	A267S, RGSPAGMSVQPK465-476VAGPSNMAVQGR, NFT500-502EFA
Hu45	AAS99293.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu46	AAS99294.1	A267S, RGSPAGMSVQPK465-476VAGPSNMAVQGR, NFT500-502EFA
Hu47	AAS99295.1	A266S, S270N, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu48	AAS99296.1	A267S, RGSPAGMSVQPK465-476VAGPSNMAVQGR,NFT500-502EFA
Hu49	AAS99297.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu51	AAS99298.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu52	AAS99299.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu54	AAS99301.1	A266S, QAGPTNMSLQAK463-474VAGPSNMAVQGR, NFP498-500EFA
Hu55	AAS99302.1	A266S, QAGPTNMSLQAK463-474VAGPSNMAVQGR, NFP498-500EFA
Hu56	AAS99303.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu57	AAS99304.1	A265S, QAGASDIRDQSR463-474VAGPSNMAVQGR, YS499-500FA
Hu58	AAS99305.1	A266S, QAGASDIRDQSR464-475VAGPSNMAVQGR, YS500-502FA
Hu60	AAS99307.1	A266S, SQAGPTMNSLQAK463-475SVAGPSNMAVQGR, NFP499-501EFA
Hu61	AAS99308.1	A266S, SQAGPTMNSLQAK463-475SVAGPSNMAVQGR, NFP499-501EFA
Hu63	AAS99309.1	A266S, SQAGASDIRDQSR463-475SVAGPSNMAVQGR, YS500-501FA
Hu64	AAS99310.1	A266S, SQAGASDIRDQSR463-475SVAGPSNMAVQGR, YS500-501FA
Hu66	AAS99311.1	T270S, SQAGPANMSAQAK466-478SVAGPSNMAVQGR, N502E
Hu67	AAS99312.1	T270S, SQAGPANMSAQAK466-478SVAGPSNMAVQGR, N502E
Rh1	AAS99241.1	T270S, SQAGPSSMANQAR465-477SVAGPSNMAVQGR, N501E
Rh2	AAO88193.1	T270S, SQAGPANMSAQAK466-478SVAGPSNMAVQGR, N502E
Rh8	AAO88183.1	T270S, SQAGPSSMANQAR464-476SVAGPSNMAVQGR, N500E
Rh10	AAO88201.1	T270S, SQAGPNNMSAQAK466-478SVAGPSNMAVQGR, N502E
Rh12	AAO88200.1	T265S, SQAGPNNMSAQAK461-473SVAGPSNMAVQGR, N497E
Rh13	AAO88199.1	AT262-263SS, HQAGPNTMAEQSK457-469SVAGPSNMAVQGR, N493E
Rh14	AAO88198.1	T265S, SQAGPNNMSAQAK461-473SVAGPSNMAVQGR, N497E
Rh16	AAO88197.1	AT262-263SS, SQAGPNNMSAQAK459-471SVAGPSNMAVQGR, N495E
Rh17	AAO88196.1	AT262-263SS, SQAGPNNMSAQAK459-471SVAGPSNMAVQGR, N495E

AAV Strain	Accession No.	Mutations to Graft Galactose Binding Footprint
Rh18	AAO88195.1	T270S, SQAGPNNMSAQAK466-478SVAGPSNMAVQGR, N502E
Rh19	AAO88194.1	T270S, HQAGPNTMAEQSK464-476SVAGPSNMAVQGR, N500E
Rh22	AAO88192.1	AT262-263SS, HQAGPNTMAEQSK457-469SVAGPSNMAVQGR, N493E
Rh23	AAO88191.1	T270S, HQAGPNTMAEQSK464-476SVAGPSNMAVQGR, N500E
Rh24	AAO88190.1	T265S, SQAGPNNMSAQAK461-473SVAGPSNMAVQGR, N497E
Rh25	AAS99242.1	T270S, SQAGPNNMSAQAK466-478SVAGPSNMAVQGR, N502E
Rh26	AAO89501.1	T188S, YQGGPTTMAEQAK385-397SVAGPSNMAVQGR, N421E
Rh27	AAO89502.1	T188S, YQGGPTTMAEQAK385-397SVAGPSNMAVQGR, N421E
Rh31	AAO89500.1	T188S, YQGGPTTMAEQAK385-397SVAGPSNMAVQGR, N421E
Rh32	AAO88189.1	insert SGGSS before N259,GKIRSGDFAFYRK457-469SVAGPSNMAVQGR, NALL498-501EFAW
Rh33	AAO88188.1	insert SGGSS before N259, GKIRSGDFAFYRK457-469SVAGPSNMAVQGR, NALL498-501EFAW
Rh34	AAO88187.1	insert SGGSS before N259, GKIRSGDFAFYRK457-469SVAGPSNMAVQGR, NALL498-501EFAW
Rh35	AAO88186.1	AT263-264SS, HQAGPNTMAEQSK458-470SVAGPSNMAVQGR, N494E
Rh36	AAO88185.1	AT263-264SS, HQAGPNTMAEQSK458-470SVAGPSNMAVQGR, N494E
Rh37	AAO88184.1	AT263-264SS, HQAGPNTMAEQSK458-470SVAGPSNMAVQGR, N494E
Rh38	AAS99243.1	T270S, SQAGPANMSAQAK466-478SVAGPSNMAVQGR, N502E
Rh40	AAS99244.1	T270S. SQAGPANMSARAK466-478SVAGPSNMAVQGR, N502E
Rh43	AAS99245.1	AT268-269SS, SQGGPNTMANQAK456-477SVAGPSNMAVQGR, N501E
Rh48	AAS99246.1	T269S, YQGGPTTMAEQAK466-478SVAGPSNMAVQGR, N502E
Rh49	AAS99247.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh50	AAS99248.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh51	AAS99249.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh52	AAS99250.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh53	AAS99251.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh54	AAS99252.1	T269S, YQGGPTTMAEQAK466-478SVAGPSNMAVQGR, N502E
Rh55	AAS99253.1	T269S, YQGGPTTMAEQAK466-478SVAGPSNMAVQGR, N502E
Rh57	AAS99254.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh58	AAS99255.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh60	AAS99256.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh61	AAS99257.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E
Rh62	AAS99258.1	T269S, YQGGPTTMAEQAK466-478SVAGPSNMAVQGR, N502E
Rh64	AAS99259.1	T270S, SQAGPSNMSAQAR466-478SVAGPSNMAVQGR, N502E

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THAT WHICH IS CLAIMED IS:

1. An adeno-associated virus (AAV) capsid protein, comprising amino acid substitutions, wherein the amino acid substitutions comprise at least the substitutions at:

- amino acid 266 in AAV2, and
- at least one of amino acids 463-475 in AAV2, and
- at least one of amino acids 499-502 in AAV2,

or the corresponding amino acid positions in AAV1, AAV3a, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV10, and

wherein the substitutions introduce, from a first AAV serotype, a glycan binding site into the AAV capsid protein of a second AAV serotype that is different from said first AAV serotype.

2. The AAV capsid protein of claim 1, wherein the glycan binding site is a hexose binding site, wherein the hexose is a galactose (Gal), a mannose (Man), a glucose (Glu) or a fucose (fuc).

3. The AAV capsid protein of claim 1, wherein the glycan binding site is a sialic acid (Sia) binding site, wherein the Sia residue is N-acetylneuraminic acid (Neu5Ac) or N-Glycolylneuraminic acid (Neu5Gc).

4. The AAV capsid protein of claim 1, wherein the glycan binding site is a disaccharide binding site, wherein the disaccharide is a sialic acid linked to galactose in the form Sia(alpha2,3)Gal or Sia(alpha2,6)Gal.

5. The AAV capsid protein of claim 1, wherein the glycan binding site is a galactose binding site.

6. The AAV capsid protein of any preceding claim, wherein the serotype of the second AAV serotype is AAV serotype 1 (AAV1), AAV serotype 2 (AAV2), AAV serotype 3a (AAV3a), AAV serotype 3b (AAV3b), AAV serotype 4 (AAV4), AAV serotype 5 (AAV5), AAV serotype 6 (AAV6), AAV serotype 7 (AAV7), AAV serotype 8 (AAV8), or AAV serotype 10 (AAV10).

7. The AAV capsid protein of any preceding claim, wherein the glycan binding site is the galactose binding site from AAV serotype 9 (AAV9).

8. The AAV capsid protein of claim 1, wherein the AAV capsid protein is from AAV1, AAV2, AAV3a, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV10, and

a) the substitution at amino acid 266 is A266S;

b) the substitutions at amino acids 463-475 are SQAGASDIRDQSR463-475SX_1AGX_2S$X_3X_4X_5X_6$QX_7R, wherein X_{1-7} can be any amino acid; and

c) the substitutions at amino acids 499-502 are EYSW499-502EX_8X_9W, wherein X_{8-9} can be any amino acid;

wherein the substitutions in a) - c) are of AAV2 or the corresponding amino acid positions in AAV1, AAV3a, AAV3b, AAV4, AAV5, AAV6, AAV7, AAV8 or AAV10.

9. The AAV capsid protein of claim 8, wherein: X_1 is V; X_2 is P; X_{3-6} are NMAV; and X_7 is G and optionally wherein, X_8 is F and X_9 is A.

10. The AAV capsid protein of any preceding claim, wherein the comprising AAV capsid protein is an AAV2 capsid amino acid sequence into which the following substitutions have been introduced: A266S, Q464V, A467P, D469N, I470M, R471A, D472V, S474G, Y500F and S501A, and wherein amino acids 585-590 are substituted with QQNTAP.

11. An AAV capsid comprising the AAV capsid protein of any of claims 1-10.

12. A virus vector comprising:

(a) the AAV capsid of claim 11; and

(b) a nucleic acid comprising at least one terminal repeat sequence,

wherein the nucleic acid is encapsidated by the AAV capsid.

13. A composition comprising the AAV capsid protein of any of claims 1-10, the AAV capsid of claim 11 or the virus vector of claim 12 in a pharmaceutically acceptable carrier.

14. The AAV capsid protein of any of claims 1-10, the AAV capsid of claim 11 or the virus vector of claim 12 or the composition of claim 13 for use in delivering a heterologous nucleic acid encoding a polypeptide or functional RNA to treat and/or prevent a disease state for which it is beneficial to deliver a therapeutic polypeptide or functional RNA.

15. The AAV capsid protein of any of claims 1-10, the AAV capsid of claim 11 or the virus vector of claim 12 or the composition of claim 13 in the manufacture of a medicament for treating and/or preventing a disease state for which it is beneficial to deliver a therapeutic polypeptide or functional RNA.

16. A method of introducing a nucleic acid into a cell, comprising contacting the cell with the virus vector of claim 12 or the composition of claim 13 when comprising the virus vector.

17. The method of claim 16, wherein the cell is in a subject.

18. The method of claim 17, wherein the subject is a human subject.

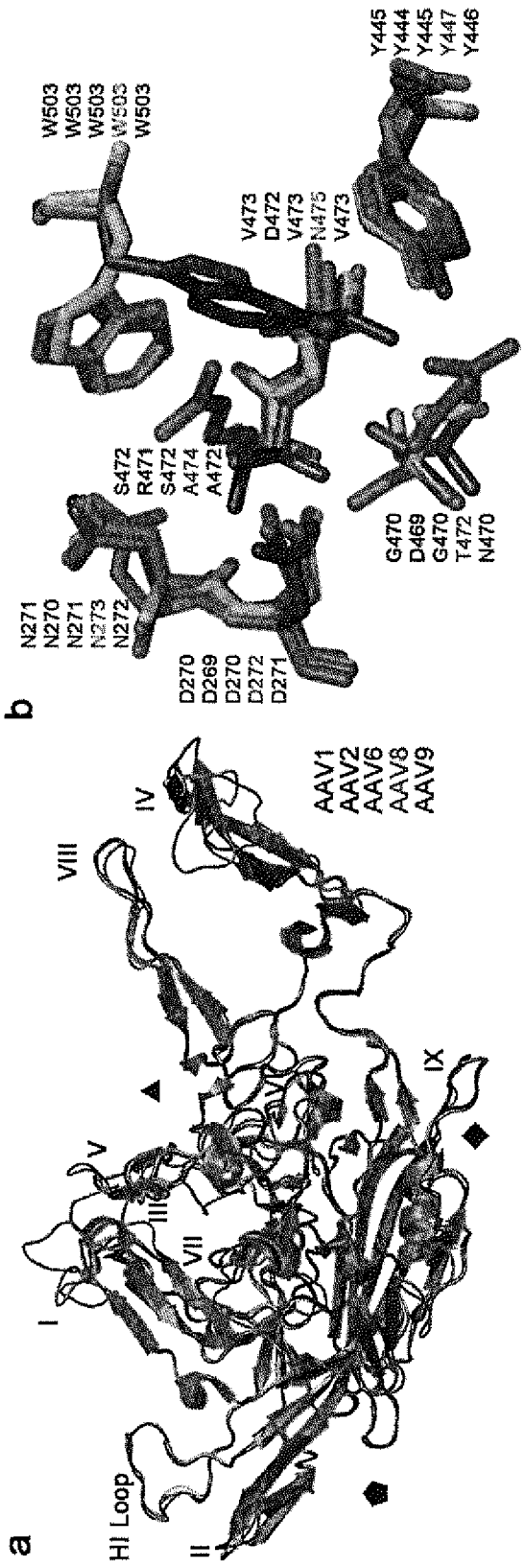
a
HI Loop
II
I
III
V
VII
VIII
IV
IX
AAV1
AAV2
AAV6
AAV8
AAV9
b
N271
N270
N271
N273
N272
D270
D269
D270
D272
D271
S472
R471
S472
A474
A472
W503
W503
W503
W503
W503
V473
D472
V473
N475
V473
Y445
Y444
Y445
Y447
Y446
G470
D469
G470
T472
N470

FIG. 1

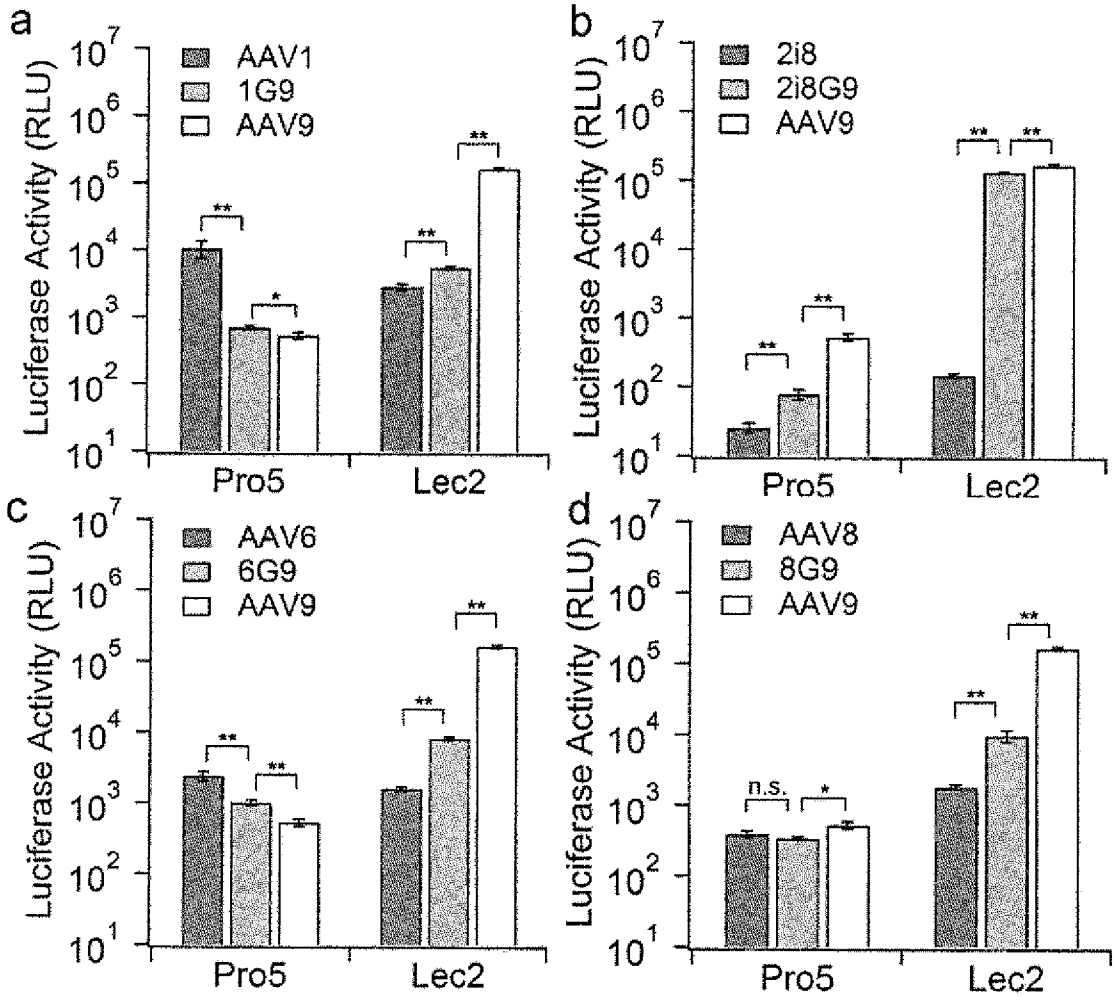
a
Luciferase Activity (RLU)
AAV1
1G9
AAV9
Pro5
Lec2
b
2i8
2i8G9
AAV9
c
AAV6
6G9
AAV9
d
AAV8
8G9
AAV9
n.s.

FIG. 2

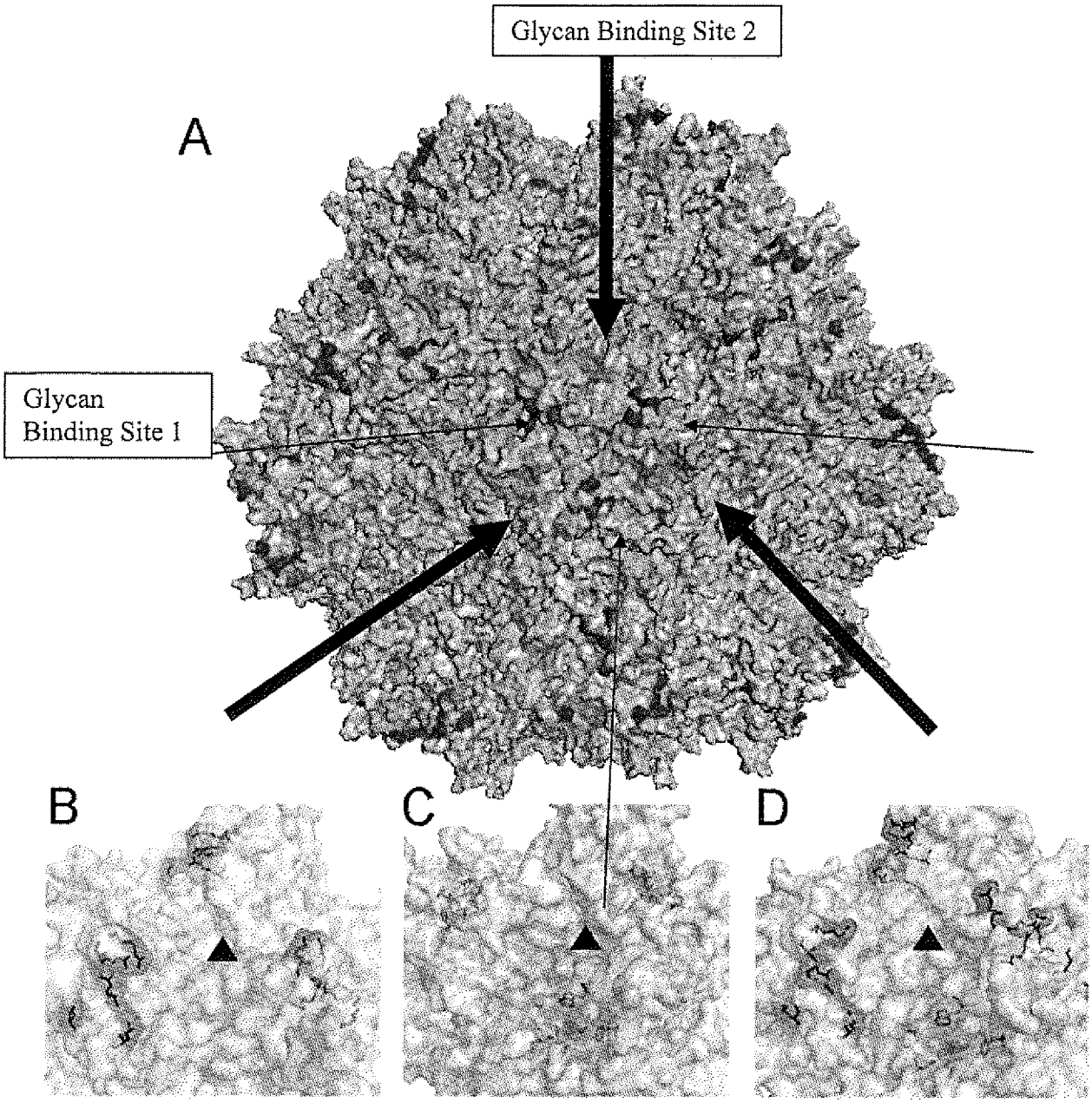

FIG. 3

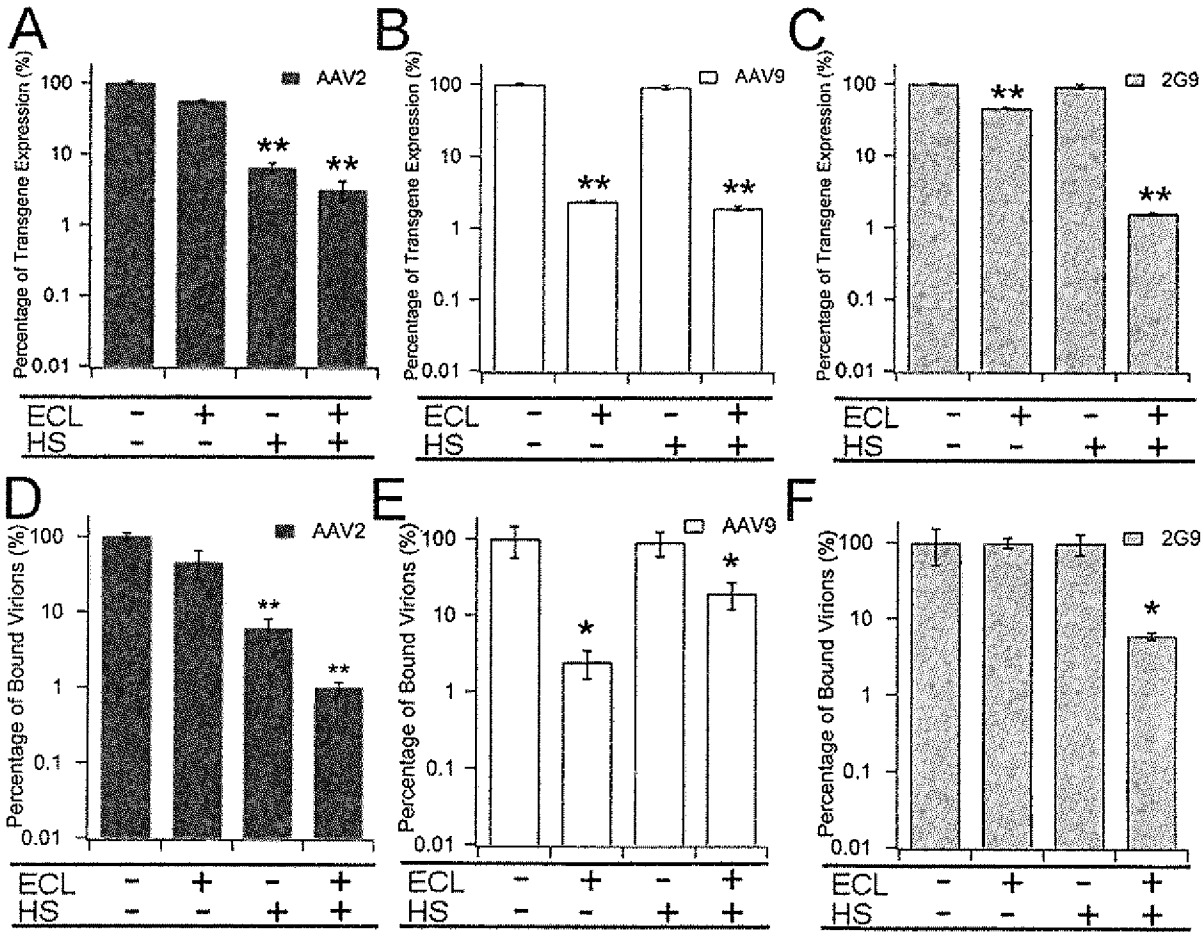
A
B
C
D
E
F
Percentage of Transgene Expression (%)
Percentage of Bound Virions (%)
AAV2
AAV9
2G9
100
10
1
0.1
0.01
**
*
ECL - + - +
HS - - + +

FIG. 4

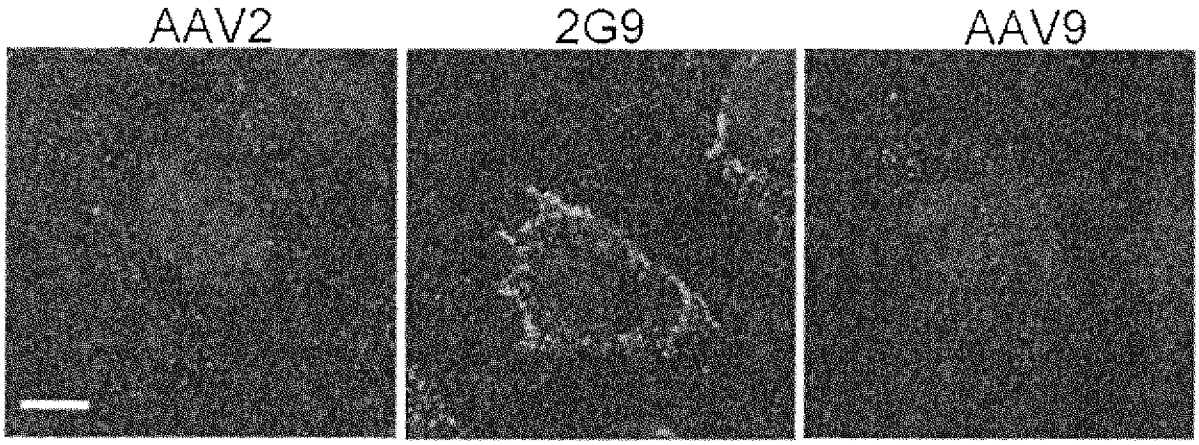

FIG. 5

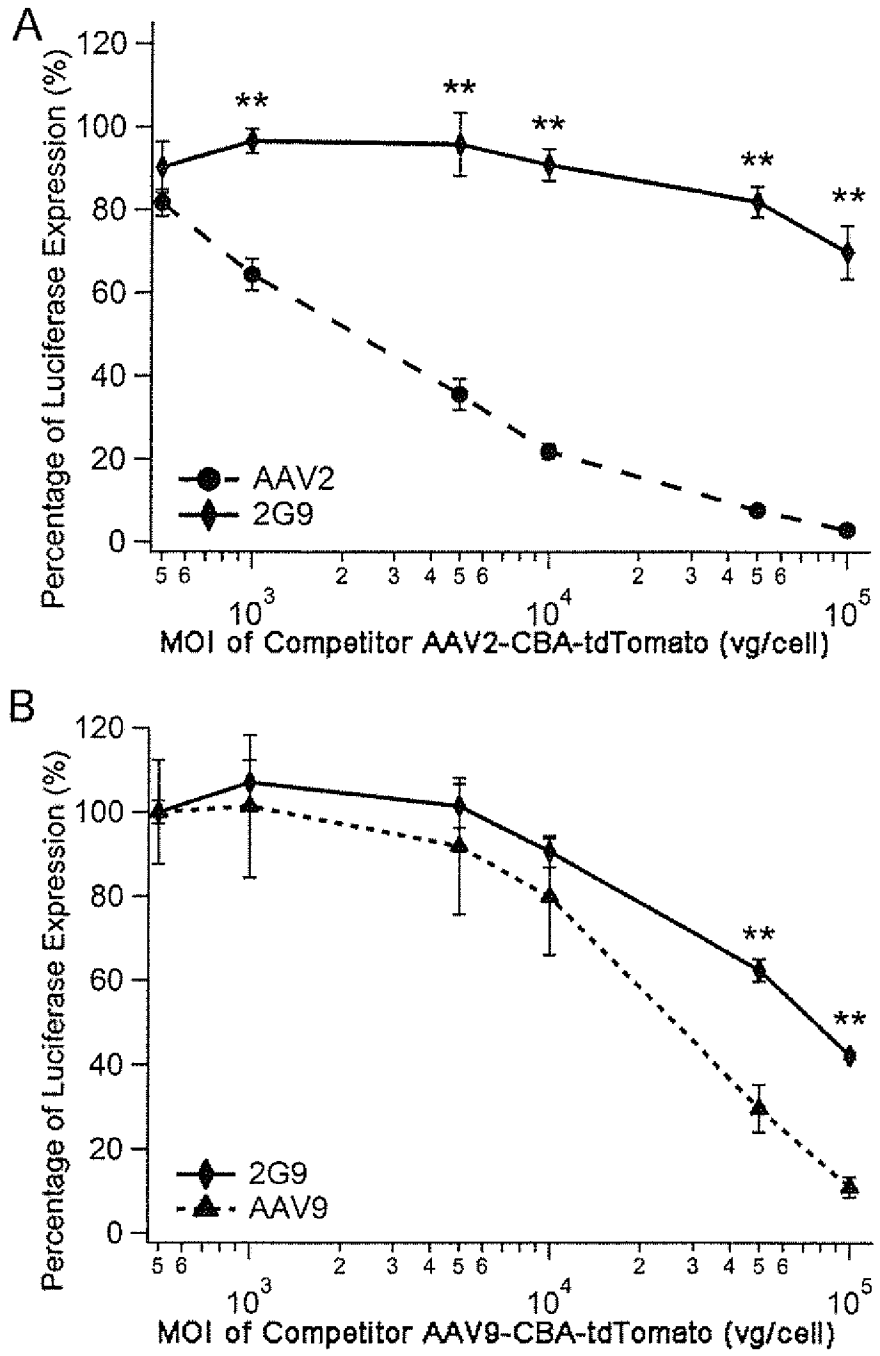
A
Percentage of Luciferase Expression (%)
120
100
80
60
40
20
0
**
**
**
**
**
AAV2
2G9
5 6
2
3
4
5 6
2
3
4
5 6
10^3
10^4
10^5
MOI of Competitor AAV2-CBA-tdTomato (vg/cell)
B
Percentage of Luciferase Expression (%)
120
100
80
60
40
20
0
**
**
2G9
AAV9
5 6
2
3
4
5 6
2
3
4
5 6
10^3
10^4
10^5
MOI of Competitor AAV9-CBA-tdTomato (vg/cell)

FIG. 6

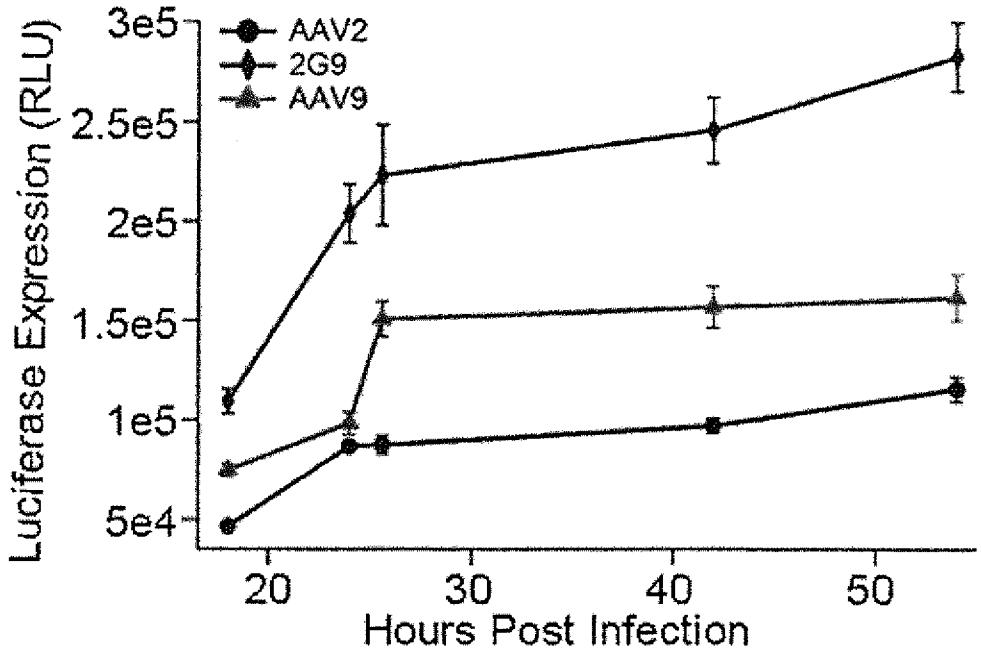
3e5
2.5e5
2e5
1.5e5
1e5
5e4
Luciferase Expression (RLU)
AAV2
2G9
AAV9
20
30
40
50
Hours Post Infection

FIG. 7

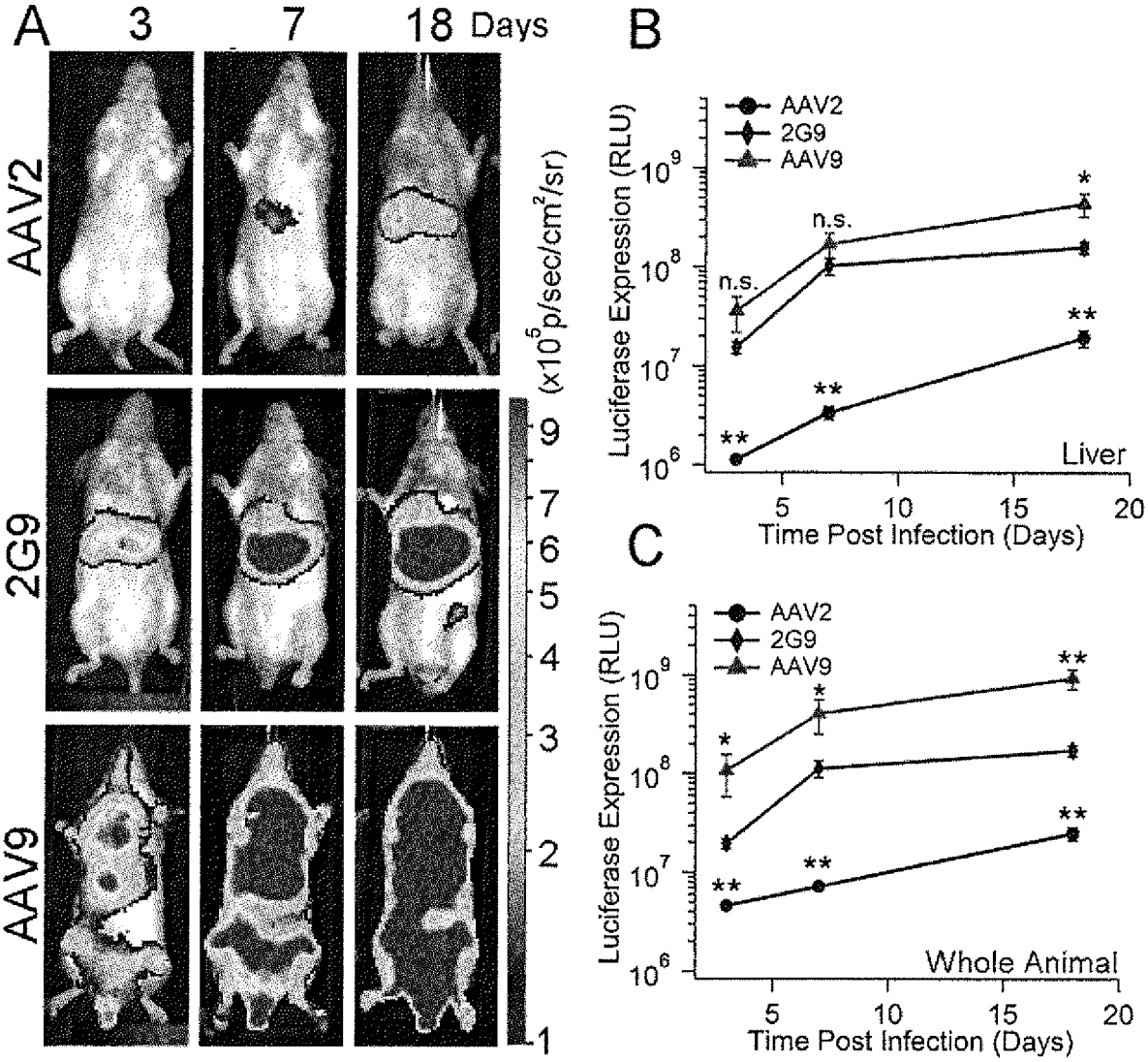
A
3
7
18 Days
AAV2
2G9
AAV9
(x10^5 p/sec/cm^2/sr)
9
7
6
5
4
3
2
1
B
AAV2
2G9
AAV9
Luciferase Expression (RLU)
10^9
10^8
10^7
10^6
n.s.
n.s.
*
**
**
**
Liver
5
10
15
20
Time Post Infection (Days)
C
AAV2
2G9
AAV9
Luciferase Expression (RLU)
10^9
10^8
10^7
10^6
*
*
**
**
**
Whole Animal
5
10
15
20
Time Post Infection (Days)

FIG. 8

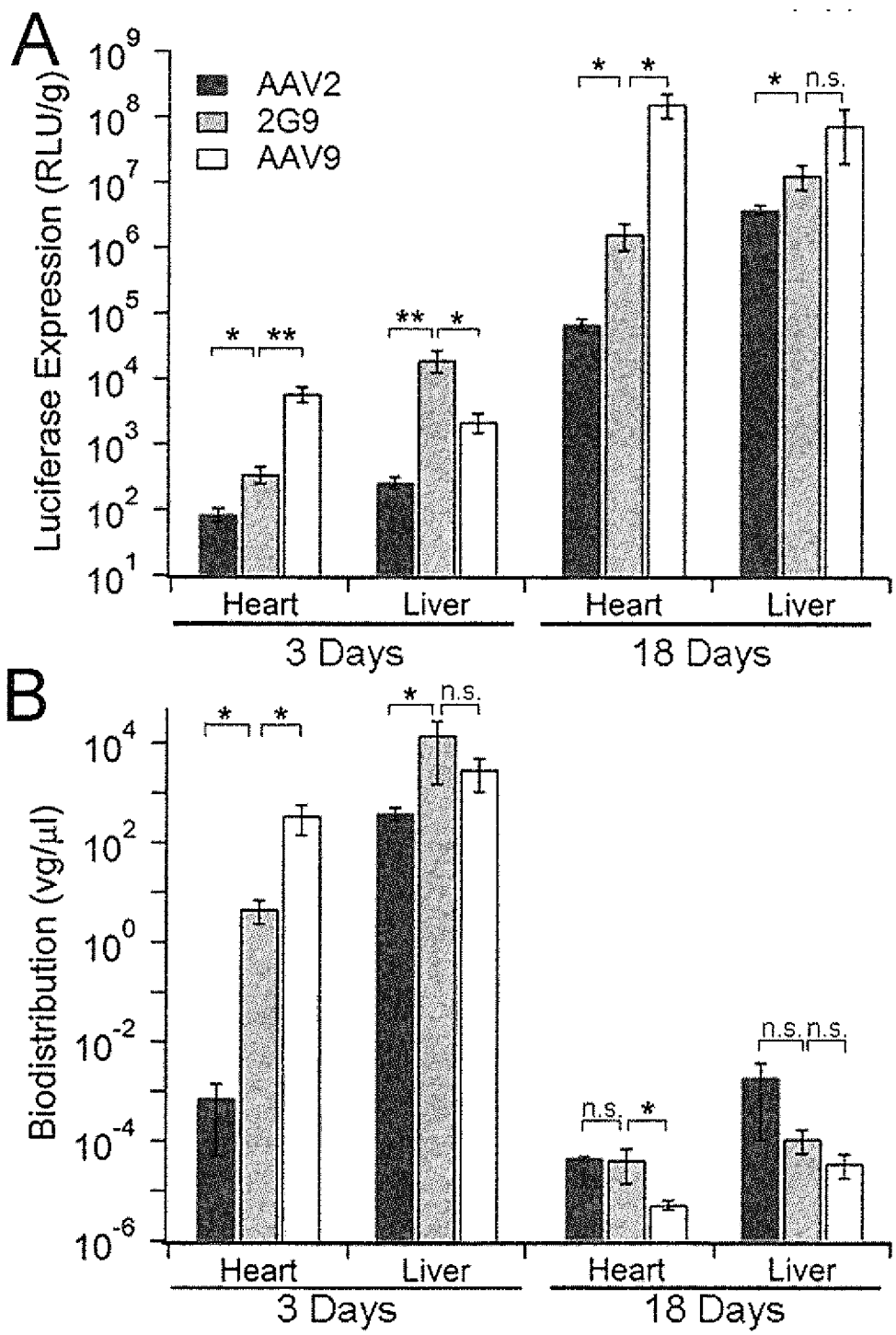
A
Luciferase Expression (RLU/g)
AAV2
2G9
AAV9
Heart
Liver
Heart
Liver
3 Days
18 Days
n.s.
B
Biodistribution (vg/μl)
Heart
Liver
Heart
Liver
3 Days
18 Days
n.s.

FIG. 9

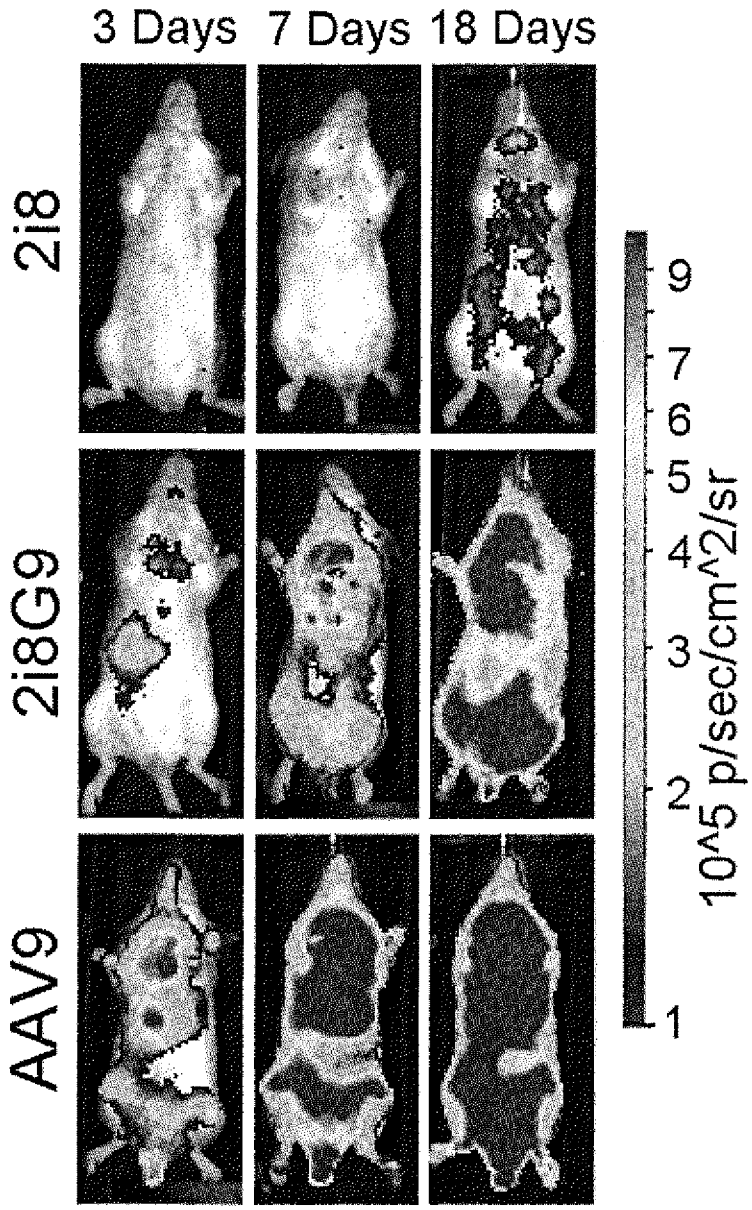

FIG. 10

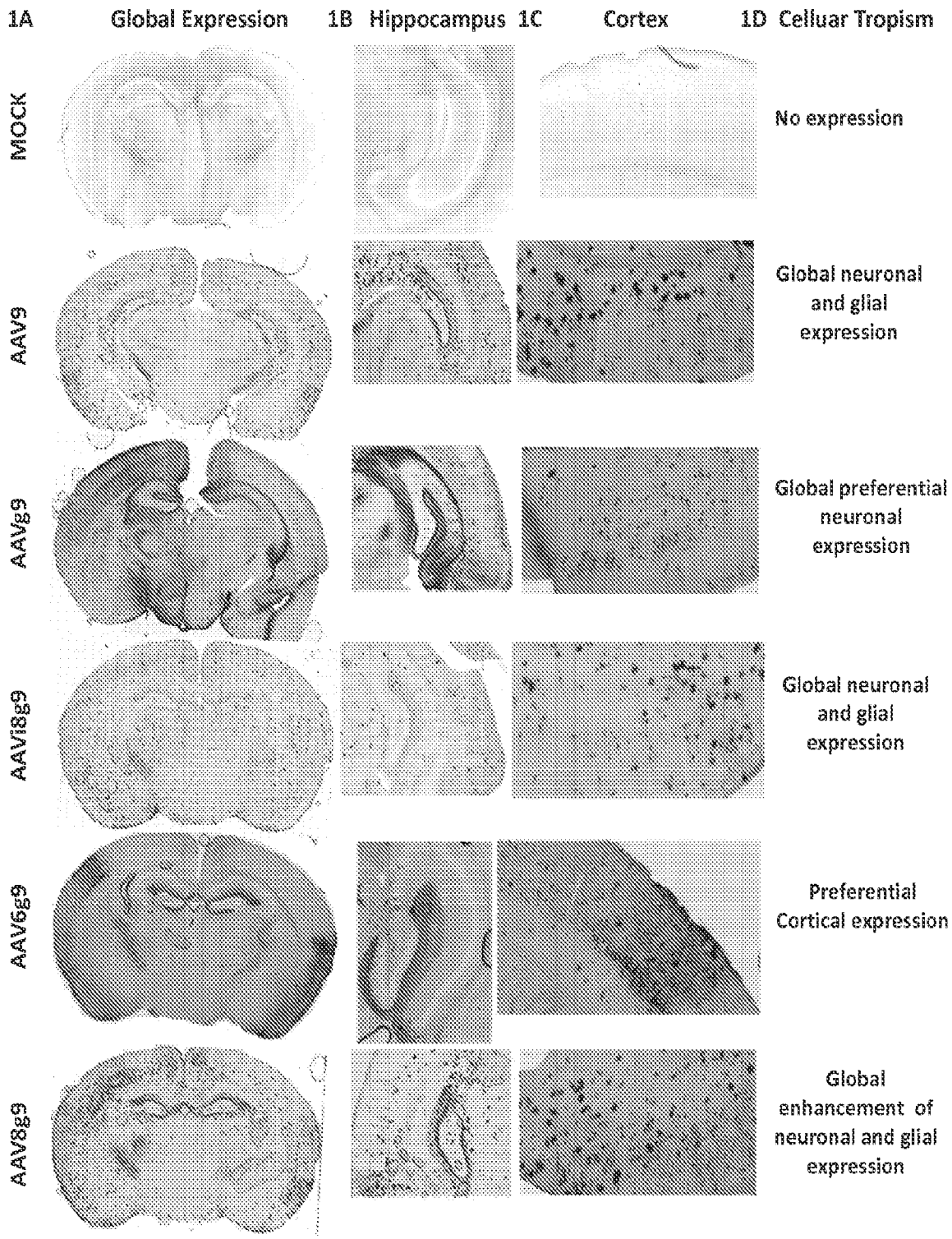
1A
Global Expression
1B
Hippocampus
1C
Cortex
1D
Celluar Tropism
MOCK
No expression
AAV9
Global neuronal and glial expression
AAVg9
Global preferential neuronal expression
AAVi8g9
Global neuronal and glial expression
AAV6g9
Preferential Cortical expression
AAV8g9
Global enhancement of neuronal and glial expression

FIG. 11