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(54) Title: VIRAL VECTORS COMPRISING ENGINEERED AAV CAPSIDS AND COMPOSITIONS CONTAINING THE SAME

HVR I AAV8 259 KQISNGTSCGA 263-267
AAV3B 259 KQISS—QSSA

HVR II AAV8 325 EVTONEGTKTANNLTS 330-333
AAV3B 322 EVTQNDGTTTANNLTS

HVR IV AAV8 446 NYLSRTQ—TGGTANTOTLQFSGGPNTMANG 449-475
AAV3B 443 NYLNRQGTTCGTTNQSRELFSGAGPGMSLQ

HVR V AAV8 488 GQRVSTTTCGNNNSNFAWTAGTKYHE 491-509
AAV3B 486 GQRLSKTANDNNNSNFWAASKYHE

HVR III, AAV3B = AAV8

HVR VI AAV8 537 EPSSNGTLE 539-542
AAV3B 535 EPMHGNLE

HVR VII AAV8 544 IEGKQNAARDNADYSDVM 548-559
AAV3B 542 IEGKREGTTASNAELDNVM

HVR VIII AAV8 582 VADNLQOONTAPQIGTVNSQGA 584-600
AAV3B 580 VANNLQSSNTAPTTRTVNDQGA

HVR IX AAV8 706 INYYKSTVDFAVNTGCVYSE 708-720
AAV3B 704 INYNKSVNVDVDTGTVYSE

FIG. 1

(57) Abstract: Provided herein is an artificial AAV capsid for delivery of an AAV vector genome carrying a transgene with an improved translatibility among species. The artificial AAV capsid is also useful in generation of recombinant AAV comprising the same. Methods of increasing transduction of rAAV in a species and delivering therapeutic and other molecules thereto are also provided.



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VIRAL VECTORS COMPRISING ENGINEERED AAV CAPSIDS AND COMPOSITIONS CONTAINING THE SAME

BACKGROUND OF THE INVENTION

5 Adeno-associated viruses (AAV) hold great promise in human gene therapy and have been widely used to target liver, muscle, heart, brain, eye, kidney and other tissues in various studies due to its ability to provide long-term gene expression and lack of pathogenicity. AAVs belong to the parvovirus family and each contains a single strand DNA flanked by two inverted terminal repeats. Dozens of naturally occurring AAV capsids have been
10 reported their unique capsid structures enable them to recognize and transduce different cell types, organs and species.

 The ever-accumulating safety records of AAV vector in clinical trials, combined with demonstrated efficacy, show that AAV is a good platform to work with. Another attractive feature is that AAV is relatively easy to be manipulated as AAV is a single-
15 stranded DNA virus with a small genome (~4.7 kb) and simple genetic components – inverted terminal repeats (ITR), the *Rep* and *Cap* genes. Only the ITRs and AAV capsid protein are required in AAV vectors, with the ITRs serving as replication and packaging signals for vector production and the capsid proteins playing a central role by forming capsids to accommodate vector genome DNA, determining tissue tropism as well as species
20 preference and delivering vector genomic DNA into target cells. There have been mainly four ways to obtain AAV capsid: isolating AAVs from cultures or tissues samples, AAV directed evolution, shuffling, and rational design.

 AAV8 has been shown to effectively transduce liver, muscle. In addition, AAV8-mediated hFIX gene transfer by a single peripheral-vein infusion consistently leads to long-
25 term expression of the FIX transgene at therapeutic levels without acute or long-lasting toxicity in patients with severe hemophilia B. Based on promising pre-clinical studies in mice and monkeys, several groups conducted clinical trials with AAV8 based vectors in patients with hemophilia B. The St. Jude's/UCL trial achieved stable expression but low level of factor IX without dose limiting toxicities (Nathwani, AC, et al, N Engl J Med., 365:
30 2357 - 2365 (2011); Nathwani, AC, et al, N Engl J Med, 371: 1994-2004 (2014)).

 In another aspect, recombinant adeno-associated virus serotype 3B (rAAV3B) can transduce cultured human liver cancer cells and primary human hepatocytes efficiently.

Systemically delivered capsid-optimized rAAV3B vectors can specifically target cancer cells in a human liver cancer xenograft model, suggesting their potential use for human liver-directed gene therapy. However, current data indicate that *in vivo* rAAV3B transduces mouse hepatocytes much less (~20-fold) efficiently than rAAV8. See, LI, et al. (2015). "Efficient and targeted transduction of nonhuman primate liver with systemically delivered optimized AAV3B vectors." *Molecular Therapy* 23(12): 1867-1876; LISOWSKI, et al. (2014). "Selection and evaluation of clinically relevant AAV variants in a xenograft liver model." *Nature* 506(7488): 382-386.

Thus, an AAV comprising capsid with improved translatability from one species to another, especially from the non-clinical studies to the clinical ones is needed.

SUMMARY OF THE INVENTION

Described herein are engineered adeno-associated virus (AAV) capsids useful in targeting non-human primate and human cells. Also provided herein are recombinant AAV (rAAV) vectors comprising these engineered AAV capsids having improved transduction and/or expression efficiencies for primate cells, including both non-human primate and human cells.

In one embodiment, a recombinant adeno-associated virus (rAAV) having an AAV capsid is provided. In certain embodiments, the AAV capsid is produced from an AAV VP nucleic acid sequence having a mutation in at least one nucleotide within a codon resulting in an amino acid change in: one or more amino acid residues in hypervariable region (HVR) VIII or HVR IX of a parental AAV. In certain embodiments, an AAV3B is mutated in the HVR VIII or HVR IX region. In certain embodiments, an AAV8 is mutated in at least the HVR IX region. In certain embodiments, an AAV1 is mutated in at least the HVR VIII or HVR IX region. In certain embodiments, the HVR VIII mutations are selected from one or more of: (a) amino acid (aa) 582; (b) aa 586; (c) aa 587; (d) aa 592; (e) aa 593; (f) aa 594; and (g) aa 598; and the HVR IX mutations are selected from one or more of: (h) aa 706; (i) aa 709; (j) aa 710; (k) aa 714; (l) aa 716; and (m) aa 718.

In one aspect, an engineered adeno-associated virus (AAV) capsid comprising a mutation in one or more amino acid residues in hypervariable region (HVR) VIII or HVR IX of a parental AAV is provided. Such engineered capsids are produced using, at a minimum, an engineered AAV capsid nucleic acid sequence which has mutations resulting in at least one amino acid change in the HVR VIII region selected from one or more of the codons

encoding: (a) aa 582; (b) aa 586 to aa 587; (c) aa 592 to aa 594; or (d) aa 598; and/or at least one amino acid change in the HVR IX region selected from one or more of the codons encoding: (e) aa 706; or (h) aa 710, wherein the amino acid residue positions are determined based in an alignment using the numbering of SEQ ID NO: 4 (AAV3B). In certain

5 embodiments, the mutation comprises one or more of: (i) replacement at aa 582 with an Asn(N); (ii) insertion or substitution of one, two or three of amino acids at aa 592 to aa 594 with one, two or three of Thr-Thr-Arg (TTR); (iii) substitution at aa 706 with Y; or (iv) substitution at aa 710 with S. In a further embodiment, the invention provides an adeno-associated virus (AAV) capsid comprising an AAV3B HVR VIII having the sequence of aa

10 582 to aa 598 of SEQ ID NO: 4. In one embodiment, the AAV capsid comprises an AAV3B HVR VI having the sequence of aa 537 to aa 540 of SEQ ID NO: 4. In another embodiment, the AAV capsid comprises an AAV3B HVR IV having the sequence of aa 446 to aa 473 of SEQ ID NO: 4.

In another aspect, an adeno-associated virus (AAV) capsid comprises a mutation in

15 the following locations of HVR IX as compared to encoded AAV8 capsid (SEQ ID NO: 2) for improving or increasing the transduction efficiency in rodent: aa 708; aa 711; aa 712; aa 716; aa 718; or aa 720. In one embodiment, the mutated aa 708 is Y. In another embodiment, the mutated aa 712 is S. In a further embodiment, the invention provides an adeno-associated virus (AAV) capsid comprising an AAV8 HVR IX having the sequence of aa 708 to aa 720

20 of SEQ ID NO: 2.

An adeno-associated virus (AAV) comprising AAV inverted terminal repeats, a transgene operably linked to regulatory sequences which direct expression of a product encoded by said transgene in a target host cell, and the capsid as described herein is provided. A composition comprising the AAV described herein and a physiologically

25 compatible carrier is provided. A method of delivering a transgene to a cell is provided, wherein said method comprising the step of contacting the cell with the recombinant AAV described herein, wherein said AAV comprises the transgene. In a further aspect, the invention involves transducing hepatocytes of a species. In yet a further aspect, the invention provides a method of modulating, altering, improving, increasing or decreasing transduction

30 of an AAV in a species by utilizing an engineered AAV capsid protein described herein.

A nucleic acid molecule comprising a nucleic acid sequence encoding the AAV capsid described herein, or a nucleic acid sequence sharing at least about 75%, or about 80%, or about 85%, or about 90%, or about 95%, or about 97%, or about 98%, or about 99%

identity therewith is provided. In another aspect, the invention provides a pharmaceutical composition comprising said molecule and a physiologically compatible carrier. In still a further aspect, the invention provides a packaging host cell comprising a nucleic acid molecule as described herein. In yet another aspect, method for generating a recombinant

5 adeno-associated virus (rAAV) comprises the steps of culturing a packaging host cell containing: (a) a nucleic acid molecule as described herein; (b) a molecule encoding a functional *rep*; (c) a vector genome comprising AAV inverted terminal repeats (ITRs) and a transgene; and (d) sufficient helper functions to permit packaging of the vector genome into the AAV capsid protein.

10 Still other aspects and advantages of the invention will be apparent from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 provides alignments of the AAV8 and AAV3B capsid proteins in the regions

15 of HVRs I, II, IV, V, VI, VII, VIII and IX and the adjacent amino acids thereof. AAV8 and AAV3B share one HVR III with an identical sequence. The regions of said HVRs are labelled on the right of the corresponding alignment as compared to SEQ ID NO: 2.

FIG. 2 provides result of transduction in human Huh7 cells with rAAVs comprising indicated capsid. Transduction rate of the rAAV with AAV8 capsid was set as 100% and

20 served as a standard reference. Chimeric capsids were constructed as described in Examples by swapping of an AAV3B HVR indicated on the X axis to the backbone of AAV8. The transduction efficiency was plotted as first bar of each group. AAV8.3BVR_x, wherein x indicates the number of the swapped HVR, is used to refer to the constructed capsid. Furthermore, capsids with double swaps were constructed by swapping of an AAV3B HVR

25 indicated on the X axis to the backbone of AAV8.3BVR8. The transduction thereof was plotted as second bar of each group if applicable. AAV8.3BVR8.3BVR_x, wherein x indicates the number of the swapped HVR other than HVR VIII, is used to refer to the capsid with double swaps. Triple swapped capsids were constructed by swapping of an AAV3B HVR indicated on the X axis to the backbone of AAV8.3BVR8.3BVR6. The transduction

30 there of was plotted as third bar of each group if applicable. AAV8.3BVR8.3BVR6.3BVR_x is used to refer to the capsid with triple swaps.

FIG. 3 provides result of transduction in human Huh7 cells with rAAVs comprising indicated capsid. Transduction rate of the rAAV with AAV3B capsid was set as 100% and

served as a standard reference. Engineered capsids were constructed as described in Examples by swapping of an AAV8 HVR indicated on the X axis to the backbone of AAV3B. Transductions of rAAV with native AAV3B or AAV8 were supplied as controls.

FIG. 4A provides an alignment of AAV8 and AAV3B capsids in HVR8 and the adjacent amino acid sequence thereof. Four regions which are different between AAV and AAV3B capsids were identified and assigned as “Patches 1 to 4” (i.e., Mutations 1 to 4) as indicated.

FIG. 4B provides result of transduction in human Huh7 cells with rAAVs comprising indicated capsid. Transduction rate of the rAAV with AAV3B capsid was set as 100% and served as a standard reference. Engineered capsids were constructed as described in Examples by swapping of AAV8 HVR region 1, 2, 3 or 4 as indicated on the X axis to the backbone of AAV3B.

FIGs. 5A and 5B provide result of transduction in human Huh7 cells (FIG. 5A) or mouse MC57G cells (FIG. 5B) with rAAVs comprising AAV8 or AAV3B capsid. The transduction rate was measured in luciferase assay and the results were plotted in Y axis as relative light unites per second or RLU/s. The multiplicity of infection (MOI or M.O.I, which is the ratio of AAV to infection targets) was also calculated and recorded. The MOI of rAAV with AAV8 capsid is about 159 while the MOI of rAAV with AAV3B capsid is about 9.

FIG. 6A shows result of transduction in human Huh7 cells (right bar of each group) or in mouse MC57G cells (left bar of each group) with rAAVs comprising indicated capsid. The transduction rate was measured in luciferase assay and the results were plotted in Y axes as relative light unites per second (RLU/s). MOI utilized is about 100. Engineered capsids were constructed as described in Examples by swapping of an AAV3B HVR as indicated on the X axis to the backbone of AAV8.3BVR8 (AAV8 with HVR VIII replaced with that of AAV3B, also indicated as AAV8.8). rAAV with AAV8 capsid was supplied as a control. FIG. 6B provides result of transduction in human Huh7 cells (right bar of each group) or in mouse H2.35 cells (left bar of each group) with rAAVs comprising indicated capsid. The transduction rate was measured in luciferase assay and the results were plotted in Y axes as relative light unites per second (RLU/s). MOI utilized is about 100. Engineered capsids were constructed as described in Examples by swapping of an AAV8 HVR as indicated on the X axis to the backbone of AAV3B. rAAVs with AAV8 or AAV3B capsid was supplied as controls.

FIG. 7 provides result of *in vivo* transduction in mice. One animal in AAV3B group is below detection. Experiment was performed as described in Examples. Briefly, 1×10^{11} GC of rAAV.humanFactorIX per mouse was administrated into C57BL/6J mice i.v.. The capsid protein is AAV3B or AAV3B.8VR9 (AAV3B with HVR IX replaced with that of AAV8, also noted as AAV3B.IX=AAV8). Samples were collected and concentration of human Factor IX was assessed and plotted as ng/mL on the Y axis. Samples from one animal in the AAV3B group was below detection range thus is not shown in FIG. 7.

FIG. 8 provides evaluation on yield of rAAVs with the indicated capsids measured in the lysate of host cells cultured in 6-well plate. Average yield of the rAAV with AAV3B.8VR9 capsid (noted as rAAV.AAV3B.8VR9) was set as 100 and served as a reference for normalization. Engineered capsids were constructed as described in Examples by swapping of one of the AAV3B HVR IX Mutations 1 to 6 back to the backbone of AAV3B.8VR9. rAAV with AAV8, AAV3B or AAV3B.8VR9 capsid was supplied as a control. No obvious difference in yield was detected.

FIGs. 9A to 9B provide result of transduction in human Huh7 cells (FIG. 9A) or in mouse H2.35 cells (FIG. 9B) with rAAVs comprising indicated capsid. Average transduction efficiency of the rAAV with AAV3B.8VR9 capsid (noted as rAAV.AAV3B.8VR9) was set as 100 and served as a reference for normalization. Engineered capsids were constructed as described in Examples by swapping of one of the AAV3B HVR IX Mutations 1 to 6 back to the backbone of AAV3B.8VR9. rAAV with AAV8, AAV3B or AAV3B.8VR9 capsid was supplied as a control.

FIG. 10 provides an alignment of AAV8, AAV3B, LK03, AAV2 and AAVDJ capsid proteins within HVR IX and the adjacent amino acid sequence thereof. Six amino acid differences were identified and assigned as Mutations 1 to 6 as indicated.

FIGs. 11A and 11B provide comparison of transduction efficiency in mouse liver using AAV1 capsid or AAV1.8VR9 capsid. FIG. 11 A provides representative fluorescent images of mouse livers. FIG. 11B is a plot showing quantification of vectors as GC/ μ g liver DNA. Mice were administrated i.v. with rAAV with eGFP as transgene and AAV1 or AAV1.8VR9 capsid at 1×10^{11} GC per mouse.

FIG. 12 provides expression of hF9 in male B6 mice administrated with rAAV vectors having a vector genome of TBG.hF9 and a capsid selected from AAV8 (inverted triangles), AAV8.8.6 (triangles), AAV8.8.4 (squares) and AAV8.8 (circles). See Example 6 for more details.

FIG. 13 provides representative microscopic images of human Huh7 cells transduced with 1×10^7 GC/well, 1×10^8 GC/well, or 1×10^9 GC/well of AAV8.TBG.eGFP or AAV8.8.6.TBG.eGFP as described in Example 7.

FIG. 14 provides a comparison of yields (measured by ddPCR) of rAAV vectors
5 having a TBG.eGFP genome vector packaged in various capsids, including AAV8.8.6 (dot pointed via an arrow head) and AAV8 (dot pointed via an arrow) as discussed in Example 7.

FIGs. 15A through 15F provide eGFP images of livers from mice administrated i.v. with AAV8. TBG.eGFP (FIGs 15A to 15C) or AAV8.8.6. TBG.eGFP (FIGs 15D to 15F) at a dose of 3×10^{10} GC per mouse. See Example 7 for more discussion.

10 FIG. 16 provides a scheme of morphometric analysis performed to evaluate transduction efficiency in chimeric liver as discussed in Example 7.

FIGs. 17A through 17I provide representative images from three fields (FIGs 17A to 17C; 17D to 17F; and 17G to 17I) of FAH staining (FIGs 17A, 17D and 17G), eGFP expression (FIGs 17B, 17E and 17H), or both (FIGs 17C, 17F and 17I)

15 FIG. 18 provides a scheme of analysis via flow cytometry. Cells of bigger size (e.g., hepatocytes) are gated for further analysis of HLA (human-specific marker), H2kb (mouse-specific marker) and GFP expression.

FIG. 19 provides a scheme of analysis via flow cytometry. Cells of all sizes are gated for further analysis of HLA (human-specific marker), H2kb (mouse-specific marker) and
20 GFP expression.

FIG. 20 provides a bar graph showing transduction efficiency of various AAV capsids obtained via the morphology analysis. For each sample tested, two bars are presented: the left one represents GFP+ human cells and the right one represents GFP+ mouse cells.

25 FIG. 21 provides a bar graph showing transduction efficiency of various AAV capsids obtained via flow cytometry. For each sample tested, two bars are presented: the left one represents GFP+ human cells and the right one represents GFP+ mouse cells.

FIGs. 22A through 22I provide representative image of iPSC-derived hepatocytes. FIGs. 22A to 22C show GFP expression in a monolayer of the generated hepatocytes. Other exposure times, 10 ms (FIGs. 22 D, 22E and 22F) and 35 ms (FIGs. 22G, 22H and 22I), were
30 used to reveal larger bodies formed by hepatocytes which resemble cell organization in the liver. FIGs 22A, 22D and 22G are images of cells transduced with AAV6.2.TBG.eGFP.

FIGs 22B, 22E and 22H are images of cells transduced with AAV8.TBG.eGFP. FIGs 22C, 22F and 22I are images of cells transduced with AAV8.8.6.TBG.eGFP.

DETAILED DESCRIPTION OF THE INVENTION

5 Adeno-associated virus (AAV)-based gene therapy is showing increasing promise, stimulated by encouraging results from clinical trials in recent years. Until now, AAV vectors utilizing the capsid have shown a tremendous potential for *in vivo* gene delivery with nearly complete transduction of many tissues in rodents, non-human primates, human and various species after intravascular infusion. To advance the platform, provided herein are
10 mutated or engineered capsids and recombinant AAVs comprising the same capsids having a high transduction efficiency in a species and/or an improved translatability from one species to another, especially from the non-clinical studies to clinical studies. The compositions, regimens, and methods of generating and producing such rAAVs as well as those of utilizing said rAAVs to treat various conditions are also provided.

15 In one embodiment, a recombinant adeno-associated virus (rAAV) having an AAV capsid is provided. In certain embodiments, the AAV capsid is produced from an AAV VP nucleic acid sequence having a mutation in at least one nucleotide within a codon resulting in an amino acid change in: one or more amino acid residues in hypervariable region (HVR) VIII or HVR IX of a parental AAV. In certain embodiments, an AAV3B is mutated in the
20 HVR VIII or HVR IX region. In certain embodiments, an AAV8 is mutated in at least the HVR IX region. In certain embodiments, an AAV1 is mutated in at least the HVR VIII or HVR IX region. Other AAVs and other mutations are set forth below. When reference is made to amino acid mutations and positions throughout the specification, it will be understood that the mutation is made in the nucleic acid sequence encoding the amino acids
25 at the position, with the numbering being based on the amino acid sequence of the full-length VP1 protein.

 In certain embodiments, the HVR VIII mutations are selected from one or more of: (a) amino acid (aa) 582; (b) aa 586; (c) aa 587; (d) aa 592; (e) aa 593; (f) aa 594; and (g) aa 598; and the HVR IX mutations are selected from one or more of: (h) aa 706; (i) aa 709; (j)
30 aa 710; (k) aa 714; (l) aa 716; and (m) aa 718.

 Unless defined otherwise, 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 and by reference to published texts, which provide one skilled in the art with a

general guide to many of the terms used in the present application. The following definitions are provided for clarity only and are not intended to limit the claimed invention.

As used herein, the term “a” or “an”, refers to one or more, for example, “a mutation” is understood to represent one or more mutations. As such, the terms “a” (or “an”), “one or more,” and “at least one” are used interchangeably herein.

An ordinal number, such as “first” “second” “third” “fourth” or the term “additional” are used throughout this specification as reference terms to distinguish between various forms and components of the compositions and methods.

As used herein, the term “about” means a variability of 10 % from the reference given, unless otherwise specified.

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

As used herein, the term “subject” includes any mammal in need of the methods of treatment described herein or prophylaxis, including particularly humans. Other mammals in need of such treatment or prophylaxis include dogs, cats, or other domesticated animals, horses, livestock, laboratory animals, including non-human primates, etc. The subject may be male or female.

As used herein, the term “species” refers to a group of closely related subjects that are very similar to each other and are usually capable of interbreeding and producing fertile offspring. In certain embodiments, a species may be human, a non-human primate, a primate, rat, mouse, rodent, dog, cat, or pig.

While various embodiments in the specification are presented using “comprising” language, under other circumstances, a related embodiment is also intended to be interpreted and described using “consisting of” or “consisting essentially of” language.

With regard to the following description, it is intended that each of the compositions herein described, is useful, in another embodiment, in the methods of the invention. In addition, it is also intended that each of the compositions herein described as useful in the methods, is, in another embodiment, itself an embodiment of the invention.

A. The AAV capsid

An adeno-associated virus (AAV, recombinant AAV, rAAV) comprises an AAV vector genome and an AAV capsid. An AAV vector genome, which is packaged within an AAV capsid, comprises a nucleic acid molecule containing a 5' AAV ITR, an expression

cassette, and a 3' AAV ITR. As described herein, an expression cassette contains a transgene operably linked to regulatory elements which direct expression thereof in a transduced host cell (e.g., a hepatocyte).

As used herein, the term “operably linked” refers to both expression control sequences that are contiguous with the gene of interest (transgene) and expression control sequences that act in trans or at a distance to control the gene of interest (transgene). The transgene may encode any biologically active product or other product, e.g., a product desirable for study.

In one aspect, an engineered adeno-associated virus (AAV) capsid comprising a mutation in one or more amino acid residues in hypervariable region (HVR) VIII or HVR IX of a parental AAV is provided. Such engineered capsids are produced using, at a minimum, an engineered AAV capsid nucleic acid sequence. The AAV capsid nucleic acid sequence is mutated in the HVR VIII region to encode a different amino acid at one or more of the following positions: (a) aa 582; (b) aa 586 to aa 587; (c) aa 592 to aa 594; or (d) aa 598. Additionally, or alternatively, the AAV capsid nucleic acid sequence is mutated in the HVR IX mutations are selected from one or more of: (e) aa 706; or (h) aa 710. These amino acid residue positions are determined based in an alignment using the numbering of SEQ ID NO: 4 (encoded amino acid of AAV3B). In certain embodiments, the mutation comprises one or more of: (i) replacement at aa 582 with an Asn (N); (ii) insertion or substitution of one, two or three of amino acids at aa 592 to aa 594 with one, two or three of Thr-Thr-Arg (TTR); (iii) substitution at aa 706 with Y; or (iv) substitution at aa 710 with S. In a further embodiment, the invention provides an adeno-associated virus (AAV) capsid comprising an HVR VIII having the sequence of aa 582 to aa 598 of SEQ ID NO: 4. In one embodiment, the AAV capsid comprises an HVR VI having the sequence of aa 537 to aa 540 of SEQ ID NO: 4. In another embodiment, the AAV capsid comprises an HVR IV having the sequence of aa 446 to aa 473 of SEQ ID NO: 4.

In certain embodiment, when the parental AAV is AAV8, the VP protein comprise a mutation in one or more of the amino acid residues in HVR IX.

In another aspect, an engineered adeno-associated virus (AAV) capsid comprising a mutation in one or more amino acid residues in HVR IX of a parental AAV is provided. Such engineered capsids are produced using, at a minimum, an engineered AAV capsid nucleic acid sequence which has mutations resulting in at least one amino acid change in the HVR IX region selected from one or more of the codons encoding: aa 708; aa 711; aa 712;

aa 716; aa 718; or aa 720, wherein the locations of HVR IX is compared to encoded AAV8 capsid (SEQ ID NO: 2). In certain embodiment, such capsid improves or increases the transduction efficiency in rodent. In one embodiment, the mutated aa 708 is Y. In another embodiment, the mutated aa 712 is S. In a further embodiment, the invention provides an adeno-associated virus (AAV) capsid comprising an HVR IX having the sequence of aa708 to aa 720 of SEQ ID NO: 2.

As used herein, a VP protein may refer to a VP1 protein, a VP2 protein, or a VP3 protein, unless particularly specified.

In certain embodiments, an engineered adeno-associated virus (AAV) capsid is provided herein which comprises a VP protein comprising a mutation in one or more amino acid residues in hypervariable region (HVR) VIII or HVR IX of a parental AAV is provided. The HVR VIII mutations are selected from one or more of: amino acid (aa) 582; aa 586; aa 587; aa 592; aa 593; aa 594; and aa 598. The HVR IX mutations are selected from one or more of: aa 706; aa 709; aa 710; aa 714; aa 716; and aa 718. These amino acids are numbered as in SEQ ID NO: 4. Corresponding amino acid position in the engineered AAV capsid protein indicated by amino acid number can be determined via an alignment between the engineered AAV capsid protein and SEQ ID NO: 4.

In certain embodiments, the mutation comprises one or more of: (i) substitution at aa 582 with an amino acid produced from nucleotide (nt) 1744 to nt 1746 of SEQ ID NO: 3; (ii) substitution at aa 586 with an amino acid produced from nt 1756 to nt 1758 of SEQ ID NO: 3; (iii) substitution at aa 587 with an amino acid produced from nt 1759 to nt 1761 of SEQ ID NO: 3; (iv) substitution at aa 592 with an amino acid produced from nt 1774 to nt 1776 of SEQ ID NO: 3; (v) substitution at aa 593 with an amino acid produced from nt 1777 to nt 1779 of SEQ ID NO: 3; (vi) substitution at aa 594 with an amino acid produced from nt 1780 to nt 1782 of SEQ ID NO: 3; (vii) substitution at aa 706 with an amino acid produced from nt 2122 to nt 2124 of SEQ ID NO: 1; (viii) substitution at aa 709 with an amino acid produced from nt 2131 to nt 2133 of SEQ ID NO: 1; (ix) substitution at aa 710 with an amino acid produced from nt 2134 to nt 2136 of SEQ ID NO: 1; (x) substitution at aa 714 with an amino acid produced from nt 2146 to nt 2148 of SEQ ID NO: 1; (xi) substitution at aa 716 with an amino acid produced from nt 2152 to nt 2154 of SEQ ID NO: 1; and (xii) substitution at aa 718 with an amino acid produced from nt 2158 to nt 2160 of SEQ ID NO: 1.

In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein comprising an exogenous AAV3B HVR VIII having an amino acid

sequence produced from nt 1744 to nt 1794 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein comprising an exogenous AAV3B HVR VI having an amino acid sequence produced from nt 1609 to nt 1620 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided

5 herein which comprises a VP protein comprising an exogenous AAV3B HVR IV having an amino acid sequence produced from nt 1336 to nt 1419 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein comprising an exogenous AAV3B HVR I having an amino acid sequence produced from nt 784 to nt 792 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is

10 provided herein which comprises a VP protein comprising an exogenous AAV3B HVR II having an amino acid sequence produced from nt 979 to nt 990 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein comprising an exogenous AAV3B HVR V having an amino acid sequence produced from nt 1465 to nt 1521 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is

15 provided herein which comprises a VP protein comprising an exogenous AAV3B HVR VII having an amino acid sequence produced from nt 1636 to nt 1671 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein comprising an exogenous AAV3B HVR IX having an amino acid sequence produced from nt 2116 to nt 2154 of SEQ ID NO: 3. In certain embodiments, an engineered AAV

20 capsid is provided herein which comprises a VP protein having any two, any three, any four, any five, any six, any seven, or all eight of the AAV 3B HVR I, II, IV, V, VI, VII, VIII, and IX. For example, the engineered AAV capsid may be AAV8.8.6 (an AAV8 backbone swapped with HVR8 and HVR6 of AAV3B), AAV8.8.6.7 (an AAV8 backbone swapped with HVR8, HVR7, and HVR6 of AAV3B), AAV8.8.6.9 (an AAV8 backbone swapped with

25 HVR9, HVR8, and HVR6 of AAV3B), AAV8.8.7 (an AAV8 backbone swapped with HVR8, and HVR7 of AAV3B), AAV8.8.9 (an AAV8 backbone swapped with HVR8 and HVR9 of AAV3B).

In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence

30 comprising a nucleic acid sequence which encodes aa 262 to aa 264 of SEQ ID NO: 4 (AAV3B HVR I). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 327 to aa 330 of SEQ ID

NO: 4 (AAV3B HVR II). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 446 to aa 473 of SEQ ID NO: 4 (AAV3B HVR IV). In certain embodiments, an engineered AAV capsid is

5 provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 489 to aa 507 of SEQ ID NO: 4 (AAV3B HVR V). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which is encodes aa 537 to aa 540

10 of SEQ ID NO: 4 (AAV3B HVR VI). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 546 to aa 557 of SEQ ID NO: 4 (AAV3B HVR VII). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered

15 nucleic acid sequence comprising a nucleic acid sequence which encodes aa 582 to aa 598 of SEQ ID NO: 4 (AAV3B HVR VIII). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 706 to aa 718 of SEQ ID NO: 4 (AAV3B HVR IX). In certain embodiments, an engineered AAV capsid is

20 provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes any two, any three, any four, any five, any six, any seven, or all eight of the AAV 3B HVR I, II, IV, V, VI, VII, VIII, and IX, in the VP protein. For example, the engineered AAV capsid may be AAV8.8.6 (an AAV8 backbone swapped with HVR8 and HVR6 of AAV3B), AAV8.8.6.7 (an AAV8

25 backbone swapped with HVR8, HVR7, and HVR6 of AAV3B), AAV8.8.6.9 (an AAV8 backbone swapped with HVR9, HVR8, and HVR6 of AAV3B), AAV8.8.7 (an AAV8 backbone swapped with HVR8, and HVR7 of AAV3B), AAV8.8.9 (an AAV8 backbone swapped with HVR8 and HVR9 of AAV3B).

In certain embodiments, an engineered AAV capsid is provided herein which

30 comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 263 to aa 267 of SEQ ID NO: 2 (AAV8 HVR I). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid

sequence comprising a nucleic acid sequence which encodes aa 330 to aa 333 of SEQ ID NO: 2 (AAV8 HVR II). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 446 to aa 475 of SEQ ID NO: 2 (AAV8 HVR IV). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 491 to aa 509 of SEQ ID NO: 2 (AAV8 HVR V). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 539 to aa 542 of SEQ ID NO: 2 (AAV8 HVR VI). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 548 to aa 559 of SEQ ID NO: 2 (AAV8 HVR VII). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 584 to aa 600 of SEQ ID NO: 2 (AAV8 HVR VIII). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes aa 708 to aa 720 of SEQ ID NO: 2 (AAV8 HVR IX). In certain embodiments, an engineered AAV capsid is provided herein which comprises a VP protein produced using, at a minimum, an engineered nucleic acid sequence comprising a nucleic acid sequence which encodes any two, any three, any four, any five, any six, any seven, or all eight of the AAV8 HVR I, II, IV, V, VI, VII, VIII, and IX, in the VP protein.

In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR VIII having an amino acid sequence produced from nt 1750 to nt 1800 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR VI having an amino acid sequence produced from nt 1615 to nt 1626 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR IV having an amino acid sequence produced from nt 1345 to nt 1425 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR I

having an amino acid sequence produced from nt 787 to nt 801 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR II having an amino acid sequence produced from nt 988 to nt 999 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR V having an amino acid sequence produced from nt 1471 to nt 1527 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR VII having an amino acid sequence produced from nt 1642 to nt 1677 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which comprises a VP protein comprising an exogenous AAV8 HVR IX having an amino acid sequence produced from nt 2122 to nt 2160 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid may comprises a VP protein having any two, any three, any four, any five, any six, any seven, or all eight of the AAV8 HVR I, II, IV, V, VI, VII, VIII, and IX.

In certain embodiments, the engineered AAV capsid comprises a VP protein having an amino acid sequence produced from a nucleic acid sequence selected from SEQ ID NO: 20, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 42, SEQ ID NO: 50, SEQ ID NO: 54, SEQ ID NO: 56, SEQ ID NO: 58, SEQ ID NO: 64, SEQ ID NO: 72, or SEQ ID NO: 74, and SEQ ID NO: 76.

The engineered capsid comprises at least 1 amino acid (aa) which is different from the parental capsid. In certain embodiment, the engineered capsid protein comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 amino acid(s) which is/are different from the parental capsid protein. In certain embodiment, the engineered capsid protein is from at least about 99%, at least about 98%, at least about 97%, at least about 95%, at least about 90%, at least about 85%, at least about 80%, at least about 75%, to 100% identical to the parental capsid protein. In certain embodiment, the engineered capsid protein is about 99%, about 98%, about 97%, about 95%, about 90%, about 85%, about 80%, about 75% identical to the parental capsid protein.

As used herein, the term “backbone” or “parental” capsid refers to an AAV capsid for generating the mutant or chimeric capsid having improved transduction in a species or a good translatability between species as described herein. In one embodiment, the source of the parental AAV capsid is Clade A, Clade B (represented by AAV2), Clade C (represented by the AAV2-AAV3 hybrid), Clade D (represented by AAV7), Clade E (represented by

AAV8), and Clade F capsids (represented by human AAV9). Functional clade A AAVs include AAV6, AAV 1, hu44R2, and hu48R3. Other clade A AAVs include hu. 44, hu.46, hu.43, and hu.48, amongst others [WO 2005/033321 and WO 2006/110689]. Further discussion of AAV clades is provided in G. Gao, et al., J. Virol., 78(12):6381-6388 (June 2004) and International Patent Publication Nos. WO 2004/028817 and WO 2005/033321. In a further embodiment, the parental AAV capsid may be a clade A capsid, a clade B capsid, a clade D capsid, AAV2-3 hybrids, AAV3 capsids. Further AAV include, without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, LK03, AAVDJ, rh.8, rh.10, rh.20, hu.37, rh.2R, rh.43, rh.46, rh.64R1, hu.48R3, cy.5R4, rh.32, rh.64. However, it is not intended that only naturally occurring AAV capsid be the source of the parental capsids. Useful herein are non-naturally occurring AAV capsid, including, without limitation, recombinant, modified or altered, shuffled, chimeric, hybrid, evolved, synthetic, artificial, etc., AAV capsid. This includes AAV capsid with mutations or region replacements described herein, provided they are used as the “starting sequence” for generating the mutant or chimeric capsid as described while not having a sequence of said mutant or chimeric capsid. As such, the terms “backbone” and “parental” are used interchangeably herein. In certain embodiments, the parental AAV capsid protein is produced in a packaging host cell which comprises the AAV capsid nucleic acid sequence of SEQ ID NO: 3, or a nucleic acid at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% identical thereto. In certain embodiments, the parental AAV capsid protein is produced in a packaging host cell which comprises the AAV capsid nucleic acid sequence of SEQ ID NO: 1, or a nucleic acid at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% identical thereto. In certain embodiments, the parental AAV capsid has an amino acid sequence produced in a packaging host cell which comprises the AAV capsid nucleic acid sequence of SEQ ID NO: 140, or a nucleic acid at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 97%, or at least about 99% identical thereto.

As used herein, an AAV3B capsid is a self-assembled AAV capsid composed of approximately 60 AAV3B vp proteins (AAV3B vp1, vp2 and vp3 proteins). The AAV3B vp proteins are typically produced in a packaging host as alternative splice variants comprising a nucleic acid sequence of SEQ ID NO: 3 or a sequence at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% thereto, which

encodes the vp1 amino acid sequence of SEQ ID NO: 4 (GenBank accession: AAB95452.1a US 6156305, Rutledge and Russell, J Virol, 72(1): 309-319 (1999) for the amino acid sequence of AAV3B; and Lerch, et al, 2010, Virology 403 (1), 26–36 for the crystal structure of AAV3B). In certain embodiment, AAV3B also refers to an AAV3B variant, including but not limited to AAV3B.ST [produced by nt sequence encoding S663V+T492V modified AAV3B, SEQ ID NO: 132; Li Zhong et al, Abstract 240. American Society of Gene & Cell Therapy 17th Annual Meeting, 2014, Mol Therapy, Vol 22 (Suppl 1) May 2014, p. S91]; LK03 [produced by nt sequence encoding SEQ ID NO: 134 encoding SEQ ID NO: 133, which is similar to SEQ ID NO: 4 with only 8 amino acid differences between the two capsids, See, e.g., US 2013/0059732], LK03 I125 [a variant of LK03, produced by nt sequence encoding SEQ ID NO: 135 in which the Leu located at position 125 is substituted with an Ile (called AAVLK03.L125I)]. In certain embodiments, the term “capsid protein” and “capsid” are used interchangeably. In certain embodiment, initiation codon or the sequence of about 500 base pairs (bp) upstream to about 500 bp downstream, about 100 base pairs (bp) upstream to about 100 bp downstream, about 20 base pairs (bp) upstream to about 20 bp downstream, or about 10 base pairs (bp) upstream to about 10 bp downstream of the initiation codon of a VP protein (vp1, vp2, vp3) may be optimized for a higher or lower translational efficiency in a host cell. It would be understood by one of skill in the art would understand that ATG at nt 1 to nt 3 of SEQ ID NO: 3 is considered as an initiation codon for VP1 produced by SEQ ID NO: 3 in a host cell; ACT at nt 412 to nt 414 of SEQ ID NO: 3 is considered as an initiation codon for VP2 produced by SEQ ID NO: 3 or any fragment thereof in a host cell; and that ATG at nt 607 to nt 609 of SEQ ID NO: 3 is considered as an initiation codon for VP3 produced by SEQ ID NO: 3 or any fragment thereof in a AAV packaging host cell.

As used herein, the term “fragment” refers to a continuous section of the reference sequence, wherein a fragment is shorter than the reference sequence in length. In certain embodiments, a fragment of a VP1 coding sequence may be part of the VP1 coding sequence which produces a VP2 or VP3 protein in a host cell. In one example, a fragment of the VP1 protein may be the VP1-unique region (e.g., the portion of VP1 which is not present in VP2 or VP3).

An AAV8 capsid is a self-assembled AAV capsid composed of approximately 60 AAV8 vp proteins (AAV8 vp1, vp2 and vp3 proteins). The AAV8 vp proteins are typically produced in a packaging host cell as alternative splice variants, which comprises a nucleic

acid sequence of SEQ ID NO: 1 or a sequence at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% thereto, which encodes the vp1 amino acid sequence of SEQ ID NO: 2 (GenBank accession: AAN03857.1, WO2003052051 and Gao et al, PNAS USA, 99(18): 11854-11859 (2002)). In certain

5 embodiment, initiation codon or the sequence of about 500 base pairs (bp) upstream to about 500 bp downstream, about 100 base pairs (bp) upstream to about 100 bp downstream, about 20 base pairs (bp) upstream to about 20 bp downstream, or about 10 base pairs (bp) upstream to about 10 bp downstream of the initiation codon of a VP protein (vp1, vp2, vp3) may be optimized for a higher or lower translational efficiency in a host cell. Furthermore, one of

10 skill in the art would understand that ATG at nt 1 to nt 3 of SEQ ID NO: 1 is considered as an initiation codon for VP1; ACT at nt 412 to nt 414 of SEQ ID NO: 1 is considered as an initiation codon for VP2; and that ATG at nt 610 to nt 612 of SEQ ID NO: 1 is considered as an initiation codon for VP1. Initiation codon of another nucleic acid sequence producing a capsid vp protein can be readily determined by one of skill in the art, e.g., via alignment with

15 SEQ ID NO: 1 or SEQ ID NO: 3. Similar alterations to the start codons may be made to the other AAV types described herein.

An AAV1 capsid is a self-assembled AAV capsid composed of approximately 60 AAV1 vp proteins (AAV1 vp1, vp2 and vp3 proteins). The AAV1 vp proteins are typically produced in a packaging host cell as alternative splice variants which comprises a nucleic

20 acid sequence of SEQ ID NO: 140 or a sequence at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% thereto, which encodes the vp1 amino acid sequence of SEQ ID NO: 141 (NCBI Reference Sequence: NP_049542.1, and GenBank: AAD27757.1).

Further, the sequences of AAV capsid such as rh10 have been described in, e.g., US

25 Patent 7790449; US Patent 7282199, WO 2003/042397, and a variety of databases. Still other AAV sources may include, e.g., AAV9 [US 7,906,111; US 2011-0236353-A1], and/or hu37 [see, e.g., US 7,906,111; US 2011-0236353-A1], AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV6.2, AAV7, AAV8, [US Patent 7790449; US Patent 7282199] and others. See, e.g., WO 2003/042397; WO 2005/033321, WO 2006/110689; US Patent

30 7790449; US Patent 7282199; US 7588772B2 for sequences of these and other suitable AAV, as well as for methods for generating AAV vectors. Still other AAV may be selected.

The term "serotype" is a distinction with respect to an AAV having a capsid which is serologically distinct from other AAV serotypes. Serologic distinctiveness is determined on

the basis of the lack of cross-reactivity between antibodies to the AAV as compared to other AAV. Cross-reactivity is typically measured in a neutralizing antibody assay.

The AAV capsid consists of three main splice variants, which vary in length due to alternative start codon usage. These proteins are referred to as VP1, VP2 and VP3, with VP1 being the longest and VP3 being the shortest. The AAV particle consists of all three capsid proteins at a ratio of ~1:1:10 (VP1:VP2:VP3). VP3, which is comprised in VP1 and VP2 at the N-terminus, is the main structural component that builds the particle. The capsid protein can be referred to using several different numbering systems. For convenience, as used herein, the AAV sequences are referred to using VP1 numbering, which starts with aa 1 for the first residue of VP1. However, the capsid proteins described herein include VP1, VP2 and VP3 (used interchangeably herein with vp1, vp2 and vp3) with mutations or replacements in the corresponding region of the protein. In SEQ ID NO: 2, the VP proteins include VP1 (aa 1 to 738), VP2 (aa 138 to 738), and VP3 (aa 204 to 738) using the numbering of the full length VP1. In SEQ ID NO: 4, the variable proteins correspond to VP1 (aa 1 to 736), VP2 (aa 138 to 736), and VP3 (aa 203 to 736) using the numbering of the full length VP1.

As used herein, the term “native”, “native capsid”, or “native AAV capsid” refers to a protein with an amino acid sequence encoded by nucleic acid sequence which produces the capsid protein. The term “encode” or any grammatical variation thereof refers to a process of converting a nucleic acid sequence to an amino acid sequence based on the standard genetic code (see, e.g., en.wikipedia.org/wiki/DNA_codon_table). As used herein, a “codon” refers to three nucleotides that encode a predicted amino acid. As provided herein, a mutation in a codon may be a change in one or more nucleotide bases which changes the predicted amino acid encoded by the codon.

As used herein, in certain embodiments, the encoded sequence shown in SEQ ID NO: 2 is referred as AAV8, AAV8 capsid, native AAV8 capsid, or AAV8 vp1. Similarly, the encoded sequence shown in SEQ ID NO: 4 is referred as AAV3B, AAV3B capsid, native AAV3B capsid, or AAV3B vp1. On the other hand, the term “produce” or any grammatical variation thereof refers to a process of generating a protein from a nucleic acid sequence in a target or host cell, wherein the protein may have an amino acid sequence encoded by the nucleic acid sequence, or may be about 60%, about 70%, about 80%, about 90%, about 95% identical to the encoded aa sequence. This variance from the encoded protein may occur due to transcriptional, translational, and/or post-translational regulation/modification.

The AAV capsid contains 9 hypervariable regions (HVRs) which show the most sequence divergence throughout AAV isolates. See, Govindasamy, Lakshmanan, et al. "Structurally mapping the diverse phenotype of adeno-associated virus serotype 4." Journal of virology 80.23 (2006): 11556-11570. Epub 2006 Sep 13, which is incorporated herein by reference. Thus, when rationally designing new vectors, the HVRs are a rich target. In AAV8, the HVRs are identified as follows using the numbering of the full length AAV8 VP1 (SEQ ID NO: 2) as well as shown in FIG. 1: HVR I, aa 263 to aa 267; HVR II, aa 330 to aa 333; HVR III, aa 383 to aa 391; HVR IV, aa 449 to aa 475; HVR V, aa 491 to aa 509; HVR VI, aa 539 to aa 542; HVR VII, aa 548 to aa 559; HVR VIII, aa 584 to aa 600; and HVR IX, aa 708 to aa 720. In AAV3B, the HVRs are identified as follows using the numbering of the full length AAV3B VP1 (SEQ ID NO: 4) as well as shown in FIG. 1: HVR I, aa 262 to aa 264; HVR II, aa 327 to aa 330; HVR III, aa 380 to aa 388 ; HVR IV, aa 446 to aa 473; HVR V, aa 489 to aa 507; HVR VI, aa 537 to aa 540; HVR VII, aa 546 to aa 557; HVR VIII, aa 582 to aa 598; and HVR IX, aa 706 to aa 718. In AAV2, the HVRs are identified as follows using the numbering of the full length AAV2 VP1 (SEQ ID NO: 5): HVR I, aa 262 to aa 264; HVR II, aa 327 to aa 330; HVR III, aa 380 to aa 388 ; HVR IV, aa 446 to aa 472; HVR V, aa 488 to aa 506; HVR VI, aa 536 to aa 539; HVR VII, aa 545 to aa 556; HVR VIII, aa 581 to aa 597; and HVR IX, aa 705 to aa 717.

The AAV HVR VIII contains 4 mutations which show differences between amino acid sequences of AAV3B and AAV8 capsids. In AAV8, the four Mutations of HVR VIII are identified as follows using the numbering of the full length AAV8 VP1 (SEQ ID NO: 2) as well as shown in FIG. 4A: HVR VIII Mutation 1 (VR8P1, 8VR8P1), aa 584; HVR VIII Mutation 2 (VR8P2, 8VR8P2), aa 588-589; HVR VIII Mutation 3 (VR8P3, 8VR8P3), aa 594 to aa 596; and HVR VIII Mutation 4 (VR8P4, 8VR8P4), aa 600. In AAV3B, the four Mutations of HVR VIII are identified as follows using the numbering of the full length AAV3B VP1 (SEQ ID NO: 4) as well as shown in FIG. 4A: HVR VIII Mutation 1 (VR8P1, 3BVR8P1), aa 582; HVR VIII Mutation 2 (VR8P2, 3BVR8P2), aa 586-587; HVR VIII Mutation 3 (VR8P3, 3BVR8P3), aa 592 to aa 594; and HVR VIII Mutation 4 (VR8P4, 3BVR8P4), aa 598.

The AAV HVR IX contains 6 Mutations which show differences between amino acid sequences of AAV3B and AAV8 capsids. In AAV8, the six Mutations of HVR IX are identified as follows using the numbering of the full length AAV8 VP1 (SEQ ID NO: 2) as well as shown in FIG. 10: HVR IX Mutation 1 (VR9P1, 8VR9P1), aa 708; HVR IX

Mutation 2 (VR9P2, 8VR9P2), aa 711; HVR IX Mutation 3 (VR9P3, 8VR9P3), aa 712; HVR IX Mutation 4 (VR9P4, 8VR9P4), aa 716; HVR IX Mutation 5 (VR9P5, 8VR9P5), aa 718; and HVR IX Mutation 6 (VR9P6, 8VR9P6), aa 720. In AAV3B, the four Mutations of HVR IX are identified as follows using the numbering of the full length AAV3B VP1 (SEQ ID NO: 4) as well as shown in FIG. 10: HVR IX Mutation 1 (VR9P1, 3BVR9P1), aa 706; HVR IX Mutation 2 (VR9P2, 3BVR9P2), aa 709; HVR IX Mutation 3 (VR9P3, 3BVR9P3), aa 710; HVR IX Mutation 4 (VR9P4, 3BVR9P4), aa 714; HVR IX Mutation 5 (VR9P5, 3BVR9P5), aa 716; and HVR IX Mutation 6 (VR9P6, 3BVR9P6), aa 718.

Methods of aligning AAV to determine the proper amino acid region in the AAV capsid for mutation or replacement as described herein have been described in the literature and/or are available through commercial vendors and web-based applications. See, e.g., discussion of multiple sequence alignment programs provided above in this document. Typically, when an alignment is prepared based upon the AAV capsid vp1 protein, the alignment contains insertions and deletions which are so identified with respect to a reference AAV sequence (e.g., AAV3B or AAV8 or AAV2) and the numbering of the amino acid residues is based upon a reference scale provided for the alignment. However, any given AAV sequence may have fewer amino acid residues than the reference scale. In the present invention, when discussing the parental AAV and the sequences of the reference library, the term “the same region” or the “corresponding region” refers to the amino acid(s) located at the same residue number in each of the sequences, with respect to the reference scale for the aligned sequences. However, when taken out of the alignment, each of the AAV vp1 proteins may have these amino acids located at different residue numbers.

As used herein, the term “transduction” refers to the process by which the vector genome carrying transgene as described herein is introduced into the target host cells or the target tissue. The term “transduction rate” or “transduction efficiency” refers to the levels of the transgene delivered to the target host cells or the target tissue via the AAV as described herein. Thus, “transduction rate” and “transduction efficiency” are used interchangeably herein. Typically, transduction efficiency is measured by assessing gene product expressed in the target tissue or the target host cells. In a further embodiment, transduction efficiency is measured by assessing circulating transgene product in the case of said product secreted from a transduced target host cell or target tissue. In yet another embodiment, transduction efficiency is measured by quantifying percentages of transduced cells (e.g., hepatocytes) in the target host cells or target tissue. Transduction efficiency can be evaluated by a variety of

methods, which is known by one of skills in the art. Such methods include but are not limited to the following: flow cytometric analysis of isolated hepatocytes (FACS), fluorescent or immunohistochemical imaging of a cell/tissue/organ, and luciferase assay. In one embodiment, the rAAV comprising the mutant or chimeric capsid described herein may

5 result in an increase or decrease of transduction efficiency in a species, as compared to the transduction efficiency of the rAAV comprising the corresponding parental capsid or the corresponding native capsid. In one embodiment, the increase is at least about 50 folds, or about 20 folds to about 50 folds, or about 10 folds to about 20 folds, or about 5 folds to about 10 folds, or about 2 folds to about 5 folds, or about 1 fold to about 2 folds, or about 1 fold, or

10 about 2 folds, or about 3 folds, or about 4 folds, or about 5 folds, or about 6 folds, or about 7 folds, or about 8 folds, or about 9 folds, or about 10 folds, or about 15 folds, or about 20 folds, or about 30 folds or about 40 folds, or about 50 folds or about 75 folds, or about 100 folds, or about 10%, or about 20%, or about 30 %, or about 40%, or about 50%, or about 60%, or about 70% or about 80% or about 90%, or about 10% to about 20%, or about 20%

15 to about 30%, or about 30% to about 40%, or about 40% to about 50%, or about 50% to about 60%, or about 60% to about 70%, or about 70% to about 80%, or about 80% to about 90%, or about 90% to about 100%, as compared to the transduction efficiency of the rAAV comprising the corresponding parental capsid or the corresponding native capsid.

As used herein, the “translatability” negatively correlates to the variability in

20 transduction efficiency of a rAAV comprising the capsid described herein among different species. Thus, the term “good translatability” refers to a low variability in transduction efficiency of a rAAV comprising the capsid described herein among species. In one embodiment, the low variability is less than about 1%, or about 3%, or about 5 %, or about 10%, or about 15%, or about 20%, or about 25%, or about 30%, or about 40% or about 50%

25 or about 60% of the transduction efficiency. As used herein, the term “improved translatability” refers to a reduced variability in transduction efficiency among species via a rAAV comprising the mutant or chimeric capsid as described herein, as compared to that via a rAAV comprising the corresponding parental or native capsid.

In one embodiment, the species are selected from human, non-human primate,

30 rodent, cat, dog, goat, donkey, pig, cow, other laboratory animals, horses, other domesticated animals, and livestock. In a further embodiment, rodent is selected from mouse, rat, or guinea pig.

As used herein, the term "target tissue" can refer to any tissue which is intended to be transduced by a rAAV comprising the capsid described herein. The term may refer to any one or more of muscle, liver (hepatocytes), spleen, lung, airway epithelium, neurons, eye (ocular cells), or heart. In one embodiment, the target tissue is liver. In another
 5 embodiment, the target tissue is spleen.

As used herein, the term "host cell", "packaging cell" or "packaging host cell" (used interchangeably herein) may refer to the cell in which the rAAV comprising the capsid described herein is produced. In certain embodiment, the term "host cell", "target cell" or "target host cell" (used interchangeably herein) may refer to the cell in which expression of
 10 the transgene is desired. In one embodiment, the packaging cell is a HEK 293 cell. In one embodiment, the target cell is a hepatocyte. In another embodiment, the target cell is Huh7 cell. In yet another embodiment, the target cell is MC57G cell or H2.35 cell.

In one aspect, the AAV capsid has a mutation in the HVR VIII. In one embodiment, an AAV capsid is provided which has a mutation in aa 584 (HVR VIII Mutation 1), aa 588 -
 15 aa 589 (HVR VIII Mutation 2), aa 594 - aa 596 (HVR VIII Mutation 3), or aa 590 (HVR VIII Mutation 4) using numbering of the AAV8 native sequence. In a further embodiment, the AAV capsid is provided herein, wherein the mutation comprises N at aa 584; SS at aa 588 to aa 589; TTR at aa 594 to aa 596; or D at aa 600, using the numbering of the native AAV8 capsid (SEQ ID NO: 2). In another embodiment, the AAV capsid has a mutation in
 20 the HVR IX region. In one embodiment, an adeno-associated virus (AAV) capsid comprises a mutation in the following Mutations of the HVR VIII using numbering of the native AAV3B capsid (SEQ ID NO: 4) for improving or increasing the transduction efficiency in a species: i. aa 582 (HVR VIII Mutation 1); ii. aa 586 to aa 587 (HVR VIII Mutation 2); iii. aa 592 to aa 594 (HVR VIII Mutation 3); or iv. aa 598 (HVR VIII Mutation 4). In one
 25 embodiment, the species is human. In one embodiment, the AAV capsid comprises a mutation selected from: i. G, A, V, L, I, P, F, Y, W, S, T, C, M, Q, K, R, H, or E at aa 582; ii. N at aa 582; ii. three amino acids other than QIG at aa 592 to aa 594; or iv. TTR at aa 592 to aa 594, using the numbering of the native AAV3B capsid (SEQ ID NO: 2). In one
 30 embodiment, the mutated aa 582 is N. In another embodiment, the mutated aa 592 to aa 594 is TTR. In a further embodiment, the invention provides an adeno-associated virus (AAV) capsid comprising an AAV3B HVR VIII having the sequence of aa 582 to aa 598 of SEQ ID NO: 4. In one embodiment, the AAV capsid comprises an AAV3B HVR VI having the sequence of aa 537 to aa 540 of SEQ ID NO: 4. In another embodiment, the AAV capsid

comprises an AAV3B HVR IV having the sequence of aa 446 to aa 473 of SEQ ID NO: 4. In one embodiment, the HVR VIII of an AAV capsid is replaced by an AAV3B HVR VIII having the sequence of 582 to aa 598 of SEQ ID NO: 4. In one embodiment, the HVR VI of an AAV capsid is replaced by an AAV3B HVR VI having the sequence of aa 537 to aa 540 of SEQ ID NO: 4. In one embodiment, the HVR IV of an AAV capsid is replaced by an AAV3B HVR IV having the sequence of aa 446 to aa 473 of SEQ ID NO: 4. In one embodiment, the AAV capsid comprises an amino acid sequence selected from SEQ ID NO: 43 (AAV8.3BVR8), SEQ ID NO: 51 (AAV8.3BVR8.3BVR4), SEQ ID NO: 55 (AAV8.3BVR8.3BVR6) or SEQ ID NO: 65 (AAV8.3BVR8.3BVR4.3BVR6), SEQ ID NO: 73 (AAV8.3BVR8P1), SEQ ID NO: 75 (AAV8.3BVR8P3) or SEQ ID NO: 77 (AAV8.3BVR8P1.3BVR8P3).

In one aspect, an AAV capsid is provided which has a mutation in aa 706 (HVR IX Mutation 1), aa 709 (HVR IX Mutation 2), aa 710 (HVR IX Mutation 3), aa 714 (HVR IX Mutation 4), aa 716 (HVR IX Mutation 5), or aa 718 (HVR IX Mutation 6) using the numbering of the AAV3B native sequence. In a further embodiment, the AAV capsid is provided, wherein the mutation comprises Y at aa 706; T at aa 709; S at aa 710; A at aa 714; N at aa 716; or E at aa 718 as compared to native AAV3B capsid (SEQ ID NO: 4). In one embodiment, an adeno-associated virus (AAV) capsid comprises a mutation in the following Mutations of HVR IX using the numbering of the native AAV8 capsid (SEQ ID NO: 2) for improving or increasing the transduction efficiency in a species: i. aa 708 (HVR IX Mutation 1); ii. aa 711 (HVR IX Mutation 2); iii. aa 712 (HVR IX Mutation 3); iv. aa 716 (HVR IX Mutation 4); v. aa 718 (HVR IX Mutation 5); or vi. aa 720 (HVR IX Mutation 6). In one embodiment, the species is a rodent. In a further embodiment, the species is a mouse. In one embodiment, the AAV capsid comprises a mutation in aa 708 (VR9P1) or aa 711 (VR9P3), using the numbering of the native AAV8 capsid (SEQ ID NO: 2). In one embodiment, the AAV capsid comprises a mutation selected from: i. G, A, V, L, I, P, F, Y, W, S, T, C, M, Q, K, R, H, or E at aa 708; ii. Y at aa 708; iii. G, A, V, L, I, P, F, Y, W, T, C, M, Q, K, R, H, E, or Y at aa 712; or iv. S at aa 712, using the numbering of the native AAV8 capsid (SEQ ID NO: 2). In one embodiment, the mutated aa 708 is Y. In another embodiment, the mutated aa 712 is S. In a further embodiment, the invention provides an adeno-associated virus (AAV) capsid comprising an AAV8 HVR IX having the sequence of aa 708 to aa 720 of SEQ ID NO: 2. In a further embodiment, the parental AAV capsid is a capsid other than AAV2. Previous AAV2 study (TENNEY, et al. (2014). "AAV8 capsid variable regions at

the two-fold symmetry axis contribute to high liver transduction by mediating nuclear entry and capsid uncoating." Virology 454-455: 227-236) showed that replacing AAV2' HVR.IX (its sequence is the same as AAV3B) with that from AAV8 increases its murine liver transduction significantly. In one embodiment, the HVR IX of an AAV capsid is replaced
 5 by an AAV8 HVR IX having the sequence of aa 708 to aa 720 of SEQ ID NO: 2. In one embodiment, the AAV capsid comprises an amino acid sequence selected from SEQ ID NO: 21(AAV3B.8VR9, AAV3B with its HVR.IX replaced with that of AAV8), SEQ ID NO: 79 (AAV3B.8VR9P1, AAV3B with its HVR.IX Mutation 1 replaced with that of AAV8), SEQ ID NO: 81 (AAV3B.8VR9P3, AAV3B with its HVR.IX Mutation 3 replaced with that of
 10 AAV8), SEQ ID NO: 83 (AAV3B.8VR9P3, AAV3B with its HVR.IX Mutations 1 & 3 replaced with that of AAV8), SEQ ID NO: 136 (AAV3B.8VR9.3BVR9P2, AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 2 stays as that of AAV3B), SEQ ID NO: 137 (AAV3B.8VR9.3BVR9P4, AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 4 stays as that of AAV3B), SEQ ID NO: 138
 15 (AAV3B.8VR9.3BVR9P5, AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 5 stays as that of AAV3B), or SEQ ID NO: 139 (AAV3B.8VR9.3BVR9P6, AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 6 stays as that of AAV3B).

In certain embodiments, an engineered AAV capsid is provided which has AAV VP
 20 proteins comprising an exogenous AAV8 HVR VIII Mutation 1 having an amino acid sequence produced from nt 1750 to nt 1752 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR VIII Mutation 2 having an amino acid sequence produced from nt 1762 to nt 1767 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided
 25 which has AAV VP proteins comprising an exogenous AAV8 HVR VIII Mutation 3 having an amino acid sequence produced from nt 1780 to nt 1788 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR VIII Mutation 4 having an amino acid sequence produced from nt 1798 to nt 1800 of SEQ ID NO: 1. In certain embodiments, an engineered
 30 AAV capsid is provided which has AAV VP proteins comprises any two, any three, or all four of the AAV8 HVR VIII Mutations. In certain embodiments, an engineered AAV capsid is provided which has an AAV VP protein which further comprises any one two, any three,

any four, any five, any six, or all seven of the AAV8 HVR I, II, IV, V, VI, VII, and IX, or any other mutations or substitutions as described herein.

In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR VIII Mutation 1 having an amino acid sequence produced from nt 1744 to nt 1746 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR VIII Mutation 2 having an amino acid sequence produced from nt 1756 to nt 1761 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR VIII Mutation 3 having an amino acid sequence produced from nt 1774 to nt 1782 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR VIII Mutation 4 having an amino acid sequence produced from nt 1792 to nt 1794 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising any two, any three, or all four of the AAV3B HVR VIII Mutations. In certain embodiments, an engineered AAV capsid is provided which has an AAV VP protein which may further comprise any one two, any three, any four, any five, any six, or all seven of the AAV3B HVR I, II, IV, V, VI, VII, and IX, or any other mutations or substitutions as described herein.

In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR IX Mutation 1 having an amino acid sequence produced from nt 2116 to nt 2118 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR IX Mutation 2 having an amino acid sequence produced from nt 2125 to nt 2127 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR IX Mutation 3 having an amino acid sequence produced from nt 2128 to nt 2130 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR IX Mutation 4 having an amino acid sequence produced from nt 2140 to nt 2142 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR IX Mutation 5 having an amino acid sequence produced from nt 2146 to nt 2148 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV3B HVR IX Mutation 6 having an amino

acid sequence produced from nt 2152 to nt 2154 of SEQ ID NO: 3. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising any two, any three, any four, any five, or all six of the AAV3B HVR IX Mutations. In certain embodiments, an engineered AAV capsid is provided which has an AAV VP protein which may further comprise any one two, any three, any four, any five, any six, or all seven of the AAV3B HVR I, II, IV, V, VI, VII, and VIII, or any other mutations or substitutions as described herein.

In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR IX Mutation 1 having an amino acid sequence produced from nt 2122 to nt 2124 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR IX Mutation 2 having an amino acid sequence produced from nt 2131 to nt 2133 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR IX Mutation 3 having an amino acid sequence produced from nt 2134 to nt 2136 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR IX Mutation 4 having an amino acid sequence produced from nt 2146 to nt 2148 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR IX Mutation 5 having an amino acid sequence produced from nt 2152 to nt 2154 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising an exogenous AAV8 HVR IX Mutation 6 having an amino acid sequence produced from nt 2158 to nt 2160 of SEQ ID NO: 1. In certain embodiments, an engineered AAV capsid is provided which has AAV VP proteins comprising any two, any three, any four, any five, or all six of the AAV8 HVR IX Mutations. In certain embodiments, an engineered AAV capsid is provided which has an AAV VP protein further comprising any one two, any three, any four, any five, any six, or all seven of the AAV3B HVR I, II, IV, V, VI, VII, and VIII, or any other mutations or substitutions as described herein.

In one aspect, a region of the AAV capsid described herein is replaced with the corresponding region from a different capsid to improve or increase the transduction efficiency in a species. In one embodiment, the HVR region of an AAV capsid is replaced the corresponding HVR region of AAV8. In one embodiment, the HVR IX of an AAV capsid is replaced AAV8 HVR IX. In one embodiment, a HVR region of AAV3B is replaced

a HVR region of AAV8. In another embodiment, a HVR region of an AAV is replaced the corresponding HVR region of AAV3B. In one embodiment, the HVR VI of an AAV is replaced with the HVR VI of AAV3B. In one embodiment, the HVR IV of an AAV is replaced with the HVR IV of AAV3B. In one embodiment, the HVR VIII of an AAV is replaced with the HVR VIII of AAV3B. In one embodiment, the HVR VIII of AAV8 (amino acid 584 to 600) is swapped with the corresponding portion of another capsid. The source of the HVR region may be of the same or different amino acid lengths. For example, in AAV3B, the HVR VIII region spans amino acids 582 to 598 of that sequence. See, Limberis et al, Mol Ther. 2009 Feb; 17(2): 294–301 (which is incorporated herein by reference). In yet another embodiment, the HVR region of AAV3B is replaced the corresponding HVR region of AAV8 (AAV3B.8VRn, AAV3B with its HVR.n replaced with that of AAV8). In another embodiment, a HVR region of AAV1, AAV2, AAV6, AAV9, LK03, AAVDJ, rh.8, rh.10, rh.20, hu.37, rh.2R, rh.43, rh.46, rh.64R1, hu.48R3, or cy.5R4 is replaced the corresponding HVR region of AAV8 or AAV3B. In a further embodiment, the parental AAV capsid is a capsid other than AAV2. The HVR regions can be readily determined based on alignments available in the art, which are also provided in FIG. 1, FIG. 4A (HVR VIII), and FIG. 10 (HVR IX).

The term “functional” refers to a product (e.g., a protein or peptide) which performs its native function, although not necessarily at the same level as the native product. The term “functional” may also refer to a gene which encodes a product and from which a desired product can be expressed.

Unless otherwise specified (as above), the term fragments include peptides at least 8 amino acids in length, at least 15 amino acids in length, at least 25 amino acids in length. However, fragments of other desired lengths may be readily utilized depending upon the desired context. Such fragments may be produced recombinantly or by other suitable means, e.g., by chemical synthesis.

The terms “percent (%) identity”, “sequence identity”, “percent sequence identity”, or “percent identical” in the context of amino acid sequences refers to the residues in the two sequences which are the same when aligned for correspondence. Percent identity may be readily determined for amino acid sequences over the full-length of a protein, polypeptide, about 32 amino acids, about 330 amino acids, or a peptide fragment thereof or the corresponding nucleic acid sequence coding sequencers. A suitable amino acid fragment may be at least about 8 amino acids in length, and may be up to about 700 amino acids.

Generally, when referring to “identity”, “homology”, or “similarity” between two different sequences, “identity”, “homology” or “similarity” is determined in reference to “aligned” sequences. “Aligned” sequences or “alignments” refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, e.g., the “Clustal Omega”, “Clustal X”, “MAP”, “PIMA”, “MSA”, “BLOCKMAKER”, “MEME”, and “Match-Box” programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, e.g., J. D. Thomson et al, Nucl. Acids. Res., “A comprehensive comparison of multiple sequence alignments”, 27(13):2682-2690 (1999).

As shown in the examples below, swapping to AAV3B HVR.VIII increased AAV8’s transduction in human by about 3 times. Swapping to AAV3B HVR.VI alone did not enhance AAV8’s transduction, but it demonstrated a synergetic enhancement with replacement with AAV3B HVR.VIII. Intriguingly, HVR.VI is not surface-exposed. Thus, HVR VIII is the determining HVR for AAV3B’s high transduction efficiency in human cells. Aa 582 and aa 592 - 594 of AAV3B are critical to AAV3B’s high transduction efficiency in human, demonstrating that charges of accessible amino acids at HVR VIII of an AAV is critical to its transduction. Altering, increasing or decreasing the charges of the amino acids at HVR VIII of an AAV is provided as a method to improving or increasing the transduction efficiency in a species. The methods of altering, increasing or decreasing the charges of the amino acid of an AAV is known to one skilled in the art. As used herein, the term “swapping” or any grammatical variation thereof refers to a process of inserting the indicated sequence into the parental sequence, and deleting sequence in the parental which corresponds with the inserted indicated sequence. The correspondence may be the same HVR, or part of HVR, which can be determined by sequence alignments.

In another embodiment, AAV3B preforms poorly in mouse cells. HVR IX of AAV8 was the only HVR that increase parental capsid’s (e.g., AAV3B’s) transduction in mouse cells. This is also true in mouse liver transduction *in vivo*, demonstrating that HVR IX is a major region to determine the species-specificity of AAV3B thus provides a powerful tool

for evaluating as well as constructing better gene therapy vectors. It is known to one of skill in the art that there are at least 53 AAVs carrying this HVR IX sequence in Genbank. Only very few of them have had a chance to be evaluated due to the previously reported poor performance of AAV2 and AAV3 in mice.

5 In one aspect, the invention provides a method of evaluating the transduction efficiency in a species or translatability among species of the AAV comprising an AAV capsid via comparing the amino acid sequence of said capsid with that of AAV8 or AAV3B in the region of HVR VIII and/or HVR IX.

 In one embodiment, an AAV capsid is provided which has the sequence shown in
10 SEQ ID NO: 43 (AAV8.3BVR8, AAV8 with its HVR VIII replaced with that of AAV3B). In one embodiment, an AAV capsid is provided which has the sequence shown in SEQ ID NO: 51 (AAV8.3BVR8.3BVR4, AAV8 with its HVR VIII and HVR IV replaced with those of AAV3B). In one embodiment, an AAV capsid is provided which has the sequence shown in SEQ ID NO: 55 (AAV8.3BVR8.3BVR6, AAV8 with its HVR VIII and HVR VI
15 replaced with those of AAV3B). In one embodiment, an AAV capsid is provided which has the sequence shown in SEQ ID NO: 65 (AAV8.3BVR8.3BVR4.3BVR6, AAV8 with its HVR VIII, HVR VI and HVR IV replaced with those of AAV3B). In one embodiment, an AAV capsid is provided which has the sequence shown in SEQ ID NO: 73 (AAV8.3BVR8P1, AAV8 with its HVR VIII Mutation 1 replaced with that of AAV3B). In
20 one embodiment, an AAV capsid is provided which has the sequence shown in SEQ ID NO: 75 (AAV8.3BVR8P3, AAV8 with its HVR VIII Mutation 3 replaced with that of AAV3B). In one embodiment, an AAV capsid is provided which has the sequence shown in SEQ ID NO: 77 (AAV8.3BVR8P1.3BVR8P3, AAV8 with its HVR VIII Mutation 1 and HVR VIII Mutation 3 replaced with those of AAV3B). In one embodiment, an AAV capsid is provided
25 which has the sequence shown in SEQ ID NO: 21 (AAV3B.8VR9, AAV3B with its HVR IX replaced with that of AAV8). In another embodiment, an AAV capsid comprises a vp1, vp2, vp3 or a fragment of any capsid described herein.

 In one embodiment, the engineered AAV capsid comprises a VP protein which is produced by a sequence of SEQ ID NO: 7 or any fragment thereof. In a further embodiment,
30 a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 6. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 9. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 8. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID

NO: 11. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 10. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 13. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 12. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 15. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 14. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 17. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 16. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 19. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 18. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 21. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 20. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 23. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 22. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 25. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 24. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 27. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 26. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 29. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 28. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 31. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 30. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 33. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 32. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 35. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 34. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 37. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 36. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 39. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 38. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 41. In a further embodiment, a nucleic acid sequence encoding said capsid is

reproduced in SEQ ID NO: 40. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 43. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 42. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 45. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 44. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 47. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 46. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 49. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 48.

10 In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 51. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 50. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 53. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 52. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 55. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 54. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 57. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 56. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 59. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 58. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 61. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 60. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 63. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 62.

20 In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 65. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 64. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 67. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 66. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 69. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 68. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 71. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 70. In one embodiment, the engineered AAV capsid

comprises a sequence of SEQ ID NO: 73. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 72. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 75. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 74. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 77. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 76. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 79. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 78. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 81. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 80. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 83. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 82. In one embodiment, the engineered AAV capsid comprises a sequence of SEQ ID NO: 133, SEQ ID NO: 134, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, SEQ ID NO: 138, or SEQ ID NO: 139. In a further embodiment, a nucleic acid sequence encoding said capsid is reproduced in SEQ ID NO: 134. In one embodiment, a nucleic acid sequence encoding SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, SEQ ID NO: 138, or SEQ ID NO: 139 is provided.

In one embodiment, the capsid is produced in a cell using, at a minimum, a sequence of SEQ ID NO: 6. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 8. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 10. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 12. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 14 which encodes an amino acid sequence of SEQ ID NO: 15. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 16. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 18. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 20. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 22. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 24. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 26. In one embodiment, the capsid is produced in a host cell using, at a minimum, a

sequence of SEQ ID NO: 28. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 30. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 32. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 34. In one
5 embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 36. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 38. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 40. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 42. In one
10 embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 44. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 46. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 48. In one embodiment, the capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 50. In one embodiment, the
15 capsid is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 52. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 54. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 56. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 58. In one
20 embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 60. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 62. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 64. In one
embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence
25 of SEQ ID NO: 66. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 68. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 70. In one
embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 72. In one embodiment, the capsid protein is produced in a host cell using,
30 at a minimum, a sequence of SEQ ID NO: 74. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 76. In one
embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 78. In one embodiment, the capsid protein is produced in a host cell using,

at a minimum, a sequence of SEQ ID NO: 80. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 82. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence of SEQ ID NO: 134. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence which can encode an amino acid sequence of SEQ ID NO: 135. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence which can encode an amino acid sequence of SEQ ID NO: 136. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence which can encode an amino acid sequence of SEQ ID NO: 137. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence which can encode an amino acid sequence of SEQ ID NO: 138. In one embodiment, the capsid protein is produced in a host cell using, at a minimum, a sequence which can encode an amino acid sequence of SEQ ID NO: 139.

In one embodiment, the capsid is produced in a cell using, at a minimum, a sequence which can encode an amino acid sequence selected from: SEQ ID NOs: 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 135, 136, 137, 138, and 139.

It should be understood that the compositions described herein are intended to be applied to other compositions, regimens, aspects, embodiments and methods described across the Specification.

B. Nucleic Acid Sequences Encoding the AAV Capsids and Host Cells

In certain embodiments, a nucleic acid sequence useful in producing an engineered AAV capsid protein is provided, which comprises a nucleic acid sequence encoding aa 262 to aa 264 of SEQ ID NO: 4 (HVR I). In certain embodiments, the invention provides a nucleic acid sequence producing an engineered AAV capsid protein, and comprising a nucleic acid encoding aa 327 to aa 330 of SEQ ID NO: 4 (HVR II). In certain embodiments, the invention provides a nucleic acid sequence producing an engineered AAV capsid protein, and comprising a nucleic acid encoding aa 446 to aa 473 of SEQ ID NO: 4 (HVR IV). In certain embodiments, the invention provides a nucleic acid sequence producing an engineered AAV capsid protein, and comprising a nucleic acid encoding aa 489 to aa 507 of SEQ ID NO: 4 (HVR V). In certain embodiments, the invention provides a nucleic acid sequence producing an engineered AAV capsid protein, and comprising a nucleic acid

encoding aa 537 to aa 540 of SEQ ID NO: 4 (HVR VI). In certain embodiments, the invention provides a nucleic acid sequence producing an engineered AAV capsid protein, and comprising a nucleic acid encoding aa 546 to aa 557 of SEQ ID NO: 4 (HVR VII). In certain embodiments, the invention provides a nucleic acid sequence producing an engineered AAV capsid protein, and comprising a nucleic acid encoding aa 582 to aa 598 of SEQ ID NO: 4 (HVR VIII). In certain embodiments, the invention provides a nucleic acid sequence producing an engineered AAV capsid protein, and comprising a nucleic acid encoding aa 706 to aa 718 of SEQ ID NO: 4 (HVR IX). In certain embodiments, a nucleic acid sequence producing an engineered AAV capsid protein, may comprises any two, any three, any four, any five, any six, any seven, or all eight of the nucleic acids encoding AAV 3B HVR I, II, IV, V, VI, VII, VIII, and IX. For example, the nucleic acid sequence may produce AAV8.8.6 (an AAV8 backbone swapped with HVR8 and HVR6 of AAV3B), AAV8.8.6.7 (an AAV8 backbone swapped with HVR8, HVR7, and HVR6 of AAV3B), AAV8.8.6.9 (an AAV8 backbone swapped with HVR9, HVR8, and HVR6 of AAV3B), AAV8.8.7 (an AAV8 backbone swapped with HVR8, and HVR7 of AAV3B), AAV8.8.9 (an AAV8 backbone swapped with HVR8 and HVR9 of AAV3B).

In certain embodiment, provided herein is a nucleic acid sequence producing or encoding an engineered capsid as described herein.

Nucleic acid sequence encoding an amino acid sequence may be generated via tools for reverse-translation, e.g., www.ebi.ac.uk/Tools/st/, www.ebi.ac.uk/Tools/st/emboss_transeq/, www.ebi.ac.uk/Tools/st/emboss_sixpack/, www.ebi.ac.uk/Tools/st/emboss_backtranseq/, and www.ebi.ac.uk/Tools/st/emboss_backtranambig/. Furthermore, the coding sequences might be codon-optimized for expression in a subject, e.g., human, mice, rat or a non-human primate.

A nucleic acid molecule comprising a nucleic acid sequence producing an engineered AAV capsid protein or a fragment thereof. In one embodiment, the nucleic acid molecule further comprises a sequence encoding a functional AAV rep or a fragment thereof. In one embodiment, the nucleic acid molecular further comprises a reporter sequence as described herein. In another embodiment, the nucleic acid molecule is a plasmid. In yet another embodiment, the nucleic acid molecule comprises a regulatory element. In yet another embodiment, two or more of the nucleic acid sequences share a regulatory element. In yet another embodiment, the nucleic acid sequence sharing the same regulatory element(s) are

separated by a separator. In a further embodiment, the separator is an internal ribozyme entry site (IRES). As an alternative to an IRES, the nucleic acid sequences may be separated by sequences encoding a 2A peptide, which self-cleaves in a post-translational event. See, e.g., M.L. Donnelly, et al, J. Gen. Virol., 78(Pt 1):13-21 (Jan 1997); Furler, S., et al, Gene Ther., 8(11):864-873 (June 2001); Klump H., et al., Gene Ther., 8(10):811-817 (May 2001). This 2A peptide is significantly smaller than an IRES, making it well suited for use when length of the nucleic acid molecule is a limiting factor.

Reporters include sequences encoding geneticin, hygromycin or purimycin resistance, among others. Such selectable reporters or marker genes can be used to signal the presence of the plasmids in bacterial cells, such as ampicillin resistance. Other components of the plasmid may include an origin of replication. Selection of these and other promoters and vector elements are conventional, and many such sequences are available.

In the nucleic acid molecular wherein nucleic acid sequences encoding both rep and capsid (cap) are provided, the AAV rep and AAV cap sequences can both be of one serotype origin, e.g., all AAV8 origin. Alternatively, vectors may be used in which the rep sequences are from an AAV which differs from the AAV providing the cap sequences. In one embodiment, the rep and cap sequences are expressed from separate sources (e.g., separate nucleic acid molecules). In another embodiment, a rep sequence is fused in frame to a cap sequence of a different AAV serotype to form a chimeric AAV, such as AAV2/8 described in US Patent No. 7,282,199, which is incorporated by reference herein. Optionally, the nucleic acid molecule further contains a vector genome comprising a transgene which is flanked by AAV 5' ITR and AAV 3' ITR.

In certain embodiments, the nucleic acid sequence may be codon-optimized.

In another aspect, the invention provides a composition comprising a nucleic acid molecule described herein and a physiologically compatible carrier. In another embodiment, the carrier is saline, which may be formulated with a variety of buffering solutions (e.g., phosphate buffered saline). Other exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextran, agar, pectin, peanut oil, sesame oil, and water.

Optionally, the compositions may contain other conventional pharmaceutical ingredients,

such as preservatives, or chemical stabilizers. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable chemical stabilizers include gelatin and albumin. In another embodiment, the composition comprising a carrier described

herein as well as in Section D- Pharmaceutical Compositions and Administration of the detailed description of the invention.

In still a further aspect, the invention provides a packaging host cell comprising a nucleic acid molecule as described herein. In one embodiment, the host cell is a HEK 293 cell. In one embodiment, the host cell is an insect cell, such as Sf9. Also provided herein are host cells transfected with a nucleic acid molecule as described herein. Most suitably, such a stable host cell contains the required component(s) under the control of an inducible promoter. However, the required component(s) may be under the control of a constitutive promoter. Examples of suitable inducible and constitutive promoters are known to one of skills in the art and also provided herein, in the discussion below of regulatory elements suitable for use with the transgene. In still another alternative, a selected stable host cell may contain selected component(s) under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters. For example, a stable host cell may be generated which is derived from HEK 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contains the rep and/or cap under the control of inducible promoters. Still other stable host cells may be generated by one of skill in the art. Preferably, the molecule carrying the vector genome is transfected into the packaging cell, where it may exist transiently. Alternatively, the vector genome may be stably integrated into the genome of the packaging host cell, either chromosomally or as an episome. In certain embodiments, the vector genome may be present in multiple copies, optionally in head-to-head, head-to-tail, or tail-to-tail concatamers. Suitable transfection techniques are known and may readily be utilized to deliver the expression cassette to the host cell. Generally, when delivering the vector genome comprising the expression cassette by transfection, the vector is delivered in an amount from about 5 μ g to about 100 μ g DNA, about 10 μ g to about 50 μ g DNA to about 1×10^4 cells to about 1×10^{13} cells, or about 1×10^5 cells. However, the relative amounts of vector DNA to host cells may be adjusted by one of ordinary skill in the art, who may take into consideration such factors as the selected vector, the delivery method and the host cells selected.

In yet another aspect, a method of generating a recombinant adeno-associated virus is provided. In one embodiment, the method for generating a recombinant adeno-associated virus (rAAV) comprises the steps of culturing a packaging host cell containing: (a) a nucleic acid molecule as described herein; (b) a molecule encoding a functional rep; (c) a vector genome comprising AAV inverted terminal repeats (ITRs) and a transgene; and (d) sufficient

helper functions to permit packaging of the vector genome into the AAV capsid protein. In one embodiment, described herein are molecules which utilize the AAV capsid sequences described herein, including fragments thereof, for production in packaging cells useful in delivery of a transgene or other nucleic acid sequences to a target cell.

5 The components required to culture the host cell to package a vector genome in an AAV capsid may be provided in the host cell in trans. Alternatively, any one or more of the required components (e.g., vector genome, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required components using methods known to those of skill in the art.

10 The vector genome, *rep* sequences, *cap* sequences, and helper functions required for producing the rAAV described herein may be delivered to the packaging host cell in the form of any genetic element which transfers the sequences carried thereon. The selected genetic element may be delivered by any suitable method, including those described herein. The methods used to construct any embodiment of this invention are known to those with
15 skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY. Similarly, methods of generating rAAV are well known and the selection of a suitable method is not a limitation on the present invention. See, e.g., K. Fisher et al, 1993 *J. Virol.*, 70:520-532 and US Patent
20 5,478,745, among others. These publications are incorporated by reference herein.

 The rAAV may be generated using methods described herein, or other methods described in the art, and purified as described. See, e.g., M. Mietzsch et al, "OneBac: Platform for Scalable and High-Titer Production of Adeno-Associated Virus Serotype 1-12 Vectors for Gene Therapy, *Hum Gene Ther.* 2014 Mar 1; 25(3): 212–222. See, also, Smith
25 RH, et al, *Mol Ther*, 2009 Nov; 17(11): 1888-96 (2009), describing a simplified baculovirus-AAV vector expression system coupled with one-step affinity purification. For example, lysates or supernatants (e.g., treated, freeze-thaw supernatants or media containing secreted rAAV), may be purified using one-step AVB sepharose affinity chromatography using 1 ml prepacked HiTrap columns on an ACTA purifier (GE Healthcare) as described by
30 manufacturer, or in M. Mietzsch, et al, cited above. In one embodiment, an affinity capture method is performed using an antibody-capture affinity resin. See, e.g. WO 2017/015102. Alternatively, the rAAV used herein may be purified using other techniques known in the art.

Methods of preparing AAV-based vectors are known. See, e.g., US Published Patent Application No. 2007/0036760 (February 15, 2007), which is incorporated by reference herein. The use of AAV capsids having tropism for muscle cells and/or cardiac cells are particularly well suited for the compositions and methods described herein. However, other targets may be selected. The sequences of AAV9 and methods of generating vectors based on the AAV9 capsid are described in US 7,906,111; US2015/0315612; WO 2012/112832; and WO2017160360A3, which are incorporated herein by reference. In certain embodiments, the sequences of AAV1, AAV5, AAV6, AAV9, AAV8triple, Anc80, Anc81 and Anc82 are known and may be used to generate AAV vector. See, e.g., US 7186552, WO 2017/180854, US 7,282,199 B2, US 7,790,449, and US 8,318,480, which are incorporated herein by reference. The sequences of a number of such AAV are provided in the above-cited US Patent 7,282,199 B2, US 7,790,449, US 8,318,480, US Patent 7,906,111, WO/2003/042397, WO/2005/033321, WO/2006/110689, US 8,927,514, US 8,734,809; WO2015054653A3, WO-2016065001-A1, WO-2016172008-A1, WO-2015164786-A1, US-2010186103-A1, WO-2010138263-A2, and WO 2016/049230A1, and/or are available from GenBank. Corresponding methods have been described for AAV1, AAV8, and AAVrh10-like vectors. See, WO2017100676 A1; WO2017100674A1; and WO2017100704A1.

The recombinant adeno-associated virus (AAV) described herein may be generated using techniques which are known. See, e.g., WO 2003/042397; WO 2005/033321, WO 2006/110689; US 7588772 B2. Such a method involves culturing a host cell which contains a nucleic acid sequence encoding an AAV capsid; a functional rep gene; an expression cassette composed of, at a minimum, AAV inverted terminal repeats (ITRs) and a transgene; and sufficient helper functions to permit packaging of the expression cassette into the AAV capsid protein. The host cell may be a 293 cell or a suspension 293 cell. See, e.g., Zinn, E., et al., as cited herein; Joshua C Grieger et al. Production of Recombinant Adeno-associated Virus Vectors Using Suspension HEK293 Cells and Continuous Harvest of Vector From the Culture Media for GMP FIX and FLT1 Clinical Vector. Mol Ther. 2016 Feb; 24(2): 287–297. Published online 2015 Nov 3. Prepublished online 2015 Oct 6. doi: 10.1038/mt.2015.187; Laura Adamson-Small, et al. Sodium Chloride Enhances Recombinant Adeno-Associated Virus Production in a Serum-Free Suspension Manufacturing Platform Using the Herpes Simplex Virus System. Hum Gene Ther Methods. 2017 Feb 1; 28(1): 1–14. Published online 2017 Feb 1. doi: 10.1089/hgtb.2016.151; US20160222356A1; and Chahal PS et al. Production of adeno-associated virus (AAV)

serotypes by transient transfection of HEK293 cell suspension cultures for gene delivery. *J Virol Methods*. 2014 Feb;196:163-73. doi: 10.1016/j.jviromet.2013.10.038. Epub 2013 Nov 13.

Other methods of producing rAAV available to one of skill in the art may be utilized.

- 5 Suitable methods may include without limitation, baculovirus expression system (e.g., baculovirus-infected-insect-cell system) or production via yeast. See, e.g., WO2005072364A2; WO2007084773A2; WO2007148971A8; WO2017184879A1; WO2014125101A1; US6723551B2; Bryant, L.M., et al., Lessons Learned from the Clinical Development and Market Authorization of Glybera. *Hum Gene Ther Clin Dev*, 2013; Robert M. Kotin, Large-scale recombinant adeno-associated virus production. *Hum Mol Genet*. 2011 Apr 15; 20(R1): R2–R6. Published online 2011 Apr 29. doi: 10.1093/hmg/ddr141; Aucoin MG et al., Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios. *Biotechnol Bioeng*. 2006 Dec 20;95(6):1081-92; SAMI S. THAKUR, Production of Recombinant Adeno-associated viral
10 vectors in yeast. Thesis presented to the Graduate School of the University of Florida, 2012; Kondratov O et al. Direct Head-to-Head Evaluation of Recombinant Adeno-associated Viral Vectors Manufactured in Human versus Insect Cells, *Mol Ther*. 2017 Aug 10. pii: S1525-0016(17)30362-3. doi: 10.1016/j.ymthe.2017.08.003. [Epub ahead of print]; Mietzsch M et al, OneBac 2.0: Sf9 Cell Lines for Production of AAV1, AAV2, and AAV8 Vectors with
15 Minimal Encapsidation of Foreign DNA. *Hum Gene Ther Methods*. 2017 Feb;28(1):15-22. doi: 10.1089/hgtb.2016.164.; Li L et al. Production and characterization of novel recombinant adeno-associated virus replicative-form genomes: a eukaryotic source of DNA for gene transfer. *PLoS One*. 2013 Aug 1;8(8):e69879. doi: 10.1371/journal.pone.0069879. Print 2013; Galibert L et al, Latest developments in the large-scale production of adeno-
20 associated virus vectors in insect cells toward the treatment of neuromuscular diseases. *J Invertebr Pathol*. 2011 Jul;107 Suppl:S80-93. doi: 10.1016/j.jip.2011.05.008; and Kotin RM, Large-scale recombinant adeno-associated virus production. *Hum Mol Genet*. 2011 Apr 15;20(R1):R2-6. doi: 10.1093/hmg/ddr141. Epub 2011 Apr 29.

i. Rep and Cap Sequences

- 30 The host cell contains the sequences which drive expression of a AAV capsid protein of the invention (or a capsid protein comprising a fragment thereof) in the host cell and rep sequences of the same source as the source of the AAV ITRs found in the expression cassette, or a cross-complementing source. The AAV cap and rep sequences may be

independently obtained from an AAV source as described above and may be introduced into the host cell in any manner known to one in the art as described above. Additionally, when pseudotyping an AAV vector, the sequences encoding each of the essential rep proteins may be supplied by different AAV sources (e.g., AAV2, AAV3, AAV4, AAV5, AAV7, AAV8, AAV9, or one of the other AAV sequences described herein or known in the art). For example, the rep78/68 sequences may be from AAV2, whereas the rep52/40 sequences may be from AAV8.

In one embodiment, the host cell stably contains the capsid protein under the control of a suitable promoter, such as those described above. Most desirably, in this embodiment, the capsid protein is expressed under the control of an inducible promoter. In another embodiment, the capsid protein is supplied to the host cell in trans. When delivered to the host cell in trans, the capsid protein may be delivered via a plasmid which contains the sequences necessary to direct expression of the selected capsid protein in the host cell. Most desirably, when delivered to the host cell in trans, the plasmid carrying the capsid protein also carries other sequences required for packaging the rAAV, e.g., the rep sequences.

In another embodiment, the host cell stably contains the rep sequences under the control of a suitable promoter, such as those described above. Most desirably, in this embodiment, the essential rep proteins are expressed under the control of an inducible promoter. In another embodiment, the rep proteins are supplied to the host cell in trans. When delivered to the host cell in trans, the rep proteins may be delivered via a plasmid which contains the sequences necessary to direct expression of the selected rep proteins in the host cell.

Thus, in one embodiment, the rep and cap sequences may be transfected into the host cell on a single nucleic acid molecule and exist stably in the cell as an episome. In another embodiment, the rep and cap sequences are stably integrated into the chromosome of the cell. Another embodiment has the rep and cap sequences transiently expressed in the host cell. For example, a useful nucleic acid molecule for such transfection comprises, from 5' to 3', a promoter, an optional spacer interposed between the promoter and the start site of the rep gene sequence, an AAV rep gene sequence, and an AAV cap gene sequence.

In certain embodiments, various methods may be used to adjust ratio of VP1, VP2, VP3 in a produced AAV vector. Such methods may be transfecting an additional nucleic acid sequence encoding a VP protein into host cells, and selecting a suitable regulatory element for each nucleic acid sequence encoding a VP. Further methods may include

optimizing the coding sequence of a VP protein for expression in a host cell, for example, codon-optimization based on species-preferred codon usage bias (e.g., en.wikipedia.org/wiki/Codon_usage_bias, Athey J et al. A new and updated resource for codon usage tables. BMC Bioinformatics. 2017 Sep 2;18(1):391. doi: 10.1186/s12859-017-1793-7.) of the host cell, or variant translational efficiency of different initiation codon and sequence operably linked thereto (see, e.g., Sean M. O'Donnell et al. J Bacteriol. 2001 Feb; 183(4): 1277–1283; Chen SJ et al. Translational efficiency of redundant ACG initiator codons is enhanced by a favorable sequence context and remedial initiation. J Biol Chem. 2009 Jan 9;284(2):818-27. doi: 10.1074/jbc.M804378200. Epub 2008 Nov 14; and Chen SJ et al. Translational efficiency of a non-AUG initiation codon is significantly affected by its sequence context in yeast. J Biol Chem. 2008 Feb 8;283(6):3173-80. Epub 2007 Dec 7.).

Optionally, the rep and/or cap sequences may be supplied on a vector that contains other nucleic acid sequences that are to be introduced into the host cells. The vector may comprise one or more of the genes encoding the helper functions, e.g., the adenoviral proteins E1, E2a, and E4 ORF6, and the gene for VAI RNA.

Preferably, the promoter used in this construct may be any of the constitutive, inducible or native promoters known to one of skill in the art or as discussed above. In one embodiment, an AAV P5 promoter sequence is employed. The selection of the AAV to provide any of these sequences does not limit the invention.

In another preferred embodiment, the promoter for rep is an inducible promoter, such as are discussed above in connection with the transgene regulatory elements. One preferred promoter for rep expression is the T7 promoter. The vector comprising the rep gene regulated by the T7 promoter and the cap gene, is transfected or transformed into a cell which either constitutively or inducibly expresses the T7 polymerase. See International Patent Publication No. WO 98/10088, published March 12, 1998.

The spacer is an optional element in the design of the vector. The spacer is a DNA sequence interposed between the promoter and the rep gene ATG start site. The spacer may have any desired design; that is, it may be a random sequence of nucleotides, or alternatively, it may encode a gene product, such as a marker gene. The spacer may contain genes which typically incorporate start/stop and polyA sites. The spacer may be a non-coding DNA sequence from a prokaryote or eukaryote, a repetitive non-coding sequence, a coding sequence without transcriptional controls or a coding sequence with transcriptional controls. Two exemplary sources of spacer sequences are the λ phage ladder sequences or yeast ladder

sequences, which are available commercially, e.g., from Gibco or Invitrogen, among others. The spacer may be of any size sufficient to reduce expression of the rep78 and rep68 gene products, leaving the rep52, rep40 and cap gene products expressed at normal levels. The length of the spacer may therefore range from about 10 bp to about 10.0 kbp, preferably in the range of about 100 bp to about 8.0 kbp. To reduce the possibility of recombination, the spacer is preferably less than 2 kbp in length; however, the invention is not so limited.

Although the molecule(s) providing rep and cap may exist in the host cell transiently (i.e., through transfection), it is preferred that one or both of the rep and cap proteins and the promoter(s) controlling their expression be stably expressed in the host cell, e.g., as an episome or by integration into the chromosome of the host cell. The methods employed for constructing embodiments of this invention are conventional genetic engineering or recombinant engineering techniques such as those described in the references above. While this specification provides illustrative examples of specific constructs, using the information provided herein, one of skill in the art may select and design other suitable constructs, using a choice of spacers, P5 promoters, and other elements, including at least one translational start and stop signal, and the optional addition of polyadenylation sites.

In another embodiment of this invention, the rep or cap protein may be provided stably by a host cell.

ii. The Helper Functions

The packaging host cell also requires helper functions in order to package the rAAV. Optionally, these functions may be supplied by a herpesvirus. Most desirably, the necessary helper functions are each provided from a human or non-human primate adenovirus source, such as those described above and/or are available from a variety of sources, including the American Type Culture Collection (ATCC), Manassas, VA (US). In one embodiment, the host cell is provided with and/or contains an E1a gene product, an E1b gene product, an E2a gene product, and/or an E4 ORF6 gene product. The host cell may contain other adenoviral genes such as VAI RNA, but these genes are not required. In a preferred embodiment, no other adenovirus genes or gene functions are present in the host cell.

The adenovirus E1a, E1b, E2a, and/or E4ORF6 gene products, as well as any other desired helper functions, can be provided using any means that allows their expression in a cell. Each of the sequences encoding these products may be on a separate vector, or one or more genes may be on the same vector. The vector may be any vector known in the art or disclosed above, including plasmids, cosmids and viruses. Introduction into the host cell of

the vector may be achieved by any means known in the art or as disclosed above, including transfection, infection, electroporation, liposome delivery, membrane fusion techniques, high velocity DNA-coated pellets, viral infection and protoplast fusion, among others. One or more of the adenoviral genes may be stably integrated into the genome of the host cell, stably expressed as episomes, or expressed transiently. The gene products may all be expressed transiently, on an episome or stably integrated, or some of the gene products may be expressed stably while others are expressed transiently. Furthermore, the promoters for each of the adenoviral genes may be selected independently from a constitutive promoter, an inducible promoter or a native adenoviral promoter. The promoters may be regulated by a specific physiological state of the organism or cell (i.e., by the differentiation state or in replicating or quiescent cells) or by other means, e.g., by exogenously added factors.

iii. Host Cells and Packaging Cell Lines

The host cell itself may be selected from any biological organism, including prokaryotic (e.g., bacterial) cells, and eukaryotic cells, including, insect cells, yeast cells and mammalian cells. Particularly desirable host cells are selected from among any mammalian species, including, without limitation, cells such as A549, WEHI, 3T3, 10T1/2, BHK, MDCK, COS 1, COS 7, BSC 1, BSC 40, BMT 10, VERO, WI38, HeLa, 293 cells (which express functional adenoviral E1), Saos, C2C12, L cells, HT1080, HepG2 and primary fibroblast, hepatocyte and myoblast cells derived from mammals including human, monkey, mouse, rat, rabbit, and hamster. The selection of the mammalian species providing the cells is not a limitation of this invention; nor is the type of mammalian cell, i.e., fibroblast, hepatocyte, tumor cell, etc. The requirements for the cell used is that it not carry any adenovirus gene other than E1, E2a and/or E4 ORF6; it not contain any other virus gene which could result in homologous recombination of a contaminating virus during the production of rAAV; and it is capable of infection or transfection of DNA and expression of the transfected DNA. In a preferred embodiment, the host cell is one that has rep and cap stably transfected in the cell.

One host cell useful in the present invention is a host cell stably transformed with the sequences encoding rep and cap, and which is transfected with the adenovirus E1, E2a, and E4ORF6 DNA and a construct carrying the expression cassette as described above. Stable rep and/or cap expressing cell lines, such as B-50 (International Patent Application Publication No. WO 99/15685), or those described in US Patent No. 5,658,785, may also be similarly employed. Another desirable host cell contains the minimum adenoviral DNA

which is sufficient to express E4 ORF6. Yet other cell lines can be constructed using the novel singleton-corrected AAV cap sequences of the invention.

The preparation of a host cell according to this invention involves techniques such as assembly of selected DNA sequences. This assembly may be accomplished utilizing conventional techniques. Such techniques include cDNA and genomic cloning, which are well known and are described in Sambrook et al., cited above, use of overlapping oligonucleotide sequences of the adenovirus and AAV genomes, combined with polymerase chain reaction, synthetic methods, and any other suitable methods which provide the desired nucleotide sequence.

Introduction of the molecules (as plasmids or viruses) into the host cell may also be accomplished using techniques known to the skilled artisan and as discussed throughout the specification. In preferred embodiment, standard transfection techniques are used, e.g., CaPO₄ transfection or electroporation, and/or infection by hybrid adenovirus/AAV vectors into cell lines such as the human embryonic kidney cell line HEK 293 (a human kidney cell line containing functional adenovirus E1 genes which provides trans-acting E1 proteins).

It should be understood that the rAAV compositions described herein are intended to be applied to other regimens, aspects, embodiments and methods described across the specification.

C. The recombinant AAV (rAAV) and Compositions

An adeno-associated virus (AAV) comprising an engineered capsid is provided. Also provided herein is an adeno-associated virus (AAV) comprising AAV inverted terminal repeats, a transgene operably linked to regulatory sequences which direct expression of a product encoded by said transgene in a target host cell or in a target tissue and an engineered capsid is provided. A composition comprising the AAV described herein and a physiologically compatible carrier is provided. A composition comprises said AAV and a physiologically compatible carrier, buffers, adjuvants, and/or diluent.

In another embodiment, the AAV is a self-complementary AAV (sc-AAV) (See, US 2012/0141422 which is incorporated herein by reference). Self-complementary vectors package an inverted repeat genome that can fold into dsDNA without the requirement for DNA synthesis or base-pairing between multiple vector genomes. Because scAAV have no need to convert the single-stranded DNA (ssDNA) genome into double-stranded DNA (dsDNA) prior to expression, they are more efficient vectors. However, the trade-off for this

efficiency is the loss of half the coding capacity of the vector, ScAAV are useful for small protein-coding genes (up to ~55 kd) and any currently available RNA-based therapy.

The rAAV described herein also comprise a vector genome. As used herein, a “vector genome” refers to the nucleic acid sequence packaged inside a vector. The vector genome is composed of, at a minimum, a transgene and its regulatory sequences, and 5’ and 3’ AAV inverted terminal repeats (ITRs). It is this vector genome which is packaged into a capsid and delivered to a selected target cell or target tissue.

i. ITR

Unless otherwise specified, the AAV ITRs, and other selected AAV components described herein, may be individually selected from among any AAV serotype, including, without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 or other known and unknown AAV serotypes. In one desirable embodiment, the ITRs of AAV serotype 2 are used. However, ITRs from other suitable serotypes may be selected. These ITRs or other AAV components may be readily isolated using techniques available to those of skill in the art from an AAV serotype. Such AAV may be isolated or obtained from academic, commercial, or public sources (e.g., the American Type Culture Collection, Manassas, VA). Alternatively, the AAV sequences may be obtained through synthetic or other suitable means by reference to published sequences such as are available in the literature or in databases such as, e.g., GenBank, PubMed, or the like.

ii. Transgene

The transgene is a nucleic acid sequence, heterologous to the vector ITR sequences flanking the transgene, which encodes a polypeptide, protein, or other product, of interest. The nucleic acid coding sequence (i.e., transgene) is operatively linked to regulatory components in a manner which permits transgene transcription, translation, and/or expression in a target cell. The heterologous nucleic acid sequence (transgene) can be derived from any organism. The AAV may comprise one or more transgenes.

The composition of the transgene sequence will depend upon the use to which the resulting vector will be put. For example, one type of transgene includes a reporter sequence, which upon expression produces a detectable signal. Such reporter sequences include, without limitation, DNA sequences encoding β -lactamase, β -galactosidase (LacZ), alkaline phosphatase, thymidine kinase, green fluorescent protein (GFP), enhanced GFP (eGFP), chloramphenicol acetyltransferase (CAT), luciferase, membrane bound proteins

including, for example, CD2, CD4, CD8, the influenza hemagglutinin protein, and others well known in the art, to which high affinity antibodies directed thereto exist or can be produced by conventional means, and fusion proteins comprising a membrane bound protein appropriately fused to an antigen tag domain from, among others, hemagglutinin or Myc.

5 These reporter sequences, when associated with regulatory elements which drive their expression, provide signals detectable by conventional means, including enzymatic, radiographic, colorimetric, fluorescence or other spectrographic assays, fluorescent activating cell sorting assays and immunological assays, including enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and immunohistochemistry. For
10 example, where the marker sequence is the LacZ gene, the presence of the vector carrying the signal is detected by assays for beta-galactosidase activity. Where the transgene is green fluorescent protein or luciferase, the vector carrying the signal may be measured visually by color or light production in a luminometer.

 However, desirably, the transgene is a non-marker sequence encoding a product
15 which is useful in biology and medicine, such as proteins, peptides, RNA, enzymes, dominant negative mutants, or catalytic RNAs. Desirable RNA molecules include tRNA, dsRNA, ribosomal RNA, catalytic RNAs, siRNA, small hairpin RNA, trans-splicing RNA, and antisense RNAs. One example of a useful RNA sequence is a sequence which inhibits or extinguishes expression of a targeted nucleic acid sequence in the treated animal.
20 Typically, suitable target sequences include oncologic targets and viral diseases. See, for examples of such targets the oncologic targets and viruses identified below in the section relating to immunogens.

 The transgene may be used to correct or ameliorate gene deficiencies, which may include deficiencies in which normal genes are expressed at less than normal levels or
25 deficiencies in which the functional gene product is not expressed. Alternatively, the transgene may provide a product to a cell which is not natively expressed in the cell type or in the host. A preferred type of transgene sequence encodes a therapeutic protein or polypeptide which is expressed in a host cell. The invention further includes using multiple transgenes. In certain situations, a different transgene may be used to encode each subunit of
30 a protein, or to encode different peptides or proteins. This is desirable when the size of the DNA encoding the protein subunit is large, *e.g.*, for an immunoglobulin, the platelet-derived growth factor, or a dystrophin protein. In order for the cell to produce the multi-subunit protein, a cell is infected with the recombinant virus containing each of the different

subunits. Alternatively, different subunits of a protein may be encoded by the same transgene. In this case, a single transgene includes the DNA encoding each of the subunits, with the DNA for each subunit separated by an internal ribozyme entry site (IRES). This is desirable when the size of the DNA encoding each of the subunits is small, *e.g.*, the total size of the DNA encoding the subunits and the IRES is less than five kilobases. As an alternative to an IRES, the DNA may be separated by sequences encoding a 2A peptide, which self-cleaves in a post-translational event. See, *e.g.*, M.L. Donnelly, *et al*, *J. Gen. Virol.*, 78(Pt 1):13-21 (Jan 1997); Furler, S., *et al*, *Gene Ther.*, 8(11):864-873 (June 2001); Klump H., *et al.*, *Gene Ther.*, 8(10):811-817 (May 2001). This 2A peptide is significantly smaller than an IRES, making it well suited for use when space is a limiting factor. More often, when the transgene is large, consists of multi-subunits, or two transgenes are co-delivered, rAAV carrying the desired transgene(s) or subunits are co-administered to allow them to concatamerize *in vivo* to form a single vector genome. In such an embodiment, a first AAV may carry a vector genome which expresses a single transgene and a second AAV may carry a vector genome which expresses a different transgene for co-expression in the host cell. However, the selected transgene may encode any biologically active product or other product, *e.g.*, a product desirable for study.

Useful therapeutic products encoded by the transgene include hormones and growth and differentiation factors including, without limitation, insulin, glucagon, growth hormone (GH), parathyroid hormone (PTH), growth hormone releasing factor (GRF), follicle stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (hCG), vascular endothelial growth factor (VEGF), angiopoietins, angiostatin, granulocyte colony stimulating factor (GCSF), erythropoietin (EPO), connective tissue growth factor (CTGF), basic fibroblast growth factor (bFGF), acidic fibroblast growth factor (aFGF), epidermal growth factor (EGF), transforming growth factor α (TGF α), platelet-derived growth factor (PDGF), insulin growth factors I and II (IGF-I and IGF-II), any one of the transforming growth factor β superfamily, including TGF β , activins, inhibins, or any of the bone morphogenic proteins (BMP) BMPs 1-15, any one of the heregluin/neuregulin/ARIA/neu differentiation factor (NDF) family of growth factors, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins NT-3 and NT-4/5, ciliary neurotrophic factor (CNTF), glial cell line derived neurotrophic factor (GDNF), neurturin, agrin, any one of the family of semaphorins/collapsins, netrin-1 and

netrin-2, hepatocyte growth factor (HGF), ephrins, noggin, sonic hedgehog and tyrosine hydroxylase.

Other useful transgene products include proteins that regulate the immune system including, without limitation, cytokines and lymphokines such as thrombopoietin (TPO), interleukins (IL) IL-1 through IL-25 (including, IL-2, IL-4, IL-12, and IL-18), monocyte chemoattractant protein, leukemia inhibitory factor, granulocyte-macrophage colony stimulating factor, Fas ligand, tumor necrosis factors α and β , interferons α , β , and γ , stem cell factor, flk-2/flt3 ligand. Gene products produced by the immune system are also useful in the invention. These include, without limitations, immunoglobulins IgG, IgM, IgA, IgD and IgE, chimeric immunoglobulins, humanized antibodies, single chain antibodies, T cell receptors, chimeric T cell receptors, single chain T cell receptors, class I and class II MHC molecules, as well as engineered immunoglobulins and MHC molecules. Useful gene products also include complement regulatory proteins such as complement regulatory proteins, membrane cofactor protein (MCP), decay accelerating factor (DAF), CR1, CF2 and CD59.

Still other useful gene products include any one of the receptors for the hormones, growth factors, cytokines, lymphokines, regulatory proteins and immune system proteins. Receptors for cholesterol regulation, including the low density lipoprotein (LDL) receptor, high density lipoprotein (HDL) receptor, the very low density lipoprotein (VLDL) receptor, and the scavenger receptor may be selected. Other suitable gene products may encompass gene products such as members of the steroid hormone receptor superfamily including glucocorticoid receptors and estrogen receptors, Vitamin D receptors and other nuclear receptors. In addition, useful gene products include transcription factors such as *jun*, *fos*, *max*, *mad*, serum response factor (SRF), AP-1, AP2, *myb*, MyoD and myogenin, ETS-box containing proteins, TFE3, E2F, ATF1, ATF2, ATF3, ATF4, ZF5, NFAT, CREB, HNF-4, C/EBP, SP1, CCAAT-box binding proteins, interferon regulation factor (IRF-1), Wilms tumor protein, ETS-binding protein, STAT, GATA-box binding proteins, e.g., GATA-3, and the forkhead family of winged helix proteins.

Other useful gene products include, carbamoyl synthetase I, ornithine transcarbamylase, arginosuccinate synthetase, arginosuccinate lyase, arginase, fumarylacetic acid hydrolase, phenylalanine hydroxylase, alpha-1 antitrypsin, glucose-6-phosphatase, porphobilinogen deaminase, factor VIII, factor IX, cystathione beta-synthase, branched chain ketoacid decarboxylase, albumin, isovaleryl-coA dehydrogenase, propionyl

CoA carboxylase, methyl malonyl CoA mutase, glutaryl CoA dehydrogenase, insulin, beta-glucosidase, pyruvate carboxylate, hepatic phosphorylase, phosphorylase kinase, glycine decarboxylase, H-protein, T-protein, a cystic fibrosis transmembrane regulator (CFTR) sequence, and a dystrophin cDNA sequence. Still other useful gene products include enzymes such as may be useful in enzyme replacement therapy, which is useful in a variety of conditions resulting from deficient activity of enzyme. For example, enzymes that contain mannose-6-phosphate may be utilized in therapies for lysosomal storage diseases (e.g., a suitable gene includes that encodes β -glucuronidase (GUSB)).

Other useful gene products include non-naturally occurring polypeptides, such as chimeric or hybrid polypeptides having a non-naturally occurring amino acid sequence containing insertions, deletions or amino acid substitutions. For example, single-chain engineered immunoglobulins could be useful in certain immunocompromised patients. Other types of non-naturally occurring gene sequences include antisense molecules and catalytic nucleic acids, such as ribozymes, which could be used to reduce overexpression of a target.

Reduction and/or modulation of expression of a gene is particularly desirable for treatment of hyperproliferative conditions characterized by hyperproliferating cells, as are cancers and psoriasis. Target polypeptides include those polypeptides which are produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include polypeptides encoded by oncogenes such as myb, myc, fyn, and the translocation gene bcr/abl, ras, src, P53, neu, trk and EGRF. In addition to oncogene products as target antigens, target polypeptides for anti-cancer treatments and protective regimens include variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas which, in some embodiments, are also used as target antigens for autoimmune disease. Other tumor-associated polypeptides can be used as target polypeptides such as polypeptides which are found at higher levels in tumor cells including the polypeptide recognized by monoclonal antibody 17-1A and folate binding polypeptides.

Other suitable therapeutic polypeptides and proteins include those which may be useful for treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce self-directed antibodies. T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis

(MS), Sjögren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors (TCRs) that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases.

Alternatively, or in addition, the rAAV comprises a transgene encoding a peptide, polypeptide or protein which induces an immune response to a selected immunogen. For example, immunogens may be selected from a variety of viral families. Example of desirable viral families against which an immune response would be desirable include, the picornavirus family, which includes the genera rhinoviruses, which are responsible for about 50% of cases of the common cold; the genera enteroviruses, which include polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus; and the genera aphthoviruses, which are responsible for foot and mouth diseases, primarily in non-human animals. Within the picornavirus family of viruses, target antigens include the VP1, VP2, VP3, VP4, and VPG. Another viral family includes the calcivirus family, which encompasses the Norwalk group of viruses, which are an important causative agent of epidemic gastroenteritis. Still another viral family desirable for use in targeting antigens for inducing immune responses in humans and non-human animals is the togavirus family, which includes the genera alphavirus, which include Sindbis viruses, Ross River virus, and Venezuelan, Eastern & Western Equine encephalitis, and rubivirus, including Rubella virus. The flaviviridae family includes dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses. Other target antigens may be generated from the Hepatitis C or the coronavirus family, which includes a number of non-human viruses such as infectious bronchitis virus (poultry), porcine transmissible gastroenteric virus (pig), porcine hemagglutinating encephalomyelitis virus (pig), feline infectious peritonitis virus (cats), feline enteric coronavirus (cat), canine coronavirus (dog), and human respiratory coronaviruses, which may cause the common cold and/or non-A, B or C hepatitis. Within the coronavirus family, target antigens include the E1 (also called M or matrix protein), E2 (also called S or Spike protein), E3 (also called HE or hemagglutinin-esterase) glycoprotein (not present in all coronaviruses), or N (nucleocapsid). Still other antigens may be targeted against the rhabdovirus family, which includes the genera vesiculovirus (e.g., Vesicular Stomatitis Virus), and the general lyssavirus (e.g., rabies). Within the rhabdovirus family,

suitable antigens may be derived from the G protein or the N protein. The family filoviridae, which includes hemorrhagic fever viruses such as Marburg and Ebola virus may be a suitable source of antigens. The paramyxovirus family includes parainfluenza Virus Type 1, parainfluenza Virus Type 3, bovine parainfluenza Virus Type 3, rubulavirus (mumps virus, parainfluenza Virus Type 2, parainfluenza virus Type 4, Newcastle disease virus (chickens), rinderpest, morbillivirus, which includes measles and canine distemper, and pneumovirus, which includes respiratory syncytial virus. The influenza virus is classified within the family orthomyxovirus and is a suitable source of antigen (e.g., the HA protein, the N1 protein). The bunyavirus family includes the genera bunyavirus (California encephalitis, La Crosse), phlebovirus (Rift Valley Fever), hantavirus (puremala is a hemahagin fever virus), nairovirus (Nairobi sheep disease) and various unassigned bungaviruses. The arenavirus family provides a source of antigens against LCM and Lassa fever virus. The reovirus family includes the genera reovirus, rotavirus (which causes acute gastroenteritis in children), orbiviruses, and cultivirus (Colorado Tick fever, Lebombo (humans), equine encephalosis, blue tongue).

The retrovirus family includes the sub-family oncorivirinal which encompasses such human and veterinary diseases as feline leukemia virus, HTLVI and HTLVII, lentivirinal (which includes human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus, and spumavirinal). Between the HIV and SIV, many suitable antigens have been described and can readily be selected. Examples of suitable HIV and SIV antigens include, without limitation the gag, pol, Vif, Vpx, VPR, Env, Tat and Rev proteins, as well as various fragments thereof. In addition, a variety of modifications to these antigens have been described. Suitable antigens for this purpose are known to those of skill in the art. For example, one may select a sequence encoding the gag, pol, Vif, and Vpr, Env, Tat and Rev, amongst other proteins. See, e.g., the modified gag protein which is described in US Patent 5,972,596. See, also, the HIV and SIV proteins described in D.H. Barouch et al, J. Virol., 75(5):2462-2467 (March 2001), and R.R. Amara, et al, Science, 292:69-74 (6 April 2001). These proteins or subunits thereof may be delivered alone, or in combination via separate vectors or from a single vector.

The papovavirus family includes the sub-family polyomaviruses (BKU and JCU viruses) and the sub-family papillomavirus (associated with cancers or malignant progression of papilloma). The adenovirus family includes viruses (EX, AD7, ARD, O.B.)

which cause respiratory disease and/or enteritis. The parvovirus family feline parvovirus (feline enteritis), feline panleucopeniavirus, canine parvovirus, and porcine parvovirus. The herpesvirus family includes the sub-family alphaherpesvirinae, which encompasses the genera simplexvirus (HSV1, HSV2), varicellovirus (pseudorabies, varicella zoster) and the sub-family betaherpesvirinae, which includes the genera cytomegalovirus (HCMV, 5 muromegalovirus) and the sub-family gammaherpesvirinae, which includes the genera lymphocryptovirus, EBV (Burkitt's lymphoma), infectious rhinotracheitis, Marek's disease virus, and rhadinovirus. The poxvirus family includes the sub-family chordopoxvirinae, which encompasses the genera orthopoxvirus (Variola (Smallpox) and Vaccinia (Cowpox)), 10 parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, and the sub-family entomopoxvirinae. The hepadnavirus family includes the Hepatitis B virus. One unclassified virus which may be suitable source of antigens is the Hepatitis delta virus. Still other viral sources may include avian infectious bursal disease virus and porcine respiratory and reproductive syndrome virus. The alphavirus family includes equine arteritis virus and 15 various Encephalitis viruses.

The present invention may also encompass immunogens which are useful to immunize a human or non-human animal against other pathogens including bacteria, fungi, parasitic microorganisms or multicellular parasites which infect human and non-human vertebrates, or from a cancer cell or tumor cell. Examples of bacterial pathogens include 20 pathogenic gram-positive cocci include pneumococci; staphylococci; and streptococci. Pathogenic gram-negative cocci include meningococcus; gonococcus. Pathogenic enteric gram-negative bacilli include enterobacteriaceae; pseudomonas, acinetobacteria and eikenella; melioidosis; salmonella; shigella; haemophilus; moraxella; *H. ducreyi* (which causes chancroid); brucella; *Francisella tularensis* (which causes tularemia); yersinia 25 (pasteurella); streptobacillus moniliformis and spirillum; Gram-positive bacilli include listeria monocytogenes; erysipelotheix rhusiopathiae; *Corynebacterium diphtheria* (diphtheria); cholera; *B. anthracis* (anthrax); donovanosis (granuloma inguinale); and bartonellosis. Diseases caused by pathogenic anaerobic bacteria include tetanus; botulism; other clostridia; tuberculosis; leprosy; and other mycobacteria. Pathogenic spirochetal 30 diseases include syphilis; treponematoses: yaws, pinta and endemic syphilis; and leptospirosis. Other infections caused by higher pathogen bacteria and pathogenic fungi include actinomycosis; nocardiosis; cryptococcosis, blastomycosis, histoplasmosis and coccidioidomycosis; candidiasis, aspergillosis, and mucormycosis; sporotrichosis;

paracoccidioidomycosis, petriellidiosis, torulopsosis, mycetoma and chromomycosis; and dermatophytosis. Rickettsial infections include Typhus fever, Rocky Mountain spotted fever, Q fever, and Rickettsialpox. Examples of mycoplasma and chlamydial infections include: mycoplasma pneumoniae; lymphogranuloma venereum; psittacosis; and perinatal chlamydial infections. Pathogenic eukaryotes encompass pathogenic protozoans and helminths and infections produced thereby include: amebiasis; malaria; leishmaniasis; trypanosomiasis; toxoplasmosis; *Pneumocystis carinii*; *Trichans*; *Toxoplasma gondii*; babesiosis; giardiasis; trichinosis; filariasis; schistosomiasis; nematodes; trematodes or flukes; and cestode (tapeworm) infections.

Many of these organisms and/or toxins produced thereby have been identified by the Centers for Disease Control [(CDC), Department of Health and Human Services, USA], as agents which have potential for use in biological attacks. For example, some of these biological agents, include, *Bacillus anthracis* (anthrax), *Clostridium botulinum* and its toxin (botulism), *Yersinia pestis* (plague), variola major (smallpox), *Francisella tularensis* (tularemia), and viral hemorrhagic fever, all of which are currently classified as Category A agents; *Coxiella burnetti* (Q fever); Brucella species (brucellosis), *Burkholderia mallei* (glanders), *Ricinus communis* and its toxin (ricin toxin), *Clostridium perfringens* and its toxin (epsilon toxin), *Staphylococcus* species and their toxins (enterotoxin B), all of which are currently classified as Category B agents; and Nipah virus and hantaviruses, which are currently classified as Category C agents. In addition, other organisms, which are so classified or differently classified, may be identified and/or used for such a purpose in the future. It will be readily understood that the viral vectors and other constructs described herein are useful to deliver antigens from these organisms, viruses, their toxins or other by-products, which will prevent and/or treat infection or other adverse reactions with these biological agents.

Administration of the vectors of the invention to deliver immunogens against the variable region of the T cells elicit an immune response including CTLs to eliminate those T cells. In rheumatoid arthritis (RA), several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include V-3, V-14, V-17 and V α -17. Thus, delivery of a nucleic acid sequence that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in RA. In multiple sclerosis (MS), several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V-7 and V α -10. Thus, delivery of

a nucleic acid sequence that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in MS. In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V-6, V-8, V-14 and V α -16, V α -3C, V α -7, V α -14, V α -15, V α -16, V α -28 and V α -12.

5 Thus, delivery of a nucleic acid molecule that encodes at least one of these polypeptides will elicit an immune response that will target T cells involved in scleroderma.

In another embodiment, the transgene is selected for use in gene augmentation therapy, i.e., to provide replacement copy of a gene that is missing or defective. In this embodiment, the transgene may be readily selected by one of skill in the art to provide the necessary

10 replacement gene.

In another embodiment, the transgene is selected for use in gene suppression therapy, i.e., expression of one or more native genes is interrupted or suppressed at transcriptional or translational levels. This can be accomplished using short hairpin RNA (shRNA) or other techniques well known in the art. See, e.g., Sun et al, Int J Cancer. 2010 Feb 1;126(3):764-74 and O'Reilly M, et al. Am J Hum Genet. 2007 Jul;81(1):127-35, which are incorporated

15 herein by reference. In this embodiment, the transgene may be readily selected by one of skill in the art based upon the gene which is desired to be silenced.

In another embodiment, the transgene comprises more than one transgene. This may be accomplished using a single vector carrying two or more heterologous sequences or using two or more AAV each carrying one or more heterologous sequences. In one embodiment, the AAV is used for gene suppression (or knockdown) and gene augmentation co-therapy. In knockdown/augmentation co-therapy, the defective copy of the gene of interest is silenced and a non-mutated copy is supplied. In one embodiment, this is accomplished using two or more co-administered vectors. See, Millington-Ward et al, Molecular Therapy, April 2011, 19(4):642–649 which is incorporated herein by reference. The transgenes may be readily

25 selected by one of skill in the art based on the desired result.

In another embodiment, the transgene is selected for use in gene correction therapy. This may be accomplished using, e.g., a zinc-finger nuclease (ZFN)-induced DNA double-strand break in conjunction with an exogenous DNA donor substrate. See, e.g., Ellis et al, Gene Therapy (epub January 2012) 20:35-42 which is incorporated herein by reference. The transgenes may be readily selected by one of skill in the art based on the desired result.

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In one embodiment, the transgene described herein are useful in the CRISPR-Cas dual vector system described in US Provisional Patent Application Nos. 61/153,470,

62/183,825, 62/254,225 and 62/287,511, each of which is incorporated herein by reference. The capsids are also useful for delivery homing endonucleases or other meganucleases.

Desirably, the transgene encodes a product which is useful in biology and medicine, such as proteins, peptides, RNA, enzymes, or catalytic RNAs. Desirable RNA molecules include shRNA, tRNA, dsRNA, ribosomal RNA, catalytic RNAs, and antisense RNAs. One example of a useful RNA sequence is a sequence which extinguishes expression of a targeted nucleic acid sequence in the treated animal.

iii. Regulatory Elements.

The regulatory sequences include conventional control elements which are operably linked to the transgene in a manner which permits its transcription, translation and/or expression in a cell transfected/transduced with the vector or infected with the virus produced as described herein. As used herein, "operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in *trans* or at a distance to control the gene of interest.

Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A great number of expression control sequences, including promoters, are known in the art and may be utilized.

The regulatory sequences useful in the constructs provided herein may also contain an intron, desirably located between the promoter/ enhancer sequence and the gene. One desirable intron sequence is derived from SV-40, and is a 100 bp mini-intron splice donor/splice acceptor referred to as SD-SA. Another suitable sequence includes the woodchuck hepatitis virus post-transcriptional element. (See, e.g., L. Wang and I. Verma, 1999 Proc. Natl. Acad. Sci., USA, 96:3906-3910). PolyA signals may be derived from many suitable species, including, without limitation SV-40, human and bovine.

Another regulatory component of the rAAV useful in the methods described herein is an internal ribosome entry site (IRES). An IRES sequence, or other suitable systems, may be used to produce more than one polypeptide from a single gene transcript. An IRES (or other suitable sequence) is used to produce a protein that contains more than one polypeptide chain or to express two different proteins from or within the same cell. An exemplary IRES

is the poliovirus internal ribosome entry sequence, which supports transgene expression in photoreceptors, RPE and ganglion cells. Preferably, the IRES is located 3' to the transgene in the rAAV vector.

In one embodiment, the AAV comprises a promoter (or a functional fragment of a promoter). The selection of the promoter to be employed in the rAAV may be made from among a wide number of constitutive or inducible promoters that can express the selected transgene in the desired target cell. In one embodiment, the target cell is a liver cell. The promoter may be derived from any species, including human and rodent. Desirably, in one embodiment, the promoter is "cell specific". The term "cell-specific" means that the particular promoter selected for the recombinant vector can direct expression of the selected transgene in a particular cell tissue. In one embodiment, the promoter is specific for expression of the transgene in muscle cells. In another embodiment, the promoter is specific for expression in lung. In another embodiment, the promoter is specific for expression of the transgene in liver cells. In another embodiment, the promoter is specific for expression of the transgene in airway epithelium. In another embodiment, the promoter is specific for expression of the transgene in neurons. In another embodiment, the promoter is specific for expression of the transgene in heart.

The expression cassette typically contains a promoter sequence as part of the expression control sequences, *e.g.*, located between the selected 5' ITR sequence and the transgene. In one embodiment, expression in liver is desirable. Thus, in one embodiment, a liver-specific promoter is used. Tissue specific promoters, constitutive promoters, regulatable promoters [*see, e.g.*, WO 2011/126808 and WO 2013/04943], or a promoter responsive to physiologic cues may be used may be utilized in the vectors described herein. In another embodiment, expression in muscle is desirable. Thus, in one embodiment, a muscle-specific promoter is used. In one embodiment, the promoter is an MCK based promoter, such as the dMCK (509-bp) or tMCK (720-bp) promoters (*see, e.g.*, Wang et al, Gene Ther. 2008 Nov;15(22):1489-99. doi: 10.1038/gt.2008.104. Epub 2008 Jun 19, which is incorporated herein by reference). Another useful promoter is the SPc5-12 promoter (*see* Rasowo et al, European Scientific Journal June 2014 edition vol.10, No.18, which is incorporated herein by reference). In one embodiment, the promoter is a CMV promoter. In another embodiment, the promoter is a TBG promoter. In another embodiment, a CB7 promoter is used. CB7 is a chicken β -actin promoter with cytomegalovirus enhancer elements. Alternatively, other liver-specific promoters may be used [*see, e.g.*, The Liver

Specific Gene Promoter Database, Cold Spring Harbor, rulai.schl.edu/LSPD, alpha 1 anti-trypsin (A1AT); human albumin Miyatake et al., J. Virol., 71:5124-32 (1997), humAlb; and hepatitis B virus core promoter, Sandig *et al.*, Gene Ther., 3:1002-9 (1996)]. TTR minimal enhancer/promoter, alpha-antitrypsin promoter, LSP (845 nt)²⁵(requires intron-less scAAV).

5 The promoter(s) can be selected from different sources, *e.g.*, human cytomegalovirus (CMV) immediate-early enhancer/promoter, the SV40 early enhancer/promoter, the JC polymovirus promoter, myelin basic protein (MBP) or glial fibrillary acidic protein (GFAP) promoters, herpes simplex virus (HSV-1) latency associated promoter (LAP), rouse sarcoma virus (RSV) long terminal repeat (LTR) promoter, neuron-specific promoter (NSE), platelet
10 derived growth factor (PDGF) promoter, hSYN, melanin-concentrating hormone (MCH) promoter, CBA, matrix metalloprotein promoter (MPP), and the chicken beta-actin promoter.

Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the
15 cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart et al, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the β -actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1 promoter [Invitrogen]. Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the
20 presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied
25 compounds, include, the zinc-inducible sheep metallothionein (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system [International Patent Publication No. WO 98/10088]; the ecdysone insect promoter [No et al, Proc. Natl. Acad. Sci. USA, 93:3346-3351 (1996)], the tetracycline-repressible system [Gossen et al, Proc. Natl. Acad. Sci. USA, 89:5547-5551
30 (1992)], the tetracycline-inducible system [Gossen et al, Science, 268:1766-1769 (1995), see also Harvey et al, Curr. Opin. Chem. Biol., 2:512-518 (1998)], the RU486-inducible system [Wang et al, Nat. Biotech., 15:239-243 (1997) and Wang et al, Gene Ther., 4:432-441 (1997)] and the rapamycin-inducible system [Magari et al, J. Clin. Invest., 100:2865-2872

(1997)]. Other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

The expression cassette may contain at least one enhancer, *i.e.*, CMV enhancer. Still
5 other enhancer elements may include, *e.g.*, an apolipoprotein enhancer, a zebrafish enhancer, a GFAP enhancer element, and brain specific enhancers such as described in WO 2013/1555222, woodchuck post hepatitis post-transcriptional regulatory element. Additionally, or alternatively, other, *e.g.*, the hybrid human cytomegalovirus (HCMV)-immediate early (IE)-PDGR promoter or other promoter - enhancer elements may be
10 selected. Other enhancer sequences useful herein include the IRBP enhancer (Nicoud 2007, J Gene Med. 2007 Dec;9(12):1015-23), immediate early cytomegalovirus enhancer, one derived from an immunoglobulin gene or SV40 enhancer, the cis-acting element identified in the mouse proximal promoter, etc.

In addition to a promoter, an expression cassette and/or a vector genome may contain
15 other appropriate transcription initiation, termination, enhancer sequences, efficient RNA processing signals such as splicing and polyadenylation (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*i.e.*, Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. A variety of suitable polyA are known. In
20 one example, the polyA is rabbit beta globin, such as the 127 bp rabbit beta-globin polyadenylation signal (GenBank # V00882.1). In other embodiments, an SV40 polyA signal is selected. Still other suitable polyA sequences may be selected. In certain embodiments, an intron is included. One suitable intron is a chicken beta-actin intron. In one embodiment, the intron is 875 bp (GenBank # X00182.1). In another embodiment, a
25 chimeric intron available from Promega is used. However, other suitable introns may be selected. In one embodiment, spacers are included such that the vector genome is approximately the same size as the native AAV vector genome (e.g., between 4.1 and 5.2 kb). In one embodiment, spacers are included such that the vector genome is approximately 4.7 kb. See, Wu et al, Effect of Genome Size on AAV Vector Packaging, Mol Ther. 2010
30 Jan; 18(1): 80–86, which is incorporated herein by reference.

Selection of these and other common vector and regulatory elements are conventional, and many such sequences are available. See, e.g., Sambrook et al, and references cited therein at, for example, pages 3.18-3.26 and 16.17-16.27 and Ausubel et al.,

Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1989. Of course, not all vectors and expression control sequences will function equally well to express all of the transgenes as described herein. However, one of skill in the art may make a selection among these, and other, expression control sequences without departing from the scope of this invention.

Methods of altering the AAV vector genome and/or capsid may involve a variety of techniques, which techniques are known to those of skill in the art. For example, site directed mutagenesis may be performed at the level of the nucleic acids encoding one or more amino acids to be altered. Typically, the site-directed mutagenesis is performed using as few steps as required to obtain the desired codon for the conserved amino acid residue. The site-directed mutagenesis may be performed on the AAV genomic sequence. Alternatively, an insertion or a deletion of one or more amino acids (e.g., 2, 3, 4, 5 or more) may be made at the target region within the AAV capsid. Techniques of swapping the desired regions is known in the art. This typically involves excising the corresponding variable loop regions of the heterologous capsid sequence. Such methods are well known to those of skill in the art and can be performed using published methods and/or commercially available kits [e.g., available from Stratagene and Promega]. Alternatively, one of skill in the art can alter the parental AAV using other techniques known to those of skill in the art, e.g., inserting a chemically synthesized peptide, and the like. Still other suitable techniques may be selected. See, e.g., Green and Sambrook, "Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press; 4th Edition (June 15, 2012).

It should be understood that the compositions described herein are intended to be applied to other compositions, regimens, aspects, embodiments and methods described across the specification.

D. Pharmaceutical Compositions and Administration

In one embodiment, the recombinant AAV containing the desired transgene and capsid is optionally assessed for contamination by conventional methods and then formulated into a pharmaceutical composition intended for administration to a subject in need thereof. Such formulation involves the use of a pharmaceutically and/or physiologically acceptable vehicle or carrier, such as buffered saline or other buffers, e.g., HEPES, to maintain pH at appropriate physiological levels, and, optionally, other medicinal agents, pharmaceutical agents, stabilizing agents, buffers, carriers, adjuvants, diluents, etc.

For injection, the carrier will typically be a liquid. Exemplary physiologically acceptable carriers include sterile, pyrogen-free water and sterile, pyrogen-free, phosphate buffered saline. A variety of such known carriers are provided in US Patent Publication No. 7,629,322, incorporated herein by reference. In one embodiment, the carrier is an isotonic sodium chloride solution. In another embodiment, the carrier is balanced salt solution. In one embodiment, the carrier includes tween. If the virus is to be stored long-term, it may be frozen in the presence of glycerol or Tween20. In another embodiment, the pharmaceutically acceptable carrier comprises a surfactant, such as perfluorooctane (Perfluron liquid). The vector is formulated in a buffer/carrier suitable for infusion in human subjects. The buffer/carrier should include a component that prevents the rAAV from sticking to the infusion tubing but does not interfere with the rAAV binding activity *in vivo*.

The compositions described herein are designed for delivery to subjects (e.g., human patients) in need thereof by any suitable route or a combination of different routes. In certain embodiments of the methods described herein, the pharmaceutical composition described above is administered to the subject intramuscularly. In other embodiments, the pharmaceutical composition is administered by intravenously. For treatment of liver disease, direct or intrahepatic delivery to the liver is desired and may optionally be performed via intravascular delivery, e.g., via the portal vein, hepatic vein, bile duct, or by transplant. Other forms of administration that may be useful in the methods described herein include, but are not limited to, direct delivery to a desired organ (e.g., the eye), including subretinal or intravitreal delivery, oral, inhalation, intranasal, intratracheal, intravenous, intramuscular, subcutaneous, intradermal, intraarterial, intraocular, and other parenteral routes of administration. Routes of administration may be combined, if desired. Intravenous delivery may be selected for delivery to proliferating, progenitor and/or stem cells. Alternatively, another route of delivery may be selected. Optionally, the rAAV described herein may be delivered in conjunction with other viral vectors, or non-viral DNA or RNA transfer moieties. The vectors (or other transfer moieties) can be formulated with a physiologically acceptable carrier for use in gene transfer and gene therapy applications.

Furthermore, the composition may be delivered in a volume of from about 0.1 μ L to about 10 mL, including all numbers within the range, depending on the size of the area to be treated, the viral titer used, the route of administration, and the desired effect of the method. In one embodiment, the volume is about 50 μ L. In another embodiment, the volume is about 70 μ L. In another embodiment, the volume is about 100 μ L. In another embodiment, the

volume is about 125 μL . In another embodiment, the volume is about 150 μL . In another embodiment, the volume is about 175 μL . In yet another embodiment, the volume is about 200 μL . In another embodiment, the volume is about 250 μL . In another embodiment, the volume is about 300 μL . In another embodiment, the volume is about 450 μL . In another embodiment, the volume is about 500 μL . In another embodiment, the volume is about 600 μL . In another embodiment, the volume is about 750 μL . In another embodiment, the volume is about 850 μL . In another embodiment, the volume is about 1000 μL . In another embodiment, the volume is about 1.5 mL. In another embodiment, the volume is about 2 mL. In another embodiment, the volume is about 2.5 mL. In another embodiment, the volume is about 3 mL. In another embodiment, the volume is about 3.5 mL. In another embodiment, the volume is about 4 mL. In another embodiment, the volume is about 5 mL. In another embodiment, the volume is about 5.5 mL. In another embodiment, the volume is about 6 mL. In another embodiment, the volume is about 6.5 mL. In another embodiment, the volume is about 7 mL. In another embodiment, the volume is about 8 mL. In another embodiment, the volume is about 8.5 mL. In another embodiment, the volume is about 9 mL. In another embodiment, the volume is about 9.5 mL. In another embodiment, the volume is about 10 mL.

An effective concentration of a recombinant adeno-associated virus carrying a transgene under the control of the regulatory elements desirably ranges from about 10^7 and 10^{14} vector genomes per milliliter (vg/mL) (also called genome copies/mL (GC/mL)). In one embodiment, the rAAV vector genomes are measured by real-time PCR. In another embodiment, the rAAV vector genomes are measured by digital PCR. See, Lock et al, Absolute determination of single-stranded and self-complementary adeno-associated viral vector genome titers by droplet digital PCR, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14, which are incorporated herein by reference. In another embodiment, the rAAV infectious units are measured as described in S.K. McLaughlin et al, 1988 J. Virol., 62:1963, which is incorporated herein by reference. Preferably, the concentration is from about 1.5×10^9 vg/mL to about 1.5×10^{13} vg/mL, and more preferably from about 1.5×10^9 vg/mL to about 1.5×10^{11} vg/mL. In one embodiment, the effective concentration is about 1.4×10^8 vg/mL. In one embodiment, the effective concentration is about 3.5×10^{10} vg/mL. In another embodiment, the effective concentration is about 5.6×10^{11} vg/mL. In another embodiment, the effective concentration is about 5.3×10^{12} vg/mL. In yet another embodiment, the effective concentration is about 1.5×10^{12}

vg/mL. In another embodiment, the effective concentration is about 1.5×10^{13} vg/mL. All ranges described herein are inclusive of the endpoints.

In one embodiment, the dosage is from about 1.5×10^9 vg (vector genome, GC)/kg of body weight to about 1.5×10^{13} vg/kg, and more preferably from about 1.5×10^9 vg/kg to about 1.5×10^{11} vg/kg. In one embodiment, the dosage is about 1.4×10^8 vg/kg. In one embodiment, the dosage is about 3.5×10^{10} vg/kg. In another embodiment, the dosage is about 5.6×10^{11} vg/kg. In another embodiment, the dosage is about 5.3×10^{12} vg/kg. In yet another embodiment, the dosage is about 1.5×10^{12} vg/kg. In another embodiment, the dosage is about 1.5×10^{13} vg/kg. In another embodiment, the dosage is about 3.0×10^{13} vg/kg. In another embodiment, the dosage is about 1.0×10^{14} vg/kg. All ranges described herein are inclusive of the endpoints.

In one embodiment, the effective dosage (total genome copies delivered) is from about 10^7 to 10^{13} vector genomes. In one embodiment, the total dosage is about 10^8 genome copies. In one embodiment, the total dosage is about 10^9 genome copies. In one embodiment, the total dosage is about 10^{10} genome copies. In one embodiment, the total dosage is about 10^{11} genome copies. In one embodiment, the total dosage is about 10^{12} genome copies. In one embodiment, the total dosage is about 10^{13} genome copies. In one embodiment, the total dosage is about 10^{14} genome copies. In one embodiment, the total dosage is about 10^{15} genome copies.

It is desirable that the lowest effective concentration of AAV be utilized in order to reduce the risk of undesirable effects, such as toxicity. Still other dosages and administration volumes in these ranges may be selected by the attending physician, taking into account the physical state of the subject, preferably human, being treated, the age of the subject, the particular disorder and the degree to which the disorder, if progressive, has developed. Intravenous delivery, for example may require doses on the order of 1.5×10^{13} vg/kg.

It should be understood that the compositions described herein are intended to be applied to other compositions, regimens, aspects, embodiments and methods described across the Specification.

E. Methods

The rAAV comprising a capsid or fragment thereof as described herein are capable of increasing or decreasing transduction efficiency in a species or improving translatability among species. As used herein, the term “translatability” or “improved transduction

efficiency” refers to transduction efficiency of an rAAV in a first species is about one tenth to about 10 times, about one fifth to about five times, about one third to about three times, about one half to about twice of that in a second species. In one embodiment, the rAAV comprising a capsid or fragment thereof as described herein increases transduction efficiency in a subject. In another embodiment the rAAV comprising a capsid or fragment thereof as described herein decreases transduction efficiency in a species. In a further embodiment, the species is human. In a further embodiment, the subject is a non-human primate. In yet another embodiment, the subject is a rodent, such as mouse or rat. Thus, provided herein is a method of delivering a transgene to a target cell or a target tissue in a subject. The method includes contacting the cell with a rAAV having a capsid described herein and a vector genome comprising a transgene. In another aspect, the use of a rAAV having a capsid and a vector genome comprising a transgene is provided for delivering the transgene in a target cell or a target tissue in a subject. In one embodiment, the target tissue is liver.

A method is provided for delivering a transgene to a cell, said method comprising the step of contacting the cell with an AAV described herein, wherein said rAAV comprises the transgene. In yet a further aspect, the invention provides a method of modulating, altering, improving, increasing or decreasing transduction efficiency of an AAV in a species by utilizing the capsid as described herein.

A variety of different diseases and conditions may be treated using the method described herein. Examples of such conditions may include, e.g., alpha-1-antitrypsin deficiency, liver conditions (e.g., biliary atresia, Alagille syndrome, alpha-1 antitrypsin, tyrosinemia, neonatal hepatitis, Wilson disease), metabolic conditions such as biotinidase deficiency, carbohydrate deficient glycoprotein syndrome (CDGS), Crigler-Najjar syndrome, diabetes insipidus, Fabry, galactosemia, glucose-6-phosphate dehydrogenase (G6PD), fatty acid oxidation disorders, glutaric aciduria, hypophosphatemia, Krabbe, lactic acidosis, lysosomal storage diseases, mannosidosis, maple syrup urine, mitochondrial, neuro-metabolic, organic acidemias, PKU, purine, pyruvate dehydrogenase deficiency, urea cycle conditions, vitamin D deficient, and hyperoxaluria. Urea cycle disorders include, e.g., N-acetylglutamate synthase deficiency, carbamoyl phosphate synthetase I deficiency, ornithine transcarbamylase deficiency, “AS deficiency” or citrullinemia, “AL deficiency” or argininosuccinic aciduria, and “arginase deficiency” or argininemia.

Other diseases may also be selected for treatment according to the method described herein. Such diseases include, e.g., cystic fibrosis (CF), hemophilia A (associated with

defective factor VIII), hemophilia B (associated with defective factor IX),
 mucopolysaccharidosis (MPS) (e.g., Hunter syndrome, Hurler syndrome, Maroteaux-Lamy
 syndrome, Sanfilippo syndrome, Scheie syndrome, Morquio syndrome, other, MPSI, MPSII,
 MPSIII, MSIV, MPS 7); ataxia (e.g., Friedreich ataxia, spinocerebellar ataxias, ataxia
 5 telangiectasia, essential tremor, spastic paraplegia); Charcot-Marie-Tooth (e.g., peroneal
 muscular atrophy, hereditary motor sensory neuropathy), glycogen storage diseases (e.g.,
 type I, glucose-6-phosphatase deficiency, Von Gierke), II (alpha glucosidase deficiency,
 Pompe), III (debrancher enzyme deficiency, Cori), IV (brancher enzyme deficiency,
 Anderson), V (muscle glycogen phosphorylase deficiency, McArdle), VII (muscle
 10 phosphofructokinase deficiency, Tauri), VI (liver phosphorylase deficiency, Hers), IX (liver
 glycogen phosphorylase kinase deficiency). This list is not exhaustive and other genetic
 conditions are identified, e.g., www.kumc.edu/gec/support; www.genome.gov/10001200;
 and www.ncbi.nlm.nih.gov/books/NBK22183/, which are incorporated herein by reference.

It should be understood that the compositions and methods described in the Methods
 15 section are intended to be applied to other compositions, regimens, aspects, embodiments
 and methods described across the Specification.

The following examples are illustrative only and are not a limitation on the invention
 described herein.

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EXAMPLES

Example 1 – Summary of Engineered AAV

The table below provides a summary of AAV capsids constructed, yields thereof,
 25 and transduction results observed. As used in the form, “up”, “down”, or “OK” indicate a
 decrease, an increase, or not significantly different in yield or transduction level compared to
 rAAV having the backbone capsid without any swapping, respectively. Detailed result as
 well as methods and materials are illustrated in the later Examples.

AAV3B Backbone with AAV8 HVRs

Name	Alternative Name	Yield	Huh7 Transduction	B6 Liver Transduction	FRG Mice	Non- human Primate (NHP)
AAV3B.1	AAV3B.8VR1	OK	OK	-	-	-
AAV3B.2	AAV3B.8VR2	OK	OK	-	-	-
AAV3B.4	AAV3B.8VR4	OK	OK	-	-	-
AAV3B.5	AAV3B.8VR5	OK	down	-	-	-
AAV3B.6	AAV3B.8VR6	OK	OK	-	-	-
AAV3B.7	AAV3B.8VR7	OK	OK	-	-	-
AAV3B.8	AAV3B.8VR8	OK	down	-	-	-
AAV3B.8-1	AAV3B.8VR8P1	OK	down	-	-	-
AAV3B.8-2	AAV3B.8VR8P2	OK	OK	-	-	-
AAV3B.8-3	AAV3B.8VR8P3	OK	down	-	-	-
AAV3B.8-4	AAV3B.8VR8P4	OK	OK	-	-	-
AAV3B.9	AAV3B.8VR9	OK	OK	up	-	down
AAV8 Backbone with AAV3B HVRs						
Name	Alternative Name	Yield	Huh7 Transduction	B6 Liver Transduction	FRG Mice	NHP
AAV8.1	AAV8.3BVR1	OK	down	-	-	-
AAV8.2	AAV8.3BVR2	down	down	-	-	-
AAV8.4	AAV8.3BVR4	OK	OK	-	-	-
AAV8.5	AAV8.3BVR5	OK	down	-	-	-
AAV8.6	AAV8.3BVR6	OK	OK	-	-	-
AAV8.7	AAV8.3BVR7	OK	OK	-	-	-
AAV8.8	AAV8.3BVR8	OK	up	OK	-	-
AAV8.8.1	AAV8.3BVR8.3B VR1	OK	OK	-	-	-
AAV8.8.2	AAV8.3BVR8.3B VR2	down	OK	-	-	-

AAV3B Backbone with AAV8 HVRs						
Name	Alternative Name	Yield	Huh7 Transduction	B6 Liver Transduction	FRG Mice	Non-human Primate (NHP)
AAV8.8.4	AAV8.3BVR8.3B VR4	OK	up	OK	-	-
AAV8.8.5	AAV8.3BVR8.3B VR5	OK	OK	-	-	-
AAV8.8.6	AAV8.3BVR8.3B VR6	OK	up	OK	Species preference different from clade E members	
AAV8.8.6.1	AAV8.3BVR8.3B VR6.3BVR1	OK	OK	-	-	-
AAV8.8.6.2	AAV8.3BVR8.3B VR6.3BVR2	down	OK	-	-	-
AAV8.8.6.4	AAV8.3BVR8.3B VR6.3BVR4	OK	up	-	-	-
AAV8.8.6.5	AAV8.3BVR8.3B VR6.3BVR5	OK	OK	-	-	-
AAV8.8.6.7	AAV8.3BVR8.3B VR6.3BVR7	OK	up	-	-	-
AAV8.8.6.9	AAV8.3BVR8.3B VR6.3BVR9	OK	up	-	-	-
AAV8.8.7	AAV8.3BVR8.3B VR7	OK	up	-	-	-
AAV8.8.9	AAV8.3BVR8.3B VR9	OK	up	-	-	-
AAV8.9	AAV8.3BVR9	OK	down	-	-	-

Example 2 – Hypervariable Region IX Is a Major Domain Responsible for Adeno-Associated Virus 3B's Species Specificity.

Adeno-associated virus 3B (AAV3B) transduces human hepatocyte cell line Huh7 very well and has a good performance of gene deliver in nonhuman primate liver; in contrast, its murine liver transduction is very poor. To find out which parts of AAV3B capsid are responsible for the stark difference, a serial of domain swapping was conducted between hyper-viable regions (HVRs) of AAV3B and AAV8 (FIG. 1). Swapping AAV3B's HVR.VIII into AAV8 increases the Huh7 transduction by around 3 folds while other HVRs have no effect (FIG. 2). Additionally, swapping HVR.VI into AAV8 carrying AAV3B's HVR.VIII has a Huh7 transduction around 10 times AAV8, as well as HVR.IV (to a less degree), while other HVRs have no such effect (FIG. 2). A third swapping HVR.IV into AAV8 with AAV3B-derived HVRs VIII and VI provides a Huh7 transduction which is more than 15 times of AAV8 (FIG. 2). Intriguingly, HVR.VI is not exposed on AAV8 capsid surface.

The importance of HVR.VIII on AAV3B's Huh7 transduction was further confirmed by swapping AAV8's HVR.VIII into AAV3B that demonstrated a dramatic decrease of Huh7 transduction (down ~50 folds, FIG. 3). The HVR.VIII of AAV3B differs from AAV8 by seven amino acids clustering into four Mutations (FIG. 4A). By turning respectively each of the four Mutations of AAV3B capsid into AAV8, we showed that surface-exposed charges at this region are playing a major role in AAV3B's high Huh7 transduction (FIG. 4B). Mutation 1 or 3 of AAV8 HVR VIII was swapped to AAV3B capsid and demonstrated a significant decrease of Huh7 transduction, while swaps with Mutation 2 or 4 of AAV8 HVR VIII did not show a significant change in Huh7 transduction (FIG. 4B).

AAV3B transduces murine cell lines poorer (FIG. 5B) while it transduces human cells better compared to AAV8 (FIG. 5A). By comparing the transduction on human cell line and murine cell lines among AAV3B HVR swapping mutants (that is, HVR.I-IX of AAV8 were swapped into AAV3B individually; FIG. 1), we found that HVR.IX region is the key to control AAV3B's species difference. Replacement experiments for HVR IX showed *in vitro* (FIG. 6B) and *in vivo* (FIG. 7) that HVR.IX region is the key to control AAV3B's species specificity. It was confirmed by *in vivo* study (C57BL/6 male mice, TBG promoter, I.V. injection, transgene expression of AAV3B with its HVR.IX replaced with that from AAV8 was more than 20 times that of AAV3B, FIG. 7). The *in vivo* data corroborates the previous AAV2 study (TENNEY, et al. (2014). "AAV8 capsid variable regions at the two-fold

symmetry axis contribute to high liver transduction by mediating nuclear entry and capsid uncoating." Virology 454-455: 227-236) showing that replacing AAV2' HVR.IX (its sequence is the same as AAV3B) with that from AAV8 increases its murine liver transduction significantly. HVR replacement is used to control species specific transduction by AAV thus improving translatability of the non-clinical studies from one species to another. This approach allows us to have control of species-specific transduction.

The importance of the discoveries lies in the fact that there are at least 53 AAVs in Genbank, all carrying the HVR.IX sequences same as AAV3B. It should be careful if those AAVs are to be evaluated in mice.

Example 2 – Vector Production and Transduction Evaluation

Triple-transfection was used in the experiments. On the 6-well-plate scale, those swapping mutants were constructed on a trans-plasmid backbone, pAAV2/8 or pAAV2/3B. Then, those trans-plasmids carrying those mutants were transfected into HEK293 cells with pDeltaF6 (helper plasmid) and a cis-plasmid (the cis was pAAV.CB7.CI.fluciferase.RBG or pAAV.eGFP). The triple-transfection produced AAV vectors with the capsid protein from the trans-plasmids and the DNA sequence from the cis-plasmid.

The lysate was then tested with Huh7 for the transduction capability of the capsids.

The mutant AAV3B.8VR9 was then used to package TBG.human F9 for mouse study. Its yield was comparable to AAV3B while the expression thereof was more than 10 of that of AAV3B.TBG.F9 (FIG. 7).

Example 3 – Other AAV Serotypes with 8VR9

8VR9 means the HVR9 region was placed by that from AAV8. The transduction of AAV1.8VR9 equals 4.1 times of that of AAV1 in terms of vector genome copies per μg of mouse liver DNA. Transduction of AAV4.8VR9 in HEK293 cell decreased compared to that of AAV4. Transduction of AAV5.8VR9 in HEK293 cell decreased compared to that of AAV5. AAV6.2.8VR9 and AAV7.8VR9 are under investigation in mice. rh.32.8VR9 was constructed.

rAAV.eGFP was prepared with various capsids, such as AAV1, or AAV1.8VR9 (AAV1 capsid with replaced HVR IX from AAV8). B6 mice were administrated i.v. with rAAV with eGFP as transgene, TBG as promoter, and said capsid at 1×10^{11} GC per mouse.

Two weeks later, liver samples were harvested and processed for fluorescent images. Representative images are shown in FIG. 11A while numbers of genome copy per μg liver DNA is plotted in FIG. 11B. P value of the data acquired from mice treated with rAAV having AAV1 or AAV1.8VR9 capsid equals 0.0217. The result demonstrated that swapping
5 with region described above improved transduction in murine liver.

rAAV packaged in AAV7.8VR9 (AAV7 with replaced HVR IX from that of AAV8),
, and AAV6.2.8VR9 (AAV6.2 with replaced HVR IX from that of AAV8) are constructed and the transductions thereof are under investigation.

10 Example 4 – *In Vitro* Transduction in human and murine cells

In vitro transduction was evaluated via luciferase assay in human Huh7 cells or in mouse MC57G cells with rAAVs comprising capsid described below and the result is shown in FIG. 6A. Chimeric capsids were constructed by swapping of AAV3B HVR I, II, IV, V, VI, VII, or IX individually to the backbone of AAV8.3BVR8 (AAV8 with HVR VIII
15 replaced with that of AAV3B, also indicated as AAV8.8). rAAV with AAV8 or AAV8.3BVR8 capsid was supplied as controls.

Consistent with the result shown in precious Examples, rAAV with AAV8 capsid demonstrated a high transduction in mouse cells but a low transduction in human cells. After replacing AAV8's HVR VIII with that of AAV3B, the transduction in human cells increased
20 significantly. With additional swapping with corresponding HVR from AAV3B into AAV8.3BVR8, several chimeric capsids demonstrated elevated transduction in human cells and/or murine cells (FIG. 6A). For example, AAV8.3BVR8.3BVR4 displays an increase in transduction in both human Huh7 cells and murine MC57G cells while there was still difference of transduction between species. Yet, increased transduction was overserved in
25 cells of both species if AAV8.3BVR8.3BVR4 was applied while the difference in transduction between species is not significant, indicating AAV8.3BVR8.3BVR4 could serve as a good candidate for pharmaceutical use with a high translatability between non-clinical studies and clonal ones.

In another aspect, additional *in vitro* transduction in human Huh7 cells or in mouse
30 H2.35 cells with rAAVs comprising capsid indicated below. Swapping of AAV8 HVR I, II, IV, V, VI, VII, VIII or IX to the backbone of AAV3B was performed. rAAVs with AAV8 or AAV3B capsid was supplied as controls. AAV3B.8VR9 capsid revealed a high transduction

in both human and murine cells (FIG. 6B). This result provides another candidate for pharmaceutical and clinical use.

Example 5 – Critical Amino Acid in HVR IX

5 Chimeric capsids were constructed as described by swapping of one of the AAV3B HVR IX Mutations 1 to 6 back to the backbone of AAV3B.8VR9. rAAV with AAV8, AAV3B or AAV3B.8VR9 capsid was supplied as a control. No obvious difference in yield was detected (FIG. 8). Transduction of rAAV with the capsid described above in this Example is evaluated in human Huh7 cells and in mouse H2.35 cells. The results are shown
10 in FIG. 9A and FIG. 9B respectively. Average transduction efficiency of the rAAV with AAV3B.8VR9 capsid (noted as rAAV.AAV3B.8VR9) was set as 100 and served as a reference for normalization. The result showed that one AAV3B-derived Mutation of HVR IX did not reduce the transduction of rAAV with said capsid compared to that with AAV3B.8VR9. However, the transduction of rAAV in murine cells varied.
15 AAB3B.8VR9.3BVR9P1 showed a low transduction which is comparable to AAV3B while AAV3B.8VR9.3BVR9P3 showed a transduction higher than AAV3B but still lower compared to AAV8. The rest of the capsids described in the Example, including AAB3B.8VR9.3BVR9P2, AAB3B.8VR9.3BVR9P4, AAB3B.8VR9.3BVR9P5, and AAB3B.8VR9.3BVR9P6 exhibited a high transduction in murine cells which is similar to
20 AAV8 as well as that in human cells which is comparable to AAV3B, indicating a good translatability between species.

Example 6 – *In Vivo* Results

Furthermore, male B6 mice were administrated intravenously with 1×10^{10} GC/mouse
25 with rAAV carrying coding sequence of human Factor IX (hF9) under control of a TBG promoter and packed in a capsid of AAV8, AAV8.3BVR8 (AAV8.8), AAV8.3BVR8.3BVR4 (AAV8.8.4), or AAV8.3BVR8.3BVR4 (AAV8.8.6). Those rAAV vectors were produced and the titers thereof were quantified and listed in the following table. Blood sample from the mice were then acquired and assessed for hF9 expression, results of
30 which is plotted in FIG. 12. AAV8.8, AAV8.8.4 and AAV8.8.6 achieved a similar hF9 amount in blood compared to AAV8 on days 3, 7 and 14 post administration. Administration of those rAAV vectors in FRG mice is under investigation.

TBG.hF9	GC/mL (qPCR)	1CS (1 cell stack)
AAV8	1.39×10^{13}	1.60×10^{13}
AAV8.8	1.83×10^{13}	1.88×10^{13}
AAV8.8.4	1.3×10^{13}	1.20×10^{13}
AAV8.8.6	1.59×10^{13}	1.47×10^{13}

Example 7 – AAV8.8.6

rAAV vectors having an AAV8 or AAV8.8.6 capsid and packed therein a vector genome which comprises a TBG promoter and a coding sequence of eGFP were produced.

- 5 In vivo potency assay is performed. The vectors were used to transduce Huh7 cells at 1×10^7 GC/well, 1×10^8 GC/well, or 1×10^9 GC/well. Cells without transduction were served as negative control. Fluorescent microscope was used to detect the green signal from the eGFP protein expressed. Representative images are shown in FIG. 13. Cells transduced with 1×10^9 GC/well of the rAAV vectors having AAV8 capsid showed low (1×10^9 GC/well) or
10 non-detectable (all other doses and the negative control) green fluorescence. On the other hand, 1×10^8 GC/well of the rAAV vectors having AAV8.8.6 capsid demonstrated some green fluorescence while 1×10^9 GC/well shows a strong and wide-spread green fluorescence indicating a high expression of eGFP in most of the cells cultured.

- Yield of a rAAV vector having AAV8.8.6 capsid was evaluated and compared to that
15 of AAV8 or other rAAV vectors. Vector genome used in this yield evaluation is noted TBG.eGFP which comprises coding sequence for eGFP under regulation of TBG promoter. The results are plotted in FIG. 14. Dot pointed by the arrow indicates total yield in GC of rAAV vector having AAV8 capsid while dot pointed by the arrow head indicates that with AAV8.8.6 capsid. AAV8.8.6 shows a comparable yield compared to AAV8.

- 20 B6 mice were administrated i.v. with rAAV vectors (AAV8. TBG.eGFP or AAV8.8.6. TBG.eGFP) having AAV8 or AAV8.8.6 capsids and packed therein a vector genome of TBG.eGFP at 3×10^{10} GC per mouse. Two weeks later, mice livers were harvested. Fluorescent microscope was used to assess the eGFP expression. Representative images provided in FIGs 15A to 15C (AAV8. TBG.eGFP) and FIGs 15D to 15F
25 (AAV8.8.6.TBG.eGFP) shows comparable expression.

Human hepatocytes were isolated and transplanted into FRG mice to generate chimeric mice with humanized liver. Those chimeric mice were administrated i.v. with $1 \times$

10^{12} GC of AAV8.8.6.TBG.eGFP vector per mouse. 10 days later, livers were then harvested and analyzed as discussed below.

Morphometric analysis was performed to evaluate transduction efficiency. Briefly, immunohistochemistry for FAH (a human-specific marker) was performed on the liver sections and visualized via non-green fluorescence. Thus, human cells were identified as FAH-positive while mouse cells were identified as FAH-negative. rAAV-transduced human cells and rAAV-transduced mouse cells were represented as GFP+FAH+ area, and GFP+FAH- area, respectively. Such quantification strategy is further shown in FIG. 16. Representative images from three fields are provide (FIGs 17A to 17C; 17D to 17F; and 17G to 17I) while FIGs 17A, 17D and 17G show immunostaining for FAH, FIGs 17B, 17E and 17H show eGFP expression, as well as FIGs 17C, 17F and 17I show merged images. Quantification results demonstrate a 25.6% of GFP-positive human cells and 21.1% of GFP-positive mouse cells of all cells.

This morphology analysis was further performed to evaluate transduction efficiency of various AAV capsids. A summary result is presented in FIG. 20. For each sample tested, two bars are presented: the left one represents GFP+ human cells and the right one represents GFP+ mouse cells. Briefly, in this chimeric FRG mouse model, 1) rAAV comprising a AAV3B capsid shows that the transduction percentage of human hepatocytes is higher than that of mouse hepatocytes; 2) rAAV comprising a clade E capsid (e.g., AAV8, rh.10) shows that the transduction percentage of human hepatocytes is lower than that of mouse hepatocytes; and 3) rAAV comprising a AAV8.8.6 capsid shows that the transduction percentage of human hepatocytes is higher than that of mouse hepatocytes.

Furthermore, flow cytometry was used to evaluate transduction efficiency. liver cells acquired from the chimeric mice were harvested, and then stained for human-specific marker HLA along with mouse-specific marker H1kb. Bigger cells (e.g., hepatocytes, as illustrated in FIG. 18), or all cells (as illustrated in FIG. 19) were gated for further analysis of HLA, H2kb and GFP expression. A representative result showing percentage of GFP+ or GFP- cells among the total cells from each species is listed below.

Big cells gated as shown in FIG. 18		GFP +	GFP-
	H2kb(+)	42.30%	53.00%
	HLA(+)	18.70%	75.90%
All cells gated as shown in FIG. 19		GFP +	GFP-
	H2kb(+)	38.90%	55.50%

	HLA(+)	24.20%	72.70%
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This analysis via flow cytometry was also performed to evaluate transduction efficiency of various AAV capsids. A summary result is presented in FIG. 21. For each sample tested, two bars are presented: the left one represents GFP+ human cells and the right one represents GFP+ mouse cells. Briefly, both AAV3B capsid and clade E members show that the transduction percentage of human hepatocytes is higher than that of mouse hepatocytes. However, rAAV having AAV8.8.6 capsid demonstrates a higher transduction percentage in human compared to mouse.

Example 8 – iPSC-derived hepatocytes transduced with rAAV having AAV8.8.6 capsid.

Induced Pluripotent Stem Cells (iPSC) were used to generate hepatocytes. Such iPSC-derived hepatocytes were transduced with rAAV having AAV8.8.6 capsid and TBG.eGFP vector genome at 5×10^4 GC per well. 96 hours later, the cells were assessed for eGFP expression. Representative images are presented in FIGs 22A through 22I. FIGs. 22A to 22C show GFP expression from a monolayer of the generated hepatocytes via using an exposure time of 300ms. Other exposure times (and optionally with different amplification), 10 ms (FIGs. 22 D, 22E and 22F) and 35 ms (FIGs. 22G, 22H and 22I), were used to reveal organizing of the iPSC-derived hepatocytes. FIGs 22A, 22D and 22G are images of cells transduced with AAV6.2.TBG.eGFP. FIGs 22B, 22E and 22H are images of cells transduced with AAV8.TBG.eGFP. FIGs 22C, 22F and 22I are images of cells transduced with AAV8.8.6.TBG.eGFP. Results show that cells transduced with AAV8.8.6.TBG.eGFP form larger bodies and resemble the organization of hepatocytes in the liver compared to other tested vector.

Example 9 – Material and Methods

A. Cloning of chimeric AAV capsid trans-packaging plasmids

Chimeric capsid sequences were cloned using splicing by overlap extension (SOE) PCR (HORTON, et al. (1990). "Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction." *Biotechniques* 8(5): 528-535). Other suitable techniques may be used, including the SOE (gene splicing overlap) method and/or a mutagenesis kit (e.g., QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, CA), following the manufacturer's instructions.) Chimeric *cap* genes were subsequently cloned

onto a packaging plasmid containing AAV2 *rep* by digestion with restriction endonucleases *Hind*III and *Spe*I followed by ligation with T4 ligase. Competent Stbl3 *Escherichia coli* (Invitrogen, Grand Island, NY) were transformed with ligated plasmids, and resulting single colonies were amplified by Megaprep (QIAGEN, Germany).

- 5 All chimeric *cap* gene sequences were confirmed by full-length DNA sequencing (QIAGEN) prior to vector production for *in vivo* studies.

The domain swapping was carried out with QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The templates were pAAV2/8 and pAAV2/3B, which carry AAV8 capsid gene and AAV3B capsid gene. The variants with multiple domain swapped were generated by multiple rounds of mutagenesis with the kit with one domain swapped each round and then sequenced to make sure the sequences were correct before going into the next round of mutagenesis. The primers for the mutagenesis were in the following table.

Mutagenesis Primer	SEQ ID NO
AGGAGGTCACGCAGAATGATGGCACGACGACCATCGCCAATAACCT	84
AGGTTATTGGCGATGGTCGTCGTGCCATCATTCTGCGTGACCTCCT	85
GCCAACAACGCGTCTCAAAGACTGCTAACGACAACAACAATAGCAACTT	86
AAGTTGCTATTGTTGTTGTCGTTAGCAGTCTTTGAGACGCGTTGTTGGC	87
AGGAGCGTTTTTTTCCCATGCACGGCAATCTGATTTTTGGCAAACA	88
TGTTTGCCAAAAATCAGATTGCCGTGCATGGGAAAAAACGCTCCT	89
GGATCCTGATTTTTGGCAAAGAAGGGACAACGGCAAGTAACGCAGAATTAGATA ATGTCATGCTCACCAGCGAGGA	90
TCCTCGCTGGTGAGCATGACATTATCTAATTCTGCGTTACTTGCCGTTGTCCCTTC TTTGCCAAAAATCAGGATCC	91
AGGAATACGGTATCGTGCCAAATAACTGCAGAGCTCAAATACAGCTCCACGA CTAGAACTGTCAATGATCAGGGGGCCTTACCCGGTAT	92
ATACCGGGTAAGGCCCCCTGATCATTGACAGTTCTAGTCGTGGGAGCTGTATTTG AGCTCTGCAAGTTATTTGCCACGATACCGTATTCCT	93
TCCAGTACACCTCCAACCTACAACAAGTCTGTTAATGTGGACTTTACTGTAGACAC TAATGGCGTGTACTCTGAACCCCG	94

Mutagenesis Primer	SEQ ID NO
CGGGGTTTCAGAGTACACGCCATTAGTGTCTACAGTAAAGTCCACATTAACAGAC TTGTTGTAGTTGGAGGTGTACTGGA	95
AGCAGTATGGAAGTGTGGCAGATAACTTGCAGCAGCAAAACACGGCTCCTCAA TTGGAACTGTCAACAGCCAGGGGGCCTTACCTGGCAT	96
ATGCCAGGTAAGGCCCCCTGGCTGTTGACAGTTCCAATTTGAGGAGCCGTGTTTT GCTGCTGCAAGTTATCTGCCACAGTTCCATACTGCT	97
ACAGAACGCAAGGAACAACCGGAGGCACGGCAAATACGCAGACTCTGGGCTTT AGCCAGGCTGGGCC	98
GGCCCAGCCTGGCTAAAGCCCAGAGTCTGCGTATTTGCCGTGCCTCCGGTTGTTC CTTGCGTTCTGT	99
AAGAAAAATTTTTCCCTAGTAACGGGATCCTAATATTTGGCAAAGA	100
TCTTTGCCAAATATTAGGATCCCGTTACTAGGGAAAAATTTTTCTT	101
GCAATCTGATTTTTGGCAAAGAAGGGACAACGGCAAGTAACGCAGAATTAGATA ATGTCATGCTCACCAGCGAGGA	102
TCCTCGCTGGTGAGCATGACATTATCTAATTCTGCGTACTTGCCGTTGTCCCTTC TTTGCCAAAAATCAGATTGC	103
TCTACAAGCAAATCTCCAACGGGACATCGGGAGGAGCCACCAACGACAACACCT ACTTTGGCTACAGCAC	104
GTGCTGTAGCCAAAGTAGGTGTTGTCGTTGGTGGCTCCTCCCGATGTCCCGTTGG AGATTTGCTTGTAGA	105
AAGAGGTCACGCAGAACGAAGGCACCAAGACTATTGCCAATAACCT	106
AGGTTATTGGCAATAGTCTTGGTGCCTTCGTTCTGCGTGACCTCTT	107
GGCAACAGAGACTTTCAACGACAACCGGGCAAAACAACAGTAACTT	108
AAGTTACTGTTGTTGTTTTGCCCGGTTGTCGTTGAAAGTCTCTGTTGCC	109
ATCTAATATTTGGCAAACAAAATGCTGCCAGAGACAATGCGGATTACAGCGATG TAATGATTACGGATGA	110
TCATCCGTAATCATTACATCGCTGTAATCCGCATTGTCTCTGGCAGCATTTTGTTT GCCAAATATTAGAT	111

Mutagenesis Primer	SEQ ID NO
AGTACACTTCCAACACTACTACAAATCTACAAGTGTGGACTTTGCTGTTAATACAGA AGGTGTTTATAGTGAACC	112
GGTTCACATAAAACACCTTCTGTATTAACAGCAAAGTCCACACTTGTAGATTTGT AGTAGTTGGAAGTGTACT	113
ATCCTCCGACGACTTTCAACAAGGACAAGCTGAACTCATTTATCACTCAGTA	114
TACTGAGTGATAAATGAGTTCAGCTTGTCTTGTGAAAGTCGTCGGAGGAT	115
AGTATGGAAGTGTGGCAGATAACTTGCAGAGCTCAAA	116
TTTGAGCTCTGCAAGTTATCTGCCACAGTTCCATACT	117
TGGCAAATAACTTGCAGCAGCAAAATACAGCTCCCACGAC	118
GTCGTGGGAGCTGTATTTTGCTGCTGCAAGTTATTTGCCA	119
GCTCAAATACAGCTCCCCAAATTGGAAGTGTCAATGATCAGGG	120
CCCTGATCATTGACAGTTCCAATTTGGGGAGCTGTATTTGAGC	121
CGACTAGAAGTGTCAATAGCCAGGGGGCCTTACCTGG	122
CCAGGTAAGGCCCCCTGGCTATTGACAGTTCTAGTCG	123
TCTACAAGCAAATCTCCAGCCAATCAGGAGCCACCAACGACAA	124
TTGTCGTTGGTGGCTCCTGATTGGCTGGAGATTTGCTTGTAGA	125
CTCGGACTCAAACAACATCTGGAACAACCAACCAATCACGGCTGCTTTTCAGCC AAGGTGGGCC	126
GGCCACCTTGGCTGAAAAGCAGCCGTGATTGGTTGGTTGTCCAGATGTTGTTT GAGTCCGAG	127
ATCCTCCGACCACCTTCAGCCCTGCCAAGTTTGCTTCTTTTCATCACGCAATA	128
TATTGCGTGATGAAAGAAGCAAACCTTGGCAGGGCTGAAGGTGGTCGGAGGAT	129
ACTTTGCTGTTAATACAAATGGCGTGTACTCTGAACC	130
GGTTCAGAGTACACGCCATTTGTATTAACAGCAAAGT	131

B. Production of AAV vectors for preliminary *in vitro* tests.

Vector production in 6-well plates was based on the scaling down of a protocol described previously (Lock, M. et al. Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale. Hum Gene Ther 21, 1259-1271 (2010)) without purification.

Recombinant AAV vectors expressing firefly Luciferase (ffLuc) under the cytomegalovirus (CMV) promoter and flanked by AAV2 inverted terminal repeats were produced by triple calcium phosphate transfection of human embryonic kidney 293 cells (HEK293). Briefly, 3.25 µg of a *cis* plasmid containing the transgenic cDNA, 3.25 µg of a *trans*-packaging plasmid construct containing the AAV2 *rep* gene along with the AAV8, AAV2 or chimeric *cap* gene and 6.5 µg of an Adenovirus helper plasmid (pAdΔF6) were added to 6-well plates containing cultured cells at 90% confluence. Two days after transfection, cells were harvested by scraping followed by repeated freeze/thaw cycles. Lysates were cleared by centrifugation for 10 min at 4000 RPM (4 °C). Vector genome copy number per milliliter (GC/mL) was determined by real-time quantitative PCR using primers and a Taqman probe specific for the vector's polyadenylation sequence.

C. *In vitro* transduction assays

The *in vitro* infectivity assay was performed as previously described (Wang, Q. et al. Identification of an adeno-associated virus binding epitope for AVB sepharose affinity resin. Molecular Therapy - Methods & Clinical Development 2, 15040 (2015)).

Crude small-scale vector preparations of AAV vectors were added to cells seeded onto black-walled, clear-bottomed 96-well plates at an MOI as indicated. Two days after transduction, luciferase activity was measured using a luminometer after injection of 100 µL 0.15 mg/mL Luciferin into each well.

D. Scaled production and purification of vectors for animal experiments

AAV vector was made by a modified version of previously described methods (Lock et al., 2010). Briefly, a 10-layer cell stack (1.5 L total culture volume) containing 75% confluent HEK293 cell monolayers was triple-transfected using PEI. Medium was treated with turbonuclease and 0.65 M NaCl, cleared by centrifugation and subsequently concentrated by tangential flow filtration. The resulting lysate was then purified either over a single iodixanol (Optiprep; Sigma Chemical Co., St Louis, MO) gradient or by two rounds of cesium chloride centrifugation. Pooled fractions were dialyzed against PBS 35 mM NaCl and concentrated using Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA), and frozen after the addition of 5% glycerol.

E. Animal procedures

Six to eight-week old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and housed in the Animal Facility of the Translational Research Laboratories. All experimental procedures were in accordance with protocols

approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Recombinant AAV vector was injected intravenously in 100 μ L–350 μ L volumes, depending on the viral titer. All animal injections were performed by the Animal Models Core at the Gene Therapy Program of the University of Pennsylvania.

5 For the human/mice hepatocyte preference evaluation, FRG mice repopulated with human hepatocytes were purchased from Yecuris (Tualatin, OR). The purified AAV vectors used in the animal experiment were produced by Penn Vector Core. The protocol of the animal study and analysis were performed as previously described (Wang, L.L. et al. Comparative Study of Liver Gene Transfer With AAV Vectors Based on Natural and
10 Engineered AAV Capsids. *Mol Ther* 23, 1877-1887 (2015)).

All animal related work followed proper protocols, guidelines and policies of University of Pennsylvania.

F. Factor IX Activity Assay.

The ELISA for measuring F9 was modified from a protocol described by Wang et al
15 (Wang, L.L. et al. Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated, liver-directed gene therapy. *Blood* 105, 3079-3086 (2005)).

Blood samples were collected from the retroorbital plexus into 0.1 volume of 3.2% sodium citrate 1 week post injection. After two sequential centrifugation steps (2,500 x g and 20,000 x g), plasma was stored at -70°C. Factor IX activity was determined by activated
20 partial thromboplastin time (APTT) assays as follows. Fifty microliters of APTT reagent (Dade, Miami, FL), 50 ml of factor IX-deficient human plasma (George King Biomedical, Overland, KS), and 50 ml of a 1:10 dilution of mouse test plasma in Hepes buffer (50 mM Hepes/100 mM NaCl/0.02% NaN₃, pH 7.4), were incubated at 37°C in an ST4 coagulometer (American Bioproducts, Parsippany, NJ). After 3 min, clotting was initiated by the addition
25 of 50 ml of 33 mM CaCl₂ in Hepes buffer. Factor IX activity of duplicate samples was determined from a log–log standard curve that was constructed from the APTT results for dilution (1:5 to 1:640) of pooled plasma from 15 normal (129Sv) mice.

G. AAV Genome Quantification

Three methods of quantifying genome copies (GCs) of AAV were evaluated,
30 including Traditional qPCR, optimized qPCR (oqPCR) and droplet digital PCR (ddPCR). As described previously by Lock M et al (*Hum Gene Ther Methods*. 2014 Apr;25(2):115-25), addition of surfactant in reagent and Proteinase K pretreatment are used in oqPCR, while addition of surfactant (Pluronic F-68) is performed in ddPCR. Generally, number of genome

copies obtained via oqPCR is comparable to that via ddPCR, and about 2 times to about 4 times of that obtained via qPCR. Examples of result obtained are listed in the following table.

Lot #	Vector Name	Titer (GC/ml)		
		ddPCR	qPCR	oqPCR
WL1120CS	AAV3B. hFVIII	7.74×10^{11}	1.798×10^{13}	2.54×10^{13}
WL1123CS	AAV3B. hFVIII	9.26×10^{11}	1.705×10^{13}	3.69×10^{13}
WL1721CS	AAV3B-8VR9. hFVIII	3.67×10^{12}		2.41×10^{13}

- 5 All publications, references to GenBank and other sequences cited in this specification and the appended Sequence Listing are incorporated herein by reference, as is US Provisional Patent Application No. 62/488,808, filed April 23, 2017. While the invention has been described with reference to particular embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such
- 10 modifications are intended to fall within the scope of the appended claims.

(Sequence Listing Free Text)

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

15

SEQ ID NO: (containing free text)	Free text under <223>
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	<223> AAV8 VP1 <220> <221> misc_feature <222> (787)..(801) <223> AAV8 HVR I <220> <221> misc_feature <222> (988)..(999) <223> AAV8 HVR II <220> <221> misc_feature <222> (1345)..(1425) <223> AAV8 HVR IV <220> <221> misc_feature <222> (1471)..(1527) <223> AAV8 HVR V <220> <221> misc_feature <222> (1615)..(1626) <223> AAV8 HVR VI <220> <221> misc_feature <222> (1642)..(1677) <223> AAV8 HVR VII
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	<220> <221> misc_feature <222> (1750)..(1800) <223> AAV8 HVR VIII <220> <221> misc_feature <222> (1750)..(1752) <223> AAV8 HVR VIII Patch 1 <220> <221> misc_feature <222> (1762)..(1767) <223> AAV8 HVR VIII Patch 2 <220> <221> misc_feature <222> (1780)..(1788) <223> AAV8 HVR VIII Patch 3 <220> <221> misc_feature <222> (1798)..(1800) <223> AAV8 HVR VIII Patch 4 <220> <221> misc_feature <222> (2122)..(2160) <223> AAV8 HVR IX
2	<223> encoded amino acid sequence of AAV8 Capsid VP1 protein

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7	<223> encoded amino acid sequence of AAV3B.8VR1, The backbone is AAV3B. The HVR.1 is AAV8.
8	<223> nucleic acid sequence producing AAV3B.8VR2, The backbone is AAV3B. The HVR.2 is AAV8.
9	<223> encoded amino acid sequence of AAV3B.8VR2, The backbone is AAV3B. The HVR.2 is AAV8.
10	<223> nucleic acid sequence producing AAV3B.8VR4, The backbone is AAV3B. The HVR.4 is AAV8.
11	<223> encoded amino acid sequence of AAV3B.8VR4, The backbone is AAV3B. The HVR.4 is AAV8.
12	<223> nucleic acid sequence producing AAV3B.8VR5, The backbone is AAV3B. The HVR.5 is AAV8.
13	<223> encoded amino acid sequence of AAV3B.8VR5, The backbone is AAV3B. The HVR.5 is AAV8.
14	<223> nucleic acid sequence producing AAV3B.8VR6, The backbone is AAV3B. The HVR.6 is AAV8.
15	<223> encoded amino acid sequence of AAV3B.8VR6, The backbone is AAV3B. The HVR.6 is AAV8.
16	<223> nucleic acid sequence producing

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17	<223> encoded amino acid sequence of AAV3B.8VR7, The backbone is AAV3B. The HVR.7 is AAV8.
18	<223> nucleic acid sequence producing AAV3B.8VR8, The backbone is AAV3B. The HVR.8 is AAV8.
19	<223> encoded amino acid sequence of AAV3B.8VR8, The backbone is AAV3B. The HVR.8 is AAV8.
20	<223> nucleic acid sequence producing AAV3B.8VR9, The backbone is AAV3B. The HVR.9 is AAV8.
21	<223> encoded amino acid sequence of AAV3B.8VR9, The backbone is AAV3B. The HVR.9 is AAV8.
22	<223> nucleic acid sequence producing AAV3B.8VR8P1, The backbone is AAV3B. The HVR.8 patch 1 is AAV8.
23	<223> encoded amino acid sequence of AAV3B.8VR8P1, The backbone is AAV3B. The HVR.8 patch 1 is AAV8.
24	<223> nucleic acid sequence producing AAV3B.8VRP2, The backbone is AAV3B. The HVR.8 Patch 2 is AAV8.
25	<223> encoded amino acid sequence of AAV3B.8VRP2, The backbone is AAV3B. The HVR.8 Patch 2 is AAV8.
26	<223> nucleic acid sequence producing AAV3B.8VR8P3, The backbone is AAV3B. The HVR.8 patch 3 is AAV8.

27	<223> encoded amino acid sequence of AAV3B.8VR8P3, The backbone is AAV3B. The HVR.8 patch 3 is AAV8.
28	<223> nucleic acid sequence producing AAV3B.8VR8P4, The backbone is AAV3B. The HVR.8 patch 4 is AAV8.
29	<223> encoded amino acid sequence of AAV3B.8VR8P4, The backbone is AAV3B. The HVR.8 patch 4 is AAV8.
30	<223> nucleic acid sequence producing AAV8.3BVR1, The backbone is AAV8. The HVR.1 is AAV3B.
31	<223> encoded amino acid sequence of AAV8.3BVR1, The backbone is AAV8. The HVR.1 is AAV3B.
32	<223> nucleic acid sequence producing AAV8.3BVR2, The backbone is AAV8. The HVR.2 is AAV3B.
33	<223> encoded amino acid sequence of AAV8.3BVR2, The backbone is AAV8. The HVR.2 is AAV3B.
34	<223> nucleic acid sequence producing AAV8.3BVR4, The backbone is AAV8. The HVR.4 is AAV3B.
35	<223> encoded amino acid sequence of AAV8.3BVR4, The backbone is AAV8. The HVR.4 is AAV3B.
36	<223> nucleic acid sequence producing AAV8.3BVR5, The backbone is AAV8. The HVR.5 is AAV3B.
37	<223> encoded amino acid sequence of AAV8.3BVR5, The backbone is AAV8.

	The HVR.5 is AAV3B.
38	<223> nucleic acid sequence producing AAV8.3BVR6. The backbone is AAV8. The HVR.6 is AAV3B.
39	<223> encoded amino acid sequence of AAV8.3BVR6. The backbone is AAV8. The HVR.6 is AAV3B.
40	<223> nucleic acid sequence producing AAV8.3BVR7, The backbone is AAV8. The HVR.7 is AAV3B.
41	<223> encoded amino acid sequence of AAV8.3BVR7, The backbone is AAV8. The HVR.7 is AAV3B.
42	<223> nucleic acid sequence producing AAV8.3BVR8, The backbone is AAV8. The HVR.8 is AAV3B.
43	<223> encoded amino acid sequence of AAV8.3BVR8, The backbone is AAV8. The HVR.8 is AAV3B.
44	<223> nucleic acid sequence producing AAV8.3BVR9, The backbone is AAV8. The HVR.9 is AAV3B.
45	<223> encoded amino acid sequence of AAV8.3BVR9, The backbone is AAV8. The HVR.9 is AAV3B.
46	223> nucleic acid sequence producing AAV8.3BVR8.3BVR1, The backbone is AAV8.3BVR8. The HVR.1 is AAV3B.
47	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR1, The backbone is AAV8.3BVR8. The HVR.1 is AAV3B.
48	<223> nucleic acid sequence producing

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49	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR2, The backbone is AAV8.3BVR8. The HVR.2 is AAV3B.
50	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR4, The backbone is AAV8.3BVR8. The HVR.4 is AAV3B.
51	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR4, The backbone is AAV8.3BVR8. The HVR.4 is AAV3B.
52	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR5, The backbone is AAV8.3BVR8. The HVR.5 is AAV3B.
53	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR5, The backbone is AAV8.3BVR8. The HVR.5 is AAV3B.
54	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR6, The backbone is AAV8.3BVR8. The HVR.6 is AAV3B.
55	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR6, The backbone is AAV8.3BVR8. The HVR.6 is AAV3B.
56	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR7, The backbone is AAV8.3BVR8. The HVR.7 is AAV3B.
57	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR7, The backbone is AAV8.3BVR8. The HVR.7 is AAV3B.
58	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR9, The backbone is AAV8.3BVR8. The HVR.9 is AAV3B.

59	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR9, The backbone is AAV8.3BVR8. The HVR.9 is AAV3B.
60	<223> Anucleic acid sequence producing AV8.3BVR8.3BVR6.3BVR1, The backbone is AAV8.3BVR8.3BVR6. The HVR.1 is AAV3B.
61	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR6.3BVR1, The backbone is AAV8.3BVR8.3BVR6. The HVR.1 is AAV3B.
62	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR6.3BVR2, The backbone is AAV8.3BVR8.3BVR6. The HVR.2 is AAV3B.
63	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR6.3BVR2, The backbone is AAV8.3BVR8.3BVR6. The HVR.2 is AAV3B.
64	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR6.3BVR4, The backbone is AAV8.3BVR8.3BVR6. The HVR.4 is AAV3B.
65	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR6.3BVR4, The backbone is AAV8.3BVR8.3BVR6. The HVR.4 is AAV3B.
66	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR6.3BVR5, The backbone is AAV8.3BVR8.3BVR6. The HVR.5 is AAV3B.
67	<223> encoded amino acid sequence of

	AAV8.3BVR8.3BVR6.3BVR5, The backbone is AAV8.3BVR8.3BVR6. The HVR.5 is AAV3B.
68	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR6.3BVR7, The backbone is AAV8.3BVR8.3BVR6. The HVR.7 is AAV3B.
69	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR6.3BVR7, The backbone is AAV8.3BVR8.3BVR6. The HVR.7 is AAV3B.
70	<223> nucleic acid sequence producing AAV8.3BVR8.3BVR6.3BVR9, The backbone is AAV8.3BVR8.3BVR6. The HVR.9 is AAV3B.
71	<223> encoded amino acid sequence of AAV8.3BVR8.3BVR6.3BVR9, The backbone is AAV8.3BVR8.3BVR6. The HVR.9 is AAV3B.
72	<223> nucleic acid sequence producing AAV8.3BVR8P1, The backbone is AAV8. The HVR 8 Patch 1 is AAV3B.
73	<223> encoded amino acid sequence of AAV8.3BVR8P1, The backbone is AAV8. The HVR 8 Patch 1 is AAV3B.
74	<223> nucleic acid sequence producing AAV8.3BVR8P3, The backbone is AAV8. The HVR 8 Patch 3 is AAV3B.
75	<223> encoded amino acid sequence of AAV8.3BVR8P3, The backbone is AAV8. The HVR 8 Patch 3 is AAV3B.
76	<223> nucleic acid sequence producing

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77	<223> encoded amino acid sequence of AAV8.3BVR8P1.3BVR8P3, The backbone is AAV8. The HVR 8 Patches 1 and 3 are AAV3B.
78	<223> nucleic acid sequence producing AAV3B.8VR9P1, The backbone is AAV3B. The HVR 9 Patch 1 is AAV8.
79	<223> encoded amino acid sequence of AAV3B.8VR9P1, The backbone is AAV3B. The HVR 9 Patch 1 is AAV8.
80	<223> nucleic acid sequence producing AAV3B.8VR9P3, The backbone is AAV3B. The HVR 9 Patch 3 is AAV8.
81	<223> encoded amino acid sequence of AAV3B.8VR9P3, The backbone is AAV3B. The HVR 9 Patch 3 is AAV8.
82	<223> nucleic acid sequence producing AAV3B.8VR9P1.8VR9P3, The backbone is AAV3B. The HVR 9 Patches 1 and 3 are AAV8.
83	<223> encoded amino acid sequence of AAV3B.8VR9P1.8VR9P3, The backbone is AAV3B. The HVR 9 Patches 1 and 3 are AAV8.
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132	<223> encoded AAV3B variant with S663V and T492V
133	<223> encoded amino acid sequence of AAV3B variant LK03
134	<223> nucleic acid sequence producing AAV3B variant LK03
135	<223> encoded amino acid sequence of AAV variant LK03.L125I.
136	<223> encoded sequence of AAV3B.8VR9.3BVR9P2 (AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 2 stays as that of AAV3B)
137	<223> encoded amino acid sequence of AAV3B.8VR9.3BVR9P4 (AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 4 stays as that of AAV3B).
138	<223> encoded amino acid sequence of

	AAV3B.8VR9.3BVR9P5 (AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 5 stays as that of AAV3B)
139	<223> encoded amino acid sequence of AAV3B.8VR9.3BVR9P6 (AAV3B with its HVR.IX replaced with that of AAV8 while its HVR IX Mutation 6 stays as that of AAV3B)
140	<223> necleic acid sequence producing AAV1 capsid protein
141	<223> encoded AAV1 capsid protein vp1.

CLAIMS:

1. A recombinant adeno-associated virus (rAAV) having an AAV capsid, wherein the AAV capsid is produced from an AAV VP nucleic acid sequence having a mutation in a codon resulting in an amino acid change in: one or more amino acid residues in hypervariable region (HVR) VIII or HVR IX of a parental AAV, wherein the HVR VIII mutations are selected from one or more of:

- (a) amino acid (aa) 582;
- (b) aa 586;
- (c) aa 587;
- (d) aa 592;
- (e) aa 593;
- (f) aa 594; and
- (g) aa 598;

and the HVR IX mutations are selected from one or more of:

- (h) aa 706;
- (i) aa 709;
- (j) aa 710;
- (k) aa 714;
- (l) aa 716; and
- (m) aa 718.

wherein the numbering of the amino acid is based on SEQ ID NO: 4 and corresponding amino acid positions in the engineered AAV capsid can be determined via an alignment between the engineered AAV capsid and SEQ ID NO: 4.

2. The rAAV according to claim 1, wherein when the parental AAV is AAV8, the VP protein comprise a mutation in one or more of the amino acid residues in HVR IX.

3. The rAAV according to claim 1 or 2, wherein the mutation comprises one or more of:

- (i) substitution at aa 582 with an amino acid Asn (N);
- (ii) substitution at aa 586 with an amino acid Ser (S);

- (iii) substitution at aa 587 with an amino acid Ser (S);
- (iv) substitution at aa 592 with an amino acid Thr (T);
- (v) substitution at aa 593 with an amino acid Thr (T);
- (vi) substitution at aa 594 with an amino acid Arg (R);
- (vii) substitution at aa 706 with an amino acid Tyr (Y);
- (viii) substitution at aa 709 with an amino acid Thr (T);
- (ix) substitution at aa 710 with an amino acid Ser (S);
- (x) substitution at aa 714 with an amino acid Ala (A);
- (xi) substitution at aa 716 with an amino acid Asn (N); and
- (xii) substitution at aa 718 with an amino acid Glu (E).

4. The rAAV according to any of claim 1 to 3, wherein HVR VIII of the AAV capsid is replaced with an exogenous HVR VIII having an amino acid sequence of aa 582 to aa 598 of SEQ ID NO: 4.

5. The rAAV according to any of claims 1 to 4, wherein HVR VI of the AAV capsid is further replaced with an exogenous HVR VI having an amino acid sequence of aa 537 to aa 540 of SEQ ID NO: 4.

6. The rAAV according to any of claims 1 to 5, wherein HVR IV of the AAV capsid is further replaced by an exogenous HVR IV having an amino acid sequence of aa 446 to aa 473 of SEQ ID NO: 4.

7. The rAAV according to any of claim 1 to 6, wherein HVR IX of the AAV capsid is replaced with an exogenous HVR IX having an amino acid sequence of aa 708 to aa 720 of SEQ ID NO: 2.

8. The rAAV capsid according to any of claims 1, 3 and 7, wherein the parental AAV capsid protein has an amino acid sequence produced from the nucleic acid sequence of SEQ ID NO: 3, or a nucleic acid sequence at least 95% identical thereto.

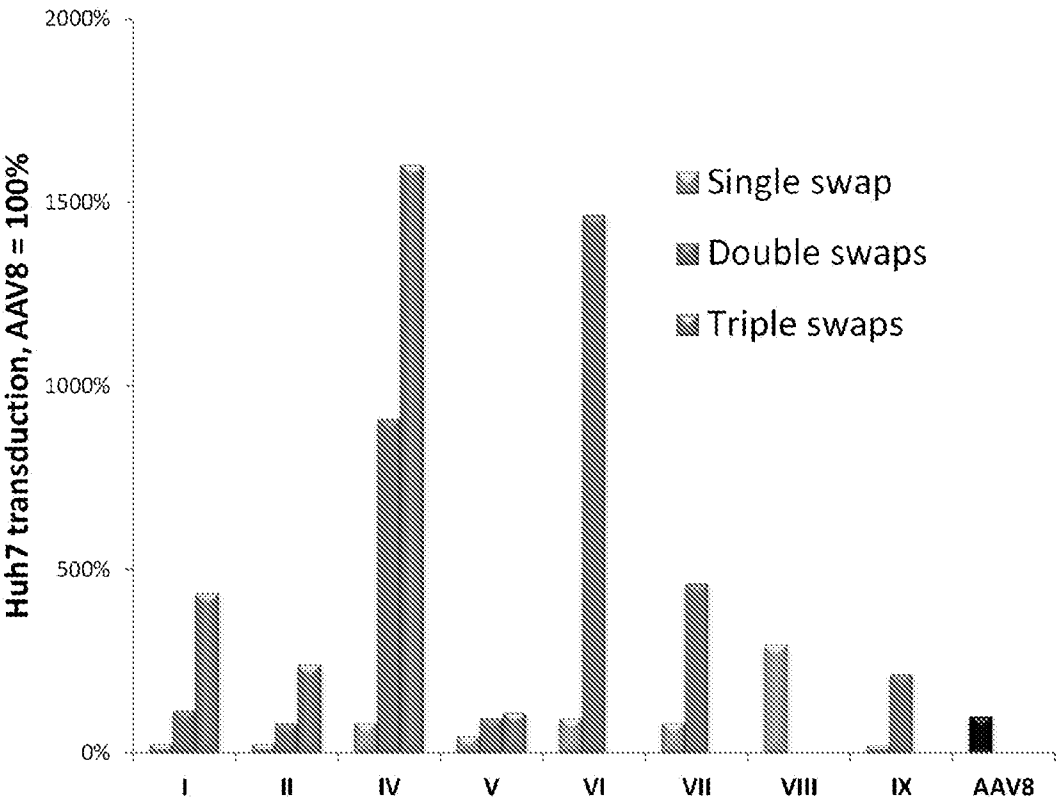
9. The rAAV according to any of claims 1 to 6, wherein the parental AAV capsid protein has an amino acid sequence produced from the nucleic acid sequence of SEQ ID NO: 1, or a nucleic acid sequence at least 95% identical thereto.
10. The rAAV according to any of claims 1 to 9, comprising an amino acid sequence produced from a nucleic acid sequence selected from SEQ ID NO: 20, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 42, SEQ ID NO: 50, SEQ ID NO: 54 or SEQ ID NO: 64, SEQ ID NO: 72, or SEQ ID NO: 74, and SEQ ID NO: 76.
11. The rAAV according to any of claims 1 to 10, further comprising AAV inverted terminal repeats, a transgene operably linked to regulatory sequences which direct expression of a product encoded by said transgene in a target cell.
12. A composition comprising the rAAV according to any of claim 1 to 11 and a physiologically compatible carrier, excipient and/or preservative.
13. A use of a composition comprising the rAAV according to any of claim 1 to 11 for delivering a transgene to a target cell.
14. A method of delivering the transgene to a target cell, said method comprising treating a subject with the rAAV according to any of claim 1 to 11.
15. A nucleic acid molecule comprising a nucleic acid sequence producing the rAAV according to any of claims 1 to 11.
16. The nucleic acid molecule according to claim 15, comprising a sequence producing a functional AAV rep protein.
17. The nucleic acid molecule according to claim 15 or 16, wherein said molecule is a plasmid.
18. A packaging host cell comprising the nucleic acid molecule according to any of claims 15 to 17.

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HVR.I	<u>AAV8</u> 259 <u>KQISNGTSGGA</u>	263-267
	<u>AAV3B</u> 258 <u>KQISS---QSGA</u>	
HVR.II	<u>AAV8</u> 325 <u>EVTQNEGTKTIANNLTS</u>	330-333
	<u>AAV3B</u> 322 <u>EVTQNDGTTTIANNLTS</u>	
HVR.IV	<u>AAV8</u> 446 <u>YYLSRTQ - TTGGTANTQTLGFSQGGPNTMANQ</u>	449-475
	<u>AAV3B</u> 443 <u>YYLNRTQGTTSGTTNQSRLIFSQAGPQSMSLQ</u>	
HVR.V	<u>AAV8</u> 488 <u>QQRVSTTTGQNNNSNEFAWTAGTKYHL</u>	491-509
	<u>AAV3B</u> 486 <u>QQRLSKTANDNNNSNEFPWTAASKYHL</u>	
HVR.III, AAV3B = AAV8		
HVR.VI	<u>AAV8</u> 537 <u>FPSNG I LIF</u>	539-542
	<u>AAV3B</u> 535 <u>FPMHGNLIF</u>	
HVR.VII	<u>AAV8</u> 544 <u>IFGKQNAARDNADYSDVM</u>	548-559
	<u>AAV3B</u> 542 <u>IFGKEGTTASNAELDNVM</u>	
HVR.VIII	<u>AAV8</u> 582 <u>VADNLQQQNTAPQIGTVNSQGA</u>	584-600
	<u>AAV3B</u> 580 <u>VANNLQSSNTAPTTRTVNDQGA</u>	
HVR.IX	<u>AAV8</u> 706 <u>NYYKSTSVDFAVNTEGVYSE</u>	708-720
	<u>AAV3B</u> 704 <u>NYNKSVNVDFTVDTNGVYSE</u>	

FIG. 1

FIG. 2



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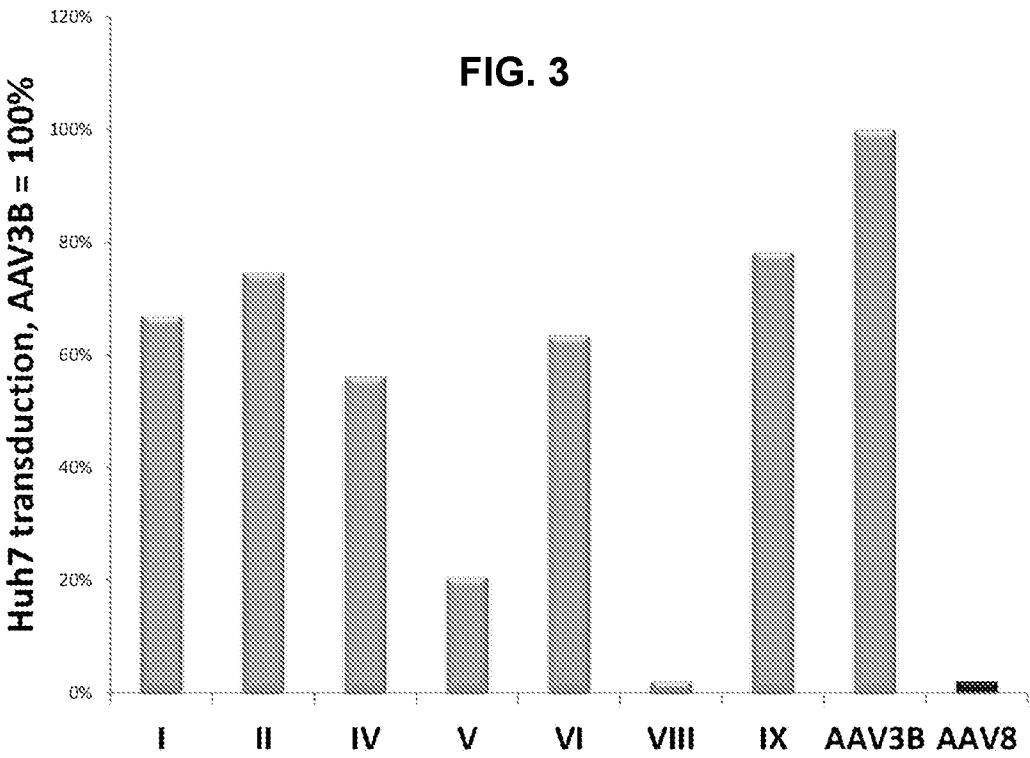


FIG. 4A

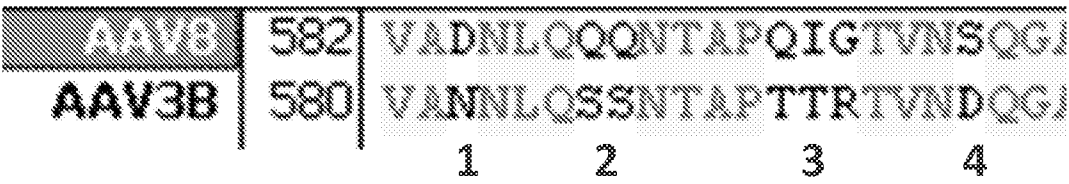
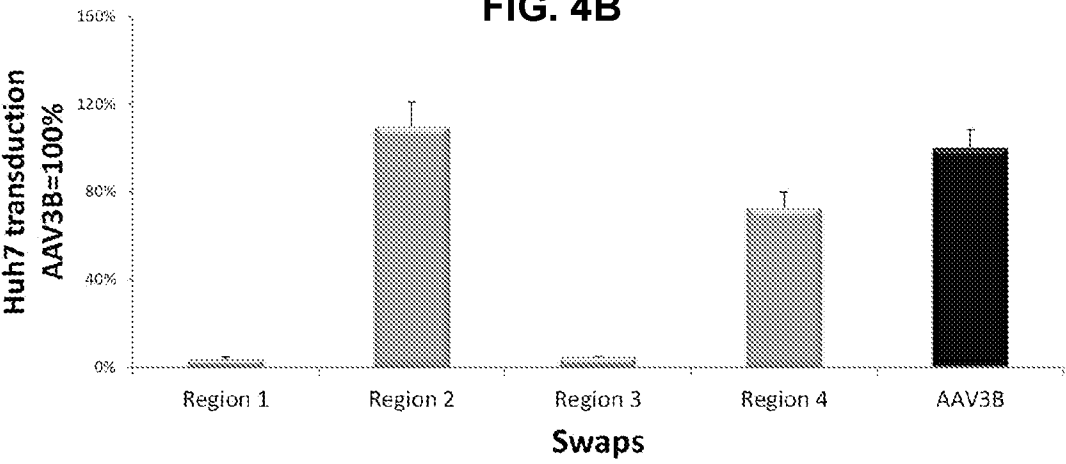
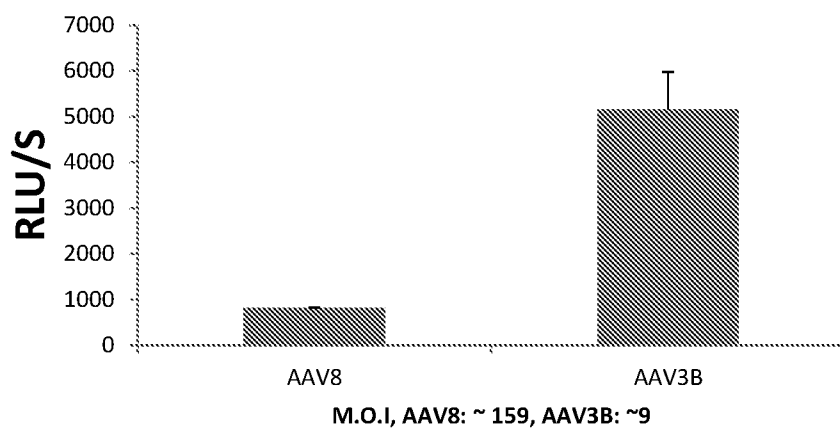
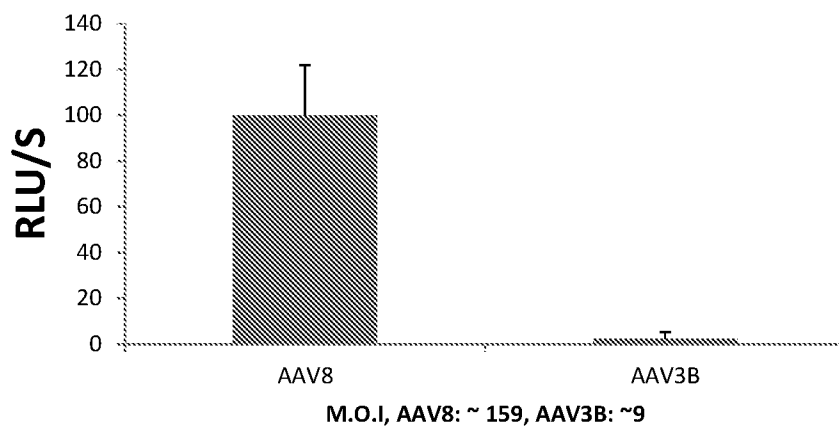


FIG. 4B



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FIG. 5A**Huh7****FIG. 5B****MC57G**

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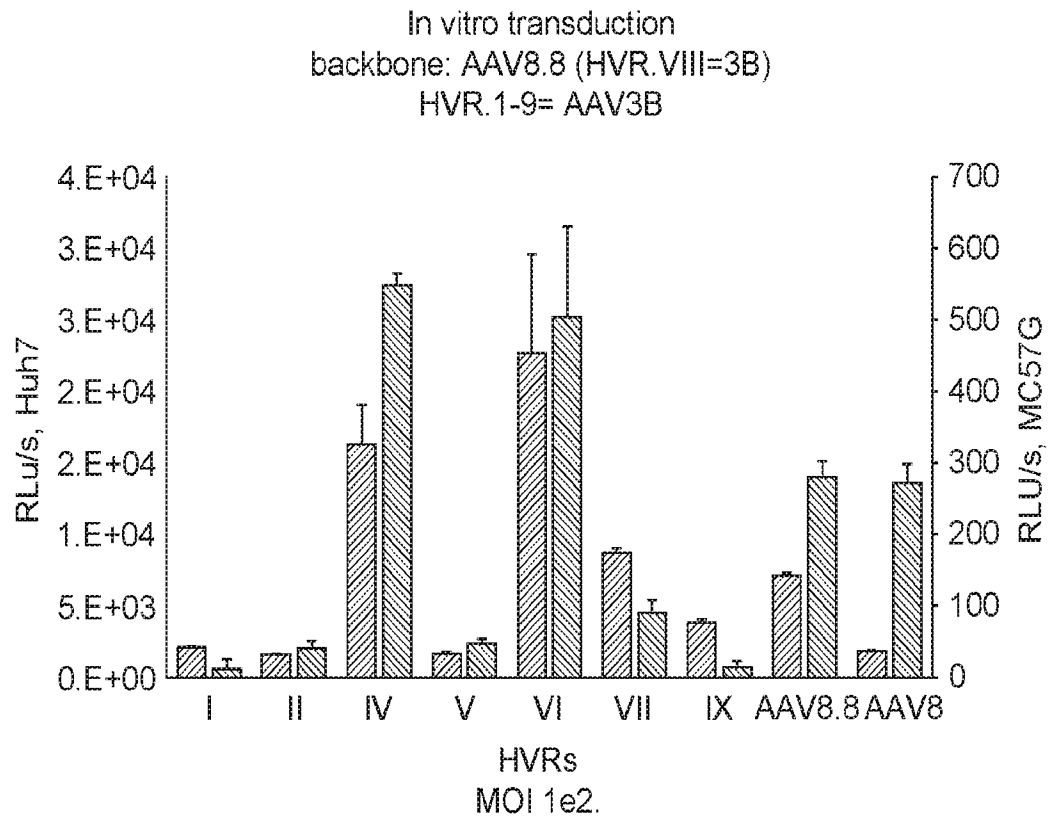


FIG. 6A

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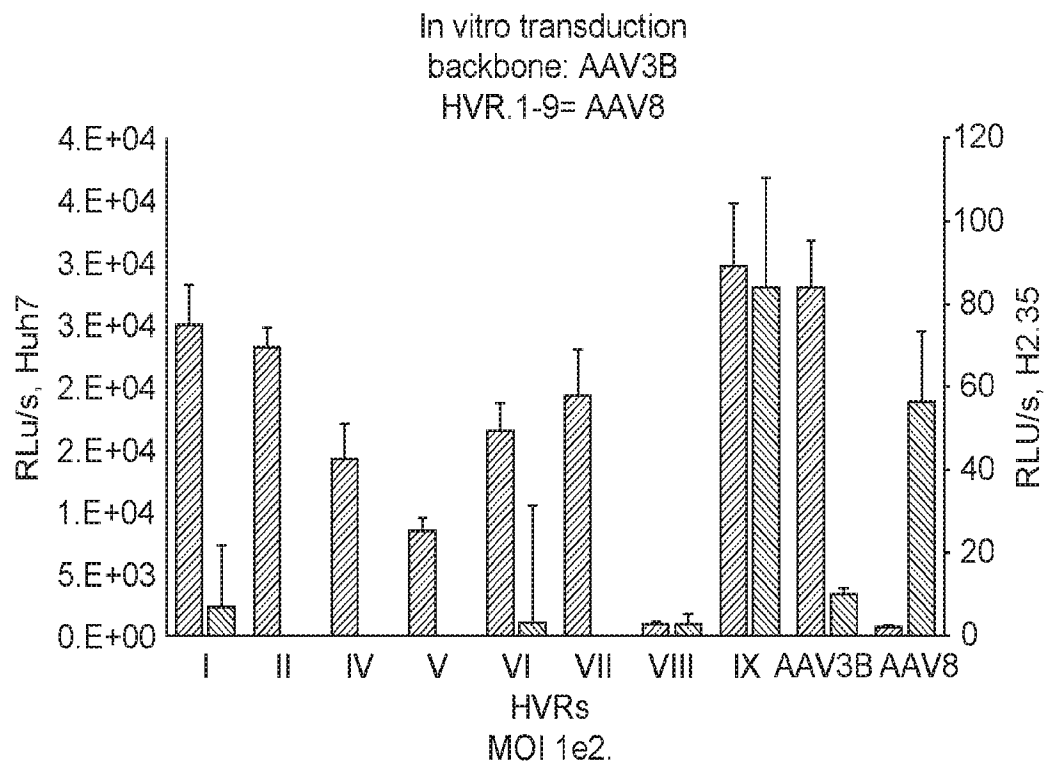
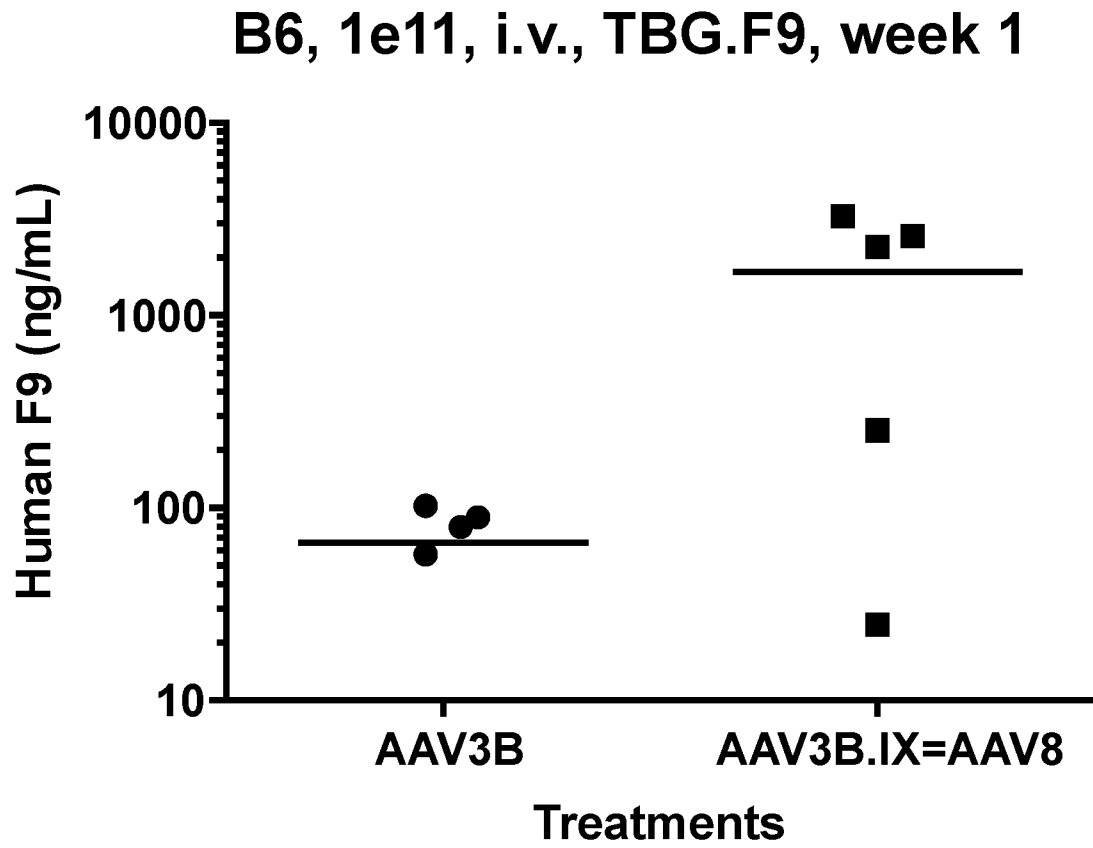


FIG. 6B

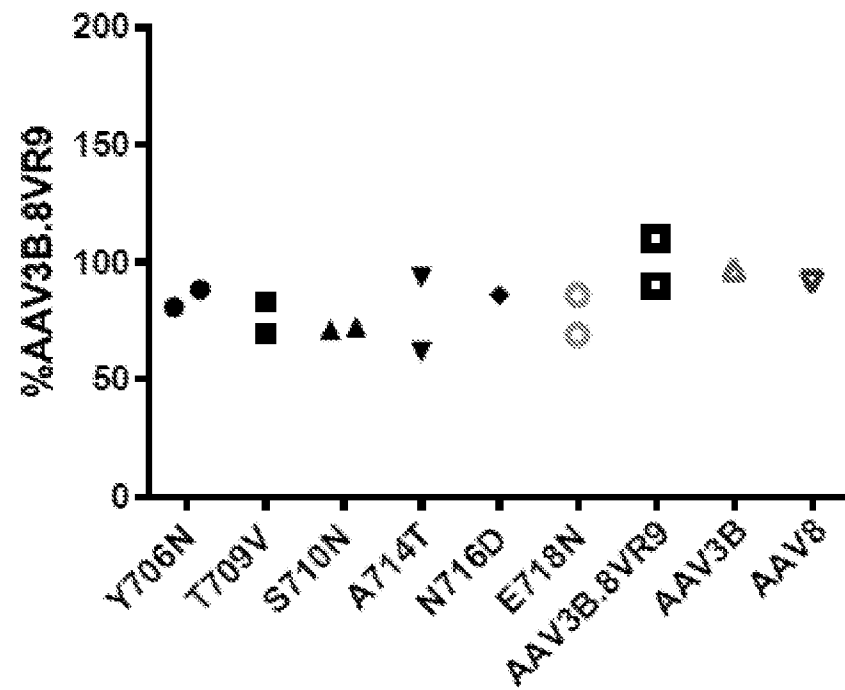
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FIG. 7



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FIG. 8



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FIG. 9A

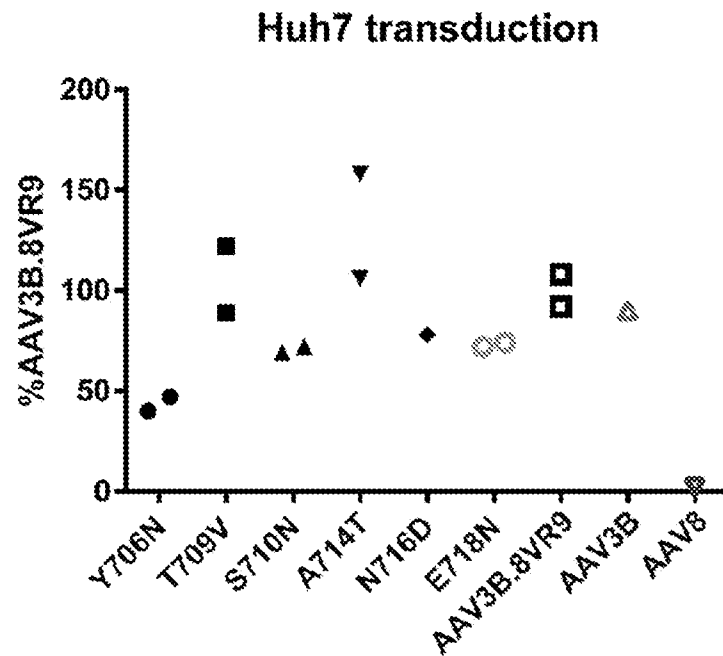
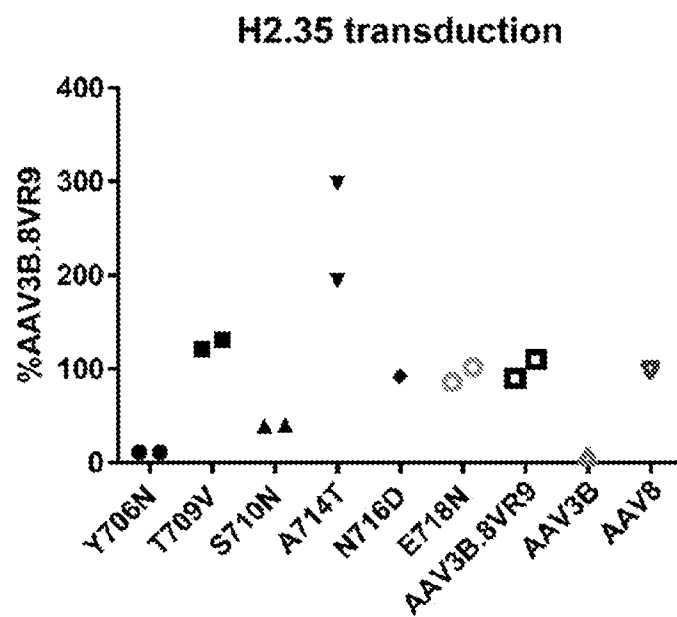


FIG. 9B



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FIG. 10

AAV8(706)	NYYKSTSVDFAVNTEGV
AAV3B(704)	NYNKSVNVDFFTVDTNGV
LK03(704)	NYNKSVNVDFFTVDTNGV
AAV2(703)	NYNKSVNVDFFTVDTNGV
AAVDJ(705)	NYYKSTSVDFAVNTEGV
	1 2 3 4 5 6

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FIG. 11A

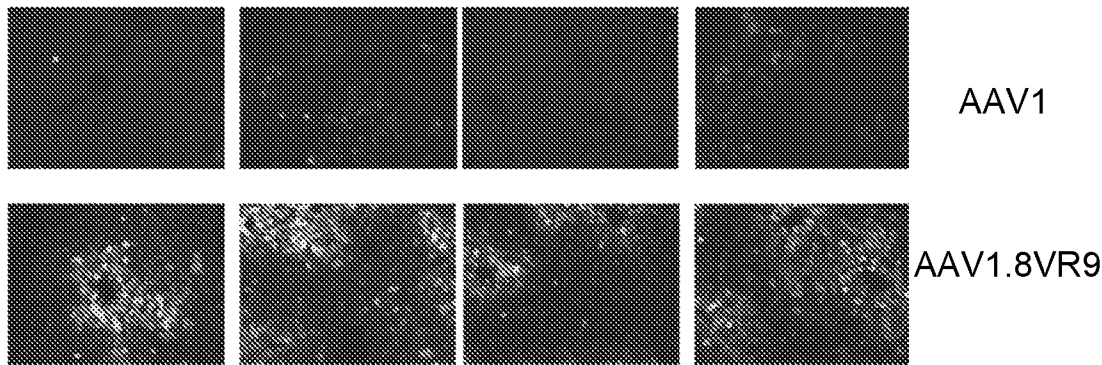
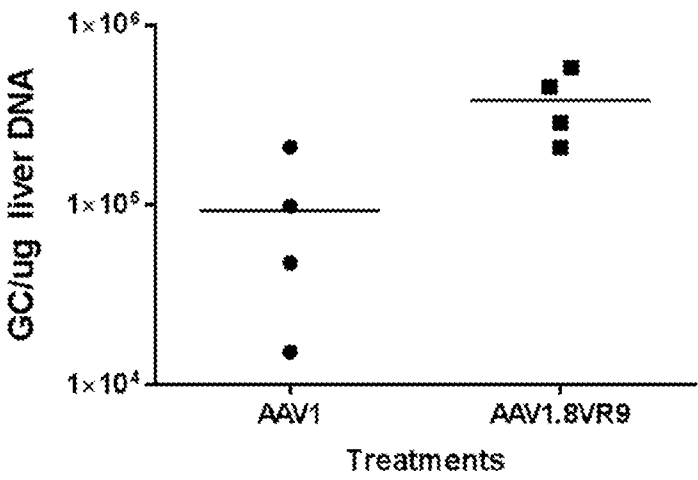


FIG. 11B



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FIG. 12

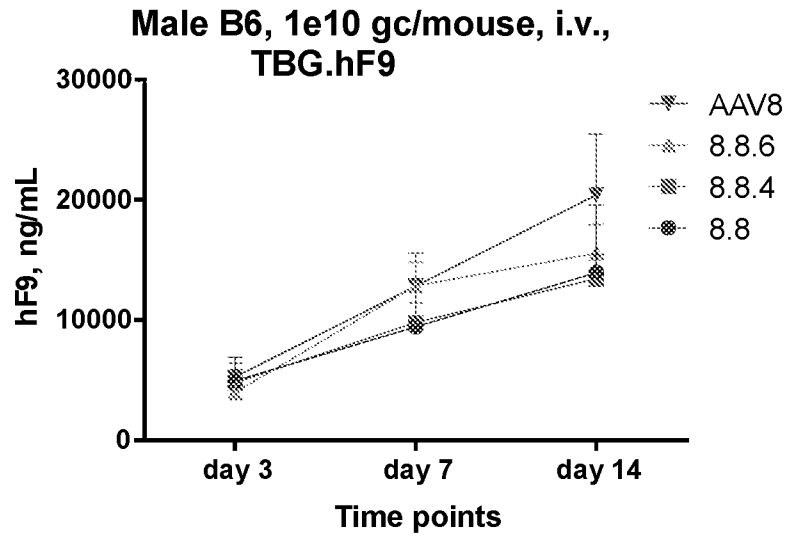
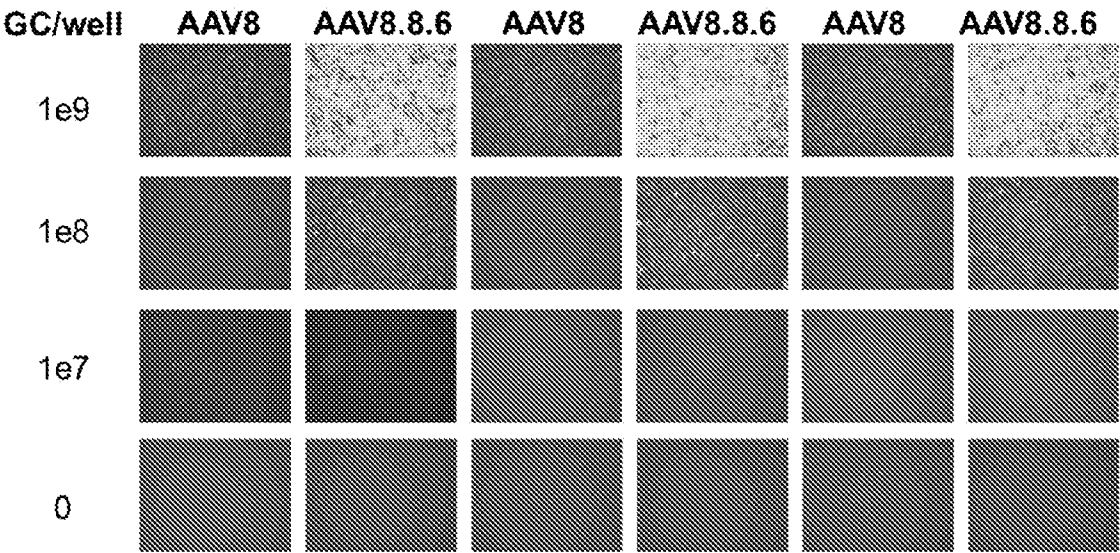
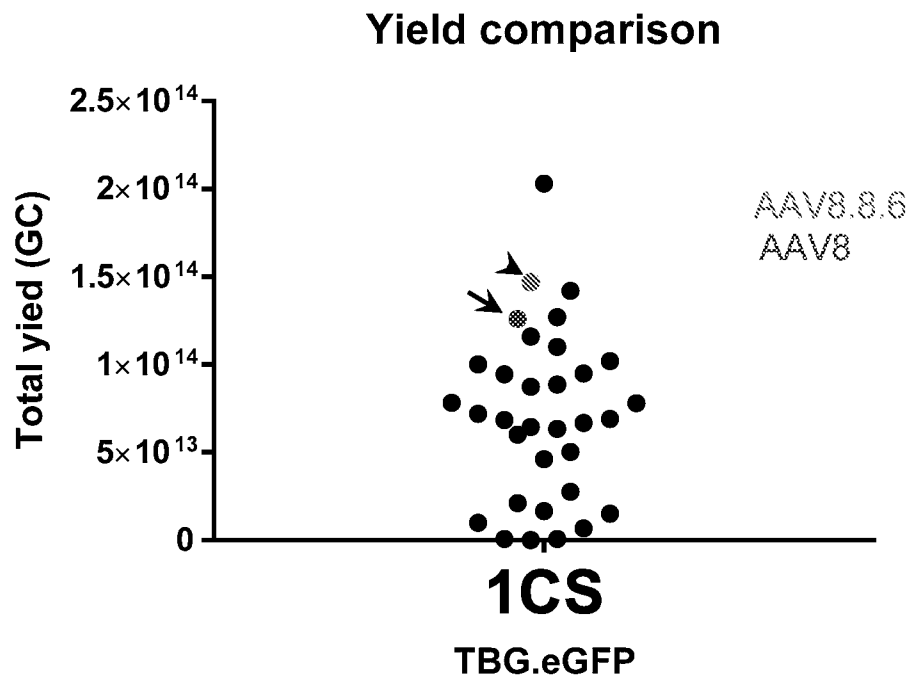
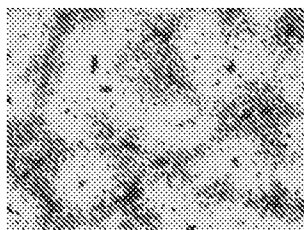
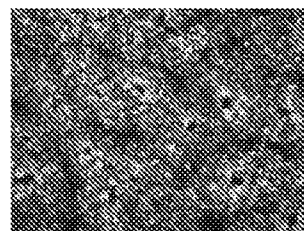


FIG. 13

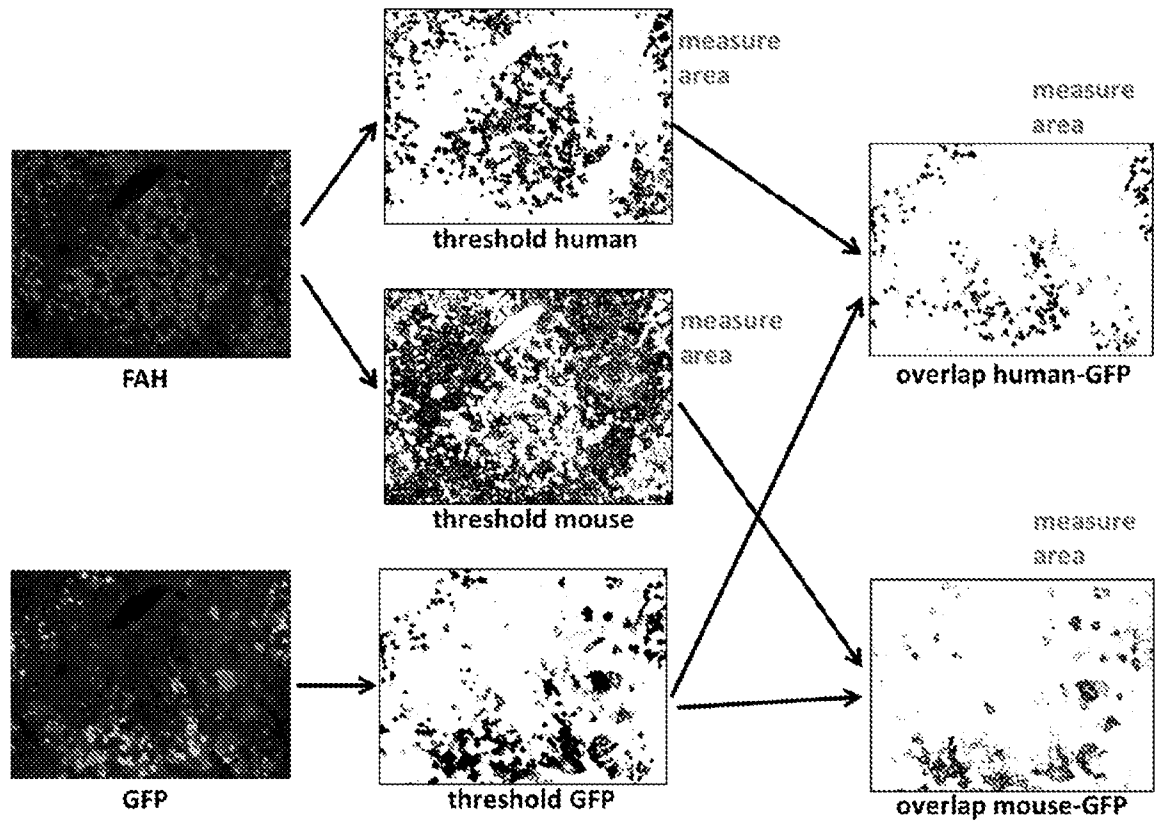


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FIG. 14**FIG. 15A****FIG. 15B****FIG. 15C****FIG. 15D****FIG. 15E****FIG. 15F**

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FIG. 16



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FIG. 17A

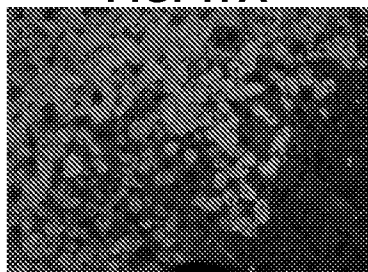


FIG. 17B



FIG. 17C

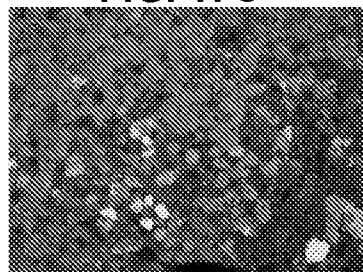


FIG. 17D

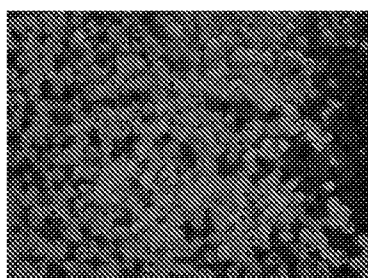


FIG. 17E

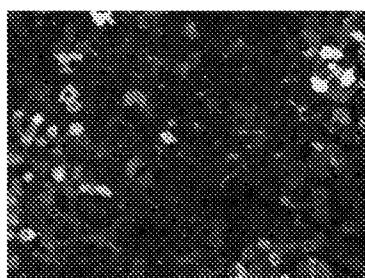


FIG. 17F

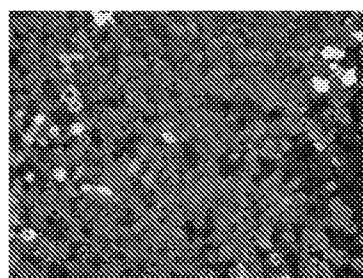


FIG. 17G

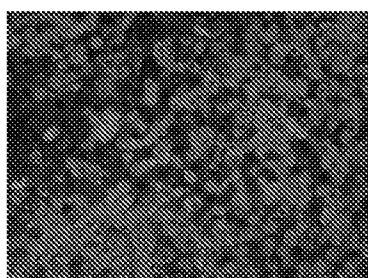


FIG. 17H

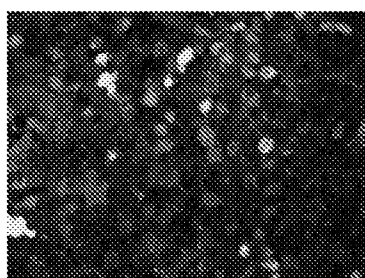
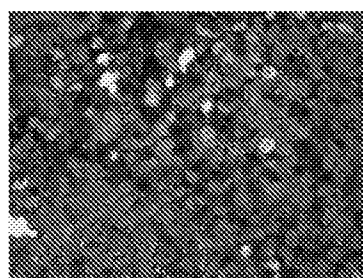


FIG. 17I



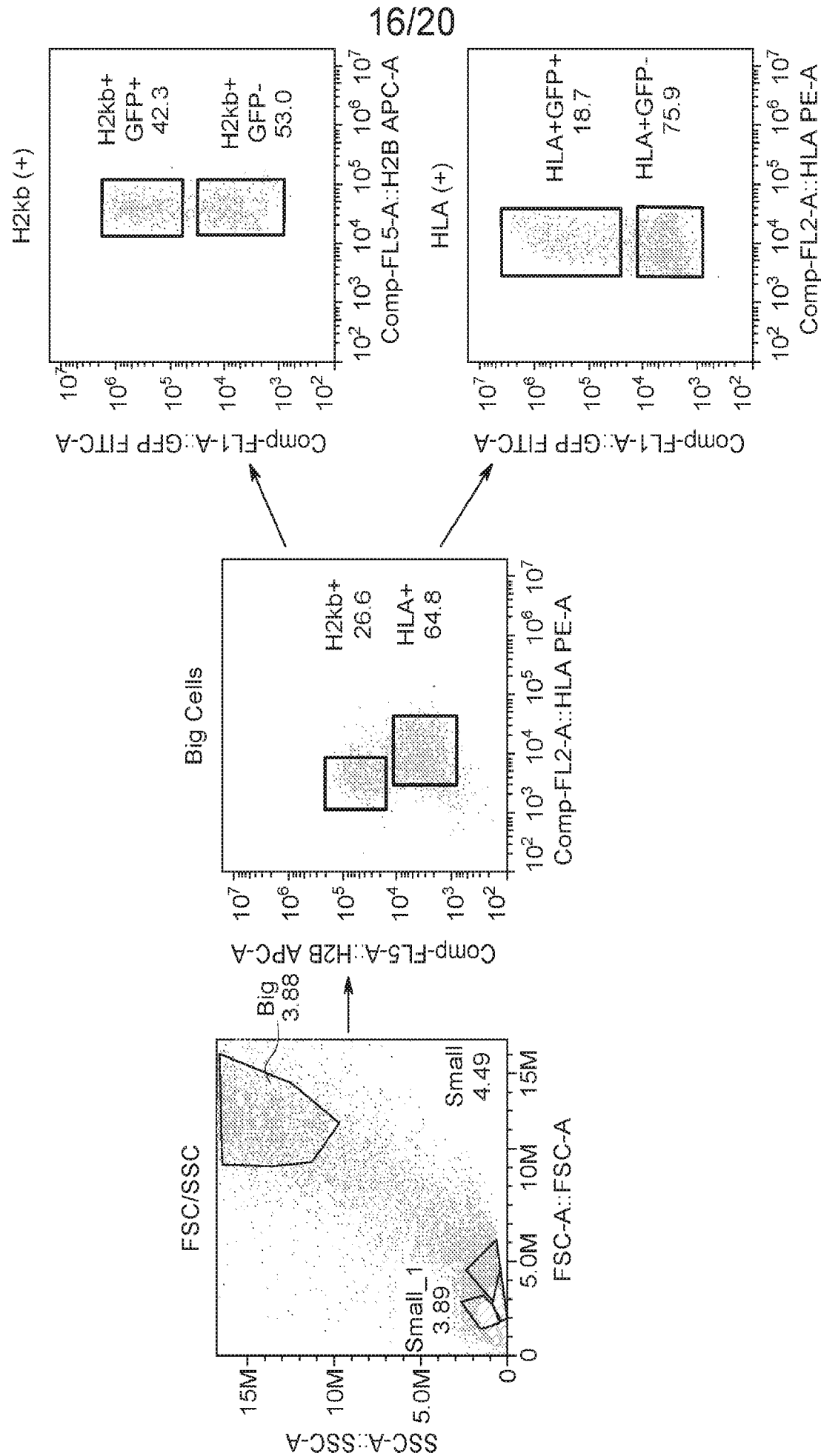


FIG. 18

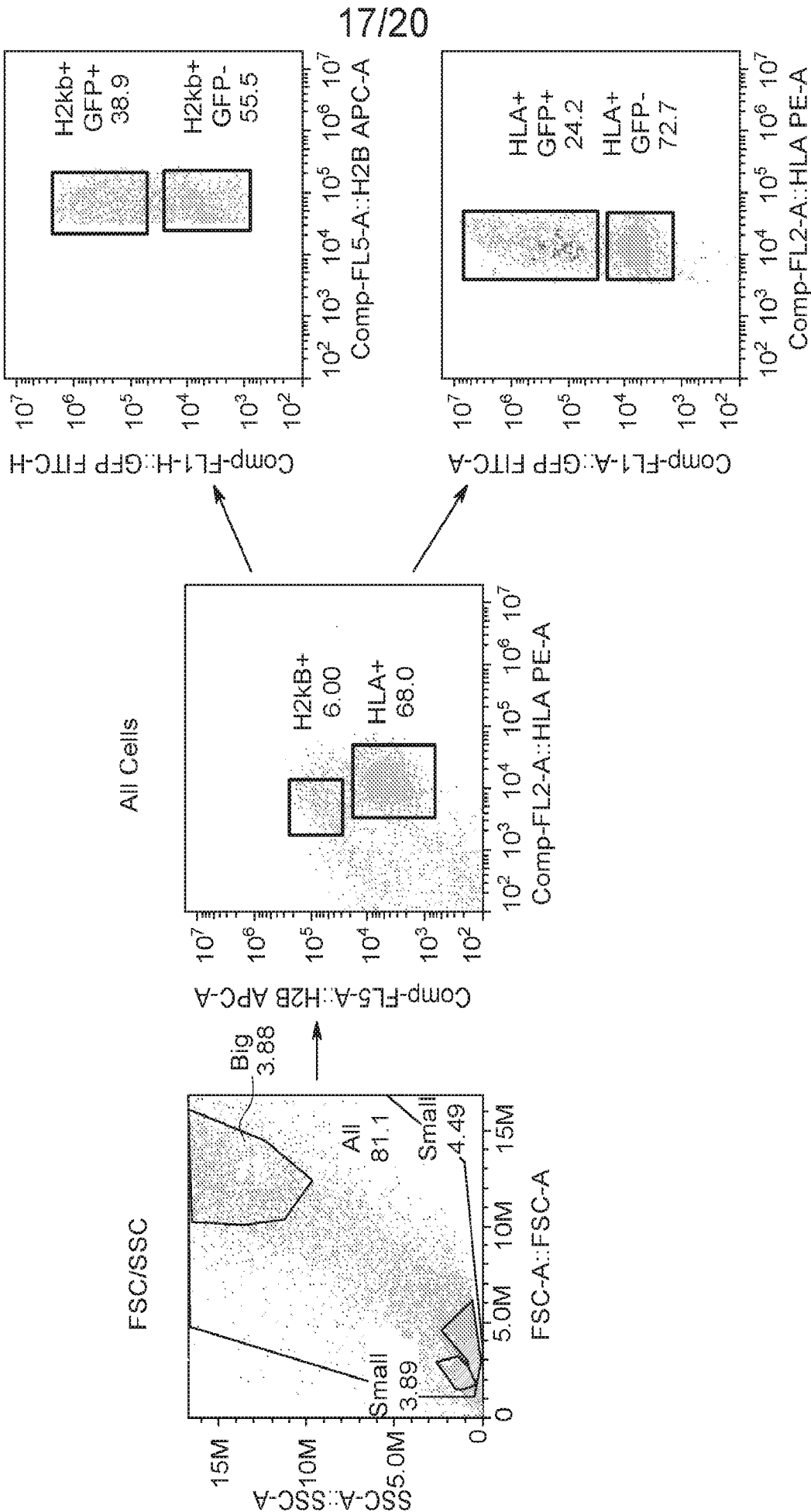
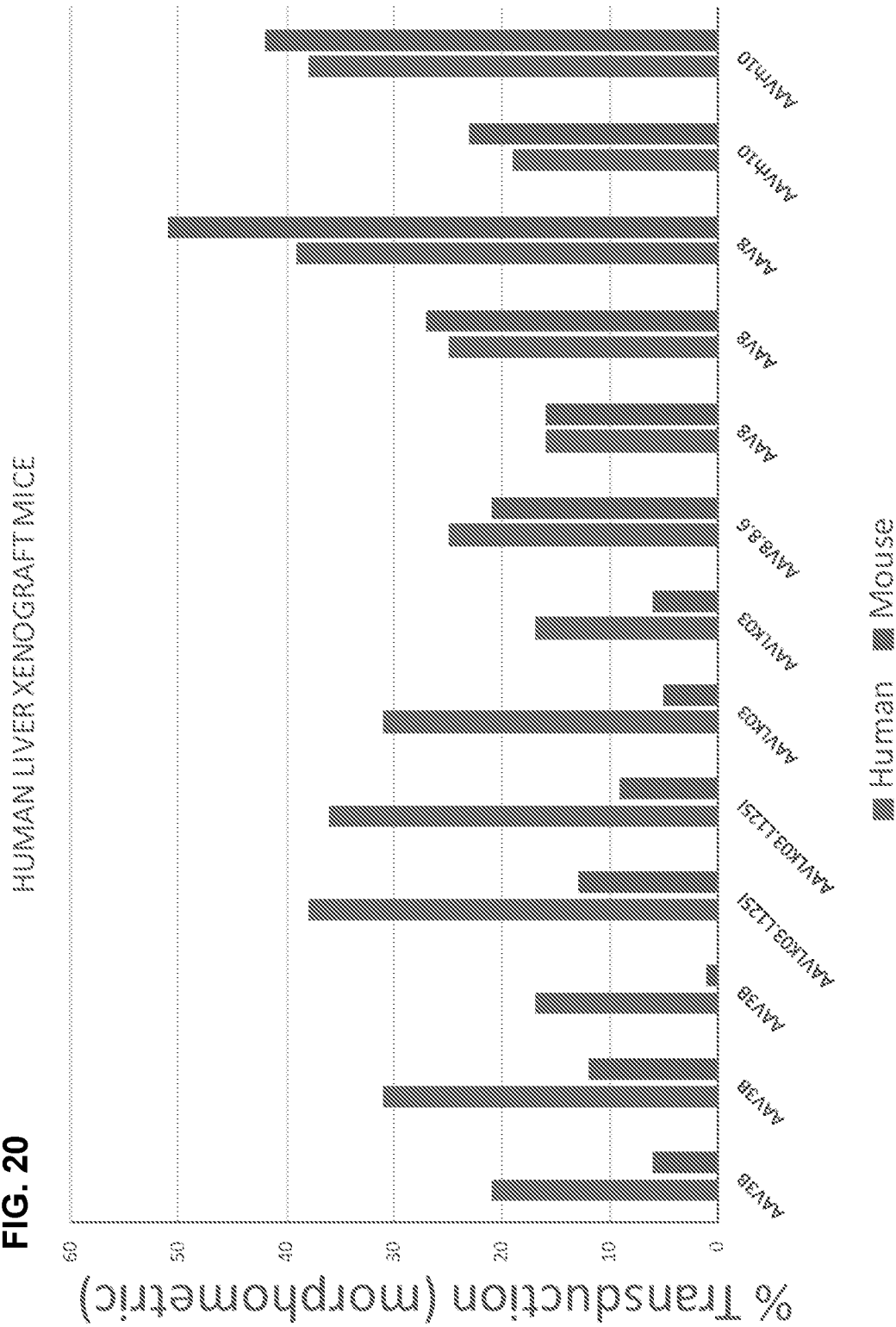
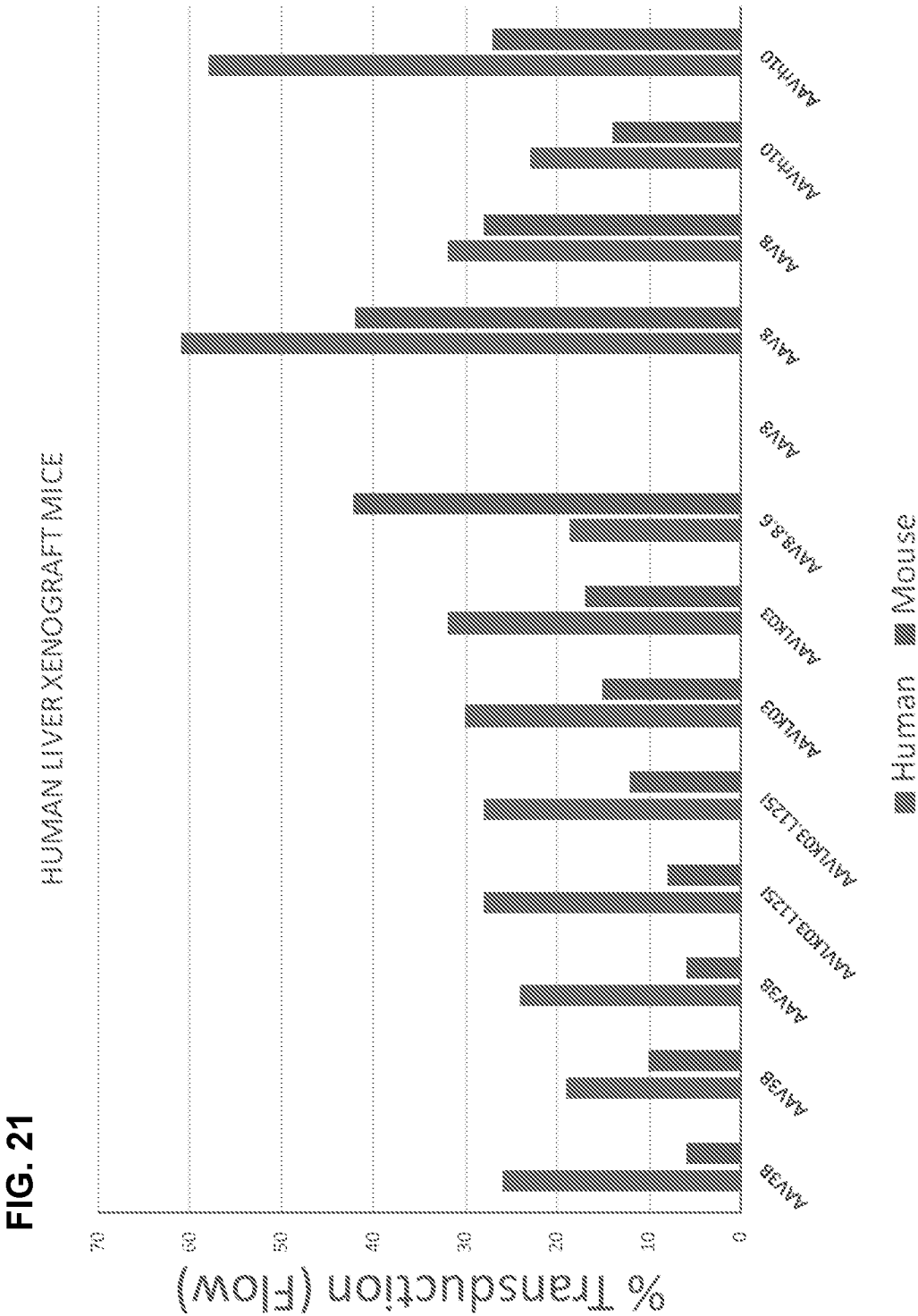


FIG. 19



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FIG. 22A

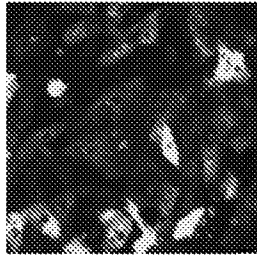


FIG. 22B

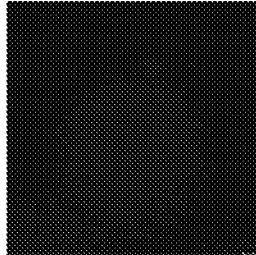


FIG. 22C

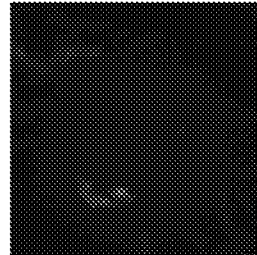


FIG. 22D

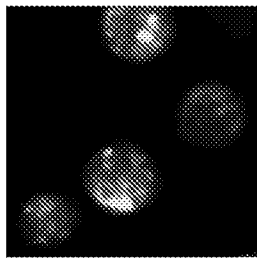


FIG. 22E

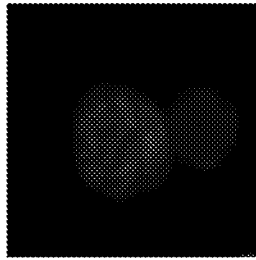


FIG. 22F

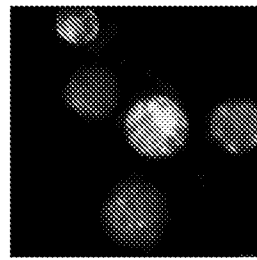


FIG. 22G

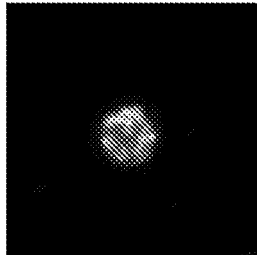


FIG. 22H

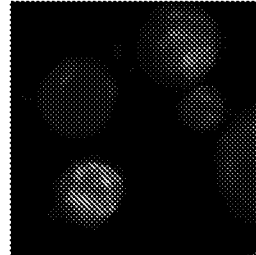
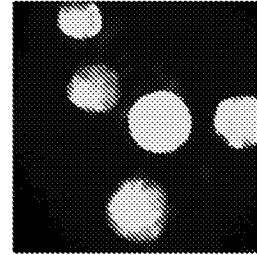


FIG. 22I



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/28954

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 1/15, 1/19, 1/21, 15/09, 5/10, 15/86, 15/861, 15/864; A61K 35/76, 35/761, 38/16 (2018.01)
 CPC - A61K 35/76, 35/761, 38/16, 38/162, 48/0075; C07K 14/005; C12N 15/09, 5/10, 15/86, 15/861, 15/864, 15/8645

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

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

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0096683 A1 (GENZYME CORPORATION) 06 April 2017; paragraphs [0007], [0024], [0031], [0097]-[0098], [0100], [0150], [0354]	1, 3
A	WO 2017/015102 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 26 January 2017; entire document	1, 3
A	(GURDA, BL et al.) Capsid Antibodies to Different Adeno-Associated Virus Serotypes Bind Common Regions. Journal of Virology. 12 June 2013, Vol. 87, No. 16, pages 9111-9124; DOI: 10.1128/JVI.00622-13	1, 3
A	WO 2016/065001 A1 (UNIVERSITY OF MASSACHUSETTS) 28 April 2016; entire document	1, 3

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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

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

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

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

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

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

13 August 2018 (13.08.2018)

Date of mailing of the international search report

07 SEP 2018

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
 P.O. Box 1450, Alexandria, Virginia 22313-1450
 Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US18/28954

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

---Please See Supplemental Page---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
claims 1, 3; mutation of amino acid 582 to Asn (N)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/28954

---Continued from Box No. III: Observations where unity of invention is lacking---

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-3 and a mutation of amino acid 582 to Asn (N) (mutation) are directed toward a recombinant adeno-associated virus having a capsid amino acid mutation in a hypervariable region.

The virus will be searched to the extent it encompasses a mutation of amino acid 582 to Asn (N) (first exemplary mutation). Applicant is invited to elect additional specified mutation(s) to be searched. Additional mutation(s) will be searched upon the payment of additional fees. It is believed that claims 1 (in-part) and 3 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass a mutation of amino acid 582 to Asn (N) (mutation). Applicants must specify the claims that encompass any additionally elected mutation(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be a mutation of amino acid 586 to Ser (mutation).

No technical features are shared between the mutation(s) of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a recombinant adeno-associated virus (rAAV) having an AAV capsid, wherein the AAV capsid is produced from an AAV VP nucleic acid sequence having a mutation in a codon resulting in an amino acid change in: one or more amino acid residues in hypervariable region (HVR) VIII or HVR IX of a parental AAV; wherein the numbering of the amino acid is based on SEQ ID NO: 4; these shared technical features are previously disclosed by US 2017/0096683 A1 to Genzyme Corporation (hereinafter 'Genzyme').

Genzyme discloses a recombinant adeno-associated virus (rAAV) (a recombinant adeno-associated virus (rAAV); abstract) having an AAV capsid, wherein the AAV capsid is produced from an AAV VP nucleic acid sequence having a mutation in a codon resulting in an amino acid change (having an AAV capsid, wherein the AAV capsid is produced from an AAV VP nucleic acid sequence having a mutation in a codon resulting in an amino acid change; paragraph [0008]) in: one or more amino acid residues in hypervariable region (HVR) VIII of a parental AAV (to amino acids including R585 or R588 (in: one or more amino acid residues in hypervariable region (HVR) VIII of a parental AAV); paragraph [0008]); wherein the numbering of the amino acid is based on SEQ ID NO: 4 (wherein the numbering of the amino acid is based on SEQ ID NO: 7 (SEQ ID NO: 4); paragraph [0150]; wherein SEQ ID NO: 7 is 100% identical to Applicants' SEQ ID NO: 4).

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Genzyme reference, unity of invention is lacking.