ADENO-ASSOCIATED VIRUS WITH ENGINEERED CAPSID

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority of U.S. Provisional Patent Application Serial No. 63/012,703 filed on April 20, 2020, the contents of which are hereby incorporated by reference in their entireties.

FIELD OF THE INVENTION

[0002] The present disclosure relates generally to gene therapy with adeno-associated virus vectors. In particular, the disclosure relates to recombinant adeno-associated virus virions having an engineered capsid protein.

REFERENCE TO SEQUENCE LISTING

[0003] This application is being filed electronically via EFS-Web and includes an electronically submitted sequence listing in .txt format. The .txt file contains a sequence listing entitled "TENA_019_01WO_SeqList_ST25.txt" created on April 13, 2021 and having a size of ~ 479 kilobytes. The sequence listing contained in this .txt file is part of the specification and is incorporated herein by reference in its entirety.

BACKGROUND

[0004] Adeno-associated virus (AAV) holds promise for gene therapy and other biomedical applications. In particular, AAV can be used to deliver gene products to various tissues and cells, both *in vitro* and *in vivo*. The capid proteins of AAV largely determine the immunogenicity and tropism of AAV vectors.

[0005] For cardiac tissues, AAV subtype 9 (AAV9) is a prefered AAV vector due to its ability to transduce the heart following systemic delivery. While AAV9 can achieve moderate transduction of the heart, the majority of vector trafficks to the liver. Moreover, in order to achieve therapeutic levels of transduction in the heart, relatively high systemic doses are required, potentially leading to systemic inflammation and in turn, toxicity.

[0006] There is a need for developing an Adeno-associated virus with engineered capsid protein that achieves improved cardiac tropism, and optionally improved selectivity of cardiac tissues over liver. The present disclosure provides variants of the AAV9 capsid and/or chimeric AAV5/AAV9 capsid that form rAAV virions capable of transducing cardiac tissues and/or cell

types for more efficiently and/or with more selectivity than rAAV virions comprising wild-type AAV9 capsid proteins, which can be used for safe and efficacious cardiac gene therapy.

SUMMARY OF THE DISCLOSURE

[0007] In one aspect, the present disclosure provides recombinant adeno-associated virus (rAAV) capsid proteins comprising a variant polypeptide sequence at one or more of a VR-IV site, a VR-V site, a VR-VII site, and a VR-VIII site of a parental sequence, wherein the parental sequence comprises a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463. In some embodiments, the variant polypeptide sequence is a cardiotrophic variant polypeptide sequence.

[0008] In some embodiments, the capsid protein of the disclosure comprises a variant polypeptide at the VR-IV site of the parental sequence. In some embodiments, the variant polypeptide at the VR-IV site has a sequence:

$$-X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-$$

wherein: X_1 is G, S or V; X_2 is Y, Q or I; X_3 is H, W, V or I; X_4 is K or N; X_5 is S, G or I; X_6 is G or R; X_7 is A, P or V; X_8 is A or R; and X_9 is Q or D (SEQ ID NO: 477). In some embodiments, the variant polypeptide at the VR-IV site comprises an amino acid sequence selected from SEQ ID NOs: 6-104. In some embodiments, the variant polypeptide at the VR-IV site comprises an amino acid sequence selected from GYHKSGAAQ (SEQ ID NO: 6), VIIKSGAAQ (SEQ ID NO: 7), GYHKIGAAQ (SEQ ID NO: 8), SQVNGRPRD (SEQ ID NO: 33) and GYHKSGVAQ (SEQ ID NO: 9). In some embodiments, the variant polypeptide at the VR-IV site comprises the amino acid sequence GYHKSGAAQ (SEQ ID NO: 6) or a sequence comprising at most 1, 2, 3, or 4 amino-acid substitutions relative to GYHKSGAAQ (SEQ ID NO: 6).

[0009] In some embodiments, the capsid protein of the disclosure comprises a variant polypeptide at the VR-V site of the parental sequence. In some embodiments, the variant polypeptide at the VR-V site has a sequence:

$$-X_1-X_2-X_3-X_4-X_5-X_6-$$

wherein: X_1 is S, L, H, N, or A; X_2 is T, M, K, G, or N; X_3 is S, T, M or I; X_4 is S, P, F, M, or N; X_5 is F, S, P or L; and X_6 is I, V, or T (SEQ ID NO: 474). In some embodiments, the variant polypeptide at the VR-V site comprises an amino acid sequence selected from SEQ ID NOs: 105-203. In some embodiments, the variant polypeptide at the VR-V site comprises an amino acid sequence selected from LNSMLI (SEQ ID NO: 105), NGMSFT (SEQ ID NO: 106) , HKTFSI (SEQ ID NO: 107) and SMSNFV (SEQ ID NO: 108). In some embodiments, the variant polypeptide at the VR-V site comprises the amino acid sequence LNSMLI (SEQ ID NO: 105) or a sequence comprising at most 1, 2, 3, or 4 amino-acid substitutions relative to LNSMLI (SEQ ID NO: 105).

[0010] In some embodiments, the capsid protein of the disclosure comprises a variant polypeptide at the VR-VII site of the parental sequence. In some embodiments, the variant polypeptide at the VR-VII site has a sequence:

$$-X_1-X_2-X_3-X_4-X_5-$$

wherein: X_1 is V, L, Q, C, or R; X_2 is S, H, G, C, or D; X_3 is Y, S, L, G, or N; X_4 is S, L, H, Q, or N; and X_5 is V, I, or R (SEQ ID NO: 475). In some embodiments, the variant polypeptide at the VR-VII site comprises an amino acid sequence selected from SEQ ID NOs: 204-302. In some embodiments, the variant polypeptide at the VR-VII site comprises an amino acid sequence selected from RGNQV (SEQ ID NO: 204), VSLNR (SEQ ID NO: 205), CDYSV (SEQ ID NO: 206), and QHGHI (SEQ ID NO: 207). In some embodiments, the variant polypeptide at the VR-VII site comprises the amino acid sequence RGNQV (SEQ ID NO: 204) or a sequence comprising at most 1, 2, or 3 amino-acid substitutions relative to RGNQV (SEQ ID NO: 204).

[0011] In some embodiments, the capsid protein of the disclosure comprises a variant polypeptide at the VR-VII site of the parental sequence. In some embodiments, the variant polypeptide at the VR-VIII site has a sequence:

$$-X_1-X_2-X_3-X_4-$$

wherein: X_1 is S, N, or A; X_2 is V, M, N, or A; X_3 is Y, V, S, or G; and X_4 is Y, T, M, G, or N (SEQ ID NO: 476). In some embodiments, the variant polypeptide at the VR-VIII site comprises an amino acid sequence selected from SEQ ID NOs: 303-401. In some embodiments, the variant

polypeptide at the VR-VIII site comprises an amino acid sequence selected from ANYG (SEQ ID NO: 305), NVSY (SEQ ID NO: 303), SMVN (SEQ ID NO: 304), and NVGT (SEQ ID NO: 306). In some embodiments, the variant polypeptide at the VR-VIII site comprises the amino acid sequence ANYG (SEQ ID NO: 305) or a sequence comprising at most 1 or 2 amino-acid substitutions relative to ANYG (SEQ ID NO: 305). In some embodiments, the variant polypeptide at the VR-VIII site comprises the amino acid sequence NVSY (SEQ ID NO: 303) or a sequence comprising at most 1 or 2 amino-acid substitutions relative to NVSY (SEQ ID NO: 303).

[0012] In one aspect, the present disclosure provides recombinant adeno-associated virus (rAAV) capsid proteins comprising a variant polypeptide sequence at one or more of a VR-IV site, a VR-V site, a VR-VII site, and a VR-VIII site of a parental sequence, wherein the parental sequence comprises a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463. In some embodiments, the capsid protein comprises an amino acid sequence at least 95% identical to a sequence selected from SEQ ID NOs: 402-410. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 402. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 403. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 404. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 406. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 409. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to 482. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to 483. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to 484. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to 485.

[0013] In some embodiments, the capsid protein is a AAV5/AAV9 chimeric capsid protein. In some embodiments, the capsid protein comprises at least one segment from an AAV5 capsid protein. In some embodiments, the capsid protein comprises: a) a first segment comprising a sequence at least 95% identical to SEQ ID NO: 411 or SEQ ID NO: 412; b) a

second segment comprising a sequence at least 95% identical to SEQ ID NO: 413 or SEQ ID NO: 414; c) a third segment comprising a sequence at least 95% identical to SEQ ID NO: 415 or SEQ ID NO: 416; d) a fourth segment comprising a sequence at least 95% identical to SEQ ID NO: 417 or SEQ ID NO: 418; e) a fifth segment comprising a sequence at least 95% identical to SEQ ID NO: 419 or SEQ ID NO: 420; wherein at least one segment is from an AAV5 capsid protein and at least one segment is from an AAV9 capsid protein. In some embodiments, the chimeric capsid protein comprises an amino acid sequence at least 95% identical to a sequence selected from SEQ ID NOs: 445-462. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 457. In some embodiments, capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 459. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 445. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 446. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 447. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 448.

[0014] In one aspect, the present disclosure provides recombinant adeno-associated virus (rAAV) capsid proteins comprising a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463. In some embodiments, the variant polypeptide sequence is a cardiotrophic variant polypeptide sequence. In some embodiments, the capsid protein comprises at least one segment from an AAV5 capsid protein. In some embodiments, the capsid protein comprises:

a) a first segment comprising a sequence at least 95% identical to SEQ ID NO: 411 or SEQ ID NO: 412;
b) a second segment comprising a sequence at least 95% identical to SEQ ID NO: 413 or SEQ ID NO: 414;
c) a third segment comprising a sequence at least 95% identical to SEQ ID NO: 415 or SEQ ID NO: 416;
d) a fourth segment comprising a sequence at least 95% identical to SEQ ID NO: 417 or SEQ ID NO: 418;

e) a fifth segment comprising a sequence at least 95% identical to SEQ ID NO: 419 or SEQ ID NO: 420.

wherein at least one segment is from an AAV5 capsid protein and at least one segment from an AAV9 capsid protein. In some embodiments, the chimeric capsid protein comprising a sequence at least 95% identical to a sequence selected from SEQ ID NOs: 421-444. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 434. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 438. In some embodiments, the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 441.

[0015] In one aspect, the present disclosure provides recombinant adeno-associated virus (rAAV) virions comprising a capsid protein of the disclosure and a heterologous nucleic acid comprising a nucleotide sequence encoding one or more gene products. In some embodiments, the rAAV virion comprises a capsid protein comprising an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 404. In some embodiments, the rAAV virion comprises a capsid protein comprising an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 483. In some embodiments, the rAAV virion exhibits increased transduction efficiency in cardiac cells compared to an AAV virion comprising the parental sequence. In some embodiments, the cardiac cells are located in the left ventricle of the heart. In some embodiments, the rAAV virion exhibits increased transduction efficiency in induced pluripotent stem cell-derived cardiomyocyte (iPS-CM) cells compared to an AAV virion comprising the parental sequence. In some embodiments, the rAAV virion exhibits increased transduction efficiency in human cardiac fibroblast (hCF) cells compared to an AAV virion comprising the parental sequence. In some embodiments, the rAAV virion exhibits at least 2-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 100,000. In some embodiments, the rAAV virion exhibits at least 2-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 75,000. In some embodiments, the rAAV virion exhibits at least 2-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 2.5E+11 vg/mouse. In some embodiments, the rAAV virion exhibits at least 1.5-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 2E+11

vg/mouse. In some embodiments, the rAAV virion exhibits at least 2-fold increased cardiac transduction efficiency in a C57BL/6J mouce, wherein the mouse is injected with a virion dosage of 1E+11 vg/mouse. In some embodiments, the rAAV virion exhibits decreased transduction efficiency in liver cells compared to an AAV virion comprising the parental sequence. In some embodiments, the rAAV virion exhibits improved NAb evasion compared to an AAV virion comprising the parental sequence. In some embodiments, the rAAV virion exhibits increased selectivity of the rAAV virion for cardiac cells over liver cells. In some embodiments, the rAAV virion exhibits increased selectivity of the rAAV virion for iPS-CM cells over liver cells.

[0016] In one aspect, the present disclosure provides pharmaceutical compositions comprising an rAAV virion of the disclosure and a pharmaceutically acceptable carrier.

[0017] In one aspect, the present disclosure provides polynucleotides encoding the capsid protein of the disclosure. In some embodiments, the polynucleotide comprises a sequence encoding a capsid protein comprising an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 404. In some embodiments, the polynucleotide comprises a sequence encoding a capsid protein comprising an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 483.

[0018] In one ~~aspect~~embodiment, the present disclosure provides methods of transducing a cardiac cell, comprising contacting the cardiac cell with an rAAV virion of the disclosure, wherein the rAAV virion transduces the cardiac cell. In some embodiments, the cardiac cell is a cardiomyocyte. In some embodiments, the rAAV virion exhibits increased transduction efficiency in the cell compared to an AAV virion comprising AAV9 capsid protein sequence. In some embodiments, the rAAV virion exhibits at least 2-fold increased transduction efficiency in the cell compared to an AAV viron comprising AAV9 capsid protein sequence at a multiplicity of infection (MOI) of 75,000.

[0019] In one ~~aspect~~embodiment, the present disclosure provides methods of delivering one or more gene products to a cardiac cell, comprising contacting the cardiac cell with an rAAV virion of the disclosure. In some embodiments, the cardiac cell is a cardiomyocyte.

[0019a] In one aspect, the present disclosure provides *in vitro* or *ex vivo* methods of transducing, or delivering one or more gene products to, a cardiac cell or a cardiomyocyte, comprising contacting the cardiac cell or the cardiomyocyte with an rAAV of the disclosure.

[0020] In one ~~aspect~~embodiment, the present disclosure provides methods of treating a cardiac pathology in a subject in need thereof, comprising administering a therapeutically effective amount of a rAAV virion of the disclosure or a pharmaceutical composition of the disclosure to the subject, wherein the rAAV virion transduces cardiac tissue. In some embodiments, the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding one or more gene products. In some embodiments, the one or more gene products comprise MYBPC3, DWORF, KCNH2, TRPM4, DSG2, PKP2 and/or ATP2A2. In some embodiments, the one or more gene products comprise CACNA1C, DMD, DMPK, EPG5, EVC, EVC2, FBN1, NF1, SCN5A, SOS1, NPR1, ERBB4, VIP, MYH7, and/or Cas9. In some embodiments, the one or more gene products comrpise MYOCD, ASCL1, GATA4, MEF2C, TBX5, miR-133, and/or MESP1.

[0020a] In one aspect, the present disclosure provides an rAAV virion or the pharmaceutical composition of the disclosure for use as a medicament for treating a cardiac pathology in a subject in need thereof, wherein a therapeutically effective amount of the rAAV virion is to be administered to the subject, wherein the rAAV virion transduces cardiac tissue.

[0020b] In one aspect, the present disclosure provides the use of the rAAV virion of the disclosure in the manufacture of a medicament for transducing, or delivering one or more gene products to, a cardiac cell or a cardiomyocyte.

[0020c] In one aspect, the present disclosure provides the use of the rAAV virion of the disclosure in the manufacture of a medicament for treating a cardiac pathology in a subject in need thereof, wherein a therapeutically effective amount of the medicament is to be administered to the subject, wherein the rAAV virion transduces cardiac tissue.

[0021] In one embodiment~~aspect~~, the present disclosure provides kits comprising a pharmaceutical composition of the disclosure and instructions for use.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] **FIG. 1** depicts the AAV9 capsid highlighting amino acids in selected AAV9 variable regions (VR-IV, VR-V, VR-VII and VR-VIII site).

[0023] **FIG. 2** shows a schematic of directed evolution selection strategy and variant characterization. Following library generation, each library was subjected to one round of selection in hiPSC-CMs and two rounds of selection in a mouse model. Top variants from each

library were characterized through evaluation of *in vitro* transduction (hiPSC-CMs) and *in vivo* transduction (heart and liver) following systemic delivery of virus. Top variants were screened for the ability to escape human NAb inhibition.

[0024] **FIG. 3**. shows graphic representation of VR-modified libraries following three rounds of library screening. Graphical representation of library complexity for VR-IV (**FIG. 3A**), VR-V (**FIG. 3B**), VR-VII (**FIG. 3C**) and VR-VIII (**FIG. 3D**) in the parental viral library and after three rounds of selection are shown, where each read in the image correlates to one pixel in the image. Protein sequences are given a unique color based on the hydrophobicity of the sequence and reads of the same sequence are clustered together.

[0025] **FIG. 4** depicts protein motifs identified in VR-IV (**FIG. 4A**), VR-V (**FIG. 4B**), VR-VII (**FIG. 4C**) and VR-VIII (**FIG. 4D**) site following the directed evolution.

[0026] **FIG. 5** shows that AAV VR-IV modified variants exhibit superior hiPSC-CM transduction compared to AAV9. Human IPSC-derived cardiomyocytes were infected at a MOI of 100,000 with AAV9 or various evolved capsid variants packaging a ubiquitously expressing GFP reporter. Three days following infection, GFP expression was quantified by flow cytometry. CR9-01 (129-fold), CR9-07 (16-fold) and CR9-13 (9-fold) displayed significantly improved transduction compared to AAV9, while CR9-10, CR9-13 and CR9-14 showed a modest increase in transduction. Lower half of the figure shows representative images of AAV9 and CR9-01 showing a dramatic increase in transduction.

[0027] **FIG. 6** shows *in vivo* characterization of novel AAV variants. AAV9:CAG-GFP or CAG-GFP packaged in a novel capsid were retro-orbitally injected in C57BL/6J at 2.5x1011 vg/mouse (n=2-3 mice per group). **FIG. 6A** shows that CR9-07, CR9-10, CR9-13 and CR9-14 exhibited significantly higher heart transduction than AAV9 as determined by ELISA ($p < 0.05$, One-way ANOVA; Dunnett's multiple comparison test). **FIG. 6B** shows representative cross sections of the heart for the top transducing variants. **FIG. 6C** shows that CR9-10 and CR9-14 demonstrated significantly decreased liver tropism compared to AAV9 ($p < 0.03$, One-way ANOVA; Dunnett's multiple comparison test). **FIG. 6D** shows representative IHC images of the liver for the top variants.

[0028] **FIGS. 7A-7C** shows susceptibility of novel AAV variants to NAb inhibition against pooled human IgG. **FIG. 7A** is a graphical illustration of the experimental design to assess neutralizing antibody evasion at various doses of pooled human IgG (~2000 patients) ranging from 0-600 μg/mL. **FIG. 7B** shows a dose response curve demonstrating decreased

neutralization of CR9-07 and CR9-13 at IgG concentrations above 300 μg/mL. **FIG. 7C** shows that CR9-07 and CR9-13 have significantly decreased neutralization compared to AAV9 ($p < 0.0001$, One-way ANOVA; Dunnett's multiple comparison test)

[0029] **FIG. 8** shows a visual depiction of AAV5/9 chimeric library generation through DNA shuffling. AAV9 was codon modified to improve homology to AAV5 and both Cap genes were fragmented by DNaseI digestion and re-assembled via PCR on the basis of partial homology between the two Cap genes.

[0030] **FIG. 9** shows that library complexity of the AAV5/9 chimeric library was significantly reduced following one round of in vivo screening. The sequences of individual AAV chimeras are depicted graphically.

[0031] **FIG. 10** shows in vitro characterization of the top AAV5/9 chimeras. **FIG. 10A** shows transduction efficiency of hiPSC-CMs at a MOI of 75,000. ZC44 exhibits improved hiPSC-CM transduction compared to AAV9. **FIG. 10B** shows evaluation of NAb evasion of the top AAV5/9 chimeras at 1mg/mL of pooled human IgG. ZC44 shows enhanced NAb evasion compared to AAV9.

[0032] **FIG. 11** shows *in vivo* characterization of chimeric AAV variants. AAV9:CAG-GFP or CAG-GFP packaged in a chimeric capsid were retro-orbitally injected in C57BL/6J at 2x1011 vg/mouse (n=3 mice per group). 14 days following injection, the heart (**FIG. 11A**) and liver (**FIG. 11B**) were harvested and GFP expression was assessed to evaluate the transfection efficiency and specificity. ZC40 and ZC47 demonstrated improved cardiac specificity compared to wildtype AAV9.

[0033] **FIG. 12** depicts the generation of combinatory AAV variants by combining the top AAV5/9 chimeras and AAV9 VR-modified variants.

[0034] **FIG. 13** shows the assessment of manufacturability of VR-modified and combinatory AAV capsid variants. Midi-scale vector production was conducted to assess the manufacturability of AAV capsid variants. CR9-07, CR9-10 and TN40-14 had improved manufacturability when compared to AAV9.

[0035] **FIG. 14** demonstrates that combinatory AAV variants have significantly improved hiPSC-CM transduction. Human IPSC-derived cardiomyocytes were infected at a MOI of 100,000 with AAV9 or various combinatory capsid variants packaging a ubiquitously expressing GFP reporter. Five days following infection, GFP expression was quantified using

the Cytation 5 cell imaging reader. TN44-07 and TN47-07 had significantly improved transduction (>15-fold) of hiPSC-CMs compared to AAV9.

[0036] **FIG. 15** shows thre result of in vivo characterization of novel AAV variants. AAV9:CAG-GFP or CAG-GFP packaged in a novel capsid were retro-orbitally injected in make C57BL/6J at 1x1011 vg/mouse (n=4 mice per group). 14 days following injection the heart and liver were harvested and GFP expression was assessed. **FIG. 15A** shows the transduction efficiency of each capsid variant in heart. **FIG. 15B** shows the transduction efficiency of each capsid variant in liver. **FIG. 15C** shows the ratio of transduction efficiency in heart/transduction efficiency in liver. TN44-07 and TN47-10 displayed enhanced heart transduction compared to AAV9, while TN47-14 was detargeted from the liver and had a meaningfully higher heart to liver transduction ratio than AAV9.

[0037] **FIG. 16** shows the evaluation of human NAb evasion of the top combinatory capsid variants. All combinatory AAV capsid variants demonstrated improved evasion from neturalizing antibodies in pooled human IgG, with TN44-07 being the most stealth capsid, having a highly significant reduction of NAb neutralization ($p = 0.0002$, t-test, Welch's correction) compared to AAV9.

[0038] **FIG. 17A** shows the relative transduction of rAAV virions with different engineered capsids in the left ventricle of cynomolgus macaques benchmarked against a standard AAV9 capsid. Two capsid variants TN3 and TN6 exhibited significant improvement in left ventricle transduction. **FIG. 17B** shows the transduction profile of the capsid variants in the liver. The majority of the capsid variants demonstrate decreased liver tropism compared to AAV9. **FIG. 17C** shows the relative heart to liver transduction ratio normalized to AAV9 capsid. TN3 revealed a 5-fold increase cardiac to liver specificity, when compared to AAV9.

DETAILED DESCRIPTION

[0039] The disclosure provides recombinant adeno-associated virus (rAAV) virions. In particular, the disclosure provides engineered capsid proteins (including chimeric capsid proteins), methods of identifying them, and methods of using them. The methods of identifying new capsid proteins disclosed herein have wide applicability for any serotype of AAV, including chimeric capsid proteins. In addition, they can be applied to iteratively improve capsid proteins that have mutations from this or other methods. In general, the methods of the disclosure relate to preparation of randomized or semi-randomized libraries of AAV capsids in

the form of *cap* gene polynucleotides, preparation of AAV virions comprising such capsids (either by incorporating the *cap* gene library into an AAV genome or providing it *in trans* such as on a plasmid transfected into the packaging line), positively or negatively selecting the AAV virions, and recovering the *cap* gene for sequencing. In some embodiments, the recovery and sequencing include nanopore sequencing. Other high-throughput or next-generation-sequencing (NGS) methods can be used.

[0040] In some embodiments, the present disclosure provides recombinant adeno-associated virus (rAAV) virions comprising:

a) a capsid protein as described herein; and

b) a heterologous nucleic acid comprising a nucleotide sequence encoding one or more gene products.

[0041] In some embodiments, the rAAV virions disclosed herein comprise an AAV9 capsid protein as disclosed herein. In some embodiments, the rAAV virions disclosed herein comprise a chimeric AAV5/AAV9 capsid protein as disclosed herein. In some embodiments, the rAAV virions disclosed herein comprise a combinatory capsid protein as disclosed herein.

[0042] In some embodiments, the AAV9 capsid protein comprises a sequence that shares at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 1, as shown below. The N-terminal residue of VP1, VP2, and VP3, as well as the VR sites (VR-IV, VR-V, VR-VII and VR-VIII), are indicated in the sequence of full-length VP1 (SEQ ID NO: 1) below.

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VP1-->
MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPGYKYLGPGNGLDKGE
PVNAADAAALEHDKAYDQQLKAGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQAKKRLL
            VP2-->
EPLGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPAKKRLNFGQTGDTESVPDPQPIG
                                                  VP3-->
EPPAAPSGVGSLTMASGGGAPVADNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRTWALPTY
NNHLYKQISNSTSGGSSNDNAYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNF
KLFNIQVKEVTDNNGVKTIANNLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQYG
YLTLNDGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRLMNPLID
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VR-IV VR-V

QYLYYLSKTI**NGSGQNQQT**LKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTTVTQN**NNSEFA**WP

VR-VII

GASSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGT**GRDNV**DADKVMITNEEEIK

VR-VIII

TTNPVATESYGQVATNHQ**SAQA**QAQTGWVQNQGILPGMVWQDRDVYLQGPIWAKIPHTDGNFH

PSPLMGGFGMKHPPPQILIKNTPVPADPPTAFNKDKLNSFITQYSTGQVSVEIEWELQKENSK

RWNPEIQYTSNYYKSNNVEFAVNTEGVYSEPRPIGTRYLTRNL

(SEQ ID NO: 1)

Capsid Protein with Variant Polypeptide Sequence at VR Sites

[0043] In one aspect, the present disclosure provides AAV9 capsid proteins, wherein the capsid protein comprises variant polypeptide sequences with respect to the parental sequence at one or more sites of the parental sequence. In some embodiments, the one or more sites of the parental sequence are selected from the group consisting of VR-IV site, VR-V site, VR-VII site and VR-VIII site. As labeled in the SEQ ID NO: 1 above, the VR-IV site is between residues 452 and 460 in the parental sequence ("NGSGQNQQT", SEQ ID NO: 2); the VR-V site is between residues 497 and 502 in the parental sequence ("NNSEFA", SEQ ID NO: 3); the VR-VII site is between residues 549 and 553 in the parental sequence ("GRDNV", SEQ ID NO: 4); the VR-VIII site is between residues 586 and 589 in the parental sequence ("SAQA", SEQ ID NO: 5). In some embodiments, the AAV9 capsid protein comprises a sequence that shares at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to SEQ ID NO: 1, exluding the VR-IV site, VR-V site, VR-VII site and/or the VR-VIII site. In some embodiments, the AAV9 capsid protein comprises a variant polypeptide sequence at one or more of a VR-IV site, a VR-V site, a VR-VII site, and a VR-VIII site of a parental sequence, wherein the parental sequence comprises a sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463. (In SEQ ID NO:463, the amino acids residues labled "X" are excluded from sequence identity calculation.)

[0044] In some embodiments, the AAV9 capsid protein comprises a variant polypeptide sequence that are either rationally designed; introduced by mutagenesis; or randomized through generating a library of sequences with random codon usage at one or more sites. The capsid proteins of the disclosure include any variant polypeptide sequences identified as enriched by directed evolution followed by sequencing, as shown in, but not limited to, the Examples.

Without being limited to any particular substitution site, in some embodiments, one or more sites selected from the group consisting of the VR-IV site, the VR-V site, the VR-VII site and VR-VIII site have the amino acid substitutions as described herein.

[0045] In some embodiments, the capsid protein of the present disclosure comprises a variant polypeptide sequence at the VR-IV site. In some embodiments, the entire VR-IV site ("NGSGQNQQT", SEQ ID NO: 2) is substituted by a peptide of formula:

$$-(X)_n-$$

wherein *n* is 7-11, and X represents any of the 20 standard amino acids (SEQ ID NO: 478).

[0046] In some embodiments, the variant polypeptide seqeuence at the VR-IV site is:

$$-X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9- .$$

[0047] In some embodiments, the variant polypeptide seqeuence at the VR-IV site is:

$$-X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-$$

Wherein X_1 is G, S or V; X_2 is Y, Q or I; X_3 is H, W, V, or I; X_4 is K or N; X_5 is S, G or I; X_6 is G or R; X_7 is A, P or V; X_8 is A or R; and/or X_9 is Q or D (SEQ ID NO: 477).

[0048] In some embodiments, the variant popypeptide sequence at the VR-IV site comprises or consists of a sequence selected from GYHKSGAAQ (SEQ ID NO: 6), VIIKSGAAQ (SEQ ID NO: 7), GYHKIGAAQ (SEQ ID NO: 8), GYHKSGVAQ (SEQ ID NO: 9), VYHKSGAAQ (SEQ ID NO: 10), GYHKISAAQ (SEQ ID NO: 11), TTVPSSSRY (SEQ ID NO: 12), VIIRVVRLS (SEQ ID NO: 13), TVLGQNQQT (SEQ ID NO: 14), IYHKSGAAQ (SEQ ID NO: 15), TVLDKNQQT (SEQ ID NO: 16), YSGTDVRYK (SEQ ID NO: 17), VTASGKEHR (SEQ ID NO: 18), GYRKSGAAQ (SEQ ID NO: 19), NRTVSNGSE (SEQ ID NO: 20), TVLDRINKT (SEQ ID NO: 21), TGVGHLTSA (SEQ ID NO: 22), GYHKGGAAQ (SEQ ID NO: 23), VIAKSGAAQ (SEQ ID NO: 24), GYHKSGAAH (SEQ ID NO: 25), FIIKSGAAQ (SEQ ID NO: 26), GYHKVVRLS (SEQ ID NO: 27), GATRSAVES (SEQ ID NO: 28), TVSGQNQQT (SEQ ID NO: 29), LSHKSGAAQ (SEQ ID NO: 30), SSSGQNQQT (SEQ ID NO: 31), SGSGQNQQT (SEQ ID NO: 32), SQVNGRPRD (SEQ ID NO: 33), GYHKEWCGS (SEQ ID NO: 34), VVSSKSLNS (SEQ ID NO: 35), GYHKSGAAP (SEQ ID

NO: 36), DASSREKVR (SEQ ID NO: 37), SYHKSGAAQ (SEQ ID NO: 38), TANGSQKYL (SEQ ID NO: 39), VIIRVGAAQ (SEQ ID NO: 40), SSTNKISTA (SEQ ID NO: 41), TVLDRIQQT (SEQ ID NO: 42), GYHKSGAVQ (SEQ ID NO: 43), TVLDQNQQT (SEQ ID NO: 44), VNMSSPIKT (SEQ ID NO: 45), AAYNSNSAF (SEQ ID NO: 46), GYHKSGAAR (SEQ ID NO: 47), VIIRVVRLQ (SEQ ID NO: 48), RFWTQNQQT (SEQ ID NO: 49), SSPRASSAL (SEQ ID NO: 50), IIIRVVRLS (SEQ ID NO: 51), KSSNLTAMP (SEQ ID NO: 52), NLNSDRHSA (SEQ ID NO: 53), LSLKSGAAQ (SEQ ID NO: 54), TVLDRNQQT (SEQ ID NO: 55), GSERVSNSG (SEQ ID NO: 56), VIAKIGAAQ (SEQ ID NO: 57), VYHKIGAAQ (SEQ ID NO: 58), LSYKSGAAQ (SEQ ID NO: 59), STVSQPVRT (SEQ ID NO: 60), GHHKSGAAQ (SEQ ID NO: 61), YAGIDPRYH (SEQ ID NO: 62), DRSRKSMCD (SEQ ID NO: 63), VIIRSGAAQ (SEQ ID NO: 64), GYHKSGGSA (SEQ ID NO: 65), VIIKIGAAQ (SEQ ID NO: 66), GYHKVVQLS (SEQ ID NO: 67), VIIKLVAAQ (SEQ ID NO: 68), KVSSHSVCD (SEQ ID NO: 69), GYHKRVRLS (SEQ ID NO: 70), GYHKSSAAQ (SEQ ID NO: 71), GYRKIGAAQ (SEQ ID NO: 72), GYHKSGAAC (SEQ ID NO: 73), GYRQSGAAQ (SEQ ID NO: 74), VIIKLIAAQ (SEQ ID NO: 75), VIIRVVRAQ (SEQ ID NO: 76), GYHKSGAAW (SEQ ID NO: 77), GYHKSGAVS (SEQ ID NO: 78), GYHKEWCSS (SEQ ID NO: 79), SSSSNRLAD (SEQ ID NO: 80), SNNSSSAKF (SEQ ID NO: 81), VKLSSTSSS (SEQ ID NO: 82), GYHKEWCAQ (SEQ ID NO: 83), AGSGQNQQT (SEQ ID NO: 84), NPHGTATYL (SEQ ID NO: 85), NGSGQNQHT (SEQ ID NO: 86), GYHKVGAAQ (SEQ ID NO: 87), VIIRVVRLK (SEQ ID NO: 88), NSIPSTSKW (SEQ ID NO: 89), VIIRVVQLQ (SEQ ID NO: 90), SQVNGRPQD (SEQ ID NO: 91), NGSGQDQQT (SEQ ID NO: 92), GLNSSDRRL (SEQ ID NO: 93), IYHKIGAAQ (SEQ ID NO: 94), YHKSGAAQL (SEQ ID NO: 95), YSGTDVQYK (SEQ ID NO: 96), LGSGQNQQT (SEQ ID NO: 97), PVSSGADRR (SEQ ID NO: 98), EHSTKLNAC (SEQ ID NO: 99), NGSDRINKR (SEQ ID NO: 100), VIIKGGAAQ (SEQ ID NO: 101), GYHRVVRLS (SEQ ID NO: 102), VIIRVVRLL (SEQ ID NO: 103), and VILKSGAAQ (SEQ ID NO: 104).

[0049] In some embodiments, the variant polypeptide seqeuence at the VR-IV site comprises, consists essentially of, or consists of a polypeptide sequence at least about 60%, 70%, 80%, 90%, or 100% identical to one of SEQ ID NOs: 6-104.

[0050] In some embodiments, the variant polypeptide seqeuence at the VR-IV site comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to GYHKSGAAQ (SEQ ID NO: 6). In some embodiments, the variant polypeptide seqeuence at the VR-IV site comprises, consists essentially of, or consists of a

sequence consisting of at most 1, 2, 3, or 4 amino-acid substitutions relative to GYHKSGAAQ (SEQ ID NO: 6). In some embodiments, the variant polypeptide seqeuence at the VR-IV site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 conservative amino-acid substitutions relative GYHKSGAAQ (SEQ ID NO: 6). In some embodiments, the variant polypeptide seqeuence at the VR-IV site is GYHKSGAAQ (SEQ ID NO: 6).

[0051] In some embodiments, the variant polypeptide seqeuence at the VR-IV site comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to SQVNGRPRD (SEQ ID NO: 33). In some embodiments, the variant polypeptide seqeuence at the VR-IV site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 amino-acid substitutions relative to SQVNGRPRD (SEQ ID NO: 33). In some embodiments, the variant polypeptide seqeuence at the VR-IV site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 conservative amino-acid substitutions relative SQVNGRPRD (SEQ ID NO: 33). In some embodiments, the variant polypeptide seqeuence at the VR-IV site is SQVNGRPRD (SEQ ID NO: 33).

[0052] In some embodiments, the capsid protein of the present disclosure comprises a variant polypeptide sequence at the VR-V site. In some embodiments, the entire VR-V site ("NNSEFA", SEQ ID NO: 3) is substituted by a peptide of formula:

$$-(X)_n-$$

wherein *n* is 4-8, and X represents any of the 20 standard amino acids (SEQ ID NO: 479).

[0053] In some embodiments, the variant polypeptide seqeuence at the VR-V site is:

$$-X_1-X_2-X_3-X_4-X_5-X_6-$$

[0054] In some embodiments, the variant polypeptide seqeuence at the VR-V site is:

$$-X_1-X_2-X_3-X_4-X_5-X_6-$$

Wherein X_1 is S, L, H, N, or A; X_2 is T, M, K, G, or N; X_3 is S, T, M or I; X_4 is S, P, F, M, or N; X_5 is F, S, P or L; and X_6 is I, V, or T (SEQ ID NO: 474).

[0055] In some embodiments, the variant popypeptide sequence at the VR-V site comprises or consists of a sequence selected from LNSMLI (SEQ ID NO: 105), NGMSFT

(SEQ ID NO: 106), HKTFSI (SEQ ID NO: 107), SMSNFV (SEQ ID NO: 108), ATIPPI (SEQ ID NO: 109), SSTHFD (SEQ ID NO: 110), NNQFSY (SEQ ID NO: 111), NMGHYS (SEQ ID NO: 112), SKQMFQ (SEQ ID NO: 113), WPSAGV (SEQ ID NO: 114), NGGYQC (SEQ ID NO: 115), STSPIV (SEQ ID NO: 116), SQSGLW (SEQ ID NO: 117), VNSQFS (SEQ ID NO: 118), SGIEFR (SEQ ID NO: 119), SASKFT (SEQ ID NO: 120), QLNWTS (SEQ ID NO: 121), SMGFPV (SEQ ID NO: 122), SSFMGL (SEQ ID NO: 123), GSNFHV (SEQ ID NO: 124), DMTLYA (SEQ ID NO: 125), MGCLFT (SEQ ID NO: 126), ALAFNS (SEQ ID NO: 127), SKFLFA (SEQ ID NO: 128), QDAGLL (SEQ ID NO: 129), QDASLL (SEQ ID NO: 130), RDDMFS (SEQ ID NO: 131), LSRCFQ (SEQ ID NO: 132), LSRDFQ (SEQ ID NO: 133), QGLTPV (SEQ ID NO: 134), QWDVFT (SEQ ID NO: 135), PRVSFA (SEQ ID NO: 136), QSYYNP (SEQ ID NO: 137), RASHLG (SEQ ID NO: 138), IILFVP (SEQ ID NO: 139), IISFSY (SEQ ID NO: 140), LDSMLI (SEQ ID NO: 141), NIGHYS (SEQ ID NO: 142), NRMSFT (SEQ ID NO: 143), NGMSFA (SEQ ID NO: 144), IILLLP (SEQ ID NO: 145), RMRSLL (SEQ ID NO: 146), RRRCRF (SEQ ID NO: 147), PKQMFQ (SEQ ID NO: 148), LMSNFV (SEQ ID NO: 149), GASHLG (SEQ ID NO: 150), CASISW (SEQ ID NO: 151), SMTTFR (SEQ ID NO: 152), AAIPPI (SEQ ID NO: 153), PGCESL (SEQ ID NO: 154), SMGFAC (SEQ ID NO: 155), FLPSLM (SEQ ID NO: 156), NGISFT (SEQ ID NO: 157), ESSRWA (SEQ ID NO: 158), QLYFVP (SEQ ID NO: 159), SSNFHV (SEQ ID NO: 160), LEFMLI (SEQ ID NO: 161), QFDSFD (SEQ ID NO: 162), SPVFAC (SEQ ID NO: 163), VRLIFD (SEQ ID NO: 164), NGMSFI (SEQ ID NO: 165), LLFPPI (SEQ ID NO: 166), GAGVTG (SEQ ID NO: 167), QWMSFT (SEQ ID NO: 168), SIGFPV (SEQ ID NO: 169), RMQSLL (SEQ ID NO: 170), TSALQV (SEQ ID NO: 171), SLTHFD (SEQ ID NO: 172), QELPFL (SEQ ID NO: 173), LYFLLP (SEQ ID NO: 174), LSFFFA (SEQ ID NO: 175), LSRIFQ (SEQ ID NO: 176), DEVILF (SEQ ID NO: 177), RAGVAG (SEQ ID NO: 178), NGMSLP (SEQ ID NO: 179), PFEDFQ (SEQ ID NO: 180), QYGSLF (SEQ ID NO: 181), NYTFVL (SEQ ID NO: 182), MSGYQC (SEQ ID NO: 183), NYAFVP (SEQ ID NO: 184), RAGVTG (SEQ ID NO: 185), WNSMLI (SEQ ID NO: 186), IRRFSI (SEQ ID NO: 187), NGMSFY (SEQ ID NO: 188), IIQFSY (SEQ ID NO: 189), NGCLFT (SEQ ID NO: 190), RDASLL (SEQ ID NO: 191), ADSMLI (SEQ ID NO: 192), VDSQFS (SEQ ID NO: 193), SIGNFV (SEQ ID NO: 194), NGMSLL (SEQ ID NO: 195), NYTFVP (SEQ ID NO: 196), IRRLVF (SEQ ID NO: 197), PMSNFV (SEQ ID NO: 198), LWVFPV (SEQ ID NO: 199), VRLHFD (SEQ ID NO: 200), SMSNLF (SEQ ID NO: 201), STSLIV (SEQ ID NO: 202), and HKTFGI (SEQ ID NO: 203).

[0056] In some embodiments, the variant polypeptide seqeuence at the VR-V site comprises, consists essentially of, or consists of a polypeptide sequence at least about 60%, 70%, 80%, 90%, or 100% identical to one of SEQ ID NOs: 105-203.

[0057] In some embodiments, the variant polypeptide seqeuence at the VR-V site comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to LNSMLI (SEQ ID NO: 105). In some embodiments, the variant polypeptide seqeuence at the VR-V site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 amino-acid substitutions relative to LNSMLI (SEQ ID NO: 105). In some embodiments, the variant polypeptide seqeuence at the VR-V site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 conservative amino-acid substitutions relative LNSMLI (SEQ ID NO: 105). In some embodiments, the variant polypeptide seqeuence at the VR-V site is LNSMLI (SEQ ID NO: 105).

[0058] In some embodiments, the capsid protein of the present disclosure comprises a variant polypeptide sequence at the VR-VII site. In some embodiments, the entire VR-VII site ("GRDNV", SEQ ID NO: 4) is substituted by a peptide of formula:

$$-(X)_n-$$

wherein n is 3-7, and X represents any of the 20 standard amino acids (SEQ ID NO: 480).

[0059] In some embodiments, the variant polypeptide seqeuence at the VR-VII site is:

$$-X_1-X_2-X_3-X_4-X_5-$$

[0060] In some embodiments, the variant polypeptide seqeuence at the VR-VII site is:

$$-X_1-X_2-X_3-X_4-X_5-$$

Wherein X_1 is V, L, Q, C, or R; X_2 is S, H, G, C, or D; X_3 is Y, S, L, G, or N; X_4 is S, L, H, Q, or N; and X_5 is V, I, or R (SEQ ID NO: 475).

[0061] In some embodiments, the variant popypeptide sequence at the VR-VII site comprises or consists of a sequence selected from RGNQV (SEQ ID NO: 204), VSLNR (SEQ ID NO: 205), CDYSV (SEQ ID NO: 206), QHGHI (SEQ ID NO: 207), LCSLV (SEQ ID NO: 208), PTIYV (SEQ ID NO: 209), DVIHI (SEQ ID NO: 210), AEFYA (SEQ ID NO: 211),

NSVVC (SEQ ID NO: 212), VRSNC (SEQ ID NO: 213), LANNI (SEQ ID NO: 214), NLQFM (SEQ ID NO: 215), EFRDL (SEQ ID NO: 216), DFGSL (SEQ ID NO: 217), VTNYC (SEQ ID NO: 218), WNTNA (SEQ ID NO: 219), TESTC (SEQ ID NO: 220), SGAAV (SEQ ID NO: 221), GGCDI (SEQ ID NO: 222), SGSVV (SEQ ID NO: 223), SSNAC (SEQ ID NO: 224), YNTTV (SEQ ID NO: 225), SKCLA (SEQ ID NO: 226), SAYTV (SEQ ID NO: 227), VRDTV (SEQ ID NO: 228), WRSMV (SEQ ID NO: 229), AYHGV (SEQ ID NO: 230), GMNTI (SEQ ID NO: 231), AETSL (SEQ ID NO: 232), TLVYV (SEQ ID NO: 233), NHDWI (SEQ ID NO: 234), TVGIV (SEQ ID NO: 235), SLPTV (SEQ ID NO: 236), TGILC (SEQ ID NO: 237), TDTYI (SEQ ID NO: 238), LPVTY (SEQ ID NO: 239), GDVYI (SEQ ID NO: 240), LYGTV (SEQ ID NO: 241), GCEFI (SEQ ID NO: 242), SAGLL (SEQ ID NO: 243), IKSNI (SEQ ID NO: 244), VTTSL (SEQ ID NO: 245), AVTSV (SEQ ID NO: 246), RDIHI (SEQ ID NO: 247), SAISL (SEQ ID NO: 248), VASTC (SEQ ID NO: 249), IKGLL (SEQ ID NO: 250), GSYHT (SEQ ID NO: 251), RIGFV (SEQ ID NO: 252), NDIYI (SEQ ID NO: 253), AVSCV (SEQ ID NO: 254), QHNLL (SEQ ID NO: 255), VSSCV (SEQ ID NO: 256), LNLDV (SEQ ID NO: 257), LGATI (SEQ ID NO: 258), PVLCV (SEQ ID NO: 259), SARHI (SEQ ID NO: 260), RATLI (SEQ ID NO: 261), PYNHA (SEQ ID NO: 262), IGDSI (SEQ ID NO: 263), SPMLC (SEQ ID NO: 264), YDSTL (SEQ ID NO: 265), ALKHV (SEQ ID NO: 266), ADLLT (SEQ ID NO: 267), NNGHL (SEQ ID NO: 268), INSEV (SEQ ID NO: 269), SNKTT (SEQ ID NO: 270), GSTGL (SEQ ID NO: 271), DSDMI (SEQ ID NO: 272), TSNFI (SEQ ID NO: 273), RNFTT (SEQ ID NO: 274), SHKYS (SEQ ID NO: 275), VSDIV (SEQ ID NO: 276), RVVQA (SEQ ID NO: 277), AACAV (SEQ ID NO: 278), RGRQI (SEQ ID NO: 279), AVANI (SEQ ID NO: 280), AGYDL (SEQ ID NO: 281), LSEAA (SEQ ID NO: 282), MSNYL (SEQ ID NO: 283), NFSDN (SEQ ID NO: 284), SCCDV (SEQ ID NO: 285), LASSV (SEQ ID NO: 286), PDHAV (SEQ ID NO: 287), KFDII (SEQ ID NO: 288), NSSSA (SEQ ID NO: 289), HTMHV (SEQ ID NO: 290), TLSYC (SEQ ID NO: 291), ADTHR (SEQ ID NO: 292), SMYSV (SEQ ID NO: 293), SVNLV (SEQ ID NO: 294), MSGHL (SEQ ID NO: 295), KISDT (SEQ ID NO: 296), TGLLA (SEQ ID NO: 297), AWTTS (SEQ ID NO: 298), GGALI (SEQ ID NO: 299), SCIEV (SEQ ID NO: 300), PPVIC (SEQ ID NO: 301), and GTYNL (SEQ ID NO: 302).

[0062] In some embodiments, the variant polypeptide seqeuence at the VR-VII site comprises, consists essentially of, or consists of a polypeptide sequence at least about 60%, 70%, 80%, 90%, or 100% identical to one of SEQ ID NOs: 204-302.

[0063] In some embodiments, the variant polypeptide seqeuence at the VR-VII site comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%,

or 100% identical to RGNQV (SEQ ID NO: 204). In some embodiments, the variant polypeptide seqeuence at the VR-VII site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 amino-acid substitutions relative to RGNQV (SEQ ID NO: 204). In some embodiments, the variant polypeptide seqeuence at the VR-VII site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 conservative amino-acid substitutions relative RGNQV (SEQ ID NO: 204). In some embodiments, the variant polypeptide seqeuence at the VR-VII site is RGNQV (SEQ ID NO: 204).

[0064] In some embodiments, the capsid protein of the present disclosure comprises a variant polypeptide sequence at the VR-VII site. In some embodiments, the entire VR-VIII site ("SAQA", SEQ ID NO: 5) is substituted by a peptide of formula:

$$-(X)_n-$$

wherein *n* is 2-6, and X represents any of the 20 standard amino acids (SEQ ID NO: 481).

[0065] In some embodiments, the variant polypeptide seqeuence at the VR-VIII site is:

$$-X_1-X_2-X_3-X_4-$$

[0066] In some embodiments, the variant polypeptide seqeuence at the VR-VIII site is:

$$-X_1-X_2-X_3-X_4-$$

Wherein X_1 is S, N, or A; X_2 is V, M, N, or A; X_3 is Y, V, S, or G; and X_4 is Y, T, M, G, or N (SEQ ID NO: 476).

[0067] In some embodiments, the variant popypeptide sequence at the VR-VIII site comprises or consists of a sequence selected from NVSY (SEQ ID NO: 303), SMVN (SEQ ID NO: 304), ANYG (SEQ ID NO: 305), NVGT (SEQ ID NO: 306), SAYM (SEQ ID NO: 307), EKVT (SEQ ID NO: 308), TTPG (SEQ ID NO: 309), GVYS (SEQ ID NO: 310), SYVG (SEQ ID NO: 311), LQYN (SEQ ID NO: 312), DPAK (SEQ ID NO: 313), THFS (SEQ ID NO: 314), IGGV (SEQ ID NO: 315), SSWN (SEQ ID NO: 316), SVYV (SEQ ID NO: 317), TLNG (SEQ ID NO: 318), NTSN (SEQ ID NO: 319), VQYA (SEQ ID NO: 320), DQYR (SEQ ID NO: 321), MPVS (SEQ ID NO: 322), SAQA (SEQ ID NO: 323), MTVA (SEQ ID NO: 324), TVMG (SEQ ID NO: 325), FSSI (SEQ ID NO: 326), SLRL (SEQ ID NO: 327), SAMG (SEQ ID NO: 328), YIKL (SEQ ID NO: 329), LMTM (SEQ ID NO: 330), QVHL (SEQ ID NO: 331), YNSV

(SEQ ID NO: 332), CVIS (SEQ ID NO: 333), RLDG (SEQ ID NO: 334), AIMV (SEQ ID NO: 335), GTTG (SEQ ID NO: 336), ASYT (SEQ ID NO: 337), LHVG (SEQ ID NO: 338), LQFA (SEQ ID NO: 339), VRGD (SEQ ID NO: 340), NVMI (SEQ ID NO: 341), SLYG (SEQ ID NO: 342), GTVG (SEQ ID NO: 343), FNSV (SEQ ID NO: 344), TRLG (SEQ ID NO: 345), LKVL (SEQ ID NO: 346), SIRV (SEQ ID NO: 347), KIQG (SEQ ID NO: 348), QILG (SEQ ID NO: 349), QRDA (SEQ ID NO: 350), EAVR (SEQ ID NO: 351), AITV (SEQ ID NO: 352), KESI (SEQ ID NO: 353), LMVN (SEQ ID NO: 354), INLS (SEQ ID NO: 355), GQVS (SEQ ID NO: 356), TSLL (SEQ ID NO: 357), SSTL (SEQ ID NO: 358), YEKF (SEQ ID NO: 359), DGKL (SEQ ID NO: 360), QVYS (SEQ ID NO: 361), QKEG (SEQ ID NO: 362), ARDM (SEQ ID NO: 363), DNFR (SEQ ID NO: 364), SHGL (SEQ ID NO: 365), VSVN (SEQ ID NO: 366), GLKD (SEQ ID NO: 367), QPVF (SEQ ID NO: 368), VYSM (SEQ ID NO: 369), VMAQ (SEQ ID NO: 370), FVGM (SEQ ID NO: 371), WSTP (SEQ ID NO: 372), SYPV (SEQ ID NO: 373), TTYS (SEQ ID NO: 374), TVTT (SEQ ID NO: 375), KDKT (SEQ ID NO: 376), YREL (SEQ ID NO: 377), LSHF (SEQ ID NO: 378), SPGT (SEQ ID NO: 379), LMGT (SEQ ID NO: 380), AASL (SEQ ID NO: 381), FSNN (SEQ ID NO: 382), QARL (SEQ ID NO: 383), YHIA (SEQ ID NO: 384), ARQD (SEQ ID NO: 385), VAYT (SEQ ID NO: 386), TPSY (SEQ ID NO: 387), MILH (SEQ ID NO: 388), LGNV (SEQ ID NO: 389), TSIS (SEQ ID NO: 390), TMVY (SEQ ID NO: 391), LVVG (SEQ ID NO: 392), SPLY (SEQ ID NO: 393), YKSE (SEQ ID NO: 394), FTRL (SEQ ID NO: 395), VSYN (SEQ ID NO: 396), ERTP (SEQ ID NO: 397), FRSE (SEQ ID NO: 398), NYTE (SEQ ID NO: 399), QTIN (SEQ ID NO: 400), and DVHR (SEQ ID NO: 401).

[0068] In some embodiments, the variant polypeptide seqeuence at the VR-VIII site comprises, consists essentially of, or consists of a polypeptide sequence at least about 60%, 70%, 80%, 90%, or 100% identical to one of SEQ ID NOs: 303-401.

[0069] In some embodiments, the variant polypeptide seqeuence at the VR-VIII site comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to ANYG (SEQ ID NO: 305) . In some embodiments, the variant polypeptide seqeuence at the VR-VIII site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, or 3 amino-acid substitutions relative to ANYG (SEQ ID NO: 305) . In some embodiments, the variant polypeptide seqeuence at the VR-VIII site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, or 3 conservative amino-acid substitutions relative ANYG (SEQ ID NO: 305) . In some embodiments, the variant polypeptide seqeuence at the VR-VIII site is ANYG (SEQ ID NO: 305) .

[0070] In some embodiments, the variant polypeptide seqeuence at the VR-VIII site comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to NVSY (SEQ ID NO: 303). In some embodiments, the variant polypeptide seqeuence at the VR-VIII site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, or 3 amino-acid substitutions relative to NVSY (SEQ ID NO: 303). In some embodiments, the variant polypeptide seqeuence at the VR-VIII site comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, or 3 conservative amino-acid substitutions relative NVSY (SEQ ID NO: 303). In some embodiments, the variant polypeptide seqeuence at the VR-VIII site is NVSY (SEQ ID NO: 303).

[0071] In some embodiments, the capsid protein comprises, consists essentially of, or consists of a polypeptide sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to one of SEQ ID NOs: 402-410 and 464-468, or a functional fragment thereof.

Table 1. Capsid Protein Sequences

Name / Alternate Name	SEQ ID NO:
CR9-01 / TN1	402
CR9-07	403
CR9-07-A / TN5	482
CR9-07-E / TN6	483
CR9-08	464
CR9-09	465
CR9-10 / TN3	404
CR9-11	466
CR9-13	405
CR9-14 / TN4	406
CR9-15	467
CR9-16	468
CR9-17	407
CR9-20	408
CR9-21	409
CR9-22	410
HV1 / TN7	484
HV2 / TN11	485

Chimeric AAV5/AAV9 Capsid

[0072] The present disclosure also provides recombinant adeno-associated virus (rAAV) capsid proteins comprising a sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463. (In SEQ ID NO:463, the amino acids residues labled "X" are excluded from sequence identity calculation.) In some embodiments, the capsid protein is an AAV5/AAV9 chimeric capsid protein. In some embodiments, the AAV5/AAV9 chimeric capsid protein sequence is more than about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 99.5% identical to the AAV9 capsid protein sequence (SEQ ID NO: 1). In some embodiments, the C-terminal 500 residues of the AAV5/AAV9 chimeric capsid protein sequence is at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to the C-terminal 500 residues of the AAV9 capsid protein sequence (SEQ ID NO: 1). In some embodiments, the residue at the position equivalent to Q688 of the AAV9 capsid protein sequence (SEQ ID NO: 1) is a lysine (K) in the chimeric capsid protein.

[0073] In some embodiments, the chimeric capsid protein comprises at least 1, 2, 3, 4, 5 or more polypeptide segments that are derived from AAV5 capsid protein. In some embodiments, the chimeric capsid protein comprises at least 1, 2, 3, 4, 5 or more polypeptide segments that are derived from AAV9 capsid protein. In some embodiments, at least one polypeptide segment is derived from the AAV5 capsid protein and at least one polypeptide segment is derived from the AAV9 capsid protein.

[0074] In some embodiments, the first 250 residues at the N-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, the first 225 residues at the N-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, the first 200 residues at the N-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, the first 150 residues at the N-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, the first 100 residues at the N-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, the first 50 residues at the N-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, each of the one or more AAV5 capsid derived polypeptide segments has at least about 80%, 85%, 90%,

95%, 96%, 97%, 98%, 99%, 99.5%, or 100% sequence identity to the corresponding AAV5 capsid sequence.

[0075] In some embodiments, residues 50-250 of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, residues 50-200 of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, residues 50-150 of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, residues 100-250 of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, residues 100-200 of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, residues 150-250 of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, each of the one or more AAV5 capsid derived polypeptide segments has at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% sequence identity to the corresponding AAV5 capsid sequence.

[0076] In some embodiments, the last 100 residues at the C-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, the last 50 residues at the C-terminus of the chimeric capsid protein comprise one or more AAV5 capsid derived polypeptide segments. In some embodiments, each of the one or more AAV5 capsid derived polypeptide segments has at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% sequence identity to the corresponding AAV5 capsid sequence. In some embodiments, the chimeric capsid protein comprises one or more AAV5 capsid derived polypeptide segments at or near the N-terminus of the chimeric capsid protein, as described above, and one or more AAV5 capsid derived polypeptide segments at or near the C-terminus of the chimeric capsid protein, as described in this paragraph.

[0077] In some embodiments, the chimeric capsid protein comprises, in N-terminal to C-terminal order, a first polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 411 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 412; a second polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 413 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 414; a third polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%,

or 100% identical to SEQ ID NO: 415 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 416; a fourth polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 417 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 418; and a fifth polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 419 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 420. In some embodiments, at least one polypeptide segment is derived from the AAV5 capsid protein and at least one polypeptide segment is derived from the AAV9 capsid protein.

[0001] AAV9 derived polypeptide segment 1:

[0002] MAADGYLPDWLEDNLSEGIREWWALKPGAPQPKANQQHQDNARGLVLPG Y (SEQ ID NO: 411)

[0003] Sequence of AAV5 derived polypeptide segment 1:

[0004] MSFVDHPPDWLEEVGEGLREFLGLEAGPPKPKPNQQHQDQARGLVLPGY (SEQ ID NO: 412)

[0005] Sequence of AAV9 derived polypeptide segment 2:

[0006] KYLGPGNGLDKGEPVNAADAAALEHDKAYDQQLK (SEQ ID NO: 413)

[0007] Sequence of AAV5 derived polypeptide segment 2:

[0008] NYLGPGNGLDRGEPVNRADEVAREHDISYNEQLE (SEQ ID NO: 414)

[0009] Sequence of AAV9 derived polypeptide segment 3:

[0010] AGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQAKKRLLEP (SEQ ID NO: 415)

[0011] Sequence of AAV5 derived polypeptide segment 3:

[0012] AGDNPYLKYNHADAEFQEKLADDTSFGGNLGKAVFQAKKRVLEP (SEQ ID NO: 416)

[0013] Sequence of AAV9 derived polypeptide segment 4:

[0014] LGLVEEAAKTAPGKKRPVEQSPQEPDSSAGIGKSGAQPAKKRLNFGQTGDT ESVPDPQPIGEPPAAPSGVGSLTMASGGGAPVA (SEQ ID NO: 417)

[0015] Sequence of AAV5 derived polypeptide segment 4:

[0016] FGLVEEGAKTAPTGKRIDDHFPKRKKARTEEDSKPSTSSDAEAGPSGSQQLQ IPAQPASSLGADTMSAGGGGPLG (SEQ ID NO: 418)

[0017] Sequence of AAV9 derived polypeptide segment 5:

[0018] DNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISN STSGGSSNDNAYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFN IQVKEVTDNNGVKTIANNLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQY GYLTLNDGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRL MNPLIDQYLYYLSKTINGSGQNQQTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTT VTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTg rdnvDADKVMITNEEEIKTTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMV WQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTPVPADPPTAFN KDKLNSFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGV YSEPRPIGTRYLTRNL (SEQ ID NO: 419)

[0019] Sequence of AAV9 derived polypeptide segment 5 with Q688K mutation:

[0020] DNNEGADGVGSSSGNWHCDSQWLGDRVITTSTRTWALPTYNNHLYKQISN STSGGSSNDNAYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFN IQVKEVTDNNGVKTIANNLTSTVQVFTDSDYQLPYVLGSAHEGCLPPFPADVFMIPQY GYLTLNDGSQAVGRSSFYCLEYFPSQMLRTGNNFQFSYEFENVPFHSSYAHSQSLDRL MNPLIDQYLYYLSKTINGSGQNQQTLKFSVAGPSNMAVQGRNYIPGPSYRQQRVSTT VTQNNNSEFAWPGASSWALNGRNSLMNPGPAMASHKEGEDRFFPLSGSLIFGKQGTg rdnvDADKVMITNEEEIKTTNPVATESYGQVATNHQSAQAQAQTGWVQNQGILPGMV WQDRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGMKHPPPQILIKNTPVPADPPTAFN KDKLNSFITQYSTGQVSVEIEWELKKENSKRWNPEIQYTSNYYKSNNVEFAVNTEGV YSEPRPIGTRYLTRNL (SEQ ID NO: 420)

[0078] In some embodiments, the chimeric capsid protein comprises, consists essentially of, or consists of a polypeptide sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to one of SEQ ID NOs: 421-444, or a functional fragment thereof.

Table 2. Capsid Protein Sequences

Name / Alternate Name	SEQ ID NO:
ZC23	421
ZC24	422
ZC25	423
ZC26	424
ZC27	425
ZC28	426
ZC29	427

ZC30	428
ZC31	429
ZC32	430
ZC33	431
ZC34	432
ZC35	433
ZC40 / TN8	434
ZC41	435
ZC42	436
ZC43	437
ZC44 / TN10	438
ZC45	439
ZC46	440
ZC47 / TN14	441
ZC48	442
ZC49	443
ZC50	444

Combinatory Capsid Protein

[0079] In one aspect, the present disclosure provides combinatory capsid proteins. As used herein, "combinatory capsid protein" refers to a AAV5/AAV9 chimeric capsid protein as described in the present disclosre, which further comprises amino acid variations with respect to the chimeric parental sequence at one or more sites. In some embodiments, the one or more sites of the chimeric parental sequence are selected from those equivalent to the VR-IV site, the VR-V site, the VR-VII site and the VR-VIII site of the AAV9 capsid protein.

[0080] The combinatory capsid proteins of the present disclosure include any variant polypeptide sequences identified as shown in, but not limited to, the Examples. Without being limited to any particular example, in some embodiments, the combinatory capsid protein comprises a chimeric AAV5/AAV9 capsid protein backbone, and further comprises the variant polypeptide sequence at one or more sites selected from the group consisting of those equivalent to the VR-IV site, the VR-V site, the VR-VII site and the VR-VIII site of the AAV9 capsid protein as described herein.

[0081] In some embodiments, the combinatory capsid protein comprises, in N-terminal to C-terminal order, a first polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 411 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 412; a

second polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 413 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 414; a third polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 415 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 416; a fourth polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 417 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 418; and a fifth polypeptide segment having sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 419 or at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to SEQ ID NO: 420 (here, regions equivalent to the VR-IV site, the VR-V site, the VR-VII site and the VR-VIII site of the AAV9 capsid protein are excluded in the sequence identity calculation of the fifth polypeptide segment). In some embodiments, the combinatory capsid protein comprises a variant polypeptide sequence at one or more of a VR-IV site, a VR-V site, a VR-VII site, and a VR-VIII site of a parental sequence, wherein the parental sequence comprises a sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463. (In SEQ ID NO:463, the amino acids residues labled “X” are excluded from sequence identity calculation.)

[0082] In some embodiments, at least one polypeptide segment is derived from the AAV5 capsid protein and at least one polypeptide segment is derived from the AAV9 capsid protein.

[0083] In some embodiments, the combinatory capsid protein further comprises variant polypeptide sequence at one or more sites selected from those equivalent to the VR-IV site, the VR-V site, the VR-VII site and the VR-VIII site of the AAV9 capsid protein.

[0084] In some embodiments, the combinatory capsid protein has a variant polypeptide seqeuence at the site equivalent to the VR-IV site of the AAV9 capsid protein, which comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to GYHKSGAAQ (SEQ ID NO: 6). In some embodiments, the variant polypeptide seqeuence at the site equivalent to the VR-IV site of the AAV9 capsid protein comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 conservative amino-acid substitutions relative GYHKSGAAQ (SEQ ID NO: 6).

[0085] In some embodiments, the combinatory capsid protein has a variant polypeptide seqeuence at the site equivalent to the VR-V site of the AAV9 capsid protein, which comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to LNSMLI (SEQ ID NO: 105). In some embodiments, the variant polypeptide seqeuence at the site equivalent to the VR-V site of the AAV9 capsid protein comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 conservative amino-acid substitutions relative LNSMLI (SEQ ID NO: 105).

[0086] In some embodiments, the combinatory capsid protein has a variant polypeptide seqeuence at the site equivalent to the VR-VIII site of the AAV9 capsid protein, which comprises, consists essentially of, or consists of a sequence at least about 60%, 70%, 80%, 90%, or 100% identical to ANYG (SEQ ID NO: 305) or NVSY (SEQ ID NO: 303). In some embodiments, the variant polypeptide seqeuence at the site equivalent to the VR-VIII site of the AAV9 capsid protein comprises, consists essentially of, or consists of a sequence consisting of at most 1, 2, 3, or 4 conservative amino-acid substitutions relative ANYG (SEQ ID NO: 305) or NVSY (SEQ ID NO: 303).

[0087] In some embodiments, the residue at the position equivalent to Q688 of the AAV9 capsid protein sequence (SEQ ID NO: 1) is a lysine (K) in the combinatory capsid protein.

[0088] In some embodiments, the combinatory capsid protein comprises, consists essentially of, or consists of a polypeptide sequence at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identical to one of SEQ ID NOs: 445-462, or a functional fragment thereof.

Table 3. Capsid Protein Sequences

Name / Alternate Name	SEQ ID NO:
TN47-07	445
TN47-10 / TN12	446
TN47-13	447
TN47-14	448
TN47-17	449
TN47-22	450
TN40-07	451
TN40-10	452
TN40-13	453
TN40-14	454

TN40-17	455
TN40-22	456
TN44-07 / TN13	457
TN44-10	458
TN44-13	459
TN44-14	460
TN44-17	461
TN44-22	462

Additional Mutations

[0089] Additional amino acid substitutions may be incorporated, for example, to further improve transduction efficiency or tissue selectivity. Exemplary non-limiting substitutions include, but are not limited to, S651A, T578A or T582A relative to the sequence of AAV5, in either an AAV5 or AAV9-based capsid.

[0090] In some embodiments, the capsid protein comprises a mutation selected from S651A, T578A, T582A, K251R, Y709F, Y693F, or S485A relative to the sequence of AAV5, in either an AAV5 or AAV9-based capsid. In some embodiments, the capsid protein comprises a mutation selected from K251R, Y709F, Y693F, or S485A relative to the sequence of AAV5, in either an AAV5 or AAV9-based capsid.

Transduction Efficiency, Tropism, and NAb Evasion

[0091] Transduction efficiency can be determined using methods known in the art or those described in the Examples. In some embodiments, the rAAV virion with engineered capsid protein exhibits increased transduction efficiency in cardiac cells compared to an AAV virion comprising the parental sequence.

[0092] In some embodiments, the rAAV virion exhibits increased transduction efficiency in human cardiac fibroblast (hCF) cells compared to an AAV virion comprising the parental sequence. In some embodiments, the human cardiac fibroblasts are located in the left ventricle of the heart.

[0093] In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold increased transduction efficiency in hCF cells at a multiplicity of infection (MOI) of 100,000. In some embodiments, the rAAV virion exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about

10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold increased transduction efficiency in hCF cells at a multiplicity of infection (MOI) of 100,000. In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10, 11-, 12-, 13-, 14, or 15-fold increased transduction efficiency in hCF cells at a multiplicity of infection (MOI) of 100,000. In some embodiments, the rAAV virion exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% increased transduction efficiency in hCF cells at a multiplicity of infection (MOI) of 100,000.

[0094] In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold increased transduction efficiency in hCF cells at a multiplicity of infection (MOI) of 1,000. In some embodiments, the rAAV virion exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold increased transduction efficiency in hCF cells at a multiplicity of infection (MOI) of 1,000. In some embodiments, the rAAV virion exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% increased transduction efficiency in hCF cells at a multiplicity of infection (MOI) of 1,000.

[0095] In some embodiments, the rAAV virion exhibits increased transduction efficiency in induced pluripotent stem cell-derived cardiomyocyte (iPS-CM) cells compared to an AAV virion comprising the parental sequence.

[0096] In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 100,000. In some embodiments, the rAAV virion exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 100,000. In some embodiments, the rAAV virion exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 100,000.

[0097] In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 75,000. In some embodiments, the rAAV virion exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 75,000. In some embodiments, the rAAV virion exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 75,000.

[0098] In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 1,000. In some embodiments, the rAAV virion exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 1,000. In some embodiments, the rAAV virion exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 1,000.

[0099] In some embodiments, the rAAV virion comprising the engineered capsid protein of the present disclosure exhibits increased transduction efficiency in heart compared to an AAV virion comprising the parental sequence. In some embodiments, transduction efficiency in heart is monitored by injecting C57BL/6J mice with either AAV9:CAG-GFP or CAG-GFP encapsulated by the engineered capsid protein of the present disclosure. In some embodiments, the injection dosage is 2.5E+11 vg/mouse. In some embodiments, the injection dosage is 2E+11 vg/mouse. In some embodiments, the injection dosage is 1E+11 vg/mouse. In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold increased transduction efficiency in heart. In some embodiments, the rAAV virion exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold increased transduction efficiency in heart. In some embodiments, the rAAV virion exhibits about 20% to 30%, about 30% to 40%, about 40% to

50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% increased transduction efficiency in heart.

[0100] In some embodiments, the rAAV virion comprising the engineered capsid protein of the present disclosure exhibits decreased transduction efficiency in liver cells compared to an AAV virion comprising the parental sequence. In some embodiments, liver transduction efficiency is monitored by injecting C57BL/6J mice with either AAV9:CAG-GFP or CAG-GFP encapsulated by the engineered capsid protein of the present disclosure. In some embodiments, the injection dosage is 2.5E+11 vg/mouse. In some embodiments, the injection dosage is 2E+11 vg/mouse. In some embodiments, the injection dosage is 1E+11 vg/mouse. In some embodiments, the rAAV virion exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold decreased transduction efficiency in liver. In some embodiments, the rAAV virion exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold decreased transduction efficiency in liver. In some embodiments, the rAAV virion exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, or about 80% to 100 decreased transduction efficiency in liver.

[0101] Selectivity for a cell type and/or a tissue/organ type is increased when the ratio of the transduction efficiencies for one cell/tissue/organ type over another is increased for rAAV virions comprising the engineered capsid protein of the present disclosure compared to an AAV virion comprising the parental sequence. In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits increased selectivity for iPS-CM cells over liver cells. In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits increased selectivity for heart over liver when injected *in vivo*. In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits increased selectivity for the left ventricle of the heart over liver when injected *in vivo*.

[0102] In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14, or 15-fold increased selectivity of iPS-CM cells over liver cells and/or heart over liver. In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold increased selectivity of iPS-CM cells over liver cells and/or heart over liver. In some embodiments, the

rAAV virion comprising the engineered capsid protein exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% increased selectivity of iPS-CM cells over liver cells and/or heart over liver.

[0103] In some embodiments, the rAAV virion comprising the engineered capsid protein of the present disclosure exhibits improved ability to evade human NAb (neturalizing antibodies) compared to an AAV virion comprising the parental sequence. In some embodiments, the ability to evade hman NAb is measured via an NAb inhibition assay. Non-limiting examples of NAb inhibition assays are described in the Example section of the present disclosure. In some embodiments, NAb inhibition assays are performed by incubating AAV virions with pooled human NAb (*e.g.*, IgG) before treating a target cell at a pre-determined MOI and measure the decrease of transduction efficiency compared to AAV virions not incubated with pooled human NAb. Less NAb inhibition indicates improved ability of the AAV virion to evade human NAb. In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits at least 2-, 3-, 4-, 5-, 6, 7-, 8-, 9-, 10, 11-, 12-, 13-, 14, or 15-fold improved ability to evade human NAb. In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits about 2- to about 16-fold, about 2- to about 14-fold, about 2- to about 12-fold, about 2- to about 10-fold, about 2- to about 8-fold, about 2- to about 6-fold, about 2- to about 4-fold, or about 2- to about 3-fold improved ability to evade human NAb. In some embodiments, the rAAV virion comprising the engineered capsid protein exhibits about 20% to 30%, about 30% to 40%, about 40% to 50%, about 50% to 80%, about 80% to 100%, about 100% to 125%, about 125% to 150%, about 150% to 175%, or about 175% to 200% improved ability to evade human NAb.

[0104] The polynucleotide encoding the capsid protein can comprise a sequence comprising either the native codons of the wild-type *cap* gene, or alternative codons selected to encode the same protein. The codon usage of the insertion can be varied. It is within the skill of those in the art to select appropriate nucleotide sequences and to derive alternative nucleotide sequences to encode any capsid protein of the disclosure. Reverse translation of the protein sequence can be performed using the codon usage table of the host organism, *i.e.* Eukaryotic codon usage for humans.

[0105] In some embodiments, the disclosure provides a polynucleotide encoding an AAV9 derived capsid protein comprising a sequence at least 80%, 85%, 90%, 95%, 99%, or 100% identical to any one of SEQ ID NOs: 402-410 and 464-468.

[0106] In some embodiments, the disclosure provides a polynucleotide encoding an AAV5/AAV9 chimeric capsid protein comprising a sequence at least 80%, 85%, 90%, 95%, 99%, or 100% identical to any one of SEQ ID NOs: 421-444.

[0107] In some embodiments, the disclosure provides a polynucleotide encoding an combinatory capsid protein comprising a sequence at least 80%, 85%, 90%, 95%, 99%, or 100% identical to any one of SEQ ID NO: 445-462.

[0108] In some embodiments, the disclosure provides an AAV9, AAV5/AAV9 chimeric, or combinatory capsid protein comprising a sequence at least 80%, 85%, 90%, 95%, 99%, or 100% identical to a modified capsid selected from SEQ ID NOs: 402-410, 421-462, 464-468, wherein the amino acid substitutions, optionally conservative substitutions, with the specified percent identity level are tolerated.

Gene Product

[0109] In some embodiments, the rAAV virion of the present disclosure comprises a heterologous nucleic acid comprising a nucleotide sequence that encodes one or more gene products selected from MYBPC3, KCNH2, TRPM4, DSG2, ATP2A2, CACNA1C, DMD, DMPK, EPG5, EVC, EVC2, FBN1, NF1, SCN5A, SOS1, NPR1, ERBB4, VIP, MYH7, or a mutant, variant, or fragment thereof.

[0110] In some embodiments, the rAAV virion of the present disclosure comprises a heterologous nucleic acid comprising a nucleotide sequence that encodes one or more gene products selected from ASCL1, MYOCD, MEF2C, and TBX5. In some embodiments, the rAAV virion of the present disclosure comprises a heterologous nucleic acid comprising a nucleotide sequence that encodes one or more gene products selected from ASCL1, MYOCD, MEF2C, AND TBX5, CCNB1, CCND1, CDK1, CDK4, AURKB, OCT4, BAF60C, ESRRG, GATA4, GATA6, HAND2, IRX4, ISLL, MESP1, MESP2, NKX2.5, SRF, TBX20, ZFPM2, and MIR-133.

[0111] In some embodiments, the rAAV virion of the present disclosure comprises a heterologous nucleic acid comprising a nucleotide sequence that encodes one or more gene products selected from MYBPC3, DWORF, KCNH2, TRPM4, DSG2, PKP2 and ATP2A2.

[0112] In some embodiments, the rAAV virion of the present disclosure comprises a heterologous nucleic acid comprising a nucleotide sequence that encodes one or more gene products selected from CACNA1C, DMD, DMPK, EPG5, EVC, EVC2, FBN1, NF1, SCN5A, SOS1, NPR1, ERBB4, VIP, MYH7, and Cas9.

[0113] In some embodiments, the rAAV virion of the present disclosure comprises a heterologous nucleic acid comprising a nucleotide sequence that encodes one or more gene products selected from MYOCD, ASCL1, GATA4, MEF2C, TBX5, miR-133, and MESP1.

Definitions

[0114] Unless the context indicates otherwise, the features of the invention can be used in any combination. Any feature or combination of features set forth can be excluded or omitted. Certain features of the invention, which are described in separate embodiments may also be provided in combination in a single embodiment. Features of the invention, which are described in a single embodiment may also be provided separately or in any suitable sub-combination. All combinations of the embodiments are disclosed herein as if each and every combination were individually disclosed. All sub-combinations of the embodiments and elements are disclosed herein as if every such sub-combination were individually disclosed.

[0115] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The detailed description is divided into sections only for the reader's convenience and disclosure found in any section may be combined with that in another section. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the exemplary methods and materials are now described. All publications mentioned herein are incorporated by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. Reference to a publication is not an admission that the publication is prior art.

[0116] The singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. For example, reference to "a recombinant AAV virion" includes a plurality of such virions and reference to "the cardiac cell" includes one or more cardiac cells.

[0117] The conjunction "and/or" means both "and" and "or," and lists joined by "and/or" encompasses all possible combinations of one or more of the listed items.

[0118] The term "vector" refers to a macromolecule or complex of molecules comprising a polynucleotide or protein to be delivered to a cell.

[0119] "AAV" is an abbreviation for adeno-associated virus. The term covers all subtypes of AAV, except where a subtype is indicated, and to both naturally occurring and recombinant forms. The abbreviation "rAAV" refers to recombinant adeno-associated virus. "AAV" includes AAV or any subtype. "AAV5" refers to AAV subtype 5. "AAV9" refers to AAV subtype 9. The genomic sequences of various serotypes of AAV, as well as the sequences of the native inverted terminal repeats (ITRs), Rep proteins, and capsid subunits may be found in the literature or in public databases such as GenBank. *See, e.g.*, GenBank Accession Numbers NC_002077 (AAV1), AF063497 (AAV1), NC_001401 (AAV2), AF043303 (AAV2), NC_001729 (AAV3), NC_001829 (AAV4), U89790 (AAV4), NC_006152 (AAV5), AF513851 (AAV7), AF513852 (AAV8), NC_006261 (AAV8), and AY530579 (AAV9). Publications describing AAV include Srivistava et al. (1983) *J. Virol.* 45:555; Chiorini et al. (1998) *J. Virol.* 71:6823; Chiorini et al. (1999) *J. Virol.* 73:1309; Bantel-Schaal et al. (1999) *J. Virol.* 73:939; Xiao et al. (1999) *J. Virol.* 73:3994; Muramatsu et al. (1996) *Virol.* 221:208; Shade et al. (1986) *J. Virol.* 58:921; Gao et al. (2002) *Proc. Nat. Acad. Sci. USA* 99: 11854; Moris et al. (2004) *Virology* 33:375-383; Int'l Pat. Publ Nos. WO2018/222503A1, WO2012/145601A2, WO2000/028061A2, WO1999/61601A2, and WO1998/11244A2; U.S. Pat. Appl. Nos. 15/782,980 and 15/433,322; and U.S. Pat. Nos. 10,036,016, 9,790,472, 9,737,618, 9,434,928, 9,233,131, 8,906,675, 7,790,449, 7,906,111, 7,718,424, 7,259,151, 7,198,951, 7,105,345, 6,962,815, 6,984,517, and 6,156,303.

[0120] An "AAV vector" or "rAAV vector" as used in the art to refer either to the DNA packaged into in the rAAV virion or to the rAAV virion itself, depending on context. As used herein, unless otherwise apparent from context, rAAV vector refers to a nucleic acid (typically a plasmid) comprising a polynucleotide sequence capable of being packaged into an rAAV virion, but with the capsid or other proteins of the rAAV virion. Generally an rAAV vector comprises a heterologous polynucleotide sequence (*i.e.*, a polynucleotide not of AAV origin) and one or two AAV inverted terminal repeat sequences (ITRs) flanking the heterologous polynucleotide sequence. Only one of the two ITRs may be packaged into the rAAV and yet infectivity of the resulting rAAV virion may be maintained. *See* Wu et al. (2010) *Mol Ther.* 18:80. An rAAV vector may be designed to generate either single-stranded (ssAAV) or self-complementary (scAAV). *See* McCarty D. (2008) *Mo. Ther.* 16:1648-1656; WO2001/11034; WO2001/92551; WO2010/129021.

[0121] An "rAAV virion" refers to an extracellular viral particle including at least one viral capsid protein (*e.g.* VP1) and an encapsidated rAAV vector (or fragment thereof), including the capsid proteins.

[0122] For brevity and clarity, the disclosure refers to "capsid protein" or "capsid proteins." Those skilled in the art understand that such references refer to VP1, VP2, or VP3, or combinations of VP1, VP2, and VP3. As in wild-type AAV and most recombinant expression systems VP1, VP2, and VP3 are expressed from the same open reading frame, engineering of the sequence that encodes VP3 inevitably alters the sequences of the C-terminal domain of VP1 and VP2. One may also express the capsid proteins from different open reading frames, in which case the capsid of the resulting rAAV virion could contain a mixture of wild-type and engineered capsid proteins, and mixtures of different engineered capsid proteins.

[0123] The term "inverted terminal repeats" or "ITRs" as used herein refers to AAV viral cis-elements named so because of their symmetry. These elements are essential for efficient multiplication of an AAV genome. Without being bound by theory, it is believed that the minimal elements indispensable for ITR function are a Rep-binding site and a terminal resolution site plus a variable palindromic sequence allowing for hairpin formation. The disclosure contemplates that alternative means of generating an AAV genome may exist or may be prospectively developed to be compatible with the capsid proteins of the disclosure.

[0124] "Helper virus functions" refers to functions encoded in a helper virus genome which allow AAV replication and packaging.

[0125] "Packaging" refers to a series of intracellular events that result in the assembly of an rAAV virion including encapsidation of the rAAV vector. AAV "*rep*" and "*cap*" genes refer to polynucleotide sequences encoding replication and encapsidation proteins of adeno-associated virus. AAV *rep* and *cap* are referred to herein as AAV "packaging genes." Packaging requires either a helper virus itself or, more commonly in recombinant systems, helper virus function supplied by a helper-free system (*i.e.* one or more helper plasmids).

[0126] A "helper virus" for AAV refers to a virus that allows AAV (*e.g.* wild-type AAV) to be replicated and packaged by a mammalian cell. The helper viruses may be an adenovirus, herpesvirus or poxvirus, such as vaccinia.

[0127] An "infectious" virion or viral particle is one that comprises a competently assembled viral capsid and is capable of delivering a polynucleotide component into a cell for

which the virion is tropic. The term does not necessarily imply any replication capacity of the virus.

[0128] "Infectivity" refers to a measurement of the ability of a virion to inflect a cell. Infectivity can be expressed as the ratio of infectious viral particles to total viral particles. Infectivity is general determined with respect to a particular cell type. It can be measured both *in vivo* or *in vitro*. Methods of determining the ratio of infectious viral particle to total viral particle are known in the art. *See, e.g.*, Grainger et al. (2005) *Mol. Ther.* 11:S337 (describing a $TCID_{50}$ infectious titer assay); and Zolotukhin et al. (1999) *Gene Ther.* 6:973.

[0129] The terms "parental capsid" or "parental sequence" refer to a reference sequence from which a particle capsid or sequence is derived. Unless otherwise specified, parental sequence refers to the sequence of the wild-type capsid protein of the same serotype as the engineered capsid protein.

[0130] A "replication-competent" virus (*e.g.* a replication-competent AAV) refers to a virus that is infectious, and is also capable of being replicated in an infected cell (*i.e.* in the presence of a helper virus or helper virus functions). In some embodiments, the rAAV virion of the disclosure comprises a genome that lacks the *rep* gene, or both the *rep* and *cap* genes, and therefore is replication incompetent.

[0131] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of tissue culture, immunology, molecular biology, cell biology and recombinant DNA, which are within the skill of the art. *See*, *e.g.*, Sambrook and Russell eds. (2001) Molecular Cloning: A Laboratory Manual, 3rd edition; Ausubel et al. eds. (2007) Current Protocols in Molecular Biology; Methods in Enzymology (Academic Press, Inc., N.Y.); MacPherson et al. (1991) PCR 1: A Practical Approach (IRL Press at Oxford University Press); MacPherson et al. (1995) PCR 2: A Practical Approach; Harlow and Lane eds. (1999) Antibodies, A Laboratory Manual; Freshney (2005) Culture of Animal Cells: A Manual of Basic Technique, 5thedition; Gait ed. (1984) Oligonucleotide Synthesis; U.S. Pat. No. 4,683,195; Hames and Higgins eds. (1984) Nucleic Acid Hybridization; Anderson (1999) Nucleic Acid Hybridization; Hames and Higgins eds. (1984) Transcription and Translation; IRL Press (1986) Immobilized Cells and Enzymes; Perbal (1984) A Practical Guide to Molecular Cloning; Miller and Calos eds. (1987) Gene Transfer Vectors for Mammalian Cells (Cold Spring Harbor Laboratory); Makrides ed. (2003) Gene Transfer and Expression in Mammalian Cells; Mayer and Walker eds. (1987) Immunochemical Methods in Cell and

Molecular Biology (Academic Press, London); Herzenberg et al. eds (1996) Weir's Handbook of Experimental Immunology; Manipulating the Mouse Embryo: A Laboratory Manual, 3rd edition (2002) Cold Spring Harbor Laboratory Press; Sohail (2004) Gene Silencing by RNA Interference: Technology and Application (CRC Press); and Sell (2013) Stem Cells Handbook.

[0132] The terms "nucleic acid" and "polynucleotide" are used interchangeably and refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Non-limiting examples of polynucleotides include linear and circular nucleic acids, messenger RNA (mRNA), cDNA, recombinant polynucleotides, vectors, probes, and primers. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[0133] The terms "polypeptide" and "protein," are used interchangeably herein and refer to a polymeric form of amino acids of any length, which can include genetically coded and non-genetically coded amino acids, chemically or biochemically modified or derivatized amino acids, and polypeptides having modified peptide backbones. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, phosphorylation, or conjugation with a labeling component.

[0134] The term "peptide" refers to a short polypeptide, *e.g.* a peptide having between about 4 and 30 amino acid residues.

[0135] The term "isolated" means separated from constituents, cellular and otherwise, in which the virion, cell, tissue, polynucleotide, peptide, polypeptide, or protein is normally associated in nature. For example, an isolated cell is a cell that is separated form tissue or cells of dissimilar phenotype or genotype.

[0136] As used herein, "sequence identity" or "identity" refers to the percentage of number of amino acids that are identical between a sequence of interest and a reference sequence. Generally identity is determined by aligning the the sequence of interest to the reference sequence, determining the number of amino acids that are identical between the aligned sequences, dividing that number by the total number of amino acids in the reference sequence, and multiplying the result by 100 to yield a percentage. Sequences can be aligned using various computer programs, such BLAST, available at ncbi.nlm.nih.gov. Other techniques for alignment are described in Methods in Enzymology, vol. 266: Computer

Methods for Macromolecular Sequence Analysis (1996); and *Meth. Mol. Biol.* 70: 173-187 (1997); *J. Mol. Biol.* 48: 44. Skill artisans are capable of choosing an appropriate alignment method depending on various factors including sequence length, divergence, and the presence of absence of insertions or deletions with respect to the reference sequence.

[0137] "Recombinant," as applied to a polynucleotide means that the polynucleotide is the product of various combinations of cloning, restriction or ligation steps, and other procedures that result in a construct that is distinct from a polynucleotide found in nature, or that the polynucleotide is assembled from synthetic oligonucleotides. A "recombinant" protein is a protein produced from a recombinant polypeptide. A recombinant virion is a virion that comprises a recombinant polynucleotide and/or a recombinant protein, *e.g.* a recombinant capsid protein.

[0138] A "gene" refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated. A "gene product" is a molecule resulting from expression of a particular gene. Gene products may include, without limitation, a polypeptide, a protein, an aptamer, an interfering RNA, or an mRNA. Gene-editing systems (*e.g.* a CRISPR/Cas system) may be described as one gene product or as the several gene products required to make the system (*e.g.* a Cas protein and a guide RNA).

[0139] A "short hairpin RNA," or shRNA, is a polynucleotide construct used to express an siRNA.

[0140] A "control element" or "control sequence" is a nucleotide sequence involved in an interaction of molecules that contributes to the functional regulation of a polynucleotide, including replication, duplication, transcription, splicing, translation, or degradation of the polynucleotide. The regulation may affect the frequency, speed, or specificity of the process, and may be enhancing or inhibitory in nature. Control elements include transcriptional regulatory sequences such as promoters and/or enhancers.

[0141] A "promoter" is a DNA sequence capable under certain conditions of binding RNA polymerase and initiating transcription of a coding region usually located downstream (in the 3' direction) from the promoter. The term "tissue-specific promoter" as used herein refers to a promoter that is operable in cells of a particular organ or tissue, such as the cardiac tissue.

[0142] "Operatively linked" or "operably linked" refers to a juxtaposition of genetic elements, wherein the elements are in a relationship permitting them to operate in the expected manner. For instance, a promoter is operatively linked to a coding region if the promoter helps initiate transcription of the coding sequence. There may be intervening residues between the promoter and coding region so long as this functional relationship is maintained.

[0143] An "expression vector" is a vector comprising a coding sequence which encodes a gene product of interest used to effect the expression of the gene product in target cells. An expression vector comprises control elements operatively linked to the coding sequence to facilitate expression of the gene product.

[0144] The term "expression cassette" refers to a heterologous polynucleotide comprising a coding sequence which encodes a gene product of interest used to effect the expression of the gene product in target cells. Unless otherwise specified, the expression cassette of an AAV vector include the polynucleotides between (and not including) the ITRs.

[0145] The term "gene delivery" or "gene transfer" as used herein refers to methods or systems for reliably inserting foreign nucleic acid sequences, *e.g.*, DNA, into host cells. Such methods can result in transient expression of non-integrated transferred DNA, extra-chromosomal replication and expression of transferred replicons (*e.g.*, episomes), or integration of transferred genetic material into the genomic DNA of host cells.

[0146] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared. For example, a polynucleotide introduced by genetic engineering techniques into a plasmid or vector derived from a different species is a heterologous polynucleotide. A promoter removed from its native coding sequence and operatively linked to a coding sequence with which it is not naturally found linked is a heterologous promoter. Thus, for example, an rAAV that includes a heterologous nucleic acid is an rAAV that includes a nucleic acid not normally included in a naturally-occurring AAV.

[0147] The terms "genetic alteration" and "genetic modification" (and grammatical variants thereof), are used interchangeably herein to refer to a process wherein a genetic element (*e.g.*, a polynucleotide) is introduced into a cell other than by mitosis or meiosis. The element may be heterologous to the cell, or it may be an additional copy or improved version of an element already present in the cell. Genetic alteration may be effected, for example, by transfecting a cell with a recombinant plasmid or other polynucleotide through any process known in the art, such as electroporation, calcium phosphate precipitation, or contacting with a

polynucleotide-liposome complex. Genetic alteration may also be effected, for example, by transduction or infection with a vector.

[0148] A cell is said to be "stably" altered, transduced, genetically modified, or transformed with a polynucleotide sequence if the sequence is available to perform its function during extended culture of the cell *in vitro*. Generally, such a cell is "heritably" altered (genetically modified) in that a genetic alteration is introduced which is also inheritable by progeny of the altered cell.

[0149] The term "transfection" is as used herein refers to the uptake of an exogenous nucleic acid molecule by a cell. A cell has been "transfected" when exogenous nucleic acid has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. *See, e.g.*, Graham et al. (1973) *Virology*, 52:456, Sambrook et al. (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York, Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier, and Chu et al. (1981) *Gene* 13:197. Such techniques can be used to introduce one or more exogenous nucleic acid molecules into suitable host cells.

[0150] The term "transduction" is as used herein refers to the transfer of an exogenous nucleic acid into a cell by a recombinant virion, in contrast to "infection" by a wild-type virion. When infection is used with respect to a recombinant virion, the terms "transduction" and "infectious" are synomymous, and therefore "infectivity" and "transduction efficiency" are equivalent and can be determined using similar methods.

[0151] Unless otherwise specified, all medical terminology is given the ordinary meaning of the term used by medical professional as, for example, in *Harrison's Principles of Internal Medicine*, 15ed., which is incorporated by reference in its entirety for all purposes, in particular the chapters on cardiac or cardiovascular diseases, disorders, conditions, and dysfunctions.

[0152] "Treatment," "treating," and "treat" are defined as acting upon a disease, disorder, or condition with an agent to reduce or ameliorate harmful or any other undesired effects of the disease, disorder, or condition and/or its symptoms.

[0153] "Administration," "administering" and the like, when used in connection with a composition of the invention refer both to direct administration (administration to a subject by a medical professional or by self-administration by the subject) and/or to indirect administration

(prescribing a composition to a patient). Typically, an effective amount is administered, which amount can be determined by one of skill in the art. Any method of administration may be used. Administration to a subject can be achieved by, for example, intravenous, intrarterial, intramuscular, intravascular, or intramyocardial delivery.

[0154] As used herein the term "effective amount" and the like in reference to an amount of a composition refers to an amount that is sufficient to induce a desired physiologic outcome (*e.g.*, reprogramming of a cell or treatment of a disease). An effective amount can be administered in one or more administrations, applications or dosages. Such delivery is dependent on a number of variables including the time period which the individual dosage unit is to be used, the bioavailability of the composition, the route of administration, etc. It is understood, however, that specific amounts of the compositions (*e.g.*, rAAV virions) for any particular subject depends upon a variety of factors including the activity of the specific agent employed, the age, body weight, general health, sex, and diet of the subject, the time of administration, the rate of excretion, the composition combination, severity of the particular disease being treated and form of administration.

[0155] The terms "individual," "subject," and "patient" are used interchangeably herein, and refer to a mammal, including, but not limited to, human and non-human primates (*e.g.*, simians); mammalian sport animals (*e.g.*, horses); mammalian farm animals (*e.g.*, sheep, goats, etc.); mammalian pets (*e.g.*, dogs, cats, etc.); and rodents (*e.g.*, mice, rats, etc.).

[0156] The terms "cardiac pathology" or "cardiac dysfunction" are used interchangeably and refer to any impairment in the heart's pumping function. This includes, for example, impairments in contractility, impairments in ability to relax (sometimes referred to as diastolic dysfunction), abnormal or improper functioning of the heart's valves, diseases of the heart muscle (sometimes referred to as cardiomyopathies), diseases such as angina pectoris, myocardial ischemia and/or infarction characterized by inadequate blood supply to the heart muscle, infiltrative diseases such as amyloidosis and hemochromatosis, global or regional hypertrophy (such as may occur in some kinds of cardiomyopathy or systemic hypertension), and abnormal communications between chambers of the heart.

[0157] As used herein, the term "cardiomyopathy" refers to any disease or dysfunction that affects myocardium directly. The etiology of the disease or disorder may be, for example, inflammatory, metabolic, toxic, infiltrative, fibroplastic, hematological, genetic, or unknown in origin. Two fundamental forms are recognized (1) a primary type, consisting of heart muscle

disease of unknown cause; and (2) a secondary type, consisting of myocardial disease of known cause or associated with a disease involving other organ systems. "Specific cardiomyopathy" refers to heart diseases associated with certain systemic or cardiac disorders; examples include hypertensive and metabolic cardiomyopathy. The cardiomyopathies include dilated cardiomyopathy (DCM), a disorder in which left and/or right ventricular systolic pump function is impaired, leading to progressive cardiac enlargement; hypertrophic cardiomyopathy, characterized by left ventricular hypertrophy without obivious causes such as hypertension or aortic stenosis; and restrictive cardiomyopathy, characterized by abnormal diastolic function and excessively rigid ventricular walls that impede ventricular filling. Cardiomyopathies also include left ventricular non-compaction, arrhythmogenic right ventricular cardiomyopathy, and arrhythmogenic right ventricular dysplasia.

[0158] "Heart failure" referes to the pathological state in which an abnormality of cardiac function is responsible for failure of the hear to pump blood at a rate commensurate with the requirements of the metabolizing tissues and/or allows the heart to do so only from an abnormally elevated diastolic volume. Heart failure includes systolic and diastotic failure. Patient with heart failure are classified into those with low cardiac output (typically secondary to ischemic heart disease, hypertension, dialated cardiomyopathy, and/or valvular or pericardial disease) and those with elevated cardiac output (typically due to hyperthyroidism, anemia, pregnancy, arteriovenous fistulas, beriberi, and Paget's disease). Heart failure includes heart failure with reduced ejection fraction (HFrEF) and heart failure with preserved ejection fraction (HFpEF).

[0159] The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[0160] The term "purified" as used herein refers to material that has been isolated under conditions that reduce or eliminate the presence of unrelated materials, *i.e.* impurities, including native materials from which the material is obtained. For example, purified rAAV vector DNA is preferably substantially free of cell or culture components, including tissue culture components, contaminants, and the like.

[0161] The terms "regenerate," "regeneration" and the like as used herein in the context of injured cardiac tissue shall be given their ordinary meanings and shall also refer to the process of growing and/or developing new cardiac tissue in a heart or cardiac tissue that has been injured, for example, injured due to ischemia, infarction, reperfusion, or other disease. In some embodiments, cardiac tissue regeneration comprises generation of cardiomyocytes.

[0162] The term "therapeutic gene" as used herein refers to a gene that, when expressed, confers a beneficial effect on the cell or tissue in which it is present, or on a mammal in which the gene is expressed. Examples of beneficial effects include amelioration of a sign or symptom of a condition or disease, prevention or inhibition of a condition or disease, or conferral of a desired characteristic. Therapeutic genes include genes that partially or wholly correct a genetic deficiency in a cell or mammal.

[0163] As used herein, the term "functional cardiomyocyte" refers to a differentiated cardiomyocyte that is able to send or receive electrical signals. In some embodiments, a cardiomyocyte is said to be a functional cardiomyocyte if it exhibits electrophysiological properties such as action potentials and/or Ca^{2+} transients.

[0164] As used herein, a "differentiated non-cardiac cell" can refer to a cell that is not able to differentiate into all cell types of an adult organism (*i.e.*, is not a pluripotent cell), and which is of a cellular lineage other than a cardiac lineage (*e.g.*, a neuronal lineage or a connective tissue lineage). Differentiated cells include, but are not limited to, multipotent cells, oligopotent cells, unipotent cells, progenitor cells, and terminally differentiated cells. In particular embodiments, a less potent cell is considered "differentiated" in reference to a more potent cell.

[0165] A "somatic cell" is a cell forming the body of an organism. Somatic cells include cells making up organs, skin, blood, bones and connective tissue in an organism, but not germ cells.

[0166] As used herein, the term "totipotent" means the ability of a cell to form all cell lineages of an organism. For example, in mammals, only the zygote and the first cleavage stage blastomeres are totipotent.

[0167] As used herein, the term "pluripotent" means the ability of a cell to form all lineages of the body or soma. For example, embryonic stem cells are a type of pluripotent stem cells that are able to form cells from each of the three germs layers, the ectoderm, the mesoderm,

and the endoderm. Pluripotent cells can be recognized by their expression of markers such as Nanog and Rex1.

[0168] As used herein, the term "multipotent" refers to the ability of an adult stem cell to form multiple cell types of one lineage. For example, hematopoietic stem cells are capable of forming all cells of the blood cell lineage, *e.g.*, lymphoid and myeloid cells.

[0169] As used herein, the term "oligopotent" refers to the ability of an adult stem cell to differentiate into only a few different cell types. For example, lymphoid or myeloid stem cells are capable of forming cells of either the lymphoid or myeloid lineages, respectively.

[0170] As used herein, the term "unipotent" means the ability of a cell to form a single cell type. For example, spermatogonial stem cells are only capable of forming sperm cells.

[0171] As used herein, the term "reprogramming" or "transdifferentiation" refers to the generation of a cell of a certain lineage (*e.g.*, a cardiac cell) from a different type of cell (*e.g.*, a fibroblast cell) without an intermediate process of de-differentiating the cell into a cell exhibiting pluripotent stem cell characteristics.

[0172] As used herein the term "cardiac cell" refers to any cell present in the heart that provides a cardiac function, such as heart contraction or blood supply, or otherwise serves to maintain the structure of the heart. Cardiac cells as used herein encompass cells that exist in the epicardium, myocardium or endocardium of the heart. Cardiac cells also include, for example, cardiac muscle cells or cardiomyocytes, and cells of the cardiac vasculatures, such as cells of a coronary artery or vein. Other non-limiting examples of cardiac cells include epithelial cells, endothelial cells, fibroblasts, cardiac stem or progenitor cells, cardiac conducting cells and cardiac pacemaking cells that constitute the cardiac muscle, blood vessels and cardiac cell supporting structure. Cardiac cells may be derived from stem cells, including, for example, embryonic stem cells or induced pluripotent stem cells.

[0173] The term "cardiomyocyte" or "cardiomyocytes" as used herein refers to sarcomere-containing striated muscle cells, naturally found in the mammalian heart, as opposed to skeletal muscle cells. Cardiomyocytes are characterized by the expression of specialized molecules *e.g.*, proteins like myosin heavy chain, myosin light chain, cardiac α-actinin. The term "cardiomyocyte" as used herein is an umbrella term comprising any cardiomyocyte subpopulation or cardiomyocyte subtype, *e.g.*, atrial, ventricular and pacemaker cardiomyocytes.

[0174] The term "cardiomyocyte-like cells" is intended to mean cells sharing features with cardiomyocytes, but which may not share all features. For example, a cardiomyocyte-like cell may differ from a cardiomyocyte in expression of certain cardiac genes.

[0175] The term "culture" or "cell culture" means the maintenance of cells in an artificial, in vitro environment. A "cell culture system" is used herein to refer to culture conditions in which a population of cells may be grown as monolayers or in suspension. "Culture medium" is used herein to refer to a nutrient solution for the culturing, growth, or proliferation of cells. Culture medium may be characterized by functional properties such as, but not limited to, the ability to maintain cells in a particular state (*e.g.*, a pluripotent state, a quiescent state, etc.), to mature cells - in some instances, specifically, to promote the differentiation of progenitor cells into cells of a particular lineage (*e.g.*, a cardiomyocyte).

[0176] As used herein, the term "expression" or "express" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell. The expression level of a gene may be determined by measuring the amount of mRNA or protein in a cell or tissue sample.

[0177] The term "induced cardiomyocyte" or the abbreviation "iCM" refers to a non-cardiomyocyte (and its progeny) that has been transformed into a cardiomyocyte (and/or cardiomyocyte-like cell). The methods of the present disclosure can be used in conjunction with any methods now known or later discovered for generating induced cardiomyocytes, for example, to enhance other techniques.

[0178] The term "induced pluripotent stem cell-derived cardiomyocytes" as used herein refers to human induced pluripotent stem cells that have been differentiated into cardiomyocyte-like cells. Exemplary methods for prepared iPS-CM cells are provided by Karakikes et al. *Circ Res.* 2015 Jun 19; 117(1): 80–88.

[0179] The terms "human cardiac fibroblast" and "mouse cardiac fibroblast" as used herein refer to primary cell isolated from the ventricles of the adult heart of a human or mouse, respectively, and maintain in culture *ex vivo*.

[0180] The term "non-cardiomyocyte" as used herein refers to any cell or population of cells in a cell preparation not fulfilling the criteria of a "cardiomyocyte" as defined and used

herein. Non-limiting examples of non-cardiomyocytes include somatic cells, cardiac fibroblasts, non-cardiac fibroblasts, cardiac progenitor cells, and stem cells.

[0181] As used herein "reprogramming" includes transdifferentiation, dedifferentiation and the like.

[0182] As used herein, the term "reprogramming efficiency" refers to the number of cells in a sample that are successfully reprogrammed to cardiomyocytes relative to the total number of cells in the sample.

[0183] The term "reprogramming factor" as used herein includes a factor that is introduced for expression in a cell to assist in the reprogramming of the cell from one cell type into another. For example, a reprogramming factor may include a transcription factor that, in combination with other transcription factors and/or small molecules, is capable of reprogramming a cardiac fibroblast into an induced cardiomyocyte. Unless otherwise clear from context, a repogramming factor refers to a polypeptide that can be encoded by an AAV-delivered polynucleotide. Reprogramming factors may also include small molecules.

[0184] The term "stem cells" refer to cells that have the capacity to self-renew and to generate differentiated progeny. The term "pluripotent stem cells" refers to stem cells that can give rise to cells of all three germ layers (endoderm, mesoderm and ectoderm), but do not have the capacity to give rise to a complete organism.

[0185] As used herein, the term "equivalents thereof" in reference to a polypeptide or nucleic acid sequence refers to a polypeptide or nucleic acid that differs from a reference polypeptide or nucleic acid sequence, but retains essential properties (*e.g.*, biological activity). A typical variant of a polynucleotide differs in nucleotide sequence from another, reference polynucleotide. Changes in the nucleotide sequence of the variant may or may not alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Nucleotide changes may result in amino acid substitutions, deletions, additions, fusions and truncations in the polypeptide encoded by the reference sequence. Generally, differences are limited so that the sequences of the reference polypeptide and the variant are closely similar overall and, in many regions, identical.

[0186] As used herein, the term "progenitor cell" refers to a cell that is committed to differentiate into a specific type of cell or to form a specific type of tissue. A progenitor cell,

like a stem cell, can further differentiate into one or more kinds of cells, but is more mature than a stem cell such that it has a more limited/restricted differentiation capacity.

[0187] The term "genetic modification" refers to a permanent or transient genetic change induced in a cell following introduction of new nucleic acid (*i.e.*, nucleic acid exogenous to the cell). Genetic change can be accomplished by incorporation of the new nucleic acid into the genome of the cardiac cell, or by transient or stable maintenance of the new nucleic acid as an extrachromosomal element. Where the cell is a eukaryotic cell, a permanent genetic change can be achieved by introduction of the nucleic acid into the genome of the cell. Suitable methods of genetic modification include viral infection, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate precipitation, direct microinjection, and the like.

[0188] The term "stem cells" refer to cells that have the capacity to self-renew and to generate differentiated progeny. The term "pluripotent stem cells" refers to stem cells that can give rise to cells of all three germ layers (endoderm, mesoderm and ectoderm), but do not have the capacity to give rise to a complete organism. In some embodiments, the compositions for inducing cardiomycocyte phenotype can be used on a population of cells to induce reprogramming. In other embodiments, the compositions induce a cardiomycocyte phenotype.

[0189] The term "induced pluripotent stem cells" shall be given its ordinary meaning and shall also refer to differentiated mammalian somatic cells (*e.g.*, adult somatic cells, such as skin) that have been reprogrammed to exhibit at least one characteristic of pluripotency. *See*, for example, Takahashi et al. (2007) *Cell* 131(5):861-872, Kim et al. (2011) *Proc. Natl. Acad. Sci.* 108(19): 7838-7843, Sell (2013) Stem Cells Handbook.

[0190] The term "transduction efficiency" refers to the percentage of cells transduced with at least one AAV genome. For example, if 1 x 10^6 cells are exposed to a virus and 0.5 x 10^6 cells are determined to contain at least one copy of the AAV genome, then the transduction efficiency is 50%. An illustrative method for determining transduction efficiency is flow cytometry. For example, the percentage of GFP+ cells is a measure of transduction efficieny when the AAV genome comprises a polynucleotide encoding green fluorescence protein (GFP).

[0191] The term "selectivity" refers to the ratio of transduction efficiency for one cell type over another, or over all other cells types.

[0192] The term "infectivity" refers to the ability of an AAV virion to infect a cell, in particularly an *in vivo* cell. Infectivity therefore is a function of, at least, biodistribution and neutralizing antibody escape.

[0193] Unless stated otherwise, the abbreviations used throughout the specification have the following meanings: **AAV**, adeno-associated virus, **rAAV**, recombinant adeno-associated virus; **AHCF**, adult human cardiac fibroblast; **APCF**, adult pig cardiac fibroblast, **a-MHC-GFP;** alpha-myosin heavy chain green fluorescence protein; **CF**, cardiac fibroblast; cm, centimeter; **CO**, cardiac output; **EF**, ejection fraction; **FACS**, fluorescence activated cell sorting; **GFP**, green fluorescence protein; **GMT**, Gata4, Mef2c and Tbx5; **GMTc**, Gata4, Mef2c, Tbx5, TGF-βi, WNTi; **GO**, gene ontology; **hCF**, human cardiac fibroblast; **iCM**, induced cardiomyocyte; **kg**, killigram; **µg**, microgram; **µl**, microliter; **mg**, milligram; **ml**, milliliter; **MI**, myocardial infarction; **msec**, millisecond; **min**, minute; **MyAMT**, Myocardin, Ascl1, Mef2c and Tbx5; **MyA**, Myocardin and Ascl1; **MyMT**, Myocardin, Mef2c and Tbx5; **MyMTc**, Myocardin, Mef2c, Tbx5, TGF-βi, **WNTi**; MRI, magnetic resonance imaging; **PBS**, phosphate buffered saline; **PBST**, phosphate buffered saline, triton; **PFA**, paraformaldehyde; **qPCR**, quantitative polymerase chain reaction; **qRT-PCR**, quantitative reverse transcriptase polymerase chain reaction; **RNA**, ribonucleic acid; **RNA-seq**, RNA sequencing; **RT-PCR**, reverse transcriptase polymerase chain reaction; **sec**, second; **SV**, stroke volume; **TGF-β**, transforming growth factor beta; **TGF-βi**, transforming growth factor beta inhibitor; **WNT**, wingless-Int; **WNTi**, wingless-Int inhibitor; **YFP**, yellow fluorescence protein; **4F**, Gata4, Mef2c, TBX5, and Myocardin; **4Fc**, Gata4, Mef2c, TBX5, and Myocardin + TGF-βi and WNTi; **7F**, Gata4, Mef2c, and Tbx5, Essrg, Myocardin, Zfpm2, and Mesp1; **7Fc**, Gata4, Mef2c, and Tbx5, Essrg, Myocardin, Zfpm2, and Mesp1 + TGF-β and WNTi.

[0194] The term "conservative amino-acid substitutions" refers to substutions of amino acid residues that share similar sidechain physical properties with the residues being substituted. Conservative substitutions include polar for polar residues, non-polar for non-polar residues, hydrophobic for hydrophobic residues, small for small residues, and large for large residues. Conservative substitutions further comprise substitutions within the following groups: {S, T}, {A, G}, {F, Y}, {R, H, K, N, E}, {S, T, N, Q}, {C, U, G, P, A}, and {A, V, I, L, M, F, Y, W}.

Compositions

[0195] Efforts to identify capsid variants with properties useful for gene therapy have included shuffling the DNA of AAV2 and AAV5 *cap* genes as described in U.S. Pat. No.

9,233,131; as well as directed evolution as described in Int'l Pat. Appl. Nos. WO2012/145601A2 and WO2018/222503A1. The disclosures of these documents are incorporated here for all purposes, and particularly for the methods of making and using AAV virions and for the polynucleotide sequences and gene products therein disclosed, as well as for the combinations of transcription factors useful in treating cardiac diseases or disorders.

[0196] The AAV capsid is encoded by the *cap* gene of AAV, which is also termed the right open-reading frame (ORF) (in contrast to the left ORF, *rep*). The structures of representative AAV capsids are described in various publications including Xie et al. (2002) *Proc. Natl. Acad. Sci USA* 99:10405-1040 (AAV2); Govindasamy et al. (2006) *J. Virol.* 80:11556-11570 (AAV4); Nam et a. (2007) *J. Virol*. 81:12260-12271 (AAV8) and Govindasamy et al. (2013) *J. Virol.* 87:11187-11199 (AAV5).

[0197] The AAV capsid contain 60 copies (in total) of three viral proteins (VPs), VP1, VP2, and VP3, in a predicted ratio of 1:1:10, arranged with T=1 icosahedral symmetry. The three VPs are translated from the same mRNA, with VP1 containing a unique N-terminal domain in addition to the entire VP2 sequence at its C-terminal region. VP2 contains an extra N-terminal sequence in addition to VP3 at its C terminus. In most crystal structures, only the C-terminal polypeptide sequence common to all the capsid proteins (~530 amino acids) is observed. The N-terminal unique region of VP1, the VP1-VP2 overlapping region, and the first 14 to 16 N-terminal residues of VP3 are thought to be primarily disordered. Cryo-electron microscopy and image reconstruction data suggest that in intact AAV capsids, the N-terminal regions of the VP1 and VP2 proteins are located inside the capsid and are inaccessible for receptor and antibody binding. Thus, receptor attachment and transduction phenotypes are, generally, determined by the amino acid sequences within the common C-terminal domain of VP1, VP2 and VP3

[0198] In some embodiments, the one or more amino acid insertions, substitutions, or deletions is/are in the GH loop, or loop IV, of the AAV capsid protein, *e.g.*, in a solvent-accessible portion of the GH loop, or loop IV, of the AAV capsid protein. For the GH loop/loop IV of AAV capsid, *see, e.g.*, van Vliet et al. (2006) *Mol. Ther.* 14:809; Padron et al. (2005) *Virol.* 79:5047; and Shen et al. (2007) *Mol. Ther.* 15: 1955. In some embodiments, a "parental" AAV capsid protein is a wild-type AAV5 capsid protein. In some embodiments, a "parental" AAV capsid protein is a chimeric AAV capsid protein. Amino acid sequences of various AAV capsid proteins are known in the art. *See, e.g.*, GenBank Accession No. NP_049542 for AAV1;

GenBank Accession No. NP_044927 for AAV4; GenBank Accession No. AAD13756 for AAV5; GenBank Accession No. AAB95450 for AAV6; GenBank Accession No. YP_077178 for AAV7; GenBank Accession No. YP_077180 for AAV 8; GenBank Accession No. AAS99264 for AAV9 and GenBank Accession No. AAT46337 for AAV10. *See, e.g.*, Santiago-Ortiz et al. (2015) *Gene Ther.* 22:934 for a predicted ancestral AAV capsid.

[0199] Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including two 145 nucleotide inverted terminal repeat (ITRs). There are multiple serotypes of AAV. The nucleotide sequences of the genomes of the AAV serotypes are known. For example, the AAV5 genome is provided in GenBank Accession No. AF085716. The life cycle and genetics of AAV are reviewed in Muzyczka, *Current Topics in Microbiology and Immunology*, 158: 97-129 (1992). Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692. Other types of rAAV variants, for example rAAV with capsid mutations, are also contemplated. See, for example, Marsic et al., *Molecular Therapy*, 22(11): 1900-1909 (2014). Illustrative AAV vectors are provided in US 7,105,345; US 15/782,980; US 7,259,151; US 6,962,815; US 7,718,424; US 6,984,517; US 7,718,424; US 6,156,303; US 8,524,446; US 7,790,449; US 7,906,111; US 9,737,618; US App 15/433,322; US 7,198,951, each of which is incorporated by reference in its entirety for all purposes.

[0200] The rAAV virions of the disclosure comprise a heterologous nucleic acid comprising a nucleotide sequence encoding one or more gene product. The gene product(s) may be either a polypeptide or an RNA, or both. When the gene product is a polypeptide, the nucleotide sequence encodes a messenger RNA, optionally with one or more introns, which is translated into the gene product polypeptide. The nucleotide sequence may encode one, two, three, or more gene products (though the number is limited by the packaging capacity of the rAAV virion, typically about 5.2 kb). The gene products may be operatively linked to one promoter (for a single transcriptional unit) or more than one. Multiple gene products may also be produced using internal ribosome entry signal (IRES) or a self-cleaving peptide (*e.g.*, a 2A peptide).

[0201] In some embodiments, the gene product is a polypeptide. In some embodiments, the polypeptide gene product is a polypeptide that induces reprogramming of a cardiac fibroblast, to generate an induced cardiomyocyte-like cell (iCM). In some embodiments, the polypeptide gene product is a polypeptide that enhances the function of a cardiac cell. In some

embodiments, the polypeptide gene product is a polypeptide that provides a function that is missing or defective in the cardiac cell. In some embodiments, the polypeptide gene product is a genome-editing endonuclease.

[0202] In some embodiments, the gene product comprises a fusion protein that is fused to a heterologous polypeptide. In some embodiments, the gene product comprises a genome editing nuclease fused to an amino acid sequence that provides for subcellular localization, *i.e.*, the fusion partner is a subcellular localization sequence (*e.g.*, one or more nuclear localization signals (NLSs) for targeting to the nucleus, two or more NLSs, three or more NLSs, etc.).

[0203] In general, a viral vector is produced by introducing a viral DNA or RNA construct into a "producer cell" or "packaging cell" line. Packaging cell lines include but are not limited to any easily-transfectable cell line. Packaging cell lines can be based on HEK291, 293T cells, NIH3T3, COS, HeLa or Sf9 cell lines. Examples of packaging cell lines include but are not limited to: Sf9 (ATCC® CRL-1711™). Exemplary packing cell lines and methods for generating rAAV virions are provided by Int'l Pat. Pub. Nos. WO2017075627, WO2015/031686, WO2013/063379, WO2011/020710, WO2009/104964, WO2008/024998, WO2003/042361, and WO1995/013392; U.S. Pat. Nos. US9441206B2, US8679837, and US7091029B2.

[0204] In some embodiments, the gene product is a functional cardiac protein. In some embodiments, the gene product is a genome-editing endonuclease (optionally with a guide RNA, single-guide RNA, and/or repair template) that replaces or repairs a non-functional cardiac protein into a functional cardiac protein. Functional cardiac proteins include, but are not limited to cardiac troponin T; a cardiac sarcomeric protein; β-myosin heavy chain; myosin ventricular essential light chain 1; myosin ventricular regulatory light chain 2; cardiac a-actin; a-tropomyosin; cardiac troponin I; cardiac myosin binding protein C; four-and-a-half LIM protein 1; titin; 5'-AMP-activated protein kinase subunit gamma-2; troponin I type 3, myosin light chain 2, actin alpha cardiac muscle 1; cardiac LIM protein; caveolin 3 (CAV3); galactosidase alpha (GLA); lysosomal-associated membrane protein 2 (LAMP2); mitochondrial transfer RNA glycine (MTTG); mitochondrial transfer RNA isoleucine (MTTI); mitochondrial transfer RNA lysine (MTTK); mitochondrial transfer RNA glutamine (MTTQ); myosin light chain 3 (MYL3); troponin C (TNNC1); transthyretin (TTR); sarcoendoplasmic reticulum calcium-ATPase 2a (SERCA2a); stromal-derived factor-1 (SDF-1); adenylate cyclase-6 (AC6); beta-ARKct (β-adrenergic receptor kinase C terminus); fibroblast growth

factor (FGF); platelet-derived growth factor (PDGF); vascular endothelial growth factor (VEGF); hepatocyte growth factor; hypoxia inducible growth factor; thymosin beta 4 (TMSB4X); nitric oxide synthase-3 (NOS3); unocartin 3 (UCN3); melusin; apoplipoprotein-E (ApoE); superoxide dismutase (SOD); and S100A1 (a small calcium binding protein; *see, e.g.*, Ritterhoff and Most (2012) *Gene Ther.* 19:613; Kraus et al. (2009) *Mol. Cell. Cardiol.* 47:445).

[0205] In some embodiments, the gene product is a gene product whose expression complements a defect in a gene responsible for a genetic disorder. The disclosure provides rAAV virions comprising a polynucleotide encoding one or more of the following—*e.g.*, for use, without limitation, in the disorder indicated in parentheses, or for other disorders caused by each: TAZ (Barth syndrome); FXN (Freidrich's Ataxia); CASQ2 (CPVT); FBN1 (Marfan); RAF1 and SOS1s (Noonan); SCN5A (Brugada); KCNQ1 and KCNH2s (Long QT Syndrome); DMPK (Myotonic Dystrophy 1); LMNA (Limb Girdle Dystrophy Type 1B); JUP (Naxos); TGFBR2 (Loeys-Dietz); EMD (X-Linked EDMD); and ELN (SV Aortic Stenosis). In some embodiments, the rAAV virion comprises a polynucleotide encording one or more of cardiac troponin T (TNNT2); BAG family molecular chaperone regulator 3 (BAG3); myosin heavy chain (MYH7); tropomyosin 1 (TPM1); myosin binding protein C (MYBPC3); 5'-AMP-activated protein kinase subunit gamma-2 (PRKAG2); troponin I type 3 (TNNI3); titin (TTN); myosin, light chain 2 (MYL2); actin, alpha cardiac muscle 1 (ACTC1); potassium voltage-gated channel, KQT- like subfamily, member 1 (KCNQ1); plakophilin 2 (PKP2); myocyte enhancer factor 2c (MEF2C); and cardiac LIM protein (CSRP3).

[0206] In some embodiments, the gene products of the disclosure are polypeptide reprogramming factors. Reprogramming factors are desirable as means to convert one cell type into another. Non-cardiomyocytes cells can be differentiated into cardiomyocytes cells *in vitro* or *in vivo* using any method available to one of skill in the art. For example, see methods described in Ieda et al. (2010) *Cell* 142:375-386; Christoforou et al. (2013) *PLoS ONE* 8:e63577; Addis et al. (2013) *J. Mol. Cell Cardiol.* 60:97-106; Jayawardena et al. (2012) *Circ. Res.* 110: 1465-1473; Nam Y et al. (2003) *PNAS USA* 110:5588-5593; Wada R et al. (2013) *PNAS USA* 110: 12667-12672; and Fu J et al. (2013) *Stem Cell Reports* 1:235-247.

[0207] In cardiac context, the reprogramming factors may be capable of converting a cardiac fibroblast to a cardiac myocyte either directly or through an intermediate cell type. In particular, direct regramming is possible, or reprogramming by first converting the fibroblast to a pluripotent or totipotent stem cell. Such a pluripotent stem cell is termed an induced

pluripotent stem (iPS) cell. An iPS cell that is subsequently converted to a cardiac myocyte (CM) cell is termed an iPS-CM cell. In the examples, iPS-CM derived *in vitro* from cardiac fibroblasts are used *in vivo* to select capsid proteins of interest. The disclosure also envisions using the capsid proteins disclosure to in turn generate iPS-CM cells *in vitro* but, particular, *in vivo*, as part of a therapeutic gene therapy regimen. Induced cardiomyocyte-like (iCM) cells refer to cells directly reprogrammed into cardiomyocytes.

[0208] Induced cardiomyocytes express one or more cardiomyocyte-specific markers, where cardiomyocyte-specific markers include, but are not limited to, cardiac troponin I, cardiac troponin-C, tropomyosin, caveolin-3, myosin heavy chain, myosin light chain-2a, myosin light chain-2v, ryanodine receptor, sarcomeric a-actinin, Nkx2.5, connexin 43, and atrial natriuretic factor. Induced cardiomyocytes can also exhibit sarcomeric structures. Induced cardiomyocytes exhibit increased expression of cardiomyocyte-specific genes ACTC1 (cardiac a-actin), ACTN2 (actinin a2), MYH6 (a-myosin heavy chain), RYR2 (ryanodine receptor 2), MYL2 (myosin regulatory light chain 2, ventricular isoform), MYL7 (myosin regulatory light chain, atrial isoform), TNNT2 (troponin T type 2, cardiac), and NPPA (natriuretic peptide precursor type A), PLN (phospholamban). Expression of fibroblasts markers such as Colla2 (collagen la2) is downregulated in induced cardiomyocytes, compared to fibroblasts from which the iCM is derived.

[0209] Reprogramming methods involving polypeptide reprogramming factors (in some cases supplemented by small-molecule reprogramming factors supplied in conjuction with the rAAV) include those described in US2018/0112282A1, WO2018/005546, WO2017/173137, US2016/0186141, US2016/0251624, US2014/0301991, and US2013/0216503A1, which are incorporated in their entirety, particularly for the reprogramming methods and factors disclosed.

[0210] In some embodiments, cardiac cells are reprogrammed into induced cardiomyocyte-like (iCM) cells using one or more reprogramming factors that modulate the expression of one or more polynucleotides or proteins of interest, such as Achaete-scute homolog 1 (ASCL1), Myocardin (MYOCD), myocyte-specific enhancer factor 2C (MEF2C), and/or T-box transcription factor 5 (TBX5). In some embodiments, the one or more reprogramming factors are provided as a polynucleotide (*e.g.*, an RNA, an mRNA, or a DNA polynucleotide) that encode one or more polynucleotides or proteins of interest. In some embodiments, the one or more reprogramming factors are provided as a protein.

[0211] In some embodiments, the reprogramming factors are microRNAs or microRNA antagonists, siRNAs, or small molecules that are capable of increasing the expression of one or more polynucleotides or proteins of interest. In some embodiments, expression of a polynucleotides or proteins of interest is increased by expression of a microRNA or a microRNA antagonist. For example, endogenous expression of an Oct polypeptide can be increased by introduction of microRNA-302 (miR-302), or by increased expression of miR-302. *See, e.g.*, Hu et al., *Stem Cells* 31(2): 259-68 (2013), which is incorporated herein by reference in its entirety. Hence, miRNA-302 can be an inducer of endogenous Oct polypeptide expression. The miRNA-302 can be introduced alone or with a nucleic acid that encodes the Oct polypeptide. In some embodiments, a suitable nucleic acid gene product is a microRNA. Suitable micrRNAs include, *e.g.*, mir-1, mir-133, mir-208, mir-143, mir-145, and mir-499.

[0212] In some embodiments, the methods of the disclosure comprise administering an rAAV virion of the disclosure before, during, or after administration of the small-molecule reprogramming factor. In some embodiments, the small-molecule reprogramming factor is a small molecule selected from the group consisting of SB431542, LDN-193189, dexamethasone, LY364947, D4476, myricetin, IWR1, XAV939, docosahexaenoic acid (DHA), S-Nitroso-TV-acetylpenicillamine (SNAP), Hh-Agl.5, alprostadil, cromakalim, MNITMT, A769662, retinoic acid p-hydoxyanlide, decamethonium dibromide, nifedipine, piroxicam, bacitracin, aztreonam, harmalol hydrochloride, amide-C2 (A7), Ph-C12 (CIO), mCF3-C-7 (J5), G856-7272 (A473), 5475707, or any combination thereof.

[0213] In some embodiments, the gene products comprise reprogramming factors that modulate the expression of one or more proteins of interest selected from ASCL1, MYOCD, MEF2C, and TBX5. In some embodiments, the gene products comprise one or more reprogramming factors selected from ASCL1, MYOCD, MEF2C, AND TBX5, CCNB1, CCND1, CDK1, CDK4, AURKB, OCT4, BAF60C, ESRRG, GATA4, GATA6, HAND2, IRX4, ISLL, MESP1, MESP2, NKX2.5, SRF, TBX20, ZFPM2, and miR-133.

[0214] In some embodiments, the gene products comprise GATA4, MEF2C, and TBX5 (*i.e.*, GMT). In some embodiments, the gene products comprise MYOCD, MEF2C, and TBX5 (*i.e.*, MyMT). In some embodiments, the gene products comprise MYOCD, ASCL1, MEF2C, and TBX5 (*i.e.*, MyAMT). In some embodiments, the gene products comprise MYOCD and ASCL1 (*i.e.*, MyA). In some embodiments, the gene products comprise GATA4, MEF2C, TBX5, and MYOCD (*i.e.*, 4F). In other embodiments, the gene products comprise GATA4,

MEF2C, TBX5, ESSRG, MYOCD, ZFPM2, and MESP1 (*i.e.*, 7F). In some embodiments, the gene products comprise one or more of ASCL1, MEF2C, GATA4, TBX5, MYOCD, ESRRG, AND MESPL.

[0215] In some embodiments, the rAAV virions generate cardiac myocytes *in vitro* or *in vivo*. Cardiomyocytes or cardiac myocytes are the muscle cells that make up the cardiac muscle. Each myocardial cell contains myofibrils, which are long chains of sarcomeres, the contractile units of muscle cells. Cardiomyocytes show striations similar to those on skeletal muscle cells, but unlike multinucleated skeletal cells, they contain only one nucleus. Cardiomyocytes have a high mitochondrial density, which allows them to produce ATP quickly, making them highly resistant to fatigue. Mature cardiomyocytes can express one or more of the following cardiac markers: α-Actinin, MLC2v, MY20, cMHC, NKX2-5, GATA4, cTNT, cTNI, MEF2C, MLC2a, or any combination thereof. In some embodiments, the mature cardiomyocytes express NKX2-5, MEF2C or a combination thereof. In some embodiments, cardiac progenitor cells express early stage cardiac progenitor markers such as GATA4, ISL1 or a combination thereof.

[0216] In some embodiments, the gene product is a polynucleotide. In some embodiments, as described below, the gene product is a guide RNA capable of binding to an RNA-guided endonuclease. In some embodiments, the gene product is an inhibitory nucleic acid capable of reducing the level of an mRNA and/or a polypeptide gene product, *e.g.*, in a cardiac cell. For example, in some embodiments, the polnucleotide gene product is an interfering RNA capable of selectively inactivating a transcript encoded by an allele that causes a cardiac disease or disorder. As an example, the allele is a myosin heavy chain 7, cardiac muscle, beta (MYH7) allele that comprises a hypertrophic cardiomyopathy-causing mutation. Other examples include, *e.g.*, interfering RNAs that selectively inactivate a transcript encoded by an allele that causes hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM) or Left Ventricular Non-Compaction (LVNC), where the allele is a MYL3 (myosin light chain 3, alkali, ventricular, skeletal slow), MYH7, TNNI3 (troponin I type 3 (cardiac)), TNNT2 (troponin T type 2 (cardiac)), TPMl (tropomyosin 1 (alpha)) or ACTCl allele comprising an HCM-causing, a DCM-causing or a LVNC-causing mutation. *See, e.g.*, U.S. Pat. Pub. No. 2016/0237430 for examples of cardiac disease-causing mutations.

[0217] In some embodiments, the gene product is a polypeptide-encoding RNA. In some embodiments, the gene product is an interfering RNA. In some embodiments, the gene

product is an aptamer. In some embodiments, the gene product is a polypeptide. In some embodiments, the gene product is a therapeutic polypeptide, *e.g.*, a polypeptide that provides clinical benefit. In some embodiments, the gene product is a site-specific nuclease that provide for site-specific knock-down of gene function. In some embodiments, the gene product is an RNA-guided endonuclease that provides for modification of a target nucleic acid. In some embodiments, the gene products are: i) an RNA-guided endonuclease that provides for modification of a target nucleic acid; and ii) a guide RNA that comprises a first segment that binds to a target sequence in a target nucleic acid and a second segment that binds to the RNA-guided endonuclease. In some embodiments, the gene products are: i) an RNA-guided endonuclease that provides for modification of a target nucleic acid; ii) a first guide RNA that comprises a first segment that binds to a first target sequence in a target nucleic acid and a second segment that binds to the RNA- guided endonuclease; and iii) a first guide RNA that comprises a first segment that binds to a second target sequence in the target nucleic acid and a second segment that binds to the RNA-guided endonuclease.

[0218] A nucleotide sequence encoding a heterologous gene product in an rAAV virion of the present disclosure can be operably linked to a promoter. For example, a nucleotide sequence encoding a heterologous gene product in an rAAV virion of the present disclosure can be operably linked to a constitutive promoter, a regulatable promoter, or a cardiac cell-specific promoter. Suitable constitutive promoters include a human elongation factor 1 α subunit (EFlα) promoter, a β-actin promoter, an α-actin promoter, a β-glucuronidase promoter, CAG promoter, super core promoter, and a ubiquitin promoter. In some embodiments, a nucleotide sequence encoding a heterologous gene product in an rAAV virion of the present disclosure is operably linked to a cardiac-specific transcriptional regulator element (TRE), where cardiac-specific TREs include promoters and enhancers. Suitable cardiac-specific TREs include, but are not limited to, TREs derived from the following genes: myosin light chain-2 (MLC-2), a- myosin heavy chain (a-MHC), desmin, AE3, cardiac troponin C (cTnC), and cardiac actin. Franz et al. (1997) *Cardiovasc. Res.* 35:560-566; Robbins et al. (1995) *Ann. NY. Acad. Sci.* 752:492-505; Linn et al. (1995) *Circ. Res.* 76:584-591; Parmacek et al. (1994) *Mol. Cell. Biol.* 14: 1870-1885; Hunter et al. (1993) *Hypertension* 22:608-617; and Sartorelli et al. (1992) *Proc. Natl. Acad. Sci.* USA 89:4047-4051. *See also*, Pacak et al. (2008) *Genet Vaccines Ther.* 6:13. In some embodiments, the promoter is an α-MHC promoter, an MLC-2 promoter, or cTnT promoter.

[0219] The polynucleotide encoding a gene product is operably linked to a promoter and/or enhancer to facilitate expression of the gene product. Depending on the host/vector

system utilized, any of a number of suitable transcription and translation control elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the rAAV virion (*e.g.*, Bitter et al. (1987) *Methods in Enzymology*, 153:516-544).

[0220] Separate promoters and/or enhancers can be employed for each of the polynucleotides. In some embodiments, the same promoter and/or enhance is used for two or more polynucleotides in a single open reading frame. Vectors employing this configuration of genetic elements are termed "polycistronic." An illustrative example of a polycistronic vector comprises an enhancer and a promoter operatively linked to a single open-reading frame comprising two or more polynucleotides linked by 2A region(s), whereby expression of the open-reading frame result in multiple polypeptides being generated co-translationally. The 2A region is believed to mediate generation of multiple polypeptide sequences through codon skipping; however, the present disclosure relates also to polycistronic vectors that employ post-translational cleavage to generate two or more proteins of interest from the same polynucleotide. Illustrative 2A sequences, vectors, and associated methods are provided in US20040265955A1, which is incorporated herein by reference.

[0221] Non-limiting examples of suitable eukaryotic promoters (promoters functional in a eukaryotic cell) include CMV, CMV immediate early, HSV thymidine kinase, early and late SV40, long terminal repeats (LTRs) from retrovirus, and mouse metallothionein-I. In some embodiments, promoters that are capable of conferring cardiac specific expression will be used. Non-limiting examples of suitable cardiac specific promoters include desmin (Des), alpha-myosin heavy chain (a-MHC), myosin light chain 2 (MLC-2), cardiac troponin T (cTnT) and cardiac troponin C (cTnC). Non-limiting examples of suitable neuron specific promoters include synapsin I (SYN), calcium/calmodulin-dependent protein kinase II, tubulin alpha I, neuron-specific enolase and platelet-derived growth factor beta chain promoters and hybrid promoters by fusing cytomegalovirus enhancer (E) to those neuron-specific promoters.

[0222] Examples of suitable promoters for driving expression reprogramming factors include, but are not limited to, retroviral long terminal repeat (LTR) elements; constitutive promoters such as CMV, HSV1-TK, SV40, EF-la, β-actin, phosphoglycerol kinase (PGK); inducible promoters, such as those containing Tet- operator elements; cardiac specific promoters, such as desmin (DES), alpha-myosin heavy chain (a-MHC), myosin light chain 2 (MLC-2), cardiac troponin T (cTnT) and cardiac troponin C (cTnC); neural specific promoters,

such as nestin, neuronal nuclei (NeuN), microtubule-associate protein 2 (MAP2), beta III tubulin, neuron specific enolase (NSE), oligodendrocyte lineage (Oligl/2), and glial fibrillary acidic protein (GFAP); and pancreatic specific promoters, such as Pax4, Nkx2.2, Ngn3, insulin, glucagon, and somatostatin.

[0223] In some embodiments, a polynucleotide is operably linked to a cell type-specific transcriptional regulator element (TRE), where TREs include promoters and enhancers. Suitable TREs include, but are not limited to, TREs derived from the following genes: myosin light chain-2, a-myosin heavy chain, AE3, cardiac troponin C, and cardiac actin. Franz et al. (1997) *Cardiovasc. Res.* 35:560-566; Robbins et al. (1995) *Ann. N. Y. Acad. Sci.* 752:492-505; Linn et al. (1995) *Circ. Res.* 76:584-591; Parmacek et al. (1994) *Cell. Biol.* 14:1870-1885; Hunter et al. (1993) *Hypertension* 22:608-617; and Sartorelli et al. (1992) *PNAS USA* 89:4047-4051.

[0224] The promoter can be one naturally associated with a gene or nucleic acid segment. Similarly, for RNAs (*e.g.*, microRNAs), the promoter can be one naturally associated with a microRNA gene (*e.g.*, an miRNA-302 gene). Such a naturally associated promoter can be referred to as the "natural promoter" and may be obtained by isolating the 5′ non-coding sequences located upstream of the coding segment and/or exon. Similarly, an enhancer may be one naturally associated with a nucleic acid sequence. However, the enhancer can be located either downstream or upstream of that sequence.

[0225] Alternatively, certain advantages will be gained by positioning the coding nucleic acid segment under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with a nucleic acid in its natural environment. A recombinant or heterologous enhancer refers also to an enhancer not normally associated with a nucleic acid sequence in its natural environment. Such promoters or enhancers can include promoters or enhancers of other genes, and promoters or enhancers isolated from any other prokaryotic, viral, or eukaryotic cell, and promoters or enhancers not "naturally occurring," *i.e.*, containing different elements of different transcriptional regulatory regions, and/or mutations that alter expression. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including PCR™, in connection with the compositions disclosed herein (see U.S. Pat. No. 4,683,202, U.S. Pat. No. 5,928,906, each incorporated herein by reference).

[0226] The promoters employed may be constitutive, inducible, developmentally-specific, tissue-specific, and/or useful under the appropriate conditions to direct high level expression of the nucleic acid segment. For example, the promoter can be a constitutive promoter such as, a CMV promoter, a CMV cytomegalovirus immediate early promoter, a CAG promoter, an EF-1α promoter, a HSV1-TK promoter, an SV40 promoter, a β-actin promoter, a PGK promoter, or a combination thereof. Examples of eukaryotic promoters that can be used include, but are not limited to, constitutive promoters, *e.g.*, viral promoters such as CMV, SV40 and RSV promoters, as well as regulatable promoters, *e.g.*, an inducible or repressible promoter such as the tet promoter, the hsp70 promoter and a synthetic promoter regulated by CRE. In certain embodiments, cell type-specific promoters are used to drive expression of reprogramming factors in specific cell types. Examples of suitable cell type-specific promoters useful for the methods described herein include, but are not limited to, the synthetic macrophage-specific promoter described in He et al (2006), *Human Gene Therapy* 17:949-959; the granulocyte and macrophage-specific lysozyme M promoter (see, *e.g.*, Faust et al (2000), *Blood* 96(2):719-726); and the myeloid-specific CD11b promoter (see, *e.g.*, Dziennis et al (1995), *Blood* 85(2):319-329). Other examples of promoters that can be employed include a human EF1α elongation factor promoter, a CMV cytomegalovirus immediate early promoter, a CAG chicken albumin promoter, a viral promoter associated with any of the viral vectors described herein, or a promoter that is homologous to any of the promoters described herein (*e.g.*, from another species). Examples of prokaryotic promoters that can be used include, but are not limited to, SP6, T7, T5, *tac*, *bla*, *trp*, *gal*, *lac*, or maltose promoters.

[0227] In some embodiments, an internal ribosome entry sites (IRES) element can be used to create multigene, or polycistronic, messages. IRES elements are able to bypass the ribosome scanning model of 5′-methylated Cap dependent translation and begin translation at internal sites (Pelletier and Sonenberg, *Nature* 334(6180):320-325 (1988)). IRES elements from two members of the picornavirus family (polio and encephalomyocarditis) have been described (Pelletier and Sonenberg, *Nature* 334(6180):320-325 (1988)), as well an IRES from a mammalian message (Macejak & Samow, *Nature* 353:90-94 (1991)). IRES elements can be linked to heterologous open reading frames. Multiple open reading frames can be transcribed together, each separated by an IRES, creating polycistronic messages. By virtue of the IRES element, each open reading frame is accessible to ribosomes for efficient translation. Multiple genes can be efficiently expressed using a single promoter/enhancer to transcribe a single message (see U.S. Patent Nos. 5,925,565 and 5,935,819, herein incorporated by reference).

[0228] In some embodiments, a nucleotide sequence is operably linked to a polyadenylation sequence. Suitable polyadenylation sequences include bovine growth hormone polyA signal (bGHpolyA) and short poly A signal. Optionally the rAAV vectors of the disclosure compriset the Woodchuck Post-transcriptional Regulatory Element (WPRE). In some embodiments, the polynucleotide encoding gene products are join by sequences include so-called self-cleaving peptide, *e.g.* P2A peptides.

[0229] In some embodiments, the gene product comprises a site-specific endonuclease that provides for site-specific knock-down of gene function, *e.g.*, where the endonuclease knocks out an allele associated with a cardiac disease or disorder. For example, where a dominant allele encodes a defective copy of a gene that, when wild-type, is a cardiac structural protein and/or provides for normal cardiac function, a site-specific endonuclease can be targeted to the defective allele and knock out the defective allele. In some embodiments, a site-specific endonuclease is an RNA-guided endonuclease.

[0230] In addition to knocking out a defective allele, a site-specific nuclease can also be used to stimulate homologous recombination with a donor DNA that encodes a functional copy of the protein encoded by the defective allele. For example, a subject rAAV virion can be used to deliver both a site-specific endonuclease that knocks out a defective allele a functional copy of the defective allele (or fragment thereof), resulting in repair of the defective allele, thereby providing for production of a functional cardiac protein (*e.g.*, functional troponin, etc.). In some embodiments, a subject rAAV virion comprises a heterologous nucleotide sequence that encodes a site-specific endonuclease and a heterologous nucleotide sequence that encodes a functional copy of a defective allele, where the functional copy encodes a functional cardiac protein. Functional cardiac proteins include, *e.g.*, troponin, a chloride ion channel, and the like.

[0231] Site-specific endonucleases that are suitable for use include, *e.g.*, zinc finger nucleases (ZFNs); meganucleases; and transcription activator-like effector nucleases (TALENs), where such site-specific endonucleases are non-naturally occurring and are modified to target a specific gene. Such site-specific nucleases can be engineered to cut specific locations within a genome, and non-homologous end joining can then repair the break while inserting or deleting several nucleotides. Such site-specific endonucleases (also referred to as "INDELs") then throw the protein out of frame and effectively knock out the gene. *See, e.g.*, U.S. Pat. Pub. No. 2011/0301073. Suitable site-specific endonucleases include engineered meganuclease re-engineered homing endonucleases. Suitable endonucleases include an I-Tevl

nuclease. Suitable meganucleases include I-SceI (see, *e.g.*, Bellaiche et al. (1999) *Genetics* 152: 1037); and I-CreI (see, *e.g.*, Heath et al. (1997) *Nature Sructural Biology* 4:468). Site-specific endonucleases that are suitable for use include CRISPRi systems and the Cas9-based SAM system.

[0232] In some embodiments, the gene product is an RNA-guided endonuclease. In some embodiments, the gene product comprises an RNA comprising a nucleotide sequence encoding an RNA-guided endonuclease. In some embodiments, the gene product is a guide RNA, *e.g.*, a single -guide RNA. In some embodiments, the gene products are: 1) a guide RNA; and 2) an RNA-guided endonuclease. The guide RNA can comprise: a) a protein-binding region that binds to the RNA-guided endonuclease; and b) a region that binds to a target nucleic acid. An RNA-guided endonuclease is also referred to herein as a "genome editing nuclease."

[0233] Examples of suitable genome editing nucleases are CRISPR/Cas endonucleases (*e.g.*, class 2 CRISPR/Cas endonucleases such as a type II, type V, or type VI CRISPR/Cas endonucleases). A suitable genome editing nuclease is a CRISPR/Cas endonuclease (*e.g.*, a class 2 CRISPR/Cas endonuclease such as a type II, type V, or type VI CRISPR/Cas endonuclease). In some embodiments, the gene product comprises a class 2 CRISPR/Cas endonuclease. In some embodiments, the gene product comprises a class 2 type II CRISPR/Cas endonuclease (*e.g.*, a Cas9 protein). In some embodiments, the gene product comprises a class 2 type V CRISPR/Cas endonuclease (*e.g.*, a Cpfl protein, a C2cl protein, or a C2c3 protein). In some embodiments, the gene product comprises a class 2 type VI CRISPR/Cas endonuclease (*e.g.*, a C2c2 protein; also referred to as a "Casl3a" protein). In some embodiments, the gene product comprises a CasX protein. In some embodiments, the gene product comprises a CasY protein.

Methods of Use

[0234] In some embodiments, the disclosure provides methods of identifying AAV capsid proteins that confer on rAAV virions increased transduction efficiency in target cells. The methods comprise providing a population of rAAV virions whose rAAV genomes comprise a library of *cap* polynucleotides encoding variant AAV capsid proteins; optionally contacting the population with non-target cells for a time sufficient to permit attachment of undesired rAAV virions to the non-target cells; contacting the population with target cells for a time sufficient to permit transduction of the *cap* polynucleotide into the target cells by the rAAV virions; and sequencing the *cap* polynucleotides from the target cells, thereby identifying AAV

capsid proteins that confer increased transduction efficiency in the target cells. In some embodiments, the method further comprises depleting the population of rAAV virions by contacting the population with non-target cells for time sufficient to permit attachment of the rAAV virions to the non-target cells. Non-limiting examples of such identifications methods are provided in the Examples.

[0235] The disclosure provides methods for generating cardiomyocytes and/or cardiomyocyte-like cells *in vitro* using an rAAV virion. Selected starting cells are transduced with an rAAV and optionally exposed to small-molecule reprogramming factors (before, during, or after transduction) for a time and under conditions sufficient to convert the starting cells across lineage and/or differentiation boundaries to form cardiac progenitor cells and/or cardiomyocytes. In some embodiments, the starting cells are fibroblast cells. In some embodiments, the starting cells express one or more markers indicative of a differentiated phenotype. The time for conversion of starting cells into cardiac progenitor and cardiomyocyte cells can vary. For example, the starting cells can be incubated after treatment with one or more polynucleotides or proteins of interest until cardiac or cardiomyocyte cell markers are expressed. Such cardiac or cardiomyocyte cell markers can include any of the following markers: α-GATA4, TNNT2, MYH6, RYR2, NKX2-5, MEF2C, ANP, Actinin, MLC2v, MY20, cMHC, ISL1, cTNT, cTNI, and MLC2a, or any combination thereof. In some embodiments, the induced cardiomycocyte cells are negative for one or more neuronal cells markers. Such neuronal cell markers can include any of the following markers: DCX, TUBB3, MAP2, and ENO2.

[0236] Incubation can proceed until cardiac progenitor markers are expressed by the starting cells. Such cardiac progenitor markers include GATA4, TNNT2, MYH6, RYR2, or a combination thereof. The cardiac progenitor markers such as GATA4, TNNT2, MYH6, RYR2, or a combination thereof can be expressed by about 8 days, or by about 9 days, or by about 10 days, or by about 11 days, or by about 12 days, or by about 14 days, or by about 15 days, or by about 16 days, or by about 17 days, or by about 18 days, or by about 19 days, or by about 20 days after starting incubation of cells in the compositions described herein. Further incubation of the cells can be performed until expression of late stage cardiac progenitor markers such as NKX2-5, MEF2C or a combination thereof occurs.

[0237] Reprogramming efficiency may be measured as a function of cardiomyocyte markers. Such pluripotency markers include, but are not limited to, the expression of

cardiomyocyte marker proteins and mRNA, cardiomyocyte morphology and electrophysiological phenotype. Non-limiting examples of cardiomyocyte markers include, a-sarcoglycan, atrial natriuretic peptide (ANP), bone morphogenetic protein 4 (BMP4), connexin 37, connexin 40, crypto, desmin, GATA4, GATA6, MEF2C, MYH6, myosin heavy chain, NKX2.5, TBX5, and Troponin T.

[0238] The expression of various markers specific to cardiomyocytes may be detected by conventional biochemical or immunochemical methods (*e.g.*, enzyme- linked immunosorbent assay, immunohistochemical assay, and the like). Alternatively, expression of a nucleic acid encoding a cardiomyocyte- specific marker can be assessed. Expression of cardiomyocyte-specific marker-encoding nucleic acids in a cell can be confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) or hybridization analysis, molecular biological methods which have been commonly used in the past for amplifying, detecting and analyzing mRNA coding for any marker proteins. Nucleic acid sequences coding for markers specific to cardiomyocytes are known and are available through public databases such as GenBank. Thus, marker-specific sequences needed for use as primers or probes are easily determined.

[0239] Cardiomyocytes exhibit some cardiac-specific electrophysiological properties. One electrical characteristic is an action potential, which is a short-lasting event in which the difference of potential between the interior and the exterior of each cardiac cell rises and falls following a consistent trajectory. Another electrophysiological characteristic of cardiomyocytes is the cyclic variations in the cytosolic-free Ca^{2+} concentration, named as Ca^{2+} transients, which are employed in the regulation of the contraction and relaxation of cardiomyocytes. These characteristics can be detected and evaluated to assess whether a population of cells has been reprogrammed into cardiomyocytes.

[0240] The present disclosure provides a method of delivering a gene product to a cardiac cell, *e.g.*, a cardiac fibroblast. The methods generally involve infecting a cardiac cell (*e.g.*, a cardiac fibroblast) with an rAAV virion, where the gene product(s) encoded by the heterologous nucleic acid present in the rAAV virion is/are produced in the cardiac cell (*e.g.*, cardiac fibroblast). Delivery of gene product(s) to a cardiac cell (*e.g.*, cardiac fibroblast) can provide for treatment of a cardiac disease or disorder. Delivery of gene product(s) to a cardiac cell (*e.g.*, cardiac fibroblast) can provide for generation of an induced cardiomyocyte-like (iCM)

cell from the cardiac fibroblast. Delivery of gene product(s) to a cardiac cell (*e.g.*, cardiac fibroblast) can provide for editing of the genome of the cardiac cell (*e.g.*, cardiac fibroblast).

[0241] In some embodiments, infecting or transducing a cardiac cell (*e.g.*, cardiac fibroblast) is carried out *in vitro*. In some embodiments, infecting or transducing a cardiac cell (*e.g.*, cardiac fibroblast) is carried out *in vitro*; and the infected/transduced cardiac cell (*e.g.*, cardiac fibroblast) is introduced into (*e.g.*, transfused into or implanted into) an individual in need thereof, *e.g.*, directly into cardiac tissue of an individual in need thereof. For *in vitro* transduction, an effective amount of rAAV virions to be delivered to cells is from about 10^5 to about 10^{13} of the rAAV virions. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

[0242] In some embodiments, infecting a cardiac cell (*e.g.*, cardiac fibroblast) is carried out *in vivo*. For example, in some embodiments, an effective amount of an rAAV virion of the present disclosure is administered directly into cardiac tissue of an individual in need thereof. An "effective amount" will fall in a relatively broad range that can be determined through experimentation and/or clinical trials. For example, for in vivo injection, *i.e.*, injection directly into cardiac tissue, a therapeutically effective dose will be on the order of from about 10^6 to about 10^{15} of the rAAV virions, *e.g.*, from about 10^5 to 10^{12} rAAV virions, of the present disclosure. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via intramyocardial injection through the epicardium. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via vascular delivery through the coronary artery. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through the superior vena cava. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through a peripheral vein.

[0243] For example, from about 10^4 to about 10^5, from about 10^5 to about 10^6, from about 10^6 to about 10^7, from about 10^6 to about 10^7, from about 10^7 to about 10^8, from about 10^8 to about 10^9, from about 10^9 to about 10^{10}, from about 10^{10} to about 10^{11}, to about 10^{11}, from about 10^{11} to about 10^{12}, from about 10^{12} to about 10^{13}, from about 10^{13} to about 10^{14}, from about 10^{14} to about 10^{15} genome copies, or more than 10^{15} genome copies, of an rAAV virion of the present disclosure are administered to an individual, *e.g.*, are administered directly into cardiac tissue in the individual, or are administered via another route. The number of rAAV virions administered to an individual can be expressed in viral genomes (vg) per kilogram (kg)

body weight of the individual. In some embodiments, and effective amount of an rAAV virion of the present disclosure is from about 10^2 vg/kg to 10^4 vg/kg, from about 10^4 vg/kg to about 10^6 vg/kg, from about 10^6 vg/kg to about 10^8 vg/kg, from about 10^8 vg/kg to about 10^{10} vg/kg, from about 10^{10} vg/kg to about 10^{12} vg/kg, from about 10^{12} vg/kg to about 10^{14} vg/kg, from about 10^{14} vg/kg to about 10^{16} vg/kg, from about 10^{16} vg/kg to about 10^{18} vg/kg, or more than 10^{18} vg/kg.

[0244] In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via intramyocardial injection through the epicardium. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via vascular delivery through the coronary artery. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through the superior vena cava. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through a peripheral vein.

[0245] In some embodiments, more than one administration (*e.g.*, two, three, four or more administrations) may be employed to achieve the desired level of gene expression. In some embodiments, the more than one administration is administered at various intervals, *e.g.*, daily, weekly, twice monthly, monthly, every 3 months, every 6 months, yearly, etc. In some embodiments, multiple administrations are administered over a period of time of from 1 month to 2 months, from 2 months to 4 months, from 4 months to 8 months, from 8 months to 12 months, from 1 year to 2 years, from 2 years to 5 years, or more than 5 years.

[0246] The present disclosure provides a method of reprogramming a cardiac fibroblast to generate an induced cardiomyocyte-like cell (iCM). The method generally involves infecting a cardiac fibroblast with an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding one or more reprogramming factors.

[0247] The expression of various markers specific to cardiomyocytes is detected by conventional biochemical or immunochemical methods (*e.g.*, enzyme-linked immunosorbent assay; immunohistochemical assay; and the like). Alternatively, expression of nucleic acid encoding a cardiomyocyte-specific marker can be assessed. Expression of cardiomyocyte-specific marker-encoding nucleic acids in a cell can be confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) or hybridization analysis, molecular biological methods which have been commonly used in the past for amplifying, detecting and analyzing mRNA

coding for any marker proteins. Nucleic acid sequences coding for markers specific to cardiomyocytes are known and are available through public data bases such as GenBank; thus, marker-specific sequences needed for use as primers or probes is easily determined.

[0248] Induced cardiomyocytes can also exhibit spontaneous contraction. Whether an induced cardiomyocyte exhibits spontaneous contraction can be determined using standard electrophysiological methods (*e.g.*, patch clamp).

[0249] In some embodiments, induced cardiomyocytes can exhibit spontaneous Ca^{2+} oscillations. Ca^{2+} oscillations can be detected using standard methods, *e.g.*, using any of a variety of calcium-sensitive dyes, intracellular Ca^{2+} ion-detecting dyes include, but are not limited to, fura-2, bis-fura 2, indo-1, Quin-2, Quin-2 AM, Benzothiaza-1, Benzothiaza-2, indo-5F, Fura-FF, BTC, Mag-Fura-2, Mag-Fura-5, Mag-Indo-1, fluo-3, rhod-2, rhod-3, fura-4F, fura-5F, fura-6F, fluo-4, fluo-5F, fluo-5N, Oregon Green 488 BAPTA, Calcium Green, Calcein, Fura-C18, Calcium Green-C18, Calcium Orange, Calcium Crimson, Calcium Green-5N, Magnesium Green, Oregon Green 488 BAPTA-1, Oregon Green 488 BAPTA-2, X-rhod-1, Fura Red, Rhod-5F, Rhod-5N, X-Rhod-5N, Mag-Rhod-2, Mag-X- Rhod-1, Fluo-5N, Fluo-5F, Fluo-4FF, Mag-Fluo-4, Aequorin, dextran conjugates or any other derivatives of any of these dyes, and others (see, *e.g.*, the catalog or Internet site for Molecular Probes, Eugene, see, also, Nuccitelli, ed., Methods in Cell Biology, Volume 40: A Practical Guide to the Study of Calcium in Living Cells, Academic Press (1994); Lambert, ed., Calcium Signaling Protocols (Methods in Molecular Biology Volume 114), Humana Press (1999); W. T. Mason, ed., Fluorescent and Luminescent Probes for Biological Activity. A Practical Guide to Technology for Quantitative Real-Time Analysis, Second Ed, Academic Press (1999); Calcium Signaling Protocols (Methods in Molecular Biology), 2005, D.G. Lamber, ed., Humana Press.).

[0250] In some embodiments, an iCM is generated *in vitro*; and the iCM is introduced into an individual, *e.g.*, the iCM is implanted into a cardiac tissue of an individual in need thereof. A method of the present disclosure can comprise infecting a population of cardiac fibroblasts in vitro, to generate a population of iCMs; and the population of iCMs is implanted into a cardiac tissue of an individual in need thereof.

[0251] In some embodiments, an iCM is generated *in vivo*. For example, in some embodiments, an rAAV virion of the present disclosure that comprises a heterologous nucleic acid comprising a nucleotide sequence encoding one or more reprogrammning factors is administered to an individual. In some embodiments, the rAAV virion is administered directly

into cardiac tissue of an individual in need thereof. In some embodiments, from about 10^6 to about 10^5, from about 10^5 to about 10^9, from about 10^9 to about 10^{10}, from about 10^{10} to about 10^{11}, from about 10^{11} to about 10^{12}, from about 10^{12} to about 10^{13}, from about 10^{13} to about 10^{14}, from about 10^{14} to about 10^{15} genome copies, or more than 10^{15} genome copies, of an rAAV virion of the present disclosure that comprises a heterologous nucleic acid comprising a nucleotide sequence encoding one or more reprogramming factors are administered to an individual, *e.g.*, are administered directly into cardiac tissue in the individual or via another route of administration. The number of rAAV virions administered to an individual can be expressed in viral genomes (vg) per kilogram (kg) body weight of the individual. In some embodiments, and effective amount of an rAAV virion of the present disclosure is from about 10^2 vg/kg to 10^4 vg/kg, from about 10^4 vg/kg to about 10^6 vg/kg, from about 10^6 vg/kg to about 10^8 vg/kg, from about 10^8 vg/kg to about 10^{10} vg/kg, from about 10^{10} vg/kg to about 10^{12} vg/kg, from about 10^{12} vg/kg to about 10^{14} vg/kg, from about 10^{14} vg/kg to about 10^{14} vg/kg, from about 10^{14} vg/kg to about 10^{16} vg/kg, or more than 10^{16} vg/kg. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via intramyocardial injection through the epicardium. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via vascular delivery through the coronary artery. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through the superior vena cava. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through a peripheral vein.

[0252] The present disclosure provides a method of modifying ("editing") the genome of a cardiac cell. The present disclosure provides a method of modifying ("editing") the genome of a cardiac fibroblast. The present disclosure provides a method of modifying ("editing") the genome of a cardiomyocyte. The methods generally involve infecting a cardiac cell (*e.g.*, a cardiac fibroblast or a cardiomyocyte) with an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding a genome-editing endonuclease. In some embodiments, the method comprises infecting a cardiac fibroblast or a cardiomyocyte with an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding an RNA-guided genome -editing endonuclease. In some embodiments, the method comprises infecting a cardiac fibroblast or a cardiomyocyte with an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence

encoding: i) an RNA-guided genome-editing endonuclease; and ii) one or more guide RNAs. In some embodiments, the method comprises infecting a cardiac fibroblast or a cardiomyocyte with an rAAV virion of the present disclosure, where the rAAV virion comprises a heterologous nucleic acid comprising a nucleotide sequence encoding: i) an RNA-guided genome-editing endonuclease; ii) a guide RNAs; and iii) a donor template DNA. Suitable RNA-guided genome-editing endonucleases are described above.

[0253] In some embodiments, infecting a cardiac cell (*e.g.*, cardiac fibroblast; a cardiomyocyte) is carried out *in vitro*. In some embodiments, infecting a cardiac cell (*e.g.*, cardiac fibroblast; a cardiomyocyte) is carried out in vitro; and the infected cardiac cell (*e.g.*, cardiac fibroblast) is introduced into (*e.g.*, implanted into) an individual in need thereof, *e.g.*, directly into cardiac tissue of an individual in need thereof. For in vitro transduction, an effective amount of rAAV virions to be delivered to cells will be on the order of from about 10^s to about 10^{13} of the rAAV virions. Other effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves.

[0254] In some embodiments, infecting a cardiac cell (*e.g.*, cardiac fibroblast; a cardiomyocyte) is carried out in vivo. For example, in some embodiments, an effective amount of an rAAV virion of the present disclosure is administered directly into cardiac tissue of an individual in need thereof. An "effective amount" will fall in a relatively broad range that can be determined through experimentation and/or clinical trials. For example, for *in vivo* injection, *i.e.*, injection directly into cardiac tissue, a therapeutically effective dose will be on the order of from about 10^6 to about 10^{15} of the rAAV virions, *e.g.*, from about 10^{11} to 10^{12} rAAV virions, of the present disclosure. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via intramyocardial injection through the epicardium. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via vascular delivery through the coronary artery. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through the superior vena cava. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through a peripheral vein.

[0255] For example, from about 10^6 to about 10^7, from about 10^7 to about 10^8, from about 10^8 to about 10^9, from about 10^9 to about 10^{10}, from about 10^{10} to about 10^{11}, from about $l0^{11}$ to about 10^{12}, from about 10^{12} to about 10^{13}, from about 10^{13} to about 10^{14}, from about 10^{14} to about 10^{15} genome copies, or more than 10^{15} genome copies, of an rAAV virion of the present

disclosure are administered to an individual, *e.g.*, are administered directly into cardiac tissue in the individual. The number of rAAV virions administered to an individual can be expressed in viral genomes (vg) per kilogram (kg) body weight of the individual. In some embodiments, and effective amount of an rAAV virion of the present disclosure is from about 10^2 vg/kg to 10^4 vg/kg, from about 10^4 vg/kg to about 10^6 vg/kg, from about 10^6 vg/kg to about 10^8 vg/kg, from about 10^8 vg/kg to about 10^{10} vg/kg, from about 10^{10} vg/kg to about 10^{12} vg/kg, from about 10^{12} vg/kg to about 10^{14} vg/kg, from about 10^{14} vg/kg to about 10^{16} vg/kg, from about 10^{16} vg/kg to about 10^{18} vg/kg, or more than 10^{18} vg/kg. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via intramyocardial injection through the epicardium. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via vascular delivery through the coronary artery. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through the superior vena cava. In some embodiments, an effective amount of an rAAV virion of the present disclosure is administered via systemic delivery through a peripheral vein.

[0256] In some embodiments, the genome editing comprises homology-directed repair (HDR). In some embodiments, the HDR corrects a defect in an endogenous target nucleic acid in the cardiac fibroblast or the cardiomyocyte, where the defect is associated with, or leads to, a defect in structure and/or function of the cardiac fibroblast or the cardiomyocyte, or a component of the cardiac fibroblast or the cardiomyocyte.

[0257] In some embodiments, the genome editing comprises non-homologous end joining (NHEJ). In some embodiments, the NHEJ deletes a defect in an endogenous target nucleic acid in the cardiac fibroblast or the cardiomyocyte, where the defect is associated with, or leads to, a defect in structure and/or function of the cardiac fibroblast or the cardiomyocyte, or a component of the cardiac fibroblast or the cardiomyocyte.

[0258] A method of the present disclosure for editing the genome of a cardiac cell can be used to correct any of a variety of genetic defects that give rise to a cardiac disease or disorder. Mutations of interest include mutations in one or more of the following genes: cardiac troponin T (TNNT2); myosin heavy chain (MYH7); tropomyosin 1 (TPM1); myosin binding protein C (MYBPC3); 5'-AMP-activated protein kinase subunit gamma-2 (PRKAG2); troponin I type 3 (TNNI3); titin (TTN); myosin, light chain 2 (MYL2); actin, alpha cardiac muscle 1 (ACTC1); potassium voltage-gated channel, KQT- like subfamily, member 1 (KCNQ1);

plakophilin 2 (PKP2); myocyte enhancer factor 2c (MEF2C); and cardiac LIM protein (CSRP3). Specific mutations of interest include, without limitation, MYH7 R663H mutation; TNNT2 R173W; PKP2 2013delC mutation; PKP2 Q617X mutation; and KCNQ1 G269S missense mutation. Mutations of interest include mutations in one or more of the following genes: MYH6, ACTN2, SERCA2, GATA4, TBX5, MYOCD, NKX2-5, NOTCH1, MEF2C, HAND2, and HAND1. In some embodiments, the mutations of interest include mutations in the following genes: MEF2C, TBX5, and MYOCD. Cardiac diseases and disorders that can be treated with a method of the present disclosure include coronary heart disease, cardiomyopathy, endocarditis, congenital cardiovascular defects, and congestive heart failure. Cardiac diseases and disorders that can be treated with a method of the present disclosure include hypertrophic cardiomyopathy; a valvular heart disease; myocardial infarction; congestive heart failure; long QT syndrome; atrial arrhythmia; ventricular arrhythmia; diastolic heart failure; systolic heart failure; cardiac valve disease; cardiac valve calcification; left ventricular non-compaction; ventricular septal defect; and ischemia.

Methods of Treatment

[0259] The disclosure provides a methods of treating a cardiac pathology in a subject in need thereof, comprising administering a therapeutically effective amount of a pharmaceutical composition comprising an rAAV virion to the subject, wherein the rAAV virion transduces cardiac tissue.

[0260] Subjects in need of treatment using compositins and methods of the present disclosure include, but are not limited to, individuals having a congenital heart defect, individuals suffering from a degenerative muscle disease, individuals suffering from a condition that results in ischemic heart tissue (*e.g.*, individuals with coronary artery disease), and the like. In some examples, a method is useful to treat a degenerative muscle disease or condition (*e.g.*, familial cardiomyopathy, dilated cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, or coronary artery disease with resultant ischemic cardiomyopathy). In some examples, a subject method is useful to treat individuals having a cardiac or cardiovascular disease or disorder, for example, cardiovascular disease, aneurysm, angina, arrhythmia, atherosclerosis, cerebrovascular accident (stroke), cerebrovascular disease, congenital heart disease, congestive heart failure, myocarditis, valve disease coronary, artery disease dilated, diastolic dysfunction, endocarditis, high blood pressure (hypertension), cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, coronary artery disease with

resultant ischemic cardiomyopathy, mitral valve prolapse, myocardial infarction (heart attack), or venous thromboembolism.

[0261] Subjects suitable for treatment using the compositions, cells and methods of the present disclosure include individuals (*e.g.*, mammalian subjects, such as humans, non-human primates, domestic mammals, experimental non- human mammalian subjects such as mice, rats, etc.) having a cardiac condition including but limited to a condition that results in ischemic heart tissue (*e.g.*, individuals with coronary artery disease) and the like.

[0262] In some examples, an individual suitable for treatment suffers from a cardiac or cardiovascular disease or condition, *e.g.*, cardiovascular disease, aneurysm, angina, arrhythmia, atherosclerosis, cerebrovascular accident (stroke), cerebrovascular disease, congenital heart disease, congestive heart failure, myocarditis, valve disease coronary, artery disease dilated, diastolic dysfunction, endocarditis, high blood pressure (hypertension), cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, coronary artery disease with resultant ischemic cardiomyopathy, mitral valve prolapse, myocardial infarction (heart attack), or venous thromboembolism. In some examples, individuals suitable for treatment with a subject method include individuals who have a degenerative muscle disease, *e.g.*, familial cardiomyopathy, dilated cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, or coronary artery disease with resultant ischemic cardiomyopathy.

[0263] For example, the cardiac pathology can be selected from the group consisting of congestive heart failure, myocardial infarction, cardiac ischemia, myocarditis and arrhythmia. In some embodiments, the subject is diabetic. In some embodiments, the subject is non-diabetic. In some embodiments, the subject suffers from diabetic cardiomyopathy.

[0264] For therapy, the rAAV virions of the disclosure and/or pharmaceutical compositions thereof can be administered locally or systemically. An rAAV virion can be introduced by injection, catheter, implantable device, or the like. An rAAV virion can be administered in any physiologically acceptable excipient or carrier that does not adversely affect the cells. For example, rAAV virions of the disclosure and/or pharmaceutical compositions thereof can be administered intravenously or through an intracardiac route (*e.g.*, epicardially or intramyocardially). Methods of administering rAAV virions of the disclosure and/or pharmaceutical compositions thereof to subjects, particularly human subjects include injection or infusion of the pharmaceutical compositions (*e.g.*, compositions comprising rAAV virions). Injection may include direct muscle injection and infusion may include intravascular infusion.

The rAAV virions or pharmaceutical compositions can be inserted into a delivery device which facilitates introduction by injection into the subjects. Such delivery devices include tubes, *e.g.*, catheters, for injecting cells and fluids into the body of a recipient subject. The tubes can additionally include a needle, *e.g.*, a syringe, through which the cells of the invention can be introduced into the subject at a desired location.

[0265] In some embdoiments, the rAAV virion is administered by subcutaneous, intravenous, intramuscular, intraperitoneal, or intracardiac injection or by intracardiac catheterization. In some embdoiments, the rAAV virion is administered by direct intramyocardial injection or transvascular administration. In some embdoiments, the rAAV virion is administered by direct intramyocardial injection, antegrade intracoronary injection, retrograde injection, transendomyocardial injection, or molecular cardiac surgery with recirculating delivery (MCARD).

[0266] The rAAV virions can be inserted into such a delivery device, *e.g.*, a syringe, in different forms. The rAAV virion can be supplied in the form of a pharmaceutical composition. Such a composition can include an isotonic excipient prepared under sufficiently sterile conditions for human administration. For general principles in medicinal formulation, the reader is referred to *Cell Therapy: Stem Cell Transplantation*, *Gene Therapy*, *and Cellular Immunotherapy*, by G. Morstyn & W. Sheridan eds, Cambridge University Press, 1996; and *Hematopoietic Stem Cell Therapy*, E. D. Ball, J. Lister & P. Law, Churchill Livingstone, 2000. The choice of the excipient and any accompanying constituents of the composition can be adapted to optimize administration by the route and/or device employed.

[0267] Recombinant AAV may be administered locally or systemically. Recombinant AAV may be engineered to target specific cell types by selecting the appropriate capsid protein of the disclosure. To determine the suitability of various therapeutic administration regimens and dosages of AAV virion compositions, the rAAV virionscan first be tested in a suitable animal model. At one level, recombinant AAV are assessed for their ability to infect target cells *in vivo*. Recombinant AAV can also be assessed to ascertain whether it migrates to target tissues, whether they induce an immune response in the host, or to determine an appropriate number, or dosage, of rAAV virions to be administered. It may be desirable or undesirable for the recombinant AAV to generate an immune response, depending on the disease to be treated. Generally, if repeated administration of a virion is required, it will be advantageous if the virion is not immunogenic. For testing purposes, rAAV virion compositions can be administered to

immunodeficient animals (such as nude mice, or animals rendered immunodeficient chemically or by irradiation). Target tissues or cells can be harvested after a period of infection and assessed to determine if the tissues or cells have been infected and if the desired phenotype (*e.g.* induced cardiomyocyte) has been induced in the target tissue or cells.

[0268] Recombinant AAV virions can be administered by various routes, including without limitation direct injection into the heart or cardiac catetherization. Alternatively, the rAAV virions can be administered systemically such as by intravenous infusion. When direct injection is used, it may be performed either by open-heart surgey or by minimally invasive surgery. In some embodiments, the recombinant viruses are delivered to the pericardial space by injection or infusion. Injected or infused recombinant viruses can be traced by a variety of methods. For example, recombinant AAV labeled with or expressing a detectable label (such as green fluorescent protein, or beta-galactosidase) can readily be detected. The recombinant AAV may be engineered to cause the target cell to express a marker protein, such as a surface-expressed protein or a fluorescent protein. Alternatively, the infection of target cells with recombinant AAV can be detected by their expression of a cell marker that is not expressed by the animal employed for testing (for example, a human-specific antigen when injecting cells into an experimental animal). The presence and phenotype of the target cells can be assessed by fluorescence microscopy (*e.g.*, for green fluorescent protein, or beta-galactosidase), by immunohistochemistry (*e.g.*, using an antibody against a human antigen), by ELISA (using an antibody against a human antigen), or by RT-PCR analysis using primers and hybridization conditions that cause amplification to be specific for RNA indicative of a cardiac phenotype.

Pharmaceutical Compositions

[0269] The present disclosure provides pharmaceutical composition comprising an rAAV virion of the disclosure. The pharmaceutical composition may include one or more of a pharmaceutically acceptable carrier, diluent, excipient, and buffer. In some embodiments, the pharmaceutically acceptable carrier, diluent, excipient, or buffer is suitable for use in a human. Such excipients, carriers, diluents, and buffers include any pharmaceutical agent that can be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as pH

buffering substances may be present in such vehicles. A wide variety of pharmaceutically acceptable excipients are known in the art and need not be discussed in detail herein. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, 20th edition, Lippincott, Williams, & Wilkins; *Pharmaceutical Dosage Forms and Drug Delivery Systems* (1999) H.C. Ansel et al., eds., 7th ed., Lippincott, Williams, & Wilkins; and *Handbook of Pharmaceutical Excipients* (2000) A.H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc.

[0270] To prepare the composition, rAAV virion is generated and purified as necessary or desired. The rAAV can be mixed with or suspended in a pharmaceutically acceptable carrier. These rAAV can be adjusted to an appropriate concentration, and optionally combined with other agents. The concentation of rAAV virion and/or other agent included in a unit dose can vary widely. The dose and the number of administrations can be optimized by those skilled in the art. For example, about 10^2-10^{10} vector genomes (vg) may be administered. In some embdoiments, the dose be at least about 10^2 vg, about 10^3 vg, about 10^4 vg, about 10^5 vg, about 10^6 vg, about 10^7 vg, about 10^8 vg, about 10^9 vg, about 10^{10} vg, or more vector genomes. Daily doses of the compounds can vary as well. Such daily doses can range, for example, from at least about 10^2 vg/day, about 10^3 vg/day, about 10^4 vg/day, to about 10^5 vg/day, about 10^6 vg/day, about 10^7 vg/day, about 10^8 vg/day, about 10^9 vg/day, about 10^{10} vg/day, or more vector genomes per day.

[0271] In certain embodiments, the method of treatment is enhanced by the administration of one or more anti-inflammatory agents, *e.g.*, an anti-inflammatory steroid or a nonsteroidal antiinflammatory drug (NSAID).

[0272] Anti-inflammatory steroids for use in the invention include the corticosteroids, and in particular those with glucocorticoid activity, *e.g.*, dexamethasone and prednisone. Nonsteroidal anti-inflammatory drugs (NSAIDs) for use in the invention generally act by blocking the production of prostaglandins that cause inflammation and pain, cyclooxygenase-1 (COX- 1) and/or cyclooxygenase-2 (COX-2). Traditional NSAIDs work by blocking both COX-1 and COX-2. The COX-2 selective inhibitors block only the COX-2 enzyme. In certain embodiment, the NSAID is a COX-2 selective inhibitor, *e.g.*, celecoxib (Celebrex®), rofecoxib (Vioxx), and valdecoxib (B extra). In certain embodiments, the anti-inflammatory is an NSAID prostaglandin inhibitor, *e.g.*, Piroxicam.

[0273] The amount of rAAV virion for use in treatment will vary not only with the particular carrier selected but also with the route of administration, the nature of the condition being treated and the age and condition of the patient. Ultimately, the attendant health care provider may determine proper dosage. A pharmaceutical composition may be formulated with the appropriate ratio of each compound in a single unit dosage form for administration with or without cells. Cells or vectors can be separately provided and either mixed with a liquid solution of the compound composition, or administered separately.

[0274] Recombinant AAV can be formulated for parenteral administration (*e.g.*, by injection, for example, bolus injection or continuous infusion) and may be presented in unit dosage form in ampoules, prefilled syringes, small volume infusion containers or multi-dose containers with an added preservative. The pharmaceutical compositions can take the form of suspensions, solutions, or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Suitable carriers include saline solution, phosphate buffered saline, and other materials commonly used in the art.

[0275] The compositions can also contain other ingredients such as agents useful for treatment of cardiac diseases, conditions and injuries, such as, for example, an anticoagulant (*e.g.*, dalteparin (fragmin), danaparoid (orgaran), enoxaparin (lovenox), heparin, tinzaparin (innohep), and/or warfarin (coumadin)), an antiplatelet agent (*e.g.*, aspirin, ticlopidine, clopidogrel, or dipyridamole), an angiotensin-converting enzyme inhibitor (*e.g.*, Benazepril (Lotensin), Captopril (Capoten), Enalapril (Vasotec), Fosinopril (Monopril), Lisinopril (Prinivil, Zestril), Moexipril (Univasc), Perindopril (Aceon), Quinapril (Accupril), Ramipril (Altace), and/or Trandolapril (Mavik)), angiotensin II receptor blockers (*e.g.*, Candesartan (Atacand), Eprosartan (Teveten), Irbesartan (Avapro), Losartan (Cozaar), Telmisartan (Micardis), and/or Valsartan (Diovan)), a beta blocker (*e.g.*, Acebutolol (Sectral), Atenolol (Tenormin), Betaxolol (Kerlone), Bisoprolol/hydrochlorothiazide (Ziac), Bisoprolol (Zebeta), Carteolol (Cartrol), Metoprolol (Lopressor, Toprol XL), Nadolol (Corgard), Propranolol (Inderal), Sotalol (Betapace), and/or Timolol (Blocadren)), Calcium Channel Blockers (*e.g.*, Amlodipine (Norvasc, Lotrel), Bepridil (Vascor), Diltiazem (Cardizem, Tiazac), Felodipine (Plendil), Nifedipine (Adalat, Procardia), Nimodipine (Nimotop), Nisoldipine (Sular), Verapamil (Calan, Isoptin, Verelan), diuretics (e.g, Amiloride (Midamor), Bumetanide (Bumex), Chlorothiazide (Diuril), Chlorthalidone (Hygroton), Furosemide (Lasix), Hydrochlorothiazide (Esidrix, Hydrodiuril), Indapamide (Lozol) and/or Spironolactone (Aldactone)), vasodilators (*e.g.*, Isosorbide dinitrate (Isordil), Nesiritide (Natrecor), Hydralazine

(Apresoline), Nitrates and/or Minoxidil), statins, nicotinic acid, gemfibrozil, clofibrate, Digoxin, Digitoxin, Lanoxin, or any combination thereof.

[0276] Additional agents can also be included such as antibacterial agents, antimicrobial agents, anti-viral agents, biological response modifiers, growth factors; immune modulators, monoclonal antibodies and/or preservatives. The compositions of the invention may also be used in conjunction with other forms of therapy.

[0277] The rAAV virions described herein can be administered to a subject to treat a disease or disorder. Such a composition may be in a single dose, in multiple doses, in a continuous or intermittent manner, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is in response to traumatic injury or for more sustained therapeutic purposes, and other factors known to skilled practitioners. The administration of the compounds and compositions of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated. In some embodiments, localized delivery of rAAV virion is achieved. In some embodiments, localized delivery of rAAV virions is used to generate a population of cells within the heart. In some embodiments, such a localized population operates as "pacemaker cells" for the heart. In some embodiments, the rAAV virions are used to generate, regenerate, repair, replace, and/or rejunevate one or more of a sinoatrial (SA) node, an atrioventricular (AV) node, a bindle of His, and/or Purkinje fibres.

[0278] To control tonicity, an aqueous pharmaceutical composition can comprise a physiological salt, such as a sodium salt. Sodium chloride (NaCl) is preferred, which may be present at between 1 and 20 mg/ml. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride and calcium chloride.

[0279] Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer; or a citrate buffer. Buffers will typically be included at a concentration in the 5-20 mM range. The pH of a composition will generally be between 5 and 8, and more typically between 6 and 8 *e.g.* between 6.5 and 7.5, or between 7.0 and 7.8.

[0280] The composition is preferably sterile. The composition is preferably gluten free. The composition is preferably non-pyrogenic.

[0281] In some embodiments, a composition comprising cells may include a cryoprotectant agent. Non-limiting examples of cryoprotectant agents include a glycol (*e.g.*, ethylene glycol, propylene glycol, and glycerol), dimethyl sulfoxide (DMSO), formamide, sucrose, trehalose, dextrose, and any combinations thereof.

[0282] One or more of the following types of compounds can also be present in the composition with the rAAV virions: a WNT agonist, a GSK3 inhibitor, a TGF-beta signaling inhibitor, an epigenetic modifier, LSD1 inhibitor, an adenylyl cyclase agonist, or any combination thereof.

Kits

[0283] A variety of kits are described herein that include any of composition (*e.g.* rAAV virions) described herein. The kit can include any of compositions described herein, either mixed together or individually packaged, and in dry or hydrated form. The rAAV virions and/or other agents described herein can be packaged separately into discrete vials, bottles or other containers. Alternatively, any of the rAAV virions and/or agents described herein can be packaged together as a single composition, or as two or more compositions that can be used together or separately. The compounds and/or agents described herein can be packaged in appropriate ratios and/or amounts to facilitate conversion of selected cells across differentiation boundaries to form cardiac progenitor cells and/or cardiomyocytes.

[0284] The kit can include instructions for administering those compositions, compounds and/or agents. Such instructions can provide the information described throughout this application. The rAAV virion or pharmaceutical composition can be provided within any of the kits in the form of a delivery device. Alternatively a delivery device can be separately included in the kits, and the instructions can describe how to assemble the delivery device prior to administration to a subject.

[0285] Any of the kits can also include syringes, catheters, scalpels, sterile containers for sample or cell collection, diluents, pharmaceutically acceptable carriers, and the like. The kits can provide other factors such as any of the supplementary factors or drugs described herein for the compositions in the preceding section or other parts of the application.

[0286] The following non-limiting Examples illustrate some of the experimental work involved in developing the invention.

EXAMPLES

Example 1: Identification of Variable Region Modified AAV9 Capsid

Library Generation and AAV Selection

[0287] Variable regions (VR-IV, VR-V, VR-VII and VR-VIII sites) on the AAV9 capsid are shown in **FIG. 1**. A library screening strategy was employed to generate highly diverse libraries through randomly altering residues within each site of AAV9 capsid to identify AAV variants with improved transduction efficiency and/or selectivity towards cardiac tissue, as shown in **FIG. 2**. Briefly, individual libraries were generated for each variable region along with a library consisting of combinations of all VR-modified libraries. These libraries were independently subjected to three rounds of directed evolution. The first round of evolution was performed in hiPSC-CMs in order to select for human trophic variants. The remaining two rounds of directed evolution were carried through systemic delivery of the libraries in αMHC-Cre mice in order to select for cardiotrophic variants that can transcytose through the endothelial barrier of the heart and transduce cardiomyocytes following systemic delivery. Following three rounds of directed evolution, each library exhibited varying levels of convergence, as shown in **FIG. 3A-3D** and **4A-4D**.

Confirmation of Increased Transduction Efficiency In Vitro and In Vivo

[0288] The top variants from each library were first assessed for their ability to transduce human iPSC-CMs in vitro. Cells were infected with each variant packaging a ubiquitously expressing GFP reporter at a MOI of 100,000. VR-IV modified AAV capsids exhibited significantly enhanced hiPSC-CM transduction compared to AAV9, with CR9-01 displaying a 129-fold increase in transduction efficiency (**FIG. 5**). Subsequently, variants were assessed *in vivo* for their ability to transduce the heart following systemic delivery. To this end, C57BL/6J mice were injected with 2.5E+11 vg/mouse of either AAV9:CAG-GFP or CAG-GFP encapsulated by a novel capsid variant. Seven days following injection, animals were sacrificed, and the heart and liver were recovered for GFP expression analysis by ELISA. Variants from each library displayed increased cardiac transduction compared to AAV9 (**FIGs. 6A-6B**). Most variants with increased cardiac transduction also exhibited reduced liver tropism (**FIGs. 6C-6D**) leading to the identification of capsids with improved cardiac specificity. Finally, the top performing AAV capsid variants were assessed for their ability to evade human NAbs. Variants packaging CAG-GFP were incubated with increasing concentrations of pooled human IgG for 30 minutes before treating HEK293T cells at a MOI of 100,000. Fourty-eigth hours post-

infection, GFP expression was assessed by flow cytometry (**FIG. 7A**). Two variants, CR9-07 and CR9-13 displayed improved NAb evasion compared to unmodified AAV9 (**FIG. 7B-7C**).

Example 2: Identification of AAV5/9 Chimeric Capsid

[0289] Capsid shuffling was used to identify AAV5/9 chimeric protein variants with improved cardiomyocyte tropism. An AAV5/9 chimeric library was generated in order to identify chimeras with the favorable properties of AAV5 and AAV9 (decreased liver tropism, low NAb susceptibility, transcytosis and/or cardiac transduction), as shown in **FIG. 8**. Following one round of *in vivo* selection, the library complexity was dramatically reduced, resulting in less than 100 variants that were capable of transducing the heart following systemic delivery of the parental library (**FIG. 9**). The majority of crossover events occurred within the VP1 region of the capsid, with the majority of variants having AAV9 dominated VP3 regions. hiPSC-CMs were then infected at a MOI of 75,000 either by AAV9:CAG-GFP or CAG-GFP encapsulated by a chimeric AAV5/9 capsid. Seventy-two hours after infection, cells were harvested, and the transduction efficiency of each capsid was evaluated using flow cytometry (**FIG. 10A**). ZC44 exhibited a improvement in transduction efficiency in hiPSC-CM cells compared to AAV9.

[0290] AAV5/9 chimeric capsids were assessed for their ability to evade human NAbs. Here, variants packaging CAG-GFP were incubated with 1 mg/mL of pooled human IgG for 30 minutes before treating HEK293T cells at a MOI of 100,000. 48 post-infection, GFP expression was assessed by flow cytometry.In addition to increased transduction efficiency, ZC44 showed decreased susceptibility to NAbs compared to AAV9 (**FIG. 10B**).

[0291] The top chimeric variants were assessed *in vivo* for their ability to transduce heart and liver following systemic delivery. C57BL/6J mice were injected with 2E+11 vg/mouse of either AAV9:CAG-GFP or CAG-GFP encapsulated by a novel capsid variant. Fourteen days following injection, animals were sacrificed, and the heart and the liver were recovered for GFP expression analysis by ELISA. ZC47 displayed increased heart transduction compared to AAV9; ZC40, ZC44, and ZC49 retained an ability to transduce heart tissue *in vivo* equivalent to parental AAV9 (**FIG. 11A**). Z40, Z41, Z46, and Z47 each exhibited liver de-targeting as evidenced by the pronounced reduction in liver transduction following systemic delivery (**FIG. 11B**).

Example 3: Identification of Combinatory Capsid

[0292] Modifications within the variable region modified capsids and the chimeric capsids were combined to generate additional combinatory capsid variants. A total of 18 combinatory variants containing AAV5-derieved VP1 sequences and modified variable regions were generated (**FIG. 12**). Midi-scale vector production was produced to assess the manufacturability of the AAV capsid variants (**FIG. 13**). The transduction efficiency of the combinatory variants in hiPSC-CMs were then evaluated as follows: Cells were infected at a MOI of 75,000 either by AAV9:CAG-GFP or a combinatory AAV capsid packaging CAG-GFP. 5 days following infection, cells were harvested, and the transduction efficiency of each capsid was evaluated using Cytation 5 cell imaging reader. TN44-07 and TN47-07 displayed transduction superior to AAV9 (>15-fold) (**FIG. 14**).

[0293] Combinatory variants were assessed *in vivo* for their ability to transduce the heart following systemic delivery. C57BL/6J mice were injected with 1E+11 vg/mouse of either AAV9:CAG-GFP or a combinatory capsid variant packaging the same transgene cassette. Fourteen days following injection, animals were sacrificed, and the heart and liver were recovered for GFP expression analysis by ELISA. TN44-07 and TN47-10, showed improvement in cardiac transduction compared to AAV9; TN47-14 transduced the heart at a similar level as AAV9 (**FIG. 15A**). Remarkably, TN47-14 lost almost all tropism for the liver, as evidenced by the highly significant reduction in GFP expression in the liver (**FIG. 15B**). Both TN47-10 and TN47-14 have an improved ratio of heart to liver transduction compared to AAV9 following systemic delivery (**FIG. 15C**). Selected combinatory capsid variants were also evaluated for human NAb evasion. AAV9 or a capsid variant packaging CAG-GFP was incubated for 30 minutes at 37 ^{o}C in the absence or presence (600 ug/mL) of pooled human IgG from ~2500 patients. Following the incubation, virus was incubated with HEK293T cells at an MOI of 100,000. The following day, media was replenished, and the cells were incubated for an additional 24 hours to allow for adequate GFP expression. GFP expression was quantified by flow cytometry and transduction was normalized to the no IgG control. All these combinatory AAV capsid variants (TN44-07, TN47-07, TN47-10, TN47-13 and TN47-14) demonstrated improved evasion from neturalizing antibodies in pooled human IgG. Among them, virions containing TN44-07 combinatory capsid were the most stealth, having a highly significant reduction of NAb neutralization ($p = 0.0002$, t-test, Welch's correction) compared to AAV9 (**FIG. 16**).

Example 4: Testing in Non-Human Primates

[0294] Transduction of recombinant AAV mediated by a panel of engineered capsids was assessed in male cynomolgus macaques (*Macaca fascicularis*) following intravenous delivery of each capsid packaging CAG-GFP at a dose of $1x10^{12}$ vg/kg (n=3/group). Thirty days following dosing, animals were euthanized and organs harvested for analysis of specific transduction.

[0295] In order to evaluate the organ-specific transduction profile of novel engineered capsid variants in a high throughput manner, an approach using RNA barcode sequencing was employed. Each capsid (two parental & 12 novel variants) was assigned a unique barcode that was placed in the 3'-UTR of a ubiquitously expressing GFP cassette (CAG-GFP). Each barcode selected was experimentally determined to have no effect on protein expression/RNA stability. rAAV was individually manufactured for each capsid in order to link the barcode to the capsid and all rAAV preparations were pooled together at equal concentrations. Male cynomolgus macaques (2-5 years in age) were dosed with either $1x10^{13}$ vg/kg of the pooled viral library (n=3) or sham treated with HBSS (n=1). Thirty days following the injection date, animals were sacrificed, and tissue collected for RNA analysis. The relative abundance of each barcode was determined through next-generation sequencing of GFP-barcode transcripts using the Illumina NextSeq550 and a custom python script. Relative expression of the barcodes for each tissue was normalized to AAV9. The results are shown in **FIG. 17**.

[0296] Transduction of rAAV containing engineered capsid was assessed in the left ventricle and liver of the animals. The TN3 and TN6 engineered capsids showed the highest transduction in the left ventricle compared to AAV9 (**FIG. 17A**). While TN6 engineered capsid showed liver transduction comparable to AAV9, TN3 showed substantially less transduction in liver relative to AAV9 (**FIG. 17B**). The left ventricle (LV) to liver transduction ratio for each engineered capsid is shown in **FIG. 17C**. The TN3 engineered capsid shows the most favorable transduction profile. It has high LV transduction while maintaining low transduction in the liver. rAAV-induced toxicity due to off-target transduction in the liver can be a challenge in clinical applications, and the transduction profile shown by the TN3 engineered capsid may overcome this limitation. A broader transduction profile was generated by analyzing rAAV transduction mediated by the panel of engineered capsids in a range of tissues (**FIG. 17D**).

Claims

What is claimed is:

1. A recombinant adeno-associated virus (rAAV) capsid protein, comprising a variant polypeptide sequence at one or more of a VR-IV site, a VR-V site, a VR-VII site, and a VR-VIII site of a parental sequence, wherein the parental sequence comprises a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463.

2. The capsid protein of claim 1, wherein the variant polypeptide sequence is a cardiotrophic variant polypeptide sequence.

3. The capsid protein of claim 1 or 2, wherein the capsid protein comprises a variant polypeptide at the VR-IV site of the parental sequence.

4. The capsid protein of claim 3, wherein the variant polypeptide at the VR-IV site has a sequence:

$$-X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-$$

wherein:

a) X_1 is G, S or V;
b) X_2 is Y, Q or I;
c) X_3 is H, W, V or I;
d) X_4 is K or N;
e) X_5 is S, G or I;
f) X_6 is G or R;
g) X_7 is A, P or V;
h) X_8 is A or R; and
i) X_9 is Q or D (SEQ ID NO: 477).

5. The capsid protein of claim 3, wherein the variant polypeptide at the VR-IV site comprises an amino acid sequence selected from SEQ ID NOs: 6-104.

6. The capsid protein of claim 3, wherein the variant polypeptide at the VR-IV site comprises an amino acid sequence selected from GYHKSGAAQ (SEQ ID NO: 6),

~~VIIKSGAAQ (SEQ ID NO: 7), GYHKIGAAQ (SEQ ID NO: 8), SQVNGRPRD (SEQ ID NO: 33) and GYHKSGVAQ (SEQ ID NO: 9).~~

~~7. The capsid protein of claim 3, wherein the variant polypeptide at the VR-IV site comprises the amino acid sequence GYHKSGAAQ (SEQ ID NO: 6) or a sequence comprising at most 1, 2, 3, or 4 amino acid substitutions relative to GYHKSGAAQ (SEQ ID NO: 6).~~

~~8. The capsid protein of any one of claims 1-7, wherein the capsid protein comprises a variant polypeptide at the VR-V site of the parental sequence.~~

~~9. The capsid protein of claim 8, wherein the variant polypeptide at the VR-V site has a sequence:~~

~~X_1-X_2-X_3-X_4-X_5-X_6-~~

~~wherein:~~

~~a) X_1 is S, L, H, N, or A;~~
~~b) X_2 is T, M, K, G, or N;~~
~~c) X_3 is S, T, M or I;~~
~~d) X_4 is S, P, F, M, or N;~~
~~e) X_5 is F, S, P or L; and~~
~~f) X_6 is I, V, or T (SEQ ID NO: 474).~~

~~10. The capsid protein of claim 8, wherein the variant polypeptide at the VR-V site comprises an amino acid sequence selected from SEQ ID NOs: 105-203.~~

~~11. The capsid protein of claim 8, wherein the variant polypeptide at the VR-V site comprises an amino acid sequence selected from LNSMLI (SEQ ID NO: 105), NGMSFT (SEQ ID NO: 106) , HKTFSI (SEQ ID NO: 107) and SMSNFV (SEQ ID NO: 108).~~

~~12. The capsid protein of claim 8, wherein the variant polypeptide at the VR-V site comprises the amino acid sequence LNSMLI (SEQ ID NO: 105) or a sequence comprising at most 1, 2, 3, or 4 amino acid substitutions relative to LNSMLI (SEQ ID NO: 105).~~

13. The capsid protein of any one of claims 1-12, wherein the capsid protein comprises a variant polypeptide at the VR-VII site of the parental sequence.

14. The capsid protein of claim 13, wherein the variant polypeptide at the VR-VII site has a sequence:

$-X_1-X_2-X_3-X_4-X_5-$

wherein:

a) X_1 is V, L, Q, C, or R;
b) X_2 is S, H, G, C, or D;
c) X_3 is Y, S, L, G, or N;
d) X_4 is S, L, H, Q, or N; and
e) X_5 is V, I, or R (SEQ ID NO: 475).

15. The capsid protein of claim 13, wherein the variant polypeptide at the VR-VII site comprises an amino acid sequence selected from SEQ ID NOs: 204-302.

16. The capsid protein of claim 13, wherein the variant polypeptide at the VR-VII site comprises an amino acid sequence selected from RGNQV (SEQ ID NO: 204), VSLNR (SEQ ID NO: 205), CDYSV (SEQ ID NO: 206), and QHGHI (SEQ ID NO: 207).

17. The capsid protein of claim 13, wherein the variant polypeptide at the VR-VII site comprises the amino acid sequence RGNQV (SEQ ID NO: 204) or a sequence comprising at most 1, 2, or 3 amino acid substitutions relative to RGNQV (SEQ ID NO: 204).

18. The capsid protein of any one of claims 1-17, wherein the capsid protein comprises a variant polypeptide at the VR-VII site of the parental sequence.

19. The capsid protein of claim 18, wherein the variant polypeptide at the VR-VIII site has a sequence:

$-X_1-X_2-X_3-X_4-$

wherein:

a) X_1 is S, N, or A;

b) X_2 is V, M, N, or A;

c) X_3 is Y, V, S, or G; and

d) X_4 is Y, T, M, G, or N (SEQ ID NO: 476).

20. The capsid protein of claim 18, wherein the variant polypeptide at the VR-VIII site comprises an amino acid sequence selected from SEQ ID NOs: 303-401.

21. The capsid protein of claim 18, wherein the variant polypeptide at the VR-VIII site comprises an amino acid sequence selected from ANYG (SEQ ID NO: 305), NVSY (SEQ ID NO: 303), SMVN (SEQ ID NO: 304), and NVGT (SEQ ID NO: 306).

22. The capsid protein of claim 18, wherein the variant polypeptide at the VR-VIII site comprises the amino acid sequence ANYG (SEQ ID NO: 305) or a sequence comprising at most 1 or 2 amino acid substitutions relative to ANYG (SEQ ID NO: 305).

23. The capsid protein of claim 18, wherein the variant polypeptide at the VR-VIII site comprises the amino acid sequence NVSY (SEQ ID NO: 303) or a sequence comprising at most 1 or 2 amino acid substitutions relative to NVSY (SEQ ID NO: 303).

24. The capsid protein of claim 1, wherein the capsid protein comprises an amino acid sequence at least 95% identical to a sequence selected from SEQ ID NOs: 402-410.

25. The capsid protein of claim 1, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 402.

26. The capsid protein of claim 1, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 403.

27. The capsid protein of claim 1, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 404.

28. The capsid protein of claim 1, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 406.

~~29. The capsid protein of claim 1, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 409.~~

~~30. The capsid protein of claim 1, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 483.~~

~~31. The capsid protein of any one of claims 1-23, wherein the capsid protein is a AAV5/AAV9 chimeric capsid protein.~~

~~32. The capsid protein of claim 31, wherein the capsid protein comprises at least one segment from an AAV5 capsid protein.~~

~~33. The capsid protein of claim 31 or 32, wherein the capsid protein comprises:~~

~~a) a first segment comprising a sequence at least 95% identical to SEQ ID NO: 411 or SEQ ID NO: 412;~~
~~b) a second segment comprising a sequence at least 95% identical to SEQ ID NO: 413 or SEQ ID NO: 414;~~
~~c) a third segment comprising a sequence at least 95% identical to SEQ ID NO: 415 or SEQ ID NO: 416;~~
~~d) a fourth segment comprising a sequence at least 95% identical to SEQ ID NO: 417 or SEQ ID NO: 418;~~
~~e) a fifth segment comprising a sequence at least 95% identical to SEQ ID NO: 419 or SEQ ID NO: 420;~~

~~wherein at least one segment is from an AAV5 capsid protein and at least one segment is from an AAV9 capsid protein.~~

~~34. The capsid protein of claim 31, wherein the chimeric capsid protein comprises an amino acid sequence at least 95% identical to a sequence selected from SEQ ID NOs: 445-462.~~

~~35. The capsid protein of claim 31, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 457.~~

~~36. The capsid protein of claim 31, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 459.~~

~~37. The capsid protein of claim 31, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 445.~~

~~38. The capsid protein of claim 31, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 446.~~

~~39. The capsid protein of claim 31, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 447.~~

~~40. The capsid protein of claim 31, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 448.~~

~~41. A recombinant adeno-associated virus (rAAV) capsid protein, comprising a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463.~~

~~42. The capsid protein of claim 41, wherein the variant polypeptide sequence is a cardiotrophic variant polypeptide sequence.~~

~~43. The capsid protein of claim 41 or 42, wherein the capsid protein comprises at least one segment from an AAV5 capsid protein.~~

~~44. The capsid protein of any one of claims 41-43, wherein the capsid protein comprises:~~

- ~~f) a first segment comprising a sequence at least 95% identical to SEQ ID NO: 411 or SEQ ID NO: 412;~~
- ~~g) a second segment comprising a sequence at least 95% identical to SEQ ID NO: 413 or SEQ ID NO: 414;~~
- ~~h) a third segment comprising a sequence at least 95% identical to SEQ ID NO: 415 or SEQ ID NO: 416;~~
- ~~i) a fourth segment comprising a sequence at least 95% identical to SEQ ID NO: 417 or SEQ ID NO: 418;~~
- ~~j) a fifth segment comprising a sequence at least 95% identical to SEQ ID NO: 419 or SEQ ID NO: 420.~~

~~wherein at least one segment is from an AAV5 capsid protein and at least one segment from an AAV9 capsid protein.~~

~~45. The capsid protein of any one of claims 41-44, wherein the chimeric capsid protein comprising a sequence at least 95% identical to a sequence selected from SEQ ID NOs: 421-444.~~

~~46. The capsid protein of claim 45, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 434.~~

~~47. The capsid protein of claim 45, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 438.~~

~~48. The capsid protein of claim 45, wherein the capsid protein comprises an amino acid sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 441.~~

~~49. A recombinant adeno-associated virus (rAAV) virion, comprising:~~

~~a) the capsid protein of any one of claims 1 to 48; and~~

~~b) a heterologous nucleic acid comprising a nucleotide sequence encoding one or more gene products.~~

~~50. The rAAV virion of claim 49, wherein the rAAV virion exhibits increased transduction efficiency in cardiac cells compared to an AAV virion comprising the parental sequence.~~

~~51. The rAAV virion of claim 49 or 50, wherein the rAAV virion exhibits increased transduction efficiency in induced pluripotent stem cell-derived cardiomyocyte (iPS-CM) cells compared to an AAV virion comprising the parental sequence.~~

~~52. The rAAV virion of any one of claims 49-51, wherein the rAAV virion exhibits increased transduction efficiency in human cardiac fibroblast (hCF) cells compared to an AAV virion comprising the parental sequence.~~

~~53. The rAAV virion of claim 52, wherein the human cardiac fibroblasts are located in the left ventricle of the heart.~~

~~54. The rAAV virion of claim 51, wherein the rAAV virion exhibits at least 2-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 100,000.~~

~~55. The rAAV virion of claim 51, wherein the rAAV virion exhibits at least 2-fold increased transduction efficiency in iPS-CM cells at a multiplicity of infection (MOI) of 75,000.~~

~~56. The rAAV virion of any one of claims 49-55, wherein the rAAV virion exhibits at least 2-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 2.5E+11 vg/mouse.~~

~~57. The rAAV virion of any one of claims 49-55, wherein the rAAV virion exhibits at least 1.5-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 2E+11 vg/mouse.~~

~~58. The rAAV virion of any one of claims 49-55, wherein the rAAV virion exhibits at least 2-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 1E+11 vg/mouse.~~

~~59. The rAAV virion of any one of claims 49-58, wherein the rAAV virion exhibits decreased transduction efficiency in liver cells compared to an AAV virion comprising the parental sequence.~~

~~60. The rAAV virion of any one of claims 49-59, wherein the rAAV virion exhibits improved NAb evasion compared to an AAV virion comprising the parental sequence.~~

~~61. The rAAV virion of any one of claims 49-60, wherein the rAAV virion exhibits increased selectivity of the rAAV virion for cardiac cells over liver cells.~~

~~62. The rAAV virion of any one of claims 49-61, wherein the rAAV virion exhibits increased selectivity of the rAAV virion for iPS-CM cells over liver cells.~~

~~63. The rAAV virion of any one of claims 49-62, wherein the capsid protein comprises a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 404.~~

~~64. The rAAV virion of any one of claims 49-62, wherein the capsid protein comprises a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 483.~~

~~65. A pharmaceutical composition comprising the rAAV virion of any one of claims 49-64 and a pharmaceutically acceptable carrier.~~

66. A polynucleotide encoding the capsid protein of any one of claims 1-64.

67. A method of transducing a cardiac cell, comprising contacting the cardiac cell with an rAAV virion according to any one of claims 49-64, wherein the rAAV virion transduces the cardiac cell.

68. The method of claim 67, wherein the cardiac cell is a cardiomyocyte.

69. The method of claim 67 or 68, wherein the rAAV virion exhibits increased transduction efficiency in the cell compared to an AAV virion comprising AAV9 capsid protein sequence.

70. The method of any one of claims 67-69, wherein the rAAV virion exhibits at least 2-fold increased transduction efficiency in the cell compared to an AAV viron comprising AAV9 capsid protein sequence at a multiplicity of infection (MOI) of 75,000.

71. A method of delivering one or more gene products to a cardiac cell, comprising contacting the cardiac cell with an rAAV virion according to any one of claims 49-64.

72. The method of claim 71, wherein the cardiac cell is a cardiomyocyte.

73. A method of treating a cardiac pathology in a subject in need thereof, comprising administering a therapeutically effective amount of a rAAV virion of any one of claims 49-64 or a pharmaceutical composition of claim 61 to the subject, wherein the rAAV virion transduces cardiac tissue.

74. The method of claim 73, wherein the one or more gene products comprise MYBPC3, DWORF, KCNH2, TRPM4, DSG2, PKP2 and/or ATP2A2.

75. The method of claim 73, wherein the one or more gene products comprise CACNA1C, DMD, DMPK, EPG5, EVC, EVC2, FBN1, NF1, SCN5A, SOS1, NPR1, ERBB4, VIP, MYH7, and/or Cas9.

76. The method of claim 73, wherein the one or more gene products comrpise MYOCD, ASCL1, GATA4, MEF2C, TBX5, miR-133, and/or MESP1.

77. A kit comprising the pharmaceutical composition of claim 67 and instructions for use.

1. A recombinant adeno-associated virus (rAAV) capsid protein, comprising a variant polypeptide sequence at one or more of a VR-IV site, a VR-V site, a VR-VII site, and a VR-VIII site of a parental sequence, wherein the parental sequence comprises a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 463.

2. The capsid protein of claim 1, wherein the variant polypeptide sequence (i) is a cardiotrophic variant polypeptide sequence, (ii) exhibits increased transduction efficiency in cardiac cells compared to the parental sequence, (iii) exhibits decreased transduction efficiency in liver cells compared to the parental sequence, and/or (iv) exhibits increased selectivity for the cardiac cells over liver cells compared to the parental sequence.

3. The capsid protein of claim 1 or 2, wherein the capsid protein comprises a variant polypeptide at the VR-IV site of the parental sequence.

4. The capsid protein of claim 3, wherein the variant polypeptide at the VR-IV site has a sequence:

$$-X_1-X_2-X_3-X_4-X_5-X_6-X_7-X_8-X_9-$$

wherein:

a) X_1 is G, S or V;
b) X_2 is Y, Q or I;
c) X_3 is H, W, V or I;
d) X_4 is K or N;
e) X_5 is S, G or I;
f) X_6 is G or R;
g) X_7 is A, P or V;
h) X_8 is A or R; and
i) X_9 is Q or D (SEQ ID NO: 477);

or wherein the variant polypeptide at the VR-IV site comprises an amino acid sequence selected from SEQ ID NOs: 6-104;

or wherein the variant polypeptide at the VR-IV site comprises an amino acid sequence selected from GYHKSGAAQ (SEQ ID NO: 6), VIIKSGAAQ (SEQ ID NO: 7), GYHKIGAAQ (SEQ ID NO: 8), SQVNGRPRD (SEQ ID NO: 33),GYHKSGVAQ (SEQ ID NO: 9), and

a sequence comprising at most 1, 2, 3, or 4 amino-acid substitutions relative to GYHKSGAAQ (SEQ ID NO: 6).

5. The capsid protein of claim 1 or 2, wherein the capsid protein comprises a variant polypeptide at the VR-V site of the parental sequence.

6. The capsid protein of claim 5, wherein the variant polypeptide at the VR-V site has a sequence:

$$-X_1-X_2-X_3-X_4-X_5-X_6-$$

wherein:

a) X_1 is S, L, H, N, or A;
b) X_2 is T, M, K, G, or N;
c) X_3 is S, T, M or I;
d) X_4 is S, P, F, M, or N;
e) X_5 is F, S, P or L; and
f) X_6 is I, V, or T (SEQ ID NO: 474);

or wherein the variant polypeptide at the VR-V site comprises an amino acid sequence selected from SEQ ID NOs: 105-203;

or wherein the variant polypeptide at the VR-V site comprises an amino acid sequence selected from LNSMLI (SEQ ID NO: 105), NGMSFT (SEQ ID NO: 106) , HKTFSI (SEQ ID NO: 107), SMSNFV (SEQ ID NO: 108), and

a sequence comprising at most 1, 2, 3, or 4 amino-acid substitutions relative to LNSMLI (SEQ ID NO: 105).

7. The capsid protein of claim 1 or 2, wherein the capsid protein comprises a variant polypeptide at the VR-VII site of the parental sequence.

8. The capsid protein of claim 7, wherein the variant polypeptide at the VR-VII site has a sequence:

$$-X_1-X_2-X_3-X_4-X_5-$$

wherein:

a) X_1 is V, L, Q, C, or R;
b) X_2 is S, H, G, C, or D;
c) X_3 is Y, S, L, G, or N;
d) X_4 is S, L, H, Q, or N; and
e) X_5 is V, I, or R (SEQ ID NO: 475);

or wherein the variant polypeptide at the VR-VII site comprises an amino acid sequence selected from SEQ ID NOs: 204-302;

or wherein the variant polypeptide at the VR-VII site comprises an amino acid sequence selected from RGNQV (SEQ ID NO: 204), VSLNR (SEQ ID NO: 205), CDYSV (SEQ ID NO: 206), QHGHI (SEQ ID NO: 207), and

a sequence comprising at most 1, 2, or 3 amino-acid substitutions relative to RGNQV (SEQ ID NO: 204).

9. The capsid protein of claim 1 or 2, wherein the capsid protein comprises a variant polypeptide at the VR-VIII site of the parental sequence.

10. The capsid protein of claim 9, wherein the variant polypeptide at the VR-VIII site has a sequence:

$$-X_1-X_2-X_3-X_4-$$

wherein:

a) X_1 is S, N, or A;
b) X_2 is V, M, N, or A;
c) X_3 is Y, V, S, or G; and
d) X_4 is Y, T, M, G, or N (SEQ ID NO: 476);

or wherein the variant polypeptide at the VR-VIII site comprises an amino acid sequence selected from SEQ ID NOs: 303-401;

or wherein the variant polypeptide at the VR-VIII site comprises an amino acid sequence selected from ANYG (SEQ ID NO: 305), NVSY (SEQ ID NO: 303), SMVN (SEQ ID NO: 304), NVGT (SEQ ID NO: 306),

a sequence comprising at most 1 or 2 amino-acid substitutions relative to ANYG (SEQ ID NO: 305), and

a sequence comprising at most 1 or 2 amino-acid substitutions relative to NVSY (SEQ ID NO: 303).

11. The capsid protein of claim 1 or 2, wherein the capsid protein comprises an amino acid sequence at least 95% identical to a sequence selected from SEQ ID NOs: 402-410 and SEQ ID NO:483, optionally wherein the sequence is SEQ ID NO:402, SEQ ID NO:403, SEQ ID NO:404, SEQ ID NO:406, SEQ ID NO:409, or SEQ ID NO:483.

12. The capsid protein of claim 1 or 2, wherein the capsid protein is a AAV5/AAV9 chimeric capsid protein, optionally wherein the capsid protein comprises at least one segment from an AAV5 capsid protein.

13. The capsid protein of claim 12, wherein the capsid protein comprises:

a) a first segment comprising a sequence at least 95% identical to SEQ ID NO: 411 or SEQ ID NO: 412;
b) a second segment comprising a sequence at least 95% identical to SEQ ID NO: 413 or SEQ ID NO: 414;
c) a third segment comprising a sequence at least 95% identical to SEQ ID NO: 415 or SEQ ID NO: 416;
d) a fourth segment comprising a sequence at least 95% identical to SEQ ID NO: 417 or SEQ ID NO: 418;
e) a fifth segment comprising a sequence at least 95% identical to SEQ ID NO: 419 or SEQ ID NO: 420;

wherein at least one segment is from an AAV5 capsid protein and at least one segment is from an AAV9 capsid protein.

14. The capsid protein of claim 12, wherein the chimeric capsid protein comprises an amino acid sequence at least 95% identical to a sequence selected from SEQ ID NOs: 445-463, optionally wherein the capsid protein comprises an amino acid sequence at least 95% identical to SEQ ID NO:457, SEQ ID NO:459, SEQ ID NO:445, SEQ ID NO;446, SEQ ID NO:447, SEQ ID NO:448 or SEQ ID NO:463;

and/or wherein the chimeric capsid protein comprising an amino acid sequence at least 95% identical to a sequence selected from SEQ ID NOs: 421-444, optionally wherein the capsid protein comprises an amino acid sequence at least 95% identical to SEQ ID NO:434, SEQ ID NO:438, or SEQ ID NO:441.

15. A recombinant adeno-associated virus (rAAV) virion, comprising:
a) the capsid protein of any one of claims 1 to 14; and
b) a heterologous nucleic acid comprising a nucleotide sequence encoding one or more gene products.

16. The rAAV virion of claim 15, wherein the rAAV virion exhibits increased transduction efficiency in cardiac cells compared to an AAV virion comprising the parental sequence;

and/or wherein the rAAV virion exhibits increased transduction efficiency in induced pluripotent stem cell-derived cardiomyocyte (iPS-CM) cells compared to an AAV virion comprising the parental sequence, optionally wherein the rAAV virion exhibits at least 2-fold increased transduction efficiency in iPS-CM cells at (i) a multiplicity of infection (MOI) of 100,000, or (ii) a multiplicity of infection (MOI) of 75,000;

and/or wherein the rAAV virion exhibits increased transduction efficiency in human cardiac fibroblast (hCF) cells compared to an AAV virion comprising the parental sequence,

optionally wherein the human cardiac fibroblasts are located in the left ventricle of the heart.

17. The rAAV virion of claim 15 or 16, wherein:

(i) the rAAV virion exhibits at least 2-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 2.5E+11 vg/mouse;

(ii) the rAAV virion exhibits at least 1.5-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 2E+11 vg/mouse; or

(iii) the rAAV virion exhibits at least 2-fold increased cardiac transduction efficiency in a C57BL/6J mouse, wherein the mouse is injected with a virion dosage of 1E+11 vg/mouse.

18. The rAAV virion of any one of claims 15 to 17, wherein the rAAV virion exhibits decreased transduction efficiency in liver cells compared to an AAV virion comprising the parental sequence;

or wherein the rAAV virion exhibits improved NAb evasion compared to an AAV virion comprising the parental sequence;

or wherein the rAAV virion exhibits increased selectivity of the rAAV virion for (i) cardiac cells over liver cells, or

(ii) iPS-CM cells over liver cells.

19. The rAAV virion of any one of claims claim 15 to 18, wherein the capsid protein comprises a sequence at least 95%, at least 98%, at least 99%, or 100% identical to SEQ ID NO: 404 or SEQ ID NO: 483.

20. A pharmaceutical composition comprising the rAAV virion of any one of claims 15 to 19 and a pharmaceutically acceptable carrier.

21. A polynucleotide encoding the capsid protein of any one of claims 1 to 14.

22. An *in vitro* or *ex vivo* method of transducing, or delivering one or more gene products to, a cardiac cell or a cardiomyocyte, comprising contacting the cardiac cell or the cardiomyocyte with an rAAV virion according to any one of claims 15 to 19.

23. The method of claim 22, wherein the rAAV virion exhibits increased transduction efficiency in the cell compared to an AAV virion comprising AAV9 capsid protein sequence, optionally

wherein the rAAV virion exhibits at least 2-fold increased transduction efficiency in the cell compared to an AAV viron comprising AAV9 capsid protein sequence at a multiplicity of infection (MOI) of 75,000.

24. The rAAV virion of any one of claims 15 to 19 or the pharmaceutical composition of claim 20 for use as a medicament for treating a cardiac pathology in a subject in need thereof, wherein a therapeutically effective amount of the rAAV virion is to be administered to the subject, wherein the rAAV virion transduces cardiac tissue.

25. The rAAV virion or the pharmaceutical composition of claim 24, wherein the one or more gene products comprise MYBPC3, DWORF, KCNH2, TRPM4, DSG2, PKP2 and/or ATP2A2;

or wherein the one or more gene products comprise CACNA1C, DMD, DMPK, EPG5, EVC, EVC2, FBN1, NF1, SCN5A, SOS1, NPR1, ERBB4, VIP, MYH7, and/or Cas9;

or wherein the one or more gene products comprise MYOCD, ASCL1, GATA4, MEF2C, TBX5, miR-133, and/or MESP1.

26. Use of the rAAV virion according to any one of claims 15 to 19 in the manufacture of a medicament for transducing, or delivering one or more gene products to, a cardiac cell or a cardiomyocyte.

27. Use of the rAAV virion of any one of claims 15 to 19 in the manufacture of a medicament for treating a cardiac pathology in a subject in need thereof, wherein a therapeutically effective amount of the medicament is to be administered to the subject, wherein the rAAV virion transduces cardiac tissue.