

**COMPOSITION AND METHODS FOR IDENTIFICATION OF NOVEL AAV VARIANTS
FOR DIFFERENTIATED GENE DELIVERY TO CARDIOMYOCYTES AND/OR SKELETAL
MYOFIBERS**

FIELD OF INVENTION

5 The present invention relates to the provision of recombinant adeno-associated virus (rAAV)
-based delivery vectors comprising short peptide inserts of at least 5 amino acid length, which
show enhanced transduction efficiency in one or more muscle cells, particularly in
cardiomyocytes and/or skeletal muscle cells. The rAAV vectors of the present disclosure are
suitable for use in the treatment and/or prophylaxis of a cardiac-related and/or muscle-related
10 disease or disorder and/or for use in gene therapy. Also provided are methods of treating
cardiac-related and/or muscle-related diseases or disorders.

BACKGROUND

Adeno-associated viruses (AAVs) have attracted widespread interest and are rapidly
emerging as new therapeutic delivery vectors for monogenic disorders due to their established
15 safety profile, as exemplified by various AAV-based therapies approved by the FDA in recent
years.

The efficient delivery of genes via AAV into specific cell types is necessary for therapeutic
effect, but some cell types are currently not efficiently targeted. There are many attempts in
the last decade to improve AAV vectors for tissue targeting as well as de-targeting from the
20 liver to improve gene delivery efficacy and to decrease the chance of toxicity and
immunological rejection. To date, there are only a limited number of AAV vectors with validated
improved performance to target tissues. As highlighted by the results from a recent clinical
trial in patients with cardiac diseases, the bio-distribution analyses of AAV1-SERCA2a
suggest that less than 1% of cardiomyocytes contained the vector genome, implying that the
25 AAV1 vector applied via antegrade intracoronary infusion at a dose of 10^{13} viral genomic
sequence (vgs) transduces cardiomyocytes at very low efficiency (Greenberg B et al., 2016).
Furthermore, there are still concerns over the immunogenicity and toxicity of high AAV vector
doses which may result in complications and failure of therapy in some patients. To improve
on the safety and efficacy profile, more efficient AAV capsids which can allow lower dose
30 usage and lower risk of complications or failure of the gene therapy in patients are needed.

Inherited cardiomyopathy and skeletal muscle dystrophies are some of the diseases that have
yet to benefit clinically from improved performance of gene delivery vectors. Monogenic
cardiovascular diseases (MCVDs) can be severe and life threatening. They include

arrhythmias, such as Brugada syndrome caused by mutations in the SCN5A gene, and cardiomyopathies, such as arrhythmogenic right ventricular cardiomyopathy (ARVC), caused by mutations in DSC2, DSG2, DSP, JUP, PKP2, and TMEM43. Diseases affecting skeletal muscles, such as Duchenne muscular dystrophy (DMD), also typically affect the heart as the disease progresses. However, severe cardiac involvement is rare in some dystrophies such as limb girdle muscle dystrophy (LGMD), which is caused by mutations in the CAPN3 gene. Developing tissue-specific vectors will also help to reduce unwanted off-target transduction in non-target tissues.

Accordingly, there is a significant need to provide improved AAV-based therapeutic delivery vectors in the treatment and management of monogenic disorders that overcome, or at least ameliorate, one or more of the disadvantages described above.

SUMMARY OF INVENTION

The present invention relates to the provision of improved recombinant AAV (rAAV) based vectors suitable for use in the treatment and/or prophylaxis of cardiac-related and/or muscle-related diseases or disorders.

Accordingly in a first aspect, there is provided a recombinant adeno-associated virus (rAAV) particle comprising a viral shell comprising a modified AAV capsid protein, wherein the modified AAV capsid protein comprises an extraneous peptide sequence comprising a length of at least 5 amino acids, wherein the extraneous peptide sequence enhances/increases the transduction efficiency of the rAAV particle into one or more muscle cell(s), compared to the transduction efficiency of a wild-type AAV particle not comprising the extraneous peptide sequence.

In some embodiments, the rAAV particle of the present disclosure may be a recombinant adeno-associated virus serotype 1 (rAAV1) particle and the extraneous peptide sequence may substitute and replace the amino acid residue at position 590 of the AAV capsid protein.

Further, the one or more muscle cell(s) may be one or more cardiomyocyte(s) and/or one or more skeletal myofiber cell(s). In this regard, the extraneous peptide sequence may preferably comprise the amino acid sequence set forth in any one of SEQ ID NO: 1 to SEQ ID NO: 65.

According to a second aspect, there is provided a recombinant polynucleic acid molecule encoding a modified AAV capsid protein, wherein the modified AAV capsid protein comprises an extraneous peptide sequence having a length of at least 5 amino acids. In some embodiments, the recombinant polynucleic acid molecule may be operatively linked to a mammalian promoter sequence.

According to a third aspect, there is provided a pharmaceutical composition comprising the rAAV particle according to the first aspect and a pharmaceutically acceptable carrier.

According to a fourth aspect, there is provided the rAAV particle according to the first aspect, for use in medicine.

- 5 According to a fifth aspect, there is provided the rAAV particle according to the first aspect, for use in treating a cardiac-related and/or muscle-related disease or disorder.

According to a sixth aspect, there is provided a method of treating a disease or disorder, the method comprises administering the rAAV particle according to the first aspect, to a subject in need thereof. Preferably, the disease or disorder is a cardiac-related and/or muscle-related
10 disease or disorder.

According to a seventh aspect, there is provided a use of the rAAV particle according to the first aspect in the manufacture of a medicament for the treatment of a cardiac-related and/or muscle-related disease or disorder in a subject.

In this regard, the cardiac-related and/or muscle-related disease or disorder may comprise
15 inherited cardiomyopathy, skeletal muscle dystrophy, monogenic cardiovascular diseases (MCVDs) such as arrhythmia and Brugada syndrome, cardiomyopathies such as arrhythmogenic right ventricular cardiomyopathy (ARVC), Duchenne muscular dystrophy (DMD) and limb girdle muscle dystrophy (LGMD).

According to an eighth aspect, there is provided a method of delivering a gene product to one
20 or more cell(s), the method comprising contacting the rAAV particle according to the first aspect with the cell, optionally wherein the method is an *in vitro* method.

According to a ninth aspect, there is provided a use of the rAAV particle according to the first aspect for the preparation of a pharmaceutical composition for gene therapy.

Advantageously, the rAAV particles of the present disclosure provide enhanced transduction
25 efficiency into muscle cells, particularly in cardiomyocytes, skeletal myofibers or both. More advantageously, the improved rAAV particles of the present disclosure may reduce off-target transduction, and thus may allow for lower dose application and may lower the risk of adverse effects (such as immune responses against the engineered vectors) and/or failure of gene therapy. These and other advantages of the present disclosure will become readily apparent
30 to those skilled in this art from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

The accompanying drawings illustrate disclosed embodiments and serve to explain the principles of the disclosed embodiments. It is to be understood, however, that the drawings are designed for purposes of illustration only, and not as a definition of the limits of the invention.

FIGS. 1A-1G depict the construction of AAV1 variant libraries and diversity analysis. (A) Workflow for the construction and production of AAV1 diverse variants library, followed by *in vivo* selection of variants for improved transduction performance for cardiomyocytes and skeletal myofibers. (B) Schematic of Counting Peptide Inserts (CoPI) pipeline for short peptide insert analysis as applied for AAVs. (C) Extracted reads from flanking anchor sequences. Highlighted sequences (grey, underlined) are the seq_start and seq_end within the reference. The sequence in-between the anchor sequence are added to the count and cached for further downstream analyses. (D) Plots generated from CoPI for distribution of peptide insert lengths to estimate percentage representation of wild-type AAV1, 7-mer and 10-mer AAV1 capsid variants within the constructed library. (E) In-frame amino acid distribution of the inserts for estimation of depth of diversity for the 7-mer random peptides in the AAV1 virus library. (F) In-frame amino acid distribution of the inserts for estimation of depth of diversity for the 10-mer random peptides in the AAV1 virus library. (G) AAV1 variants selection and bioinformatics analysis. Formulas for calculation of transduction fold change from NGS data.

FIGS. 2A – 2B show tables comparing selected engineered AAV variants of the present disclosure against engineered AAVs of the prior art for (A) cardiomyocytes and (B) skeletal muscles. The selected AAV1 variants of the present disclosure for cardiomyocytes tropism performed better, up to 102-fold over parent and with improved cardio/muscle specificity of up to 212-fold and muscle de-targeting of up to 0.17-fold. In most of the cases in the prior art as listed in Fig. 2A, the transduction performance improved by not more than 25-fold and with improved cardio/muscle specificity of not more than 6.25-fold, and without muscle de-targeting being investigated. Similarly, the selected AAV1 variants of the present disclosure for skeletal muscles tropism also performed better, up to 108-fold over parent and with improved muscle/cardio specificity of up to 43.21-fold and cardiomyocytes de-targeting of up to 0.57-fold. In most of the other cases in the prior art as listed in Fig. 2B, the transduction performance improved by not more than 23-fold and with improved muscle/cardio specificity of not more than 0.16-fold, and without cardiomyocytes de-targeting being investigated.

DETAILED DESCRIPTION OF THE INVENTION

Bibliographic references mentioned in the present specification are for convenience listed in the form of a list of references and added at the end of the examples. The whole content of such bibliographic references is herein incorporated by reference.

5 Definitions

For convenience, certain terms employed in the specification, examples and appended claims are collected here.

In general, technical, scientific and medical terminologies used herein has the same meaning as understood by those skilled in the art to which this invention belongs. Further, the following
10 technical comments and definitions are provided. These definitions should in no way limit the scope of the present invention to those terms alone, but are put forth for a better understanding of the following description.

As used herein, “a” or “an” may mean one or more than one unless indicated to the contrary or otherwise evident from the context.

15 As used herein, the term “comprising” or “including” is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. However, in context with the present disclosure, the term “comprising” or “including” also includes “consisting of”. The variations of the word “comprising”, such as
20 “comprise” and “comprises”, and “including”, such as “include” and “includes”, have correspondingly varied meanings.

As used herein, the term “peptide” and “polypeptide” are used interchangeably to denote a polymer of at least two amino acids covalently linked by an amide bond, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

25 Accordingly, the term “extraneous peptide sequence” as used herein refers to any peptide sequence which is foreign from and not found in the known wild-type AAV capsid protein, which is then introduced into said AAV capsid protein. In this regard, the extraneous peptide sequence may be of any length, for example, at least 5 amino acids in length or more. The extraneous peptide sequence may also comprise amino acid sequences which are randomly
30 generated.

As used herein, the term "subject" is herein defined as vertebrate, particularly mammal, more particularly human. For purposes of research, the subject may particularly be at least one animal model, e.g., a mouse, rat and the like. In particular, for treatment or prophylaxis of cardiac-related and/or muscle-related diseases or disorders, the subject may be a human.

- 5 The term "treatment", as used in the context of the invention refers to ameliorating, therapeutic or curative treatment.

A description of exemplary, non-limiting embodiments of the invention follows.

- The present invention is based, in part, on the discovery that insertion of a short peptide sequence into a viral capsid protein may mediate viral re-targeting to specific cell types and/or
10 improve the efficiency of transduction. In this regard, the inventors have successfully generated a panel of recombinant AAV vectors comprising short extraneous peptide inserts with enhanced specific-tissue transduction efficacy into muscle cells, particularly cardiomyocytes and/or skeletal myofibers, suitable for use in the treatment and/or prophylaxis of cardiac-related and/or muscle-related diseases or disorders.

- 15 Accordingly in one aspect, there is provided a recombinant adeno-associated virus (rAAV) particle comprising a viral shell comprising a modified AAV capsid protein, wherein the modified AAV capsid protein comprises an extraneous peptide sequence comprising a length of at least 5 amino acids, wherein the extraneous peptide sequence enhances/increases the transduction efficiency of the rAAV particle into one or more muscle cell(s), compared to the
20 transduction efficiency of a wild-type AAV particle not comprising the extraneous peptide sequence.

- In this regard, the rAAV particle described herein may be derived from an AAV particle of different serotypes, for example, AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, or AAV9 serotype. In some embodiments, the rAAV particle may be an rAAV1, rAAV6, rAAV7, rAAV8,
25 or a rAAV9 particle. Preferably, the rAAV particle is a rAAV1 particle.

- It will be appreciated that an AAV particle may comprise a capsid (which is the protein shell of the virus) enclosing a nucleic acid molecule(s) within. For the purposes of the present invention, the capsid protein of a rAAV particle disclosed herein may include an extraneous peptide sequence not found in a known wild-type AAV capsid sequence. For example, a
30 rAAV1 particle of the present invention may comprise an extraneous peptide sequence not found in a wild-type AAV1 particle.

Accordingly in some embodiments, the extraneous peptide sequence may substitute and replace the amino acid residue at position 590 of the wild-type AAV capsid protein. For example, the extraneous peptide sequence may substitute and replace the amino acid residue D590 of the wild-type AAV1 capsid protein (SEQ ID NO: 66).

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- It will be appreciated that the extraneous peptide sequence may vary in length. For the purposes of the present invention, the extraneous peptide sequence may comprise a length of at least 5 amino acids. In some embodiments, the extraneous peptide sequence may comprise a length of at least 7 amino acids. In some embodiments, the extraneous peptide sequence may comprise a length of 5 amino acids, 6 amino acids, 7 amino acids, 8 amino
- 10
- acids, 9 amino acids, 10 amino acids, 11 amino acids, 12 amino acids, 13 amino acids, 14 amino acids, or 15 amino acids. Preferably, the extraneous peptide sequence may comprise a length of 7 amino acids or a length of 10 amino acids.

Table 1. Amino acid sequences of the extraneous peptide sequences of the present invention

SEQ ID NO:	AMINO ACID SEQUENCE	SEQ ID NO:	AMINO ACID SEQUENCE
1	GINIGVA	34	SCKQRVC
2	KDPGPCG	35	GMLYTLY
3	DICRQRL	36	DYVPLGL
4	ATVTWGS	37	GGNVRLS
5	KWRVDSV	38	LYYMFNE
6	AWCQCES	39	RPFVSS
7	LIVIGAG	40	EGYSNGV
8	VNIRVDV	41	LCVSRYR
9	DVRGAGG	42	DVILVGA
10	HGCVSYA	43	HREELVL
11	FYANSWG	44	IRCGCLR
12	NRGHEWM	45	DTFCGYV
13	CDEGKPA	46	ERFLSCT
14	MFVWSWC	47	GARDITS

15	LGRSRDE	48	WRGGLGR
16	QGEEASE	49	SVERNCS
17	DLRNVVV	50	IVITDLW
18	RLVFTQQ	51	TGSATLG
19	VGTHSPV	52	LVEPLHD
20	RVFDSKS	53	VDLTETM
21	LIGKSMR	54	DDHSNGR
22	EAQPVLF	55	SLFSTIS
23	LERCQLL	56	KGWVRFC
24	AHAIWIG	57	ALESVTW
25	CFWSGKL	58	VHKGHSEYPL
26	MNLTEHG	59	NGGVICISMS
27	GNDLDKG	60	GSRLLPKIS
28	IGKGQFV	61	LLGTDILFP
29	YSVHDWK	62	VSAETESWVS
30	EANPTEY	63	EEPLPVLRGF
31	NQQSQSG	64	IFIHRCFSHL
32	IGSCKGK	65	HYILTLRHYG
33	CRISHLD		

Table 2. Amino acid sequence of AAV1 wild-type capsid protein

AAV1 wild-type capsid protein SEQ ID NO. 66 (736 amino acids length)	MAADGYLPDW	LEDNLSEGIR	EWDDLKPGAP	KPKANQOKQD
	DGRGLVLPGY	KYLGPFNGLD	KGEPVNAADA	AALEHDKAYD
	QQLKAGDNPY	LRYNHADAEF	QERLQEDTSF	GGNLGRAVFQ
	AKKRVLEPLG	LVEEGAKTAP	GKKRPVEQSP	QEPDSSSGIG
	KTGQQPAKKR	LNFGQTGDSE	SVPDPQPLGE	PPATPAAVGP
	TTMASGGGAP	MADNNEGADG	VGNASGNWHC	DSTWLGDRI
	TTSTRTWALP	TYNNHLYKQI	SSASTGASND	NHYFGYSTPW
	GYFDENRFHC	HFSPRDWQRL	INNNGWFRPK	RLNFKLFNIQ
	VKEVTTNDGV	TTIANNLTST	VQVFSDSEYQ	LPYVLGSAHQ
	GCLPPFPADV	FMIPQYGYLT	LNNGSQAVGR	SSFYCLEYFP

	SQMLRTGNNF	TFSYTFEEVP	FHSSYAHSQS	LDRLMNPLID
	QYLYYLNRTQ	NQSGSAQNKD	LLFSRGSPAG	MSVQPKNWLP
	GPCYRQQRVS	KTKTDNNNSN	FTWTGASKYN	LNGRESIINP
	GTAMASHKDD	EDKFFPMSGV	MIFGKESAGA	SNTALDNVMI
	TDEEEIKATN	PVATERFGTV	AVNFQSSSTD	PATGDVHAMG
	ALPGMVWQDR	DVYLQGPIWA	KIPHTDGHFH	PSPLMGGFGL
	KNPPPQILIK	NTPVPANPPA	EFSA TKFASF	ITQYSTGQVS
	VEIEWELQKE	NSKRWNPEVQ	YTSNYAKSAN	VDFTVDNNGL
	YTEPRPIGTR	YLTRPL		

Advantageously, the rAAV particles disclosed herein provide enhanced transduction efficiency in muscle cells, and particularly in cardiomyocytes, skeletal myofibers, or both. Accordingly in some embodiments, the one or more muscle cell(s) may comprise one or more cardiomyocyte(s) and/or one or more skeletal myofiber cell(s). In some embodiments, the one or more muscle cell(s) may comprise one or more mammalian muscle cell(s).

In some embodiments, the extraneous peptide sequence may comprise the amino acid sequence set forth in any one of SEQ ID NO: 1 to SEQ ID NO: 65.

In certain embodiments, the rAAV particles may provide enhanced transduction efficiency in cardiomyocytes. Accordingly, the one or more muscle cell(s) may comprise one or more cardiomyocyte(s). Preferably, the extraneous peptide sequence in these embodiments may comprise the amino acid sequence set forth in any one of SEQ ID NO: 1 to SEQ ID NO: 32, or SEQ ID NO: 58 to SEQ ID NO: 62.

In other particular embodiments, the rAAV particles may provide enhanced transduction efficiency in skeletal myofibers. Accordingly, the one or more muscle cell(s) may comprise one or more skeletal myofiber cell(s). Preferably, the extraneous peptide sequence in these particular embodiments may comprise the amino acid sequence set forth in any one of SEQ ID NO: 33 to SEQ ID NO: 54, or SEQ ID NO: 63 to SEQ ID NO: 65.

In other further embodiments, the rAAV particles may provide enhanced transduction efficiency in both cardiomyocytes and skeletal myofibers. As such, the one or more muscle cell(s) may comprise one or more cardiomyocyte(s) and skeletal myofiber cell(s). In this regard, the extraneous peptide sequence in these further embodiments may preferably comprise the amino acid sequence set forth in any one of SEQ ID NO: 55 to SEQ ID NO: 57.

According to another aspect, there is provided a recombinant polynucleic acid molecule encoding a modified AAV capsid protein, wherein the modified AAV capsid protein comprises an extraneous peptide sequence having a length of at least 5 amino acids.

5 Preferably, the extraneous peptide sequence in the modified AAV capsid protein encoded by the recombinant polynucleic acid molecule comprises a peptide sequence having a length of at least 7 amino acids. More preferably, the extraneous peptide sequence comprises a peptide sequence having a length of 7 amino acids or a length of 10 amino acids. More preferably, the extraneous peptide sequence comprises the amino acid sequence set forth in any one of **SEQ ID NO: 1 to SEQ ID NO: 65**.

10 In some embodiments, the recombinant polynucleic acid molecule may be operatively linked to a mammalian promoter sequence.

It will further be appreciated that the nucleic acid molecule within the rAAV particle may comprise a recombinant nucleic acid sequence comprising an extraneous nucleic acid sequence encoding the capsid protein with the extraneous peptide sequence. It will also
15 further be appreciated that the nucleic acid molecule of the rAAV particle may further comprise further extraneous nucleic acid sequence(s); for example for gene therapy.

According to another aspect, there is provided a pharmaceutical composition comprising the rAAV particle disclosed herein and a pharmaceutically acceptable carrier.

Suitable pharmaceutical carriers typically will contain inert ingredients that do not interact with
20 the agent or active ingredient. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% 10 mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's lactate and the like. Formulations can also include small amounts of substances that enhance the effectiveness of the active ingredient (e.g., emulsifying agents, solubilizing agents, pH
25 buffering agents, wetting agents). Methods of encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art. For inhalation, the agent can be solubilized and loaded into a suitable dispenser for administration (e.g., anatomizer or nebulizer or pressurized aerosol dispenser).

30 According to a further aspect, there is provided the rAAV particle disclosed herein for use in medicine.

According to another aspect, there is provided the rAAV particle disclosed herein for use in treating a cardiac-related and/or muscle-related disease or disorder.

According to another aspect, there is provided a method of treating a disease or disorder, the method comprises administering the rAAV particle disclosed herein, to a subject in need thereof. In some embodiments, the disease or disorder is a cardiac-related and/or muscle-related disease or disorder.

- 5 According to a further aspect, there is provided a use of the rAAV particle disclosed herein in the manufacture of a medicament for the treatment of a cardiac-related and/or muscle-related disease or disorder in a subject.

In some embodiments, the cardiac-related and/or muscle-related disease or disorder may comprise inherited cardiomyopathy, skeletal muscle dystrophy, monogenic cardiovascular
10 diseases (MCVDs) such as arrhythmia and Brugada syndrome, cardiomyopathies such as arrhythmogenic right ventricular cardiomyopathy (ARVC), Duchenne muscular dystrophy (DMD) and limb girdle muscle dystrophy (LGMD).

According to a further aspect, there is provided a method of delivering a gene product to one or more cell(s), the method comprising contacting the rAAV particle of the present disclosure
15 with the cell, optionally wherein the method is an *in vitro* method.

According to another aspect, there is provided a use of the rAAV particle of the present disclosure for the preparation of a pharmaceutical composition for gene therapy.

In some embodiments, the rAAV particle disclosed herein may be suitable for nucleic acid (prophylactics or therapeutics) delivery to treat human cardiac-related or muscle-related
20 genetic disorders.

In other embodiments, the rAAV particle disclosed herein may also be suitable for nucleic acid (prophylactics or therapeutics) delivery into other cell populations that are susceptible to AAV1 and its variants.

For *in vivo* delivery, the rAAV of the present disclosure can be delivered to a subject in need
25 thereof by a variety of routes of administration including, for example, oral, nasal, dietary, topical, transdermal, or parenteral (e.g., intra-arterial, intravenous, intramuscular, subcutaneous injection, intradermal injection) routes of administration. Administration can be local or systemic. Preferably, the medicament is formulated for subcutaneous or intravenous administration. The actual dose of a therapeutic amount of the pharmaceutical composition
30 disclosed herein and treatment regimen can be determined by a skilled physician, taking into account the nature of the condition being treated, and patient characteristics.

Unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or

subrange within the stated ranges in various embodiments, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise. "About" in reference to a numerical value generally refers to a range of values that fall within $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$ of the value unless otherwise stated or otherwise evident from the context. In any embodiment in which a numerical value is prefaced by "about", an embodiment in which the exact value is recited is provided. Where an embodiment in which a numerical value is not prefaced by "about" is provided, an embodiment in which the value is prefaced by "about" is also provided. Where a range is preceded by "about", embodiments are provided in which "about" applies to the lower limit and to the upper limit of the range or to either the lower or the upper limit, unless the context clearly dictates otherwise. Where a phrase such as "at least", "up to", "no more than", or similar phrases, precedes a series of numbers, it is to be understood that the phrase applies to each number in the list in various embodiments (it being understood that, depending on the context, 100% of a value, e.g., a value expressed as a percentage, may be an upper limit), unless the context clearly dictates otherwise. For example, "at least 1, 2, or 3" should be understood to mean "at least 1, at least 2, or at least 3" in various embodiments. It will also be understood that any and all reasonable lower limits and upper limits are expressly contemplated.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Example 1: Methods and Materials

1. Library Construction

For the construction of the current AAV1 diverse capsids libraries, random 10-mer and 7-mer peptides (IDT, Coralville, Iowa, USA) were used to substitute residues D590 in the capsid sequence, the fragments of the random insertions were cloned into the cargo capsid sequence using XcmI and SbfI restriction enzyme sites. The libraries uses a split Rep/Cap system in which the original pRepCap contains several stop codons to prevent the expression of capsid protein. In the cargo plasmid, Rep sequence containing P40 sequence and the full capsid sequence were cloned between the ITR sequences. With the non-obvious introduction of a mammalian promoter upstream of the diverse capsid sequences and a polyA tail introduced at the downstream of the diverse capsid sequences, this allow us to express the RNA transcripts of the capsid libraries in the mammalian tissues and capture them using oligodT

sequences (FIG. 1A). Capsid libraries are generated by substitution of D590 with random 21-mer or 30-mer oligonucleotides which codes for random 7-mer or 10-mer peptides. The engineered capsid sequences are packaged in AAV vectors and used for transduction of mammalian cells or tissue at low dose of 1×10^8 total viral genomes (vgs) per mice.

5 2. AAV Production

AAV variants in the library each bearing its own capsid sequence were produced as per standard protocol. Briefly, AAV were packaged via a triple transfection of 293AAV cell line (Cell Biolabs AAV-100) that were plated in a HYPERFlask 'M' (Corning) in growth media consisting of DMEM + glutaMax + pyruvate + 10%FBS (Thermo Fisher), supplemented with
10 1X MEM non-essential amino acids (Gibco). Confluency at transfection was between 70–90%. Media was replaced with fresh pre-warmed growth media before transfection. For each HYPERFlask 'M', 200 µg of pHelper (Cell Biolabs), 100 µg of pRep(stop) and 100 µg of pZac-CMV-P40-Cap1-PolyA [self-encoding diverse capsids in the libraries] were mixed in 5 ml of DMEM, and 2 mg of PEI "MAX" (Polysciences) (40 kDa, 1 mg/ml in H₂O, pH 7.1) added for
15 PEI: DNA mass ratio of 5:1. The mixture was incubated for 15 min, and transferred drop-wise to the cell media. The day after transfection, media was changed to DMEM + glutamax + pyruvate + 2%FBS. Cells were harvested 48–72 hrs after transfection by scrapping or dissociation with 1×PBS (pH7.2) + 5 mM EDTA, and pelleted at 1500 g for 12 min. Cell pellets were resuspended in 1–5 ml of lysis buffer (Tris HCl pH 7.5 + 2 mM MgCl + 150 mM NaCl),
20 and freeze-thawed 3× between dry-ice-ethanol bath and 37 °C water bath. Cell debris was clarified via 4000 g for 5 min, and the supernatant collected. The collected supernatant was treated with 50 U/ml of Benzonase (Sigma-Aldrich) and 1 U/ml of RNase cocktail (Invitrogen) for 30 min at 37 °C to remove unpackaged nucleic acids. After incubation, the lysate was loaded on top of a discontinuous density gradient consisting of 6 ml each of 15%, 25%, 40%,
25 60% Optiprep (Sigma-Aldrich) in an 29.9 ml Optiseal polypropylene tube (Beckman-Coulter). The tubes were ultra-centrifuged at 54000 rpm, at 18 °C, for 1.5 hr, on a Type 70 Ti rotor. The 40% fraction was extracted, and dialyzed with 1×PBS (pH 7.2) supplemented with 35 mM NaCl, using Amicon Ultra-15 (100 kDa MWCO) (Millipore). The titer of the purified AAV vector stocks were determined using real-time qPCR with ITR sequence- specific primers and probe
30 (Aurnhammer C. et al., 2012), referenced against the ATCC reference standard material 8 (ATCC).

3. Animal Experiment and Sequencing

All experimental procedures and animal studies were carried out according to the guidelines of the Institutional Animal Care and Use Committee (IACUC protocol #211646). Male

C57BL/6J mice 10-16 weeks old from InVivos were used. Mice were injected intra-peritoneally (IP) with 10^9 vgs of the AAV1-variants library, sacrificed 3-4 weeks after injection, and the heart and quadriceps muscle were dissected and placed on ice. Dissociation of muscle tissue into a single-cell suspension was done with the Skeletal Muscle Dissociation Kit (Miltenyi Biotec) following manufacturer's instructions. Briefly, muscle tissue were cut into 2mm pieces with a razor blade and enzymatically digested at 37°C for 1h. The cell suspensions were then strained through a 70µm cell strainer, and centrifuged at 300g for 20 min. The cell pellets were then re-suspended in Hibernate A with 10µM Calciin-AM (Invitrogen) and 1µg/ml Propidium Iodide (PI) (Stemcell), for FACS sorting. Live, single cells were selected by positive selection of Calciin-AM and negative selection of PI. Immediately after FACS sorting, cells were counted and 20,000 cells were used for generation of 10X 3' chromium libraries. Reverse transcription of the RNA transcripts followed by targeted amplification of the random peptides insert region on the capsid sequence allowed us to construct an amplicon library. The amplicon libraries constructed from the cDNA were then sequenced on an iSeq (Illumina) to determine the diversity of the libraries and to assess the comparative transduction efficiency of each variant to the mammalian cells or tissue.

4. Bioinformatics pipeline for oligonucleotide insertions analysis

Counting Peptide Inserts (CoPI) is a simple text-based search and analysis program which does quality filtering and counts the occurrences of peptide inserts in a NGS dataset. CoPI is written in Python 3.0 and executable on Windows or Linux platforms (FIG. 1). Users are able to easily modify variables specific to their experiment in a TOML formatted file. These variables include a reference sequence, two invariable anchor sequence flanking the intended engineered site ('seq_start' and 'seq_end'), and a list of other optional options (FIGS. 1B and 1C). For quality filtering, Fastp was employed, which merges paired end reads to ease downstream analyses. Fastp also removes reads with poor Qscore. While this option is optional, CoPI is able to analyze paired ends reads without the need to merge. Analysis is strand specific. If the user is unsure of the strand direction, CoPI will identify the direction of the merged and paired fastq files and subsequently analyze accordingly. All reads with the anchor sequences are included for further analysis. If one of the two anchor sequences are not found, CoPI will search for the anchor sequence tolerating 1 mismatch or indel via a shift-or algorithm (Baeza-Yates, R. & Gonnet, G., 1992). For each read with both anchor present, CoPI will determine the length of the insertion. An amplicon containing no insertion is considered as 0 indel. The insert is then translated into amino acid to count for the peptide insert occurrences. The nucleotide sequence and quality for each read is cached into memory for downstream collapse of low occurrence inserts. In order to reduce probable PCR and NGS

sequencing errors, low count inserts with nucleotide sequences within 2 Levenshtein distance are collapsed to the relatively abundant inserts. Peptide insert sequences are concatenated for indexing. A simple burrow-wheeler aligner was employed for quick query search with 1 mismatches tolerated (Li, H. & Durbin, R., 2009). CoPI ensures that the relatively abundant insert count is at least 5 times of the low insert count as current sequencing technology has an error rate of less than 20% (1-2% for Illumina platforms) (Zorita, E. et al., 2015). In the event of multiple hits found with different mismatch positions, CoPI chooses the peptide sequence with a lower hamming distance and/or relatively lower Qscore difference to collapse on. CoPI subsequently plots the distribution of the peptide lengths and the in-frame amino acid distribution of the inserts. A 100mb gzipped paired ends fastq file takes approximately 10 minutes to analyze using CoPI.

Example 2: Novel AAV1 peptide display libraries

For the construction of a novel AAV1 diverse variants library, a split Rep/Cap system was used with the AAV cargo sequences bearing self-encoding capsid sequences driven under the control of a mammalian CMV and included a polyA tail for transcript capture (FIG. 1A). Using the AAV1 capsid sequence, amino acid D590 was replaced with random 7-mer and 10-mer peptides and the final libraries consist of capsid sequences for AAV1-7mer, AAV1-10mer and AAV1-WT (FIG. 1A). After the plasmid library construction, E.coli plasmids library was produced followed by AAV1 variants production. The cargo plasmid from the AAV1 variants viruses were then extracted and used for construction of NGS library to study the diversity of the AAV1 library. Using NGS sequencing and analysis, 11453 7-mers variants and 2735 10-mer variants were identified in the AAV1 virus library after filtering out variants with only 1 read depth. Following the CoPI script processing of the NGS data (FIGS. 1B-1C), the inventors were able to confirm that the library consist of AAV1-WT (as internal control for normalization), AAV1-7mer and AAV1-10mer in the percentage of 26.67%, 64.33% and 8.92% respectively (FIG. 1D). Further analysis to look into the amino acid diversity of the 7-mer and 10-mer peptide insertions confirmed the highly diverse nature of the constructed AAV1 variants library present in the library (FIGS. 1E and 1F respectively).

Example 3: *In vivo* selection of AAV1 variants library for mouse cardiomyocytes and skeletal myofibers

To perform *in vivo* selection of AAV1 variant libraries, 1×10^9 vgs of the virus libraries were injected intra-peritoneally and the heart and skeletal muscle tissues were harvested to extract the expressed RNA transcript of the AAV1 variants in target tissue (FIG. 1A). In this way, data contamination from the DNA from the original AAV1 variants viruses that were used for the

transduction of the tissue will be avoided. Amplicon libraries constructed from the cDNA of the RNA transcripts are then sequenced on an iSeq (Illumina) and the data analyzed to identify fold change in transduction by relative abundance of script and normalization by following a series of formulaes (FIG. 1A). To identify variants for specific tissue tropism, a strict cut-off of at least 100 reads detected in original AAV1 variant panel and the targeted tissue was set to avoid skewing of data due to low read counts. The next criteria for filtering the data would be the setting of at least a 10-fold improvement in transduction of the variants for a specific tissue. This is set at 2-fold for filtering of variants that has improved transduction in both tissue types.

Table 3. 7-mer peptide variants selected for improved cardiomyocyte tropism

SEQ ID NO:	Peptide Sequence	Fold increase - Cardiomyocyte	Fold increase – Skeletal Myofibers	Cardiomyocyte/Skeletal Myofibers Ratio
1	GINIGVA	36.02	0.17	211.88
2	KDPGPCG	24.79	0.13	190.69
3	DICRQRL	26.03	0.14	185.93
4	ATVTWGS	96.94	0.6	161.57
5	KWRVDSV	20.43	0.13	157.15
6	AWCQCES	74.56	0.48	155.33
7	LIVIGAG	43.14	0.28	154.07
8	VNIRVDV	13.82	0.09	153.56
9	DVRGAGG	25.99	0.17	152.88
10	HGCVSYA	22.85	0.15	152.33
11	FYANSWG	56.22	0.37	151.95
12	NRGHEWM	30.21	0.2	151.05
13	CDEGKPA	102.28	0.68	150.41
14	MFVWSWC	126.8	0.85	149.18
15	LGRSRDE	13.36	0.09	148.44
16	QGEEASE	20.33	0.14	145.21
17	DLRNVVV	12.94	0.09	143.78
18	RLVFTQQ	79.81	0.56	142.52
19	VGTHSPV	27.07	0.19	142.47
20	RVFDSKS	14.22	0.1	142.20
21	LIGKSMR	45.32	0.32	141.63
22	EAQPVLF	16.79	0.12	139.92
23	LERCQLL	145.79	1.06	137.54
24	AHAIWIG	270.76	1.99	136.06
25	CFWSGKL	40.44	0.3	134.80
26	MNLTEHG	14.43	0.11	131.18
27	GNDLDKG	20.63	0.16	128.94
28	IGKGQFV	126.12	1	126.12
29	YSVHDWK	61.91	0.51	121.39
30	EANPTEY	18.56	0.16	116.00
31	NQQSQSG	191.08	1.65	115.81
32	IGSCKGK	68.98	1.11	62.14

Table 4. 7-mer peptide variants selected for improved skeletal myofiber tropism

SEQ ID NO:	Peptide Sequence	Fold increase - Cardiomyocyte	Fold increase – Skeletal myofiber	Skeletal Myofiber/ Cardiomyocyte Ratio
33	CRISHL D	0.57	24.63	43.21
34	SCKQRVC	0.5	19.39	38.78
35	GMLYTL Y	0.84	31.24	37.19
36	DYVPLGL	0.79	28.85	36.52
37	GGNVRLS	0.42	15.04	35.81
38	LYYMFNE	1.52	54.08	35.58
39	RPFAVSS	3.06	108.28	35.39
40	EGYSNGV	0.91	32.13	35.31
41	LCVSR YR	0.69	24.19	35.06
42	DVILVGA	0.83	27.63	33.29
43	HREELVL	1.2	39.22	32.68
44	IRCGCLR	1.45	47.3	32.62
45	DTFCGYV	0.76	24.3	31.97
46	ERFLSCT	0.76	24.19	31.83
47	GARDITS	0.42	13.11	31.21
48	WRGGLGR	0.97	29.55	30.46
49	SVERNCS	1.43	42.89	29.99
50	IVITDLW	3.26	96.35	29.56
51	TGSATLG	0.83	24.28	29.25
52	LVEPLHD	1.33	38.11	28.65
53	VDLTETM	1.98	53.73	27.14
54	DDHSNGR	1.02	26.83	26.30

Table 5. 7-mer peptide variants selected for improved transduction in both cardiomyocyte and skeletal myofiber

SEQ ID NO:	Peptide Sequence	Fold increase - Cardiomyocyte	Fold increase – Skeletal myofiber	Cardiomyocyte/Skeletal Myofibers Ratio
55	SLFSTIS	3.43	2.11	1.625592
56	KGWVRFC	3.7	2.56	1.445313
57	ALESVTW	32.3	22.99	1.404959

5

Table 6. 10-mer peptide variants selected for improved cardiomyocyte tropism

SEQ ID NO:	Peptide Sequence	Fold increase - Cardiomyocyte	Fold increase – Skeletal Myofibers	Cardiomyocyte/Skeletal Myofibers Ratio
58	VHKGHSEYPL	183.79	1.38	133.18
59	NGGVICISMS	64.24	0.33	194.67
60	GSRLLPKIS	51.59	0.27	191.07
61	LLGTDILFP	19.95	0.11	181.36
62	VSAETESWWS	15.29	0.1	152.90

Table 7. 10-mer peptide variants selected for improved skeletal myofiber tropism

SEQ ID NO:	Peptide Sequence	Fold increase - Cardiomyocyte	Fold increase – Skeletal Myofibers	Skeletal Myofiber/ Cardiomyocyte Ratio
63	EEPLPVLRGF	0.88	35	39.77
64	IFIHRCFSL	1.2	47.11	39.26
65	HYILTLRHYG	0.78	30.23	38.76

Through the filtering, thirty-two 7-mer and five 10-mer variants with at least 25-fold skewed tropism towards cardiomyocytes compared to skeletal myofibers were identified (Table 3 and 6 respectively). For AAV1 variants with skeletal myofibers tropism, twenty-two 7-mer and three 10-mer variants with at least 25-fold skewed tropism towards skeletal myofibers compared to cardiomyocytes were identified (Table 4 and 7 respectively). For AAV1 variants with dual tissue improved transduction, three 7-mer AAV1 variants and none from 10-mer variants were identified by following the strict selection criteria (Table 5).

10 Discussion

The present disclosure describes novel compositions of rAAV1 capsids for delivery of genes into specific muscle cell types. The inventors demonstrated the empirical methodology and bioinformatics pipeline for the selection of novel rAAV capsids, which significantly improves the gene transduction efficacy into cardiomyocytes, skeletal myofibers, or both muscle tissue types.

In essence, short peptides are inserted on the viral surface to mediate viral re-targeting to specific cell types and/or improve the efficiency of transduction. Capsid libraries are generated by substitution of D590 with random 21-mer or 30-mer oligonucleotides which codes for random 7-mer or 10- mer peptides. The engineered capsid sequence is then packaged in AAV1 vectors and used for transduction of mammalian cells or tissue. NGS is employed to look at the diversity of the libraries and to assess the comparative transduction efficiency of each variant to the mammalian cells or tissue. CoPI was developed, a bioinformatics pipeline that enables comprehensive analysis of short peptide inserts in any primary sequence of proteins.

Importantly, through the described methodology and bioinformatics, the present invention provides novel rAAV particles with preferential tissue tropism towards cardiomyocytes and/or skeletal muscles. Advantageously, the rAAV particles of the present disclosure provide at least 10- to 100-fold improvement in transduction efficiency. In some embodiments, the rAAV particles disclosed herein have at least 25-fold skewed tropism towards cardiomyocytes compared to skeletal muscle. Additionally in some embodiments, the rAAV particles disclosed

herein are also muscle-detargeted to various extent. As muscles made up approximately 40% of the body mass, de-targeting from the muscle can significantly reduce unnecessary AAV off-target transduction which may allow for lower dose application as well as potentially lower the immune responses against the engineered vectors. The differential improvement in delivery to the different muscle cell types will be important for a more precise and targeted gene therapy treatment, depending if the genetic disorder affect a single or both cell types.

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CLAIMS

1. A recombinant adeno-associated virus (rAAV) particle comprising a viral shell comprising a modified AAV capsid protein,
wherein the modified AAV capsid protein comprises an extraneous peptide
5 sequence comprising a length of at least 5 amino acids,
wherein the extraneous peptide sequence enhances/increases the transduction efficiency of the rAAV particle into one or more muscle cell(s), compared to the transduction efficiency of a wild-type AAV particle not comprising the extraneous peptide sequence.
- 10 2. The rAAV particle according to claim 1, wherein the rAAV particle is a recombinant adeno-associated virus serotype 1 (rAAV1) particle.
3. The rAAV particle according to claim 1 or 2, wherein the extraneous peptide sequence substitutes and replaces the amino acid residue at position 590 of the AAV capsid protein.
- 15 4. The rAAV particle according to any one of claims 1 to 3, wherein the extraneous peptide sequence comprises a peptide sequence comprising a length of at least 7 amino acids.
5. The rAAV particle according to any one of claims 1 to 4, wherein the extraneous peptide sequence comprises a peptide sequence having a length of 7 amino acids or a length of 10 amino acids.
- 20 6. The rAAV particle according to any one of claims 1 to 5, wherein the one or more muscle cell(s) comprises one or more mammalian muscle cell(s).
7. The rAAV particle according to any one of claims 1 to 6, wherein the one or more muscle cell(s) comprises one or more cardiomyocyte(s) and/or one or more skeletal myofiber cell(s).
- 25 8. The rAAV particle according to any one of claims 1 to 7, wherein the extraneous peptide sequence comprises the amino acid sequence set forth in any one of **SEQ ID NO: 1** to **SEQ ID NO: 65**.
9. The rAAV particle according to claim 8, wherein the one or more muscle cell(s) comprises one or more cardiomyocyte(s).

10. The rAAV particle according to claim 9, wherein the extraneous peptide sequence comprises the amino acid sequence set forth in any one of **SEQ ID NO: 1** to **SEQ ID NO: 32**, or **SEQ ID NO: 58** to **SEQ ID NO: 62**.
- 5 11. The rAAV particle according to claim 8, wherein the one or more muscle cell(s) comprises one or more skeletal myofiber cell(s).
12. The rAAV particle according to claim 11, wherein the extraneous peptide sequence comprises the amino acid sequence set forth in any one of **SEQ ID NO: 33** to **SEQ ID NO: 54**, or **SEQ ID NO: 63** to **SEQ ID NO: 65**.
- 10 13. The rAAV particle according to claim 8, wherein the one or more muscle cell(s) comprises one or more cardiomyocyte(s) and skeletal myofiber cell(s).
14. The rAAV particle according to claim 13, wherein the extraneous peptide sequence comprises the amino acid sequence set forth in any one of **SEQ ID NO: 55** to **SEQ ID NO: 57**.
- 15 15. A recombinant polynucleic acid molecule encoding a modified AAV capsid protein, wherein the modified AAV capsid protein comprises an extraneous peptide sequence having a length of at least 5 amino acids.
16. The recombinant polynucleic acid molecule according to claim 15, wherein the extraneous peptide sequence comprises a peptide sequence having a length of at least 7 amino acids.
- 20 17. The recombinant polynucleic acid molecule according to claim 15 or 16, wherein the extraneous peptide sequence comprises a peptide sequence having a length of 7 amino acids or a length of 10 amino acids.
18. The recombinant polynucleic acid molecule according to any one of claims 15 to 17, wherein the extraneous peptide sequence comprises the amino acid sequence set forth in any one of **SEQ ID NO: 1** to **SEQ ID NO: 65**.
- 25 19. The recombinant polynucleic acid molecule according to any one of claims 15 to 18, wherein the polynucleic acid molecule is operatively linked to a mammalian promoter sequence.
20. A pharmaceutical composition comprising the rAAV particle according to any one of claims 1 to 14 and a pharmaceutically acceptable carrier.
- 30 21. An rAAV particle according to any one of claims 1 to 14, for use in medicine.

22. An rAAV particle according to any one of claims 1 to 14, for use in treating a cardiac-related and/or muscle-related disease or disorder.
23. A method of treating a disease or disorder, the method comprises administering the rAAV particle according to any one of claims 1 to 14, to a subject in need thereof.
- 5 24. The method according to claim 23, wherein the disease or disorder is a cardiac-related and/or muscle-related disease or disorder.
25. Use of the rAAV particle according to any one of claims 1 to 14 in the manufacture of a medicament for the treatment of a cardiac-related and/or muscle-related disease or disorder in a subject.
- 10 26. The rAAV particle for use according to claim 22, the method according to claim 24 or the use according to claim 25, wherein the cardiac-related and/or muscle-related disease or disorder comprises inherited cardiomyopathy, skeletal muscle dystrophy, monogenic cardiovascular diseases (MCVDs) such as arrhythmia and Brugada syndrome, cardiomyopathies such as arrhythmogenic right ventricular cardiomyopathy (ARVC),
15 Duchenne muscular dystrophy (DMD) and limb girdle muscle dystrophy (LGMD).
27. A method of delivering a gene product to one or more cell(s), the method comprising contacting the rAAV particle according to any one of claims 1 to 14 with the cell, optionally wherein the method is an *in vitro* method.
28. Use of the rAAV particle according to any one of claims 1 to 14 for the preparation of a
20 pharmaceutical composition for gene therapy.