

INCREASED PACKAGING EFFICIENCY OF VECTOR FOR CARDIAC GENE THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 63/130,109, filed December 23, 2020, which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

10 In accordance with 37 C.F.R. 1.52(e)(5), the present specification makes reference to a Sequence Listing (submitted electronically as a .txt file named "U119770172WO00-SEQ"). The .txt file was generated on December 14, 2021, and is 57,573 bytes in size. The Sequence Listing is herein incorporated by reference in its entirety.

BACKGROUND

15 Cardiomyopathy is the second most common cause of heart disease in subjects and medical management of the secondary signs is the only therapeutic option. The prognosis for affected subjects depends on the stage of disease and, in canine subjects, the breed. Heart function is critically dependent upon calcium-dependent signaling. During heart disease,
20 malfunctioning of calcium channels within cardiac cells promotes calcium cycling abnormalities, further inhibiting heart function. Gene transfer strategies to reduce calcium cycling abnormalities have been shown to ameliorate heart disease in small and large animal models, as well as in human clinical trials.

 Dilated cardiomyopathy (DCM) is the most common type of human cardiomyopathy,
25 occurring mostly in adults 20 to 60. DCM is also present in canines (*e.g.*, dogs), particularly in large-breed canines. In both humans and canines, DCM is a disease of the heart muscle that affects the heart's ventricles and atria, the lower and upper chambers of the heart, respectively, resulting in weakened contractions and poor pumping ability. As DCM progresses, the heart chambers become enlarged, one or more valves may leak, and signs of
30 congestive heart failure develop. In canines, the cause of DCM is unclear in most cases, but certain breeds appear to have an inherited (*e.g.*, genetic) predisposition.

In humans, most forms of DCM are acquired forms from a number of causes that include coronary heart disease, heart attack, high blood pressure, diabetes, thyroid disease, viral hepatitis and viral infections that inflame the heart muscle. Alcohol abuse and certain drugs, such as cocaine and amphetamines, as well as at least two drugs used to treat cancer (doxorubicin and daunorubicin), can also lead to DCM. In addition, there are a number of genetic forms of DCM, including, but not limited to the DCM associated with Duchenne and Becker muscular dystrophies. In the case of certain forms of Becker muscular dystrophy, as well as in most cases of Duchenne muscular dystrophy, the cardiomyopathy can ultimately limit the patient's survival.

SUMMARY

In humans, dilated cardiomyopathy is the most common type of cardiomyopathy and can stem from a number of acquired as well as genetic conditions. As in dogs and other animal models, while the origins of the disease are rooted in calcium handling dysfunction, the ultimate progression of the disease is driven by mitochondrial dysfunction and/or stretch-induced apoptosis of the cardiomyocytes. While addressing calcium handling alone may be efficacious at early disease stages, addressing the combination of calcium handling, mitochondrial dysfunction, and apoptosis will be necessary to treat all forms of DCM and at all stages of disease progression.

Disclosed herein are improved gene delivery approaches for treatment of subjects with cardiomyopathy, and, in some embodiments, congestive heart failure. These approaches relate to the expression of S100A1 to address calcium handling and expression of ARC (Apoptosis Repressor with Caspase Recruitment Domain) to block all sources of apoptosis and normalize mitochondrial function. (See also US Patent Publication No. 2021/0260215, herein incorporated by reference in its entirety.) Expression of S100A1 and ARC transgenes through the disclosed self-complementary AAV vector approach, is rapid (*i.e.* within hours), which is critical in counteracting the effects of end-stage heart failure and restricted to the heart. Thus, these approaches address all three drivers of DCM onset and progression and accordingly should be applicable to any form of DCM at any stage of disease progression.

In addition, disclosed herein is a series of transgenes for gene delivery that code for the proteins ARC and S100A1. These transgenes may comprise cDNA sequences, and these sequences may be delivered and expressed using a recombinant adeno-associated virus (rAAV) vector system. The disclosed cDNA sequences may be delivered with any type of

gene delivery vector, including but not limited to all forms of AAV, lentiviruses, liposomes and exosomes.

In particular embodiments, these sequences may be expressed using a self-complementary version of AAV vector DNA that comprises i) a wild type or an optimized cDNA encoding human ARC and ii) a wild type or an optimized cDNA encoding human S100A1. The ARC and S100A1 cDNA sequences may be positioned such that ARC comprises the first cDNA and S100A1 comprises the second cDNA in the 5' to 3' direction, or vice versa. In other embodiments, these cDNA sequences may be expressed using two promoters, rather than a promoter and an IRES.

Expression of these cDNA sequences may be operably controlled by a cardiac troponin T promoter (cTnT) positioned 5' of the first cDNA, and/or an internal ribosome entry site (IRES) positioned 5' of the second cDNA. The cTnT promoter restricts expression of the two transgenes to cardiomyocytes when AAV is introduced via the circulation into the heart and other tissues, or via direct injection. The resulting expression of ARC prevents apoptosis when expressed in cardiomyocytes and helps normalize the mitochondrial membrane potential, reducing free radical generation and improving mitochondrial function. S100A1 expression leads to improved calcium pumping into the sarcoplasmic reticulum (SR) of cardiomyocytes by the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) pump (*e.g.*, the SERCA isoform 2a pump, or SERCA2a) and decreased calcium leak from the SR via the ryanodine receptor channel. The combination allows the normalization of cardiomyocyte calcium handling and improved systolic and diastolic function in the heart.

The combined effects of an absence of apoptotic effects, improved mitochondrial function, and improved calcium handling slows progression of heart failure and improves cardiac function. The disclosed rAAV vectors may be effective in all forms of human cardiomyopathy and heart failure. As such, the disclosed rAAV vectors may be delivered to subject, *e.g.*, a human subject, suffering from a disease, disorder or condition comprising poor cardiac function, *e.g.*, human cardiomyopathy and heart failure.

Accordingly, some aspects of the present disclosure provide recombinant adeno-associated virus (rAAV) vectors for delivering transgenes into the heart of a subject. In some embodiments, such rAAV vectors include at least two transgenes, one encoding an S100 family protein and one encoding an apoptotic inhibitor. Such rAAV vectors may include, from 5' to 3', in order, a first adeno-associated virus (AAV) inverted terminal repeat (ITR) sequence, a promoter operably linked to the transgenes, and a second AAV inverted terminal

repeat (ITR) sequence. In some embodiments, two transgenes are operably linked to the same single promoter. In other embodiments, each transgene is operably linked to a separate promoter. In some embodiments, the rAAV vector also includes at least one polyadenylation signal (*e.g.*, positioned 3' of two transgenes expressed from a single promoter or 3' of one or both transgenes expressed from different promoters). Aspects of the disclosure therefore provide recombinant adeno-associated virus (rAAV) nucleic acid vectors for delivering two or more transgenes into the heart of a subject, wherein said vectors comprise, from 5' to 3', a first adeno-associated virus (AAV) inverted terminal repeat (ITR) sequence, two or more transgenes and a promoter operably linked to the two or more transgenes, a polyadenylation signal, and a second AAV inverted terminal repeat (ITR) sequence, wherein the two or more transgenes encode an S100 family protein and an apoptotic inhibitor, respectively.

The transgenes of the present disclosure may encode an S100 family protein and/or an apoptotic inhibitor. For example, the S100 family protein may comprise cardiac S100 calcium-binding protein A1 (cS100A1), or a variant thereof. In another example, the apoptotic inhibitor may comprise a cardiac Apoptosis Repressor with Caspase Recruitment Domain (cARC) or a variant thereof.

In some embodiments, one or more of the transgenes of the present disclosure are naturally-occurring or wild-type sequences. In some embodiments, one or more transgenes (*e.g.* the cARC transgene) are codon-optimized for expression in humans.

In various embodiments, provided herein are rAAV vectors comprising a promoter, a polyadenylation (polyA) signal and a polynucleotide comprising two or more transgenes. In some embodiments, a first transgene encodes an S100 family protein (*e.g.* cS100A1 or variant thereof) and a second transgene encodes a cARC. In particular embodiments, the S100 family protein and cARC transgenes are derived from humans (*e.g.*, *homo sapiens*). In particular embodiments, the S100 family protein and cARC transgenes are derived from canines (*e.g.*, *canis lupus familiaris*).

In some embodiments, the first transgene (encoding an S100 family protein) of the polynucleotide comprises the nucleotide sequence set forth as SEQ ID NO: 5. Alternatively, the first transgene may comprise the nucleotide sequence set forth as SEQ ID NO: 8. In some embodiments, the first transgene may comprise the nucleotide sequence set forth as SEQ ID NO: 25. In some embodiments, the first transgene may comprise the nucleotide sequence set forth as SEQ ID NO: 26.

Accordingly, provided herein are rAAV nucleic acid vectors for delivering two or more transgenes into the heart of a subject, wherein said vectors comprise, from 5' to 3', in order, a first adeno-associated virus (AAV) inverted terminal repeat (ITR) sequence, two or more transgenes and a promoter operably linked to the two or more transgenes, a polyadenylation (polyA) signal, and a second AAV inverted terminal repeat (ITR) sequence, wherein the two or more transgenes encode an S100 family protein and an apoptotic inhibitor, respectively, and wherein the transgene encoding an S100 family protein comprises a sequence that is at least 95%, at least 98%, or at least 99.5% identical to either of the nucleotide sequences of SEQ ID NOs: 25 and 26.

10 In some embodiments, the transgene encoding an S100 family protein is positioned 5' to the transgene encoding the apoptotic inhibitor. In some embodiments, the transgene encoding the apoptotic inhibitor is positioned 5' to the transgene encoding an S100 family protein.

In some embodiments, the rAAV vector encodes a protein comprising an amino acid sequence at least 90%, at least 95%, or at least 99.5% identical to the amino acid sequence of SEQ ID NO: 13 or 14. In some embodiments, the rAAV vector encodes a protein comprising the amino acid sequence of SEQ ID NO: 13 or 14. In some embodiments, the rAAV vector encodes a protein comprising an amino acid sequence at least 90%, at least 95%, or at least 99.5% identical to the amino acid sequence of SEQ ID NO: 24 or 29. In some embodiments, the rAAV vector encodes a protein comprising the amino acid sequence of SEQ ID NO: 24 or 29.

In some embodiments, the first transgene (encoding an S100 family protein) may comprise a nucleotide sequence that is at least 90%, at least 95%, or at least 99.5% identical to any one of the nucleotide sequences of SEQ ID NOs: 19-21. The first transgene may comprise the nucleotide sequence of any one of SEQ ID NOs: 19-21. In particular embodiments, the first transgene may comprise the nucleotide sequence of SEQ ID NO: 21.

In some embodiments, the first transgene (encoding an S100 family protein) may comprise a nucleotide sequence that is at least 90%, at least 95%, or at least 99.5% identical to the nucleotide sequence of any one of SEQ ID NOs: 25 and 26. In some embodiments, the first transgene encoding the S100 family protein comprises either of the nucleotide sequences set forth as SEQ ID NOs: 25 and 26.

In some embodiments, the first transgene encoding the S100 family protein comprises a sequence having a reduced guanosine (G) and cytosine (C) (G/C) content, relative to any

one of the nucleotide sequences of SEQ ID NOs: 5, 8 or 19-20. In some embodiments, the G/C content is reduced by 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15% relative to any one of the nucleotide sequences of SEQ ID NOs: 5, 8 or 19-20. In some embodiments, the G/C content is reduced by between about 1-15%, 1-10%, 1-5%, 2-10%, 3-15%, 3-10%, 5-15%, 5-10%, 5-13%, 7-15%, or 7-10% relative to any one of the nucleotide sequences of SEQ ID NOs: 5, 8 or 19-20.

In some embodiments, the apoptotic inhibitor is cardiac Apoptosis Repressor with Caspase Recruitment Domain (ARC) or a variant thereof. In some embodiments, the second transgene encoding the apoptotic inhibitor comprises a sequence that is at least 90%, at least 95%, or at least 99.5% identical to any one of the nucleotide sequences of SEQ ID NOs: 3, 6, 7, or 15-18. In some embodiments, the transgene encoding the apoptotic inhibitor comprises any one of the nucleotide sequences of SEQ ID NOs: 3, 6, 7, or 15-18.

In some embodiments, the polyA signal comprises a nucleotide sequence that has at least 90%, 92.5%, 95%, 98%, or 99% identity to the nucleotide sequence of SEQ ID NO: 28. In some embodiments, the polyA signal comprises the nucleotide sequence of SEQ ID NO: 28.

In some embodiments, an Internal Ribosome Entry Site (IRES) is present between the two or more transgenes (*e.g.*, between the S100A1 transgene and cARC transgene). In some embodiments, the transgene encoding the S100 family protein is 5' to the transgene encoding the apoptotic inhibitor. In other embodiments, the transgene encoding the apoptotic inhibitor is 5' to the transgene encoding the S100 family protein.

In some embodiments, the promoter of the rAAV is a cardiac-restricted promoter. In some embodiments, the promoter is a cardiac-restricted promoter selected from cardiac troponin C, cardiac troponin I, and cardiac troponin T (cTnT). In some embodiments, the promoter is a cardiac-restricted promoter derived from a gene selected from the group consisting of: α -myosin heavy chain gene, 6-myosin heavy chain gene, myosin light chain 2v gene, myosin light chain 2a gene, CARP gene, cardiac α -actin gene, cardiac m2 muscarinic acetylcholine gene, ANF, cardiac sarcoplasmic reticulum Ca-ATPase gene, and skeletal α -actin; or is an artificial cardiac promoter derived from MLC-2v gene. In some embodiments, the promoter is cTnT.

In some embodiments, the rAAV vector is single-stranded. In some embodiments, the rAAV vector is self-complementary.

In some embodiments, the rAAV vector comprises a nucleotide sequence that is at least 90%, at least 95% or at least 99.5% identical to either of the nucleotide sequences of SEQ ID NOs: 22 and 23. In some embodiments, the rAAV vector comprises the nucleotide sequence of SEQ ID NO: 22. In some embodiments, the rAAV vector comprises the
5 nucleotide sequence of SEQ ID NO: 23.

Further provided herein are rAAV vectors comprising one or more transgenes (*e.g.*, an S100A1 transgene and a cARC transgene) that have improved or enhanced packaging efficiencies. In some embodiments, the transgenes are specific for *homo sapiens* or *canis lupus familiaris*. In some embodiments, these rAAV vectors have improved packaging
10 efficiencies by virtue of their shorter length, *e.g.* through a shortening of the length of the polyA sequence. In some embodiments, these rAAV vectors have improved packaging efficiencies by virtue of reduced guanine/cytosine content, *e.g.*, reduced G/C content, in one or both transgenes, relative to a wild-type transgene or to a transgene codon-optimized for expression in a specific host organism. In some embodiments, the rAAV vectors have
15 reduced G/C content in the first transgene (*e.g.*, the transgene encoding S100A1). Further provided are rAAV particles that have been packaged with any of these rAAV vectors. Increased packaging efficiencies of a vector may be measured by evaluating the vector genome titer of a resulting composition of rAAV particles packaged with this vector, or by any other method known in the art.

20 In some embodiments, the enhanced packaging efficiencies of the disclosed vectors result in compositions comprising increased rAAV particle titers relative to prior art vectors, *e.g.*, increased by about 2× to about 5×. In certain embodiments, the enhanced packaging efficiencies of the disclosed vectors result in compositions comprising rAAV particle titers of 2×10^{13} to 5×10^{13} vector genome copies/mL (vgs/mL).

25 Further provided herein are rAAV particles containing the rAAV vectors disclosed herein, encapsidated in AAV capsids. In some embodiments, the AAV capsid comprises a capsid protein derived from AAV1, AAV2, AAV3, AAV6, AAV8, AAVrh.74, AAVrh.10, AAV2/6 or AAV9 serotypes. In some embodiments, the AAV capsid comprises a capsid protein derived from AAVrh.10 serotype. In some embodiments, the AAV capsid comprises
30 a capsid protein derived from AAVrh.74 serotype.

Other aspects of the present disclosure include compositions containing the rAAV particles described herein. Such compositions may be administered to a subject for gene therapy for heart disease. Accordingly, some aspects of the disclosure contemplate a method

of treatment of a subject suffering from a heart disease comprising administering to the subject a composition containing the rAAV particles or an rAAV particle as described herein. In some embodiments, the heart disease causes heart failure in the subject. In some embodiments, the heart disease is cardiomyopathy. In other embodiments, the heart disease is hypertrophic cardiomyopathy or dilated cardiomyopathy. In other embodiments, the heart disease is acute ischemia. In some embodiments, the heart disease is cardiomyopathy or acute ischemia.

In some embodiments, provided herein are methods comprising administering to a cell (such as a cell of a subject *ex vivo* or *in vivo*) a composition containing the rAAV particles or an rAAV particle as described herein. In some embodiments, provided herein are methods comprising administering *in vitro* or *ex vivo* a composition containing the rAAV particles or an rAAV particle as described herein.

Accordingly, provided herein are methods of treatment of heart diseases, conditions or disorders, such as cardiomyopathy. In some aspects, provided herein are methods of treatment of a subject suffering from a heart disease comprising administering to the subject a composition containing the rAAV particles or an rAAV particle as described herein. Methods of administration *in vitro* and *ex vivo* are further contemplated. Uses of any of the compositions containing the rAAV particles or an rAAV particle as described herein as a medicament are further contemplated.

The compositions of the present disclosure may be administered to the subject via different routes. In some embodiments, the composition is administered via injection into the heart of the subject or intravascular injection into the coronary arteries of the subject. In some embodiments, the administration of the composition results in expression of the transgenes (*e.g.*, two or more transgenes) in the subject's heart. In various embodiments, the step of administering the composition results in improved cardiac function in the subject, such as improved cardiac function in the subject for more than 10 months. In some embodiments, administration results in improved cardiac function for more than 12 months, more than 14 months, more than 16 months, more than 17 months, more than 20 months, more than 22 months, or more than 24 months. In some embodiments, the step of administering results in improved cardiac function in the subject for more than 10 months.

In some embodiments, the subject is a mammal. In some embodiments, the mammal is a human. In some embodiments, the mammal is a companion animal. In some embodiments, the companion animal is a dog or a cat.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present disclosure. The disclosure may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements:

FIG. 1 depicts a diagram of an AAV construct of the disclosure. A first AAV inverted terminal repeat (ITR) is followed by the cardiac troponin T promoter (cTnT), then a sequence encoding S100 calcium-binding protein A1 (cS100A1), followed by an internal ribosomal entry site (IRES), followed by a sequence encoding Apoptosis Repressor with Caspase Recruitment Domain (cARC), followed by a polyadenylation (pA) sequence, and a second AAV ITR.

FIG. 2 depicts diastolic MRI imaging from a treated muscular dystrophy dog (named Calvin) at baseline and several weeks after gene delivery. The data support stable or slightly improved cardiac remodeling with a mild decrease in the diastolic left ventricular volume.

FIG. 3 depicts systolic MRI imaging from a treated muscular dystrophy dog (named Calvin) at baseline and several weeks after gene delivery. The data support stable or slightly improved left ventricular systolic function post treatment, with a mild reduction in systolic volume suggesting improved contractility and an increase in left ventricular cardiac output.

FIG. 4 shows ejection fraction, peak strain, and cardiac output of D2.mdx mice after AAVrh.10-S100A1/ARC treatment. Over a 24-week period, mice injected with the therapeutic AAV had better maintained ejection fractions, strain development, and cardiac output as compared to sham injected mice.

FIG. 5 shows S100A1 and ARC expression levels in mice treated with recombinant AAVrh.10-S100A1/ARC vector and control mice. Protein analysis (Western blots) confirmed that both S100A1 and ARC levels were elevated in the treated tissues as compared to controls (sham injected).

FIG. 6 shows the histologies of cardiomyocytes of control (left panels) and treated (right panels, "S100A1.ARC") mice under 10X and 20X magnification. Cardiac histology data indicates that the treated mice exhibited less DMD pathology, including less fibrosis of muscle tissue, as compared to control hearts.

FIG. 7 shows that the first (of two) dystrophin-deficient dogs (GRMD dogs), named Calvin, showed improved cardiac function (measured by ejection fraction) after recombinant

AAVrh.10-S100A1/ARC treatment. Both injected dogs exhibited improvements in ejection fraction and other cardiac parameters following treatment, measured by cardiac MRI and confirmed by echo data.

5 **FIG. 8** shows data that the second GRMD dog, named Sebastian, showed improved cardiac function after AAVrh.10-S100A1/ARC treatment.

FIGs. 9A to 9C show that AAV-S100A1/ARC treatment decreased serum creatine kinase (CK) levels and prevented muscle atrophy in the GRMD dogs. MRI measurements of limb muscle mass, as measured by the area of both legs (**FIG. 9A**), maximum cross-sectional area (CSA) (**FIG. 9B**), and volume of both legs (**FIG. 9C**). The results demonstrate that
10 skeletal muscle mass had either increased or remained unchanged following cardiac treatment.

FIG. 10 shows that circulating creatine kinase levels (CK) levels in skeletal muscle of the GRMD subjects were reduced after AAVrh.10-S100A1/ARC injection, indicating a reduction in ongoing muscle damage.

15 **FIG. 11** illustrates a sequence alignment between codon-optimized canine ARC cDNA sequence and codon-optimized human ARC cDNA sequence. Sequences correspond to SEQ ID NOs: 15, 16, and 6 from top to bottom.

FIG. 12 illustrates a sequence alignment between codon-optimized and native human ARC cDNA sequences. Sequences correspond to SEQ ID NOs: 17, 18, and 6 from top to
20 bottom.

FIG. 13 illustrates a sequence alignment between codon-optimized and native human S100A1 cDNA sequences. Sequences correspond to SEQ ID NOs: 19, 5, and 8 from top to bottom.

25 **FIG. 14** illustrates a sequence alignment between codon-optimized canine S100A1 cDNA sequence and codon-optimized human S100A1 cDNA sequence. Sequences correspond to SEQ ID NOs: 20, 8, and 21 from top to bottom.

FIG. 15 shows fractional shortening and ejection fraction D2.mdx mice (n=12) at 10 months of age, following AAVrh.10-S100A1/ARC treatment at 1 month of age. Both the fractional shortening and ejection fraction were not significantly different from wild type D2
30 mice in the AAVrh.10-S100A1/ARC treated mice, while the untreated D2.mdx mice had significant reductions in function.

FIG. 16 shows left ventricular volumes and diameters of D2.mdx mice (n=12) at 10 months of age, following AAVrh.10-S100A1/ARC treatment at 1 month of age. Increased

volume and diameters during both diastole and systole are indicative of dilated cardiomyopathy. Both the volume and diameter were not significantly different from wild type D2 mice in the AAVrh.10-S100A1/ARC treated mice, while the untreated D2.mdx mice had significant increases in both parameters.

FIG. 17 shows the age of 50% survival of D2.mdx.sk_utrophin mice (n = 12 per group at study initiation) that were allowed to begin exercising at 6 weeks of age, but did not receive treatment until 6 months of age. In the control group that received no treatment, median survival was 10 months of age. The group receiving AAVrh.10-ARC had a median survival of 14 months, the group receiving AAVrh.10-S100A1 had a 16-month median survival, and the group receiving AAVrh.10-S100A1/ARC had a median survival of 20 months.

FIG. 18 shows ejection fraction changes with age in the colony's untreated GRMD dogs (n=10), and the ejection fraction changes in individual GRMD dogs that received treatment with the AAVrh.10-S100A1/ARC vector at the ages indicated by the arrows (between 3 and 7 months of age). In all four of the treated dogs, the ejection fraction improved after the treatment and has been stable thereafter.

FIG. 19 shows left ventricle histology (H & E stain) of a normal 1 year-old Golden Retriever (far left panel) and a GRMD dog (WnM3/Calvin) that received treatment with the AAVrh.10-S100A1/ARC vector at 7 months of age (far right panel). The treated dog died at 34 months of age due to aspiration pneumonia, even though his cardiac function was still in the normal range (FIG. 18). The middle three panels show left ventricle histology from untreated GRMD dogs at 8, 24, and 30 months of age. There is apparent and progressive fibrosis that replaces the muscle tissue (darker shading). Note that in the treated dog panels on the far right, the histology is not discernably different from that of a normal dog, and markedly less fibrotic and more intact than an untreated GRMD dog.

FIG. 20 shows a silver-stained SDS-PAGE gel demonstrating the purity of two rAAV vectors of the present invention. SEQ ID NO: 9 is shown at left, and SEQ ID NO: 22 is shown at right.

30 DETAILED DESCRIPTION

The present disclosure relates to compositions and methods of cardiac gene therapy for heart diseases, *e.g.*, cardiomyopathy, in human and canine subjects. The methods of the present disclosure relate to the use of recombinant AAV (rAAV) particles for the concurrent

delivery and expression of two or more transgenes. The transgenes of the present disclosure encode at least two classes of proteins each having specific function to address different aspects of the heart diseases. One class of transgenes encodes proteins which regulate the calcium signaling in cardiomyocytes, *e.g.*, the S100 family proteins. The other class of transgenes encodes apoptosis repressors. In some embodiments, the transgenes may encode cardiac S100 calcium-binding protein A1 (cS100A1) or a variant thereof, and/or cardiac Apoptosis Repressor with Caspase Recruitment Domain (cARC) or a variant thereof.

The compositions and methods of the present disclosure are based on, at least in part, the synergistic effects of two transgenes, *e.g.*, transgenes which encode S100A1 and ARC, respectively, when they are delivered and expressed concurrently in the heart of the subject. The S100A1 protein improves aspects of calcium handling, including normalization of sarcoplasmic reticular calcium transients leading to normalization of contractile function. The ARC protein blocks apoptosis initiated by mitochondrial and non-mitochondrial mechanisms (such as stretch-induced apoptosis), and improves mitochondrial function. In other words, S100A1 and ARC address two separate components of cardiac failure (calcium handling dysfunction and apoptosis) with synergistic benefits, leading to better long-term therapeutic outcomes. Further, the compositions and methods of the present disclosure are effective at any disease stage of heart failure.

Further provided herein are methods of making rAAV particles suitable for delivering transgenes, *e.g.*, transgenes which encode S100A1 and/or ARC, or variants thereof, into the heart of the subject. Such rAAV particles may comprise a recombinant AAV genome, comprising nucleic acid molecules encoding the transgenes, wherein said nucleic acid molecules are encapsidated by AAV capsid proteins. In some embodiments, the rAAV particles include a recombinant adeno-associated virus (rAAV) nucleic acid vector. The recombinant AAV genome is a single-stranded DNA that may further comprise sequence elements that facilitate the integration of the AAV genome into the host genome and the expression of the transgenes. For example, the recombinant AAV genome may comprise tissue-specific promoters to ensure the expression of the transgenes in target tissues or organs. Such rAAV particles may be used in a composition for the treatment of heart conditions.

Thus, the present disclosure further provides rAAV vectors for delivering transgenes into the heart of a subject. In some embodiments, the disclosed rAAV vectors include at least two transgenes, one encoding an S100 family protein and one encoding an apoptotic

inhibitor. These rAAV vectors may include, from 5' to 3', in order, a first adeno-associated virus (AAV) inverted terminal repeat (ITR) sequence, a promoter operably linked to the transgenes, and a second AAV inverted terminal repeat (ITR) sequence. In some embodiments, two transgenes are operably linked to the same single promoter. In other
 5 embodiments, each transgene is operably linked to a separate promoter. In some embodiments, the rAAV vector also includes at least one polyadenylation signal (*e.g.*, positioned 3' of two transgenes expressed from a single promoter or 3' of one or both transgenes expressed from different promoters).

The disclosure further provides rAAV nucleic acid vectors for delivering two or more
 10 transgenes into the heart of a subject. In some embodiments, the rAAV vector comprises exactly two transgenes. In some embodiments, the rAAV vector comprises three transgenes. In some embodiments, said vector comprises, from 5' to 3', a first AAV ITR sequence, two or more transgenes and a promoter operably linked to the two or more transgenes, a polyadenylation signal, and a second AAV ITR sequence. In particular embodiments, the
 15 two or more transgenes comprise a first transgene encoding an S100 family protein and a second transgene encoding an apoptotic inhibitor.

In some embodiments, the polyA signal comprises a bovine growth hormone (BGH) signal. In some embodiments, the polyA signal comprises a variant of a BGH signal. In some embodiments, the polyA signal comprises a nucleotide sequence that differs from the
 20 sequence of SEQ ID NO: 27 by 1, 2, 3, 4, 5, or more than 5 nucleotides. In some embodiments, the polyA signal comprises a nucleotide sequence that has at least 90%, 92.5%, 95%, 98%, or 99% identity to SEQ ID NO: 27. In some embodiments, the polyA signal comprises SEQ ID NO: 27, provided below.

25 *BGH polyA signal:*

AGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCCTCCCCCGTGCCTTCC
TTGACCCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCG
CATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGG
GGAGGATTGGGAAGACAATAGCAGGCGATAAGGATC (SEQ ID NO: 27)

30

In some embodiments, the polyA signal comprises a variant (*e.g.*, a minimal variant, and/or a synthetic variant) polyA signal. In some embodiments, the variant polyA signal comprises fewer nucleotides (*e.g.*, is shorter or smaller) than a BGH polyA signal (*e.g.*, SEQ

ID NO: 27). In some embodiments, the variant polyA signal comprises a sequence comprising an AATAAA sequence and a GT/T-rich sequence. In some embodiments, the AATAAA sequence and the GT/T-rich sequence are separated by 22 nucleotides. In some embodiments, the AATAAA sequence and the GT/T-rich sequence are separated by 23
 5 nucleotides. In some embodiments, the polyA signal comprises a variant polyA signal as described in Levitt, *et al.* (1989), *Genes & Dev.* 3:1019-1025. In some embodiments, the variant polyA signal comprises a nucleotide sequence that has at least 90%, 92.5%, 95%, 98%, or 99% identity to SEQ ID NO: 28. In some embodiments, the polyA signal comprises a nucleotide sequence that differs from the sequence of SEQ ID NO: 28 by 1, 2, 3, 4, 5, or
 10 more than 5 nucleotides. In some embodiments, the variant polyA signal comprises SEQ ID NO: 28, provided below. In some embodiments, the polyA signal comprises the complement of any of SEQ ID NOs: 27 or 28.

Variant polyA signal:

15 AATAAAAGATCTTTATTTTCATTAGATCTGTGTGTTGGTTTTTGTGTG (SEQ ID NO: 28)

A “transgene”, as used herein, refers to a gene or genetic material that has been transferred naturally, or by any of a number of genetic engineering techniques from one
 20 organism to another. A transgene may encode a protein or polypeptide of interest (*e.g.*, S100A1, ARC) or express an RNA of interest (*e.g.*, a siRNA or microRNA). In some embodiments, one rAAV vector may comprise the coding sequence for one or more transgenes. For example, one rAAV vector may comprise the coding sequence for 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 transgenes. In some embodiments, the rAAV vectors of the present
 25 disclosure comprise the coding sequence of both S100A1 and ARC or variants thereof. In some embodiments, the rAAV vector further comprises a region encoding a Rep protein. The transgenes of the present disclosure encode two classes of proteins each having specific function to address different aspects of one or more heart conditions. One class of transgenes may encode a protein which regulates the calcium signaling in cardiomyocytes, *e.g.*, the S100
 30 family proteins. Another class of transgenes may encode apoptosis repressors.

As used herein, the term “variant” refers to a nucleic acid having characteristics that deviate from a reference sequence (*e.g.*, that which occurs in nature). For example, a “variant” is at least about 70% identical, at least about 80% identical, at least about 90%

identical, at least about 95% identical, at least about 96% identical, at least about 97% identical, at least about 98% identical, at least about 99% identical, at least about 99.5% identical, or at least about 99.9% identical to a reference sequence (*e.g.*, the wild type nucleic acid). In some embodiments, the reference sequence may be the wild type or naturally occurring sequence corresponding to the variant. For instance, a transgene variant is a nucleic acid comprising one or more substitutions in the nucleotides of a transgene, as compared to the wild type sequence thereof. In some embodiments, the reference sequence may be a sequence which has been codon optimized, relative to the wild type or naturally occurring sequence, for expression in a particular subject or tissue, and which, in either the wild type or codon optimized configuration, corresponds to the variant. For instance, a transgene variant is a nucleic acid comprising one or more substitutions in the nucleotides of a transgene, as compared to the codon optimized sequence thereof. These substitutions may be silent; *e.g.*, they do not modify the amino acid sequence of any encoded protein (or otherwise result in a variant amino acid sequence).

In some embodiments, provided herein are substitutions in one or more transgenes of the disclosed vectors that do not modify the amino acid sequence of the encoded protein. In some embodiments, such substitutions comprise the substitution of a guanine (G) or cytosine (C), present in the corresponding wild type and/or codon optimized nucleic acid sequence, to another nucleotide (*e.g.*, adenine (A) or tyrosine (T); or, for a G substitution, C; or, for a C substitution, G). Such substitutions reduce the quantity of G and/or C nucleotides present in the variant nucleic acid sequence (*e.g.*, variant transgene), relative to the corresponding wild type (*e.g.*, native, unmodified) and/or codon optimized nucleic acid sequence. Accordingly, such substitutions result in a “reduction of the GC content” of the variant nucleic acid sequence, relative to the corresponding wild type and/or codon optimized nucleic acid sequence. The reduction of GC content in a nucleic acid sequence may be desirable in some embodiments to increase the DNA replication rates during viral production.

In some embodiments, the GC content of the variant human S100A1 nucleic acid sequence is reduced by about 10%, relative to a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8). In some embodiments, the GC content of the variant human S100A1 nucleic acid sequence is reduced by about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% relative to a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8). In some embodiments, the GC content of the variant human S100A1 nucleic acid sequence is reduced

by 12%, relative to a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8). In some embodiments, the GC content of the variant human S100A1 nucleic acid sequence is reduced by 7%, relative to a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8).

5 In some embodiments, the G content of the variant human S100A1 nucleic acid sequence is reduced by about 5 G nucleotides, relative to number of G nucleotides present in a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8). In some
10 embodiments, the G content of the variant human S100A1 nucleic acid sequence is reduced by about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 G nucleotides, relative to number of
15 G nucleotides present in a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8). In some embodiments, the C content of the variant human S100A1 nucleic acid sequence is reduced by about 12 C nucleotides, relative to number of C nucleotides present in a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8). In some
20 embodiments, the C content of the variant human S100A1 nucleic acid sequence is reduced by about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 C nucleotides, relative to number of C nucleotides present in a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8). In some embodiments, the GC content of the variant human S100A1 nucleic acid sequence is reduced by 5 G nucleotides and/or 12 C nucleotides, relative to number of G and C nucleotides present in a codon optimized human S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 8).

 In some embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of about 50-55% (*e.g.*, 50-55% of the nucleotides in the nucleic acid sequence are G or C nucleotides). In some embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of about 30-35%, 35-40%, 40-45%, 45-50%, 50-55%, 55-60%, 60-
25 65%, or 65-70%. In some embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of 50%. In some embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of 51%. In some embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of 52%. In some embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of 53%. In some
30 embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of 54%. In some embodiments, the variant human S100A1 nucleic acid sequence comprises a GC content of 55%.

In some embodiments, the GC content of the variant canine S100A1 nucleic acid sequence is reduced by about 5%, relative to a codon optimized canine S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 21). In some embodiments, the GC content of the variant canine S100A1 nucleic acid sequence is reduced by about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 5 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, or 20% relative to a codon optimized canine S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 21). In some embodiments, the GC content of the variant canine S100A1 nucleic acid sequence is reduced by 13%, relative to a codon optimized canine S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 21). In some embodiments, the GC content of the variant canine S100A1 nucleic acid 10 sequence is reduced by 8%, relative to a codon optimized canine S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 21). In some embodiments, the GC content of the variant canine S100A1 nucleic acid sequence is reduced by 4%, relative to a codon optimized canine S100A1 nucleic acid sequence (*e.g.*, SEQ ID NO: 21).

In some embodiments, the G content of the variant canine S100A1 nucleic acid 15 sequence is reduced by about 4 G nucleotides, relative to number of G nucleotides present in a canine S100A1 codon optimized nucleic acid sequence (*e.g.*, SEQ ID NO: 21). In some embodiments, the G content of the variant canine S100A1 nucleic acid sequence is reduced by about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 G nucleotides, relative to number of G nucleotides present in a canine S100A1 codon optimized nucleic acid sequence (*e.g.*, SEQ 20 ID NO: 21). In some embodiments, the C content of the variant canine S100A1 nucleic acid sequence is reduced by about 5 C nucleotides, relative to number of C nucleotides present in a canine S100A1 codon optimized nucleic acid sequence (*e.g.*, SEQ ID NO: 21). In some 25 embodiments, the C content of the variant canine S100A1 nucleic acid sequence is reduced by about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 C nucleotides, relative to number of C nucleotides present in a canine S100A1 codon optimized nucleic acid sequence (*e.g.*, SEQ ID NO: 21). In some embodiments, the GC content of the variant canine S100A1 nucleic acid sequence is reduced by 4 G nucleotides and/or 5 C nucleotides, relative to number of G and C nucleotides present in a canine S100A1 codon optimized nucleic acid sequence (*e.g.*, SEQ ID NO: 21).

30 In some embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of about 50-55% (*e.g.*, 50-55% of the nucleotides in the nucleic acid sequence are G or C nucleotides). In some embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of about 30-35%, 35-40%, 40-45%, 45-50%, 50-55%, 55-60%, 60-

65%, or 65-70%. In some embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of 50%. In some embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of 51%. In some embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of 52%. In some embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of 53%. In some
5 embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of 54%. In some embodiments, the variant canine S100A1 nucleic acid sequence comprises a GC content of 55%.

 In some embodiments, wherein an rAAV vector comprises one or more variant
10 transgenes (*e.g.*, a first transgene encoding a variant S100A1 protein) with reduced GC content, relative to the corresponding reference (*e.g.*, codon optimized, or wild type) nucleic acid sequences, the rAAV vector will also have a reduced GC content, relative to an rAAV vector comprising two transgenes (*e.g.*, a first transgene encoding a variant S100A1 protein and a second transgene encoding a variant ARC protein) which do not comprise a reduction
15 in GC content. In some embodiments, the rAAV vector with reduced GC content is either of SEQ ID NOs: 22 or 23, and the corresponding rAAV vector which does not comprise a reduction in GC content is any one of SEQ ID NOs: 9-12. In some embodiments, the rAAV vector with reduced GC content comprises a sequence having 100% identity to SEQ ID NO: 22 or SEQ ID NO: 23. In other embodiments, rAAV vector with reduced GC content has at
20 least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to SEQ ID NO: 22 or SEQ ID NO: 23.

 In some embodiments, a transgene variant is a nucleic acid comprising one or more
25 deletions in the nucleotides of a transgene, as compared to the wild type sequence thereof. These deletions may be silent; *e.g.*, they do not modify the amino acid sequence of any encoded protein (or otherwise result in a variant amino acid sequence). In some embodiments, the deletions that do not modify the amino acid sequence of the encoded protein comprise one or more nucleotide deletions in the polyadenylation (pA) signal of the
30 rAAV vector, as compared to the corresponding rAAV vector which does not comprise such deletions. Accordingly, such deletions result in a pA signal which comprises fewer nucleotides (*e.g.*, is smaller, or is shorter, or is truncated), relative to the corresponding rAAV vector which does not comprise such deletions. The deletion of nucleotides in a pA signal

(*e.g.*, a truncated pA signal) in a nucleic acid sequence (*e.g.*, in an rAAV vector sequence) may therefore be desirable in some embodiments to reduce the size of the overall self-complimentary construct to have it under 5 kilobases (including ITRs), which will improve packaging efficiency.

5 In some embodiments, an rAAV vector of the present disclosure comprises a truncated pA signal, relative to the corresponding rAAV vector which does not comprise deletions in the pA signal. In some embodiments, the truncated pA signal comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, or more than 200 nucleotide deletions in the pA signal, relative to the
10 corresponding non-truncated pA signal. In some embodiments, the truncated pA signal comprises 1-10, 5-15, 10-20, 15-25, 20-30, 25-35, 30-40, 35-45, 40-50, 45-55, 50-60, 55-65, 60-70, 65-75, 70-80, 75-85, 80-90, 85-95, 90-100, 95-105, 100-110, 105-115, 105-115, 110-120, 115-125, 120-130, 125-135, 130-140, 135-145, 140-150, 145-155, 150-160, 155-165, 160-170, 165-175, 170-180, 175-185, 180-190, 185-195, 190-200, 195-205, or more than 200
15 nucleotide deletions relative to the corresponding non-truncated pA signal. In some embodiments, the truncated pA signal comprises about 160 or about 170 nucleotide deletions relative to the non-truncated pA signal. In some embodiments, the truncated pA signal comprises 166 nucleotide deletions relative to the non-truncated pA signal. In some
20 embodiments, the variant polyA signal comprises a nucleotide sequence that has at least 90%, 92.5%, 95%, 98%, or 99% identity to SEQ ID NO: 28. In some embodiments, the variant polyA signal comprises SEQ ID NO: 28.

In some embodiments, the rAAV vector comprising the truncated pA signal is either of SEQ ID NOs: 22 or 23, and the corresponding rAAV vector which does not comprise a truncated pA signal is any one of SEQ ID NOs: 9-12. In some embodiments, the rAAV
25 vector with a truncated pA signal comprises a sequence having 100% identity to SEQ ID NO: 22 or SEQ ID NO: 23. In other embodiments, rAAV vector with a truncated pA signal has at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about
30 99.9% identity to SEQ ID NO: 22 or SEQ ID NO: 23. In some embodiments, the rAAV vector has a nucleic acid sequence having at least about 80% identity, 90% identity, 95% identity, 96% identity, 97% identity, 98% identity, 99% identity, 99.5% identity, or 99.9% identity to SEQ ID NO: 22 or NO: 23, with the exception of comprising a domain having a

sequence having at least 99%, or 100%, identity to SEQ ID NO: 25 or 26. In some embodiments, the rAAV vector has a nucleic acid sequence having at least about 80% identity, 90% identity, 95% identity, 96% identity, 97% identity, 98% identity, 99% identity, 99.5% identity, or 99.9% identity to SEQ ID NO: 22 or NO: 23, with the exception of
5 comprising a domain having a sequence having at least 99%, or 100%, identity to SEQ ID NO: 28.

Alternatively, these substitutions may result in modifications to the amino acid sequence of an encoded protein, resulting in an encoded protein having one or more amino acid substitutions (*e.g.*, having 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 10-15, or 15-20 amino acid
10 substitutions) relative to the wild type protein sequence. These substitutions include chemical modifications as well as truncations. This term further embraces functional fragments of a wild type nucleic acid sequence. These modifications of the reference sequence may occur at the 5' or 3' ends of the reference sequence or anywhere between those positions, interspersed either individually among nucleotides in the reference sequence or in
15 one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule or polypeptide is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to, for instance, the nucleotide sequence of a transgene or encoded protein, can be determined conventionally using known computer programs. A preferred method for determining the best overall match
20 between a query sequence (*e.g.*, a sequence of the present disclosure) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB or blastn computer program based on the algorithm of Brutlag *et al.* (Comp. App. Biosci. 6:237-245 (1990)). In a sequence alignment the query and subject sequences are either both nucleotide sequences or both amino acid sequences. The result of said global sequence
25 alignment is expressed as percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. Whether a nucleotide or polypeptide is matched/aligned is
30 determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present disclosure. For subject sequences truncated at the 5'

and/or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of nucleotides of the query sequence that are positioned 5' to or 3' to the query sequence, which are not matched/aligned with a corresponding subject nucleotide, as a percent of the total bases of the query sequence. Similar procedures can be used for the comparison of amino acid sequences.

S100 family proteins that may be used in accordance to the present disclosure include, without limitation, S100A1, S100A2, S100A3, S100A4, S100A5, S100A6, S100A7 (*e.g.*, psoriasin), S100A8 (*e.g.*, calgranulin A), S100A9 (*e.g.*, calgranulin B), S100A10, S100A11, S100A12 (*e.g.*, calgranulin C), S100A13, S100A14, S100A15 (*e.g.*, koebnerisin), S100A16, S100B, S100P, and S100Z, or variants thereof.

In some embodiments, the S100 family protein may be S100 calcium-binding protein A1 (S100A1). In some embodiments, the S100A1 is cardiac S100A1 (cS100A1) or a variant thereof. The cS100A1 protein is a regulator of myocardial contractility. cS100A1 protein levels are reduced in right ventricular hypertrophied tissue in a model of pulmonary hypertension. Further, S100A1 is a regulator of the genetic program underlying cardiac hypertrophy, in that S100A1 inhibits α 1 adrenergic stimulation of hypertrophic genes, including MYH7, ACTA1 and S100B.

In cardiomyocytes, S100A1 regulates the calcium-controlled network of SR, sarcomeric, and mitochondrial function through modulation of ryanodine receptor 2 (RyR2), sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), titin, and mitochondrial F1-ATPase activity. As a result, cardiomyocytes and hearts with increased S100A1 expression show increased systolic and diastolic performance, a result of improved Ca^{2+} transient amplitudes resulting from augmented SR Ca^{2+} load and subsequent systolic Ca^{2+} release together with decreased diastolic SR Ca^{2+} leak and enhanced Ca^{2+} re-sequestration. Concurrently, S100A1 increases mitochondrial high-energy phosphate production and thus coordinates the energy supply with the increased adenosine 5'-triphosphate (ATP) demand by the enhanced cardiomyocyte Ca^{2+} turnover. Reduced S100A1 expression in cardiomyocytes is associated with reduced contractile function, corroborating the pathophysiological significance of this protein.

In some embodiments, the S100A1 cDNA (transgene) sequence of the polynucleotides of any of the disclosed rAAV vectors has 100% identity to a naturally-occurring S100A1 sequence. In some embodiments, the naturally-occurring S100A1 sequence is human-derived. In some embodiments, the naturally-occurring S100A1 sequence

is canine-derived. In other embodiments, the S100A1 cDNA sequence has at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to a

5 naturally-occurring S100A1 sequence.

In some embodiments, the S100A1 cDNA sequence is codon-optimized for expression in human cells. In some embodiments, the S100A1 cDNA (transgene) sequence comprises a sequence having 100% identity to SEQ ID NO: 5, SEQ ID NO: 8 or SEQ ID NO: 25. In some embodiments, the rAAV vector comprises an S100A1 transgene that
10 comprises SEQ ID NO: 8, which is a sequence codon-optimized for expression in humans. In some embodiments, the S100A1 cDNA sequence is SEQ ID NO: 25, which is a sequence codon-optimized for expression in humans that further has a reduced guanine and cytosine (G/C) content. In other embodiments, the S100A1 cDNA sequence has at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity,
15 at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to SEQ ID NO: 5, SEQ ID NO: 8 or SEQ ID NO: 25.

In other embodiments, the S100A1 cDNA sequence is codon-optimized for expression in canine cells. In some embodiments, the S100A1 cDNA (transgene) sequence
20 comprises a sequence having 100% identity to SEQ ID NO: 1, SEQ ID NO: 21 or SEQ ID NO: 26. In some embodiments, the rAAV vector comprises an S100A1 transgene that comprises SEQ ID NO: 21, which is a sequence codon-optimized for expression in canines. In some embodiments, the S100A1 cDNA sequence is SEQ ID NO: 26, which is a sequence codon-optimized for expression in canines that further has a reduced G/C content. In some
25 embodiments, the rAAV vector comprises an S100A1 cDNA sequence that has at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to SEQ ID NO: 1, SEQ ID NO: 21, or SEQ ID NO: 26.

30 Non-limiting examples of S100A1 cDNA sequences are set forth below.

native S100A1 (*homo sapiens*)

ATGGGCTCTGAGCTGGAGACGGCGATGGAGACCCTCATCAACGTGTTCCACGCC
 CACTCGGGCAAAGAGGGGGACAAGTACAAGCTGAGCAAGAAGGAGCTGAAAGA
 GCTGCTGCAGACGGAGCTCTCTGGCTTCCTGGATGCCCAGAAGGATGTGGATGCT
 GTGGACAAGGTGATGAAGGAGCTAGACGAGAATGGAGACGGGGAGGTGGACTT
 5 CCAGGAGTATGTGGTGCTTGTGGCTGCTCTCACAGTGGCCTGTAACAATTTCTTCT
 GGGAGAACAGTTGA (SEQ ID NO: 5)

optimized S100A1 (*homo sapiens*)

ATGGGCAGCGAGCTGGAGACCGCCATGGAGACCCTGATCAACGTGTTCCACGCC
 10 CACAGCGGCAAGGAGGGGCGACAAGTACAAGCTGAGCAAGAAGGAGCTGAAGGA
 GCTGCTGCAGACCGAGCTGAGCGGCTTCCTGGACGCCCAGAAGGACGTGGACGC
 CGTGGACAAGGTGATGAAGGAGCTGGACGAGAACGGCGACGGCGAGGTGGACT
 TCCAGGAGTACGTGGTGCTGGTGGCCGCCCTGACCGTGGCCTGCAACAACCTTCTT
 CTGGGAGAACAGCTGA (SEQ ID NO: 8)

15

optimized S100A1 with reduced G/C content (*homo sapiens*)

ATGGGCTCTGAGCTGGAGACCGCCATGGAGACCCTGATCAATGTGTTCCACGCCC
 ACTCTGGCAAGGAGGGGCGATAAGTACAAGCTGTCTAAGAAGGAGCTGAAGGAGC
 TGCTGCAGACCGAGCTGTCTGGCTTCCTGGATGCCCAGAAGGATGTGGATGCCGT
 20 GGATAAGGTGATGAAGGAGCTGGATGAGAATGGCGATGGCGAGGTGGATTTCGA
 GGAGTACGTGGTGCTGGTGGCCGCCCTGACCGTGGCCTGCAATAATTTCTTCTGG
 GAGAATTCTTGA (SEQ ID NO: 25)

25 A nucleotide sequence alignment between a codon-optimized human S100A1 cDNA
 sequence and a native human S100A1 cDNA sequence (SEQ ID NOs: 8 and 5, respectively)
 is shown in FIG. 13.

For reference, certain non-limiting examples of animal-derived S100A1 cDNA
 sequences are described below. A nucleotide sequence alignment between a codon-
 optimized canine S100A1 cDNA sequence and a codon-optimized human S100A1 cDNA
 30 sequence (SEQ ID NOs: 21 and 8, respectively) is shown in FIG. 14. SEQ ID NO: 26, which
 is a codon-optimized canine S100A1 sequence with reduced G/C content, is set forth below.

native S100A1 (*canis lupus familiaris*)

(NCBI Reference Sequence: XM_005622816.2)

ATGGGCTCTGAGCTGGAGACAGCGATGGAGACTCTCATCAATGTGTTCCATGCCC
ACTCGGGCAAGGAGGGAAACAAGTACAAGCTGAGCAAGAAGGAGCTAAAGGAG
CTGCTGCAGACTGAGCTCTCCGGCTTCCTGGACGCCCAGAAGGATGCGGATGCTG
5 TGGACAAGGTGATGAAAGAGCTAGATGAGAATGGAGATGGGGAGGTGGACTTCC
AGGAGTATGTGGTGCTGGTGGCTGCCCTCACAGTGGCCTGTAACAACCTTCTTCTG
GGAAAACAGTTGA (SEQ ID NO: 1)

optimized S100A1 (*canis lupus familiaris*)

10 ATGGGCAGCGAGCTGGAGACCGCCATGGAGACCCTGATCAACGTGTTCCACGCC
CACAGCGGCAAGGAGGGCAACAAGTACAAGCTGAGCAAGAAGGAGCTGAAGGA
GCTGCTGCAGACCGAGCTGAGCGGCTTCCTGGACGCCCAGAAGGACGCCGACGC
CGTGGACAAGGTGATGAAGGAGCTGGACGAGAACGGCGACGGCGAGGTGGACT
TCCAGGAGTACGTGGTGCTGGTGGCCGCCCTGACCGTGGCCTGCAACAACCTTCTT
15 CTGGGAGAACAGCTGA (SEQ ID NO: 21)

optimized S100A1 with reduced G/C content (*canis lupus familiaris*)

ATGGGCTCTGAGCTGGAGACCGCCATGGAGACCCTGATCAATGTGTTCCACGCCC
ACAGCGGCAAGGAGGGCAATAAGTACAAGCTGTCTAAGAAGGAGCTGAAGGAG
20 CTGCTGCAGACCGAGCTGTCTGGCTTCCTGGACGCCCAGAAGGACGCCGACGCC
GTGGACAAGGTGATGAAGGAGCTGGACGAGAAATGGCGACGGCGAGGTGGACTT
CCAGGAGTACGTGGTGCTGGTGGCCGCCCTGACCGTGGCCTGCAATAATTTCTTC
TGGGAGAATTCTTGA (SEQ ID NO: 26)

25 native S100A1 (*felis catus*)

(NCBI Reference Sequence: XM_003999773.3)

ATGGGCTCAGAGCTGGAGACGGCGATGGAGACTCTCATCAACGTGTTCCACGCC
CACTCGGGCAAGGAGGGAGACAAGTACAAGCTGAGCAAGAAGGAGCTAAAAGA
GCTGCTGCAGACCGAGCTCTCTGGCTTCCTGGACGCCCAGAAGGATGCCGACGCT
30 GTGGACAAGGTGATGAAAGAGCTAGACGAGAAATGGAGATGGGGAGGTGGACTT
CCAAGAGTATGTGGTGCTGGTGGCTGCCCTCACAGTGGCCTGTAACAACCTTTTTC
TGGGAGAACAGTTGA (SEQ ID NO: 2)

Aspects of the present disclosure provide compositions and methods that include the delivery of a transgene encoding an apoptotic inhibitor (*e.g.*, an anti-apoptotic agent). Illustrative examples of apoptotic inhibitors include fink, p35, crmA, Bcl-2, Bcl-XL, Mcl-1, E1B-19K from adenovirus, as well as antagonists of pro-apoptotic agents (*e.g.*, antisense, 5 ribozymes, antibodies, *etc.*). In some embodiments, the apoptotic inhibitor is cardiac Apoptosis Repressor with Caspase Recruitment Domain (ARC), or a variant thereof. In other embodiments, the apoptotic inhibitor is cardiac ARC or a variant thereof. In some embodiments, it may be desirable to deliver an S100 family protein and the apoptotic inhibitor separately. In certain embodiments, a transgene encoding the S100 family protein is 10 delivered concurrently or sequentially with one or more small molecule apoptotic inhibitors. Other contemplated small-molecule apoptotic inhibitors include c-Myc inhibitors, Bax inhibitors, p53 inhibitors, tBid inhibitors, caspase inhibitors, and inhibitors of pro-apoptotic BCL-2 family members.

The cARC is an apoptotic regulatory protein expressed almost exclusively in 15 myogenic cells. It contains a caspase recruitment domain (CARD) through which it blocks the activation of some initiator caspases. ARC also blocks caspase-independent events associated with apoptosis. Apoptosis caused by acute ischemia and subsequent ventricular remodeling is implicated as a mediator of heart failure. Although post-ischemic heart failure may have multiple causes, recent attention has been directed toward understanding the 20 contribution of apoptosis or programmed cell death. Apoptosis is characterized by preservation of mitochondrial and sarcolemmal membranes, nuclear chromatin condensation, and phagocytosis by macrophages or neighboring cells without triggering an inflammatory response. The activation of apoptosis is known to occur through mechanisms involving caspases, a family of cysteine proteases that are synthesized as inactive precursors and 25 proteolytically cleaved into their active form. ARC is able to block the activation of apoptosis by blocking the caspases.

Accordingly, in some aspects, provided herein are rAAV vectors (or rAAV nucleic acid vectors) comprising a polynucleotide that comprises a sequence that is at least 90%, at least 95%, or at least 99.5% identical to any one of the nucleotide sequences of SEQ ID NOs: 30 6-8, 16, 21, 25 and 26. In some embodiments, the polynucleotide sequence differs from the sequence of any one of SEQ ID NOs: 6-8, 16, 21, 25 and 26 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 nucleotides. In some embodiments, the polynucleotide sequence contains stretches of about 35, 50, 75, 100, 125, 150, 175, 200, 220, 225, 230, 235, 240, 245,

250, 255, 260, 265, 270, 275, 280 or 285 consecutive nucleotides in common with any one of SEQ ID NOs: 6-8, 16, 21, 25 and 26. In particular embodiments, any of the disclosed rAAV vectors comprise a polynucleotide that comprises any of SEQ ID NOs: 6-8, 16, 21, 25 and 26.

In some embodiments, the disclosed cARC and S100 transgene sequences comprise truncations at the 5' or 3' end relative to the wild-type or codon optimized sequences. In some embodiments, the disclosed transgene sequences comprise truncations at the 5' or 3' end relative to SEQ ID NOs: 1, 3, 5-8 and 15-21. In some embodiments, the transgene comprises a nucleotide sequence that differs from the sequence of any one of SEQ ID NOs: 1, 3, 5-8 and 15-21 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 nucleotides. In some embodiments, the transgene contains stretches of about 35, 50, 75, 100, 125, 150, 175, 200, 220, 225, 230, 235, 240, 245, 250, 255, 260, 265, 270, 275, 280, 285, 300, 350, 400, 450, 500, 550, 575, or 600 consecutive nucleotides in common with any one of SEQ ID NOs: 1, 3, 5-8 and 15-21.

In some embodiments, the cardiac ARC cDNA (or transgene) sequence of the polynucleotides of the disclosed rAAV vectors has 100% identity to a naturally-occurring cARC sequence. In other embodiments, the cARC cDNA sequence has at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to a naturally-occurring cARC sequence. In some embodiments, the naturally-occurring cARC sequence is human-derived. In some embodiments, the naturally-occurring cARC sequence is canine-derived.

In particular embodiments, the cARC cDNA sequence is codon-optimized for expression in human cells. In some embodiments, the cARC cDNA (transgene) sequence comprises a sequence having 100% identity to SEQ ID NO: 6 or SEQ ID NO: 7. In other embodiments, the cARC cDNA sequence has at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to SEQ ID NO: 6 or SEQ ID NO: 7.

In other embodiments, the cARC cDNA sequence is codon-optimized for expression in canine cells. In some embodiments, the rAAV vector comprises a cARC cDNA sequence that has at least about 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least

about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to SEQ ID NO: 16. In some embodiments, the rAAV vector comprises SEQ ID NO: 16.

Non-limiting examples of cARC cDNA sequences are described below.

5

The native ARC (*homo sapiens*) cDNA sequence is shown in SEQ ID NO: 18.

optimized ARC (*homo sapiens*)

ATGGGCAACGCCCAGGAGCGGCCAGCGAGACCATCGACCGGGAGCGGAAGCG
10 GCTGGTGGAGACCCTGCAGGCCGACAGCGGCCTGCTGCTGGACGCCCTGCTGGC
CCGGGGCGTGCTGACCGGCCCCGAGTACGAGGCCCTGGACGCCCTGCCCCACGC
CGAGCGGCGGGTGCGGCGGCTGCTGCTGCTGGTGCAGGGCAAGGGCGAGGCCGC
CTGCCAGGAGCTGCTGCGGTGCGCCCAGCGGACCGCCGGCGCCCCGACCCCGC
CTGGGACTGGCAGCACGTGGGCCCCGGCTACCGGGACCGGAGCTACGACCCCCC
15 CTGCCCCGGCCACTGGACCCCCGAGGCCCGCGCAGCGGCACCACCTGCCCCGG
CCTGCCCCGGGCCAGCGACCCCGACGAGGCCGGCGGCCCGAGGGCAGCGAGGC
CGTGCAGAGCGGCACCCCGAGGAGCCCGAGCCCGAGCTGGAGGCCGAGGCCA
GCAAGGAGGCCGAGCCCGAGCCCGAGCCCGAGCCCGAGCTGGAGCCCGAGGCC
GAGGCCGAGCCCGAGCCCGAGCTGGAGCCCGAGCCCGACCCCGAGCCCGAGCCC
20 GACTTCGAGGAGCGGGACGAGAGCGAGGACAGCTGA (SEQ ID NO: 6)

optimized ARC (*homo sapiens*)

ATGGGGAATGCCCAAGAAAGGCCTTCTGAGACTATAGACCGCGAGCGCAAGAGG
CTTGTAAGAACCTTGCAGGCGGACTCTGGTCTCTTGCTGGACGCTCTGCTTGCGC
25 GGGGTGTTCTGACTGGACCGGAGTACGAAGCATTGGATGCCCTTCCTGATGCAGA
GAGACGAGTTAGACGCCTGTTGCTTCTTGTCGAAGGCAAGGGTGAAGCCGCCTGT
CAAGAGCTCCTGAGGTGTGCTCAACGAACCGCCGGGGCGCCAGATCCGGCATGG
GATTGGCAACATGTGGGGCCCGGCTATCGGGACCGGAGCTACGATCCACCATGC
CCGGGTCAATTGGACGCCGGAGGCTCCAGGATCTGGTACAACATGCCCAGGACTC
30 CCAAGAGCCAGTGACCCCGATGAAGCTGGAGGCCCGAGGGCAGTGAAGCCGTA
CAGAGCGGTACCCAGAGAAGAACCAGAACCGGAGCTGGAGGCTGAAGCTAGTAA
AGAGGCGGAACCTGAACCCGAACCGGAGCCTGAGCTCGAGCCAGAGGCTGAGG

CCGAGCCAGAGCCTGAACTCGAACCCGAACCTGATCCAGAACCAGAGCCCCGACT
TCGAGGAACGGGATGAGTCAGAGGATTCTTGA (SEQ ID NO: 7)

A nucleotide sequence alignment between a codon-optimized human ARC cDNA
5 sequence and a native human ARC cDNA sequence (SEQ ID NOs: 6 and 18, respectively) is
shown in FIG. 12.

For reference, certain non-limiting examples of animal-derived ARC cDNA
sequences are described below. A nucleotide sequence alignment between a codon-
optimized canine ARC cDNA sequence and a codon-optimized human ARC cDNA sequence
10 (SEQ ID NOs: 16 and 6, respectively) is shown in FIG. 11.

native ARC (*canis lupus familiaris*)

(NCBI Reference Sequence: NM_001048121.1)

ATGCAGGAAGCGCCAGCCGCGCTGCCACGGAGCCGGGCCCCAGCCCCGTGCCT
15 GCCTTCCTCGGCAAGCTGTGGGCGCTGGTGGGCGACCCGGGGACCGACCACCTC
ATCCGCTGGAGCCCAGCGGGACCAGTTTCCTCGTCAGCGACCAGAGCCGCTTC
GCCAAGGAAGTGCTGCCCCAGTACTTCAAGCACAGCAACATGGCGAGCTTCGTG
CGGCAGCTCAACATGTACGGTTTTTCGGAAGGTGGTGAGCATCGAGCAGGGCGGC
CTGCTCAGGCCGGAGCGCGACCACGTCGAGTTCCAGCACCCGAGCTTCGTCCGC
20 GGCCGAGAGCAACTCCTGGAGCGCGTGCGGCGCAAGGTGCCCGCGCTGCGCAGC
GACGACGGCCGCTGGCGCCCCGAGGACCTGGGCGCGCTGCTGGGCGAGGTGCAG
GCTTTGCGGGGAGTGACAGGAGATCACCGAGGCGCGGCTGCGGGAGCTCAGGCAG
CAGAACGAGATCTTATGGAGGGAGGTGGTGACTCTGCGGCAGAGCCACGGTCAG
CAGCATCGCGTCATTGGCAAGCTGATCCAGTGCCTCTTTGGGCCACTTCAGACAG
25 GGTCCAGCGGCGCAGGAGCTAAGAGAAAGCTGTCTCTGATGCTGGATGAGGGGA
GCTCATGCCCAACACCGGCCAAATTCAACACCTGTCCTTTACCTGGTGCCCTCTT
GCAGGATCCCTACTTTATCCAGTCGCCCCCTCCCAGAGACCACCTTGGGCCTCAGC
AGCTCTCATAGGACCAGGGGCCCTATCATCTCTGACATCCATGAAGACTCTCCCT
CCCCTGATGGGACCAGGCTTTCTCCTTCCAGTGGTGGCAGGAGGGAGAAGGGCC
30 TGGCACTGCTCAAAGAAGAGCCGGCCAGCCCAGGGGGGGAAGGCGAGGCCGGG
CTGGCCCTGGCCCCAAACGAGTGTGACTTCTGCGTGACAGCCCCCCCCCACTGT
CCGTGGCTGTGGTGCAGGCCATCCTGGAAGGGAAGGGGAAGTTTCAGCCCCGAGG
GGCCAGGAATGCCCAACAGCCTGAACCAAGGGGTCCCAGGGAGGTACCTGACA

GGGGGACTCTGGGCCTGGACAGGGGGGCACGAAGCCCAGAGAATCTGCTGCCTC
CCATGCTGCTTCGGGGCCCCCCTGAAAGTGTGGAGCCTGCAGGGCCCCCTGGATGT
GCTGGGCCCCAGCCATCAAGGGCGAGAATGGACCCTGATGGACTTGGACATGGA
GCTGTCCCTGATGCAGCCCTTGGGTCCAGAGAGGAGTGAGACTGAGCTGGCGGT
5 CAAGGGGTAAATTCTCCGGGGCCAGGGAAGGACTCCACACTTGGGGCACCCT
CCTGCTCGATGTCCAAGCGGCTTTGGGAGGCCCAGCTCTCAGCCTTCCTGGAGCT
TTAACCATTACAGCACCCCTGAGAGCCGAGCCAACCTAGGCCAGGGGCC
AATCCCTCCCCCTGA (SEQ ID NO: 3)

10 optimized ARC (*canis lupus familiaris*)
ATGGGCAACAGCCAGGAGCGGCCAGCGAGACCATCGACCGGGAGCGGAAGCG
GCTGGTGGAGACCCTGCAGGCCGACAGCGGCCTGCTGCTGGACGCCCTGCTGGC
CCGGGGCGTGCTGGCCGGCCCCGAGTACGAGGCCCTGGACGCCCTGCCGACGC
CGAGCGGCGGGTGCGGCGGCTGCTGCTGCTGGTGCAGAGCAAGGGCGAGGCCGC
15 CTGCCAGGAGCTGCTGCTGTGCGCCCAGCGGACCGCCCGGGCCCCCGACCCCGC
CTGGGACTGGCAGCACGTGGGCACCGGCTACCGGGAGCGGAGCTGGGACGCCGC
CTGCGCCGGCCACTGGACCCCCGAGGCCCGGCGAGCAGCACCACTGCCCCGA
GCTGCCCCGGGCCCGCCGACTGCGGCGAGCCCGGCGCCCCCGGCGGCAGCGAGGC
CGCCCAGAGCGGCAGCCTGGAGGAGCCCGACCCGAGCTGGAGGCCGGCGCCG
20 AGCTGGAGAGCGAGCCCCAGATGGACCTGGAGCCCGAGCCCGAGGCCGAGCCC
GAGCCCGAGCTGGAGCGGGAGCCCGAGCCCGAGCCCGAGCCCGACCTGGAGGC
CGGCGACGAGAGCGAGGACAGCTGA (SEQ ID NO: 16)

native ARC (*felis catus*)

25 (NCBI Reference Sequence: XM_006941587.2)
ATGGGCAATGCGCAGGAGCGGCCCTCAGAGACGATCGATCGCGAGCGGAAACG
CCTAGTGGAGACGCTGCAGGACGACTCCGGGCTGCTGCTGGATGCACTGCTGGC
GCGCGGCGTGCTCACCGGGCCTGAGTATGAGGCGTTGGACGCGCTGCCTGATGC
CGAGCGCAGGGTGCGTCGCCTGCTGCTGCTGGTACAAAGCAAGGGCGAGGCCGC
30 CTGCCAGGAGCTGCTGCACTGCGCCCAGCGTACTACGCGCGCGCCAGACCCGGC
CTGGGACTGGCAGCACGTGGGCACTGGCTACCGGGAACGCAGCTACGACTCTCC
ATGCCCTGGCCACTGGACGCCTGAGGCACCTGACTTGAGGACCGCTTGCCCCGA
AACGCCAGAGCTTCAGACTGCGACGAGGCTGGGGTTTCAGGGGGCTCGGAGGC

AGTATCCGGAACCCTCGAGGAACTCGATCCGGAAGTGGGAAGCTGAAGTCTCTGA
 AGGGGCTGAGCCAGAGCCAGAGCCAGAGCCGACTTTGAGGCGGGTGATGAGTC
 TGAAGATTCC (SEQ ID NO: 4)

5 In other aspects, the two or more transgenes of any of the disclosed rAAV vectors
 comprise a transgene encoding a dominant negative form of Phospholamban (or dn-PLN).
 Phospholamban (PLN) is an endogenous inhibitor of the sarco/endoplasmic reticulum
 Ca²⁺ATPase 2a (SERCA2a) pump, which mediates calcium ion reuptake into the
 sarcoplasmic reticulum (SR) of cardiomyocytes. The dominant negative form is a
 10 pseudophosphorylated form that competes for binding SERCA2a with native
 phospholamban, and thereby reduces its inhibitory effect on SERCA2a (see Bish, *et al. Hum*
Gene Ther. 2011; 22(8): 969-977, herein incorporated by reference). Accordingly, in some
 embodiments, the second transgene of the disclosed rAAV vectors encodes dn-PLN. In some
 embodiments, the disclosed rAAV vectors may comprise any of the disclosed S100A1
 15 transgenes (*e.g.*, a human or canine codon-optimized S100A1 sequence) and a dn-PLN
 transgene. In some embodiments, the disclosed rAAV vectors may encode, and thereby
 deliver into a cell, a S100A1 protein and a dn-PLN protein.

In further aspects, any of the disclosed rAAV vectors may comprise three or more
 transgenes. In some embodiments, the rAAV vectors comprise three transgenes. In some
 20 embodiments, the third transgene encodes a dn-PLN sequence. Said three transgenes may
 comprise a first transgene encoding S100A1 (*e.g.*, a human or canine codon-optimized
 S100A1 sequence), a second transgene encoding cARC (*e.g.*, a human or canine codon-
 optimized cARC sequence), and a third transgene encoding dn-PLN.

In some embodiments, the disclosed rAAV vectors do not encode a protein derived
 25 from a canine or feline. In some embodiments, any of the disclosed vectors does not
 comprise a native canine or feline S100A1-encoding or ARC-encoding nucleotide sequence.
 In some embodiments, the disclosed rAAV vectors do not comprise SEQ ID NO: 1 or 2. In
 some embodiments, the disclosed rAAV vectors do not comprise SEQ ID NO: 3 or 4, or any
 of SEQ ID NOs: 1-4.

30

Recombinant AAV (rAAV) Vectors

Aspects of the present disclosure relate to recombinant AAV vectors that may be used
 for gene therapy for heart diseases. As used herein, the term “vector” may refer to a nucleic

acid vector (*e.g.*, a plasmid or recombinant viral genome), a wild-type AAV genome, or a virus that comprises a viral genome. In some embodiments, the term “vector” may refer to a viral particle, such as an AAV viral particle.

The wild-type AAV genome is a single-stranded deoxyribonucleic acid (ssDNA),
5 either positive- or negative-sensed. The genome comprises two inverted terminal repeats (ITRs), one at each end of the DNA strand, and two open reading frames (ORFs): rep and cap between the ITRs. The rep ORF comprises four overlapping genes encoding Rep proteins required for the AAV life cycle. The cap ORF comprises overlapping genes encoding capsid proteins: VP1, VP2 and VP3, which interact together to form the viral capsid. VP1, VP2 and
10 VP3 are translated from one mRNA transcript, which can be spliced in two different manners. Either a longer or shorter intron can be excised resulting in the formation of two isoforms of mRNAs: a ~2.3 kb- and a ~2.6 kb-long mRNA isoform. The capsid forms a supramolecular assembly of approximately 60 individual capsid protein subunits into a non-enveloped, T-1 icosahedral lattice capable of protecting the AAV genome. A mature AAV
15 capsid is composed of VP1, VP2, and VP3 (molecular masses of approximately 87, 73, and 62 kDa respectively) in a ratio of about 1:1:10.

Recombinant AAV (rAAV) particles may comprise a recombinant nucleic acid vector (hereafter referred to as “rAAV vector”), which may comprise at a minimum: (a) one or more heterologous nucleic acid regions comprising a transgene; and (b) one or more regions
20 comprising sequences that facilitate the integration of the heterologous nucleic acid region (optionally with the one or more nucleic acid regions comprising a sequence that facilitates expression) into the genome of the subject. In some embodiments, the sequences facilitating the integration of the heterologous nucleic acid region (optionally with the one or more nucleic acid regions comprising a sequence that facilitates expression) into the genome of the
25 subject are inverted terminal repeat (ITR) sequences (*e.g.*, wild-type ITR sequences or engineered ITR sequences) flanking the one or more nucleic acid regions (*e.g.*, heterologous nucleic acid regions).

In certain embodiments, the ITR sequences flank a nucleic acid region comprising two or more transgenes and a promoter operably linked to the two or more transgenes and a
30 polyadenylation (polyA) signal. In certain embodiments, the ITR sequences flank a nucleic acid region consisting of two or more transgenes and a promoter operably linked to the two or more transgenes and a polyA signal.

In some embodiments, the rAAV nucleic acid vector comprises one or more transgenes comprising a sequence encoding a protein or polypeptide of interest operably linked to a promoter, wherein the one or more transgenes are flanked on each side with an ITR sequence. In some embodiments, the nucleic acid vector further comprises a region
5 encoding a Rep protein as described herein, either contained within the region flanked by ITRs or outside the region or nucleic acid, operably linked to a promoter. The ITR sequences may be derived from any AAV serotype (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) or may be derived from more than one serotype. In some embodiments, the ITR sequences are derived from AAV2 or AAV6 serotypes. In some embodiments, a first serotype provided herein is not an
10 AAV2 or AAV8 serotype. In some embodiments, the ITR sequences of the first serotype are derived from AAV3, AAV5 or AAV6. In some embodiments, the ITR sequences are derived from AAV2, AAV3, AAV5 or AAV6. In some embodiments, the ITR sequences are the same serotype as the capsid (*e.g.*, AAV6 ITR sequences and AAV6 capsid, *etc.*). In some embodiments, the ITR sequences are derived from AAVrh.10 serotype.

15 ITR sequences and plasmids containing ITR sequences are known in the art and commercially available (see, *e.g.*, products and services available from Vector Biolabs, Philadelphia, PA; Cellbiolabs, San Diego, CA; Agilent Technologies, Santa Clara, Ca; and Addgene, Cambridge, MA; and Gene delivery to skeletal muscle results in sustained expression and systemic delivery of a therapeutic protein. Kessler PD, *et al. Proc Natl Acad*
20 *Sci U S A*. 1996 Nov 26;93(24):14082-7; and Curtis A. Machida. *Methods in Molecular Medicine*TM. *Viral Vectors for Gene Therapy Methods and Protocols*. 10.1385/1-59259-304-6:201 © Humana Press Inc. 2003. Chapter 10. Targeted Integration by Adeno-Associated Virus. Matthew D. Weitzman, Samuel M. Young, Jr., Toni Cathomen and Richard Jude Samulski; U.S. Pat. Nos. 5,139,941 and 5,962,313, all of which are
25 incorporated herein by reference). In some embodiments, the rAAV comprises a pTR-UF-11 plasmid backbone, which is a plasmid that contains AAV2 ITRs. This plasmid is commercially available from the American Type Culture Collection (ATCC MBA-331).

In some embodiments, the rAAV vectors of the present disclosure comprise both a cS100A1 transgene and a cARC transgene, for the concurrent delivery and expression of both
30 cS100A1 and cARC proteins in a subject. The transgene encoding the S100 family protein (*e.g.*, a cS100A1) may be positioned 5' to the transgene encoding the apoptotic inhibitor (*e.g.*, a cARC) within the herein-described rAAV nucleic acid vectors. Alternatively, the transgene

encoding the apoptotic inhibitor may be positioned 5' to the transgene encoding the S100 family protein within the described rAAV nucleic acid vectors.

Thus, in some embodiments, the rAAV vector comprises one or more regions comprising a sequence that facilitates expression of the transgene (*e.g.*, the heterologous nucleic acid), *e.g.*, expression control sequences operably linked to the nucleic acid. Numerous such sequences are known in the art. Non-limiting examples of expression control sequences include promoters, insulators, silencers, response elements, introns, enhancers, initiation sites, internal ribosome entry sites (IRES) termination signals, and poly(A) signals. Any combination of such control sequences is contemplated herein (*e.g.*, a promoter and a poly(A) signal). In some embodiments, the rAAV vectors comprise a promoter that is operably linked to the coding sequence of the transgenes and facilitates expression of the transgenes.

A “promoter”, as used herein, refers to a control region of a nucleic acid at which initiation and rate of transcription of the remainder of a nucleic acid sequence are controlled. A promoter drives transcription of the nucleic acid sequence that it regulates, thus, it is typically located at or near the transcriptional start site of a gene. A promoter may have, for example, a length of 100 to 1000 nucleotides. In some embodiments, a promoter is operably linked to a nucleic acid, or a sequence of a nucleic acid (nucleotide sequence). A promoter is considered to be “operably linked” to a sequence of nucleic acid that it regulates when the promoter is in a correct functional location and orientation relative to the sequence such that the promoter regulates (*e.g.*, to control (“drive”) transcriptional initiation and/or expression of) that sequence.

Promoters that may be used in accordance with the present disclosure may comprise any promoter that can drive the expression of the transgenes in the heart of the subject. In some embodiments, the promoter may be a tissue-specific promoter. A “tissue-specific promoter”, as used herein, refers to promoters that can only function in a specific type of tissue, *e.g.*, the heart. Thus, a “tissue-specific promoter” is not able to drive the expression of the transgenes in other types of tissues. In some embodiments, the promoter that may be used in accordance with the present disclosure is a cardiac-restricted promoter. For example, promoter is a cardiac-restricted promoter selected from cardiac troponin C, cardiac troponin I, and cardiac troponin T (cTnT).

Alternatively, the promoter may be, without limitation, a promoter from one of the following genes: α -myosin heavy chain gene, 6- myosin heavy chain gene, myosin light chain

2v (MLC-2v) gene, myosin light chain 2a gene, CARP gene, cardiac α -actin gene, cardiac m2 muscarinic acetylcholine gene, ANF, cardiac troponin C, cardiac troponin I, cardiac troponin T(cTnT), cardiac sarcoplasmic reticulum Ca-ATPase gene, skeletal α -actin; or an artificial cardiac promoter derived from MLC-2v gene.

5 In some embodiments of the disclosed rAAV vectors, the two or more transgenes are operably controlled by a single promoter. In other embodiments, each of the two or more transgenes are operably controlled by a distinct promoter.

In some embodiments, the rAAV vectors of the present disclosure further comprise an Internal Ribosome Entry Site (IRES). An IRES is a nucleotide sequence that allows for
10 translation initiation in the middle of a messenger RNA (mRNA) sequence as part of the greater process of protein synthesis. Usually, in eukaryotes, translation can be initiated only at the 5' end of the mRNA molecule, since 5' cap recognition is required for the assembly of the initiation complex. In some embodiments, the IRES is located between the transgenes. In such embodiments, the proteins encoded by different transgenes are translated individually
15 (*i.e.*, versus translated as a fusion protein).

In some embodiments, the rAAV vectors of the present disclosure further comprise a polyadenylation (pA) signal. Eukaryotic mRNAs are typically transcribed as a precursor mRNA. The precursor mRNA is processed to generate the mature mRNA, including a
20 polyadenylation process. The process of polyadenylation begins as the transcription of a gene terminates. The 3'-most segment of the newly made precursor mRNA is first cleaved off by a set of proteins. These proteins then synthesize the poly(A) tail at the RNA's 3' end. The cleavage site typically contains the polyadenylation signal, *e.g.*, AAUAAA. The poly(A) tail is important for the nuclear export, translation, and stability of mRNA.

In some embodiments, the rAAV vectors of the present disclosure comprise at least,
25 in order from 5' to 3', a first adeno-associated virus (AAV) inverted terminal repeat (ITR) sequence, a promoter operably linked to a first transgene, an IRES operably linked to a second transgene, a polyadenylation signal, and a second AAV inverted terminal repeat (ITR) sequence.

In some embodiments, the rAAV is circular. In some embodiments, the rAAV vector
30 is linear. In some embodiments, the rAAV vector is single-stranded. In some embodiments, the rAAV vector is double-stranded. In some embodiments, the rAAV vector is a self-complementary rAAV vector. Any rAAV vector described herein may be encapsidated by a viral capsid, such as an AAV6 capsid or any other serotype (*e.g.*, a serotype that is of the

same serotype as the ITR sequences). In some embodiments, the rAAV vector comprises the complement of any of the disclosed rAAV vector sequences. In some embodiments, the rAAV vector comprises the complement of any one of SEQ ID NOs: 9-12 and 22-23.

Described below are certain rAAV vectors of the present disclosure. The vectors illustrated below comprise the linearized plasmid sequences set forth as SEQ ID NOs: 9-12 and 22-23. The vectors of the disclosure may comprise nucleotide sequences that have at least 70% identity, at least about 80% identity, at least about 90% identity, at least about 95% identity, at least about 96% identity, at least about 97% identity, at least about 98% identity, at least about 99% identity, at least about 99.5% identity, or at least about 99.9% identity to the sequences set forth as SEQ ID NOs: 9-12 and 22-23. These sequences are annotated in the keys that follow.

In some embodiments, any of the disclosed rAAV nucleic acid vector sequences comprise truncations at the 5' or 3' end relative to the sequences of any one of SEQ ID NOs: 9-12, 22, and 23. In some embodiments, any of the rAAV vectors comprise a nucleotide sequence that differs from the sequence of any one of SEQ ID NOs: 9-12, 22 and 23 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, or more than 18 nucleotides. In some embodiments, any of the disclosed rAAV nucleic acid vectors has a sequence that contains stretches of about 200, 300, 400, 500, 600, 750, 1000, 1250, 1500, 1750, 2000, 2250, 2300, 2350, 2400, 2450, 2500, 2550, or 2600 consecutive nucleotides in common with any one of SEQ ID NOs: 9-12, 22 and 23.

pAAVsc.cTnT.Opt.hARC_Opt.hS100A1 (*homo sapiens*) (SEQ ID NO: 9)
ctgcgcgctcgctcgctcactgaggccgcccgggcaaaagcccgggcgctggcgacctttggtcgcccggcctcagtgagcrag
cgagcgcgcagagaggaggttagccatgctctaggaagatcaattcaattcacggtggaattcgcccttaacgggccccccctc
25 gaggtcgggataaaaagcagctctggccttcacatgacagcatctgggctcgggcagagggtcgggtccgaagcgctgccttatcag
cgccccagccctgggaggtgacagctggctggctgtctcagccctcgggcactcacgtatctccgtccgacgggttaaaatagc
aaaactctgagggcacacaatagcttggccttatatggctcctgtgggggaaggggagcacggagggggcccggggcctgct
gccaataatagcagctcacaagtgttgcattcctctctggcgccgggcacattcctgctggctctgcccggcccgggtgggcgccg
gggggaccttaagcctctgcccccaaggagccctccagacagccggcgccaccaccgctccgtgggacgatccccgaagc
30 tctagaggatccagccttaaggctagagtacttaatacgactcactataggctagcgccaccATGGGGAATGCCAAGA
AAGGCCTTCTGAGACTATAGACCGCGAGCGCAAGAGGCTTGTAGAAACCTTGCAG
GCGGACTCTGGTCTCTTGCTGGACGCTCTGCTTGCGCGGGGTGTTCTGACTGGACC
GGAGTACGAAGCATTGGATGCCCTTCCTGATGCAGAGAGACGAGTTAGACGCCTG
TTGCTTCTTGTCAGAGGCAAGGGTGAAGCCGCCTGTCAAGAGCTCCTGAGGTGTG
35 CTCAACGAACCGCCGGGGCGCCAGATCCGGCATGGGATTGGCAACATGTGGGGCC
CGGCTATCGGGACCGGAGCTACGATCCACCATGCCCGGGTCATTGGACGCCGGAG
GCTCCAGGATCTGGTACAACATGCCAGGACTCCCAAGAGCCAGTGACCCCGATG
AAGCTGGAGGCCCCGAGGGCAGTGAAGCCGTACAGAGCGGTACCCAGAAAGAACC
AGAACCGGAGCTGGAGGCTGAAGCTAGTAAAGAGGCGGAACCTGAACCCGAACCG

GAGCCTGAGCTCGAGCCAGAGGCTGAGGCCGAGCCAGAGCCTGAACTCGAACCCG
AACCTGATCCAGAACCAGAGCCCCGACTTCGAGGAACGGGATGAGTCAGAGGATTC
TTGA actagtgcgtaccaggtccctctccctccccccccctaacgttactggccgaagccgcttgaataaggccggtgtgcgtt
 5 tgtctatatgttattttccaccatattgccgtcttttggcaatgtgaggcccgaaacctggccctgtctcttgacgagcattcctagggg
tcttccctctcgccaaaggatgcaaggctctgttgaatgtcgtgaagggaagcagttcctctggaagctcttgaagacaaacaacgtct
gtagcgaccctttgcaggcagcggaaacccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataagatacacctgc
aaaggcggcacaacccagtgccacgttctgagttggatgttggaaagagtc aaatggctctctcaagcgtattcaacaagggg
ctgaaggatgccagaaggtacccattgtatggatctgatctgggacctgggtgcacatgctttacatgtgttagtcgaggttaaaa
aacgtctagggccccgaaccacggggacgtgttttctcttgaaaaacacgatgataagcttgcacaaccttgggccaccATG
 10 **GGCAGCGAGCTGGAGACCGCCATGGAGACCCTGATCAACGTNTTCCACGCC**
CACAGCGGCAAGGAGGGCGACAAGTACAAGCTGAGCAAGAAGGAGCTGAA
GGAGCTGCTGCAGACCGAGCTGAGCGGCTTCCTGGACGCCCAGAAAGGAGCT
NGACGCCGTNGACAAGGTNATGAAGGAGCTGGACGAGAACGGCGACGGCG
AGGTNGACTTCCAGGAGTACGTNGTNGTNGGTTNGCCGCCCTGACCGTNGCCT
 15 **GCAACAACTTCTTCTGGGAGAACAGCTGA** gcggccgcacgtacaccgtcgactagagctcgctgatc
agcctcgactgtgcttctagtgtccagccatctgttgttgccttccccctggccttcttgacctggaaggtgccactcccactgtcc
tttctaataaaatgaggaaattgcatcgcatctgtgtaggtgtcattctattctgggggtgggggtggggcaggacagcaagggg
gaggattgggaagacaatagcaggcgataaggatcttctagagcatggctacgtagataagtagcatggcggttaataacta
caaggaaacctagtgtatggagttgccactccctctctgcgcgtcgtcgtcactgagggcgggcgaccaaggtcgcccgac
 20 gccccggctttgccccggcgccctcagtgagcgaagcgaagcgcgag

Key:

ITRcTnT Promoter25 hARC_OptIREshS100A1_OptBGH PolyA Signal

30 pAAVsc.cTnT.Opt.hS100A1 _ Opt.hARC (*homo sapiens*) (SEQ ID NO: 10)
ctgcgcgtcgtcgtcactgagggccgcccggcgaagccccggcgctcggcgacctttggctgccccggcctcagtgagcgaag
cgagcgcgagagagggaggttagccatgctctaggaatcaattcaattacgcgtggaattcgcccttaacgggccccccctc
gaggtcgggataaaaagcagctctggcctttcacatgacagcatctggggctcgggcagagggcgggtccgaagcgctgcttatacag
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 GAGGAACGGGATGAGTCAGAGGATTCCTTGA

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20 Key:
ITR
cTnT Promoter
hS100A1_Opt
IREs
 25 hARC_Opt
BGH PolyA Signal

pAAVsc.cTnT.hS100A1.hARC_opt (*homo sapiens*) (SEQ ID NO: 11)

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15

Key:

ITRcTnT PromoterhS100A1

20

IREShARC_OptBGH PolyA Signal

pAAVsc.cTnT.hARC_Opt.hS100A1 (*homo sapiens*) (SEQ ID NO: 12)
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Key:

ITR

cTnT Promoter

15 hARC_Opt

IRES

hS100A1

BGH PolyA Signal

20

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Key:

ITR

15 Promoter

hARC-Opt

IRES

hS100A1-Optv2

Synthetic Poly A

20

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Key:
 15 ITR
Promoter
cARC-Opt
IRES
cS100A1-Opt_v2
 20 Synthetic Poly A

Recombinant AAV (rAAV) particles

Further provided herein are rAAV viral particles or rAAV preparations containing
 such particles. The rAAV particles comprise a viral capsid and an rAAV vector as described
 25 herein, which is encapsidated by the viral capsid. Methods of producing rAAV particles are
 known in the art and are commercially available (see, *e.g.*, Zolotukhin *et al.* Production and
 purification of serotype 1, 2, and 5 recombinant adeno-associated viral vectors. *Methods* 28
 (2002) 158–167; and U.S. Patent Publication Nos. US 2007/0015238 and US 2012/0322861,
 which are incorporated herein by reference; and plasmids and kits available from ATCC and
 30 Cell Biolabs, Inc.). For example, a plasmid containing the rAAV vector may be combined
 with one or more helper plasmids, *e.g.*, that contain a rep gene (*e.g.*, encoding Rep78, Rep68,
 Rep52 and Rep40) and a cap gene (encoding VP1, VP2, and VP3, including a modified VP3
 region as described herein), and transfected into a producer cell line such that the rAAV
 particle can be packaged and subsequently purified.

35 The rAAV particles or particles within an rAAV preparation disclosed herein, may be
 of any AAV serotype, including any derivative or pseudotype (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10,
 2/1, 2/5, 2/8, 2/9, 3/1, 3/5, 3/8, or 3/9). As used herein, the serotype of an rAAV an rAAV
 particle refers to the serotype of the capsid proteins of the recombinant virus. In some
 embodiments, the rAAV particle is rAAV6 or rAAV9. Non-limiting examples of derivatives
 40 and pseudotypes include AAVrh.10, AAVrh.74, AAV2/1, AAV2/5, AAV2/6, AAV2/8,

AAV2/9, AAV2-AAV3 hybrid, AAVhu.14, AAV3a/3b, AAVrh32.33, AAV-HSC15, AAV-HSC17, AAVhu.37, AAVrh.8, CHt-P6, AAV2.5, AAV6.2, AAV2i8, AAV-HSC15/17, AAVM41, AAV9.45, AAV6(Y445F/Y731F), AAV2.5T, AAV-HAE1/2, AAV clone 32/83, AAVShH10, AAV2 (Y->F), AAV8 (Y733F), AAV2.15, AAV2.4, AAVM41, AAV6(TM6) and AAVr3.45. Such AAV serotypes and derivatives/pseudotypes, and methods of producing such derivatives/pseudotypes are known in the art (see, e.g., *Mol Ther.* 2012 Apr;20(4):699-708. doi: 10.1038/mt.2011.287. Epub 2012 Jan 24. The AAV vector toolkit: poised at the clinical crossroads. Asokan A1, Schaffer DV, Samulski RJ). In particular embodiments, the capsid of any of the herein disclosed rAAV particles is of the AAVrh.10 serotype. In some embodiments, the capsid is of the AAV2/6 serotype. In some embodiments, the rAAV particle is a pseudotyped rAAV particle, which comprises (a) an rAAV vector comprising ITRs from one serotype (e.g., AAV2, AAV3) and (b) a capsid comprised of capsid proteins derived from another serotype (e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10). Methods for producing and using pseudotyped rAAV vectors are known in the art (see, e.g., Duan *et al.*, *J. Virol.*, 75:7662-7671, 2001; Halbert *et al.*, *J. Virol.*, 74:1524-1532, 2000; Zolotukhin *et al.*, *Methods*, 28:158-167, 2002; and Auricchio *et al.*, *Hum. Molec. Genet.*, 10:3075-3081, 2001).

rAAV Gene Therapy for Heart Diseases

The present disclosure is also directed to compositions comprising one or more of the disclosed rAAV particles or preparations. In some embodiments, the rAAV preparation comprises an rAAV particle comprising a rAAV vector containing ITRs of a first serotype (e.g., AAV3, AAV5, AAV6, or AAV9) and capsid proteins encapsidating the rAAV vector. In some embodiments, the capsid proteins are of the first serotype (e.g., AAV3, AAV5, AAV6, or AAV9). In some embodiments, the preparation has at least a four-fold higher transduction efficiency (e.g., in a human hepatocellular carcinoma cell line, such as Huh7) compared to a preparation prepared using a rAAV vector containing AAV2 ITRs.

As described herein, such compositions may further comprise a pharmaceutical excipient, buffer, or diluent, and may be formulated for administration to host cells *ex vivo* or *in situ* in an animal, and particularly a human being or canine. Such compositions may further optionally comprise a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle, or may be otherwise formulated for administration to the cells, tissues, organs, or body of a human or canine subject in need

thereof. Such compositions may be formulated for use in a variety of therapies, such as for example, in the amelioration, prevention, and/or treatment of conditions such as peptide deficiency, polypeptide deficiency, peptide overexpression, polypeptide overexpression, including for example, conditions which result in diseases or disorders as described herein.

5 The rAAV vectors, rAAV particles, or the composition comprising the rAAV particles of the present disclosure, may be used for gene therapy for heart diseases in a subject (*e.g.*, a human or a canine) in need thereof. Examples of heart disease that may be treated using the methods and compositions of the present disclosure include, but are not limited to, cardiomyopathy and acute ischemia. In some embodiments, the heart

10 cardiomyopathy is hypertrophic cardiomyopathy or dilated cardiomyopathy. Heart failure caused by cardiomyopathy or other heart diseases, comprise two components, calcium handling dysfunction and apoptosis. The rAAV vectors, particles, and compositions comprising the rAAV particles may be used for treatment of such heart failures when administered to a subject in need thereof, *e.g.*, via intravascular delivery into the coronary

15 arteries and/or direct injection to the heart. The rAAV vectors, particles, and compositions comprising the rAAV particles drive the concurrent expression of cS100A1 protein and ARC proteins in the cardiomyocytes of the subject. S100A1 improves aspects of calcium handling, including normalization of sarcoplasmic reticular calcium transients leading to normalization of contractile function. ARC will block apoptosis initiated by mitochondrial and non-

20 mitochondrial mechanisms (such as stretch-induced apoptosis), as well as improve mitochondrial function. Thus, the synergistic benefits of the two proteins expressed by the transgenes of the present disclosure can lead to better long-term therapeutic outcomes by targeting both aspects of cardiomyopathy.

 The amino acid sequences of the human-derived S100A1 and ARC proteins are

25 described below.

Human ARC protein:

 MGNAQERPSETIDRERKRLVETLQADSGLLLDALLARGVLTGPEYEALDALP
DAERRVRLLLLLVQGKGEEAACQELLRCAQRTAGAPDPAWDWQHVGPGYRDRSYD
30 PPCPGHWTPPEAPGSGTTCPGLPRASDPDEAGGPEGSEAVQSGTPEEPEPELEAEASKE
AEPEPEPEPELEPEAEAEPEPEPEPEPDPEPEPDFEERDESEDS (SEQ ID NO: 13)

Human S100A1 protein:

MGSELETAMETLINVFHAHSGKEGDKYKLSKKELKELLQTELSGFLDAQKDV
DAVDKVMKELDENG DGEVDFQEYVVLVAALTVACNNFFWENS (SEQ ID NO: 14)

In some aspects, the disclosed rAAV vectors encode a protein comprising an amino
5 acid sequence at least 90%, at least 95%, or at least 99.5% identical to SEQ ID NO: 13 or 14.
In some aspects, the disclosed rAAV vectors encode a first protein comprising an amino acid
sequence at least 90%, at least 95%, or at least 99.5% identical to SEQ ID NO: 13 and a
second protein comprising an amino acid sequence at least 90%, at least 95%, or at least
99.5% identical to SEQ ID NO: 14. In some embodiments, the rAAV vector encodes a
10 protein that comprises SEQ ID NO: 13. In some embodiments, the rAAV vector encodes a
protein that comprises SEQ ID NO: 14. In particular embodiments, the rAAV vector encodes
a first protein that comprises SEQ ID NO: 13 and a second protein that comprises SEQ ID
NO: 14.

In some embodiments, any of the disclosed rAAV vectors encode a first protein
15 sequence that differs from the sequence of either of SEQ ID NO: 13 or 14 by 1, 2, 3, 4, 5, 6,
7, 8, 9, 10, 11, 12, or more than 12 amino acids. In some embodiments, any of the disclosed
rAAV vectors encode proteins that are truncated by 1, 2, 3, or more than 3 amino acids at the
N- or C-terminus relative to either of SEQ ID NO: 13 or 14. In some embodiments, any of
the disclosed rAAV vectors encode one or more protein sequences that have stretches of 50,
20 75, 90, 93, 100, 125, 175, 200, or 205 amino acids in common with SEQ ID NO: 13 or 14.

The amino acid sequences of the canine-derived S100A1 and ARC proteins are
described below.

Canine ARC protein:

25 MGNSQERPSETIDRERKRLVETLQADSGLLLDALLARGVLAGPEYEALDALPDAERR
VRRLLLLVQSKGEAACQELLLCAQRTARAPDPAWDWQHVGTYRERSWDAACAG
HWTPEAPGSSTTCPELPRAADCGEPGAPGGSEAAQSGSLEEPDPELEAGAELESEPQ
MDLEPEPEAEPEPELEREPEPEPEPDLEAGDESEDS (SEQ ID NO: 24)

30 *Canine S100A1 protein:*

MGSELETAMETLINVFHAHSGKEGNKYKLSKKELKELLQTELSGFLDAQKDADA
VDKVMKELDENG DGEVDFQEYVVLVAALTVACNNFFWENS (SEQ ID NO: 29)

In some aspects, the disclosed rAAV vectors encode a protein comprising an amino acid sequence at least 90%, at least 95%, or at least 99.5% identical to SEQ ID NO: 24 or 29. In some aspects, the disclosed rAAV vectors encode a first protein comprising an amino acid sequence at least 90%, at least 95%, or at least 99.5% identical to SEQ ID NO: 24 and a
5 second protein comprising an amino acid sequence at least 90%, at least 95%, or at least 99.5% identical to SEQ ID NO: 29. In some embodiments, the rAAV vector encodes a protein that comprises SEQ ID NO: 24. In some embodiments, the rAAV vector encodes a protein that comprises SEQ ID NO: 29. In particular embodiments, the rAAV vector encodes a first protein that comprises SEQ ID NO: 24 and a second protein that comprises SEQ ID
10 NO: 29.

In some embodiments, any of the disclosed rAAV vectors encode one or more protein sequences that differ from the sequence of either of SEQ ID NO: 24 or 29 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 amino acids. In some embodiments, any of the disclosed rAAV vectors encode one or more protein sequences that are truncated by 1, 2, 3, or more
15 than 3 amino acids at the N- or C-terminus relative to either of SEQ ID NO: 24 or 29. In some embodiments, any of the disclosed rAAV vectors encode one or more protein sequences that have stretches of about 50, 75, 90, 93, 100, 125, 150, 175, or 200 consecutive amino acids in common with SEQ ID NO: 24 or 29.

Thus, other aspects of the present disclosure related to administering to a subject in
20 need thereof, the rAAV particles of the present disclosure. In some embodiments, the number of rAAV particles administered to a subject may be on the order ranging from about 10^6 to about 10^{14} particles/mL or about 10^3 to about 10^{13} particles/mL, or any values in between for either range, such as for example, about 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} , or 10^{14} particles/mL. In some embodiments, the number of rAAV particles administered to a
25 subject may be on the order ranging from about 10^6 to about 10^{14} vector genomes(vgs)/mL or 10^3 to 10^{15} vgs/mL, or any values in between for either range, such as for example, about 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} , or 10^{14} vgs/mL. The rAAV particles can be administered as a single dose or divided into two or more administrations as may be required to achieve therapy of the particular disease or disorder being treated. In some embodiments, doses
30 ranging from about 0.0001 mL to about 10 mLs are delivered to a subject.

If desired, rAAV particles and rAAV vectors may be administered in combination with other agents as well, such as, *e.g.*, proteins or polypeptides or various pharmaceutically-active agents, including one or more administrations of therapeutic polypeptides, biologically

active fragments, or variants thereof. In fact, there is virtually no limit to other components that may also be included, as long as the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The rAAV particles or preparations may thus be delivered along with various other pharmaceutically acceptable agents as
5 required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein.

Formulations comprising pharmaceutically-acceptable excipients and/or carrier solutions are well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of
10 treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, intra-articular, and intramuscular administration and formulation.

Typically, these formulations may contain at least about 0.1% of the therapeutic agent (*e.g.*, rAAV particle or preparation, and/or rAAV vector) or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1
15 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of therapeutic agent(s) in each therapeutically-useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be
20 contemplated by one skilled in the art when preparing such pharmaceutical formulations. Additionally, a variety of dosages and treatment regimens may be desirable.

In certain circumstances, it will be desirable to deliver the rAAV particles or preparations, and/or rAAV vectors in suitably formulated pharmaceutical compositions disclosed herein; either subcutaneously, intravascularly, intracardially, intraocularly,
25 intravitreally, parenterally, subcutaneously, intravenously, intracerebro-ventricularly, intramuscularly, intrathecally, orally, intraperitoneally, by oral or nasal inhalation, or by direct injection to one or more cells (*e.g.*, cardiomyocytes and/or other heart cells), tissues, or organs. In some embodiments, the rAAV particles or compositions comprising the rAAV particles of the present disclosure are administered intravascularly into the coronary arteries.
30 In other embodiments, the disclosed rAAV particles or compositions are administered by direct injection to the heart of the subject. Direct injection to the heart may comprise injection into one or more of the myocardial tissues, the cardiac lining, or the skeletal muscle surrounding the heart, *e.g.*, using a needle catheter.

The pharmaceutical formulations of the compositions suitable for injectable use include sterile aqueous solutions or dispersions. In some embodiments, the formulation is sterile and fluid to the extent that easy syringability exists. In some embodiments, the form is stable under the conditions of manufacture and storage, and is preserved against the
5 contaminating action of microorganisms, such as bacteria and fungi. The carrier may be a solvent or dispersion medium containing, for example, water, saline, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, vegetable oils or other pharmaceutically acceptable carriers such as those that are Generally Recognized as Safe (GRAS) by the United States Food and Drug Administration.
10 Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which the rAAV particle or preparation, and/or rAAV vectors is administered. Such pharmaceutical
15 carriers can be sterile liquids, such as water and oils, including those of petroleum oil such as mineral oil, vegetable oil such as peanut oil, soybean oil, and sesame oil, animal oil, or oil of synthetic origin. Saline solutions and aqueous dextrose and glycerol solutions may also be employed as liquid carriers.

For administration of an injectable aqueous solution, for example, the solution may be
20 suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, intravitreal, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 mL
25 of isotonic NaCl solution and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion, (*see*, for example, “Remington's Pharmaceutical Sciences” 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.
30 Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by, *e.g.*, FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the rAAV particles or preparations, Rep proteins, and/or rAAV vectors, in the required amount in the appropriate

solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle that contains the basic dispersion medium and the other ingredients from those enumerated above. In the case of sterile powders for the
5 preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The amount of rAAV particle or preparation, and/or rAAV vector compositions and time of administration of such compositions will be within the purview of the skilled artisan
10 having benefit of the present teachings. It is likely, however, that the administration of therapeutically-effective amounts of the compositions of the present disclosure may be achieved by a single administration, such as for example, a single injection of sufficient numbers of infectious particles to provide therapeutic benefit to the patient undergoing such treatment. Alternatively, in some circumstances, it may be desirable to provide multiple or
15 successive administrations of the rAAV particle or preparation, and/or rAAV vector compositions, either over a relatively short, or a relatively prolonged period of time, as may be determined by the medical practitioner overseeing the administration of such compositions.

The compositions of the present disclosure may include rAAV particles or
20 preparations, and/or rAAV vectors, either alone or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources or chemically synthesized. In some embodiments, rAAV particles or preparations are administered in combination, either in the same composition or administered as part of the same treatment regimen, with a proteasome inhibitor, such as Bortezomib, or hydroxyurea.

To "treat" a disease as the term is used herein, means to reduce the frequency or
25 severity of at least one sign or symptom of a disease or disorder experienced by a subject. The compositions described above are typically administered to a subject in an effective amount, which is an amount capable of producing a desired result. The desired result will depend upon the active agent being administered. For example, an effective amount of a
30 rAAV particle may be an amount of the particle that is capable of transferring a heterologous nucleic acid to a host organ, tissue, or cell.

Toxicity and efficacy of the compositions utilized in methods of the present disclosure may be determined by standard pharmaceutical procedures, using either cells in culture or

experimental animals to determine the LD₅₀ (the dose lethal to 50% of the population). The dose ratio between toxicity and efficacy the therapeutic index and it may be expressed as the ratio LD₅₀/ED₅₀. Those compositions that exhibit large therapeutic indices are preferred.

While compositions that exhibit toxic side effects may be used, care should be taken to
5 design a delivery system that minimizes the potential damage of such side effects. The dosage of compositions as described herein lies generally within a range that includes an ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

Other aspects of the present disclosure relate to methods and preparations for use with
10 a subject, such as human or non-human (*e.g.*, canine) subject, a host cell *in situ* in a subject, or a host cell derived from a subject. In some embodiments, the subject is a mammal. In some embodiments, the subject is a companion animal. "A companion animal", as used herein, refers to pets and other domestic animals. Non-limiting examples of companion animals include dogs and cats; livestock such as horses, cattle, pigs, sheep, goats, and
15 chickens; and other animals such as mice, rats, guinea pigs, and hamsters. In some embodiments, the subject is a miniature pig, or mini-pig. In some embodiments, the subject is a human subject. In some embodiments, the subject is a canine subject. In some embodiments, the canine subject is a large-breed canine. As used herein, a large-breed canine refers to a canine (*e.g.*, dog) weighing over 55 pounds, at any age. Therefore, as used herein a
20 large-breed canine may refer to a specific breed of dog which by its nature will exceed 55 pounds in weight (*e.g.*, Doberman pinschers, great Danes, Irish Wolfhounds, *etc.*), as well as dogs of any breed (specific or unknown) which may exceed 55 pounds for other reasons (*e.g.*, obesity).

In some embodiments, the subject has or is suspected of having a heart disease that
25 may be treated with gene therapy. In some embodiments, the subject is in any stages of heart failure. In some embodiments, the heart failure is caused by cardiomyopathy. In some embodiments, the heart failure is caused by hypertrophic cardiomyopathy or dilated cardiomyopathy (DCM).

The following examples are intended to be illustrative of certain embodiments of the
30 present disclosure and are intended to be non-limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this disclosure are hereby expressly

incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

EXAMPLES

5 *Example 1: Therapeutically targeting multiple aspects of heart failure*

In some aspects, the present disclosure provides compositions and methods that are useful in treating one or more heart conditions (*e.g.*, cardiomyopathy, hypertrophic cardiomyopathy, dilated cardiomyopathy, heart failure, heart disease, *etc.*). In some embodiments, compositions provided by the disclosure are administered intravascularly into
10 coronary arteries. In some embodiments, compositions can be provided to a subject via multiple direct injections into the heart. An AAV construct that could be provided to a subject is depicted in FIG. 1. In certain embodiments, such AAV construct is encapsidated by a recombinant AAV (*e.g.*, AAVrh.10 or AAV6) and comprises coding sequences of S100 calcium-binding protein A1 (S100A1) and Apoptosis Repressor with Caspase Recruitment
15 Domain (ARC) to address two separate aspects of one or more heart conditions (*e.g.*, cardiomyopathy). Both transgenes of the AAV construct in FIG. 1 are driven by the cardiac TnT promoter and thus will only express in cardiomyocytes.

S100A1 improves aspects of calcium handling, including normalization of sarcoplasmic reticular calcium transients leading to normalization of contractile function.
20 ARC will block apoptosis initiated by mitochondrial and non-mitochondrial mechanisms (*e.g.*, stretch-induced apoptosis), as well as improve mitochondrial function. These two separate components of cardiac failure (calcium handling dysfunction and apoptosis) are addressed separately, but never together. As such, the synergistic benefits of such an approach provide therapeutic options that may result in improved long-term outcomes. By
25 targeting both aspects of cardiomyopathy, compositions and methods provided by the present application may be used to address multiple heart conditions (*e.g.*, hypertrophic or dilated cardiomyopathy), and will be beneficial at any stage of heart failure.

Example 2: Gene therapy of dilated cardiomyopathy in dogs

30 Dilated cardiomyopathy (DCM) is the second most common cause of acquired heart disease in dogs, most commonly affecting large breed dogs such as Doberman pinschers, great Danes and Irish Wolfhounds. In humans affected with this disease, there are surgical options such as cardiac transplantation and left ventricular assist devices. However, in

veterinary medicine the only therapeutic option is medical management of signs associated with heart failure. The prognosis for an affected dog depends on the stage of disease and the breed. For example, most Doberman pinschers live less than 6 months after the development of congestive heart failure (CHF). In contrast other breeds such as cocker spaniels tend to survive longer. As heart disease progresses, malfunctioning of channels that regulate calcium movement within cardiac cells promotes calcium cycling abnormalities, further dysregulating contraction and relaxation of the heart. Notably, calcium transport abnormalities have been recognized in dogs with naturally occurring DCM and also occur with heart failure secondary to many different etiologies.

Gene transfer strategies designed to normalize calcium cycling abnormalities ameliorate heart disease in small and large animal models with various forms of heart disease. In fact, clinical trials are already underway in humans to test this therapeutic approach to cardiomyopathy and preliminary results are encouraging. A pilot study is evaluating the efficacy of gene delivery designed to normalize calcium handling in Doberman pinschers affected with DCM and exhibiting CHF. Doberman pinschers are utilized because DCM is widespread in this breed and the disease tends to progress quickly and uniformly in this breed once CHF has developed. Novel modalities to address DCM will have significant impact on all canine breeds predisposed to this idiopathic disease including Doberman pinschers, boxers, great Danes, German shepherds, golden retrievers, *etc.* Notably, previous investigations into myocardial protein levels in samples from dogs with the most common forms of naturally occurring heart disease (canine degenerative valve disease and DCM) found multiple protein (including S100A1) levels were abnormal (S100A1 was decreased).

These findings suggest that gene delivery targeting S100A1 may effectively treat DCM as well myocardial failure developing secondary to degenerative valve disease.

Additionally, apoptosis (programmed cell death) is more common in diseased myocardium and ARC is a potent and multifunctional inhibitor of apoptosis. Currently, the standard of care for veterinary heart failure is the medical management of fluid overload and congestion. Gene delivery techniques directed at abnormal myocardial regulatory molecules offer a mechanistic target that may allow the veterinary clinician to specifically address the myocardial disease process for the first time. Moreover, the cost associated with current vector production techniques and intramyocardial gene delivery of vector make the cost of this therapy within reach for many owners with costs expected to decrease over time.

A minimally invasive method of gene transfer using AAV 2/6 vectors resulted in transduction of >75% of myocardial cells in normal dogs (*see* Bish LT, Sleeper MM, Brainard B, *et al.* Percutaneous transendocardial delivery of self-complementary adeno-associated virus 6 achieves global cardiac gene transfer in canines. *Mol. Ther.* 16, 1953-9 (2008)). Six normal mongrel dogs were treated with either an AAV2 or an AAV6 vector encoding a dominant negative form of Phospholamban (dn-PLN) (a pseudophosphorylated form that competes with the native phospholamban therefore reducing its inhibitory effect on SERCA2a) (n=4) or AAV2/6 dn-PLN and S100A1 (n=2). All dogs remained healthy with normal cardiovascular function over 2 years post treatment, indicating that therapy did not cause myocarditis or significantly alter cardiac function, thus supporting the safety of this therapeutic approach. Cardiac function may be measured by ejection fraction, or any other method known in the art.

Indeed, over 40 normal and diseased dogs (see below) have been injected, and results to date indicate that the injection technique is well-tolerated. In addition, 20 random canine cases at the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania were sampled for antibodies to rAAV2/6 and found titers were within the acceptable range for treatment in 19 of the 20 dogs, indicating that prior immune responses will not exclude a significant proportion of therapeutic candidates. To determine if this therapeutic approach was efficacious for treatment of DCM, Portuguese water dogs with a severe form of rapidly progressing juvenile DCM were then treated. Notably, dogs injected with AAV2/6 dn-PLN exhibited a marked decrease in phosphorylated PLN, supporting the potential ability of this approach to normalize calcium cycling in this disease model. Moreover, gene delivery with a vector containing both dn-PLN and S100A1 slowed the development of CHF secondary to DCM to a greater degree than did delivery of a vector containing dn-PLN alone. The combination vector delayed onset of CHF by an average of 4 weeks as compared to dn-PLN therapy alone. For this reason, the combined vector approach is utilized in a pilot study to determine if gene therapy is effective in prolonging the life of Dobermans affected with adult-onset DCM and congestive heart failure.

The study has a blinded, placebo-controlled design. Based on the last 12 Doberman pinscher cases of DCM and CHF that have been treated, there was a mean survival of 148 days (standard deviation of 160 days). Using a power of 0.8, alpha (2 sided) of 0.05 and a ratio of cases to controls of 1, a sample size of 13 dogs in each group are required to detect a difference in 6-month survival. This calculation was determined using a parametric sample

size test. Twenty-six Doberman pinschers with DCM and controlled CHF are enrolled. In order to be eligible for enrollment, the dog must have a circulating neutralizing antibody titer to AAV2/6 of less than 1:20 and be clear of extra-cardiac disease. Additionally, dogs with concomitant congenital heart disease or evidence of primary mitral valvular disease are excluded. At baseline (time of enrollment) an antibody titer, CBC, and chemistry panel are used for screening purposes. Dogs undergo a 3-minute electrocardiogram (ECG) and a complete echocardiogram (ECHO) and owners complete a previously validated quality of life questionnaire. The ECG is evaluated for interval duration and the presence of arrhythmias. The ECHO includes 2D, M-mode and Doppler studies (including tissue Doppler). Thoracic radiographs are used to stage the disease (dogs are clinically compensated with a history of congestive heart failure).

Dogs fulfilling the requirements for enrollment are randomly assigned to the placebo arm (cardiac injection with saline) or the gene therapy group (cardiac injection with AAV2/6-ARC-s100a1). Standard medical management for DCM and congestive heart failure continues throughout the study in all dogs (pimobendan, angiotensin inhibitor and diuretic therapy). Saline instead of empty capsid is used as the sham therapy so that control dogs can undergo gene delivery if the treatment group demonstrates a significant improvement compared to the placebo group. At 2, 4, 6, 9, and 12 months following therapy ECG, ECHO, a quality of life questionnaire and laboratory analyses are repeated. Statistical analysis is performed at bi-monthly intervals.

FIGs. 2 and 3 depict diastole (relaxation) and systole (contraction) data, respectively, in a treated muscular dystrophy dog. The endocardial and epicardial contours can be seen in each of the figures. The data indicates stable or slightly improved function post treatment over several weeks as seen in Table 1. Table 1, below, shows the left ventricular mass (LVM [g]), end diastolic volume (EDV [ml]), end systolic volume (ESV [ml]), stroke volume (SV [ml]), ejection fraction (EF [%]), and cardiac output (CO [l/min]) results for the data taken at times 1 (pre-treatment) and time 2 (post-treatment).

Table 1

Acquisition Date	LVM[g]	EDV[ml]	ESV[ml]	SV[ml]	EF[%]	CO[l/min]
Time 2	91.395035	54.22289	24.595001	29.627889	54.640926	3.940509

Time 1	87.251524	57.471229	25.660014	31.811215	55.351548	3.117499
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Example 3: Assessment of dystrophy phenotypes following vector delivery into mice and dogs

Cardiac AAV gene delivery of the S100A1/ARC self-complementary vector was assessed in mouse and dog models of Duchenne muscular dystrophy (dystrophin-deficiency).

- 5 Earlier, the AAV8 (including multiple variants thereof), AAV9, and AAVrh.10 serotypes were compared in their ability to infect canine hearts, and AAVrh.10 was found to be the most efficient. For this reason, AAVrh.10 was used for all experiments described in this Example.

- 10 Mdx (dystrophin-deficient) mice on the DBA/2J background (“D2.mdx”) were injected at 4 weeks of age with recombinant AAVrh.10-S100A1/ARC vector (referred to below as the “therapeutic AAV”) and sacrificed 24 weeks later. D2.mdx mice recapitulate several human characteristics of Duchenne muscular dystrophy myopathy, such as reduced lower hind limb muscle mass, atrophied myofibers, increased fibrosis and inflammation, and muscle weakness. Over this 24-week period, the mice injected with the therapeutic AAV had
- 15 better maintained ejection fractions, strain development, and cardiac output as compared to sham injected mice (see FIG. 4), as measured by cardiac MRI. Protein analysis (Western blots) confirmed that both S100A1 and ARC levels were elevated in the treated tissues as compared to controls (sham injected) (see FIG. 5). Furthermore, cardiac histology demonstrated that the treated hearts demonstrated much less pathology as compared to
- 20 control hearts (see FIG. 6).

- Two GRMD (dystrophin-deficient) dogs, the dog model of human Duchenne muscular dystrophy, were injected with the therapeutic vector at the time of first decrease in their cardiac ejection fractions via catheter delivery into the coronary arteries. Cardiac ejection fractions represent constitute a symptom indicating onset of cardiomyopathy. Earlier
- 25 findings from a natural history study of dog subjects indicated that, as soon as ejection fractions begin to fall, they continue to fall progressively over the next year (FIG. 18). Dogs typically do not survive longer than 8-12 months after the ejection fraction begins this steady decrease.

- As shown in FIGs. 7 and 8, both subjects showed improvements in ejection fraction
- 30 and other cardiac parameters several months after treatment with AAVrh.10-S100A1/ARC, as measured by cardiac MRI and confirmed by echo measurements. Nearly 12 months after treatment, the first subject exhibited a steady ejection fraction within the normal range.

Likewise, nearly 7 months after treatment, the second subject exhibited a steady, normal ejection fraction.

Not only was cardiac function improved, but there was also a constant improvement in the exercise capacity of the dogs, as evaluated qualitatively by filming the subjects during exercise. Consistent with this improved exercise capacity, MRI measurements of the subjects' limbs demonstrated that skeletal muscle mass was either augmented or unchanged following AAV treatment (FIGs. 9A to 9C). In addition, circulating creatine kinase levels (CK) levels in skeletal muscle was reduced post-treatment (FIG. 10), indicating that a reduction in ongoing muscle damage.

Example 4: Treatment of DCM Dog Subjects

Doberman pinschers have the highest incidence of DCM of any dog breed. The genetic bases of this are known for only a subset of the dogs. This provides a large animal model of DCM and heart failure, without any other genetic complications. Only dogs that are in late stage heart failure are being treated with the same AAV.S100A1.ARC vector that is being used in the GRMD dogs. The goal of the treatment is to both improve cardiac function and prolong life.

Two Doberman pinscher subjects have been treated with AAVrh.10-S100A1/ARC via catheter delivery into the coronary arteries to date, wherein both dogs had experienced heart failure at the time of treatment. Both dogs showed rapid improvement following treatment. Each dog was assessed by two rounds of echocardiographs after treatment to evaluate cardiac structural and functional parameters, including (but not limited to) ventricular volumes, wall thickness and chamber diameters in diastole and systole, as well as fractional shortening and ejection fraction.

The first dog was treated with an ejection fraction of only 10%, and was thus close to death at the time of treatment. Within 24 hours post-treatment, the ejection fraction improved to 25%. At the dog's first follow up visit at 4 months post-treatment the ejection fraction had held steady at 26%. At the second follow up at 6 months, its ejection fraction was 32%. The dog died from congestive heart failure at 8 months of age. Thus, the treatment appeared to prolong life, but cardiac function was already too compromised at treatment to allow long-term survival.

The second treated Doberman pinscher had an ejection fraction of 32% prior to treatment—a fraction that is low, but not in immediate danger of death. The dog's ejection

fraction improved to 49% within 24 hours following treatment, which is within normal range. At the 4 months post treatment exam the dog's ejection fraction was 52%, and at the 8-month exam, the ejection fraction was 50%. Prior to return for a one-year exam, the dog was diagnosed with lung cancer and died two months later. Its cause of death was unrelated to heart failure.

Based on these findings, AAVrh.10-S100A1/ARC treatment is able to restore cardiac function in canines to normal range.

Example 5: Evaluation of Vectors Comprising Human-Derived cDNA sequences

Plasmids comprising human-derived ARC ("hARC") and S100A1 ("hS100A1") cDNA sequences were constructed for evaluation in mice. The native human cARC and S100A1 genes were codon-optimized for expression in human cells.

These plasmids comprise the nucleotide sequences of SEQ ID NOs: 9-12. Each of these plasmids comprises cARC and S100A1 cDNA sequences operably controlled by a cTnT promoter, as well as an IRES between these two sequences. All four plasmids comprise a codon-optimized human cARC (hARC) sequence. The plasmids set forth in SEQ ID NOs: 9 and 10 further comprise a codon-optimized hS100A1 sequences; and the plasmids set forth in SEQ ID NOs: 11 and 12 further comprise a wild-type hS100A1 sequence.

Each of these four plasmids was cloned into a self-complementary AAVrh.10 vector and subsequently encapsidated into an rAAV particle. The rAAVrh.10 particles comprising these vectors were administered to mice.

Expression levels of ARC and S100A1 in the mice were evaluated. Dystrophy phenotypes and cardiac function, including cardiac ejection fraction, were monitored and evaluated.

Example 6: Long-term mouse and dog studies

Dystrophic Mouse studies

AAV cardiac gene delivery of S100A1 and ARC leads to preservation of cardiac function

In addition to mouse studies previously provided (FIGs. 4-6), two types of longer-term studies were conducted, the results of which are summarized in FIGs. 15-17. Shown in FIGs. 15 and 16 are data from dystrophin-deficient mice on a severely fibrotic background, the DBA/2J (also referred to as "D2") mouse, thus creating what is referred to as the D2.mdx mouse. These mice were treated with AAV containing the dual transgene (S100A1 and ARC)

cassette, operably controlled by the cardiac troponin T (cTnT) promoter, at the age of 1 month. The mice were housed in a sedentary environment until 10 months of age, at which time their cardiac status was evaluated.

5 Superiority of AAV cardiac gene delivery of S100A1 and ARC compared to either transgene alone

In parallel with these studies, a survival study was also conducted in D2.mdx mice in which there was transgenic rescue of only the skeletal muscle by expression of utrophin under control of the skeletal muscle alpha-actin promoter (see Rafael JA, *et al.* Skeletal muscle-specific expression of a utrophin transgene rescues utrophin-dystrophin deficient mice. *Nat Genet.* 19:79-82, 1998), which are referred to as D2.mdx.sk_utrophin mice. The point was to create a mouse that had a pure dilated cardiomyopathy, leading to heart failure and death, without any disease of the pulmonary musculature, or skeletal musculature in general. This allowed for the mice to be exercised by housing them individually with running wheels. The exercise creates additional load and stress on the hearts, leading to an acceleration of the development of the cardiomyopathy and heart failure. In order to better model the clinical situation in which individuals would not receive treatment prior to the onset of measurable cardiac disease, the mice were treated at 6 months of age, at which time fibrosis is present and cardiac functional abnormalities begin to emerge.

20 To assess the potential superiority of delivery of S100A1 + ARC together in the same construct, relative to delivery of ARC or S100A1 alone, either no transgene (control) was delivered, or an rAAV vector containing the same cardiac promoter used in the dual transgene construct was delivered, but driving either the same S100A1 or ARC cDNA used in the dual cassette, but alone and not in combination. Thus, the ability of either ARC alone, S100A1 alone, or the combination of S100A1 and ARC was compared to prolong life in this heart failure model.

As shown in FIG. 17, either transgene alone was able to significantly extend lifespan, ARC by 4 months and S100A1 by 5 months. However, the combination of transgenes, S100A1 and ARC, extended lifespan by 10 months, to 20 months of age. The wild type D2 mice only have a lifespan of ~23 months, so this represented a remarkably strong rescue of the heart. The combination of the transgenes into a dual rAAV construct provided synergistic efficacy. Because administration of the vector extended the lifespan of D2 mice to approximately wild-type lifespan and extended lifespan relative to single-transgene vectors

by between 4 and 5 months, these results represent a statistically significant improvement relative to single-vector therapies.

Dystrophic Dog Studies

5 Next, a study to extend the results of the mouse treatments to a GRMD dogs was conducted. The study under way in GRMD dogs (four dogs to date) entails treatment with AAVrh.10-S100A1/ARC (dog transgene sequences) when cardiac function, as assessed by ejection fraction, drops below the levels of those of the untreated dog's natural history. Frequent echocardiographs and/or MRIs to assess cardiac structural and functional
10 parameters are used to follow the dogs' cardiac status. The first dog treated (WnM3/Calvin) was followed for more than 2 years, during which its cardiac function improved and was stable. This dog died due to complications associated with aspiration pneumonia at 34 months of age, but his cardiac function was still stable and in the normal range through that age. The other three dogs all improved following the initial treatment and have displayed stable
15 cardiac function for greater than a year. This data is depicted in FIG. 18. The death of Calvin from pneumonia allowed assessment of his cardiac histology, which is shown in FIG. 19. Remarkably, the histology of the left ventricle (FIG. 19, far right column of histology slides), appears nearly indistinguishable from that of a normal dog (FIG. 19, far left column of panels). In contrast, without treatment, the histology of the left ventricle is highly fibrotic at
20 8 months of age (one month after WnM3 received treatment) (FIG. 19, second-from-left column of panels) and even more fibrotic at two years of age (middle panels). This is evidence for nearly total cardiac rescue in a large animal model of DCM that progresses to heart failure and death between one and two years of age. There has never been an untreated dog that has survived longer than 30 months of age in this colony (FIG. 18), and the typical
25 cause of death is heart failure.

Example 7: Immune response studies

Four miniature pigs of 45-70 kgs in weight are treated with the rAAV vectors of this disclosure. Two of the pigs receive the high dose of 2×10^{14} genome copies and the other
30 two receive the lower dose of 2×10^{13} genome copies. In each case, two-thirds of the virus is delivered to the left heart, and one-third of the dose is delivered to the right heart. Pigs are screened for antibodies against AAVrh10 prior to enrollment in the study. Synchrony provides blood for this purpose.

Blood is collected in a red top tube, allowing blood to clot for approximately 30 minutes, and is spun down at 1000xG for 15 minutes and serum is aliquoted into 200µl aliquots and frozen prior to the screen. The serum is used for analysis of preexisting antibodies.

5 Immune Suppression Regimen

Starting 1 day prior to gene transfer, subjects receive 1 mg/kg of glucocorticoid (or prednisone equivalent) daily for 60 days after the infusion. At 60 days post gene transfer, a tapering dose of glucocorticoid is implemented and liver enzymes is monitored for immune response. In the event of an immune response, glucocorticoid dose and regimen is
10 administered again at the discretion of a physician.

Throughout the glucocorticoid administration, prophylactic antibiotics are administered as a precaution.

Delivery of rAAV particles to pig hearts

- Introducer placed in carotid or femoral artery
- 15 • Pigtail angiocatheter advanced into left ventricle for coronary angiogram to roadmap the coronary arteries on digital fluoroscopy
- Coronary catheters (each) flushed with heparinized saline with 1 cc of subject's blood and advanced into aortic root and left and right coronary orifices (Judkins R or L in size dependent on size of animal).
- 20 • Adenosine CRI (1 mg/kg/min) started intravenously. Vector delivered into coronary after 15 seconds of adenosine CRI. Vector administered over 5-10 seconds and CRI continued 30 seconds after delivery of vector is complete.
- 2/3 vector administered into left coronary and 1/3 vector administered into right coronary.

25 Pig subjects are monitored by weekly blood draws (serum chemistries, CBC, AAVrh10 antibodies (done by sponsor), Elis spot response) for 2 months following AAV delivery. Two months following delivery, pigs are sacrificed and hearts and other tissues (see below list) are prepared (samples taken for formalin fixative and fresh frozen) and tissues are
30 made available.

Blood draws needed:

serum chemistries 1 red top tube

CBC panel 1 EDTA purple top tube

Anti-AAVrh10 ELISA assay 1 red top tube

ELISPOT at a minimum 1 10mL EDTA purple top tube

5 Postmortem Procedures

Special Procedures

Fresh frozen Tissue Collection and Formalin Fixed Paraffin Embedded Tissues

Muscle/ Organ	Abbreviation	Left/Right
Tibialis Anterior	R/L TA	Both
Extensor Digitorum Longus	R/L EDL	Both
Soleus	R/LSOL	Both
Bicep	R/L BIC	Both
Tricep	R/ L TRI	Both
Heart	HRT	-
Lung	LUNG	-
Kidney	KIDNEY	-
Liver	LIV	-
Spleen	SPLN	-
Gonad	GND	-
Pancreas	PANC	-
Popliteal Lymph Node	POPLN	-
Inguinal Lymph Node	ING LN	-
Mesenteric Lymph Node	MES LN	-
Femoral Artery	R/L FEM ART	Both
Sural Artery	R/LSUR ART	Both
Bladder	BLADDER	-
Diaphragm	DIA	-
Stomach	STMCH	-

Brain	BRAIN	-
Spinal Cord	SPN CORD	-
Nerve	NERVE	-
Sural Nerve	SUR	L/R
Portal Vein	Port al Vein	-
Aorta	Aorta	-
Inferior Vena Cava	IVC	-
Hepatic Port al Vein	HPV	-

10

Example 8: Recombinant AAV vectors with increased packaging efficiency and higher titers.

Recombinant AAV (rAAV) vectors were designed which (1) reduced the guanosine (G) and cytosine (C) content in the S100A1 transgene and (2) included a different, smaller polyA signal than previously-designed vectors (including those set forth as SEQ ID NOs: 9-12). The S100A1 transgenes with reduced G/C content, which were previously codon optimized for expression in humans or canines, are shown in SEQ ID NOs: 25 (human) and 26 (canine). The cARC transgenes, to which no changes were made, relative to prior iterations, and which were previously codon optimized for expression in humans or dogs, are shown in SEQ ID NOs: 6 (human), 7 (human), and 16 (canine). The different, smaller polyA signal used in the newly designed vectors is shown in SEQ ID NO: 28. The newly designed full rAAV vectors are shown in SEQ ID NOs: 22 (human) and 23 (canine).

The newly designed rAAV vectors were optimized to increase packaging efficiency, resulting in higher vector titers and thus decreased production costs. To assess whether the newly designed rAAV vectors exhibited these properties relative to the analogous rAAV vectors previously designed by the inventors, a blind study was conducted in which a third-party production lab was supplied with the DNA for both a newly designed human rAAV vector with reduced G/C content in the S100A1 transgene and a smaller polyA signal (SEQ ID NO: 22), and a previously designed human rAAV vector (SEQ ID NO: 9). The third-party production lab was unaware of the differences that existed between the DNA constructs provided by the inventors. The production output for each vector can be seen in **Table 2**.

20

Table 2.

Vector Name	SEQ ID NO	Titer (genome content/mL)	Volume and Aliquot (mL)	Total genome content
AAVrh.10.cTnT.cARC-Opt_IRES_cS100A1-Opt_#1	9	1.00×10^{13}	6.8 mL, 34 tube, 34 $\times$ 200 μ L	6.80×10^{13}
AAVrh.10.cTnT.cARC-Opt_IRES_cS100A1-Opt_#2	22	2.70×10^{13}	18 mL, 6 tubes, 6 $\times$ 3 mL	4.86×10^{14}

The previously designed construct (SEQ ID NO: 9) exhibited a titer of 1.00×10^{13} genome copies per mL, as it had in previous studies. Notably, however, the newly designed vector (SEQ ID NO: 22) showed a 2.7-fold improvement in titer, and exhibited a titer of 2.7×10^{13} genome copies per mL. Additionally, the purity of the rAAV vectors was unaffected (FIG. 20). Accordingly, the newly designed human rAAV vector with reduced G/C content in

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the S100A1 transgene and a smaller polyA signal (SEQ ID NO: 22) shows a 2 to 3-fold improvement in packaging efficiency and titer over the previously designed human rAAV vector (SEQ ID NO: 9). Improving packaging efficiency and titer in the production of rAAV vectors is important, because such improvements significantly lower the overall cost of
5 rAAV production.

In some embodiments, the newly designed human rAAV vector with reduced G/C content in the S100A1 transgene and a smaller polyA signal (SEQ ID NO: 22) shows a 2 to 3-fold improvement in packaging efficiency and titer over the previously designed human rAAV vector (SEQ ID NO: 9), and no change in the functionality of the rAAV vector is
10 observed.

EQUIVALENTS

While several inventive embodiments have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or
15 structures for performing the function and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the inventive embodiments described herein. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be for descriptive purposes, and that the actual
20 parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the inventive teachings is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific inventive embodiments described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that,
25 within the scope of the appended claims and equivalents thereto, inventive embodiments may be practiced otherwise than as specifically described and claimed. Multiple embodiments of the present disclosure are directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials,
30 kits, and/or methods are not mutually inconsistent, is included within the inventive scope of the present disclosure.

All definitions, as defined and used herein, should be understood to control over dictionary definitions, definitions in documents incorporated by reference, and/or ordinary meanings of the defined terms.

5 All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles “a” and “an,” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean “at least one.”

10 The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with “and/or” should be construed in the same fashion, *i.e.*, “one or more” of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified. Thus, as a non-limiting example, a reference to “A and/or B”, when
15 used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A only (optionally including elements other than B); in another embodiment, to B only (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); *etc.*

20 As used herein in the specification and in the claims, “or” should be understood to have the same meaning as “and/or” as defined above. For example, when separating items in a list, “or” or “and/or” shall be interpreted as being inclusive, *i.e.*, the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as “only one of” or “exactly one of,” or, when used in the claims, “consisting of,” will refer to the inclusion of
25 exactly one element of a number or list of elements. In general, the term “or” as used herein shall only be interpreted as indicating exclusive alternatives (*i.e.*, “one or the other but not both”) when preceded by terms of exclusivity, such as “either,” “one of,” “only one of,” or “exactly one of.” “Consisting essentially of,” when used in the claims, shall have its ordinary
30 meaning as used in the field of patent law.

As used herein in the specification and in the claims, the phrase “at least one,” in reference to a list of one or more elements, should be understood to mean at least one element selected from any one or more of the elements in the list of elements, but not necessarily

including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements may optionally be present other than the elements specifically identified within the list of elements to which the phrase “at least one” refers, whether related or

5 unrelated to those elements specifically identified. Thus, as a non-limiting example, “at least one of A and B” (or, equivalently, “at least one of A or B,” or, equivalently “at least one of A and/or B”) can refer, in one embodiment, to at least one, optionally including more than one, A, with no B present (and optionally including elements other than B); in another embodiment, to at least one, optionally including more than one, B, with no A present (and
10 optionally including elements other than A); in yet another embodiment, to at least one, optionally including more than one, A, and at least one, optionally including more than one, B (and optionally including other elements); *etc.*

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts
15 of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as “comprising,” “including,” “carrying,” “having,” “containing,” “involving,” “holding,” “composed of,” and the like are to be understood to be open-ended, *i.e.*, to mean including
20 but not limited to. Only the transitional phrases “consisting of” and “consisting essentially of” shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03. It should be appreciated that embodiments described in this document using an open-ended transitional phrase (*e.g.*, “comprising”) are also contemplated, in alternative embodiments, as “consisting
25 of” and “consisting essentially of” the feature described by the open-ended transitional phrase. For example, if the disclosure describes “a composition comprising A and B”, the disclosure also contemplates the alternative embodiments “a composition consisting of A and B” and “a composition consisting essentially of A and B”.

30

CLAIMS

What is claimed is:

1. A recombinant adeno-associated virus (rAAV) nucleic acid vector for delivering two or more transgenes into the heart of a subject, wherein said vector comprises, from 5' to 3', in order, a first adeno-associated virus (AAV) inverted terminal repeat (ITR) sequence, two or more transgenes and a promoter operably linked to the two or more transgenes, a polyadenylation (polyA) signal, and a second AAV ITR sequence,
wherein the two or more transgenes encode an S100 family protein and an apoptotic inhibitor, respectively, and
wherein the transgene encoding an S100 family protein comprises a sequence that is at least 95%, at least 98%, or at least 99.5% identical to either of the nucleotide sequences of SEQ ID NOs: 25 and 26.
2. The rAAV nucleic acid vector of claim 1, wherein the transgene encoding an S100 family protein comprises either of the nucleotide sequences of SEQ ID NOs: 25 and 26.
3. The rAAV nucleic acid vector of claim 1 or claim 2, wherein the polyA signal comprises a nucleotide sequence that has at least 90%, 92.5%, 95%, 98%, or 99% identity to the nucleotide sequence of SEQ ID NO: 28.
4. The rAAV nucleic acid vector of any one of claims 1-3, wherein the polyA signal comprises the nucleotide sequence of SEQ ID NO: 28.
5. The rAAV nucleic acid vector of any one of claims 1-4, wherein the transgene encoding an S100 family protein comprises a sequence having a reduced guanosine (G) and cytosine (C) (G/C) content, relative to any one of the nucleotide sequences of SEQ ID NOs: 5, 8, 19, and 20.
6. The rAAV nucleic acid vector of claim 5, wherein the G/C content is reduced by 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, or 15%, relative to any one of the nucleotide sequences of SEQ ID NOs: 5, 8, 19, and 20.

7. The rAAV nucleic acid vector of any one of claims 1-6, wherein the apoptotic inhibitor is cardiac Apoptosis Repressor with Caspase Recruitment Domain (ARC) or a variant thereof.
8. The rAAV nucleic acid vector of any one of claims 1-7, wherein the transgene encoding an apoptotic inhibitor comprises a sequence that is at least 90%, at least 95%, or at least 99.5% identical to any one of the nucleotide sequences of SEQ ID NOs: 3, 6, 7, and 15-18.
9. The rAAV nucleic acid vector of any one of claims 1-8, wherein the transgene encoding an apoptotic inhibitor comprises any one of the nucleotide sequences of SEQ ID NOs: 3, 6, 7, and 15-18.
10. The rAAV nucleic acid vector of any one of claims 1-9, wherein an Internal Ribosome Entry Site (IRES) is present between the transgene encoding an S100 family protein and the transgene encoding an apoptotic inhibitor.
11. The rAAV nucleic acid vector of any one of claims 1-10, wherein the transgenes are specific for *homo sapiens* or *canis lupus familiaris*.
12. The rAAV nucleic acid vector of any one of claims 1-11, wherein the promoter is a cardiac-restricted promoter.
13. The rAAV nucleic acid vector of claim 12, wherein the promoter is a cardiac-restricted promoter selected from the group consisting of: cardiac troponin C, cardiac troponin I, and cardiac troponin T (cTnT).
14. The rAAV nucleic acid vector of claim 12 or claim 13, wherein the promoter is cTnT.
15. The rAAV nucleic acid vector of claim 12, wherein the promoter is a cardiac-restricted promoter derived from a gene selected from the group consisting of: α -myosin heavy chain gene, 6-myosin heavy chain gene, myosin light chain 2v gene, myosin light

chain 2a gene, CARP gene, cardiac α -actin gene, cardiac m2 muscarinic acetylcholine gene, ANF, cardiac sarcoplasmic reticulum Ca-ATPase gene, and skeletal α -actin; or is an artificial cardiac promoter derived from MLC-2v gene.

16. The rAAV nucleic acid vector of any one of claims 1-15, wherein the rAAV nucleic acid vector is single-stranded.

17. The rAAV nucleic acid vector of any one of claims 1-16, wherein the rAAV nucleic acid vector is self-complementary.

18. The rAAV nucleic acid vector of any one of claims 1-17, wherein the rAAV nucleic acid vector comprises a nucleotide sequence that is at least 90%, at least 95% or at least 99.5% identical to either of the nucleotide sequences of SEQ ID NOs: 22 and 23.

19. The rAAV nucleic acid vector of claim 18, wherein the rAAV nucleic acid vector comprises the nucleotide sequence of SEQ ID NO: 22.

20. The rAAV nucleic acid vector of claim 18, wherein the rAAV nucleic acid vector comprises the nucleotide sequence of SEQ ID NO: 23.

21. An rAAV particle comprising the rAAV nucleic acid vector of any one of claims 1-20 encapsidated in an AAV capsid.

22. The rAAV particle of claim 21, wherein the AAV capsid comprises a capsid protein derived from an AAV1, AAV2, AAV3, AAV6, AAV8, AAVrh.74, AAVrh.10, AAV2/6, or AAV9 serotype.

23. The rAAV particle of claim 21 or claim 22, wherein the AAV capsid comprises a capsid protein derived from an AAVrh.10 serotype.

24. A composition comprising the rAAV particle of any one of claims 21-23.

25. A method of treatment of a subject suffering from a heart disease comprising administering to the subject the composition of claim 24 or the rAAV particle of any one of claims 21-23.
26. The method of claim 25, wherein the heart disease causes heart failure in the subject.
27. The method of claim 25 or claim 26, wherein the heart disease is cardiomyopathy.
28. The method of any one of claims 25-27, wherein the heart disease is hypertrophic cardiomyopathy or dilated cardiomyopathy.
29. The method of claim 25 or claim 26, wherein the heart disease is acute ischemia.
30. The method of any one of claims 25-29, wherein the composition is administered via injection into the heart of the subject or intravascular injection into the coronary arteries of the subject.
31. The method of any one of claims 25-30, wherein the step of administering results in expression of the two or more transgenes in the subject's heart.
32. The method of any one of claims 25-31, wherein the subject is a mammal.
33. The method of claim 32, wherein the mammal is a human.
34. The method of claim 32, wherein the mammal is a companion animal.
35. The method of claim 34, wherein the companion animal is a dog or a cat.
36. The method of any one of claims 25-35, wherein the step of administering results in improved cardiac function in the subject.
37. The method of claim 36, wherein the step of administering results in improved cardiac function in the subject for more than 10 months.

38. The rAAV nucleic acid vector of any one of claims 1-20, wherein the transgene encoding an S100 family protein is positioned 5' to the transgene encoding an apoptotic inhibitor.
39. The rAAV nucleic acid vector of any one of claims 1-20, wherein the transgene encoding an apoptotic inhibitor is positioned 5' to the transgene encoding an S100 family protein.
40. The rAAV nucleic acid vector of any one of claims 1-20, 38, and 39, wherein the rAAV nucleic acid vector encodes a protein comprising an amino acid sequence at least 90%, at least 95%, or at least 99.5% identical to the amino acid sequence of SEQ ID NO: 13 or 14.
41. The rAAV nucleic acid vector of any one of claims 1-20 and 38-40, wherein the rAAV nucleic acid vector encodes a protein comprising the amino acid sequence of SEQ ID NO: 13 or 14.
42. The rAAV nucleic acid vector of any one of claims 1-20 and 38-41, wherein the rAAV nucleic acid vector encodes a protein comprising an amino acid sequence at least 90%, at least 95%, or at least 99.5% identical to the amino acid sequence of SEQ ID NO: 24 or 29.
43. The rAAV nucleic acid vector of any one of claims 1-20 and 38-42, wherein the rAAV nucleic acid vector encodes a protein comprising the amino acid sequence of SEQ ID NO: 24 or 29.