GENE THERAPY FOR TREATING GNE-RELATED DISORDERS

[0001] The present invention claims priority benefit of U.S. Provisional Application no. 63/562,412 which is incorporated by reference herein in its entirety.

Incorporation by Reference of the Sequence Listing

[0002] This application contains, as a separate part of the disclosure, a Sequence Listing in computer-readable form which is incorporated by reference in its entirety and identified as follows: 59901_Seqlisting.XML; Size: 49,617 bytes; Created: March 3, 2025.

Field

[0003] The present invention relates to methods and materials for treating GNE-related disorders such as GNE myopathy, GNE-dependent ALS, sarcopenia and aging using a dual gene recombinant adeno-associated virus comprising the GNE gene and the follistatin gene under the control of two different transcriptional control sequences.

Background

[0004] GNE Myopathy is an adult onset autosomal recessive genetic disease characterized by progressive muscle weakness that that can lead to loss of ambulation and loss of independent living. As its name implies, GNE myopathy is caused by loss of function pathogenic variants or mutations in the *GNE* gene. This disease is also known as hereditary inclusion body myopathy, quadriceps sparing myopathy, distal myopathy with rimmed vacuoles, and Nonaka myopathy. The *GNE* gene encodes a bifunctional UDP-GlcNAc-epimerase/ManNAc-6 kinase, whose enzymatic activities are essential in sialic acid biosynthetic pathway.

[0005] Sialic acid is an acidic monosaccharide that modifies non-reducing terminal carbohydrate chains on glycoproteins and glycolipids and plays an important role in different processes such as cell-adhesion and cellular interactions. Sialic acid has been implicated in health and disease and is found in terminal sugar chains of proteins modulating their cellular functions. As UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE) is the key enzyme for the biosynthesis of sialic acid. Moreover, it has been demonstrated that GNE expression is induced when myofibers are damaged or regenerating, and that GNE plays a role in muscle regeneration. Myoblasts carrying a mutated GNE gene show a reduction in their epimerase activity, whereby only the cells carrying a homozygous

epimerase mutation also present with a significant reduction in the overall membrane bound sialic acid (Pogoryleva et al., Orphanet J Rare Dis. 13: 70, 2018).

[0006] Sarcopenia is a condition marked by the wasting or loss muscle tissue and the replacement of muscle tissue with fibrosis tissue as the subject ages. The etiology of sarcopenia is complex and can be attributed to a variety of factors, including oxidative stress, inflammation, apoptosis, and mitochondrial dysregulation (Roubenoff, 2003; Fulle et al., 2004), as well as genetic factors, inadequate diet, sedentary lifestyle, and the interplay between these factors (Rolland et al., 2008; Walrand et al., 2011). Sarcopenia and age-related muscle wasting are associated with reduced GNE gene expression. Reduced GNE expression during aging is also associated with increased degeneration of brain neurons. In addition, a recessive missense mutation in the GNE gene has recently been shown to be associated with juvenile onset Amyotrophic Lateral Sclerosis (ALS) (Koroglu et al. Neurogenetics 18(4):237-243, 2017) and with infantile thrombocytopenia (Montcrieff et al. Transfusion 63:1092-1099, 2023).

[0007] GNE myopathy leads to weakness and wasting of muscles in legs and arms. First symptoms usually occur in young adults (usually in the third decade of life), but a later onset has also been observed in some patients. A diagnosis of GNE myopathy should be considered primarily in patients presenting with distal weakness (foot drop) in early adulthood (other onset symptoms are possible too). The disease slowly progresses to involve other lower and upper extremities' muscles, typically with marked sparing of the quadriceps. Characteristic findings found in biopsies of affected muscles include "rimmed" (autophagic) vacuoles, aggregation of various proteins, and fiber size variation.

[0008] Despite the fact that mutations in the *GNE* gene were shown to cause GNE myopathy in 2001, there are as yet no effective therapies for this disease. Attempts to develop slow release sialic acid therapy failed in a phase 3 clinical trial, and ManNAc glycan therapy is currently being investigated. While development of a gene therapy approach for *GNE* gene replacement might seem straightforward, it is in fact complicated by a number of unresolved issues in GNE myopathy research: First and foremost is the lack of a robust and reproducible model for the disease. While Noguchi and Nishino published several papers on a transgenic GNED176VTg $Gne^{-/-}$ mouse model showing clear aspects of disease pathology, other groups, have failed to see the same phenotypes with subsequent breeding, likely the result of genetic drift in the founder transgenic line (see Nishino et al., J. Neurol. Neurosurg, Psychiatry 86(4): 385-392). A GNE_{M712T} variant knock-in mouse model showed premature death in the first few weeks of life due to kidney disease, a clinical phenotype that is not

present in GNE Myopathy patients. Other lines of the same model were bred out to show no phenotype at all despite having the same genetic mutation. Second is a lack of measurable natural history data from the rare and geographically diverse patient population. Third, because of the late onset of disease on the highly variable disease progression, it is quite difficult to show clinical effects in GNE myopathy trials with only gene replacement, which will only slow or arrest disease progression.

[0009] Cells deficient in GNE activity can be rescued by addition of sialic acid (SA), or by addition of ManNAc, which can also be converted to ManNAc-6 phosphate, the end product of GNE activity, through GlcNAc-6 kinase activity that is not mutated in the disease. Some glycan therapies have shown efficacy in the $GNE_{D176V}{}^{Tg}Gne^{-/-}$ mouse and the Gne_{M712T} knock-in mouse. This has led to two sets of clinical trials, one using slow release SA (phase 3 completed) (Lochmuller et al., Neurology 92(18): e2109-e17, 2019) and one using ManNAc (phase 1 completed) (Xu et al., Mol. Genet. Metab., 12291-2: 126-34, 2017). While SA and ManNAc were shown to have significant therapeutic effects in mice, slow release SA therapy (ACE-ER) met no clinical milestones in a phase 3 clinical trial of GNE myopathy patients. There was no significant change from placebo for any clinical measure, and no change in walking was seen in the ManNAc clinical trial. These negative clinical findings may be due to the fact that dosing of glycan in patients is limited to a tolerable dose of 6-12g of glycan per day, while doses of 3-6g/kg doses are needed to treat the disease in mouse models. Humans average about 70kg at the time of dosing, making the needed dose 17.5 to 35 times what patients can tolerate.

[0010] The lack of efficacy for glycan therapy in GNE myopathy patients makes gene therapy a very attractive alternative. GNE myopathy is a slow and variably progressing human disease and the lack of robust short-term clinical milestones make is difficult to treat effectively. For example, a current phase 3 clinical trial was 48 weeks in duration. In that time, there was not a significant drop in any of the strength measures for the patient population from pre-treatment baseline, though some measures did trend lower.

[0011] Given the pathophysiology of the disease, recent clinical trials have evaluated the use of sialic acid or ManNAc (a precursor of sialic acid) in patients with GNE myopathy as well as early gene therapy trials. For example, AAV8 viral vectors carrying wild type human GNE cDNA have been shown to transduce murine muscle cells and human GNE myopathy-derived muscle cells in culture and to express the transgene in these cells (Mitrani-Rosenbaum et al., Neuromuscul. Disord. 22(11): 1015-24, 2012). The gene therapies in the

prior art only focus on delivering wild-type GNE gene and do not utilize the dual gene function technology disclosed herein.

[0012] The disclosure provides for gene therapies which increase muscle strength at the same time as providing a transgene for gene replacement to prevent further muscle injury or to promote muscle growth are desired. For example, gene therapy vectors that provide GNE gene replacement are likely to be one of the only ways to prove clinical effectiveness for GNE myopathy in a period shorter than 5 years, as the natural history of disease progression is slow and quite variable. It will also be the one of the only ways to show clinical efficacy in all GNE myopathy patients, many of which have lost ambulation not long after diagnosis but that can still show significant arm function, for example self-feeding, which could still be preserved or improved by such a therapy. Because this disease is a myopathy and not a dystrophy, muscle, once repaired, should remain in place permanently. In patients with GNE-dependent ALS, building new muscle mass and strength is also indicated and should benefit patients even though the disease is caused by loss of motor neurons. Motor neurons stimulate skeletal muscles to contract, and so building new contractile activity should also benefit these patients.

[0013] There is a need for more effective gene therapies for treating GNE myopathy, GNE-dependent ALS, thrombocytopenia, sarcopenia and aging.

Summary

[0014] Disclosed herein is a dual gene therapy approach that will add a muscle building component to gene replacement. Such therapies have the potential to rebuild loss muscle strength while simultaneously arresting subsequent disease progression.

[0015] The goal of the GNE therapeutic methods provided herein is to create a tandem or dual gene therapy that expresses both the normal GNE gene and a muscle building protein, such as a follistatin. Such an AAV vector will both correct the genetic defect of GNE myopathy and increase muscle strength, thus reversing rather than just arresting the decline of muscle strength clinical measures. A dual gene therapy that builds new muscle and muscle strength while also preventing further disease by adding back the normal GNE gene will be of greater benefit to patients with GNE myopathy and will provide an easier means of demonstrating clinical improvement. The dual gene therapy approach disclosed herein will also be effective for treating muscle wasting due to thrombocytopenia, sarcopenia and/or aging.

[0016] Treatment of muscle wasting may result in a reduction of muscle damage, prevention or inhibition of muscle damage and/or repair of muscle damage. In addition,

treatment of muscle wasting may build muscle mass, such as increasing the diameter of muscle fibers. For example, in mouse models of GNE myopathy, the disclosed dual gene rAAV vectors prevented muscles damage and/or built new muscle.

[0017] The rAAV vectors disclosed herein produce two mRNAs driven by two different transcriptional control sequences, e.g. promoters and/or enhancers, each with their own poly A tail (in a single AAV), and this gene therapy concept is vastly superior for producing large amounts of the second gene and the second protein, leading to improved therapeutic function. The dual gene rAAV vectors disclosed herein allow for superior gene expression and superior function in inhibiting or preventing disease and building new muscle mass and strength (which will reverse disease).

[0018] The disclosure provides for a polynucleotide sequence comprising in 5' to 3' order: an AAV ITR, a first transcriptional control sequence operably linked to a first transgene sequence encoding GNE protein, such as a protien comprising the amino acid sequence of SEQ ID NO: 4, a polyadenylation signal sequence, a second transcriptional control sequence operably linked to a second (but different) transgene sequence encoding muscle building protein, a polyadenylation signal sequence and an AAV ITR.

[0019] In an exemplary embodiment, the first transgene encodes the GNE protein. The GNE gene sequence comprises the nucleotide sequence of SEQ ID NO: 3 (or nucleotides 2897-5065 of SEQ ID NO: 1 or nucleotides 2872-5040 of SEQ ID NO: 2) or comprises a nucleotide sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotides sequence of SEQ ID NO: 3 and encodes a functional GNE protein. The GNE gene encodes a protein comprising amino acid sequence of SEQ ID NO: 4.

[0020] The disclosure provides for polynucleotides comprising a second transgene that is a gene that encodes a muscle building protein. A muscle building protein is a protein that builds new muscle mass and inducing muscle growth, including a protein that stimulates muscle growth signals or inhibit repressive muscle growth signals. Exemplary transgenes are the follistatin gene (FST), e.g. follistatin 344 (FS344), follistatin 317 (FS317) or follistatin 314, Insulin-like growth factor 1 gene (IGF1), heparin binding Epidermal Growth Factor like Growth Factor gene (HB-EGF) or Mothers against decapentaplegic homolog 7 gene (SMAD7).

[0021] In an exemplary embodiment, the second transgene is the follistatin (FST) gene that encodes protein form FS344. The FST (FS344) gene sequence comprises the nucleotide sequence of SEQ ID NO: 5 (or nucleotides 5406-6437 of SEQ ID NO: 1 or

nucleotides 5381-6412 of SEQ ID NO: 2), or comprises a nucleotide sequence at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence of SEQ ID NO: 5 and encodes a functional FS344 protein. The FST (FS344) gene encoded a protein comprising amino acid sequence of SEQ ID NO: 6.

[0022] A transcriptional control sequence is a promoter sequence that may also include an enhancer and/or intron. Exemplary transcriptional control sequences include but are not limited to, promoters, enhancers and/or polyadenylation signal sequences. Examples of transcriptional control sequences include the chicken β actin promoter (CBA), the hybrid form of the CBA promoter (Cbh), the cytomegaloviruses (CMV promoter), CMV enhancer, miniCMV promoter, MHCK7, the CK8 promoter, the CK8e promoter, the SPc5-12 promoter, a SP-301 promoter the P546 promoter simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter, the myosin promoter, the elongation factor-1a promoter, the hemoglobin promoter, and the creatine kinase promoter.

[0023] In an exemplary embodiment, the first or second transcriptional control sequence comprises one or more of the nucleotide sequences of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, and the first and second transcriptional control sequence are different.

[0024] In some embodiments, the first transcriptional control sequence comprises the one or more of the nucleotide sequences of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11. SEQ ID NO: 12, and the second transcriptional control sequence comprises the nucleotide sequence of SEQ ID NO: 13.

[0025] In an exemplary embodiment, the first or second transcriptional control sequence is a constitutive control element, such CMV or Cbh. The term “constitutive control element” refers to a nucleotide sequence that regulates expression of a coding sequence to tissues throughout the body. These control elements include enhancers and promoters.

[0026] Exemplary muscle-specific promoter include one or more of a human skeletal actin gene element, a cardiac actin gene element, a desmin promoter, a skeletal alpha-actin (ASKA) promoter, a troponin I (TNNI2) promoter, a myocyte-specific enhancer binding factor MEF binding element, a muscle creatine kinase (MCK) promoter, a truncated MCK (tMCK) promoter, a myosin heavy chain (MHC) promoter, a hybrid a-myosin heavy chain enhancer-

/MHC enhancer-promoter (MHCK7) promoter, a CK8 promoter, a CK8e promoter, a SPc5-12 promoter, a SP-301 promoter, a murine creatine kinase enhancer element, a skeletal fast-twitch troponin C gene element, a slow-twitch cardiac troponin c gene element, a slow-twitch troponin I gene element, hypoxia- inducible nuclear factor (HIF)-response element (HRE), a steroid-inducible element, and a glucocorticoid response element (GRE).

[0027] In an exemplary embodiment, the first transcriptional control element sequence comprises an enhancer comprising the nucleotide sequence of SEQ ID NO: 10 and a promoter sequence comprising the nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 9. In addition, the transcriptional control element sequence further comprises an intron. For example, the intron comprises the nucleotide sequence of SEQ ID NO: 14 or SEQ ID NO: 15.

[0028] The disclosure provides for a polynucleotide sequence comprising in 5' to 3' order: an AAV ITR, a first transcriptional control sequence operably linked to a nucleotide sequence encoding the GNE protein (nucleotides 2897-5065 of SEQ ID NO: 1), wherein the first transcriptional control element comprises the CMV enhancer and the hybrid chicken beta-actin promoter (Cbh which is a hybrid CBA promoter and an intronic sequence) (nucleotides 2060-2871 of SEQ ID NO: 1), and a second transcriptional control sequence operably that is the miniCMV promoter (nucleotides 5145-5371 of SEQ ID NO: 1) linked to a nucleotide sequence encoding the FS344 protein (nucleotides 5406-6437 of SEQ ID NO: 1), and an AAV ITR.

[0029] In addition, the disclosure provides for a polynucleotide sequence comprising in 5' to 3' order: an AAV ITR, a first transcriptional control sequence operably linked to a nucleotide sequence encoding the GNE protein (nucleotides 2872-5040 of SEQ ID NO: 2), wherein the first transcriptional control element comprises the CMV enhancer, the CMV promoter and a SV40 enhancer with an intron (nucleotides 2061-2852 of SEQ ID NO: 2), and a second transcriptional control sequence operably that is the miniCMV promoter (nucleotides 5120-5346 of SEQ ID NO: 2) linked to a nucleotide sequence encoding the FS344 protein (nucleotides 5381-6412 of SEQ ID NO: 2), and an AAV ITR.

[0030] In addition, any of the polynucleotides disclosed herein further comprise a polyadenylation signal sequence, which optionally is a synthetic polyadenylation signal sequence. Exemplary polyadenylation sequences include SEQ ID NO: 16 or SEQ ID NO: 17.

[0031] The polynucleotide sequences disclosed herein comprise an inverted terminal repeat (ITR), such as a wild type ITR or a mutant ITR. Exemplary sequences of the ITRs includes the nucleotide sequence of SEQ ID NO: 18 and SEQ ID NO: 19.

[0032] The disclosure also provides for a polynucleotide sequence that is an AAV genome. For example, the disclosure provides an AAV genome or polynucleotide sequence comprising a nucleotide sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to nucleotides 1847 to 6685 of SEQ ID NO: 1 or to nucleotides 1847 to 6660 of SEQ ID NO: 2. In addition, the disclosure provides an AAV genome or polynucleotide sequence comprising nucleotides 1847 to 6685 of SEQ ID NO: 1 or to nucleotides 1847 to 6660 of SEQ ID NO: 2.

[0033] The terms “sequence identity”, “percent sequence identity”, or “percent identical” in the context of nucleic acid or amino acid sequences refers to the residues in the two sequences which are the same when aligned for maximum correspondence. The length of sequence identity comparison may be over the full-length of the genome, the full-length of a gene coding sequence, or a fragment of at least about 500 to 5000 nucleotides, is desired. However, identity among smaller fragments, e.g. of at least about nine nucleotides, usually at least about 20 to 24 nucleotides, at least about 28 to 32 nucleotides, at least about 36 or more nucleotides, may also be desired. The percentage identity of the sequences can be determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs such as ALIGN, ClustalW2 and BLAST. In one embodiment, when BLAST is used as the alignment tool, the following default parameters: genetic code=standard; filter=none; strand=both; cutoff=60; expect=10; Matrix=BLOSUM62; Descriptions=50 sequences; sort by=HIGH SCORE; Databases=non-redundant, GenBank+EMBL+DDBJ+PDB+GenBank CDS translations+Swiss protein+Spupdate+PIR.

[0034] In addition, the rAAV genomes provided herein, hybridizes under stringent conditions to the polynucleotide sequence of nucleotides 1847 to 6685 of SEQ ID NO: 1 or to nucleotides 1847 to 6660 of SEQ ID NO: 2 or the complement thereof.

[0035] The disclosure also provides for recombinant adeno-associated virus (rAAV) comprising any of the polynucleotide sequences described herein. For example, the rAAV comprises AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13, AAV rh.74, AAV rh.10, AAVMyo3, MYOAAV capsid protein, or a variant thereof. For example, the AAV comprises is AAVrh.74 capsid protein.

[0036] The disclosure also provides for a recombinant AAV particle comprising any of the polynucleotide sequences disclosed herein or any of the rAAV disclosed herein.

[0037] In another embodiment, the disclosure provides for methods of producing a rAAV vector particle comprising culturing a cell that has been transfected with any rAAV vector of the disclosure and recovering rAAV particles from the supernatant of the transfected cells. The disclosure also provides for viral particles comprising any of the recombinant AAV vectors of the disclosure.

[0038] The disclosure also provides for compositions comprising any of the rAAV disclosed herein or any of the rAAV particles described herein. In some embodiments, the compositions further comprise a pharmaceutically acceptable carrier. The compositions may also comprise other ingredients such as diluents and adjuvants. Acceptable carriers, diluents and adjuvants are nontoxic to recipients and are preferably inert at the dosages and concentrations employed and include buffers and surfactants such as pluronics.

[0039] The disclosure also provides for methods of treating a GNE-related disorder comprising administering any of the dual gene rAAV disclosed herein or any dual gene rAAV particles disclosed herein or any of the compositions disclosed herein to a subject in need thereof. A GNE-related disorder is a disorder that is associated with a reduction the expression of the GNE gene or a dysfunction in GNE function or the subject has a mutation in the GNE gene. For example, the GNE dependent disorder is GNE-myopathy, GNE-dependent ALS, thrombocytopenia, sarcopenia or aging. In any of the disclosed methods, the dual gene rAAV, dual gene rAAV particle or the composition is administered using systemic administration, intramuscular injection or intravenous injection.

[0040] The disclosure also provides for compositions for treating a GNE-related disorder in a subject in need thereof, wherein the composition comprises any of the dual gene rAAV disclosed herein or any dual gene rAAV particles disclosed herein or any of the compositions disclosed herein. For example, the subject is suffering from GNE-myopathy, GNE-dependent ALS, sarcopenia or aging. In any of the disclosed compositions, the dual gene rAAV, dual gene rAAV particle or the composition is administered using systemic administration, intramuscular injection or intravenous injection.

[0041] The disclosure also provides for use of any of the dual gene rAAV disclosed herein or any dual gene rAAV particles disclosed herein or any of the compositions disclosed herein for the preparation of a medicament for treating GNE-related disorder in a subject in need thereof. For example, the subject is suffering from GNE-myopathy, GNE-dependent ALS, sarcopenia or aging. In any of the disclosed uses, the dual gene rAAV, dual gene rAAV

particle or the composition is administered using systemic administration, intramuscular injection or intravenous injection.

[0042] The disclosure also provides for methods of preventing or repairing muscle damage in a subject in need thereof comprising administering any of the dual gene rAAV disclosed herein or any dual gene rAAV particles disclosed herein or any of the compositions disclosed herein to a subject in need thereof. In some embodiments, the subject is suffering from a GNE-related disorder is a disorder that is associated with a reduction the expression of the GNE gene or a dysfunction in GNE function or the subject has a mutation in the GNE gene. For example, the GNE dependent disorder is GNE-myopathy, GNE-dependent ALS, thrombocytopenia, sarcopenia or aging. In any of the disclosed methods, the dual gene rAAV, dual gene rAAV particle or the composition is administered using systemic administration, intramuscular injection or intravenous injection.

[0043] The disclosure also provides for compositions for preventing or repairing muscle damage in a subject in need thereof, wherein the composition comprises any of the dual gene rAAV disclosed herein or any dual gene rAAV particles disclosed herein or any of the compositions disclosed herein. For example, the subject is suffering from GNE-myopathy, GNE-dependent ALS, sarcopenia or aging. In any of the disclosed compositions, the dual gene rAAV, dual gene rAAV particle or the composition is administered using systemic administration, intramuscular injection or intravenous injection.

[0044] The disclosure also provides for use of any of the dual gene rAAV disclosed herein or any dual gene rAAV particles disclosed herein or any of the compositions disclosed herein for the preparation of a medicament for preventing or repairing muscle damage in a subject in need thereof. For example, the subject is suffering from GNE-myopathy, GNE-dependent ALS, sarcopenia or aging. In any of the disclosed uses, the dual gene rAAV, dual gene rAAV particle or the composition is administered using systemic administration, intramuscular injection or intravenous injection.

[0045] A "subject," as used herein, can be any animal, and may also be referred to as the patient. Preferably, the subject is a vertebrate animal, and more preferably the subject is a mammal, such as a domesticated farm animal (e.g., cow, horse, pig) or pet (e.g., dog, cat). in some embodiments, the subject is a human.

Brief Description of the Drawings

[0046] Figure 1 is an annotated sequence of the plasmid sequence of pAAVCbh.GNE.spA.miniCMV.FST344.spA (SEQ ID NO: 1).

[0047] Figure 2 is an annotated sequence of the plasmid sequence of pAAVCMV.GNE.spA.miniCMV.FST344.Spa (SEQ ID NO: 2).

[0048] Figure 3 provides the muscle weights after intramuscular injection of AAV vectors expressing GNE transgene. Dual gene rAAV vectors comprising two promoters (rAAV.CMV.GNE.pA.mini (m)CMV.FST and rAAV.Cbh.GNE.pA.mCMV.FST) were able to double muscle mass two months after injection ($p<0.01$ for each compared to single gene alone or IRES), while rAAV.CMV.GNE.FL-IRES (full-length IRES).FST, rAAV.CMV.GNE, and rAAV.Cbh.GNE had no significant effect. Errors are SD for n=4-20/grp. Please note that follistatin 344 is used here in all instances.

[0049] Figure 4 provides RNA levels measured by qRT-PCR, using an 18S rRNA internal control, and provides the fold change in gene expression, relative to endogenous wild type mouse gene expression in the tibialis anterior (TA) and gastrocnemius (GS). Errors are SD for n=3-4/grp. "IRES" refers to rAAV.CMV.GNE.FL-IRES.FST, "CMV" refers to rAAV.CMVf.GNE, "Cbh" refers to rAAV.Cbh.GNE, "Bi CMV" refers to the dual gene vector (also known as bicistronic vector) rAAV.CMV.GNE.pA.mCMV.FST, and "Bi Cbh" refers to the dual gene vector (also known as bicistronic vector) rAAV.Cbh.GNE.pA.mCMV.FST.

[0050] Figure 5 demonstrates induction of myofiber growth and prevention of muscle pathology only occur simultaneously with dual gene AAV GNE/FST gene therapy in *Gne*-deficient muscle (aged $Gne^{lox/lox}$ mice). Hematoxylin and Eosin staining of myofibers sections is shown. Bar is 50μm for all panels. AAV.CMV.Cre (referred to as "CRE") was injected with or without rAAV.CBh.GNE (referred to as "Cre+GNE"), rAAV.CMV.FST (referred to as "Cre+FST") or dual gene rAAV.CBh.GNE.spA.mCMV.FST (referred to as "Cre+GNE/FST").

[0051] Figure 6 demonstrates that dual GNE/FST gene therapy prevents Cre-induced muscle damage and increases myofiber size in a dose-dependent manner. Hematoxylin and Eosin staining of myofibers sections is shown. Bar is 50μm for all panels. On day 1, adult $RosaCreER^{T2}Gne^{lox/lox}$ mice were injected intravenously with PBS, $1x10^{13}$vg/kg, $5x10^{13}$vg/kg, or $2x10^{14}$vg/kg rAAV.CBh.GNE.mCMV.FST (GNE/FST). On day 7, AAV.CMV.Cre (referred to as "CRE") was injected intramuscularly into the tibialis anterior muscle.

[0052] Figure 7 demonstrates that muscle mass is increased by dual GNE/FST gene therapy. Muscle mass, as a percentage of body weight, was significantly increased at the high dose of dual gene therapy. On day 1, adult $RosaCreER^{T2}Gne^{lox/lox}$ mice were injected intravenously with PBS, $1x10^{13}$vg/kg, $5x10^{13}$vg/kg, or $2x10^{14}$vg/kg rAAV.CBh.GNE.mCMV.FST (GNE/FST). On day 7, AAV.CMV.Cre (referred to as "CRE")

was injected intramuscularly into the tibialis anterior muscle. Significance determined by ANOVA with Tukey post-hoc test. ***p<0.001.

[0053] Figure 8 demonstrates the average myofiber diameter is increased by dual GNE/FST gene therapy. Average Mini-Feret diameter, as a percentage was significantly increased by the two higher doses of dual gene therapy. On day 1, adult RosaCreER^{T2}*Gne*$^{lox/lox}$ mice were injected intravenously with PBS, 1x10^{13}vg/kg, 5x10^{13}vg/kg, or 2x10^{14}vg/kg rAAV.CBh.GNE.mCMV.FST (GNE/FST). On day 7, AAV.CMV.Cre (referred to as "CRE") was injected intramuscularly into the tibialis anterior muscle. Significance determined by ANOVA with Tukey post-hoc test. *p<0.05, ***p<0.001, ****p<0.0001

Detailed Description

[0054] Disclosed herein is a dual gene therapy approach that will add a muscle building component to gene replacement. Such therapies have the potential to rebuild loss muscle strength while simultaneously arresting subsequent disease progression.

[0055] The concept of bicistronic gene therapy using GNE gene therapy was described in International Application No. WO 2021/127655. In that disclosure, the rAAV vectors comprised an internal ribosomal entry site (IRES) to produce a second protein from a single AAV vector where a single mRNA was made by a single promote. However, the dual gene rAAV vectors disclosed herein produce two mRNAs driven by two different promoters, each with their own poly A tail (in a single AAV), and this dual gene therapy concept is vastly superior for producing large amounts of the second gene and the second protein, leading to improved therapeutic function. The dual gene rAAV vectors disclosed herein allow for superior gene expression and superior function in preventing or inhibiting disease and building new muscle mass and strength (which will reverse disease by repairing the muscle damage).

[0056] To build new muscle mass and strength at the same time as GNE gene replacement inhibits disease, the disclosed dual gene therapy approach utilizes follistatin (FST) as a second gene component in the dual gene AAV vectors. FST encodes a secreted myostatin inhibitor protein that binds to and inhibits myostatin protein binding to its reception on muscle cells (Amthor et al, Dev Biol, 270(1): p. 19-30, 2004). Myostatin, is a secreted muscle trophic factor that negatively regulates muscle growth and strength. Elimination of myostatin in mice, cows, or humans can double the size of skeletal muscles, with minimal to no effects on cardiac muscle or non-muscle tissues. Elimination of myostatin in mdx mice significantly increased muscle size and strength but did not improve weight-normalized grip strength or specific (weight-normalized) tetanic muscle force. Thus, myostatin inhibition does

not stabilize the muscle membrane or prevent muscle damage, but instead increases muscle strength by increasing muscle mass. In this sense then, myostatin inhibition therapy alone may be ineffective over the long term, as muscles expressing the inhibitor will eventually be destroyed if effective gene replacement is not also provided.

[0057] FST protein also activates Akt and mTOR signaling via a separate pathway. Each of these properties allows for the building of new muscle mass and strength, both by making pre-existing muscles bigger and by creating new muscle fibers. As GNE myopathy is a disease where muscle mass is lost, this dual gene therapy allows patients with this disease to recover lost muscle strength, providing them, for the first time, a therapy that may recover normal, pre-disease, muscle function.

[0058] The FST gene form used in the exemplary approach described herein, FS344, has been tested in two clinical trials: IM delivery of rAAV1.CMV.FST bilaterally in the quadriceps muscles of patients with Becker Muscular Dystrophy (BMD) yielded improvements in the 6-minute walk test (6MWT, as much as 125 meters) in 4 of 6 patients at 1 year post-treatment. Treated BMD patient muscle biopsies suggested muscle hypertrophy, decreased endomysial fibrosis, and more uniform myofiber size (Mendell et al., Mol Ther, **23**(1): p. 192-201, 2015). Similar IM delivery of rAAV1.CMV.FST in patients with Inclusion Body Myositis (IBM) patients yielded an average improvement of +56 meters on the 6MWT at one year post-treatment (and as high as +153 m) in six subjects. Treated IBM patient muscle biopsies showed muscle hypertrophy, decreased fibrosis, and improved regeneration (Mendell et al., Mol. Ther. 23(1): 192-201, 2015). Thus, a major attraction to the use of FST gene therapy in dual gene vectors is that this gene has already been tested in humans and shown to be safe and effective. Another major attraction is that FST gene therapy is more potent than myostatin inhibition alone (Lee et al., PLoS One 2(8): e789, 2007) and can activate additional muscle growth signaling pathways (Winbanks et al., J. Cell Biol. 197(7):997-1008, 2012).

[0059] The dual gene therapy disclosed herein is useful for treating a GNE-related disorder, which is a disorder that is caused by GNE mutations or caused by reduced GNE expression or dysfunction in the GNE protein. For example, the dual gene therapy disclosed herein is used to treat GNE myopathy, a muscle disease caused by missense mutations in the GNE gene. Addition GNE-related disorders include GNE-dependent ALS, sarcopenia, and age-related neurodegeneration. The disclosed dual gene therapy allows for expression of GNE in all cells, and as the hybrid form of the CBA promoter (referred to as Cbh) is particularly good at inducing expression in motor neurons (compared to CMV).

[0060] Some GNE mutations are known to give rise to juvenile amyotrophic lateral sclerosis (ALS) in addition to GNE myopathy. While GNE myopathy is a muscle disease, ALS is a motor neuron disease where muscle strength is lost due to the death of motor neurons. The use of Cbh or CMV, promoters that allow for expression in all cells and tissues, provide a potential therapy for GNE dependent ALS as well as GNE myopathy. GNE is expressed normally in all tissues and cells of the body. All cells make sialic acid, and GNE is the committed step in sialic acid biosynthesis. Thus, placement of a normal copy of GNE in all cells is the most likely approach to cure both of these diseases. As FST is specific for blocking myostatin, which is a muscle-specific protein, its expression using miniCMV (mCMV), which again will lead to expression in all tissues, does not add any off-target effects and may amplify the muscle growth phenotype relative to expression only in muscle. Mini CMV (mCMV) must be used here instead of full length CMV because of the packaging limitations of the AAV capsid. Cbh (a chick beta actin with hybrid intron) promoter will likely need to be paired with mCMV to reduce the possibility of recombination for the small number of AAV vgs that may integrate into the host genome. Use of CMV together with mCMV, which shares DNA homology, might increase risk of recombination relative to the Cbh/mCMV combination. Also, Cbh is published to be superior to CMV in allowing gene expression in motor neurons.

Muscle Building Proteins

[0061] Muscle building proteins can include growth factors that induce muscle growth or increase muscle strength such as IGF, HB-EGF, Pax7, HGF (hepatocyte growth factor), HGH (human growth hormone), FGF19 (fibroblast growth factor 19), FGF21 (fibroblast growth factor 21), VEGF (vascular endothelial growth factor), IL6 (Interleukin 6), IL15 (Interleukin 15) and SMAD7 (mothers against decapentaplegic homolog 7 (MADH7)).

[0062] Growth factors that induce muscle growth or increase muscle strength also include the follistatins (FST). Follistatin is a secreted protein that inhibits the activity of TGF-β family members such as GDF-11/BMP-11. Follistatin-344 is a follistatin precursor that undergoes peptide cleavage to form the circulating Follistatin-315 isoform which includes a C-terminal acidic region. It circulates with myostatin propeptide in a complex that includes two other proteins, follistatin related gene (FLRG) and GDF associated serum protein (GASP-1). Follistatin-317 is another follistatin precursor that undergoes peptide cleavage to form the membrane-bound Follistatin-288 isoform.

[0063] The DNA and amino acid sequences of the follistatin-344 precursor are respectively set out in SEQ ID NOs: 5 and 6 (and the nucleotides 5406-6437 of SEQ ID NO:

1 or nucleotides 5381-6412 of SEQ ID NO: 2). FS344 contains a C-terminal protein domain lacking in FS288. The presence of this C-terminal domain reduces binding to activin and to heparan sulfate glycosaminoglycans, which in turn reduces non-muscle effects. The Follistatin-288 isoform, which lacks a C-terminal acidic region, exhibits strong affinity for heparin-sulfate-proteoglycans, is a potent suppressor of pituitary follicle stimulating hormone, is found in the follicular fluid of the ovary, and demonstrates high affinity for the granulose cells of the ovary. The testis also produce Follistatin-288. Lack of follistatin results in reduced muscle mass at birth.

[0064] Examples of follistatins are provided in Shimasaki et al., U.S. Patent No. 5,041,538, other follistatin-like proteins are provided in U.S. Patent Nos. 5,942,420; 6,410,232; 6,537,966; and 6,953,662), FLRG is provided in Hill *et al., J. Biol. Chem., 277*(43): 40735-40741 (2002)] and GASP-1 is provided in Hill et al., Mol Endocrinol, 17: 1144-1154 (2003).

[0065] SMAD7 is known to inhibit TGF-β-activated signaling responses by associating with the active TGF-β complex, which results in reduced TGF-β signaling. Myostatin and TGF-β signaling induces SMAD7 expression establishing a negative feedback loop to inhibit TGF-β signaling. In particular, SMAD7 is known to modulate myogenesis using this negative feedback loop (Kollias et al. Mol. Cell Biol. 26(16):6248-6260, 2006). The nucleotide sequence encoding the SMAD7 is provided in Genbank Accession No. NM_005904.4, and the amino acid sequence is provided as Genbank Accession No. NP_005895.

[0066] Transdifferentiation factors are agents that convert or induce differentiation to a non-muscle cell to muscle. For example, MyoD is known to convert a number of cell types into muscle, including dermal fibroblasts, chondrocytes, smooth muscle, retinal pigmented epithelial cells, adipocytes, and melanoma, neuroblastoma, osteosarcoma, and hepatoma cells (Abraham & Tapscott, Curr. Opin. Genet. Dev. 23(5): 568-573, 2013). Other examples of transdifferentiation factors Myocd (myocardin), Mef2C (myocyte enhancer factor 2C), Mef2B (myocyte enhancer factor 2B), Mkl1 (MKL [megakaryoblastic leukemia]/Myocd-like 1), Gata4 (GATA-binding protein 4), Gata5 (GATA-binding protein 5), Gata6 (GATA-binding protein 6), Ets1 (E26 avian leukemia oncogene 1, 5' domain).

GNE Myopathy

[0067] GNE myopathy is characterized by progressive muscle atrophy and weakness. The age of onset is typically in the third decade of life, beginning with weakness in the tibialis anterior (TA) and hamstring muscles and often rendering patient's wheelchair-bound by the

second decade after diagnosis. Patients may ultimately require assistance with daily living functions such as eating. Muscle biopsies typically shows rimmed vacuoles and inclusion bodies. GNE myopathy is caused by mutations in the GNE gene, which encodes a bifunctional UDP-GlcNAc epimerase/ManNAc-6 kinase. GNE function is required for synthesis of all sialic acid (SA). The SA biosynthetic pathway culminates in the production of CMP-SA, which is utilized by sialyltransferases to transfer SA onto glycoproteins and glycolipids in all mammalian cells.

[0068] GNE myopathy incidence has recently been estimated to between 1 and 6 per million, a rare disease. There are, however, founder effect mutations that cause GNE myopathy to occur at much higher incidence in certain human populations, for example in patients of Japanese (D176V, D207V in the new nomenclature) and Middle Eastern (M712T, M743T in the new nomenclature) descent. Disease mutation carrier frequency in one study of 1000 Iranian Jews was found to be 1 in 11. The partial reduction in GNE activity in patients leads to reduced, but not absent, SA expression.

[0069] Diminished IGF1R signaling has been shown to be a basis for muscle stem cell death in a model of GNE myopathy, making IGF1 a possible ideal growth factor element to the gene therapy design. These tandem gene vectors are expected not only to inhibit disease progression (the function of GNE gene replacement) but also induce new muscle growth (thereby increasing muscle strength) and possibly prevent stem cell death. These vectors are highly unique, as patients with GNE myopathy lose muscle and strength over decades, and the provided AAV are expected not only to slow this progression but to actually reverse it. The provided dual function AAV will be able to show clinical efficacy, as this disease shows high clinical variability (between patient disease mutations and even amongst patients with the same disease mutation) and because it is slowly progressing (with major clinical changes occurring over decades).

GNE Myopathy Mutations

[0070] In any of the provided methods of the subject is suffering from GNE myopathy. For the example, the subject has a mutation in the GNE gene that results in reduced expression of UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase. A diagnosis of GNE myopathy is confirmed in a subject by the presence of pathogenic (mostly missense) mutations in both alleles of the GNE gene. Table 1 provides of known mutations in the GNE gene that are associated with GNE myopathy is provided below. The subjects of the claimed methods may comprise a mutation set out in this table.

[0071] In Table 1 **Bold** print indicates cDNA or protein truncating variants. *Italic* print + dark gray highlight indicates 'Mild' variants. A question mark (?) indicates that the exact nomenclature could not be extracted from the reference. The DNA numbering system is based on cDNA sequence. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.

Amino Acid Substitution		**Nucleotide Substitution**	**Gene Exon**	**GNE protein domain**	**Severity Prediction**	**Ethnicity**	**Reference**
hGNE1	**hGNE2**	**mRNA Variant1**					
E2G	p.E33G	c.98A>G	3	ep	Severe	*European*	[Saechao et al., 2010]
R8*	**p.R39***	c.115C>T	3	ep	Severe	*Caucasian, Chinese, Japanese*	[Saechao et al., 2010; Lu et al., 2011; Mori-Yoshimura et al., 2012]
R11W	p.R42W	c.124C>T	3	ep	Severe	*Indian*	[Huizing et al., 2004]
C13S	p.C44S	c.131G>C	3	ep	Medium	*Chinese, Japanese, Korean*	[Kim et al., 2006; Lu et al., 2011; Park et al., 2012; Tomimitsu et al., 2004]
A26P	*p.A57P*	c.169G>C	3	ep	Mild	*Caucasian*	[Weihl et al., 2011]
P27L	p.P58L	c.173C>T	3	ep	Medium	*Japanese, Indian*	[Mori-Yoshimura et al., 2012; Nalini et al., 2013]
P27S	p.P58S	c.172C>T	3	ep	Medium	*Italian*	[Broccolini et al., 2004]
I28M	p.I59M	c.177C>G	3	ep	Medium	*Japanese*	[Cho et al., 2013]
M29T	p.M60T	c.179T>C	3	ep	Medium	*Korean, Japanese*	[Kim et al., 2006; Cho et al., 2013]
M29R	p.M60R	c.179T>G	3	ep	Severe	*Japanese*	[Cho et al., 2013]
E35K	p.E66K	c.196G>A	3	ep	Medium	*Chinese*	[Li et al., 2011; Lu et al., 2011]
P36L	p.P67L	c.200C>T	3	ep	Severe	*Italian*	[Eisenberg et al., 2003]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
E40K	p.E71K	c.211G>A	3	ep	Medium	*Japanese*	[Cho et al., 2013]
frameshift	**frameshift**	ins10 bp?	3	ep	Severe	*Japanese*	[Nishino et al., 2002]
I51M	p.I82M	c.246A>G	3	ep	Medium	*Chinese*	[Li et al., 2011; Lu et al., 2011]
exon 3 del	**exon 3 del**	?	3	ep	Severe	*Japanese*	[Cho et al., 2013]
M60V	*p.M91V*	c.271A>G	4	ep	Mild	*Portuguese*	novel
R71W	p.R102W	c.304A>T	4	ep	Severe	*Caucasian*	[Saechao et al., 2010]
G89R	p.G120R	c.358G>C	4	ep	Severe	*Thai, Japanese*	[Liewluck et al., 2006; Cho et al., 2013]
G89S	p.G120S	c.358G>A	4	ep	Medium	*Japanese*	[Mori-Yoshimura et al., 2012; Cho et al., 2013]
R101C	p.R132C	c.394C>T	4	ep	Severe	*Korean*	[Park et al., 2012]
R101H	p.R132H	c.395G>A	4	ep	Medium	*Japanese*	[Cho et al., 2013]
I106T	p.I137T	c.410T>C	4	ep	Medium	*Chinese*	[Lu et al., 2011]
I128Ifs*6	**p.I159Ifs*6**	c.476insT	4	ep-NES	Severe	*Japanese*	[Mori-Yoshimura et al., 2012; Cho et al., 2013]
R129Q	p.R160Q	c.479G>A	4	ep-NES	Medium	*Japanese*	[Mori-Yoshimura et al., 2012]
R129*	**p.R160***	c.478C>T	4	ep-NES	Severe	*Indian*	novel
H132Q	p.H163Q	c.489C>G	4	ep-NES	Medium	*Japanese*	[Nishino et al., 2002; Tomimitsu et al., 2002]
G135V	p.G166V	c.497G>T	4	ep-NES	Severe	*English, Irish, USA*	[Sparks et al., 2005]
G136R	p.G167R	c.501G>A	4	ep-NES	Severe	*Japanese*	[Cho et al., 2013]
I142T	p.I173T	c.518T>C	4	ep	Severe	*Caucasian*	[Saechao et al., 2010]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
I150V	p.I181V	c.541A>G	4	ep	Medium	*European*	[No et al., 2013]
Y156H	p.Y187H	c.559T>C	4	ep	Medium	*Indian*	novel
H157fs	**p.H188fs**	?	4	ep	Severe	*Korean*	[Sim et al., 2013]
R162C	p.R193C	c.515C>T	4	ep	Severe	*Italian, Indian*	[Del Bo et al., 2003; Nalini et al., 2013]
M171V	p.M202V	c.604A>G	4	ep	Severe	*Italian*	[Broccolini et al., 2002]
D176V	p.D207V	c.620A>T	4	ep	Medium	*Chinese, Japanese, Korean*	[Nishino et al., 2002; Tomimitsu et al., 2002, 2004; Motozaki et al., 2007; Li et al., 2011; Park et al., 2012]
R177C	p.R208C	c.622C>T	4	ep	Severe	*Japanese*	[Nishino et al., 2002; Cho et al., 2013]
I178N	p.I209N	c.626T>A	4	ep	Severe	*Japanese*	[Cho et al., 2013]
I178M	p.I209M	c.627C>G	4	ep	Medium	*Japanese*	[Cho et al., 2013]
L179F	p.L210F	c.628C>T	4	ep	Medium	*Italian*	[Grandis et al., 2010]
Y186C	p.Y217C	c.650A>G	4	ep	Severe	*Pakistani*	[No et al., 2013]
D187G	p.D218G	c.653A>G	4	ep	Severe	*Japanese*	[Mori-Yoshimura et al., 2012; Cho et al., 2013]
N194Tfs*4	**p.N225Tfs*4**	c.674delA	4	ep	Severe	*Japanese*	[Cho et al., 2013]
I200F	p.I231F	c.691A>T	4	ep	Medium	*USA*	[Eisenberg et al., 2003]
R202L	p.R233L	c.698G>T	4	ep	Medium	*Greek*	[Huizing et al., 2004]
W204*	**p.W235***	c.705G>A	4	ep	Severe	*Caucasian*	[Saechao et al., 2010]
G206S	p.G237S	c.709G>A	4	ep	Medium	*Italian*	[Broccolini et al., 2004]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
splicing	**splicing**	c.710-4A>G	in 4	ep	Splicing?	*Japanese*	[Cho et al., 2013]
G206Vfs*3	**p.G237Vfs*3**	c.710delG	5	ep	Severe	*Italian*	[Broccolini et al., 2004]
D208N	*p.D239N*	c.715G>A	5	ep	Mild	*Korean*	[Sim et al., 2013]
D213V	p.D244V	c.731A>T	5	ep	Medium	*Indian*	novel
V216A	p.V247A	c.740T>C	5	ep	Severe	*USA, German, Dutch*	[Vasconcelos et al., 2002; Huizing et al., 2004]
Q219K	p.Q250K	c.748C>A	5	ep	Medium	*Japanese*	[Cho et al., 2013]
D225N	p.D256N	c.766G>A	5	ep	Medium	*Bahamas*	[Eisenberg et al., 2001]
F233S	p.F264S	c.791T>C	5	ep	Medium	*Japanese*	[Mori-Yoshimura et al., 2012]
I241S	p.I272S	c.815T>G	5	ep	Medium	*Chinese, Taiwanese*	[Ro et al., 2005; Chu et al., 2007; Li et al., 2011; Lu et al., 2011]
R246W	p.R277W	c.829C>T	5	ep	Severe	*Caucasian, Chinese, Japanese, Italian, USA*	[Darvish et al., 2002; Ro et al., 2005; Sparks et al., 2005; Saechao et al., 2010; Stober et al., 2010; Li et al., 2011; Cho et al., 2013]
R246Q	*p.R277Q*	c.830G>A	5	ep	Mild	*Bahamas, Italian, Taiwanese, Japanese*	[Eisenberg et al., 2001; Broccolini et al., 2004; Chu et al., 2007; Saechao et al., 2010; Chai et al., 2011]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
splicing	**splicing**	c.862+4A>G	in 5	ep	Splicing	*Japanese*	[Nishino et al., 2002; Cho et al., 2013]
M261V	*p.M292V*	c.874A>G	6	ep-AR	Mild	*Korean*	[Park et al., 2012]
M261I	*p.M292I*	c.876G>?	6	ep-AR	Mild	*Korean*	[Sim et al., 2013]
M265T	p.M296T	c.887T>C	6	ep-AR	Medium	*European*	[No et al., 2013]
I270N	p.I301N	c.902T>A	6	ep-AR	Medium	*Japanese*	[Cho et al., 2013]
I270T	p.I301T	c.902T>C	6	ep-AR	Medium	*Japanese*	[Cho et al., 2013]
R277C	p.R308C	c.922C>T	6	ep-AR	Medium	*French, Japanese*	[Behin et al., 2008; Cho et al., 2013]
R277G	p.R308G	c.922C>G	6	ep-AR	Medium	*Japanese*	[Cho et al., 2013]
P283S	p.P314S	c.940C>T	6	ep-AR	Medium	*Japanese*	[Tomimitsu et al., 2004]
H293R	p.H324R	c.971A>G	6	ep-AR	Medium	*Indian*	[Kannan et al., 2012]
G295D	p.G326D	c.977G>A	6	ep-AR	Medium	*Japanese*	[Mori-Yoshimura et al., 2012]
G295R	p.G326R	c.976G>C	6	ep-AR	Medium	*Japanese*	[Cho et al., 2013]
M297T	p.M328T	c.983T>C	6	ep-AR	Medium	*Indian*	novel
I298T	p.I329T	c.986T>C	6	ep-AR	Severe	*Asian, Chinese, Indian*	[Saechao et al., 2010; Lu et al., 2011]
N300K	p.N331K	c.993C>A	6	ep-AR	Severe	*Italian*	[Tasca et al., 2012]
C303V	p.C334V	c.1000_1001TG>GT	6	ep-AR	Medium	*Japanese*	[Tomimitsu et al., 2002]
C303*	**p.C334***	c.1002T>A	6	ep-AR	Severe	*Indian*	[Eisenberg et al., 2001]
G304R	p.G335R	c.1003G>A	6	ep	Severe	*Indian*	[Nalini et al., 2013]
R306Q	p.R337Q	c.1010G>A	6	ep	Medium	*Japanese*	[Nishino et al., 2002]
A310P	p.A341P	c.1021G>C	6	ep	Severe	*Chinese*	[Ro et al., 2005; Stober et al., 2010]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
V315M	p.V346M	c.1036G>A	6	ep	Medium	*European*	[No et al., 2013]
N317D	p.N348D	c.1042A>G	6	ep	Severe	*European*	[No et al., 2013]
R321C	p.R352C	c.1054C>T	6	ep	Severe	*Japanese*	[Mori-Yoshimura et al., 2012]
splicing	**splicing**	c.1076-1delG	in 6	ep	Splicing?	*Japanese*	[Cho et al., 2013]
V331A	p.V362A	c.1085T>C	7	ep	Severe	*Japanese*	[Nishino et al., 2002]
H333R	p.H364R	c.1091A>G	7	ep	Medium	*Caucasian*	[Weihl et al., 2011]
R335W	p.R366W	c.1096C>T	7	ep	Severe	*Caucasian*	[Fisher et al., 2006; Saechao et al., 2010]
L347del; H348N	**p.L378del; p.H379N**	c.1132_1134 del;c.1135C>A	7	ep	Severe	*Caucasian*	[Fisher et al., 2006]
L347P	p.L378P	c.1133T>C	7	ep	Severe	*Japanese*	[Cho et al., 2013]
splicing	**splicing**	c.1163+2dupT	in 7	ep	Splicing	*European, Italian*	[Broccolini et al., 2004; No et al., 2013]
Y361*	**p.Y392***	c.1176T>G	8	ep	Severe	*Caucasian*	[Weihl et al., 2011]
V367I	p.V398I	c.1192G>A	8	ep	Medium	*Iranian*	[Krause et al., 2003]
I377Tfs*15	**p.I408Tfs*15**	c.1223delT	8	ep	Severe	*Italian*	[Broccolini et al., 2004]
D378Y	p.D409Y	c.1225G>T	8	UF	Severe	*European, Irish, Japanese, USA*	[Nishino et al., 2002; Eisenberg et al., 2003; No et al., 2013]
L379H	p.L410H	c.1229T>A	8	UF	Severe	*Tunisian*	[Amouri et al., 2005]
P390S	p.P421S	c.1261C>T	8	UF	Medium	*Korean*	[Sim et al., 2013]
R420*	**p.R451***	c.1351C>T	8	kin	Severe	*Japanese, Indian*	[Tomimitsu et al., 2004; Nalini et al., 2013]
V421A	p.V452A	c.1355T>C	8	kin	Medium	*Japanese*	[Tomimitsu et al., 2004; Cai et al., 2013]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
K432Rfs*16	**p.K463Rfs*16**	c.1388delA	9	kin	Severe	*Indian*	[Voermans et al., 2010]
Y434C	p.Y465C	c.1394A>G	9	kin	Medium	*Korean*	[Sim et al., 2013]
Q436*	**p.Q467***	c.1399C>T	9	kin	Severe	*Taiwanese*	[Saechao et al., 2010]
C453F	p.C484F	c.1451G>T	9	kin	Severe	*Japanese*	[Cho et al., 2013]
A460V	p.A491V	c.1472C>T	9	kin	Medium	*Japanese*	[Kayashima et al., 2002]
.L463P	p.L494P	c.1481T>C	9	ep	Severe	*Korean*	[Sim et al., 2013]
G469R	p.G500R	c.1498G>A	9	kin	Severe	*Japanese*	[Cho et al., 2013]
splicing	**splicing**	c.1504+5G>A	in 9	kin	Splicing	*Japanese*	[Cho et al., 2013]
splicing	**splicing**	c.1505-4G>A	in 9	kin	Splicing?	*Japanese*	[Cho et al., 2013]
I472T	p.I503T	c.1508T>C	10	kin	Severe	*Japanese*	[Nishino et al., 2002; Yabe et al., 2003]
W495*	**p.W526***	c.1577G>A	10	kin	Severe	*Caucasian*	[No et al., 2013]
L508S	p.L539S	c.1616T>C	10	kin	Severe	*Chinese*	[Li et al., 2011; Lu et al., 2011]
H509Y	p.H540Y	c.1618C>T	10	kin	Medium	*Chinese*	[Lu et al., 2011]
P511H	p.P542H	c.1625C>A	10	kin	Severe	*Japanese*	[Motozaki et al., 2007]
P511L	p.P542L	c.1625C>T	10	kin	Severe	*Thai*	[Liewluck et al., 2006]
W513*	**p.W544***	c.1632G>A	10	kin	Severe	*Chinese, Taiwanese, Indian*	[Ro et al., 2005; Li et al., 2011; Nalini et al., 2013]
V514del	**p.V545del**	c.1634_1637del	10	kin	Severe	*Japanese*	[Cho et al., 2013]
N519S	p.N550S	c.1649A>G	10	kin	Medium	*Italian*	[Broccolini et al., 2004]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
A524V	p.A555V	c.1664C>T	10	kin	Severe	*French, Mexican, Thai, Japanese*	[Darvish et al., 2002; Liewluck et al., 2006; Behin et al., 2008; Cho et al., 2013]
F528C	p.F559C	c.1676T>G	10	kin	Severe	*German*	[Eisenberg et al., 2003]
G545Efs*11	**p.G576Efs*11**	c.1727delG	11	kin	Severe	*Korean*	[Park et al., 2012]
L556S	p.L587S	c.1760T>C	11	kin	Severe	*Caucasian*	[Saechao et al., 2010]
I557T	p.I588T	c.1763T>C	11	kin	Medium	*Italian, Japanese*	[Eisenberg et al., 2003; Tomimitsu et al., 2004]
G559R	p.G590R	c.1768G>C	11	kin	Severe	*Japanese, Greek*	[Huizing et al., 2004; Cho et al., 2013;]
G559A	p.G590A	c.1769G>C	11	kin	Severe	*Turkish*	novel
G568S	p.G599S	c.1795G>A	11	kin	Severe	*Japanese*	[Mori-Yoshimura et al., 2012]
G568V	p.G599V	c.1796G>T	11	kin	Severe	*Indian*	[Nalini et al., 2013]
V572del	**p.V603del**	c.1806_1808del	11	kin	Severe	*Japanese*	[Cho et al., 2013]
V572L	p.V603L	c.1807G>C	11	kin	Medium	*Asian, Chinese, Japanese, Korean*	[Kayashima et al., 2002; Tomimitsu et al., 2002; Kim et al., 2006; Li et al., 2011; Park et al., 2012]
G576E	p.G607E	c.1820G>A	11	kin	Severe	*USA*	[Eisenberg et al., 2001]
C579Y	p.C610Y	c.1829G>A	11	kin	Severe	*Japanese*	[Cho et al., 2013]
C581R	p.C612R	c.1834T>C	11	kin	Severe	*Pakistani*	novel
C586*	**p.C617***	c.1850delG	11	kin	Severe	*Japanese*	[Mori-Yoshimura et al., 2012]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
I587T	p.I618T	c.1853T>C	11	kin	Medium	*Algerian, Chinese, Italian, Cajun, Japanese, Roma Gypsies*	[Tomimitsu et al., 2002; Kalaydjieva et al., 2005; Behin et al., 2008; Grandis et al., 2010; Li et al., 2011; Cho et al., 2013]
I587N	p.I618N	c.1853T>A	11	kin	Medium	*Japanese*	[Cho et al., 2013]
A591T	p.A622T	c.1864G>A	11	kin	Severe	*Chinese, Korean*	[Kim et al., 2006; Lu et al., 2011]
A600E	p.A631E	c.1892C>A	11	kin	Severe	*Japanese*	[Mori-Yoshimura et al., 2012; Cho et al., 2013]
A600T	p.A631T	c.1891G>A	11	kin	Medium	*Italian*	[Broccolini et al., 2004]
L603F	p.L634F	c.1900C>T	11	kin	Medium	*Japanese*	[Mori-Yoshimura et al., 2012]
splicing	**splicing**	c.1909+5G>A	in 11	kin	Splicing	*Indian*	[Boyden et al., 2011]
S615*	**p.S646***	c.1937C>G	12	kin	Severe	*Caucasian*	[Saechao et al., 2010]
A630Lfs*12	**p.A661Lfs*12**	c.1980delA	12	kin	Severe	*Japanese*	[Cho et al., 2013]
A630T	p.A661T	c.1981G>A	12	kin	Medium	*Japanese*	[Nishino et al., 2002; Cho et al., 2013]
A631T	p.A662T	c.1984G>A	12	kin	Severe	*Caucasian, Senegalese, USA*	[Eisenberg et al., 2001; Behin et al., 2008; No et al., 2013]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
A631V	p.A662V	c.1985C>T	12	kin	Severe	*Caucasian, Korean, Chinese, German, Irish, S. African, USA, Japanese*	[Nishino et al., 2002; Tomimitsu et al., 2002; Vasconcelos et al., 2002; Eisenberg et al., 2003; Saechao et al., 2010; Li et al., 2011; Weihl et al., 2011; Park et al., 2012]
N635K	p.N666K	c.1998T>A	12	kin	Severe	*Japanese*	[Cho et al., 2013]
N635K	p.N666K	c.1998T>G	12	kin	Severe	*Japanese*	[Cai et al., 2013]
A648V	p.A679V	c.2036C>T	13	kin	Medium	*German*	novel
I656N	p.I687N	c.2060T>A	13	kin	Severe	*Thai*	[Liewluck et al., 2006]
G669R	p.G700R	c.2098G>A	13	kin	Severe	*Japanese*	[Cho et al., 2013]
G669R	p.G700R	c.2098G>C	13	kin	Severe	*Asian, Indian, Portuguese*	[No et al., 2013]
Y675H	p.Y706H	c.2116T>C	13	kin	Medium	*Caucasian*	[Darvish et al., 2002; Saechao et al., 2010]
V679G	p.V710G	c.2129T>G	13	kin	Severe	*French*	[Behin et al., 2008]

Amino Acid Substitution		Nucleotide Substitution	Gene Exon	GNE protein	Severity Prediction	Ethnicity	Reference
V696M	p.V727M	c.2179G>A	13	kin	Medium	*Algerian, Asian, Chinese, Middle-Eastern, Indian, Pakistani, Thai, Portuguese*	[Eisenberg et al., 2001; Huizing et al., 2004; Liewluck et al., 2006; Behin et al., 2008; Saechao et al., 2010; Voermans et al., 2010; Boyden et al., 2011; Lu et al., 2011; No et al., 2013]
S699L	p.S730L	c.2189C>T	13	kin	Severe	*Middle-Eastern*	[No et al., 2013]
G708S	p.G739S	c.2215G>A	13	kin	Severe	*Japanese*	[Tomimitsu et al., 2004; Cho et al., 2013]
M712T	p.M743T	c.2228T>C	13	kin	Severe	*Egyptian-Muslim, Persian Jewish, Japanese*	[Eisenberg et al., 2001; Broccolini et al., 2002; Darvish et al., 2002; Noguchi et al., 2004; Tomimitsu et al., 2004; Amouri et al., 2005; Cho et al., 2013]
large deletion	**large deletion**	del ex2-ex10 (>35.7kb)	2–10	ep + kin	Severe	*Italian*	[Del Bo et al., 2003]

[1]Amino acid substitutions are provided in the previously used hGNE1 (NP_005467.1) and in the preferred new hGNE2 (NP_001121699.1) nomenclature [Huizing et al. 2014b]. For some variants, updated nomenclature is provided extracted from the reference.
[2]Nucleotide variants are provided in the mRNA variant 1 nomenclature (NM_001128227.2; longest mRNA spliceform; encoding hGNE2 protein).
[3]Exon numbering according to genomic sequence (NC_000009.12). in = intron.
[4]See text for details about GNE protein domains; ep = UDP-GlcNAc 2-epimerase domain; ep-NES = nuclear export signal; ep-AR: allosteric region; UF = unknown function; kin = ManNAc kinase domain; UF epimerase.
[5]Combined pathogenicity scores, Intronic variants with predicted splicing effects are listed as “splicing’, and without such effects as “splicing?”,
[6]Extracted from literature reference.

Amyotrophic lateral sclerosis

[0072] Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder whose clinical features are determined by the progressive and inexorable degeneration of motor neurons. The heterogeneity in disease progression suggests the presence of modifying factors, either genetic or environmental that could eventually help to discover new therapeutic strategies that are urgently needed.

[0073] Hallmarks of ALS are the sporadic nature, the great heterogeneity in age of onset, duration of disease and age of death. The average disease duration from diagnosis to death is 2-5 years, however, extremely aggressive or, on the opposite, mild variants where death follows as rapidly as 6 months or as late as 20 years after diagnosis, exist. ALS cases are grouped into two different categories, sporadic (sALS) and familial (fALS). Around 10% of ALS cases are classified as fALS with a predominantly autosomal dominant pattern of inheritance, while the remaining 90% occur sporadically. Among the fALS cases, about 20-25% are caused by mutations in the gene encoding for the superoxide dismutase 1 (SOD1) (Rosen et al. 1993). However, recessive missense mutation in the GNE gene p.(His705Arg) has been shown to be associated with ALS (Koroglu et al. Neurogenetics 18(4):237-243, 2017).

Sarcopenia and Aging

[0074] Sarcopenia is a condition marked by the wasting or loss muscle tissue and the replacement of muscle tissue with fibrosis tissue as the subject ages. The etiology of sarcopenia is complex and can be attributed to a variety of factors, including oxidative stress, inflammation, apoptosis, and mitochondrial dysregulation (Roubenoff, 2003; Fulle et al., 2004), as well as genetic factors, inadequate diet, sedentary lifestyle, and the interplay between these factors (Rolland et al., 2008; Walrand et al., 2011). Reduction in expression of GNE naturally occurs with aging, and $Gne^{+/-}$ mice are shown to have aging dependent loss of muscle and brain function, along with reduced sialic acid as they age.

[0075] Sarcopenia refers to the progressive deterioration in skeletal muscle mass, strength and physical function with advancing age. Patients suffering from sarcopenia have low muscle strength, muscle mass and/or physical function forms. Up to 10% of individuals aged 60–69 years are affected by sarcopenia, with this proportion rising considerably to 40% for adults over 80 years of age. The fundamental loss of independence and susceptibility to additional diseases caused by sarcopenia also places a significant burden on public health

systems worldwide. This burden is anticipated to grow considerably in coming decades, in line with increases in longevity and the consequent rise in the proportion of elderly. Thus, the consequences of age-related muscle deterioration will become increasingly relevant globally.

GNE Mouse Models

[0076] *Gne* is an essential gene in mice; deletion causes embryonic lethality between embryonic (E) day 8.5 and 9.5. The most celebrated model for GNE myopathy was made by Malicdan et al. (Hum. Mol.Genet. 16(22): 2669-82, 2007). This model constitutively expressed a mutant human GNE_{D207V} transgene (Tg) in a mouse $Gne^{-/-}$ background. By 30 weeks, $GNE_{D207V}{}^{Tg}Gne^{-/-}$ mice were reported to show significant lifespan reductions, reduced scores in rod climbing and constant speed treadmill walking, and modest elevation in serum CK activity and muscle production of Aβ1-42 peptide. By 42 weeks, muscles exhibited rimmed vacuoles with congophilic inclusion bodies, as well as pathology in respiratory and cardiac muscles that are not found in human GNE myopathy patients. Unfortunately, as these mice have been bred, most of these phenotypes have been lost from the line, such that we and others cannot find evidence of muscle pathology or muscle deficiencies at 64 weeks.

[0077] A second model, a knock-in of the M712T (now called M743T) Persian founder GNE mutation, showed perinatal lethality (by P3) due to kidney disease (Galeno et al., Clin. Invest. 117(6):1585-94, 2007). Again, others have found that this homozygous knock-in line can be bred to create a subpopulation of animals with no phenotype (Sela et al., Neuromuscular Med. 15(1): 180-91, 2013). Thus, the robustness of all pre-clinical data on this disease has been called into question due to the high phenotypic variability of the models used.

[0078] All of the pre-clinical data is highly complicated by the fact that all current mouse models of GNE myopathy show complicated and overly variable phenotypes. A GNE_{M743T} knock-in model showed early death due to kidney complications, which could be offset by ManNAc. Other strains of the same knock-in show no phenotype. A $GNE_{D207V}{}^{Tg}Gne^{-/-}$ mouse model showed clear disease phenotypes at one year of age in early studies, none of which can be repeated with mice that are currently alive. As Gne deficiency leads to embryonic death at E8.5 to E9.5 in the mouse, pure gene deletion mice are not useful, though foxed mice to deleted genes with more precision are being made by multiple groups, including ours.

[0079] Another mouse model is generated using Cas9-CRISPR, which will ultimately allow for the generation of a floxed allele into exon 3 of the mouse Gne gene, and

introduction of this allele is sufficient to allow for Cre-mediated deletion. As Gne is essential in mice, leading to lethality between E8.5 and E9, creation of a floxed allele to delete the gene in the adult mouse should allow for creation of a robust body-wide or muscle-specific phenotypes using Cre-mediated deletion. This, in turn, allows for more reproducible demonstrations of therapeutic efficacy.

[0080] Applicant developed inducible Gne gene deletion mice, *Gne*$^{lox/lox}$, as well as Rosa26CreER^{T2}*Gne*$^{lox/lox}$ mice, using Cas9-CRISPR methods. *Gne*$^{lox/lox}$ mice bear lox^{P} sites flanking exon 3 of both alleles of the mouse *Gne* gene. When the Cre recombinase is introduced, it can induce Gne gene deletion in affected cells by deleting gene sequences within the loxP sites of the Gne gene. Rosa26CreER^{T2}Gne$^{lox/lox}$ mice express a Cre-Estrogen Receptor (ER)T2 fusion protein in all tissues. This fusion protein is expressed in the cytoplasm of cells, where Cre is inactive. If tamoxifen is added, it can bind to the CreER^{T2} fusion protein and induce its migration into the nucleus, allowing for gene deletion. Both such models allow the induction of Gne gene deletion in adult mice, thereby bypassing embryonic deletion that occurs when Gne is deleted in embryonic stem cells.

AAV Gene Therapy

[0081] As used herein, the term "AAV" is a standard abbreviation for adeno-associated virus. Adeno-associated virus is a single-stranded DNA parvovirus that grows only in cells in which certain functions are provided by a co-infecting helper virus. There are currently thirteen serotypes of AAV that have been characterized General information and reviews of AAV can be found in, for] example, Carter, 1989, Handbook of Parvoviruses, Vol. 1, pp. 169-228, and Berns, 1990, Virology, pp. 1743-1764, Raven Press, (New York). However, it is fully expected that these same principles will be applicable to additional AAV serotypes since it is well known that the various serotypes are quite closely related, both structurally and functionally, even at the genetic level. (See, for example, Blacklowe, 1988, pp. 165-174 of Parvoviruses and Human Disease, J. R. Pattison, ed.; and Rose, Comprehensive Virology 3:1-61 (1974)). For example, all AAV serotypes apparently exhibit very similar replication properties mediated by homologous rep genes; and all bear three related capsid proteins such as those expressed in AAV2. The degree of relatedness is further suggested by heteroduplex analysis which reveals extensive cross-hybridization between serotypes along the length of the genome; and the presence of analogous self-annealing segments at the termini that correspond to "inverted terminal repeat sequences" (ITRs). The similar infectivity patterns also suggest that the replication functions in each serotype are under similar regulatory control.

[0082] An "AAV vector" as used herein refers to a vector comprising one or more polynucleotides of interest (or transgenes) that are flanked by AAV terminal repeat sequences (ITRs). Such AAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been transfected with a vector encoding and expressing rep and cap gene products.

[0083] An "AAV virion" or "AAV viral particle" or "AAV vector particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated polynucleotide AAV vector. If the particle comprises a heterologous polynucleotide (*i.e.* a polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as an "AAV vector particle" or simply an "AAV vector". Thus, production of AAV vector particle necessarily includes production of AAV vector, as such a vector is contained within an AAV vector particle.

[0084] Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including an inverted terminal repeat (ITRs). Exemplary ITR sequences may be 130 base pairs in length or 141 base pairs in length, such as the ITR sequence. There are multiple serotypes of AAV. The nucleotide sequences of the genomes of the AAV serotypes are known. For example, the nucleotide sequence of the AAV serotype 2 (AAV2) genome is presented in Srivastava *et al., J Virol,* 45: 555-564 (1983) as corrected by Ruffing *et al., J Gen Virol,* 75: 3385-3392 (1994). As other examples, the complete genome of AAV-1 is provided in GenBank Accession No. NC_002077; the complete genome of AAV-3 is provided in GenBank Accession No. NC_1829; the complete genome of AAV-4 is provided in GenBank Accession No. NC_001829; the AAV-5 genome is provided in GenBank Accession No. AF085716; the complete genome of AAV-6 is provided in GenBank Accession No. NC_00 1862; at least portions of AAV-7 and AAV-8 genomes are provided in GenBank Accession Nos. AX753246 and AX753249, respectively (see also U.S. Patent Nos. 7,282,199 and 7,790,449 relating to AAV-8); the AAV-9 genome is provided in Gao *et al., J. Virol., 78*: 6381-6388 (2004); the AAV-10 genome is provided in *Mol. Ther.,* 13(1): 67-76 (2006); and the AAV-11 genome is provided in *Virology,* 330(2): 375-383 (2004). Cloning of the AAVrh.74 serotype is described in Rodino-Klapac*., et al. Journal of translational medicine* **5**, 45 (2007). *Cis*-acting sequences directing viral DNA replication (rep), encapsidation/packaging and host cell chromosome integration are contained within the ITRs. Three AAV promoters (named p5, p19, and p40 for their relative map locations) drive the expression of the two AAV internal open reading frames encoding rep and cap genes. The two rep promoters (p5 and p19), coupled with the differential splicing of the single AAV intron (*e.g.*, at AAV2 nucleotides 2107

and 2227), result in the production of four rep proteins (rep 78, rep 68, rep 52, and rep 40) from the rep gene. Rep proteins possess multiple enzymatic properties that are ultimately responsible for replicating the viral genome. The cap gene is expressed from the p40 promoter and it encodes the three capsid proteins VP1, VP2, and VP3. Alternative splicing and non-consensus translational start sites are responsible for the production of the three related capsid proteins. A single consensus polyadenylation site is located at map position 95 of the AAV genome. The life cycle and genetics of AAV are reviewed in Muzyczka, *Current Topics in Microbiology and Immunology, 158*: 97-129 (1992).

[0085] AAV possesses unique features that make it attractive as a vector for delivering foreign DNA to cells, for example, in gene therapy. AAV infection of cells in culture is noncytopathic, and natural infection of humans and other animals is silent and asymptomatic. Moreover, AAV infects many mammalian cells allowing the possibility of targeting many different tissues *in vivo*. Moreover, AAV transduces slowly dividing and non-dividing cells, and can persist essentially for the lifetime of those cells as a transcriptionally active nuclear episome (extrachromosomal element). The AAV proviral genome is infectious as cloned DNA in plasmids which makes construction of recombinant genomes feasible. Furthermore, because the signals directing AAV replication, genome encapsidation and integration are contained within the ITRs of the AAV genome, some or all of the internal approximately 4.3 kb of the genome (encoding replication and structural capsid proteins, rep-cap) may be replaced with foreign DNA such as a gene cassette containing a promoter, a DNA of interest and a polyadenylation signal. The rep and cap proteins may be provided *in trans*. Another significant feature of AAV is that it is an extremely stable and hearty virus. It easily withstands the conditions used to inactivate adenovirus (56ºC to 65ºC for several hours), making cold preservation of AAV less critical. AAV may even be lyophilized. Finally, AAV-infected cells are not resistant to superinfection.

[0086] Recombinant AAV genomes of the disclosure comprise nucleic acid molecule of the disclosure and one or more AAV ITRs flanking a nucleic acid molecule. AAV DNA in the rAAV genomes may be from any AAV serotype for which a recombinant virus can be derived including, but not limited to, AAV serotypes (e.g., AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVRH10, AAVRH74, AAV11, AAV12, AAV13, or Anc80, AAV7m8 and their derivatives). Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692. Other types of rAAV variants, for example rAAV with capsid mutations, are also contemplated. See, for example, Marsic *et al., Molecular Therapy*, 22(11): 1900-1909 (2014). As noted in the Background section above, the nucleotide sequences of the genomes of various AAV serotypes are known in the art.

[0087] The provided recombinant AAV (*i.e.*, infectious encapsidated rAAV particles) comprise a rAAV genome. The term "rAAV genome" refers to a polynucleotide sequence that is derived from a native AAV genome that has been modified. In some embodiments, the rAAV genome has been modified to remove the native cap and rep genes. In some embodiments, the rAAV genome comprises the endogenous 5' and 3' inverted terminal repeats (ITRs). In some embodiments, the rAAV genome comprises ITRs from an AAV serotype that is different from the AAV serotype from which the AAV genome was derived. In some embodiments, the rAAV genome comprises a transgene of interest flanked on the 5' and 3' ends by inverted terminal repeat (ITR). In some embodiments, the rAAV genome comprises a "gene cassette." In exemplary embodiments, the genomes of both rAAV lack AAV rep and cap DNA, that is, there is no AAV rep or cap DNA between the ITRs of the genomes.

[0088] DNA plasmids of the disclosure comprise rAAV genomes of the disclosure. The DNA plasmids are transferred to cells permissible for infection with a helper virus of AAV (e.g., adenovirus, E1-deleted adenovirus or herpesvirus) for assembly of the rAAV genome into infectious viral particles. Techniques to produce rAAV particles, in which an AAV genome to be packaged, rep and cap genes, and helper virus functions are provided to a cell are standard in the art. Production of rAAV requires that the following components are present within a single cell (denoted herein as a packaging cell): a rAAV genome, AAV rep and cap genes separate from (*i.e.*, not in) the rAAV genome, and helper virus functions. The AAV rep and cap genes may be from any AAV serotype for which recombinant virus can be derived and may be from a different AAV serotype than the rAAV genome ITRs, including, but not limited to, AAV serotypes AAV-9, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAVrh.74, AAV-8, AAV-10, AAV-11, AAV-12 and AAV-13. Production of pseudotyped rAAV is disclosed in, for example, WO 01/83692 which is incorporated by reference herein in its entirety.

[0089] A method of generating a packaging cell is to create a cell line that stably expresses all the necessary components for AAV particle production. For example, a plasmid (or multiple plasmids) comprising a rAAV genome lacking AAV rep and cap genes, AAV rep and cap genes separate from the rAAV genome, and a selectable marker, such as a neomycin resistance gene, are integrated into the genome of a cell. AAV genomes have been introduced into bacterial plasmids by procedures such as GC tailing (Samulski *et al.*, 1982, *Proc. Natl. Acad. S6. USA*, 79:2077-2081), addition of synthetic linkers containing restriction endonuclease cleavage sites (Laughlin *et al.*, 1983, Gene, 23:65-73) or by direct, blunt-end ligation (Senapathy & Carter, 1984, J. Biol. Chem., 259:4661-4666). The

packaging cell line is then infected with a helper virus such as adenovirus. The advantages of this method are that the cells are selectable and are suitable for large-scale production of rAAV. Other examples of suitable methods employ adenovirus or baculovirus rather than plasmids to introduce rAAV genomes and/or rep and cap genes into packaging cells.

[0090] General principles of rAAV production are reviewed in, for example, Carter, 1992, Current Opinions in Biotechnology, 1533-539; and Muzyczka, 1992, Curr. Topics in Microbial. and Immunol., 158:97-129). Various approaches are described in Ratschin *et al.*, *Mol. Cell. Biol.* 4:2072 (1984); Hermonat *et al., Proc. Natl. Acad. Sci. USA*, 81:6466 (1984); Tratschin et al., *Mo1. Cell. Biol.* 5:3251 (1985); McLaughlin *et al., J. Virol.*, 62:1963 (1988); and Lebkowski *et al., Mol. Cell. Biol.,* 7:349 (1988). Samulski *et al.*, J. Virol., 63:3822-3828 (1989); U.S. Patent No. 5,173,414; WO 95/13365 and corresponding U.S. Patent No. 5,658.776 ; WO 95/13392; WO 96/17947; PCT/US98/18600; WO 97/09441 (PCT/US96/14423); WO 97/08298 (PCT/US96/13872); WO 97/21825 (PCT/US96/20777); WO 97/06243 (PCT/FR96/01064); WO 99/11764; Perrin *et al. Vaccine* 13:1244-1250 (1995); Paul *et al. Human Gene Therapy* 4:609-615 (1993); Clark *et al. Gene Therapy* 3:1124-1132 (1996); U.S. Patent. No. 5,786,211; U.S. Patent No. 5,871,982; and U.S. Patent. No. 6,258,595. The foregoing documents are hereby incorporated by reference in their entirety herein, with particular emphasis on those sections of the documents relating to rAAV production.

[0091] The disclosure thus provides packaging cells that produce infectious rAAV. In one embodiment packaging cells may be stably transformed cancer cells such as HeLa cells, 293 cells and PerC.6 cells (a cognate 293 line). In another embodiment, packaging cells are cells that are not transformed cancer cells, such as low passage 293 cells (human fetal kidney cells transformed with E1 of adenovirus), MRC-5 cells (human fetal fibroblasts), WI-38 cells (human fetal fibroblasts), Vero cells (monkey kidney cells) and FRhL-2 cells (rhesus fetal lung cells).

[0092] The rAAV may be purified by methods standard in the art such as by column chromatography or cesium chloride gradients. Methods for purifying rAAV vectors from helper virus are known in the art and include methods disclosed in, for example, Clark *et al., Hum. Gene Ther., 10*(6): 1031-1039 (1999); Schenpp and Clark, *Methods Mol. Med., 69* 427-443 (2002); U.S. Patent No. 6,566,118 and WO 98/09657.

[0093] In another embodiment, the disclosure contemplates compositions comprising rAAV of the present disclosure. Compositions of the disclosure comprise rAAV and a pharmaceutically acceptable carrier. The compositions may also comprise other ingredients

such as diluents and adjuvants. Acceptable carriers, diluents and adjuvants are nontoxic to recipients and are preferably inert at the dosages and concentrations employed and include, but are not limited to, buffers such as phosphate [e.g., phosphate-buffered saline (PBS)], citrate, or other organic acids; antioxidants such as ascorbic acid; low molecular weight polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, copolymers such as poloxamer 188, pluronics (e.g., Pluronic F68) or polyethylene glycol (PEG).

[0094] Sterile injectable solutions are prepared by incorporating rAAV in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by filter sterilization. Generally, dispersions are prepared by incorporating the sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze drying technique that yield a powder of the active ingredient plus any additional desired ingredient from the previously sterile-filtered solution thereof.

[0095] Titers and dosages of rAAV to be administered in methods of the invention will vary depending, for example, on the particular rAAV, the mode of administration, the treatment goal, the individual, the timing of administration, and the cell type(s) being targeted, and may be determined by methods standard in the art. Titers of rAAV may range from about $1x10^6$, about $1x10^7$, about $1x10^8$, about $1x10^9$, about $1x10^{10}$, about $1x10^{11}$, about $1x10^{12}$, about $1x10^{13}$ to about $2x10^{14}$ or more DNase resistant particles (DRP) per kg of body weight. Dosages may also be expressed in units of viral genomes (vg). These dosages of rAAV may range from about $1x10^9$ vg or more, about $1x10^{10}$ vg or more, about $1x10^{11}$ vg or more, about $1x10^{12}$ vg or more, about $6x10^{12}$ or more, about $1x10^{13}$ vg or more, about $1.3x10^{13}$ vg or more, about $1.4x10^{13}$ vg or more, about $2x10^{13}$ vg or more, about $3x10^{13}$ vg or more, about $6x10^{13}$ vg or more, about $1x10^{14}$ vg or more, about $3x10^{14}$ or more, about $6x10^{14}$ or more, about $1x10^{15}$ vg or more, about $3x10^{15}$ or more, about $6x10^{15}$ or more, about $1x10^{16}$ or more, about $3x10^{16}$ or more, or about $6x10^{16}$ or more. For a neonate, the dosages of rAAV may range from about $1x10^9$ vg or more, about $1x10^{10}$ vg or more, about $1x10^{11}$ vg or more, about $1x10^{12}$ vg or more, about $6x10^{12}$ or more, about $1x10^{13}$ vg or more, about 1.3

$x10^{13}$ vg or more, about $1.4x10^{13}$ vg or more, about $2x10^{13}$ vg or more, about $3x10^{13}$ vg or more, about $6x10^{13}$ vg or more, about $1x10^{14}$ vg or more, about $3x10^{14}$ or more, about $6x10^{14}$ or more, about $1x10^{15}$ vg or more, about $3x10^{15}$ or more, about $6x10^{15}$ or more, about $1x10^{16}$ or more, about $3x10^{16}$ or more, or about $6x10^{16}$ or more.

[0096] Methods of transducing a target cell with rAAV, *in vivo* or *in vitro*, are contemplated by the disclosure. The *in vivo* methods comprise the step of administering an effective dose, or effective multiple doses, of a composition comprising a rAAV of the disclosure to an animal (including a human being) in need thereof. If the dose is administered prior to development of a disorder/disease, the administration is prophylactic. If the dose is administered after the development of a disorder/disease, the administration is therapeutic. In embodiments of the disclosure, an effective dose is a dose that alleviates (eliminates or reduces) at least one symptom associated with the disorder/disease state being treated, that slows or prevents progression to a disorder/disease state, that slows or prevents progression of a disorder/disease state, that diminishes the extent of disease, that results in remission (partial or total) of disease, and/or that prolongs survival. Example of a disease contemplated for prevention or treatment with methods of the disclosure is limb girdle muscular dystrophy, e.g. LGMD2I, or congenital muscular dystrophy 1C.

[0097] The term “transduction” is used to refer to the administration/delivery of the coding region of the transgenes, e.g. FKRP gene and FST gene, to a recipient cell either *in vivo* or *in vitro*, via a replication-deficient rAAV of the disclosure resulting in expression of FKRP protein and FST protein in the recipient cell.

[0098] Transduction of cells with rAAV of the disclosure results in sustained expression of the proteins encoded by the first and second transgenes. The present disclosure thus provides methods of administering/delivering rAAV to an animal, preferably a human being. These methods include transducing tissues (including, but not limited to, tissues such as muscle, organs such as liver and brain, and glands such as salivary glands) with one or more rAAV of the present disclosure. Transduction may be carried out with gene cassettes comprising tissue specific control elements. For example, one embodiment of the disclosure provides methods of transducing muscle cells and muscle tissues directed by muscle specific promoter elements, including, but not limited to, those derived from the actin and myosin gene families, such as from the myoD gene family (See Weintraub *et al., Science, 251*: 761-766 (1991)), the myocyte-specific enhancer binding factor MEF-2 (Cserjesi and Olson, *Mol Cell Biol* 11: 4854-4862 (1991)), control elements derived from the human skeletal actin gene (Muscat *et al., Mol Cell Biol*, *7*: 4089-4099 (1987)), the cardiac actin

gene, muscle creatine kinase sequence elements (See Johnson *et al., Mol Cell Biol, 9*:3393-3399 (1989)) and the murine creatine kinase enhancer (MCK) element, MHCK7, control elements derived from the skeletal fast-twitch troponin C gene, the slow-twitch cardiac troponin C gene and the slow-twitch troponin I gene: hypoxia-inducible nuclear factors (Semenza *et al., Proc Natl Acad Sci USA, 88*: 5680-5684 (1991)), steroid-inducible elements and promoters including the glucocorticoid response element (GRE) (See Mader and White, *Proc. Natl. Acad. Sci. USA* 90: 5603-5607 (1993)), and other control elements.

[0099] Muscle tissue is an attractive target for *in vivo* DNA delivery, because it is not a vital organ and is easy to access. By "muscle cell" or "muscle tissue" is meant a cell or group of cells derived from muscle of any kind (for example, skeletal muscle and smooth muscle, *e.g.* from the digestive tract, urinary bladder, blood vessels or cardiac tissue). Such muscle cells may be differentiated or undifferentiated, such as myoblasts, myocytes, myotubes, cardiomyocytes and cardiomyoblasts.

[00100] Combination therapies are also contemplated by the disclosure. Combination as used herein includes both simultaneous treatment and sequential treatments. Combinations of methods of the disclosure with standard medical treatments are specifically contemplated, as are combinations with novel therapies. In some embodiments, the combination therapy comprises administering an immunosuppressing agent in combination with the gene therapy disclosed herein.

[00101] Administration of an effective dose of the compositions may be by routes standard in the art including, but not limited to, intramuscular, parenteral, intravenous, oral, buccal, nasal, pulmonary, intracranial, intraosseous, intraocular, rectal, or vaginal. Route(s) of administration and serotype(s) of AAV components of the rAAV (in particular, the AAV ITRs and capsid protein) of the disclosure may be chosen and/or matched by those skilled in the art taking into account the disease state being treated and the target cells/tissue(s) that are to express the protein encoded by the first and/or second transgene. .

[00102] The disclosure provides for local administration and systemic administration of an effective dose of rAAV and compositions of the disclosure. For example, systemic administration is administration into the circulatory system so that the entire body is affected. Systemic administration includes enteral administration such as absorption through the gastrointestinal tract and parenteral administration through injection, infusion or implantation.

Immunosuppressing Agents

[00103] The immunosuppressing agent may be administered before or after the onset of an immune response to the rAAV in the subject after administration of the gene therapy. In addition, the immunosuppressing agent may be administered simultaneously with the gene therapy or the protein replacement therapy. The immune response in a subject includes an adverse immune response or an inflammatory response following or caused by the administration of rAAV to the subject. The immune response may be the production of antibodies in the subject in response to the administered rAAV.

[00104] Exemplary immunosuppressing agents include glucocorticosteroids, janus kinase inhibitors, calcineurin inhibitors, mTOR inhibitors, cyctostatic agents such as purine analogs, methotrexate and cyclophosphamide, inosine monophosphate dehydrogenase (IMDH) inhibitors, biologics such as monoclonal antibodies or fusion proteins.

[00105] The immunosuppressing agent may be an anti-inflammatory steroid, which is a steroid that decreases inflammation and suppresses or modulates the immune system of the subject. Exemplary anti-inflammatory steroid are glucocorticoids such as prednisolone, betamethasone, dexamethasone, hydrocortisone, methylprednisolone, deflazacort, budesonide or prednisone.

[00106] Janus kinase inhibitors are inhibitors of the JAK/STAT signaling pathway by targeting one or more of the Janus kinase family of enzymes. Exemplary janus kinase inhibitors include tofacitinib, baricitinib, upadacitinib, peficitinib, and oclacitinib.

[00107] Calcineurin inhibitors bind to cyclophilin and inhibits the activity of calcineurin Exemplary calcineurine inhibitors includes cyclosporine, tacrolimus and picecrolimus.

[00108] mTOR inhibitors reduce or inhibit the serine/threonine-specific protein kinase mTOR. Exemplary mTOR inhibitors include sirolimus, everolimus, and temsirolimus.

[00109] The immunosuppressing agents include immune suppressing macrolides. The term “immune suppressing macrolides” refer to macrolide agents that suppresses or modulates the immune system of the subject. A macrolide is a class of agents that comprise a large macrocyclic lactone ring to which one or more deoxy sugars, such as cladinose or desoamine, are attached. The lactone rings are usually 14-, 15-, or 16-membered. Macrolides belong to the polyketide class of agents and may be natural products. Examples of immunosuppressing macrolides include tacrolimus, pimecrolimus, and sirolimus.

[00110] Purine analogs block nucleotide synthesis and include IMDH inhibitors. Exemplary purine analogs include azathioprine, mycophenolate and lefunomide.

[00111] Exemplary immunosuppressing biologics include abatacept, adalimumab, anakinra, certolizumab, etanercept, golimumab, infliximab, ixekizumab, natalizumab, rituximab, secukinumab, tocilizumab, ustekinenumab, vedolizumab, basiliximab, belatacep, and daclizumab.

[00112] In particular, the immunosuppressing agent is an anti-CD20 antibody. The term anti-CD20 specific antibody refers to an antibody that specifically binds to or inhibits or reduces the expression or activity of CD20. Exemplary anti-CD20 antibodies include rituximab, ocrelizumab or ofatumumab.

[00113] Additional examples of immuosuppressing antibodies include anti-CD25 antibodies (or anti-IL2 antibodies or anti-TAC antibodies) such as basiliximab and daclizumab, and anti-CD3 antibodies such as muromonab-CD3, otelixizumab, teplizumab and visilizumab, anti-CD52 antibodies such as alemtuzumab.

[00114] The following EXAMPLES are provided by way of illustration and not limitation. Described numerical ranges are inclusive of each integer value within each range and inclusive of the lowest and highest stated integer.

Examples

Example 1

Construction of Dual Gene rAAV Vector

[00115] The plasmid referred to as pAAV.CBh.GNE.spA.miniCMV.FST344.spA is set out as SEQ ID NO: 1, and the plasmid pAAV.CMV.GNE.spA.miniCMV.FST344.spA is set out as SEQ ID NO: 2. These plasmids contain an expression cassette flanked by AAV2 inverted terminal repeat sequences (ITR), these expression cassettes may also comprise a first transcriptional control sequence, operably linked to the transgene encoding the GNE protein and a second transcriptional control sequence, operably linked a transgene encoding the muscle-building gene FST344.

[00116] The dual gene expression cassette had a Kanamycin resistance gene, and an optimized Kozak sequence, which allows for more accurate and robust protein translation. rAAV vectors were produced by a modified cross-packaging approach whereby the AAV type 2 vector genome can be packaged into multiple AAV capsid serotypes [Rabinowitz *et al., J Virol.* 76 (2):791-801 (2002)]. Production was accomplished using a standard three plasmid DNA/CaPO4 precipitation method using HEK293 cells. HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin. The production plasmids were: (i) plasmids encoding the therapeutic proteins,

(ii) rep2-capX modified AAV helper plasmids encoding cap serotype AAVrh74 isolate, and (iii) an adenovirus type 5 helper plasmid (pAdhelper) expressing adenovirus E2A, E4 ORF6, and VA I/II RNA genes. A quantitative PCR-based titration method was used to determine an encapsidated vector genome (vg) titer utilizing a Prism 7500 Taqman detector system (PE Applied Biosystems). [Clark *et al., Hum Gene Ther.* 10 (6): 1031-1039 (1999)]. A final titer (vg ml^{-1}) was determined by quantitative reverse transcriptase PCR using the specific primers and probes utilizing a Prism 7500 Real-time detector system (PE Applied Biosystems, Grand Island, NY, USA). Aliquoted viruses were kept at –80 °C until production.

[00117] All plasmids used to make AAV genomes to be packaged also contain a Kanamycin resistance gene (KanR) outside of the ITR sequences used for packaging of the genome. This allows for the DNA encoding the AAV genome to be transformed into bacteria to produce large amounts of DNA in the presence of Kanamycin, which will kill all non-transformed bacteria. KanR is not packaged into the AAV capsid in the AAV genome used to treat patients, but its presence allows for DNA production in bacteria.

[00118] A description of pAAV.CBh.GNE.spA.miniCMV.FST344.spA plasmid (SEQ ID NO: 1) is provided in Table 2 below.

Element	Nucleotides in SEQ ID NO: 1	Direction
KanR	83-899	◄
Ori	1021-1609	►
5' AAV ITR	1847-2013	
CBh	2060-2871	►
CMV enhancer	2060-2363	►
Chicken beta-actin promoter	2365-2642	►
Hybrid Intron	2643-2871	►
Kozak	2888-2901	►
GNE ORF	2897-5065	►
polyA	5085-5133	►
miniCMV	5145-5371	►
Kozak	5397-5409	►
FST344	5406-6437	►
Signal Peptide	5406-5492	►
Mature FST315	5493-6437	►

Mature FST317	5493-6356	►
Forward/Reverse…5212	5975-5994	►◄
polyA	6455-6503	►
3' AAV ITR	6519-6685	
LacZ alpha	6688-6756	►
F1 ori	7074-7380	►

[00119] A description of pAAV.CMV.GNE.spA.miniCMV.FST344.spA plasmid (SEQ ID NO: 2) is provided in Table 3 below.

Element	Nucleotides in SEQ ID NO: 1	Direction
KanR	83-899	◄
Ori	1021-1609	
5' AAV ITR	1847-2013	
CMV enhancer/promoter	2061-2587	►
SV40 enhancer with intron	2657-2852	
Kozak	2862-2875	
GNE ORF	2872-5040	►
polyA	5059-5108	
miniCMV	5120-5346	
Kozak	5372-5384	
Signal Peptide of FST	5381-5467	
FST344	5381-6412	►
Mature FST317	5468-6331	
Mature FST315	5467-6412	
polyA	6430-6478	
3' AAV ITR	6494-6660	
LacZ alpha	6662-6731	►
F1 ori	7049-7355	

Example 2

[00120] Gne haploinsufficiency (*Gne*$^{+/-}$) in mice also is associated with age-dependent losses in ambulation and neurodegeneration. Thus, the disclosed rAAV are contemplated for use in treating sarcopenia, the normal age-dependent loss of muscle mass and strength,

as well as age-dependent neurodegeneration. Elderly humans lose 20-25% of their muscle mass as they age. This dramatically increases the risk of falls and bone fractures as well as impairment of ambulation as humans live past the age of 80. The gene therapy disclosed herein can be used to offset and rebuild that lost muscle strength, while at the same time preventing neuronal cell death, which can contribute to cognitive loss (e.g. memory loss). As such, the disclosed gene therapies can also be used as an anti-aging therapy in humans without a genetic disease.

[00121] The following rAAV were injected at a dose of $1x10^{11}$vg into the tibialis anterior muscle and at a dose $5x10^{11}$vg into the gastrocnemius muscle in wild type (C57Bl/6J) mice: rAAVrh74.CMV.GNE.miniCMV.FST, rAAVrh74.Cbh.GNE.miniCMV.FST344, rAAVrh74.CMV.GNE.FL-IRES(full length internal ribosomal entry site from FGF1A), FST, rAAVrh74.CMV.GNE and rAAVrh74.Cbh.GNE. Phosphate buffered saline (PBS) was injected as a control.

[00122] Mice of 2 months of age were injected bilaterally into the tibialis anterior (TA) and the gastrocnemius (GS). At 4 months of age, 2 months after treatment, mice were euthanized and muscle dissected from tendon to tendon, weighed, and snap frozen for molecular and histological studies. Muscle weights for mice injected with rAAV.CMV.GNE.FL-IRES.FST showed no growth over this short interval relative to control mice injected with PBS. The CMV or Cbh promoters were used to express the GNE gene alone (rAAV.CMV.GNE or rAAV.Cbh.GNE), and the same two promoters were used in duel gene constructs (either rAAV.CMV.GNE.pA.mCMV.FST or rAAV.Cbh.GNE.pA.mCMV.FST) to express both GNE and FST. As shown in figure 3, each of the dual gene vectors allowed for a doubling of muscle size of both the tibialis anterior muscle and the gastrocnemius muscle compared to the PBS control, single gene constructs, or the IRES construct. Thus, dual gene constructs using two promoters to drive two different mRNAs were superior to use of IRES with a single promoter.

[00123] Muscles were injected with $1x10^{11}$vg AAV in the TA or $5x10^{11}$vg in the GS. The effect of both human FST gene expression and human GNE expression, comparing the single gene and dual gene vectors was also assessed (see Fig. 4). Wild type (C57Bl/6J) mice were injected at 2 months of age, with analysis at 4 months of age, 2 months after injection. RNA levels were measured by qRT-PCR, using an 18S rRNA internal control, and fold change in gene expression, relative to endogenous wild type mouse gene expression was calculated. This number was then divided by the vector genomes per nucleus of DNA

measured as the result of the IM injection to report out the RNA/DNA ratio (or fold increase in gene expression over endogenous levels relative to copies of DNA present).

[00124] FST and GNE gene expression levels for the "IRES" construct (rAAV.CMV.GNE.FL-IRES.FST) were lower in the TA and GS than for either dual gene construct (with the exception of GNE in the gastrocnemius). Injection of rAAV.CMV.GNE (denoted as "CMV") or rAAV.Cbh.GNE (denotes as "Cbh") showed a 5-10-fold increase in *GNE* gene expression relative to endogenous mouse *Gne* levels, with Cbh being slightly stronger than CMV. Injection of either dual gene vector (also known as a bicistronic vector) rAAV.CMV.GNE.pA.mCMV.FST (denoted as "Bi CMV") or the dual gene vector (also knowns as a bicistronic vector) rAAV.Cbh.GNE.pA.mCMV.FST (denoted as "Bi Cbh") showed a dramatic elevation in FST gene expression in the TA and GS relative to the IRES alone. Bi CMV and Bi Cbh also showed an increase in GNE gene expression in the TA, but did not do so in the GS. Thus, the two promoter versions of the dual gene vectors not only built more muscle mass (Fig. 3) but amplified transgene expression (Fig. 4) relative to the IRES vector or either single gene vector.

[00125] This study demonstrated that the use of a dual gene therapy vector using two different promoters to control gene expression, such as rAAV.Cbh.GNE.pA.mCMV.FST or rAAV.CMV.GNE.pA.mCMV.FST, are preferable to other single gene vectors or bicistronic vectors comprising an IRES for the treatment of GNE myopathy, GNE-dependent ALS, sarcopenia, and aging.

Example 3

Dual gene AAV GNE/FST Gene Therapy in *Gne*-deficient Mice

[00126] Further studies were carried out in which AAV vectors were injected intramuscularly into aged Gne deletion mice ($Gne^{lox/lox}$ mice described above). On day 1, AAV.CMV.Cre (referred to as "CRE") was injected with or without rAAV.CBh.GNE (referred to as "Cre+GNE"), rAAV.CMV.FST (referred to as "Cre+FST") or dual gene rAAV.CBh.GNE.pA.mCMV.FST (referred to as "Cre+GNE/FST"). At 6 weeks (day 56), rAAV.CMV.Cre was injected again, with analysis of muscles occurring 4 weeks after the second injection (at 10 weeks, or day 70). $Gne^{lox/lox}$ mice were injected with phosphor-buffered saline (PBS) as a control that yields no gene deletion.

[00127] Figure 5 provides Hematoxylin and Eosin staining of myofibers sections obtained from the Gne-deficient treated mice. Cre-induced *Gne* gene deletion caused muscle damage, as evidenced by the loss of muscle tissue with mononuclear cell infiltrates (indicative of inflammation), necrotic myofibers (indicative of muscle cell death), split

myofibers (indicative of impaired muscle regeneration), and centrally located myofiber nuclei (indicative of a cycle of myofiber degeneration and regeneration). GNE alone stopped all muscle damage caused by Cre, but did not significantly increase average myofiber size. FST did not stop muscle damage, but modestly increased myofibers size. Dual gene GNE/FST stopped all muscle damage and dramatically increased myofiber size, indicated coordinated correction of *Gne* gene deficiency (gene replacement) and induction of muscle growth.

Example 4

Dual GNE/FST Gene Therapy prevents Cre-Induced Muscle Damage in Dose-Dependent Manner

[00128] Gene therapy was carried out on adult RosaCreER^{T2}*Gne*$^{lox/lox}$ mice. CreER^{T2} mice. Because CreER^{T2} requires tamoxifen to induce gene deletion, the RosaCreER^{T2} background did not cause any *Gne* gene deletion of its own (not shown). In this case, no tamoxifen has been added, so this line is technically no different than *Gne*$^{lox/lox}$ mice, with the exception that platelet levels are reduced somewhat due to the presence of the Rosa26 locus (not shown).

[00129] On day 1, adult RosaCreER^{T2}*Gne*$^{lox/lox}$ mice were injected intravenously (IV) with PBS, 1x10^{13} vg/kg, 5x10^{13} vg/kg, or 2x10^{14} vg/kg the dual gene therapy vector rAAV.CBh.GNE.spA.mCMV.FST (referred to as “GNE/FST”). This vector allows for expression of human GNE, which can provide gene replacement for the deleted mouse Gne gene, and human follistatin (FST), a muscle building agent. On day 7, AAV.CMV.Cre was injected intramuscularly into the tibialis anterior muscle to induce *Gne* gene deletion, which ultimately causes muscle damage unless gene replacement is provided. Muscles were injected again intramuscularly with rAAV.CMV.Cre at 6 weeks (56 days), with analysis occurring 4 weeks later (at 10 weeks, or day 70). Hematoxylin and Eosin staining of myofibers sections obtained from the treated mice is shown in Figure 6.

[00130] As shown in Figure 6, injection of Cre alone, which induces *Gne* gene deletion, resulted in muscle damage, as evidence by the presence of necrotic myofibers (indicative of muscle cell death), mononuclear infiltrates (indicative of inflammation), split myofibers (indicative of failed muscle regeneration), and myofibers with central nuclei (indicative of a cycle of myofiber degeneration and regeneration). Injection of PBS caused none of these findings and caused no *Gne* gene deletion. IV injection of dual gene AAV therapy (Cre+GNE/FST), which provides gene replacement with the human GNE gene and also expression of human follistatin (FST), a muscle building agent, prevented muscle damage at

all three doses used, with evidence of muscle growth beginning in a few myofibers at $1x10^{13}$ vg/kg and robust muscle cell growth being evident at $5x10^{13}$ vg/kg and $2x10^{14}$ vg/kg.

[00131] Figures 7 and 8 provide quantification of the data shown in the H&E stains in Figure 6. As shown in Figure 7, muscle mass, as a percentage of body weight, was significantly increased at the high dose of dual gene therapy. As shown in Figure 8, average myofiber diameter (Mini-Feret) diameter, as a percentage was significantly increased by the two higher doses of dual gene therapy.

Sequences

SEQ ID NO: 1 pAAVCbh.GNE.spA.miniCMV.FST344.spA

GTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAG
AGTTTTCGCCCCGAAGAACGAATTCTTAGAAAAACTCATCGAGCATCAAATGAAACTGC
AATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAG
GAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGAT
TCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATC
AAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCA
TTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCAT
CAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCT
GTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGC
GCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTC
CCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGA
TGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACA
TCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCC
ATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACC
CATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTAGAGCAAGACGTTTCCCGT
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CATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAA
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CACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCT
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ACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCG
CAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCC
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CGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCG
AGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTTGTAGTTAATGATT
AACCCGCCATGCTACTTATCTACGTAGCCATGCTCTAGAGTTTAAACAAGCCTAGAGTT
TAAACAAGCTTCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGA

CCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTT
TCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAA
GTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCT
GGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTA
TTAGTCATCGCTATTACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTCCCCAT
CTCCCCCCCCTCCCCACCCCCAATTTTGTATTTATTTATTTTTTAATTATTTTGTGCAGCG
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GGGCGGGGCGGGGCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCGC
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GCGCGCGGCGGGCGGGAGTCGCTGCGACGCTGCCTTCGCCCCGTGCCCCGCTCCGC
CGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCACAGGTGAG
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TCACTTTTTTTCAGGGTACCCGGCCGGCTAGCCGCCACCATGGAGAAGAATGGAAATA
ACCGAAAGCTGCGGGTTTGTGTTGCTACTTGTAACCGTGCAGATTATTCTAAACTTGCC
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RTLVLFPNIDAGSKEMVRVMRKKGIEHHPNFRAVKHVPFDQFIQLVAHAGCMIGNSSCGVR
EVGAFGTPVINLGTRQIGRETGENVLHVRDADTQDKILQALHLQFGKQYPCSKIYGDGNAV
PRILKFLKSIDLQEPLQKKFCFPPVKENISQDIDHILETLSALAVDLGGTNLRVAIVSMKGEIVK
KYTQFNPKTYEERINLILQMCVEAAAEAVKLNCRILGVGISTGGRVNPREGIVLHSTKLIQEW
NSVDLRTPLSDTLHLPVWVDNDGNCAALAERKFGQGKGLENFVTLITGTGIGGGIIHQHELI
HGSSFCAAELGHLVVSLDGPDCSCGSHGCIEAYASGMALQREAKKLHDEDLLLVEGMSVP
KDEAVGALHLIQAAKLGNAKAQSILRTAGTALGLGVVNILHTMNPSLVILSGVLASHYIHIVKD
VIRQQALSSVQDVDVVVSDLVDPALLGAASMVLDYTTRRIY

SEQ ID NO:5: FST344, Signal peptide – bold sequence (nt. 1-87)
ATGGTCCGCGCGAGGCACCAGCCGGGTGGGCTTTGCCTCCTGCTGCTGCTCTGCCAG
TTCATGGAGGACCGCAGTGCCCAGGCTGGGAACTGCTGGCTCCGTCAAGCGAAGAACGGC
CGCTGCCAGGTCCTGTACAAGACCGAACTGAGCAAGGAGGAGTGCTGCAGCACCGGCCGG
CTGAGCACCTCGTGGACCGAGGAGGACGTGAATGACAACACACTCTTCAAGTGGATGATTTT
CAACGGGGGCGCCCCCAACTGCATCCCCTGTAAAGAAACGTGTGAGAACGTGGACTGTGGA
CCTGGGAAAAAATGCCGAATGAACAAGAAGAACAAACCCCGCTGCGTCTGCGCCCCGGATT
GTTCCAACATCACCTGGAAGGGTCCAGTCTGCGGGCTGGATGGGAAAACCTACCGCAATGA
ATGTGCACTCCTAAAGGCAAGATGTAAAGAGCAGCCAGAACTGGAAGTCCAGTACCAAGGC
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GACCAATAATGCCTACTGTGTGACCTGTAATCGGATTTGCCCAGAGCCTGCTTCCTCTGAGC
AATATCTCTGTGGGAATGATGGAGTCACCTACTCCAGTGCCTGCCACCTGAGAAAGGCTACC
TGCCTGCTGGGCAGATCTATTGGATTAGCCTATGAGGGAAAGTGTATCAAAGCAAAGTCCTG
TGAAGATATCCAGTGCACTGGTGGGAAAAAATGTTTATGGGATTTCAAGGTTGGGAGAGGCC
GGTGTTCCCTCTGTGATGAGCTGTGCCCTGACAGTAAGTCGGATGAGCCTGTCTGTGCCAG
TGACAATGCCACTTATGCCAGCGAGTGTGCCATGAAGGAAGCTGCCTGCTCCTCAGGTGTG
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SEQ ID NO: 6; FST344 protein
1 mvrarhqpgg lcllllllcq fmedrsaqag ncwlrqakng rcqvlyktel skeeccstgr
61 lstswteedv ndntlfkwmi fnggapncip cketcenvdc gpgkkcrmnk knkprcvcap
121 dcsnitwkgp vcgldgktyr necallkarc keqpelevqy qgrckktcrd vfcpgsstcv
181 vdqtnnaycv tcnricpepa sseqylcgnd gvtyssachl rkatcllgrs iglayegkci
241 kakscediqc tggkkclwdf kvgrgrcslc delcpdsksd epvcasdnat yasecamkea
301 acssgvllev khsgscnsis edteeeeede dqdysfpiss ilew

SEQ ID NO: 7: Mature FST315,
GGGAACTGCTGGCTCCGTCAAGCGAAGAACGGCCGCTGCCAGGTCCTGTACAAGACCGAA
CTGAGCAAGGAGGAGTGCTGCAGCACCGGCCGGCTGAGCACCTCGTGGACCGAGGAGGA
CGTGAATGACAACACACTCTTCAAGTGGATGATTTTCAACGGGGGCGCCCCCAACTGCATCC
CCTGTAAAGAAACGTGTGAGAACGTGGACTGTGGACCTGGGAAAAAATGCCGAATGAACAA
GAAGAACAAACCCCGCTGCGTCTGCGCCCCGGATTGTTCCAACATCACCTGGAAGGGTCCA
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GTAATCGGATTTGCCCAGAGCCTGCTTCCTCTGAGCAATATCTCTGTGGGAATGATGGAGTC
ACCTACTCCAGTGCCTGCCACCTGAGAAAGGCTACCTGCCTGCTGGGCAGATCTATTGGATT
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CCTGACAGTAAGTCGGATGAGCCTGTCTGTGCCAGTGACAATGCCACTTATGCCAGCGAGT
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TTGCAACTCCATTTCGGAAGACACCGAGGAAGAGGAGGAAGATGAAGACCAGGACTACAGC
TTTCCTATATCTTCTATTCTAGAGTGG

SEQ ID NO: 8: Mature FST317,
GGGAACTGCTGGCTCCGTCAAGCGAAGAACGGCCGCTGCCAGGTCCTGTACAAGACCGAA
CTGAGCAAGGAGGAGTGCTGCAGCACCGGCCGGCTGAGCACCTCGTGGACCGAGGAGGA
CGTGAATGACAACACACTCTTCAAGTGGATGATTTTCAACGGGGGCGCCCCCAACTGCATCC
CCTGTAAAGAAACGTGTGAGAACGTGGACTGTGGACCTGGGAAAAAATGCCGAATGAACAA
GAAGAACAAACCCCGCTGCGTCTGCGCCCCGGATTGTTCCAACATCACCTGGAAGGGTCCA
GTCTGCGGGCTGGATGGGAAAACCTACCGCAATGAATGTGCACTCCTAAAGGCAAGATGTA
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TTTCTGTCCAGGCAGCTCCACATGTGTGGTGGACCAGACCAATAATGCCTACTGTGTGACCT
GTAATCGGATTTGCCCAGAGCCTGCTTCCTCTGAGCAATATCTCTGTGGGAATGATGGAGTC
ACCTACTCCAGTGCCTGCCACCTGAGAAAGGCTACCTGCCTGCTGGGCAGATCTATTGGATT
AGCCTATGAGGGAAAGTGTATCAAAGCAAAGTCCTGTGAAGATATCCAGTGCACTGGTGGGA
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CCTGACAGTAAGTCGGATGAGCCTGTCTGTGCCAGTGACAATGCCACTTATGCCAGCGAGT
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TTGCAAC

SEQ ID NO:9: CBh,
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTG
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GCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCT
TATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAGG
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TTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGGGGCGCGCGC
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CAGCCAATCAGAGCGGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCGGCGGC
GGCCCTATAAAAAGCGAAGCGCGCGGCGGGCGGGAGTCGCTGCGACGCTGCCTTCGCCC
CGTGCCCCGCTCCGCCGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACT
CCCACAGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCTGAGCAAGAG
GTAAGGGTTTAAGGGATGGTTGGTTGGTGGGGTATTAATGTTTAATTACCTGGAGCACCTGC
CTGAAATCACTTTTTTTCAG

SEQ ID NO: 10: CMV Enhancer,
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTG
ACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGG
GTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTAC
GCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCT
TATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATG

SEQ ID NO: 11: CMV Promoter
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCA
TTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACG
TCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATA
TGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGC
CCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCG
CTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGAC
TCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCA
AAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCG
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SEQ ID NO: 12: Chicken beta-actin promoter
TCGAGGTGAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCAATT
TTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGGGGGGGGGGGCG
CGCGCCAGGCGGGGCGGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGGAGAGGTGC
GGCGGCAGCCAATCAGAGCGGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGGCGGCGGCG
GCGGCGGCCCTATAAAAAGCGAAGCGCGCGGCGGGCG

SEQ ID NO: 13: miniCMV
CGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGGACTCAC
GGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAA

CGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTG
TACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCG

SEQ ID NO:14: Hybrid Intron

GGAGTCGCTGCGACGCTGCCTTCGCCCCGTGCCCCGCTCCGCCGCCGCCTCGCGCCGCC
CGCCCCGGCTCTGACTGACCGCGTTACTCCCACAGGTGAGCGGGCGGGACGGCCCTTCTC
CTCCGGGCTGTAATTAGCTGAGCAAGAGGTAAGGGTTTAAGGGATGGTTGGTTGGTGGGGT
ATTAATGTTTAATTACCTGGAGCACCTGCCTGAAATCACTTTTTTTCAG

SEQ ID NO: 15: SV40 enhancer with intron
TCTAGAGGATCCGGTACTCGAGGAACTGAAAAACCAGAAAGTTAACTGGTAAGTTTAGT
CTTTTTGTCTTTTATTTCAGGTCCCGGATCCGGTGGTGGTGCAAATCAAAGAACTGCTC
CTCAGTGGATGTTGCCTTTACTTCTAGGCCTGTACGGAAGTGTTACTTCTGCTCTAAAA
GCTGCGGAATTGTACCCG

SEQ ID NO:16: polyA,
AATAAAAGATCTTTATTTTCATTAGATCTGTGTGTTGGTTTTTTGTGTG

SEQ ID NO:17: 5' AAV ITR
TGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTT
GGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACT
AGGGGTTCCTTGTAGTTAATGATTAACCCGCCATGCTAcTTATCTACG

SEQ ID NO:18: AAV ITR
CGTAGATAAGTAGCATGGCGGGTTAATCATTAACTACAAGGAACCCCTAGTGATGGAGTTGG
CCACTCCCTCTCTGCGCGCTCgCTCGCTCACtGAGGCCGGGCGACCAAAGGTCGCCCGACG
CCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCA

SEQ ID NO: 19: KanR, reverse
CTTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCA
ATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGT
TCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAAT
ACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGT
GACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAA
CAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATT
CGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAA
CAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACC
TGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGTG
AGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAAT
TCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCA
TGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGA
TTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAA
TCGCGGCCTAGAGCAAGACGTTTCCCGTTGAATATGGCTCAT

SEQ ID NO: 20 Ori
TTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGC
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GAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAAC
TCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGG
CGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGG
TCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAA
CTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCG

GACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGG
GGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGAT
TTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAA

SEQ ID NO: 21: Kozak

gccgccaccatgg

SEQ ID NO: 22: LacZ alpha
TGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAAT
GGCGAATGG

SEQ ID NO: 23: F1 ori, (7074-7380), forward
GCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCG
CCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTC
CCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGcTTTACGGCACC
TCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAG
ACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAA
CTGGAACAA

SEQ ID NO:24: Forward/Reverse…5212, (5975-5994), forward/reverse
GACCTGTAATCGGATTTGCC

Claims

1. A polynucleotide sequence comprising in 5' to 3' order: an AAV ITR, a first transcriptional control sequence operably linked to a first transgene sequence encoding GNE protein having the amino acid sequence of SEQ ID NO:4, a polyadenylation signal sequence, a second transcriptional control sequence operably linked to a second transgene sequence encoding muscle building protein, a polyadenylation signal sequence and an AAV ITR.

2. The polynucleotide sequence of claim 1 wherein the muscle building protein is follstatin 344, follistatin 317, follistatin 315, insulin growth factor 1 (IGF1), SMAD7 or HB-IGF.

3. The polynucleotide sequcene of claim 1 or 2 wherein the muscle building protein is encoded by the nucleotide sequence of SEQ ID NO: 5, SEQ ID NO: 7 or SEQ ID NO: 8.

4. The polynucleotide sequence of claim 1 wherien the second transgene comprises the nucleotide sequece of SEQ ID NO: 5.

5. The polynucleotide sequence of any one of claim 1-4, wherein the first or second transcriptional control sequence comprises one or more of the nucleotide sequence of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13.

6. The polynucleotide sequence of any one of claims 1-4, wherein the first transcriptional control sequence comprises the one or more of the nucleotide sequences of SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11 and SEQ ID NO: 12.

7. The polynucleotide sequence of any one of claims 1-6, wherein the second transcriptional control sequence comprises the nucleotide sequence of SEQ ID NO: 13.

8. The polynucleotide sequence of any one of claim 1-4, wherein the first or second transcriptional control sequence comprises a muscle-specific promoter.

9. The polynucleotide sequence of claim 7, wherein the muscle-specific promoter comprises one or more of a human skeletal actin gene element, a cardiac actin gene element, a desmin promoter, a skeletal alpha-actin (ASKA) promoter, a troponin I (TNNI2) promoter, a myocyte-specific enhancer binding factor MEF binding element, a muscle creatine kinase (MCK) promoter, a truncated MCK (tMCK) promoter, a myosin heavy

chain (MHC) promoter, a hybrid a-myosin heavy chain enhancer-/MHC enhancer-promoter (MHCK7) promoter, a CK8 promoter, a CK8e promoter, a SPc5-12 promoter, a SP-301 promoter, a C5-12 promoter, a murine creatine kinase enhancer element, a skeletal fast-twitch troponin C gene element, a slow-twitch cardiac troponin c gene element, a slow-twitch troponin I gene element, hypoxia- inducible nuclear factor (HIF)-response element (HRE), a steroid-inducible element, and a glucocorticoid response element (GRE).

10. The polynucleotide sequence of any one of claims 1-4, wherein the first transcriptional control element sequence comprises an enhancer comprising the nucleotide sequence of SEQ ID NO: 10 and a promoter sequence comprising the nucleotide sequence of SEQ ID NO: 11.

11. The polynucleotide sequence of claim any one of claims 1-10, wherein the polynucleotide comprises an intron between the first transcriptional promoter sequence and the first transgene sequence or comprises an intron between the second transcriptional control sequence and the second transgene sequence.

12. The polynucleotide sequence of claim 11, wherein the intron comprises the nucleotide sequence of SEQ ID NO: 14 or SEQ ID NO: 15.

13. A polynucleotide sequence comprising a nucleotide sequence that is at least 95% identical to nucleotides 1847 to 6685 of SEQ ID NO: 1.

14. The polynucleotide sequence of claim 13 wherein the nucleotide sequence comprises nucleotides 1847 to 6685 of SEQ ID NO: 1.

15. A polynucleotide sequence comprising a nucleotide sequence that is at least 95% identical to nucleotides 1847 to 6660 of SEQ ID NO: 2.

16. The polynucleotide sequence of claim 16 wherein the nucleotide sequence comprises nucleotides 1847 to 6660 of SEQ ID NO: 2.

17. A recombinant adeno-associated virus (rAAV) comprising the polynucleotide sequence of any one of claims 1-16.

18. The rAAV of any one of claims 17, wherein the rAAV comprises AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13, AAV rh.74, AAV rh.10, AAVMyo3a, MYOAAV capsid protein, or a variant thereof.

19. A recombinant AAV particle comprising the polynucleotide sequence of any one of claims 1-16 or the rAAV of claim 17 or 18.

20. A composition comprising the rAAV of claim 17 or 18 or the rAAV particle of claim 19.

21. A method of treating a GNE-dependent disorder comprising administering a rAAV of claim 17 or 18, the rAAV particle of claim 19 or the composition of claim 20 to a subject in need thereof.

22. The method of claim 21, wherein the GNE-dependent disorder is GNE-myopathy, GNE-dependent ALS, sarcopenia or aging.

23. The method of claim 21 or 22, wherein the rAAV, rAAV particle or composition is administered using systemic administration, intramuscular injection or intravenous injection.

24. A composition for treating GNE-dependent disorder in a subject in need thereof, wherein the composition comprises rAAV of claim 17 or 18, the rAAV particle of claim 19 or the composition of claim 20.

25. The composition of claim 24, wherein the wherein the GNE-dependent disorder is GNE-myopathy, GNE-dependent ALS, thrombocytopenia, sarcopenia or aging.

26. The composition of claim 24 or 25, wherein the composition is formulated for systemic administration, intramuscular injection or intravenous injection.

27. Use of a rAAV of claim 17 or 18, the rAAV particle of claim 19 or the composition of claim 20 for the preparation of a medicament for treating a GNE-dependent disorder.

28. The use of claim 27, wherein the wherein the GNE-dependent disorder is GNE-myopathy, GNE-dependent ALS, thrombocytopenia, sarcopenia or aging.

29. The use of claim 27 or 28, wherein the medicament is formulated for systemic administration, intramuscular injection or intravenous injection.

30. A method of preventing or repairing muscle damage in a subject in need comprising administering a rAAV of claim 17 or 18, the rAAV particle of claim 19 or the composition of claim 20 to a subject in need thereof.

31. The method of claim 30, wherein the subject is suffering from a GNE-dependent disorder is GNE-myopathy, GNE-dependent ALS, sarcopenia or aging.

32. The method of claim 30 or 31, wherein the rAAV, rAAV particle or composition is administered using systemic administration, intramuscular injection or intravenous injection.

33. A composition for preventing or repairing muscle damage in a subject in need thereof, wherein the composition comprises rAAV of claim 17 or 18, the rAAV particle of claim 19 or the composition of claim 20.

34. The composition of claim 33, wherein the wherein the subject is suffering from a GNE-dependent disorder is GNE-myopathy, GNE-dependent ALS, thrombocytopenia, sarcopenia or aging.

35. The composition of claim 33 or 34, wherein the composition is formulated for systemic administration, intramuscular injection or intravenous injection.

36. Use of a rAAV of claim 17 or 18, the rAAV particle of claim 19 or the composition of claim 20 for the preparation of a medicament for preventing or repairing muscle damage in a subject in need thereof.

37. The use of claim 36, wherein the wherein the subject is suffering from GNE-dependent disorder is GNE-myopathy, GNE-dependent ALS, thrombocytopenia, sarcopenia or aging.

38. The use of claim 36 or 37, wherein the medicament is formulated for systemic administration, intramuscular injection or intravenous injection.