[0011] Functional improvement in patients suffering from DMD and other muscular dystrophies requires gene restoration at an early stage of disease. There is a need for treatments that increase muscle strength and protect against muscle injury in patients suffering from DMD.

SUMMARY OF INVENTION

[0012] The present invention is directed to gene therapy vectors, e.g. AAV, expressing the micro-dystrophin gene to skeletal muscles including diaphragm and cardiac muscle to protect muscle fibers from injury, increase muscle strength and reduce and/or prevent fibrosis

[0013] The disclosure provides for therapies and approaches for increasing muscular force and/or increasing muscle mass using gene therapy vectors to deliver micro-dystrophin to address the gene defect observed in DMD. As shown in Example 2, treatment with micro-dystrophin gene therapy resulted in a greater muscle force *in vivo*. Furthermore, delivery of micro-dystrophin gene therapy intramuscularly and systemically showed delivery of dystrophin to the muscles in mice models *in vivo*.

[0013a] In one aspect, the present disclosure refers to a recombinant AAVrh74 vector comprising in the 5' to 3´ direction an inverted terminal repeat (ITR), an MHCK7 muscle-specific control element, a chimeric intron sequence, the nucleotide sequence of SEQ ID NO: 1, a poly A tail, and an ITR. In another aspect, the present disclosure refers to a composition comprising the recombinant AAVrh74 vector as disclosed herein, and a pharmaceutically acceptable carrier. In another aspect, there is provided the use of a recombinant AAVrh74 vector as disclosed herein or a composition disclosed herein for the preparation of a medicament for treatment of muscular dystrophy in a subject in need thereof. In yet another aspect, there is provided the use of a recombinant AAVrh74 vector as disclosed herein or a composition as disclosed herein for the preparation of a medicament for reducing or preventing fibrosis in a subject suffering from muscular dystrophy.

[0014] In one example, the disclosure provides for a rAAV vector comprising a muscle specific control element nucleotide sequence, and a nucleotide sequence encoding the micro-dystrophin protein. For example, the nucleotide sequence encodes a functional micro-dystrophin protein, wherein the nucleotide is, e.g., at least 65%, at least 70%, at least 75%, at least 80%, 81%, 82%,

83%, 84%, 85%, 86%, 87%, 88%, or 89%, more typically at least 90%, 91%, 92%, 93%, or 94% and even more typically at least 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 1, wherein the protein retains micro-dystrophin activity. The micro-dystrophin protein provides stability to the muscle membrane during muscle contraction, e.g. micro-dystrophin acts as a shock absorber during muscle contraction.

[0015] The disclosure also provides for rAAV vectors wherein the nucleotide sequence encodes a functional micro-dystrophin protein comprising a nucleotide sequence that hybridizes under stringent conditions to the nucleic acid sequence of SEQ ID NO: 1, or compliments thereof, and encodes a functional micro-dystrophin protein.

[0016] In one example, the rAAV vector is a non-replicating, recombinant adeno-associated virus (AAV) termed rAAVrh74.MHCK7.micro-dystrophin. This

vector genome contains minimal elements required for gene expression, including AAV2 inverted terminal repeats (ITR), the micro-dystrophin, SV40 intron (SD/SA), and synthetic polyadenylation (Poly A) signal, all under the control of the MHCK7 promoter/enhancer. The schematic of the vector genome and expression cassette is shown in Figure 1. The AAVrh74 serotype can be employed to achieve efficient gene transfer in skeletal and cardiac muscle following IV administration.

[0017] The term "stringent" is used to refer to conditions that are commonly understood in the art as stringent. Hybridization stringency is principally determined by temperature, ionic strength, and the concentration of denaturing agents such as formamide. Examples of stringent conditions for hybridization and washing are 0.015 M sodium chloride, 0.0015 M sodium citrate at 65-68^{o}C or 0.015 M sodium chloride, 0.0015M sodium citrate, and 50% formamide at 42^{o}C. See Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, (Cold Spring Harbor, N.Y. 1989). More stringent conditions (such as higher temperature, lower ionic strength, higher formamide, or other denaturing agent) may also be used, however, the rate of hybridization will be affected. In instances wherein hybridization of deoxyoligonucleotides is concerned, additional exemplary stringent hybridization conditions include washing in 6x SSC 0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos).

[0018] Other agents may be included in the hybridization and washing buffers for the purpose of reducing non-specific and/or background hybridization. Examples are 0.1% bovine serum albumin, 0.1% polyvinyl-pyrrolidone, 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate, NaDodSO4, (SDS), ficoll, Denhardt's solution, sonicated salmon sperm DNA (or other non-complementary DNA), and dextran sulfate, although other suitable agents can also be used. The concentration and types of these additives can be changed without substantially affecting the stringency of the hybridization conditions. Hybridization experiments are usually carried out at pH 6.8-7.4, however, at typical ionic strength conditions, the rate of hybridization is nearly independent of pH. See Anderson *et al.*, Nucleic Acid Hybridisation: A Practical Approach, Ch. 4, IRL Press Limited (Oxford, England). Hybridization conditions can be adjusted by one skilled in the art in order to

accommodate these variables and allow DNAs of different sequence relatedness to form hybrids.

[0019] The term "muscle specific control element" refers to a nucleotide sequence that regulates expression of a coding sequence that is specific for expression in muscle tissue. These control elements include enhancers and promoters. The disclosure provides for constructs comprising the muscle specific control elements MCKH7 promoter, the MCK promoter and the MCK enhancer.

[0020] The term "operably linked" refers to the positioning of the regulatory element nucleotide sequence, e.g. promoter nucleotide sequence, to confer expression of said nucleotide sequence by said regulatory element.

[0021] In one example, the disclosure provides for a rAAV vector wherein the muscle specific control element is a human skeletal actin gene element, cardiac actin gene element, myocyte-specific enhancer binding factor (MEF), muscle creatine kinase (MCK), truncated MCK (tMCK), myosin heavy chain (MHC), hybrid α-myosin heavy chain enhancer-/MCK enhancer-promoter (MHCK7), C5-12, murine creatine kinase enhancer element, skeletal fast-twitch troponin c gene element, slow-twitch cardiac troponin c gene element, the slow-twitch troponin i gene element, hypoxia-inducible nuclear factors, steroid-inducible element or glucocorticoid response element (GRE).

[0022] For example, the muscle specific control element is the MHCK7 promoter nucleotide sequence SEQ ID NO: 2 or the muscle specific control element is MCK nucleotide sequence SEQ ID NO: 4. In addition, in any of the rAAV vectors of the disclosure, the muscle specific control element nucleotide sequence, e.g. MHCK7 or MCK nucleotide sequence, is operably linked to the nucleotide sequence encoding the micro-dystrophin protein. For example, the MHCK7 promoter nucleotide sequence (SEQ ID NO: 2) is operably linked to the human micro-dystrophin coding sequence (SEQ ID NO: 1) as set out in the construct provided in Figure 1 or Figure 10 (SEQ ID NO: 3). In another example, the MCK promoter (SEQ ID NO: 4) is operably linked to the human micro-dystrophin coding sequence (SEQ ID NO: 1) as set out in the construct provided in Figure 7 or Figure 11 (SEQ ID NO: 5). In another example, the disclosure provides for a rAAV vector comprising the nucleotide sequence of SEQ ID

NO: 1 and SEQ ID NO: 2. The disclosure also provides for a rAAV vector comprising the nucleotide sequence of SEQ ID NO: 1 and SEQ ID NO: 4.

[0023] In a further example, the disclosure provides for a rAAV vector comprising the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 5. For example, the rAAVrh74.MHCK7.microdystrophin vector comprises the nucleotide sequence of SEQ ID NO: 3 and shown in Figure 10. This rAAV vector comprises the MHCK7 promoter, a chimeric intron sequence, the coding sequence for the human micro-dystrophin gene, polyA, ampicillin resistance and the pGEX plasmid backbone with pBR322 origin or replication.

[0024] The disclosure provides for a recombinant AAV vector comprising the human micro-dystrophin nucleotide sequence of SEQ ID NO: 1 and the MHCK7 promoter nucleotide sequence of SEQ ID NO: 3. This rAAV vector is the AAV serotype AAVrh.74.

[0025] The disclosure also provides for a recombinant AAV vector comprising the pAAV.MHCK7.micro-dystrophin construct nucleotide sequence of SEQ ID NO: 3. This rAAV vector is the AAV serotype AAVrh.74.

[0026] The rAAV vectors of the disclosure may be any AAV serotype, such as the serotype AAVrh.74, AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 or AAV13.

[0027] The disclosure also provides for pharmaceutical compositions (or sometimes referred to herein as simply “compositions”) comprising any of the rAAV vectors of the disclosure.

[0028] In another example, 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.

[0029] The disclosure provides for methods of treating muscular dystrophy comprising administering a therapeutically effective amount of any of the recombinant AAV vectors of the disclosure expressing human micro-dystrophin.

[0030] The disclosure provides for methods of treating muscular dystrophy comprising administering a therapeutically effective amount of a recombinant AAV vector comprising the human micro-dystrophin nucleotide sequence of SEQ ID NO: 1 and the MHCK7 promoter nucleotide sequence of SEQ ID NO: 2.

[0031] The disclosure also provides for methods of treating muscular dystrophy comprising administering a therapeutically effective amount of a recombinant AAV vector comprising the pAAV.MHCK7.micro-dystrophin construct nucleotide sequence of SEQ ID NO: 3.

[0032] "Fibrosis" refers to the excessive or unregulated deposition of extracellular matrix (ECM) components and abnormal repair processes in tissues upon injury, including skeletal muscle, cardiac muscle, liver, lung, kidney, and pancreas. The ECM components that are deposited include fibronectin and collagen, e.g. collagen 1, collagen 2 or collagen 3.

[0033] The disclosure also provides for methods of reducing or preventing fibrosis in a subject suffering from muscular dystrophy comprising administering a therapeutically effective amount of any recombinant AAV vector of the disclosure.

[0034] In another example, the disclosure provides for methods of preventing fibrosis in a subject in need thereof, comprising administering a therapeutically effective amount of a recombinant AAV vector of the disclosure. For example, any of the rAAV of the disclosure can be administered to subjects suffering from muscular dystrophy to prevent fibrosis, e.g. the rAAV of the disclosure expressing a human micro-dystrophin protein administered before fibrosis is observed in the subject. In addition, the rAAV of the disclosure expressing a human micro-dystrophin gene can be administered to a subject at risk of developing fibrosis, such as those suffering or diagnosed with muscular dystrophy, e.g. DMD. The rAAV of the disclosure can be administered to the subject suffering from muscular dystrophy in order to prevent new fibrosis in these subjects.

[0035] The disclosure contemplates administering any of the AAV vectors of the disclosure before fibrosis is observed in the subject. In addition, the rAAV of the disclosure can be administered to a subject at risk of developing fibrosis, such as those suffering or diagnosed with muscular dystrophy, e.g. DMD. The rAAV of the

disclosure can be administered to the subject suffering from muscular dystrophy who already has developed fibrosis in order to prevent new fibrosis in these subjects.

[0036] The disclosure also provides for methods of increasing muscular force and/or muscle mass in a subject suffering from muscular dystrophy comprising administering a therapeutically effective amount of a rAAV vector of the disclosure expressing a human micro-dystrophin gene. These methods can further comprise the step of administering a rAAV expressing micro-dystrophin.

[0037] The disclosure contemplates administering any of the AAV vectors of the disclosure to patients diagnosed with DMD before fibrosis is observed in the subject or before the muscle force has been reduced or before the muscle mass has been reduced.

[0038] The disclosure also contemplates administering a AAV of the disclosure to a subject suffering from muscular dystrophy who already has developed fibrosis, in order to prevent new fibrosis in these subjects or to reduce fibrosis in these patients. The disclosure also provides for administering any of the rAAV of the disclosure to the patient suffering from muscular dystrophy who already has reduced muscle force or has reduced muscle mass in order to protect the muscle from further injury.

[0039] In any of the methods of the disclosure, the subject may be suffering from muscular dystrophy such as DMD or any other dystrophin-associated muscular dystrophy.

[0040] In another example, a rAAV vector expressing the micro-dystrophin protein comprises the coding sequence of the micro-dystrophin gene operably linked to a muscle-specific control element other than MHCK7 or MCK. For example, whrein the muscle-specific control element is human skeletal actin gene element, cardiac actin gene element, myocyte-specific enhancer binding factor MEF, tMCK (truncated MCK), myosin heavy chain (MHC), C5-12 (synthetic promoter), murine creatine kinase enhancer element, skeletal fast-twitch troponin C gene element, slow-twitch cardiac troponin C gene element, the slow-twitch troponin I gene element, hypozia-inducible nuclear factors, steroid-inducible element, or glucocorticoid response element (GRE).

[0041] In any of the methods of the disclosure, the rAAV vector or composition can be administered by intramuscular injection or intravenous injection.

[0042] In addition, in any of the methods of the disclosure, the rAAV vector or composition can be administered systemically. For example, the rAAV vector or composition can be parenterally administration by injection, infusion, or implantation.

[0043] In another example, the disclosure provides a composition comprising any of the rAAV vectors of the disclosure for reducing fibrosis in a subject in need thereof.

[0044] In addition, the disclosure provides a composition comprising any of the recombinant AAV vectors of the disclosure for preventing fibrosis in a patient suffering from muscular dystrophy.

[0045] The disclosure provides for compositions comprising any of the recombinant AAV vectors of the disclosure for treating muscular dystrophy.

[0046] The disclosure provides for compositions comprising a recombinant AAV vector comprising the human micro-dystrophin nucleotide sequence of SEQ ID NO: 1 and the MHCK7 promoter sequence of SEQ ID NO: 2 for treatment of muscular dystrophy.

[0047] The disclosure provides for a composition comprising a recombinant AAV vector comprising the pAAV.MHCK7.micro-dystrophin construct comprising the nucleotide sequence of SEQ ID NO: 3 for treatment of muscular dystrophy.

[0048] The disclosure also provides for compositions comprising any of the rAAV vectors of the disclosure for increasing muscular force and/or muscle mass in a subject suffering from muscular dystrophy. In a further example, the disclosure provides for compositions comprising any of the rAAV vectors of the disclosure for treatment of muscular dystrophy.

[0049] The compositions of the disclosure can be formulated for intramuscular injection or intravenous injection. The composition of the disclosure is also formulated for systemic administration, such as parenterally administration by injection, infusion or implantation.

[0050] In addition, any of the compositions can be formulated for administration to a subject suffering from muscular dystrophy such as DMD or any other dystrophin associated muscular dystrophy.

[0051] In a further example, the disclosure provides for use of any of the rAAV vectors of the disclosure for preparation of a medicament for reducing fibrosis in a subject in need thereof. For example, the subject in need can be suffering from muscular dystrophy, such as DMD or any other dystrophin associated muscular dystrophy.

[0052] In another example, the disclosure provides for use of a rAAV vector of the disclosure for the preparation of a medicament to prevent fibrosis in a subject suffering from muscular dystrophy.

[0053] In addition, the disclosure provides for use of a recombinant AAV vector of the disclosure to preparation of a medicament to increase muscular strength and/or muscle mass in a subject suffering from muscular dystrophy.

[0054] The disclosure also provides for use of the rAAV vectors of the disclosure for the preparation of a medicament for treatment of muscular dystrophy.

[0055] The disclosure provides for use of a recombinant AAV vector comprising the human micro-dystrophin nucleotide sequence of SEQ ID NO: 1 and the MHCK7 promoter nucleotide sequence of SEQ ID NO: 2 for preparation of a medicament for the treatment of muscular dystrophy.

[0056] The disclosure provides for use of a recombinant AAV vector comprising the pAAV.MHCK7.micro-dystrophin construct nucleotide sequence of SEQ ID NO: 3 for treatment of muscular dystrophy.

[0057] In any of the uses of the invention, the medicament can be formulated for intramuscular injection or intravenous injection. In addition, in any of the uses of the invention, the medicament can be formulated for systemic administration such as parenteral administration by injection, infusion, or implantation.

CLAIMS

What is claimed:

1. A recombinant AAVrh74 vector comprising in the 5' to 3´ direction an inverted terminal repeat (ITR), an MHCK7 muscle-specific control element, a chimeric intron sequence, the nucleotide sequence of SEQ ID NO: 1, a poly A tail, and an ITR.

2. The recombinant AAVrh74 vector of claim 1 further comprising a chimeric intron sequence between said nucleotide sequence and said MHCK7 muscle-specific control element, wherein the chimeric intron sequence is set forth as nucleotides 1046-1195 of SEQ ID NO: 3.

3. The recombinant AAVrh74 vector of claim 1 further comprising a poly A tail 3´ of said nucleotide sequence, wherein the sequence of said poly A tail is set forth as nucleotides 4787 to 4842 of SEQ ID NO: 3.

4. A composition comprising the recombinant AAVrh74 vector of any one of claims 1-3 and a pharmaceutically acceptable carrier.

5. Use of the recombinant AAVrh74 vector of any one of claims 1-3, or the composition of claim 4, for the preparation of a medicament for the treatment of muscular dystrophy in a subject in need thereof.

6. Use of the recombinant AAVrh74 vector of any one of claims 1-3 or the composition of claim 4, for the preparation of a medicament for reducing or preventing fibrosis in a subject suffering from muscular dystrophy.

7. The use of claim 5 or 6 wherein the subject is suffering from Duchenne muscular dystrophy.

8. The use of any one of claims 5-7 wherein the medicament is formulated for intramuscular injection or intravenous injection.

9. The use of any one of claims 5-7 wherein the medicament is formulated for systemic administration.

10. The use of claim 9, wherein the medicament is formulated for parenteral administration by injection, infusion, or implantation.