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(54) **ADENO-ASSOCIATED VIRUS VECTOR DELIVERY OF MUSCLE SPECIFIC MICRO-DYSTROPHIN TO TREAT MUSCULAR DYSTROPHY**

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(21) Appl. No.: **18/146,840**

(22) Filed: **Dec. 27, 2022**

Related U.S. Application Data

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A61P 21/00	(2006.01)
C12N 15/86	(2006.01)

(52) **U.S. Cl.**

CPC ***A61K 48/0058*** (2013.01); ***A61P 21/00*** (2018.01); ***C12N 15/86*** (2013.01)

(57) **ABSTRACT**

The invention provides gene therapy vectors, such as adeno-associated virus (AAV) vectors, expressing a miniaturized human micro-dystrophin gene and method of using these vectors to express micro-dystrophin in skeletal muscle including diaphragm and cardiac muscle and to protect muscle fibers from injury, increase muscle strength and reduce and/or prevent fibrosis in subjects suffering from muscular dystrophy.

Specification includes a Sequence Listing.

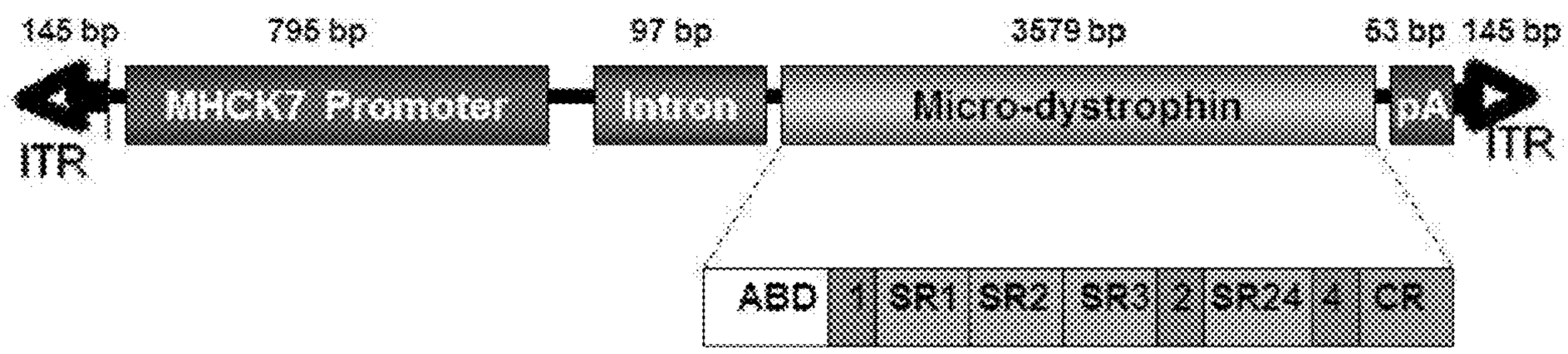
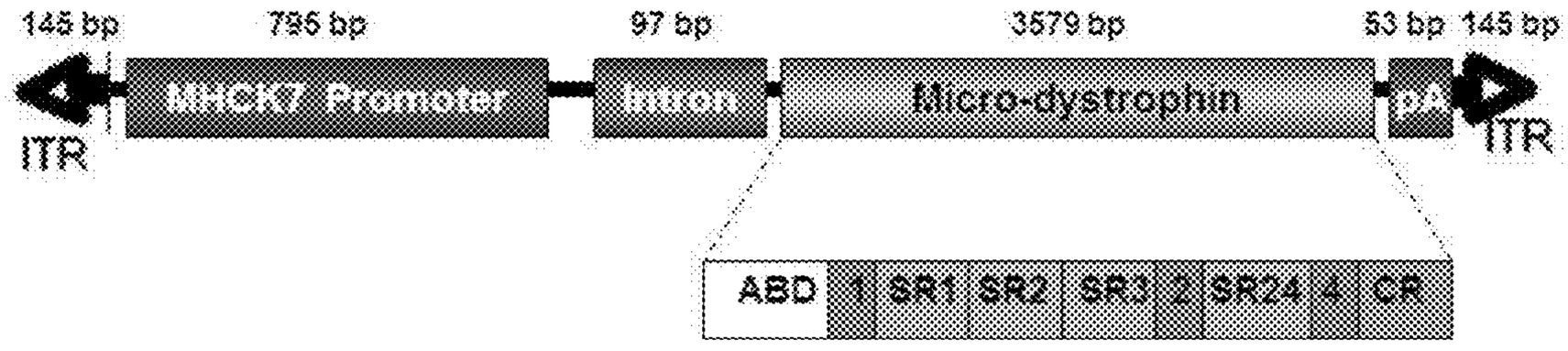


Fig. 1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795 bp
97 bp
3579 bp
53 bp
145 bp
ITR
MHCK7 Promoter
Intron
Micro-dystrophin
pA
ITR
ABD
1
SR1
SR2
SR3
2
SR24
4
CR

Fig. 2

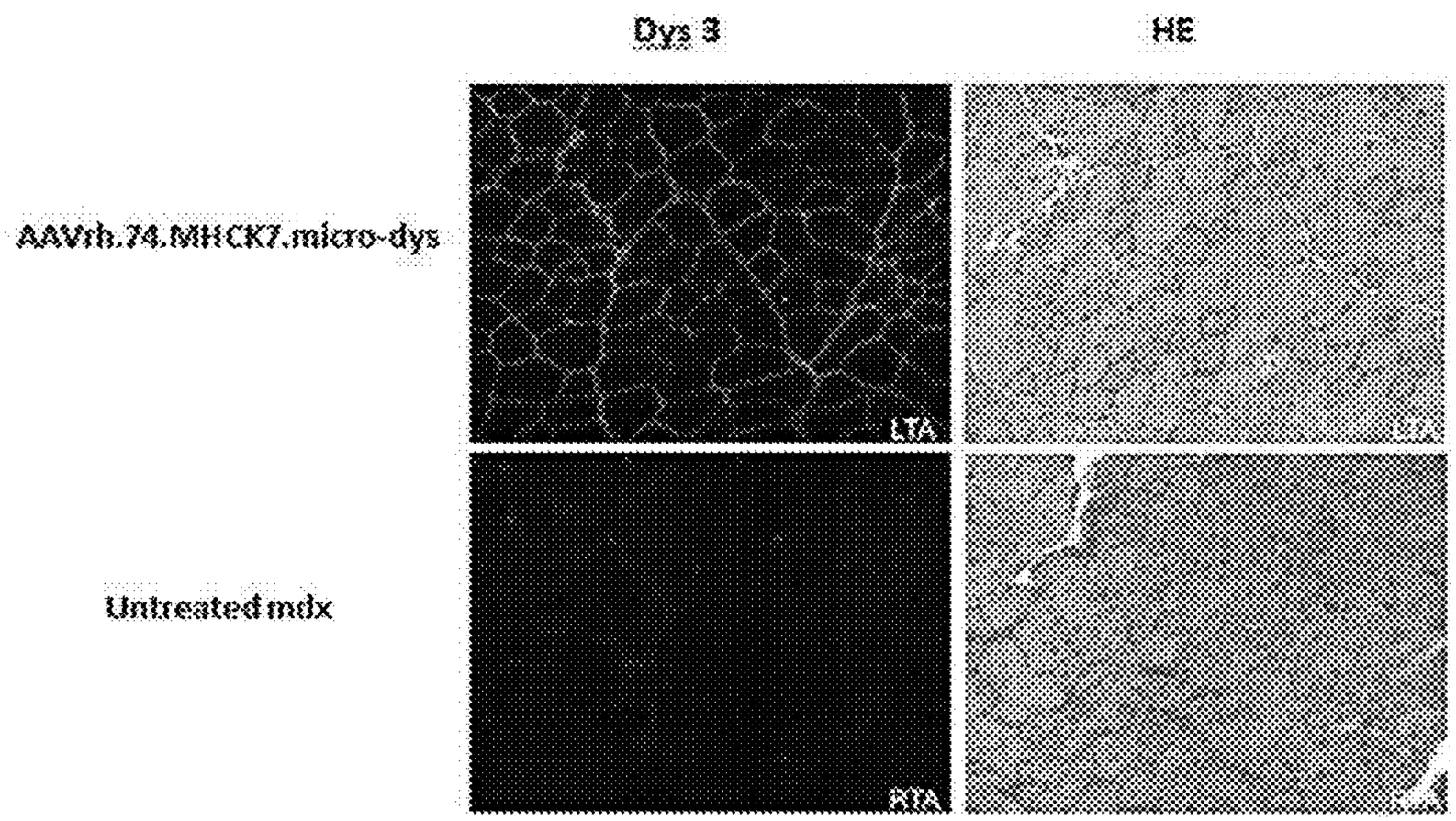

Fig. 3

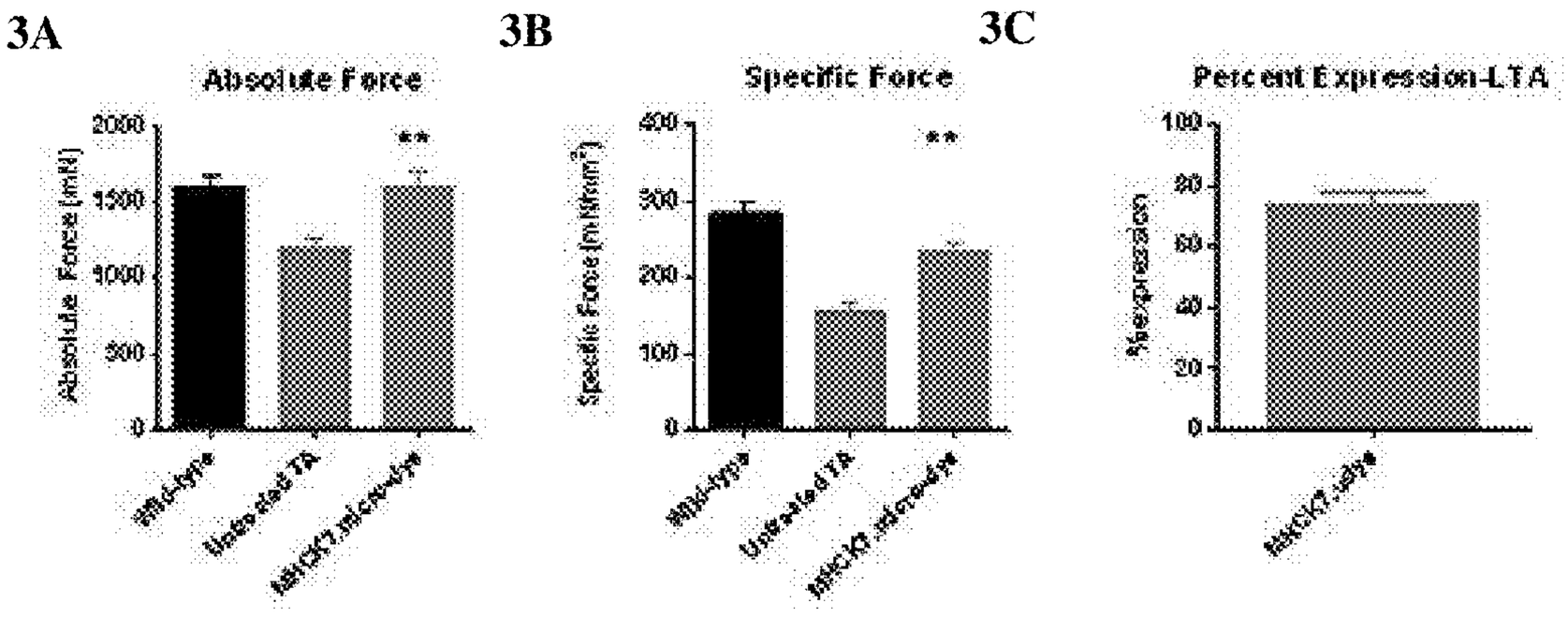
3A
Absolute Force
Absolute Force (mN)
2000
1500
1000
500
0
**
Wild-type
Untreated TA
MSTN-/- micro-dys
3B
Specific Force
Specific Force (mN/mm²)
400
300
200
100
0
**
Wild-type
Untreated TA
MSTN-/- micro-dys
3C
Percent Expression-LTA
%expression
100
80
60
40
20
0
MSTN-/- dys

Fig. 4

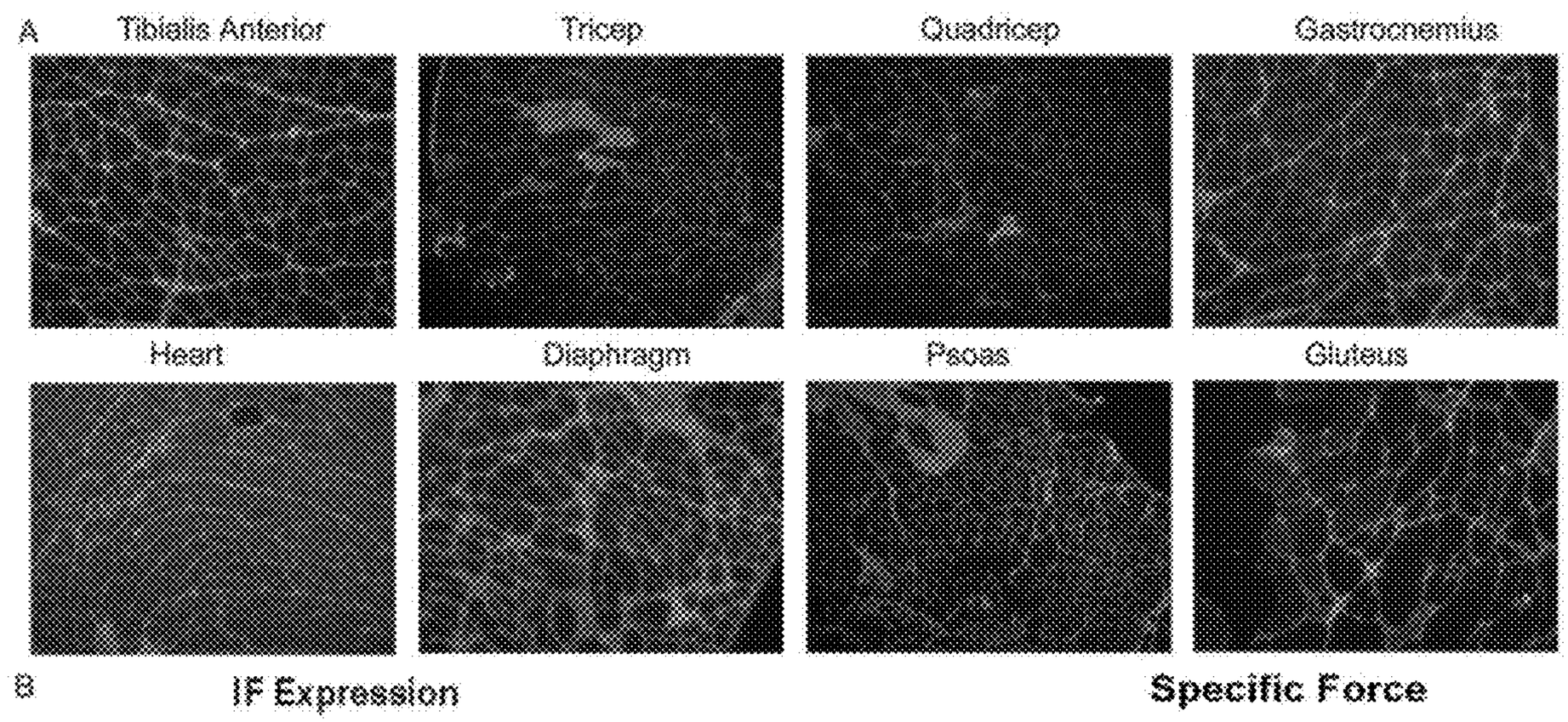
A
Tibialis Anterior
Tricep
Quadricep
Gastrocnemius
Heart
Diaphragm
Psoas
Gluteus
B
IF Expression
Specific Force
% Expression
150
100
50
0
Clinical Dose
BL6 - WT Control
TA
GAS
QD
PSO
TRI
DIA
GLUT
Specific Force (mN/mm²)
300
200
100
0
**
wt
Untreated mdx
Clinical Dose
Low Dose

Fig. 5

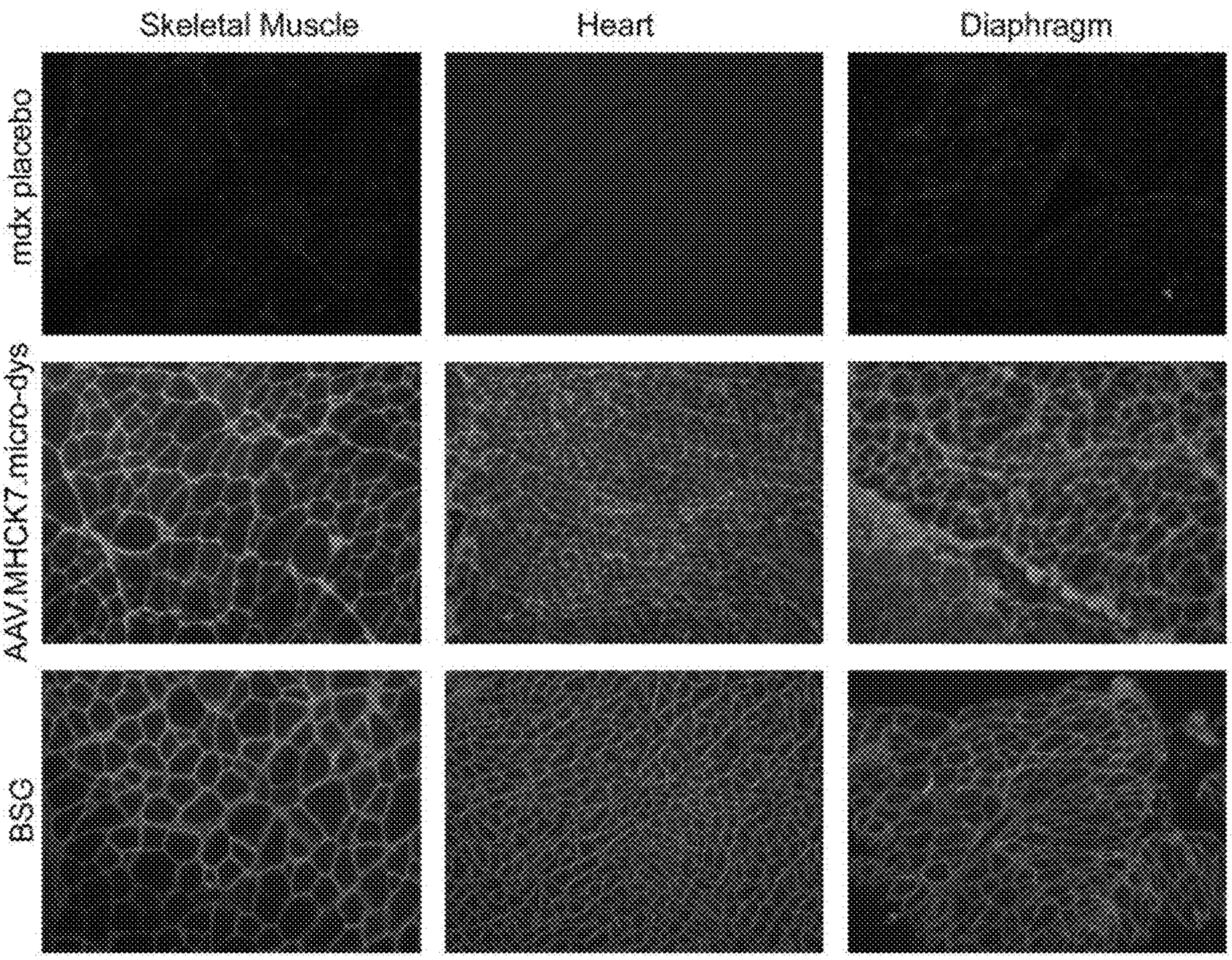
Skeletal Muscle
Heart
Diaphragm
mdx placebo
AAV.MHCK7.micro-dys
BSG

Fig. 6

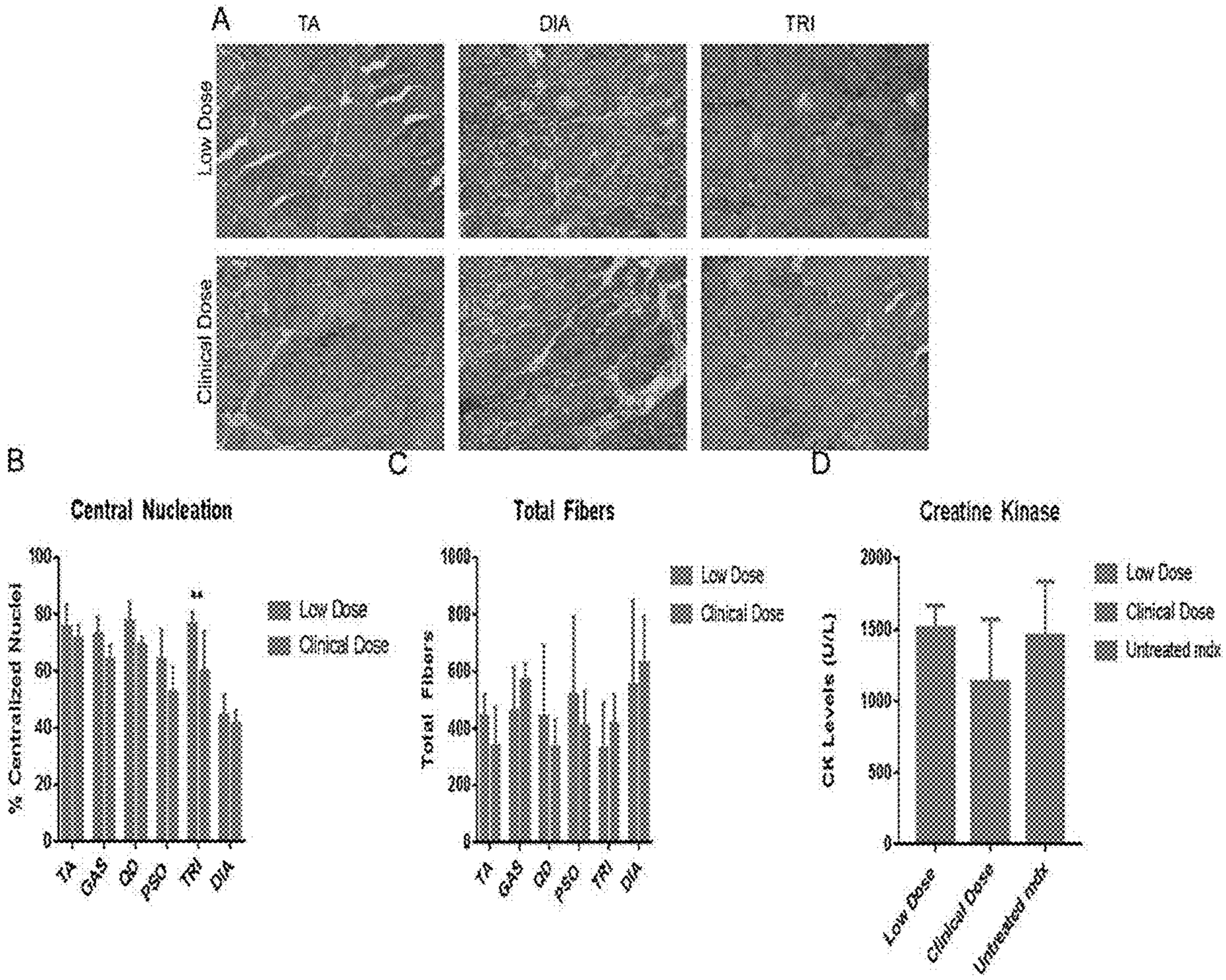
A
TA
DIA
TRI
Low Dose
Clinical Dose
B
C
D
Central Nucleation
% Centralized Nuclei
Low Dose
Clinical Dose
TA
GAS
GD
PSO
TRI
DIA
Total Fibers
Total Fibers
Low Dose
Clinical Dose
Creatine Kinase
CK Levels (U/L)
Low Dose
Clinical Dose
Untreated mdx

Fig. 7

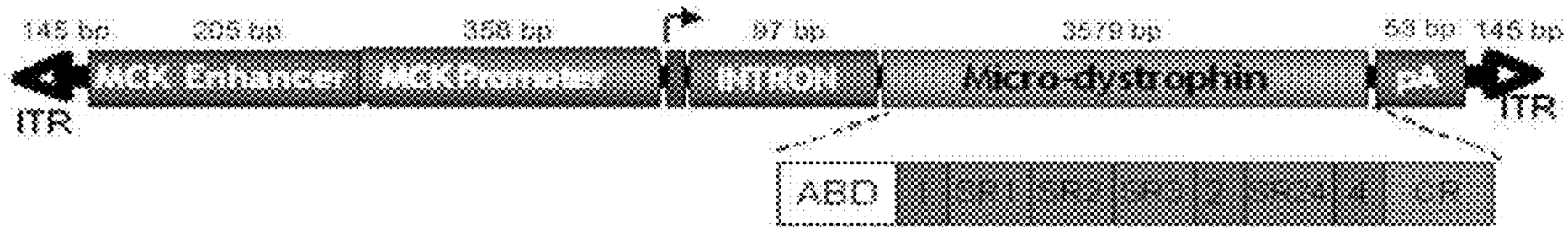
145 bp
205 bp
358 bp
97 bp
3579 bp
53 bp
146 bp
ITR
MCK Enhancer
MCK Promoter
INTRON
Micro-dystrophin
pA
ITR
ABD

Fig. 8

Cohort	Animal Identifier #	Muscle analyzed	Positive fibers/ Total fibers	% Positive	Average % Transduced
AAV8.MCK.hMicro 3e9	203	LTA	21/404	5.2	4.6%
	204	LTA	22/411	5.4	
	205	LTA	12/394	3.1	
3e10	206	LTA	299/460	65.0	65.0%
	207	LTA	282/403	69.6	
	208	LTA	213/361	60.0	
1e11	210	LTA	392/481	81.5	89.0%
	211	LTA	434/473	91.6	
	212	LTA	507/545	93.1	

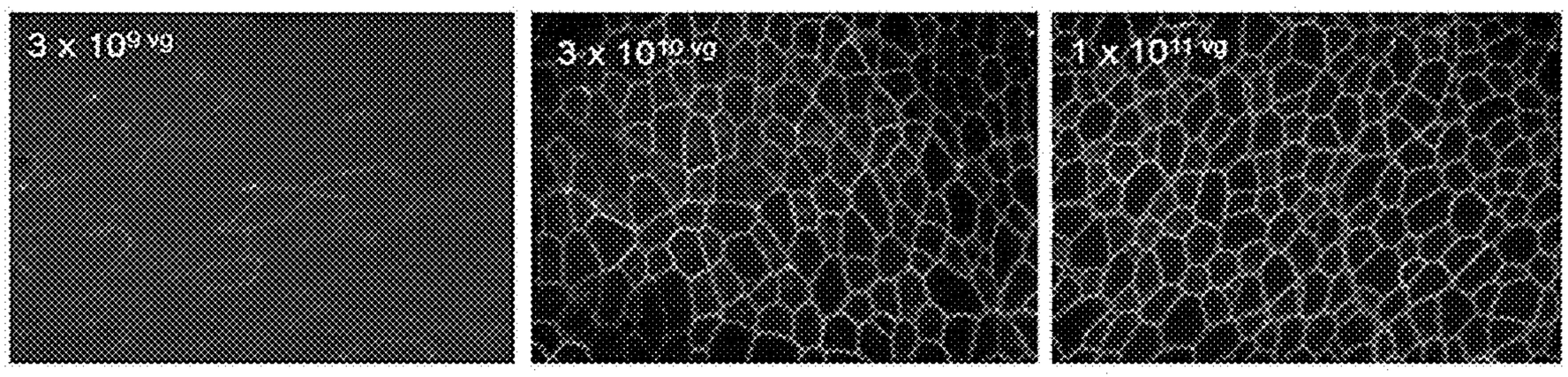

Fig. 9

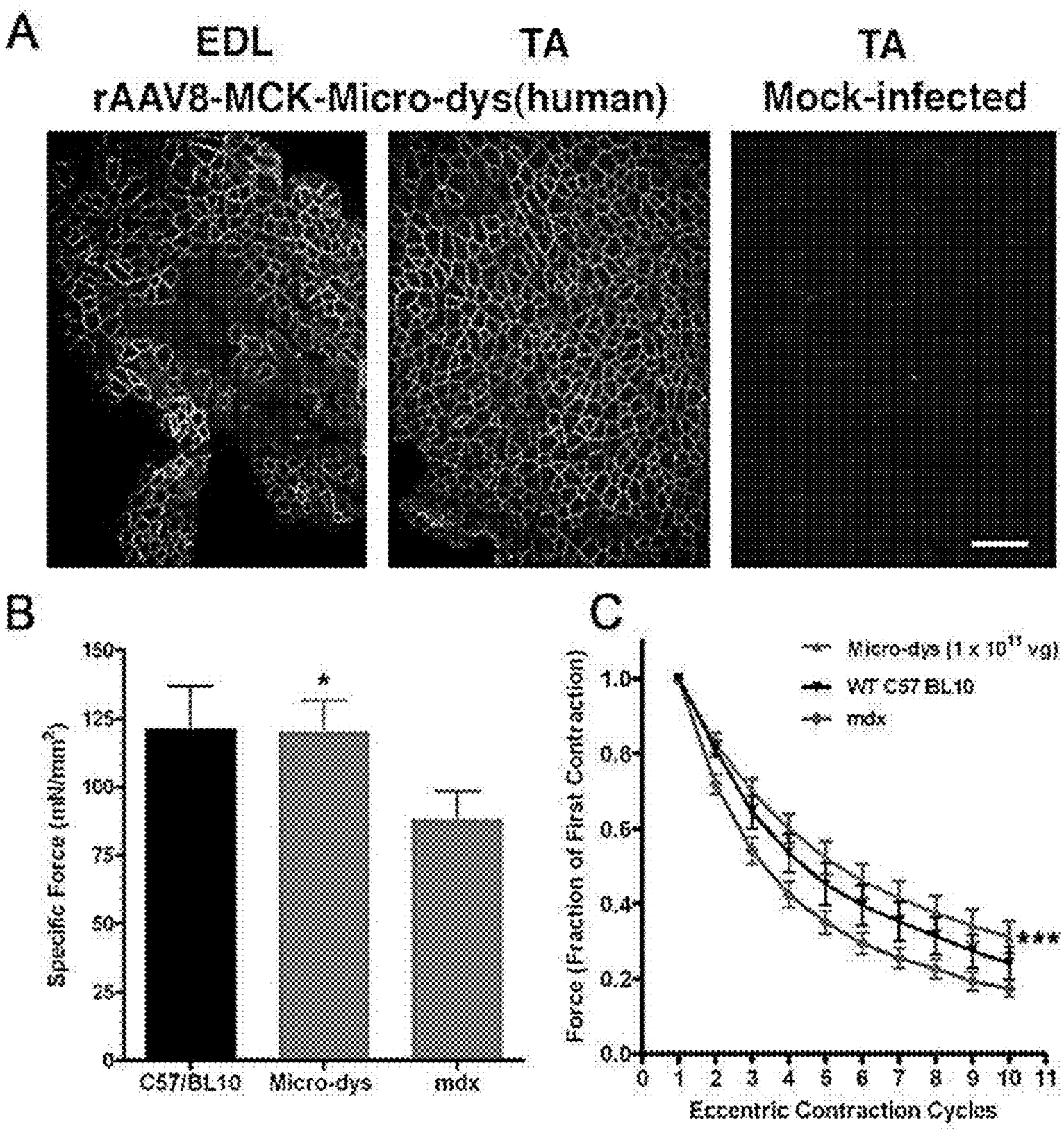
A
EDL
TA
TA
rAAV8-MCK-Micro-dys(human)
Mock-infected
B
Specific Force (mN/mm²)
150
125
100
75
50
25
0
C57/BL10
Micro-dys
mdx
C
Force (Fraction of First Contraction)
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7 8 9 10 11
Eccentric Contraction Cycles
Micro-dys (1 x 10¹³ vg)
WT C57 BL10
mdx
***

Fig. 10

SEQ ID NO: 3

rAAVrh74.MHCK7.micro-dystrophin

Main features:
MHCK7 promoter
Chimeric intron sequence
Human micro-dystrophin sequence
Poly A tail
Ampicillin resistance
pGEX plasmid backbone with pBR322 origin or replication

GCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCGCGCTCGCTCGCTCACTGA
GGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGA
GAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTTGTAGTTAATGATTAACCCGCCATGCTAATTATCTACGTAGC
CATGTCTAGAgtttaaacaagcttgcatgtctaagctagacccttcagattaaaaataactgaggtaagggcctgggtaggggaggtggtgtgag
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cgagatgcctggttataattaacccagacatgtggctgccccccccccccccaacacctgctgcctctaaaaataaccctgtccctggtggatcccctgca
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TGGTGGTGCAAATCAAAGAACTGCTCCTCAGTGGATGTTGCCTTTACTTCTAGGCCTGTACGGAAGTGTTACTTCT
GCTCTAAAAGCTGCGGAATTGTACCCGCGGCCGCCACC**ATGCTGTGGTGGGAGGAGGTGGAGGATTGTTATGAA**
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TCGAGAATCTGTTTTCCGACCTGCAGGATGGGAGACGGCTGCTGGATCTGCTGGAAGGACTGACTGGCCAGAA
GCTGCCCAAAGAGAAGGGGAGCACTAGGGTGCACGCCCTGAACAACGTGAACAAAGCTCTGAGAGTGCTGCA
GAACAACAACGTGGATCTGGTGAATATTGGCAGTACTGATATCGTGGACGGGAACCACAAACTGACACTGGGC
CTGATCTGGAACATTATTCTGCACTGGCAGGTGAAAAATGTGATGAAGAACATCATGGCCGGGCTGCAGCAGA
CCAATTCCGAGAAGATCCTGCTGTCTTGGGTGCGGCAGAGCACCCGCAACTATCCCCAGGTGAACGTGATTAAC
TTCACTACATCCTGGAGCGACGGGCTGGCCCTGAATGCTCTGATTCACAGCCACAGGCCTGATCTGTTCGACTGG
AATAGCGTGGTGTGCCAGCAGTCTGCCACACAGCGCCTGGAACATGCCTTCAATATCGCTCGGTACCAGCTGGG
GATCGAAAAACTGCTGGACCCAGAGGATGTGGACACTACATACCCAGATAAAAAGTCTATTCTGATGTACATTA
CTAGCCTGTTCCAGGTGCTGCCACAGCAGGTGTCTATTGAAGCCATTCAGGAGGTGGAAATGCTGCCCCGCCCCC
CCAAAGTGACTAAAGAGGAGCATTTTCAGCTGCATCATCAGATGCATTACAGCCAGCAGATTACCGTGAGCCTG
GCTCAGGGATATGAGCGCACCAGTAGTCCAAAACCACGGTTCAAGTCCTACGCTTATACCCAGGCTGCCTACGT
GACAACTAGCGACCCTACTAGATCCCCCTTTCCATCCCAGCACCTGGAGGCCCCAGAGGACAAGAGCTTTGGGTC
CAGCCTGATGGAAAGCGAGGTGAATCTGGATCGGTACCAGACAGCCCTGGAGGAGGTGCTGAGCTGGCTGCTG

Fig. 10 (continued)

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CCGCTGGGAGTGTCTGAGAGTGGCTAGTATGGAGAAGCAGTCCAACCTGCACCGGGTGCTGATGGACCTGCAG
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ACCGCCGCCCTGGAGGAACAGCTGAAGGTGCTGGGGGACCGGTGGGCCAACATTTGCCGGTGGACCGAGGAC
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GGCTGAGCGAGAAAGAGGACGCCGTGAACAAGATCCACACAACCGGCTTTAAGGATCAGAACGAAATGCTGTC
TAGCCTGCAGAAACTGGCTGTGCTGAAGGCCGATCTGGAGAAAAAGAAGCAGAGCATGGGCAAACTGTATAG
CCTGAAACAGGACCTGCTGAGCACCCTGAAGAACAAGAGCGTGACCCAGAAGACAGAAGCCTGGCTGGATAAC
TTTGCCCGCTGCTGGGACAACCTGGTGCAGAAACTGGAGAAAAGTACAGCTCAGATCTCTCAGGCTGTGACCAC
AACCCAGCCTAGCCTGACCCAGACAACCGTGATGGAAACCGTGACCACCGTGACAACCCGCGAACAGATCCTGG
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GCAGGAAGCCACTGACGAACTGGACCTGAAGCTGAGGCAGGCCGAAGTGATTAAGGGGTCTTGGCAGCCTGTG
GGCGATCTGCTGATTGATTCCCTGCAGGACCACCTGGAAAAGGTGAAGGCTCTGAGAGGCGAAATTGCTCCACT
GAAGGAGAACGTGAGTCATGTGAACGATCTGGCTAGACAGCTGACAACACTGGGCATCCAGCTGAGCCCATAC
AATCTGAGCACACTGGAGGACCTGAATACCAGGTGGAAGCTGCTGCAGGTGGCTGTGGAAGACCGGGTGCGG
CAGCTGCATGAGGCCCATCGCGACTTCGGACCAGCCAGCCAGCACTTTCTGAGCACATCCGTGCAGGGGCCCTG
GGAGAGGGCCATTTCTCCCAACAAGGTGCCCTACTATATTAATCACGAGACCCAGACCACTTGTTGGGACCATCC
CAAGATGACAGAACTGTACCAGTCCCTGGCCGATCTGAACAACGTGAGGTTTAGCGCTTACAGAACCGCTATGA
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CTGGAGCAGGAGCACAACAACCTGGTGAACGTGCCTCTGTGCGTGGATATGTGCCTGAACTGGCTGCTGAACGT
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GGACTGCTGCTGCATGATAGTATCCAGATTCCTAGACAGCTGGGAGAGGTGGCTAGTTTCGGAGGATCTAACAT
CGAACCCAGCGTGCGCAGCTGTTTCCAGTTTGCCAATAACAAACCTGAAATCGAGGCTGCTCTGTTCCTGGATTG
GATGCGCCTGGAACCACAGAGCATGGTGTGGCTGCCTGTGCTGCACAGAGTGGCTGCCGCCGAAACTGCCAAG
CACCAGGCTAAATGCAACATCTGCAAGGAATGTCCCATTATCGGCTTTCGCTACAGGAGTCTGAAACATTTTAAC
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GCGGCCTCAGTGAGCGAGCGAGCGCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAAC
AGTTGCGCAGCCTGAATGGCGAATGGAAGTTCCAGACGATTGAGCGTCAAAATGTAGGTATTTCCATGAGCGTTT
TTCCTGTTGCAATGGCTGGCGGTAATATTGTTCTGGATATTACCAGCAAGGCCGATAGTTTGAGTTCTTCTACTCAG
GCAAGTGATGTTATTACTAATCAAAGAAGTATTGCGACAACGGTTAATTTGCGTGATGGACAGACTCTTTTACTCG

Fig. 10 (continued)

GTGGCCTCACTGATTATAAAAACACTTCTCAGGATTCTGGCGTACCGTTCCTGTCTAAAATCCCTTTAATCGGCCTC
CTGTTTAGCTCCCGCTCTGATTCTAACGAGGAAAGCACGTTATACGTGCTCGTCAAAGCAACCATAGTACGCGCCC
TGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGC
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CCCTTTAGGGTTCCGATTTAGTGATTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGT
GGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCA
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AAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTAAATATTTGCTT
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CGTTCATCGATTCTCTTGTTTGCTCCAGACTCTCAGGCAATGACCTGATAGCCTTTGTAGAGACCTCTCAAAAATAG
CTACCCTCTCCGGCATGAATTTATCAGCTAGAACGGTTGAATATCATATTGATGGTGATTTGACTGTCTCCGGCCTT
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CCTTGCGTTGAAATAAAGGCTTCTCCCGCAAAAGTATTACAGGGTCATAATGTTTTTGGTACAACCGATTTAGCTTT
ATGCTCTGAGGCTTTATTGCTTAATTTTGCTAATTCTTTGCCTTGCCTGTATGATTTATTGGATGTTGGAAGTTCCTG
ATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGAT
GCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCAT
CCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGC
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TTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTA
CACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGT
ATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTAT
AGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGA

Fig. 10 (continued)

AAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTAT
CCCCTGATTCTGTGGATAACCGTATTACCGGGTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGC
GCAGCGAGTCAGTGAGCGACCAAGCGGAAGAGC

Fig. 11

SEQ ID NO: 5

rAAVrh74.MCK.micro-dystrophin

Main features:
MCK promoter
Chimeric intron sequence
Human codon optimized micro-dystrophin sequence -
Poly A tail
Ampicillin resistance
pGEX plasmid backbone with pBR322 origin or replication

GCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCGCGCTCGCTCGCTCACTGA
GGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGA
GAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTTGTAGTTAATGATTAACCCGCCATGCTAATTATCTACGTAGC
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TGTTCCCGGCGAAGGGCCAGCTGTCCCCCGCCAGCTAGACTCAGCACTTAGTTTAGGAACCAGTGAGCAAGTCAG
CCCTTGGGGCAGCCCATACAAGGCCATGGGGCTGGGCAAGCTGCACGCCTGGGTCCGGGGTGGGCACGGTGCCC
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CGGAATTGTACCCGCGGCCGCCACC**ATGCTGTGGTGGGAGGAGGTGGAGGATTGTTATGAAAGGGAGGACGTG**
CAGAAGAAGACTTTTACCAAGTGGGTGAACGCTCAGTTCAGCAAATTTGGGAAGCAGCACATCGAGAATCTGTT
TTCCGACCTGCAGGATGGGAGACGGCTGCTGGATCTGCTGGAAGGACTGACTGGCCAGAAGCTGCCCAAAGAG
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AGCGACGGGCTGGCCCTGAATGCTCTGATTCACAGCCACAGGCCTGATCTGTTCGACTGGAATAGCGTGGTGTG

Fig. 11 (continued)

CCAGCAGTCTGCCACACAGCGCCTGGAACATGCCTTCAATATCGCTCGGTACCAGCTGGGGATCGAAAAACTGC

TGGACCCAGAGGATGTGGACACTACATACCCAGATAAAAAGTCTATTCTGATGTACATTACTAGCCTGTTCCAGG

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GCGCACCAGTAGTCCAAAACCACGGTTCAAGTCCTACGCTTATACCCAGGCTGCCTACGTGACAACTAGCGACCC

TACTAGATCCCCCTTTCCATCCCAGCACCTGGAGGCCCCAGAGGACAAGAGCTTTGGGTCCAGCCTGATGGAAA

GCGAGGTGAATCTGGATCGGTACCAGACAGCCCTGGAGGAGGTGCTGAGCTGGCTGCTGAGTGCTGAAGACAC

ACTGCAGGCCCAGGGCGAAATTTCCAATGACGTGGAAGTGGTGAAGGATCAGTTCCACACACACGAGGGCTAT

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GAAACTGAGTGAGGACGAGGAAACAGAAGTGCAGGAGCAGATGAACCTGCTGAACAGCCGCTGGGAGTGTCT

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CCTGGAGGATCTGAAGAGACAGGTGCAGCAGCATAAGGTGCTGCAGGAGGATCTGGAACAGGAGCAGGTGCG

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GACAACCTGGTGCAGAAACTGGAGAAAAGTACAGCTCAGATCTCTCAGGCTGTGACCACAACCCAGCCTAGCCT

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TGTACCAGTCCCTGGCCGATCTGAACAACGTGAGGTTTAGCGCTTACAGAACCGCTATGAAGCTGAGACGGCTG

CAGAAGGCCCTGTGCCTGGATCTGCTGTCCCTGTCCGCCGCCTGCGATGCCCTGGATCAGCATAATCTGAAGCAG

AACGATCAGCCAATGGATATCCTGCAGATCATCAACTGCCTGACCACTATCTACGACAGGCTGGAGCAGGAGCA

Fig. 11 (continued)

CAACAACCTGGTGAACGTGCCTCTGTGCGTGGATATGTGCCTGAACTGGCTGCTGAACGTGTATGACACTGGGC

GCACCGGCCGGATCAGAGTGCTGAGTTTTAAAACTGGGATTATCTCCCTGTGTAAGGCCCACCTGGAGGACAAG

TACAGGTACCTGTTCAAGCAGGTGGCTAGTAGCACTGGATTTTGTGACCAGCGCCGCCTGGGACTGCTGCTGCA

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ACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAA

CCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTA

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TACCTACACATTACTCAGGCATTGCATTTAAAATATATGAGGGTTCTAAAAATTTTTATCCTTGCGTTGAAATAAAG

GCTTCTCCCGCAAAAGTATTACAGGGTCATAATGTTTTTGGTACAACCGATTTAGCTTTATGCTCTGAGGCTTTATT

GCTTAATTTTGCTAATTCTTTGCCTTGCCTGTATGATTTATTGGATGTTGGAAGTTCCTGATGCGGTATTTTCTCCTT

Fig. 11 (continued)

ACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCA
GCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCT
GTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTC
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GATAACCGTATTACCGGGTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTG
AGCGACCAAGCGGAAGAGC

Fig. 12

pAAV.CMV.miR29C Sequence (SEQ ID NO: 6)

Main features:
CMV promoter- 120-526
EF1a Intron- 927-1087, 1380-1854
miR-29c- 1257-1284
shRNA-miR29-c with primary seed sequence- 1088-1375
PolyA- 1896-2091

CAGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAGCCCGGGCGTCGGGCGACCTTTGGTCGCCCG
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Fig. 12 (continued)

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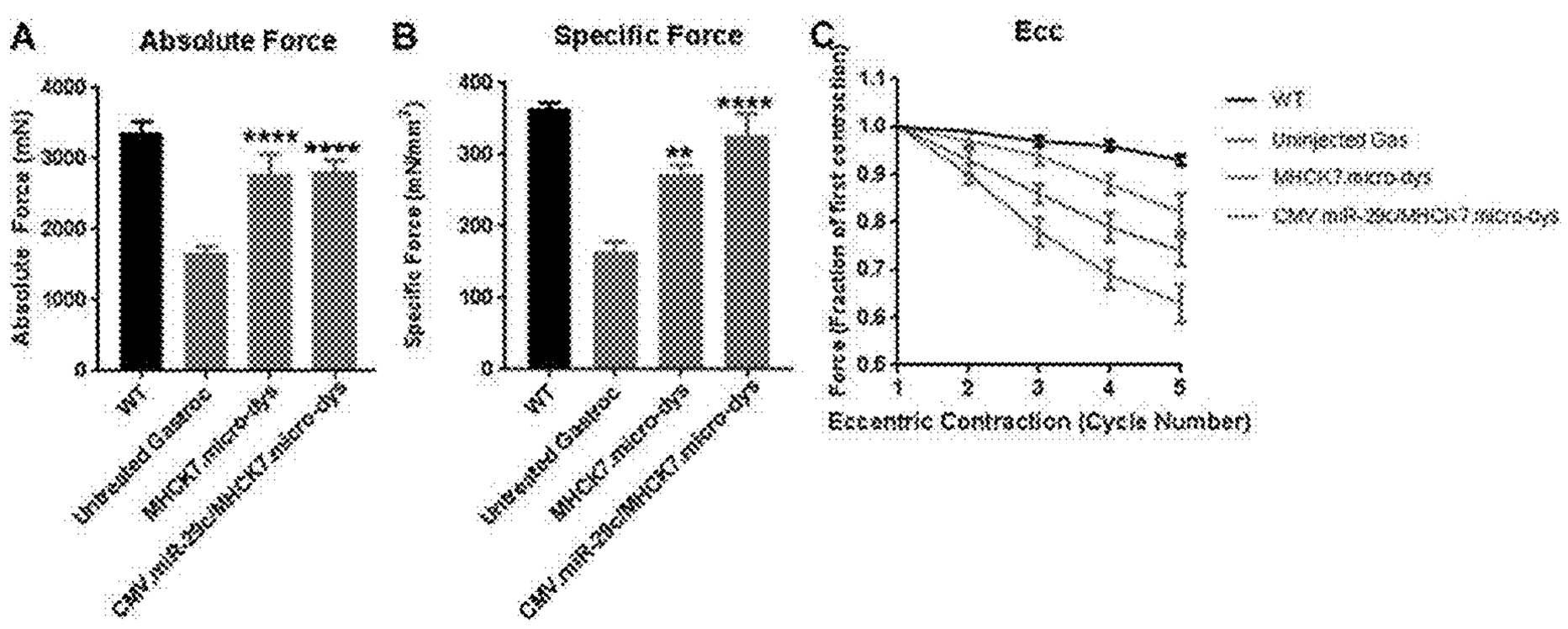
Fig. 13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Absolute Force (mN)
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3000
2000
1000
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****
****
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MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys
B
Specific Force
Specific Force (mN/mm²)
400
300
200
100
0
**
****
WT
Untreated Gastroc
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys
C
Ecc
Force (Fraction of first contraction)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
1
2
3
4
5
Eccentric Contraction (Cycle Number)
WT
Uninjected Gas
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys

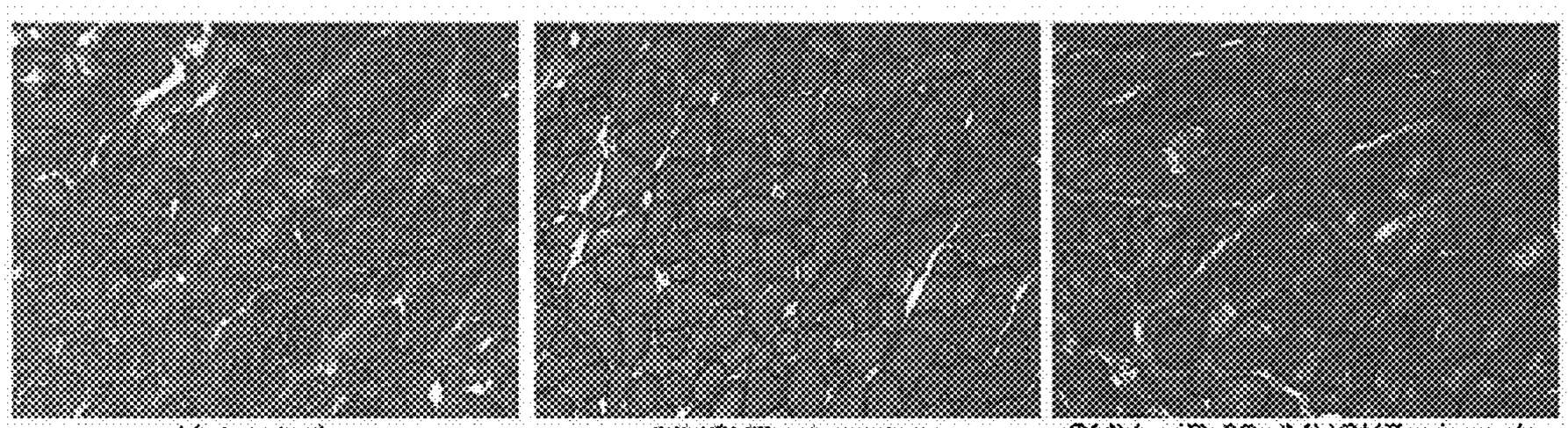
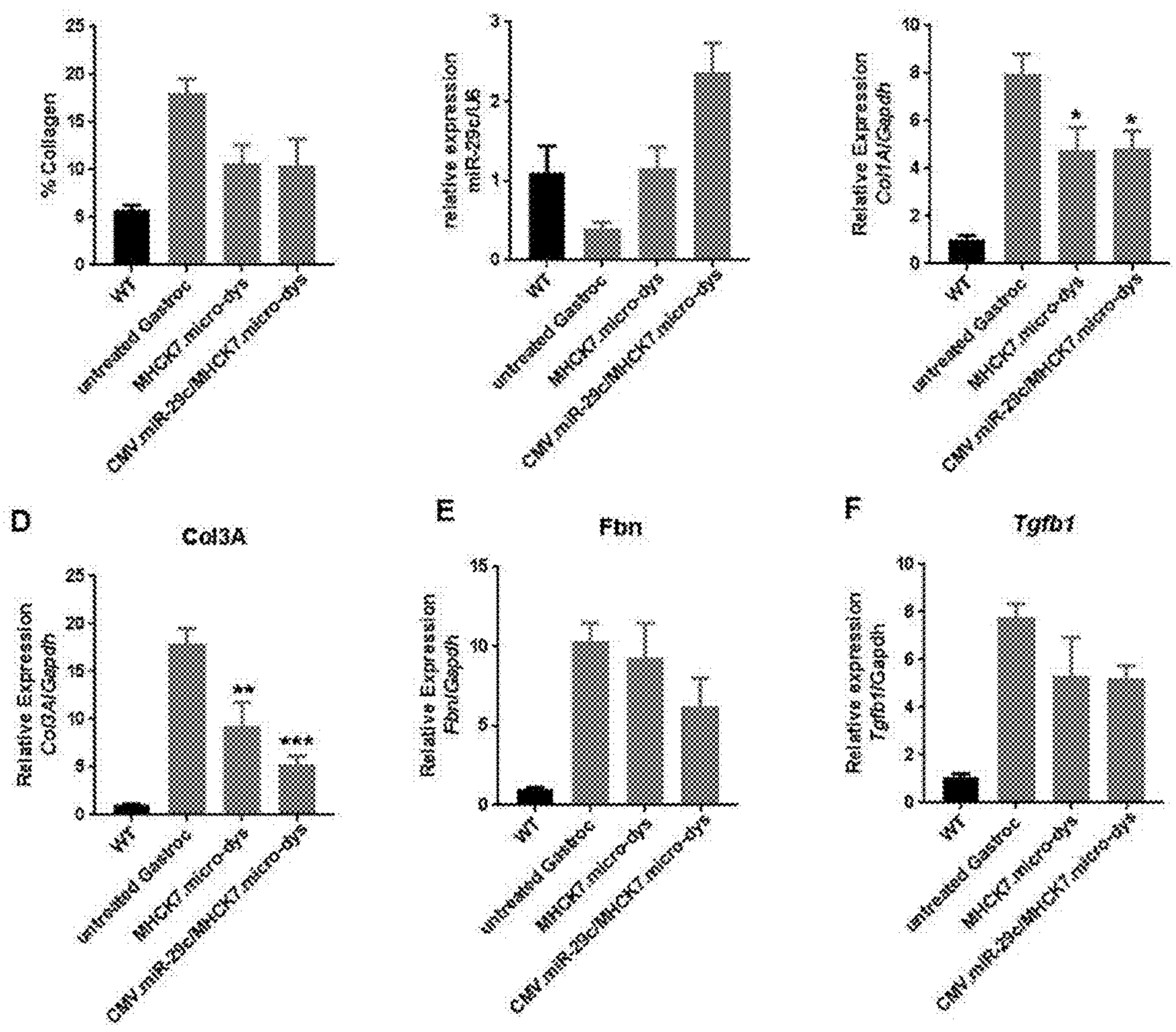

Untreated
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys

Fig. 14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% Collagen
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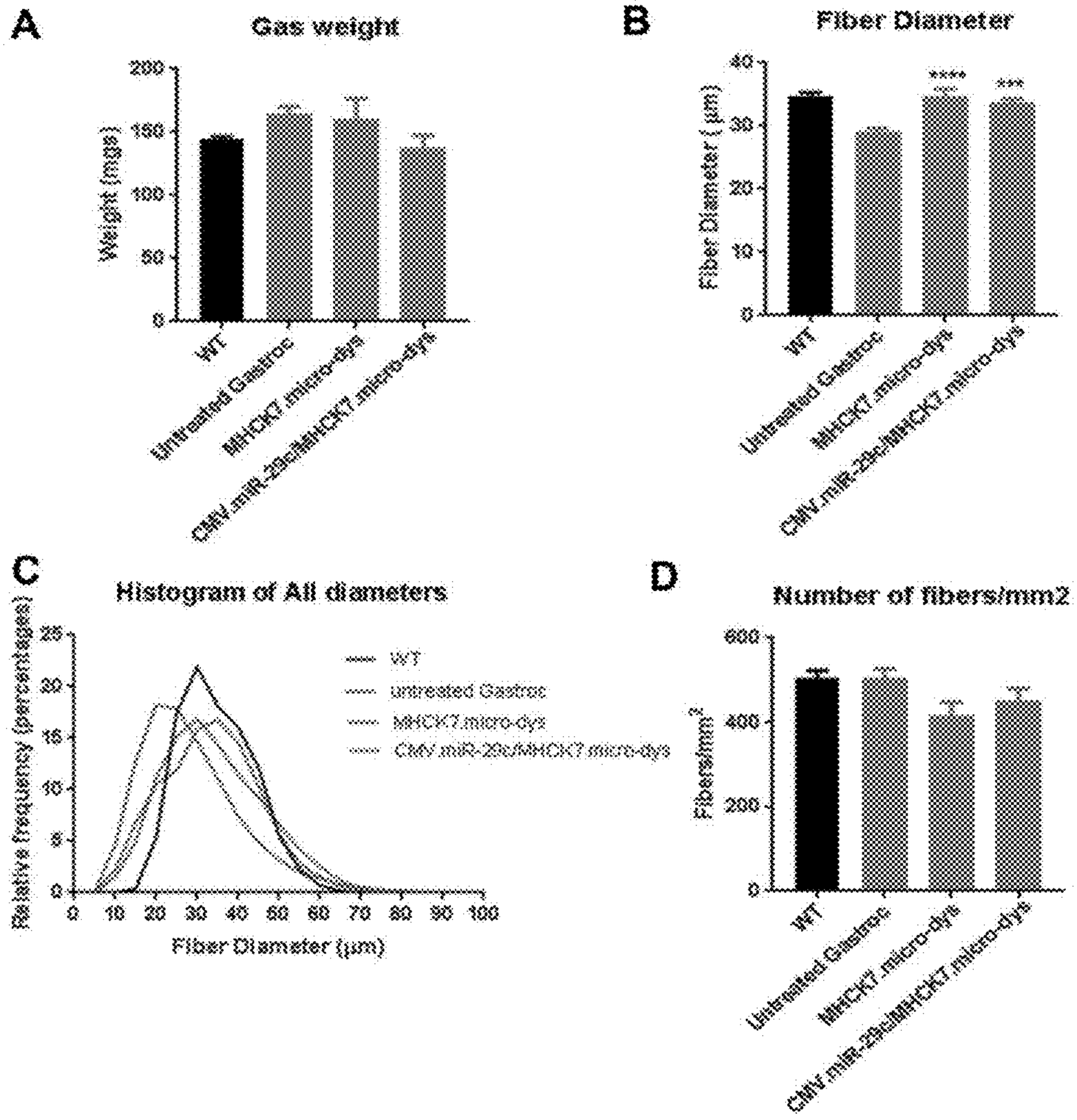
untreated Gastroc
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys
B
miR-29c levels
relative expression miR-29c/U6
C
Col1A
Relative Expression Col1A/Gapdh
D
Col3A
Relative Expression Col3A/Gapdh
E
Fbn
Relative Expression Fbn/Gapdh
F
Tgfb1
Relative expression Tgfb1/Gapdh

Fig. 15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150
100
50
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Untreated Gastroc
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys
B
Fiber Diameter
Fiber Diameter (µm)
40
30
20
10
0
****
***
WT
Untreated Gastroc
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys
C
Histogram of All diameters
Relative frequency (percentages)
25
20
15
10
5
0
WT
untreated Gastroc
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys
0 10 20 30 40 50 60 70 80 90 100
Fiber Diameter (µm)
D
Number of fibers/mm2
Fibers/mm2
600
400
200
0
WT
Untreated Gastroc
MHCK7.micro-dys
CMV.miR-29c/MHCK7.micro-dys

ADENO-ASSOCIATED VIRUS VECTOR DELIVERY OF MUSCLE SPECIFIC MICRO-DYSTROPHIN TO TREAT MUSCULAR DYSTROPHY

[0001] This application is a continuation of U.S. patent application Ser. No. 16/757,207, now U.S. Pat. No. 11,534,501, which is a national phase application of International Patent Application No. PCT/US2018/022853, filed Mar. 16, 2018, which claims priority to U.S. Provisional Patent Application No. 62/573,955, filed Oct. 18, 2017, which is incorporated by reference in its entirety.

[0002] This invention was made with government support under grant number NS055958 awarded by the National Institutes of Health/National Institute of Neurological Disorders and Stroke. The government has certain rights in the invention.

INCORPORATION BY REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0003] 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: Filename: 52822_Seqlisting.txt; Size: 39,851 bytes, created; Mar. 13, 2018.

FIELD OF INVENTION

[0004] The invention provides a combination gene therapy comprising vectors, such as adeno-associated virus (AAV) vectors, expressing a miniaturized human micro-dystrophin gene and vectors such as adeno-associated virus (AAV) vectors, expressing miR29. The invention also provides method of using this combination therapy to express micro-dystrophin and miR29 in skeletal muscles including diaphragm and cardiac muscle and to protect muscle fibers from injury, increase muscle strength and reduce and/or prevent fibrosis in subjects suffering from muscular dystrophy.

BACKGROUND

[0005] The importance of muscle mass and strength for daily activities, such as locomotion and breathing, and for whole body metabolism is unequivocal. Deficits in muscle function produce muscular dystrophies (MDs) that are characterized by muscle weakness and wasting and have serious impacts on quality of life. The most well-characterized MDs result from mutations in genes encoding members of the dystrophin-associated protein complex (DAPC). These MDs result from membrane fragility associated with the loss of sarcolemmal-cytoskeleton tethering by the DAPC. Duchenne Muscular Dystrophy (DMD) is one of the most devastating muscle diseases affecting 1 in 5000 newborn males.

[0006] DMD is caused by mutations in the DMD gene leading to reductions in mRNA and the absence of dystrophin, a 427 kD sarcolemmal protein associated with the dystrophin-associated protein complex (DAPC) (Hoffman et al., *Cell* 51(6):919-28, 1987). The DAPC is composed of multiple proteins at the muscle sarcolemma that form a structural link between the extra-cellular matrix (ECM) and the cytoskeleton via dystrophin, an actin binding protein, and alpha-dystroglycan, a laminin-binding protein. These structural links act to stabilize the muscle cell membrane during contraction and protect against contraction-induced damage. With dystrophin loss, membrane fragility results in sarcolemmal tears and an influx of calcium, triggering calcium-activated proteases and segmental fiber necrosis (Straub et al., *Curr Opin. Neurol.* 10(2): 168-75, 1997). This uncontrolled cycle of muscle degeneration and regeneration ultimately exhausts the muscle stem cell population (Sacco et al., *Cell,* 2010. 143(7): p. 1059-71; Wallace et al., *Annu Rev Physiol,* 2009. 71: p. 37-57), resulting in progressive muscle weakness, endomysial inflammation, and fibrotic scarring.

[0007] Without membrane stabilization from dystrophin or a micro-dystrophin, DMD will manifest uncontrolled cycles of tissue injury and repair ultimately replaces lost muscle fibers with fibrotic scar tissue through connective tissue proliferation. Fibrosis is characterized by the excessive deposits of ECM matrix proteins, including collagen and elastin. ECM proteins are primarily produced from cytokines such as TGFβ that is released by activated fibroblasts responding to stress and inflammation. Although the primary pathological feature of DMD is myofiber degeneration and necrosis, fibrosis as a pathological consequence has equal repercussions. The over-production of fibrotic tissue restricts muscle regeneration and contributes to progressive muscle weakness in the DMD patient. In one study, the presence of fibrosis on initial DMD muscle biopsies was highly correlated with poor motor outcome at a 10-year follow-up (Desguerre et al., *J Neuropathol Exp Neurol,* 2009. 68(7): p. 762-7). These results point to fibrosis as a major contributor to DMD muscle dysfunction and highlight the need for early intervention prior to overt fibrosis.

[0008] Most anti-fibrotic therapies that have been tested in mdx mice act to block fibrotic cytokine signaling through inhibition of the TGFβ pathway. MicroRNAs (miRNAs) are single-stranded RNAs of ~22 nucleotides that mediate gene silencing at the post-transcriptional level by pairing with bases within the 3' UTR of mRNA, inhibiting translation or promoting mRNA degradation. A seed sequence of 7 bp at the 5' end of the miRNA targets the miRNA; additional recognition is provided by the remainder of the targeted sequence, as well as its secondary structure. MiRNAs play an important role in muscle disease pathology and exhibit expression profiles that are uniquely dependent on the type of muscular dystrophy in question (Eisenberg et al. Proc Natl Acad Sci USA, 2007. 104(43): p. 17016-21). A growing body of evidence suggests that miRNAs are involved in the fibrotic process in many organs including heart, liver, kidney, and lung (Jiang et al., Proc Natl Acad Sci USA, 2007. 104(43): p. 17016-21). Recently, the down-regulation of miR-29 was shown to contribute to cardiac fibrosis (Cacchiarelli et al., Cell Metab, 2010. 12(4): p. 341-51) and reduced expression of miR-29 was genetically linked with human DMD patient muscles (Eisenberg et al. Proc Natl Acad Sci USA, 2007. 104(43): p. 17016-2). The miR-29 family consists of three family members expressed from two bicistronic miRNA clusters. MiR-29a is coexpressed with miR-29b (miR-29b-1); miR-29c is co-expressed with a second copy of miR-29b (miR-29b-2). The miR-29 family shares a conserved seed sequence and miR-29a and miR-29b each differ by only one base from miR-29c. Furthermore, electroporation of miR-29 plasmid (a cluster of miR-29a and miR-29b-1) into mdx mouse muscle reduced the expression levels of ECM components, collagen and elastin, and strongly decreased collagen deposition in muscle sections

within 25 days post-treatment (Cacchiarelli et al., Cell Metab, 2010. 12(4): p. 341-51).

[0009] Adeno-associated virus (AAV) is a replication-deficient parvovirus, the single-stranded DNA genome of which is about 4.7 kb in length including 145 nucleotide inverted terminal repeat (ITRs). There are multiple serotypes of AAV. The nucleotide sequences of the genomes of the AAV serotypes are known. For example, the 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. Pat. 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).

[0010] 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 (56° C. to 65° C. for several hours), making cold preservation of AAV less critical. AAV may even be lyophilized. Finally, AAV-infected cells are not resistant to superinfection.

[0011] Multiple studies have demonstrated long-term (>1.5 years) recombinant AAV-mediated protein expression in muscle. See, Clark et al., *Hum Gene Ther,* 8: 659-669 (1997); Kessler et al., *Proc Nat. Acad Sc. USA,* 93: 14082-14087 (1996); and Xiao et al., *J Virol,* 70: 8098-8108 (1996). See also, Chao et al., *Mol Ther,* 2:619-623 (2000) and Chao et al., *Mol Ther,* 4:217-222 (2001). Moreover, because muscle is highly vascularized, recombinant AAV transduction has resulted in the appearance of transgene products in the systemic circulation following intramuscular injection as described in Herzog et al., *Proc Natl Acad Sci USA,* 94: 5804-5809 (1997) and Murphy et al., *Proc Natl Acad Sci USA,* 94: 13921-13926 (1997). Moreover, Lewis et al., *J Virol,* 76: 8769-8775 (2002) demonstrated that skeletal myofibers possess the necessary cellular factors for correct antibody glycosylation, folding, and secretion, indicating that muscle is capable of stable expression of secreted protein therapeutics.

[0012] 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

[0013] 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

[0014] The invention 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.

[0015] In one embodiment, the invention 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 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.

[0016] The invention 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.

[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° C. or 0.015 M sodium chloride, 0.0015M sodium citrate, and 50% formamide at 42° 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 6×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 invention provides for constructs comprising the muscle specific controls element MCKH7 promoter, the MCK promoter and the MCK enhancer.

[0020] In one aspect, the invention 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).

[0021] For examples, 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 invention, 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 FIG. **1** or FIG. **10** (SEQ ID NO: 3). 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 FIG. **7** or FIG. **11** (SEQ ID NO: 5). In another aspect, the invention provides for a rAAV vector comprising the nucleotide sequence of SEQ ID NO: 1 and SEQ ID NO: 2. The invention also provides for a rAAV vector comprising the nucleotide sequence of SEQ ID NO: 1 and SEQ ID NO: 4.

[0022] In a further aspect, the invention provides for a rAAV vector comprising the nucleotide sequence of SEQ ID NO: 3 or SEQ ID NO: 5. For example, the rAAVrh74. MHCK7.micro-dystrophin vector comprises the nucleotide sequence of SEQ ID NO: 3 and shown in FIG. **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.

[0023] The invention 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.

[0024] The invention 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.

[0025] The rAAV vectors of the invention may be any AAV serotype, such as the serotype AAVrh.74, AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 or AAV13.

[0026] The invention also provides for pharmaceutical compositions (or sometimes referred to herein as simply "compositions") comprising any of the rAAV vectors of the invention.

[0027] In another embodiment, the invention provides for methods of producing a rAAV vector particle comprising culturing a cell that has been transfected with any rAAV vector of the invention and recovering rAAV particles from the supernatant of the transfected cells. The invention also provides for viral particles comprising any of the recombinant AAV vectors of the invention.

[0028] The invention provides for methods of treating muscular dystrophy comprising administering a therapeutically effective amount of any of the recombinant AAV vectors of the invention expressing human micro-dystrophin.

[0029] The invention 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.

[0030] The invention 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.

[0031] "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.

[0032] In another embodiment, the invention provides for methods of preventing or reducing fibrosis in a subject in need comprising administering a therapeutically effective amount of the any recombinant AAV vector of the invention a human micro-dystrophin protein targeted to the muscle and enhanced cardiac gene delivery and expression in the heart. For example, any of the rAAV of the invention are administered to subjects suffering from muscular dystrophy to prevent or reducing fibrosis, e.g. the rAAV of the invention expressing a human micro-dystrophin protein administered before fibrosis is observed in the subject. In addition, the rAAV of the invention expressing a human micro-dystrophin gene are 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 invention are administered to the subject suffering from muscular dystrophy in order to prevent new fibrosis in these subjects or to reduce fibrosis in these subjects.

[0033] The invention contemplates administering any of the AAV vectors of the invention before fibrosis is observed in the subject. In addition, the rAAV of the invention are 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 invention are administered to the subject suffering from muscular dystrophy who already has developed fibrosis in order to prevent new fibrosis in these subjects.

[0034] The invention 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 any of the rAAV vector of the invention expressing a human micro-dystrophin gene. These methods may further comprise the step of administering a rAAV expressing micro-dystrophin.

[0035] The invention contemplates administering any of the AAV vectors of the invention 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.

[0036] The invention also contemplates administering any of the rAAV of the invention to a subject suffering from muscular dystrophy who already has developed fibrosis, in order to prevent new fibrosis in these subjects. The invention also provides for administering any of the rAAV of the invention 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.

[0037] In any of the methods of the invention, the subject may be suffering from muscular dystrophy such as DMD or any other dystrophin-associated muscular dystrophy.

[0038] In another aspect, the rAAV vectors 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, 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, hypoxia-inducible nuclear factors, steroid-inducible element or glucocorticoid response element (GRE).

[0039] In any of the methods of the invention, the rAAV vector or composition is administered by intramuscular injection or intravenous injection.

[0040] In addition, in any of the methods of the invention, the rAAV vector or composition is administered systemically. For examples, the rAAV vector or composition is parentally administration by injection, infusion or implantation.

[0041] In another embodiment, the invention provides for composition comprising any of the rAAV vectors of the invention for reducing fibrosis in a subject in need.

[0042] In addition, the invention provides for compositions comprising any of the recombinant AAV vectors of the invention for preventing fibrosis in a patient suffering from muscular dystrophy.

[0043] The invention provides for compositions comprising any of the recombinant AAV vectors of the invention for treating muscular dystrophy.

[0044] The invention 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.

[0045] The invention provides for composition comprising a recombinant AAV vector comprising the pAAV.MHCK7.micro-dystrophin construct nucleotide sequence of SEQ ID NO: 3 for treatment of muscular dystrophy.

[0046] The invention also provides for compositions comprising any of the rAAV vectors of the invention for increasing muscular force and/or muscle mass in a subject suffering from muscular dystrophy. In a further embodiment, the invention provides for compositions comprising any of the rAAV vectors of the invention for treatment of muscular dystrophy.

[0047] The compositions of the invention are formulated for intramuscular injection or intravenous injection. The composition of the invention is also formulated for systemic administration, such as parentally administration by injection, infusion or implantation.

[0048] In addition, any of the compositions are formulated for administration to a subject suffering from muscular dystrophy such as DMD or any other dystrophin associated muscular dystrophy.

[0049] In a further embodiment, the invention provides for use of any of the rAAV vectors of the invention for preparation of a medicament for reducing fibrosis in a subject in need. For example, the subject is in need suffering from muscular dystrophy, such as DMD or any other dystrophin associated muscular dystrophy.

[0050] In another embodiment, the invention provides for provides for use of any of the rAAV vectors of the invention for the preparation of a medicament for preventing fibrosis in a subject suffering from muscular dystrophy.

[0051] In addition, the invention provides for use of the recombinant AAV vectors of the invention for the preparation of a medicament for the increasing muscular strength and/or muscle mass in a subject suffering from muscular dystrophy.

[0052] The invention also provides for use of the rAAV vectors of the invention for the preparation of a medicament for treatment of muscular dystrophy.

[0053] The invention 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.

[0054] The invention 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.

[0055] In any of the uses of the invention, the medicament is formulated for intramuscular injection or intravenous injection. In addition, in any of the uses of the invention, the medicament is formulated for systemic administration such as parental administration by injection, infusion or implantation.

[0056] Any of the medicaments may be prepared for administration to a subject suffering from muscular dystrophy such as DMD or any other dystrophin associated muscular dystrophy.

[0057] The invention also provides for combination therapy or co-therapies comprising administering a recombinant AAV vector expressing micro-dystrophin and administering a recombinant AAV vector expressing miR-29 and expression of miR-29 is controlled by a muscle-specific control element nucleotide sequence.

[0058] In one embodiment, the invention provides for methods of treating muscular dystrophy comprising administering i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of a recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

[0059] In another embodiment, the invention provides for methods of increasing muscular force or muscle mass in a subject suffering from muscular dystrophy comprising administering i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

[0060] In a further embodiment, the invention provides for methods of reducing or preventing fibrosis in a subject suffering from muscular dystrophy comprising administering i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

[0061] The invention also provides for compositions for treating muscular dystrophy comprising i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of a recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

[0062] In another embodiment, the invention provides for compositions for increasing muscular force or muscle mass in a subject suffering from muscular dystrophy comprising administering i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

[0063] In a further embodiment, the invention provides for compositions for reducing or preventing fibrosis in a subject suffering from muscular dystrophy comprising administering i) a therapeutically effective amount of i) a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

[0064] The invention also provides for use of i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin wherein the expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of recombinant AAV vector expressing miR-29c wherein the expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence for preparation of a medicament for the treatment of muscular dystrophy.

[0065] In another embodiment, the invention provides for use of i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin wherein the expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of a recombinant AAV vector expressing miR-29c wherein the expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence for the preparation of a medicament for increasing muscular force or muscle mass in a subject suffering from muscular dystrophy.

[0066] In a further embodiment, the invention provides for use of i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin wherein the expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of a recombinant AAV vector expressing miR-29c wherein the expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence for the preparation of a medicament for reducing or preventing fibrosis in a subject suffering from muscular dystrophy.

[0067] In any of the combination or co-therapy methods, compositions or uses of the invention, the muscular dystrophy is Duchenne muscular dystrophy.

[0068] In any of the combination or co-therapy methods, compositions or uses of the invention, the nucleotide sequence encoding the micro-dystrophin protein comprises a) a nucleotide sequence that is at least 85% identical to the nucleotide sequence SEQ ID NO: 1 and encodes a functional micro-dystrophin protein, or b) the nucleotide sequences of SEQ ID NO: 1.

[0069] In addition, in any of the combination or co-therapy methods, compositions or uses of the invention, the recombinant AAV vector expressing miR-29c comprises: a) the nucleotide sequences of SEQ ID NO: 8 and SEQ ID NO: 9, b) the nucleotide sequence of SEQ ID NO: 7, or c) the nucleotide sequence of SEQ ID NO: 6.

[0070] In any of the combination or co-therapy methods, compositions or uses of the invention, at least one of the muscle specific control element is 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). For example, the muscle specific control element controlling expression of micro-dystrophin comprises SEQ ID NO: 2 (MHCK7) and/or the muscle specific control element controlling expression of miR-29c comprises SEQ ID NO: 10 (CMV).

[0071] In exemplary combination or co-therapy methods, compositions or uses, the AAV vector expressing micro-dystrophin comprises i) the nucleotide sequences of SEQ ID NO: 1 (micro-dys) and ii) the nucleotide sequence of SEQ ID NO: 2 (MHCK7) or the AAV vector expressing micro-dystrophin comprises the nucleotide sequence of SEQ ID NO: 3.

[0072] In exemplary combination or co-therapy methods, compositions or uses, the AAV vector expressing miR-29c comprises i) the nucleotide sequence of SEQ ID NO: 8 or SEQ ID NO: 9 and ii) the nucleotide sequence of SEQ ID NO: 10 (CMV) or the AAV vector expressing miR-29c comprises the nucleotide sequence of SEQ ID NO: 6.

[0073] In another exemplary combination or co-therapy methods, compositions or uses, the AAV vector expressing micro-dystrophin comprises i) the nucleotide sequences of SEQ ID NO: 1 (micro-dys) and ii) the nucleotide sequence of SEQ 2 (MHCK7), and wherein the AAV vector expressing miR-29c comprises i) the nucleotide sequence of SEQ ID NO: 8 or SEQ ID NO: 9 and ii) the nucleotide sequence of SEQ ID NO: 10 (CMV).

[0074] In further combination or co-therapy methods, compositions or uses, the AAV vector expressing micro-dystrophin comprises the nucleotide sequence of SEQ ID NO: 3 and the AAV vector expressing miR-29c comprises the nucleotide sequence of SEQ ID NO: 6.

[0075] In any of the combination or co-therapy methods, compositions or uses of the invention, at least one of the recombinant AAV vectors is the serotype AAVrh.74, AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 or AAV13.

[0076] In any of the combination or co-therapy methods, compositions or uses of the invention, least one of the recombinant AAV vectors, the composition or the medicament is administered by intramuscular injection or intravenous injection.

[0077] In any of the combination or co-therapy methods, compositions or uses of the invention, at least one of the recombinant AAV vectors or the composition or the medicament is administered systemically.

[0078] In any of the combination or co-therapy methods, compositions or uses of the invention, at least one of the recombinant AAV vectors, compositions or medicaments is parenterally administered by injection, infusion or implantation.

BRIEF DESCRIPTION OF DRAWING

[0079] FIG.e **1** illustrates the pAAV.MHCK7.micro-dystrophin construct. In this construct, the cDNA expression cassette is flanked by AAV2 inverted terminal repeat sequences (ITR). The construct is characterized by an in-frame rod deletion (R4-R23), while hinges 1, 2 and 4 (H_1, H_2 and H_3) and the cysteine rich domain remain producing a 138 kDa protein. The expression of the micro-dystrophin protein (3579 bp) is guided by a MHCK7 promoter (795 bp). The intron and 5' UTR are derived from plasmid pCMVβ (Clontech). The micro-dystrophin cassette had a consensus Kozak immediately in front of the ATG start and a small 53 bp synthetic polyA signal for mRNA termination. The human micro-dystrophin cassette contained the (R4-R23/Δ71-78) domains as previously described by Harper et al. (*Nature Medicine* 8, 253-261 (2002)).

[0080] FIG. **2** demonstrates dystrophin protein expression following intramuscular delivery of AAVrh74.MHCK7 construct. The tibialis anterior muscle of mdx mice was injected with 1×10^{11} vg (n=5 per group). Six weeks later the muscles were harvested and stained for dystrophin expression with an N-terminal antibody for dystrophin and hematoxylin and eosin staining.

[0081] FIG. **3**A-FIG. **3**C provide skeletal muscle force measurements and quantification of micro-dystrophin expression following intramuscular injection of AAVrh74. MHCK7 construct. (A) The tibialis anterior muscle of mdx mice was injected with 1×10^{11} vg (n=5) with AAVrh74. MHCK7 construct. Six weeks later the tibialis anterior muscles were harvested and subjected to in vivo force measurements. The dosed cohort had significantly greater force production than untreated mdx controls.

[0082] FIG. **4**A-FIG. **4**C demonstrates widespread transduction of skeletal diaphragm and cardiac muscle fibers after systemic administration. (A) Mdx mice were treated systemically at 6 weeks of age via the tail vein with 6×10^{12} vg (2×10^{14} vg/kg) of AAVrh.74.MHCK7.micro-dys following 12 weeks of treatment. (B) Staining for micro-dystrophin demonstrates the shows quantification of the percentage of muscle fibers expressing micro-dystrophin in each tissue. (C) shows the specific force measured in the diaphragm at the low and high (planned clinical) dose. No significant difference was seen at low dose; however there was significant improvement at the high dose.

[0083] FIG. **5** demonstrates dystrophin protein expression following systemic delivery of AAVrh.74.MHCK7.micro-dys construct. Mdx mice (n=5) were treated systemically at 6 weeks of age via the tail vein with 6×10^{12}vg of

AAVrh.74.MHCK7.micro-dys following 12 weeks of treatment, all muscles were harvested and stained for dystrophin and restoration of DAPC components (beta-sarcoglycan shown).

[0084] FIG. **6**A-FIG. **6**D demonstrates the toxicology/safety of AAVrh.74.MHCK7. Hematoxylin and eosin (H&E) staining was performed on the following muscle tissues to analyze toxicity: Tibialis anterior (TA), Gastrocnemius (GAS), Quadriceps (QD), Psoas (PSO), Triceps (TRI), and Diaphragm (DIA) (FIG. **6**A). No toxicity was noted. As an indicator of efficacy, the number of muscle fibers with centrally placed nuclei (CN) was quantified (FIG. **6**B). CN are indicative of cycles of muscle degeneration and regeneration and thus reduction in CN demonstrates treatment effect. (FIG. **6**C) demonstrates the total number of fibers is unchanged with treatment. The amount of creatine kinase is provided in (D) showing improvement at high dose. Independent t-tests were used to locate differences ($p<0.05$); Data are reported as means±SEM.

[0085] FIG. **7** illustrates the pAAV.MCK.micro-dystrophin plasmid construct.

[0086] FIG. **8** provides the results of a rAAVrh74.MCK.micro-dystrophin (human) potency assay. The tibialis anterior muscle of mdx mice was injected with 3×10^9, 3×10^{10}, or 1×10^{11} vg (n=3 per group). Four weeks later the muscles were harvested and stained for dystrophin expression with the N-terminal Dys3 antibody. There was a linear correlation between expression and dose where very little expression (no effect level) at 3×10^9 vg and 89% expression at 1×10^{11} vg.

[0087] FIG. **9**A-FIG. **9**C demonstrate that Human micro-dystrophin improves force generation and protection from eccentric contraction induced injury. (A) Dystrophin protein immunostaining in the extensor digitorum longus (EDL) and TA shows expression in mdx myofibers following rAAVrh.74-MCK-Micro-dys (human) injection via the femoral artery. Mock-infected muscle was stained in an identical manner and exposures are time matched. (B) rAAVrh.74-MCK-Micro-dys significantly increased normalized specific force relative to mock-treated mdx muscles ($P<0.05$ vs. mdx). (C) mdx muscles infected with rAAVrh.74-MCK-Micro-dys (human) were compared with mock-infected contralateral mdx EDL muscles and WT (WT C57B1/10) EDL muscles for force drop during repetitive eccentric contractions at 12 wks post gene transfer. rAAVrh.74-MCK-micro-dystrophin (Micro-dys) treatment significantly protected against loss of force compared with mock-treated mdx muscles ($P<0.001$ vs. mdx). Errors are SEMs.

[0088] FIG. **10** provides the nucleic acid sequence (SEQ ID NO: 3 rAAVrh74.MHCK7. micro-dystrophin).

[0089] FIG. **11** provide the nucleic acid sequence (SEQ ID NO: 5) rAAVrh74.MCK.micro-dystrophin.

[0090] FIG. **12** provide a schematic of rAAV vector scAAVrh.74.CMV.miR29c and the nucleotide sequence of the miR-29c in a natural miR-30 backbone and the nucleotide sequence of the predicted hairpin structure.

[0091] FIG. **13**A-FIG. **13**C demonstrate that early combination therapy restores force and protects against contraction-induced damage. Measurement of absolute (A) and normalized specific force (B) following tetanic contraction demonstrated increased force with combination therapy compared to untreated mdx/utrn$^{+/-}$ muscle and micro-dystrophin therapy alone ($*p<0.05$). One-way ANOVA (C) Muscles were then assessed for loss of force following repetitive eccentric contractions. Mice co-treated with miR-29c/micro-dystrophin and micro-dystrophin alone showed a protection from loss of force compared with untreated mdx/utrn$^{+\backslash-}$ muscles (red). Two-way ANOVA. ($**p<0.01$, **** $P<0.0001$). All data represent mean±SEM (D) Sirius Red stain Representative images demonstrating muscle fibers (green) and collagen content (red).

[0092] FIG. **14**A-FIG. **14**F demonstrate that treatment of AAV.CMV.miR-29c/MHCK7.micro-dystrophin combination therapy is effective at reducing fibrosis and ECM expression. (A) Sirius Red staining shows a reduction in collagen staining in both treated cohorts. (B) qRT-PCR confirms an increase in miR-29c transcript levels in the treated cohorts (n=2-3 for all groups) One-way ANOVA. Semi-quantitative qRT-PCR shows a reduction in Col1A1 and Col3A1 (C, D), Fbn (E) and Tgfβ1 (F) levels in the AAV.CMV.miR-29c/AAV.MHCK7.micro-dystrophin treated muscle compared to the contralateral limb and the single therapy of MHCK7.micro-dystrophin, with Col1A1 and Col3A1 being significant. C-F (n=2-3 per group) One-way ANOVA. All data represent mean±SEM. ($*p<0.05$, ** $p<0.01$, *** $p<0.001$).

[0093] FIG. **15**A-FIG. **15**D demonstrate that treatment of AAV.CMV.miR-29c/MHCK7.micro-dystrophin combination therapy increased fiber diameter. (A) Treated gas weights showed no significant difference compared WT or untreated muscle. (B) miR-29c/micro-dystrophin combination treatment demonstrated an increase in average fiber size. Comparing mdx/utrn$^{+/-}$ controls with miR-29c/micro-dystrophin treated mdx/utrn$^{+/-}$, the average diameter increased from 29.02 to 33.61m (n=5-6 per group), One-way ANOVA. (C) The co-delivery produced a shift towards wild-type fiber size distribution. (D) The number of muscle fibers per mm^2 in the miR-29c/micro-dystrophin combination treatment was no different from untreated mice or WT mice. C-F (n=5-5 per group), One-way ANOVA. All data represent mean±SEM. ($***p<0.001$, $****p<0.0001$)

DETAILED DESCRIPTION

[0094] The present invention provides for gene therapy vectors, e.g. rAAV vectors, overexpressing human micro-dystrophin and methods of reducing and preventing fibrosis in muscular dystrophy patients. The present invention also provides for co-therapy (combination) gene therapy methods which comprise administering a gene therapy vector expressing miR-29 in combination with a gene therapy vector expressing micro-dystrophin that is deleted in DMD patients.

[0095] Muscle biopsies taken at the earliest age of diagnosis of DMD reveal prominent connective tissue proliferation. Muscle fibrosis is deleterious in multiple ways. It reduces normal transit of endomysial nutrients through connective tissue barriers, reduces the blood flow and deprives muscle of vascular-derived nutritional constituents, and functionally contributes to early loss of ambulation through limb contractures. Over time, treatment challenges multiply as a result of marked fibrosis in muscle. This can be observed in muscle biopsies comparing connective tissue proliferation at successive time points. The process continues to exacerbate leading to loss of ambulation and accelerating out of control, especially in wheelchair-dependent patients.

[0096] Without early treatment a parallel approach to reduce fibrosis it is unlikely that the benefits of exon

skipping, stop-codon read-through, or gene replacement therapies can ever be fully achieved. Even small molecules or protein replacement strategies are likely to fail without an approach to reduce muscle fibrosis. Previous work in aged mdx mice with existing fibrosis treated with AAV.micro-dystrophin demonstrated that we could not achieve full functional restoration (Liu, M., et al., *Mol Ther* 11, 245-256 (2005)). It is also known that progression of DMD cardiomyopathy is accompanied by scarring and fibrosis in the ventricular wall. Micro-RNA delivery is particularly innovative because of lack of immune barriers and relative ease of delivery. Micro-RNAs are small (~200 bp) and can therefore be packaged in AAV along with a therapeutic cassette to correct or bypass the genetic defect.

[0097] 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.

[0098] 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.

[0099] 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.

AAV

[0100] Recombinant AAV genomes of the invention comprise nucleic acid molecule of the invention 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 AAVrh.74, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12 and AAV-13. 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. To promote skeletal muscle specific expression, AAV1, AAV6, AAV8 or AAVrh.74 may be used.

[0101] DNA plasmids of the invention comprise rAAV genomes of the invention. 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-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAVrh.74, AAV-8, AAV-9, 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.

[0102] 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.

[0103] 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., *Mol. 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. Pat. No. 5,173,414; WO 95/13365 and corresponding U.S. Pat. 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. Pat. Nos. 5,786,211; 5,871,982; and 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.

[0104] The invention 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).

[0105] Recombinant AAV (i.e., infectious encapsidated rAAV particles) of the invention comprises a rAAV genome. 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. Examples of rAAV that may be constructed to comprise the nucleic acid molecules of the invention are set out in International Patent Application No. PCT/US2012/047999 (WO 2013/016352) incorporated by reference herein in its entirety.

[0106] 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. Pat. No. 6,566,118 and WO 98/09657.

[0107] In another embodiment, the invention contemplates compositions comprising rAAV of the present invention. Compositions of the invention 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 buffers and surfactants such as pluronics.

[0108] Titers 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, and the cell type(s) being targeted, and may be determined by methods standard in the art. Titers of rAAV may range from about 1×10^{6}, about 1×10^{7}, about 1×10^{8}, about 1×10^{9}, about 1×10^{11}, about 1×10^{11}, about 1×10^{12}, about 1×10^{13} to about 1×10^{14} or more DNase resistant particles (DRP) per ml. Dosages may also be expressed in units of viral genomes (vg).

[0109] Methods of transducing a target cell with rAAV, in vivo or in vitro, are contemplated by the invention. The in vivo methods comprise the step of administering an effective dose, or effective multiple doses, of a composition comprising a rAAV of the invention 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 invention, 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. An example of a disease contemplated for prevention or treatment with methods of the invention is FSHD.

[0110] Combination therapies or co-therapies are also contemplated by the invention. Combination as used herein includes both simultaneous treatment and sequential treatments. Combinations of methods of the invention with standard medical treatments (e.g., corticosteroids) are specifically contemplated, as are combinations with novel therapies.

[0111] 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 invention may be chosen and/or matched by those skilled in the art taking into account the infection and/or disease state being treated and the target cells/tissue(s) that are to express the micro-dystrophin protein.

[0112] The invention provides for local administration and systemic administration of an effective dose of rAAV and compositions of the invention. 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 parental administration through injection, infusion or implantation.

[0113] In particular, actual administration of rAAV of the present invention may be accomplished by using any physical method that will transport the rAAV recombinant vector into the target tissue of an animal. Administration according to the invention includes, but is not limited to, injection into muscle, the bloodstream and/or directly into the liver. Simply resuspending a rAAV in phosphate buffered saline has been demonstrated to be sufficient to provide a vehicle useful for muscle tissue expression, and there are no known restrictions on the carriers or other components that can be co-administered with the rAAV (although compositions that degrade DNA should be avoided in the normal manner with rAAV). Capsid proteins of a rAAV may be modified so that the rAAV is targeted to a particular target tissue of interest such as muscle. See, for example, WO 02/053703, the disclosure of which is incorporated by reference herein. Pharmaceutical compositions can be prepared as injectable formulations or as topical formulations to be delivered to the muscles by transdermal transport. Numerous formulations for both intramuscular injection and transdermal transport have been previously developed and can be used in the practice of the invention. The rAAV can be used with any pharmaceutically acceptable carrier for ease of administration and handling.

[0114] The dose of rAAV to be administered in methods disclosed herein will vary depending, for example, on the particular rAAV, the mode of administration, the treatment

goal, the individual, and the cell type(s) being targeted, and may be determined by methods standard in the art. Titers of each rAAV administered may range from about 1×10^6, about 1×10^7, about 1×10^8, about 1×10^9, about 1×10^{10}, about 1×10^{11}, about 1×10^{12}, about 1×10^{13}, about 1×10^{14}, or to about 1×10^{15} or more DNase resistant particles (DRP) per ml. Dosages may also be expressed in units of viral genomes (vg) (i.e., 1×10^7 vg, 1×10^8 vg, 1×10^9 vg, 1×10^{10} vg, 1×10^{11} vg, 1×10^{12} vg, 1×10^{13} vg, 1×10^{14} vg, 1×10^{15} respectively). Dosages may also be expressed in units of viral genomes (vg) per kilogram (kg) of bodyweight (i.e., 1×10^{10} vg/kg, 1×10^{11} vg/kg, 1×10^{12} vg/kg, 1×10^{13} vg/kg, 1×10^{14} vg/kg, 1×10^{15} vg/kg respectively). Methods for titering AAV are described in Clark et al., *Hum. Gene Ther.,* 10: 1031-1039 (1999).

[0115] In particular, actual administration of rAAV of the present invention may be accomplished by using any physical method that will transport the rAAV recombinant vector into the target tissue of an animal. Administration according to the invention includes, but is not limited to, injection into muscle, the bloodstream and/or directly into the liver. Simply resuspending a rAAV in phosphate buffered saline has been demonstrated to be sufficient to provide a vehicle useful for muscle tissue expression, and there are no known restrictions on the carriers or other components that can be co-administered with the rAAV (although compositions that degrade DNA should be avoided in the normal manner with rAAV). Capsid proteins of a rAAV may be modified so that the rAAV is targeted to a particular target tissue of interest such as muscle. See, for example, WO 02/053703, the disclosure of which is incorporated by reference herein. Pharmaceutical compositions can be prepared as injectable formulations or as topical formulations to be delivered to the muscles by transdermal transport. Numerous formulations for both intramuscular injection and transdermal transport have been previously developed and can be used in the practice of the invention. The rAAV can be used with any pharmaceutically acceptable carrier for ease of administration and handling.

[0116] For purposes of intramuscular injection, solutions in an adjuvant such as sesame or peanut oil or in aqueous propylene glycol can be employed, as well as sterile aqueous solutions. Such aqueous solutions can be buffered, if desired, and the liquid diluent first rendered isotonic with saline or glucose. Solutions of rAAV as a free acid (DNA contains acidic phosphate groups) or a pharmacologically acceptable salt can be prepared in water suitably mixed with a surfactant such as hydroxpropylcellulose. A dispersion of rAAV can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. In this connection, the sterile aqueous media employed are all readily obtainable by standard techniques well-known to those skilled in the art.

[0117] The pharmaceutical carriers, diluents or excipients suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating actions of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of a dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by use of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0118] 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.

[0119] Transduction with rAAV may also be carried out in vitro. In one embodiment, desired target muscle cells are removed from the subject, transduced with rAAV and reintroduced into the subject. Alternatively, syngeneic or xenogeneic muscle cells can be used where those cells will not generate an inappropriate immune response in the subject.

[0120] Suitable methods for the transduction and reintroduction of transduced cells into a subject are known in the art. In one embodiment, cells can be transduced in vitro by combining rAAV with muscle cells, e.g., in appropriate media, and screening for those cells harboring the DNA of interest using conventional techniques such as Southern blots and/or PCR, or by using selectable markers. Transduced cells can then be formulated into pharmaceutical compositions, and the composition introduced into the subject by various techniques, such as by intramuscular, intravenous, subcutaneous and intraperitoneal injection, or by injection into smooth and cardiac muscle, using e.g., a catheter.

[0121] Transduction of cells with rAAV of the invention results in sustained expression of the micro-dystrophin protein. The present invention thus provides methods of administering/delivering rAAV which express of micro-dystrophin protein 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 invention. Transduction may be carried out with gene cassettes comprising tissue specific control elements. For example, one embodiment of the invention provides methods of transducing muscle cells and muscle tissues directed by muscle specific control 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, 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.

[0122] Muscle tissue is an attractive target for in vivo DNA delivery, because it is not a vital organ and is easy to access. The invention contemplates sustained expression of micro-dystrophin from transduced myofibers.

[0123] 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.

[0124] The term "transduction" is used to refer to the administration/delivery of the coding region of the micro-dystrophin to a recipient cell either in vivo or in vitro, via a replication-deficient rAAV of the invention resulting in expression of micro-dystrophin by the recipient cell.

[0125] Thus, the invention provides methods of administering an effective dose (or doses, administered essentially simultaneously or doses given at intervals) of rAAV that encode micro-dystrophin to a patient in need thereof.

EXAMPLES

Example 1

[0126] Generation of the pAAV.MHCK7.Micro-Dystrophin Construct

[0127] The pAAV.MHCK7.micro-dystrophin plasmid contains a human micro-dystrophin cDNA expression cassette flanked by AAV2 inverted terminal repeat sequences (ITR) (see FIG. **1**). The micro-dys construct was characterized by an in-frame rod deletion (R4-R23), while hinges 1, 2 and 4 and cysteine rich domain remain producing a 138 kDa protein. The expression of the micro-dystrophin protein (3579 bp) was guided by a MHCK7 promoter (795 bp). The intron and 5' UTR are derived from plasmid pCMVβ (Clontech). The micro-dystrophin cassette had a consensus Kozak immediately in front of the ATG start and a small 53 bp synthetic polyA signal for mRNA termination. The human micro-dystrophin cassette contained the (R4-R23/Δ71-78) domains as previously described by Harper et al. (*Nature Medicine* 8, 253-261 (2002)). The complementary DNA was codon optimized for human usage and synthesized by GenScript (Piscataway, NJ) (*Mol Ther* 18, 109-117 (2010)). The only viral sequences included in this vector were the inverted terminal repeats of AAV2, which are required for both viral DNA replication and packaging. The micro-dystrophin cassette has a small 53 bp synthetic polyA signal for mRNA termination.

[0128] Previously studies have validated cardiac expression using MHCK7 promoter (Salva et al. *Mol Ther* 15, 320-329 (2007) and AAVrh74 achieving skeletal, diaphragm, and cardiac muscle expression (Sondergaard et al. *Annals of clinical and Transl Neurology* 2, 256-270 (2015)), the sequence of construct of FIG. **1** was encapsidated into AAVrh.74 virions. The molecular clone of the AAVrh.74 serotype was cloned from a rhesus macaque lymph node and is described in in Rodino-Klapac et al. *Journal of Translational medicine* 5, 45 (2007).

Example 2

[0129] Intramuscular Expression Studies Using rAAV. MHCK7.Micro-Dystrophin

[0130] Expression studies were conducted with the human micro-dystrophin construct (rAAVrh74.MHCK7. micro-dystrophin; described in Example 1) by intramuscular injection. The tibialis anterior muscle of mdx mice (spontaneous Dmd^{mdx} mutant mice that do not express dystrophin) were injected with 1×10^{11} vg of the cassette (n=5 per group). Six weeks later the muscles were harvested and stained for dystrophin (Dys3) expression with an N-terminal antibody for dystrophin and hematoxylin and eosin (HE) staining. FIG. **2** shows diffuse gene expression and reduction in centrally located nuclei with 1×10^{11} vg dose compared to the untreated muscle. Furthermore, a decrease in central nucleation with an increase in average fibers/frame was observed following treatment with micro-dystrophin construct. Expression levels of the rAAVrh74.MHCK7. micro-dystrophin construct were quantified at about 73%.

[0131] In addition to measuring micro-dystrophin localization and expression levels, skeletal muscle force was measured measurements and quantification of n following intramuscular injection of the cassette. Intramuscular expression of pAAV.MHCK7.micro-dystrophin construct resulted in significantly greater absolute and specific force production compared with untreated controls (FIG. **3**A and FIG. **3**B, respectfully).

Example 3

[0132] Systemic Delivery of rAAVrh.74.MHCK7.Micro-Dys to Mdx Mice

[0133] Cohorts of mdx mice were injected via tail vein with either 2×10^{12} vg (8×10^{13} vg/kg) or high dose (planned clinical dose) 6×10^{12} vg (2×10^{14} vg/kg) of rAAVrh.74. MHCK7.micro-dys at 6 weeks of age. Following 12 weeks of treatment, all muscles were harvested and stained for dystrophin and restoration of DAPC components. Systemically injected (tail vein) mice showed high levels of staining of dystrophin throughout all muscles. FIG. **4**A represents the widespread transduction of skeletal, diaphragm and cardiac muscle fibers after a 6×10^{12} vg (2×10^{14} vg/kg) systemic dose. FIG. **4**B shows quantification of the percentage of muscle fibers expressing micro-dystrophin in each tissue. Finally the diaphragm was tested for functional improvement (FIG. **4**C). No significant difference was seen at low dose; however there was significant improvement at the high dose. Importantly, FIG. **5** demonstrates other components of the DAPC were completely restored following micro-dystrophin delivery. Shown is Beta-sarcoglycan (B-SG).

[0134] The toxicology/safety of AAVrh.74.MHCK7.Micro-dys are evaluating by administering the vector via intravenous (i.v.) injection to the tail vein of mdx mice per Table 1. There was no evidence of toxicity in any of the muscle tissues analyzed including: Tibialis anterior (TA),

Gastrocnemius (GAS), Quadriceps (QD), Psoas (PSO), Triceps (TRI), and Diaphragm (DIA) (FIG. **6**A and FIG. B). The number of centrally placed nuclei was decreased with the high dose 6×10^{12} vg (2×10^{14} vg/kg). Historically, central nucleation of skeletal muscles in untreated age matched mdx mice are on average ~80%. Finally, the preliminary data from a small sample size (n=3) demonstrates a decrease level of CK release (U/L) in serum of high dose treated mice (D). Independent t-tests were used to locate differences ($p<0.05$); Data are reported as means±SEM.

TABLE 1

Outline of toxicology/safety study of rAAVrh.74.MHCK7.Micro-dys in mice.

Cohort Number		Study Agent	Dose (vg/kg)	Treatment Day 0	Follow-up Day 1	Sacrificial End-Point Week 6	Extra
(1)	Low Dose	AAVrh.74.MHCK7.Micro-dys	8.0×10^{13}	Single i.v. injection to the tail vein of mdx mice	24 h Weight, Clinical Observations	5M	+2
(2)	High Dose	AAVrh.74.MHCK7.Micro-dys	2.0×10^{14}			5M	+2
(3)	Control	Vehicle (LRS)	0			5M	+2
TOTAL MICE						N = 21	

Example 4

[0135] Generation of the pAAV.MCK.Micro-Dystrophin Construct

[0136] The pAAV.MCK.micro-dystrophin plasmid was constructed by inserting the MCK expression cassette driving a codon optimized human micro-dystrophin cDNA sequence into the AAV cloning vector psub201 (Samulski et al., *J. Virol.* 61(10):3096-3101). A muscle-specific regulatory element was included in the construct to drive muscle-specific gene expression. This regulatory element comprised the mouse MCK core enhancer (206 bp) fused to the 351 bp MCK core promoter (proximal). After the core promoter, the construct comprises the 53 bp endogenous mouse MCK Exon1 (untranslated) for efficient transcription initiation, followed by the SV40 late 16S/19S splice signals (97 bp) and a small 5'UTR (61 bp). The intron and 5' UTR was derived from plasmid pCMVβ (Clontech). The micro-dystrophin cassette has a consensus Kozak immediately in front of the ATG start and a small 53 bp synthetic polyA signal for mRNA termination. The human micro-dystrophin cassette contains the (R4-R23/Δ71-78) domains as previously described by Harper et al. *Nat. Med.* 8(3):253-61, 2002

[0137] The pAAV.MCK.micro-dystrophin plasmid contained the human micro-dystrophin cDNA expression cassette flanked by AAV2 inverted terminal repeat sequences (ITR) (see FIG. **7**). This sequence was encapsidated into AAVrh.74 virions. The molecular clone of the AAVrh.74 serotype was cloned from a rhesus macaque lymph node and is described in Rodino-Klapac et al. *Journal of Tran. Med.* 45 (2007).

Example 5

[0138] Potency and Dose Analysis Using rAAV.MCK.Micro-Dystrophin

[0139] Expression studies were conducted with the human micro-dystrophin construct (rAAV.MCK.micro-dystrophin; described in Example 1) by intramuscular injection. The tibialis anterior (TA) muscle of mdx mice (spontaneous Dmd^{mdx} mutant mice that do not express dystrophin) were injected with 3×10^{9}, 3×10^{10}, or 1×10^{11} vg (n=3 per group). Four weeks later the muscles were harvested and stained for dystrophin expression using an antibody specific for the N-terminal Dys3 and hematoxylin and eosin (HE) staining. FIG. **8** show a linear correlation between expression and dose where very little expression (no effect level) at 3×10^{9} vg and 89% expression at 1×10^{11} vg.

Example 6

[0140] Vascular Delivery of rAAV.MCK.micro-dystrophin to mdx Mice

[0141] Using a model of isolated limb perfusion model (Rodino-Klapac et al., *J. Trans. Med.* 5(45): 1-11, 2007), mdx mice (n=10) were injected with 1×10^{11} vg of rAAVrh.74.MCK.micro-dystrophin via the femoral artery and performed outcomes analysis was carried out. Three months post gene transfer, lower limb muscles were harvested and efficacy studies demonstrated significant improvement in both force and resistance to eccentric contraction induced injury (FIG. **9**).

[0142] Dystrophin protein immunostaining in the extensor digitorum longus (EDL) muscle and TA muscle shows expression in a mdx myofibers following rAAVrh.74-MCK-Micro-dys treatment (FIG. **9**A). Mock-infected muscle was stained in an identical manner and exposures are time matched. FIG. **9**B demonstrates that rAAVrh.74-MCK-Micro-dys significantly increased normalized specific force relative to mock-treated mdx muscles ($P<0.05$ vs. mdx). In addition, the mdx muscles infected with rAAVrh.74-MCK-Micro-dys (human) were compared with mock-infected contralateral mdx EDL muscles (blue) and Wild Type (WT C57B1/10) EDL muscles for force drop during repetitive eccentric contractions at 12 weeks post gene transfer (FIG. **9**C). rAAVrh.74-MCK-micro-dystrophin (Micro-dys) treatment significantly protected against loss of force compared with mock-treated mdx muscles ($P<0.001$ vs. mdx).

Example 7

[0143] Co-Delivery AAVrh74.MHCK7.micro-dystrophin+AAVrh74.CMV.miR29C

[0144] To determine whether miR-29c/micro-dystrophin combined gene therapy approach would be more beneficial at reducing fibrosis,4-week-old mdx/utrn $^{+\backslash-}$ mice received an intramuscular injection of AAVrh74.MHCK7.micro-dystrophin and rAAVrh74.CMV.miR-29c at 5×10^{11} vgs each to

the left gastrocnemius muscle. rAAVrh.74. MHCK7.micro-dystrophin, and rAAVrh.74. MHCK7.micro-dystrophin alone. The mice were analyzed at 12 weeks post injection which is considered early therapy.

[0145] The pAAVrh74.MHCK7.micro-dystrophin plasmid contains the human micro-dystrophin cDNA expression cassette flanked by AAV2 inverted terminal repeat sequences (ITR) as shown in FIG. **10** and is described in detail in Example 1. It is this sequence that was encapsidated into AAV rh.74 virions.

[0146] The pAAV.CMV.miR29C plasmid contains the mir29c cDNA in a miR-30 stem loop backbone flanked by AAV2 inverted terminal repeat sequences (ITR). It is this sequence that was encapsidated into AAVrh.74 virions. In addition, a few nucleotides with in the miR-29c target sequence were changed to mimic Watson-crick pairing at this site as in shRNA-miR (luc). According to ShRNA-luc design, the hairpin should be perfectly complementary throughout its length. Plus, the more changes to the passenger strand, the more likely the elimination of any endogenous mechanism that regulates miR-29 processing that could recognize the miRNA via the stem. The 19th base of the guide strand was modified to a cytosine to mimic the nucleotide that precedes the cleavage site in natural mi-29c sequence and the corresponding base on the other strand was changed to preserve pairing.as shown in FIG. **12**.

[0147] Measurement of absolute (FIG. **13**A) and normalized specific (FIG. **13**B) following tetanic contraction demonstrated increased force with combination therapy compared to untreated mdx/utrn$^{+/-}$ muscle and micro-dystrophin therapy alone (*$p<0.05$). Muscles were then assessed for loss of force following repetitive eccentric contractions. Mice co-treated with miR-29c/micro-dystrophin and micro-dystrophin alone showed a protection from loss of force compared with untreated mdx/utrn$^{+\backslash-}$ muscles (FIG. **13**C). Sirius Red stain representative images demonstrating muscle fibers (green) and collagen content (red) are shown in FIG. **13**D.

[0148] GAS muscle was analyzed 12 months post-injection to assess collagen accumulation by Sirius Red staining and subsequent quantification with ImageJ. Sirius Red staining shows a reduction in collagen staining in both treated cohorts. (FIG. **14**A). Additional outcomes included miR-29c and collagen transcript levels. qRT-PCR confirms an increase in miR-29c transcript levels in the treated cohorts (n=2-3 for all groups) One-way ANOVA (FIG. **14**B).

[0149] To further validate reduction of collagen observed by picrosirius red staining, semi-quantitative qRT-PCR was performed on the muscle to quantify transcript levels of Col1A, Col3A and also another ECM component, fibronectin (Fbn). qRT-PCR analysis detected a decrease in Col1A and Col3A following co-treatment (FIGS. **14**C and **14**D). The analysis revealed that Fbn was significantly reduced only in the co-treated cohort (FIG. **14**E).

[0150] TGF-β1 has been previously shown to be up regulated in dystrophic muscle, likely playing a role in the initiation of the fibrotic cascade. TGF-β1 is a known pro-fibrotic cytokine that down regulates miR-29c and is responsible for conversion of myoblasts to myofibroblasts with an increase in collagen and muscle fibrogenesis. qRT-PCR analysis shows that co-treated muscle had lower levels of TGF-β1 compared to uninjected muscle and either treatment alone (FIG. **14**F).

[0151] The effect of AAV.CMV.miR-29c/MHCK7.micro-dystrophin combination therapy on muscle fiber diameter was also investigated. As shown in FIG. **15**, the combination therapy increased fiber diameter. FIGS. **15**A and **15**B demonstrate that MHCK7.micro-dystrophin treated muscle gas weights show no significant difference compared WT or untreated muscle, while miR-29c/micro-dystrophin combination treatment demonstrated an increase in average fiber size. Comparing mdx/utrn$^{+/-}$ controls with miR-29c/micro-dystrophin treated mdx/utrn$^{+/-}$, the average diameter increased from 29.02 to 33.61 μm (n=5-6 per group). FIG. **15**C demonstrates that the combination therapy produced a shift towards wild-type fiber size distribution in the mdx/utrn$^{+/-}$ mice. FIG. **15**D indicates that the number of muscle fibers per mm^2 in the miR-29c/micro-dystrophin combination treatment was no different from untreated mice or WT mice.

[0152] Initial results using rAAV.miR-29c as an anti-fibrotic therapy suggest that there is beneficial effect with reduction in collagen levels, a key contributor in fibrosis. Moreover, when combined with micro-dystrophin to improve membrane stability, miR29 up regulation normalized muscle force.

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                      note = FSE-I cut site
misc_feature          9..136
                      note = miR-30 backbone
misc_feature          139..160
                      note = miR-29c target (sense) strand
misc_feature          161..175
                      note = miR-29c target (sense) strand
misc_feature          176..199
```

-continued

```
                     note = miR-30 stem loop
misc_feature         200..288
                     note = miR-29c guide (antisense) strand
misc_feature         289..296
                     note = miR-30 backbone
source               1..296
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 7
ggccggcctg tttgaatgag gcttcagtac tttacagaat cgttgcctgc acatcttgga  60
aacacttgct gggattactt cttcaggtta acccaacaga aggctcgaga aggtatattg  120
ctgttgacag tgagcgcaac cgatttcaaa tggtgctaga gtgaagccac agatgtctag  180
caccatttga aatcggttat gcctactgcc tcggaattca aggggctact ttaggagcaa  240
ttatcttgtt tactaaaact gaataccttg ctatctcttt gatacattgg ccggcc      296

SEQ ID NO: 8         moltype = DNA  length = 22
FEATURE              Location/Qualifiers
misc_feature         1..22
                     note = Synthetic oligonucleotide
source               1..22
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 8
accgatttca aatggtgcta ga                                          22

SEQ ID NO: 9         moltype = DNA  length = 24
FEATURE              Location/Qualifiers
misc_feature         1..24
                     note = Synthetic oligonucleotide
source               1..24
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 9
tctagcacca tttgaaatcg gtta                                        24

SEQ ID NO: 10        moltype = DNA  length = 407
FEATURE              Location/Qualifiers
misc_feature         1..407
                     note = CMV promotor
source               1..407
                     mol_type = other DNA
                     organism = synthetic construct
SEQUENCE: 10
ctcgttacat aacttacggt aaatggcccg cctggctgac cgcccaacga cccccgccca  60
ttgacgtcaa taatgacgta tgttcccata gtaacgccaa tagggacttt ccattgacgt  120
caatgggtgg agtatttacg gtaaactgcc cacttggcag tacatcaagt gtatcatatg  180
ccaagtacgc cccctattga cgtcaatgac ggtaaatggc ccgcctggca ttatgcccag  240
tacatgacct tatgggactt tcctacttgg cagtacatct acgtattagt catcgctatt  300
accatggtga tgcggttttg gcagtacatc aatgggcgtg gatagcggtt tgactcacgg  360
ggatttccaa gtctccaccc cattgacgtc aatgggagtt tgttttg                407
```

1. A method of treating muscular dystrophy comprising administering i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of a recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

2. A method of increasing muscular force or muscle mass in a subject suffering from muscular dystrophy comprising administering i) a therapeutically effective amount of a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

3. A method of reducing or preventing fibrosis in a subject suffering from muscular dystrophy comprising administering i) a therapeutically effective amount of i) a recombinant AAV vector expressing micro-dystrophin and expression of micro-dystrophin is controlled by a muscle specific control element nucleotide sequence and ii) a therapeutically effective amount of recombinant AAV vector expressing miR-29c and expression of miR-29c is controlled by a muscle-specific control element nucleotide sequence.

4. The method of claim **1** wherein the muscular dystrophy is Duchenne muscular dystrophy.

5. The method of claim **1** wherein the nucleotide sequence encoding the micro-dystrophin protein comprises

a) a nucleotide sequence that is at least 85% identical to the nucleotide sequence SEQ ID NO: 1 and encodes a functional micro-dystrophin protein, or

b) the nucleotide sequences of SEQ ID NO: 1.

6. The method of claim **1** wherein the recombinant AAV vector expressing miR-29c comprises:

a) the nucleotide sequences of SEQ ID NO: 8 and SEQ ID NO: 9,

b) the nucleotide sequence of SEQ ID NO: 7, or

c) the nucleotide sequence of SEQ ID NO: 6.

7. The method of claim **1** wherein at least one of the muscle specific control element is 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).

8. The method of claim **1** wherein the muscle specific control element controlling expression of micro-dystrophin comprises SEQ ID NO: 2 (MHCK7).

9. The method of claim **1** wherein the muscle specific control element controlling expression of miR-29c comprises SEQ ID NO: 10 (CMV).

10. The method of claim **1** wherein the recombinant AAV vector expressing micro-dystrophin comprises i) the nucleotide sequences of SEQ ID NO: 1 (micro-dys) and ii) the nucleotide sequence of SEQ ID NO: 2 (MHCK7).

11. The method of claim **1** wherein the recombinant AAV vector expressing micro-dystrophin comprises the nucleotide sequence of SEQ ID NO: 3.

12. The method of claim **1** wherein the recombinant AAV vector expressing miR-29c comprises i) the nucleotide sequence of SEQ ID NO: 8 or SEQ ID NO: 9 and ii) the nucleotide sequence of SEQ ID NO: 10 (CMV).

13. The method of claim **1** wherein the recombinant AAV vector expressing miR-29c comprises the nucleotide sequence of SEQ ID NO: 6.

14. The method of claim **1** wherein the recombinant AAV vector expressing micro-dystrophin comprises i) the nucleotide sequences of SEQ ID NO: 1 (micro-dys) and ii) the nucleotide sequence of SEQ 2 (MHCK7), and wherein the recombinant AAV vector expressing miR-29c comprises i) the nucleotide sequence of SEQ ID NO: 8 or SEQ ID NO: 9 and ii) the nucleotide sequence of SEQ ID NO: 10 (CMV).

15. The method of claim **1** wherein the recombinant AAV vector expressing micro-dystrophin comprises the nucleotide sequence of SEQ ID NO: 3 and the recombinant AAV vector expressing miR-29c comprises the nucleotide sequence of SEQ ID NO: 6.

16. The method of claim **1** wherein at least one of the recombinant AAV vectors is the serotype AAVrh.74, AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 or AAV13.

17. The method of claim **1** wherein at least one of the recombinant AAV vectors is administered by intramuscular injection or intravenous injection.

18. The method of claim **1** wherein at least one of the recombinant AAV vectors is administered systemically.

19. The method of claim **18**, wherein at least one of the recombinant AAV vectors is parenterally administered by injection, infusion or implantation.

20-57. (canceled)

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