US011246941B2

(12) **United States Patent**
Geall et al.

(10) **Patent No.: US 11,246,941 B2**
(45) **Date of Patent: *Feb. 15, 2022**

(54) **COMPOSITIONS AND METHODS OF TREATING MUSCLE ATROPHY AND MYOTONIC DYSTROPHY**

(71) Applicant: **Avidity Biosciences, Inc.**, La Jolla, CA (US)

(72) Inventors: **Andrew John Geall**, Carlsbad, CA (US); **Venkata Ramana Doppalapudi**, San Diego, CA (US); **David Sai-Ho Chu**, La Jolla, CA (US); **Michael Caramian Cochran**, La Jolla, CA (US); **Michael Hood**, San Diego, CA (US); **Beatrice Diana Darimont**, San Diego, CA (US); **Rob Burke**, Encinitas, CA (US); **Yunyu Shi**, San Diego, CA (US); **Gulin Erdogan Marelius**, San Diego, CA (US); **Barbora Malecova**, La Jolla, CA (US)

(73) Assignee: **AVIDITY BIOSCIENCES, INC.**, La Jolla, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **17/187,650**

(22) Filed: **Feb. 26, 2021**

(65) **Prior Publication Data**

US 2021/0187116 A1 Jun. 24, 2021

Related U.S. Application Data

(60) Division of application No. 17/024,624, filed on Sep. 17, 2020, which is a continuation of application No. 16/435,422, filed on Jun. 7, 2019, now Pat. No. 10,881,743, which is a continuation of application No. PCT/US2018/064359, filed on Dec. 6, 2018.

(60) Provisional application No. 62/725,883, filed on Aug. 31, 2018, provisional application No. 62/595,545, filed on Dec. 6, 2017.

(51) **Int. Cl.**

C07H 21/04	(2006.01)
A61K 47/68	(2017.01)
A61K 31/712	(2006.01)
A61K 31/713	(2006.01)
C07K 16/18	(2006.01)
C12N 15/113	(2010.01)
A61P 21/00	(2006.01)
A61K 39/395	(2006.01)
A61K 9/51	(2006.01)

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

CPC ***A61K 47/6807*** (2017.08); ***A61K 31/712*** (2013.01); ***A61K 31/713*** (2013.01); ***A61K 39/395*** (2013.01); ***A61K 47/6849*** (2017.08); ***A61P 21/00*** (2018.01); ***C07K 16/18*** (2013.01); ***C12N 15/113*** (2013.01); *A61K 9/5107* (2013.01); *C12N 2310/14* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/317* (2013.01); *C12N 2310/3513* (2013.01); *C12N 2310/3515* (2013.01); *C12N 2320/31* (2013.01); *C12N 2320/32* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,694,778	A	9/1987	Learn et al.
5,142,047	A	8/1992	Summerton et al.
5,185,444	A	2/1993	Summerton et al.
5,334,711	A	8/1994	Sproat et al.
5,627,053	A	5/1997	Usman et al.
5,716,824	A	2/1998	Beigelman et al.
5,736,557	A	4/1998	Hofheinz et al.
5,889,136	A	3/1999	Scaringe et al.
5,898,031	A	4/1999	Crooke
6,008,400	A	12/1999	Scaringe et al.
6,107,094	A	8/2000	Crooke
6,111,086	A	8/2000	Scaringe
6,194,551	B1	2/2001	Idusogie et al.
6,528,624	B1	3/2003	Idusogie et al.
6,538,124	B1	3/2003	Idusogie et al.
6,821,783	B1	11/2004	Comely et al.
6,849,272	B1	2/2005	Langer et al.
6,884,869	B2	4/2005	Senter et al.
6,942,972	B2	9/2005	Farooqui et al.
7,056,704	B2	6/2006	Tuschl et al.
7,078,196	B2	7/2006	Tuschl et al.
7,132,519	B2	11/2006	Monforte et al.
7,250,496	B2	7/2007	Bentwich
7,351,855	B2	4/2008	Coutts et al.
7,364,731	B2	4/2008	Idusogie et al.
7,432,249	B2	10/2008	Crooke
7,432,250	B2	10/2008	Crooke
7,452,987	B2	11/2008	Giese et al.
7,459,547	B2	12/2008	Zamore et al.

(Continued)

FOREIGN PATENT DOCUMENTS

EP	0336675 A1	10/1989
EP	0334656 B1	3/1994

(Continued)

OTHER PUBLICATIONS

Bjarne et al. ("The myotonic dystrophies: molecular, clinical, and therapeutic challenges." The Lancet Neurology 11.10 (2012): 891-905).*

(Continued)

Primary Examiner — Kimberly Chong

(74) *Attorney, Agent, or Firm* — Wilson Sonsini Goodrich & Rosati

(57) **ABSTRACT**

Disclosed herein are polynucleic acid molecules, pharmaceutical compositions, and methods for treating muscle atrophy or myotonic dystrophy.

23 Claims, 69 Drawing Sheets

Specification includes a Sequence Listing.

(56) **References Cited**

U.S. PATENT DOCUMENTS

7,498,298 B2 3/2009 Doronina et al.
7,521,232 B2 4/2009 Moon
7,595,387 B2 9/2009 Leake et al.
7,629,321 B2 12/2009 Crooke
7,691,997 B2 4/2010 Khvorova et al.
7,732,593 B2 6/2010 Zamore et al.
7,750,144 B2 7/2010 Zamore et al.
7,772,203 B2 8/2010 Zamore et al.
7,786,290 B2 8/2010 Woppmann et al.
7,829,693 B2 11/2010 Kreutzer et al.
7,833,992 B2 11/2010 Vargeese et al.
7,834,171 B2 11/2010 Khvorova et al.
7,850,975 B2 12/2010 Mullis
7,893,245 B2 2/2011 Giese et al.
7,923,547 B2 4/2011 Mcswiggen et al.
7,928,217 B2 4/2011 Vornlocher et al.
7,943,762 B2 5/2011 Weller et al.
8,084,582 B2 12/2011 Dahiyat et al.
8,084,598 B1 12/2011 Bentwich
8,090,542 B2 1/2012 Khvorova et al.
8,137,695 B2 3/2012 Rozema et al.
8,202,979 B2 6/2012 Mcswiggen et al.
8,268,986 B2 9/2012 Beigelman et al.
8,273,866 B2 9/2012 Mcswiggen et al.
8,283,329 B2 10/2012 Fire et al.
8,288,352 B2 10/2012 Doronina et al.
8,304,530 B2 11/2012 Zamore et al.
8,309,704 B2 11/2012 Zamore et al.
8,309,705 B2 11/2012 Zamore et al.
8,324,370 B2 12/2012 Giese et al.
8,324,371 B2 12/2012 Popplewell et al.
8,329,892 B2 12/2012 Zamore et al.
8,334,373 B2 12/2012 Vornlocher et al.
8,361,979 B2 1/2013 Aartsma-Rus et al.
8,362,231 B2 1/2013 Tuschl et al.
8,372,968 B2 2/2013 Tuschl et al.
8,389,710 B2 3/2013 Bruno et al.
8,420,391 B2 4/2013 Tuschl et al.
8,445,237 B2 5/2013 Tuschl et al.
8,461,325 B2 6/2013 Popplewell et al.
8,481,698 B2 7/2013 Lieberman et al.
8,501,703 B2 8/2013 Bennett et al.
8,501,930 B2 8/2013 Rozema et al.
8,546,143 B2 10/2013 Kreutzer et al.
8,552,171 B2 10/2013 Tuschl et al.
8,557,292 B2 10/2013 Davis et al.
8,591,910 B2 11/2013 Mullis
8,604,184 B2 12/2013 Mullis et al.
8,609,105 B2 12/2013 Senter et al.
8,618,277 B2 12/2013 Beigelman et al.
8,632,997 B2 1/2014 Tuschl et al.
8,648,185 B2 2/2014 Mcswigen et al.
8,691,786 B2 4/2014 Rossi et al.
8,697,688 B2 4/2014 Howard et al.
8,746,999 B2 6/2014 Davis et al.
8,765,930 B2 7/2014 Tuschl et al.
8,772,469 B2 7/2014 Uhlmann et al.
8,778,902 B2 7/2014 Tuschl et al.
8,790,922 B2 7/2014 Tuschl et al.
8,796,016 B2 8/2014 Tuschl et al.
8,796,443 B2 8/2014 Khvorova et al.
8,835,402 B2 9/2014 Kole et al.
8,846,875 B2 9/2014 Schwartz et al.
8,846,894 B2 9/2014 Mcswiggen et al.
8,853,384 B2 10/2014 Tuschl et al.
8,895,718 B2 11/2014 Tuschl et al.
8,895,721 B2 11/2014 Tuschl et al.
8,895,722 B2 11/2014 Iversen et al.
8,906,847 B2 12/2014 Cleemann et al.
8,933,044 B2 1/2015 Tuschl et al.
8,933,215 B2 1/2015 Giese et al.
8,936,910 B2 1/2015 Mitsch et al.
8,969,526 B2 3/2015 Baehner et al.
8,993,745 B2 3/2015 Tuschl et al.
9,012,138 B2 4/2015 Tuschl et al.
9,012,621 B2 4/2015 Tuschl et al.
9,078,911 B2 7/2015 Lu
9,089,614 B2 7/2015 Lin et al.
9,096,636 B2 8/2015 Baker et al.
9,096,877 B2 8/2015 Johnson et al.
9,139,828 B2 9/2015 Platenburg et al.
9,175,286 B2 11/2015 Wilton et al.
9,175,289 B2 11/2015 Khvorova et al.
9,181,551 B2 11/2015 Mcswiggen et al.
9,193,753 B2 11/2015 Tuschl et al.
9,212,364 B2 12/2015 Sah et al.
9,222,092 B2 12/2015 Giese et al.
9,228,187 B2 1/2016 Wilton et al.
9,243,251 B2 1/2016 Popplewell et al.
9,243,252 B2 1/2016 Popplewell et al.
9,249,416 B2 2/2016 Wilton et al.
9,260,471 B2 2/2016 Cancilla et al.
9,284,551 B2 3/2016 Puri et al.
9,328,345 B2 5/2016 Li et al.
9,364,553 B2 6/2016 Lee
9,416,361 B2 8/2016 Iversen et al.
9,434,948 B2 9/2016 Sazani et al.
9,441,229 B2 9/2016 Wilton et al.
9,447,415 B2 9/2016 Wilton et al.
9,447,417 B2 9/2016 Sazani et al.
9,481,905 B2 11/2016 Chen et al.
9,499,818 B2 11/2016 Van
9,528,109 B2 12/2016 De et al.
9,605,019 B2 3/2017 Verdine et al.
9,657,294 B2 5/2017 Beigelman et al.
9,695,211 B2 7/2017 Wada et al.
9,695,423 B2 7/2017 Giese et al.
9,732,344 B2 8/2017 Beigelman et al.
9,765,338 B2 9/2017 Bennett et al.
9,771,588 B2 9/2017 Mcswiggen et al.
9,796,974 B2 10/2017 Rajeev et al.
9,890,379 B2 2/2018 De et al.
9,926,557 B2 3/2018 De et al.
9,982,257 B2 5/2018 Butler et al.
10,000,754 B2 6/2018 Beigelman et al.
10,487,149 B2 11/2019 Geall et al.
10,550,188 B2 2/2020 Geall et al.
10,787,519 B2 9/2020 Geall et al.
10,881,743 B2 1/2021 Geall et al.
2002/0142980 A1 10/2002 Thompson et al.
2003/0073207 A1 4/2003 Akhtar et al.
2004/0224893 A1 11/2004 Wang et al.
2007/0004665 A1 1/2007 McSwiggen et al.
2008/0097092 A1 4/2008 Khvorova et al.
2009/0092985 A1 4/2009 Cardozo et al.
2009/0149403 A1 6/2009 Maclachlan et al.
2009/0227655 A1 9/2009 Khan
2011/0039914 A1 2/2011 Pavco et al.
2011/0081362 A1 4/2011 Elledge et al.
2011/0263680 A1 10/2011 Khvorova et al.
2011/0263686 A1 10/2011 Wilton et al.
2011/0293512 A1 12/2011 Violette et al.
2011/0294753 A1 12/2011 De et al.
2011/0301218 A1 12/2011 Bozzoni et al.
2012/0065169 A1 3/2012 Hanson et al.
2012/0094299 A1 4/2012 Ranum et al.
2012/0122800 A1 5/2012 Kadushin et al.
2012/0172415 A1 7/2012 Voit et al.
2012/0270925 A1 10/2012 Wilton et al.
2013/0028919 A1 1/2013 Howard et al.
2013/0045520 A1 2/2013 Woolf et al.
2013/0052731 A1 2/2013 Ma et al.
2013/0164366 A1 6/2013 Kreutzer et al.
2013/0172238 A1 7/2013 Mitsch et al.
2013/0177579 A1 7/2013 Lin et al.
2013/0177631 A1 7/2013 Kreutzer et al.
2013/0309256 A1 11/2013 Lyon et al.
2014/0127239 A1 5/2014 Howard
2014/0194610 A1 7/2014 Verdine et al.
2014/0286970 A1 9/2014 Jeffrey et al.
2014/0288158 A1 9/2014 Rajeev et al.
2014/0294851 A1 10/2014 Nguyen
2014/0296321 A1 10/2014 Iversen
2014/0315862 A1 10/2014 Kaye

(56) **References Cited**

U.S. PATENT DOCUMENTS

2014/0357700	A1	12/2014	Rossi et al.
2015/0018540	A1	1/2015	Prakash et al.
2015/0037360	A1	2/2015	Smith
2015/0038554	A1	2/2015	Brown
2015/0038555	A1	2/2015	Brown
2015/0056220	A1	2/2015	Chennamsetty et al.
2015/0105539	A1	4/2015	Miao et al.
2015/0105540	A1	4/2015	Miao et al.
2015/0110791	A1	4/2015	Zhang et al.
2015/0111954	A1	4/2015	Sliz et al.
2015/0141492	A1	5/2015	Tuschl et al.
2015/0211006	A1	7/2015	Butler et al.
2015/0238516	A1	8/2015	Dowdy et al.
2015/0275212	A1	10/2015	Albaek et al.
2015/0366987	A1	12/2015	Bodyak et al.
2016/0002637	A1	1/2016	Sazani et al.
2016/0030332	A1	2/2016	Lee et al.
2016/0032288	A1	2/2016	Tuschl et al.
2016/0053262	A1	2/2016	Platenburg et al.
2016/0102135	A1	4/2016	Escobar-Cabrera
2016/0102148	A1	4/2016	Park et al.
2016/0193354	A1	7/2016	Noe et al.
2016/0298111	A1	10/2016	Bestwick et al.
2016/0304864	A1	10/2016	De et al.
2016/0304874	A1	10/2016	Krauss
2016/0304877	A1	10/2016	Swayze et al.
2016/0367687	A1	12/2016	Manoharan et al.
2017/0081425	A1	3/2017	Colletti et al.
2017/0107512	A1	4/2017	De et al.
2017/0204410	A1	7/2017	Watanabe et al.
2017/0204414	A1	7/2017	Van et al.
2017/0281795	A1	10/2017	Geall et al.
2017/0342416	A1	11/2017	Mcswiggen et al.
2018/0016574	A1	1/2018	Bestwick et al.
2018/0044675	A1	2/2018	Watanabe et al.
2018/0112214	A1	4/2018	De et al.
2018/0127758	A1	5/2018	Bennett
2018/0163209	A1	6/2018	Bennett et al.
2018/0305689	A1	10/2018	Sætrom et al.
2018/0344817	A1	12/2018	Smith et al.
2018/0369400	A1	12/2018	Levin et al.
2019/0000986	A1	1/2019	Levin et al.
2019/0062435	A1	2/2019	Geall et al.
2019/0062436	A1	2/2019	Geall et al.
2019/0192681	A1	6/2019	Geall et al.
2019/0240346	A1*	8/2019	Sugo A61K 48/0091
2020/0123261	A1	4/2020	Geall et al.
2020/0282074	A1	9/2020	Levin et al.
2020/0405877	A1	12/2020	Geall et al.

FOREIGN PATENT DOCUMENTS

EP	1144623	B1	8/2002
EP	0928290	B1	3/2005
EP	1214945	B1	6/2005
EP	1579015	A2	9/2005
EP	1352061	B1	5/2006
EP	1742958	A2	1/2007
EP	1407044	B1	9/2007
EP	1068241	B1	10/2007
EP	1550719	B1	12/2008
EP	2119783	A1	11/2009
EP	1349927	B1	3/2010
EP	1409670	B1	10/2010
EP	1633890	B1	10/2010
EP	2336317	A1	6/2011
EP	2361923	A2	8/2011
EP	2049664	B1	9/2011
EP	1608733	B1	12/2011
EP	1873259	B1	1/2012
EP	2278004	B1	10/2012
EP	2514758	A1	10/2012
EP	2580326	A1	4/2013
EP	2351852	B1	10/2013
EP	2195428	B1	12/2013
EP	2028278	B1	3/2014
EP	2348133	B1	7/2014
EP	2344637	B1	12/2014
EP	1633770	B1	4/2015
EP	2340310	B1	6/2015
EP	1423406	B2	11/2015
EP	2949752	A2	12/2015
EP	2548962	B1	1/2016
EP	3031920	A1	6/2016
EP	2421971	B1	7/2016
EP	2287306	B2	10/2016
EP	3030658	A4	3/2017
EP	2813582	B1	4/2017
EP	2287305	B2	11/2017
EP	2486141	B1	1/2018
EP	2902406	B1	1/2018
EP	2595664	B1	10/2018
WO	WO-9104753	A1	4/1991
WO	WO-9207065	A1	4/1992
WO	WO-9315187	A1	8/1993
WO	WO-9726270	A2	7/1997
WO	WO-9734631	A1	9/1997
WO	WO-9813526	A1	4/1998
WO	WO-0149698	A1	7/2001
WO	WO-2006000057	A1	1/2006
WO	WO-2006006948	A2	1/2006
WO	WO-2006128138	A2	11/2006
WO	WO-2007021142	A1	2/2007
WO	WO-2008036127	A2	3/2008
WO	WO-2009099942	A2	8/2009
WO	WO-2009099991	A2	8/2009
WO	WO-2009108217	A2	9/2009
WO	WO-2009126933	A2	10/2009
WO	WO-2009129281	A2	10/2009
WO	WO-2009144481	A2	12/2009
WO	WO-2011003557	A1	1/2011
WO	WO-2011009624	A1	1/2011
WO	WO-2012012443	A2	1/2012
WO	WO-2012092373	A2	7/2012
WO	WO-2013166004	A2	11/2013
WO	WO-2013166155	A1	11/2013
WO	WO-2014080251	A1	5/2014
WO	WO-2014140317	A2	9/2014
WO	WO-2014145090	A1	9/2014
WO	WO-2014154835	A2	10/2014
WO	WO-2014177042	A1	11/2014
WO	WO-2014197854	A1	12/2014
WO	WO-2015021457	A2	2/2015
WO	WO-2015038426	A1	3/2015
WO	WO-2015057699	A2	4/2015
WO	WO-2015069587	A2	5/2015
WO	WO-2015084846	A1	6/2015
WO	WO-2015107425	A2	7/2015
WO	WO-2015113922	A1	8/2015
WO	WO-2015200223	A1	12/2015
WO	WO-2016028649	A1	2/2016
WO	WO-2016187425	A1	11/2016
WO	WO-2016207240	A1	12/2016
WO	WO-2017148879	A1	9/2017
WO	WO-2017173408	A1	10/2017
WO	WO-2017192679	A1	11/2017
WO	WO-2017221883	A1	12/2017
WO	WO-2018002812	A1	1/2018
WO	WO-2018078131	A1	5/2018
WO	WO-2018078134	A1	5/2018
WO	WO-2018129384	A1	7/2018
WO	WO-2019060775	A1	3/2019
WO	WO-2019071028	A1	4/2019
WO	WO-2019113393	A1	6/2019

OTHER PUBLICATIONS

Parmar et al. ("5'-(E)-Vinylphosphonate: A Stable Phosphate Mimic Can Improve the RNAi Activity of siRNA-GalNAc Conjugates." ChemBioChem 17.11 (2016)).*

Aartsma-Rus et al. Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. Mol Ther 17(3):548-53 (2009).

(56) **References Cited**

OTHER PUBLICATIONS

Aartsma-Rus et al. Progress in therapeutic antisense applications for neuromuscular disorders. Eur J Hum Genet 18(2):146-153 (2010).
Aartsma-Rus et al. Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. Neuromuscul Disord. 12 Suppl 1:S71-7 (2002).
Abramova et al. Novel oligonucleotide analogues based on morpholino nucleoside subunits-antisense technologies: new chemical possibilities. Indian Journal of Chemistry 48B:1721-1726 (2009).
Agarwal et al. A Pictet-Spengler ligation for protein chemical modification. PNAS 110(1):46-51 (2013).
Albarran et al. Efficient intracellular delivery of a pro-apoptotic peptide with a pH-responsive carrier. React Funct Polym 71:261-265 (2011).
Arechavala-Gomeza et al. Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during dystrophin pre-mRNA splicing in human muscle. Hum Gene Ther. 18(9)798-810 (2007).
Axup et al. Synthesis of site-specific antibody-drug conjugates using unnatural amino acids. PNAS 109(40):16101-16106 (2012).
Baumer et al. Antibody-mediated delivery of anti-KRAS-siRNA in vivo overcomes therapy resistance in colon cancer. Clin Can Res 21 (6):1383-1394 (2015).
Beduneau et al. Design of targeted lipid nanocapsules by conjugation of whole antibodies and antibody Fab' fragments. Biomaterials 28(33):4978-4990 (2007).
Beigelman et al. Chemical modification of hammerhead ribozymes. Catalytic activity and nuclease resistance. J Biol Chem 270:25702-25708 (1995).
Bell et al. Epidermal Growth Factor Receptor Mutations and Gene Amplification in Non-Small-Cell Lung Cancer: Molecular Analysis of the IDEAL/INTACT Gefitinib Trials. J Clin Oncol 23(31):8081-8092 (2005).
Bird et al. Single-chain antigen-binding proteins. Science 242:423-442 (1988).
Blaney et al. Traceless solid-phase organic synthesis. Chem. Rev. 102:2607-2024 (2002.
Bulmus et al. A new pH-responsive and glutathione-reactive, endosomal membrane-disruptive polymeric carrier for intracellular delivery of biomolecular drugs. J Controlled Release 93:105-120 (2003).
Burke et al. siRNA-mediated knockdown of P450 oxidoreductase in rats: a tool to reduce metabolism by CYPs and increase exposure of high clearance compounds. Pharm. Res. 31(12):3445-3460 (2014).
Burlina et al. Chemical engineering of RNase resistant and catalytically active hammerhead ribozymes. Bioorg Med Chem 5:1999-2010 (1997).
Carter et al. Antibody-drug conjugates for cancer therapy. Cancer J 14(3):154-69 (2008).
Casi et al. Antibody-drug conjugates: basic concepts, examples and future perspectives. J Control Release 161:422-428 (201).
Casi et al. Site-specific traceless coupling of potent cytotoxic drugs to recombinant antibodies for pharmacodelivery. J Am Chem Soc 134(13):5887-5892 (2012).
Castaneda et al. Acid-cleavable thiomaleamic acid linker for homogeneous antibody-drug conjugation, Chem. Commun. 49:8187-8189 (2013).
Certified JP2016-122187, filed on Jun. 20, 2016 (English counterpart US2019240346).
Certified U.S. Appl. No. 62/316,919, filed Apr. 1, 2016.
Chen et al. Strand-specific 5'-0-methylation of siRNA duplexes controls guide strand selection and targeting specificity. RNA 14:263-274 (2008).
Clackson et al. Making antibody fragments using phage display libraries. Nature 352(6336):624-628 (1991).
Colberre-Garapin et al. A new dominant hybrid selective marker for higher eukaryotic cells. J Mol Biol 150:1-14 (1981).
Cole et al. The EBV-hybridoma technique and its application to human lung cancer. In, Monoclonal Antibodies and Cancer Therapy (vol. 27, UCLA Symposia on Molecular and Cellular Biology, New Series) (eds. R.A. Reisfeld and S.Sell), New York: Alan R. Liss, Inc. pp. 77-96 (1985).
Crouse et al. Expression and amplification of engineered mouse dihydrofolate reductase minigenes. Mol Cell Biol 3(2):257-266 (1983).
Cuellar et al. Systematic evaluation of antibody-mediated siRNA delivery using an industrial platform of THIOMAB-siRNA conjugates. Nucleic Acids Res 43(2):1189-1203 (2015).
Dawson et al. Modulation of Reactivity in Native Chemical Ligation through the Use of Thiol Additives. J. Am. Chem. Soc. 119:4325-4329 (1997).
Dawson et al. Synthesis of proteins by native chemical ligation. Science 266(5186):776-779 (1994).
De Angelis et al. Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells. PNAS USA 99:9456-9461 (2002).
Debinski et al. Monovalent immunotoxin containing truncated form of Pseudomonas exotoxin as potent antitumor agent. Cancer Research 52(19):5379-5385 (1992).
Deleavey et al. Designing chemically modified oligonucleotides for targeted gene silencing. Chem Biol. 19(8):937-954 (2012).
Dietel et al. A 2015 update on predictive molecular pathology and its role in targeted cancer therapy: a review focussing on clinical relevance. Cancer Gene Ther 22(9):417-430 (2015).
Dimasi et al. Development of a trispecific antibody designed to simultaneously and efficiently target three different antigens on tumor cells. Mol Pharm 12(9):3490-3501 (2015).
Domingo et al. Transferrin receptor as a target for antibody—drug conjugates. Methods in Enzymology 112:238-247 (1985).
Duncan et al. A polymer-Triton X-100 conjugate capable of pH-dependent red blood cell lysis: a model system illustrating the possibility of drug delivery within acidic intracellular compartments. J Drug Target 2:341-347 (1994).
Earnshaw et al. Modified oligoribonucleotides as site-specific probes of RNA structure and function. Biopolymers (Nucleic Acid Sciences) 48:39-55 (1998).
Echigoya et al. In Silico Screening Based on Predictive Algorithms as a Design Tool for Exon Skipping Oligonucleotides in Duchenne Muscular Dystrophy. PLoS One 10(3):e0120058 (2015).
El-Sayed et al. Rational design of composition and activity correlations for pH-responsive and glutathione-reactive polymer therapeutics. J Control Release 104:417-427 (2005).
Feener et al. Alternative splicing of human dystrophin mRNA generates isoforms at the carboxy terminus. Nature 338:509-511 (Apr. 6, 1989).
Flanary et al. Antigen delivery with poly(propylacrylic acid) conjugation enhanced MHC-1 presentation and T-cell activation. Bioconjugate Chem. 20:241-248 (2009).
Gaziova et al. Chemically defined polyethylene glycol siRNA conjugates with enhanced gene silencing effect. Bioorg Med Chem 22(7):2320-2326 (2014).
Giorgetti et al. Rescue of Metabolic Alterations in AR113Q Skeletal Muscle by Peripheral Androgen Receptor Gene Silencing. Cell Rep 17(1):125-136 (2016).
Goldmacher et al. Antibody-drug conjugates: using monoclonal antibodies for delivery of cytotoxic payloads to cancer cells. Therapeutic Delivery 2:397-416 (2011).
Goldspiel et al. Human gene therapy. Clin Pharm 12:488-505 (1993).
Griffey et al. 2'-0-aminopropyl ribonucleotides: a zwitterionic modification that enhances the exonuclease resistance and biological activity of antisense oligonucleotides, J. Med. Chem. 39(26):5100-5109 (1997).
Hackeng et al. Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology. PNAS USA 96:10068-10073 (1999).
Hanes et al. In vitro selection and evolution of functional proteins by using ribosome display. PNAS USA 94:4937-4942 (1997).

(56) **References Cited**

OTHER PUBLICATIONS

Hejesen et al. A traceless aryl-triazene linker for DNA-directed chemistry. Org Biomol Chem 11(15):2493-2497 (2013).
Henry et al. pH-responsive poly(styrene-alt-maleic anhydride) alkylamide copolymers for intracellular drug delivery. Biomacromolecules 7:2407-2414 (2006).
Hitachi et al. Role of microRNAs in skeletal muscle hypertrophy. Front Physiol 16(4):408 (2014).
Hoffman et al. Restoring Dystrophin Expression in Duchenne Muscular Dystrophy Muscle: Progress in Exon Skipping and Stop Codon Read Through. Am J Pathol 179(1):12-22 (2011).
Hu et al. Site-specific Antibody-polymer Conjugates for siRNA Delivery. J Am Chem Soc 135(37):13885-13891 (2013).
Huang et al. Mechanisms of resistance to EGFR tyrosine kinase inhibitors. Acta Pharma Sinica B 5(5):390-401 (2015).
Hudson et al. Cellular delivery of hammerhead ribozymes conjugated to a transferrin receptor antibody. Int J Pharmaceuticals 182(1):49-58 (1999).
Huse et al. Generation of a large combinatorial library of the immunoglobulin repertoire in phage lambda. Science 246(4935):1275-1281 (1989).
Huston et al. Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. PNAS USA 85(16):5879-5883 (1988).
Ishikawa et al. Preparation of monomeric Fab'—horseradish peroxidase conjugate using thiol groups in the hinge and its evaluation in enzyme immunoassay and immunohistochemical staining. Ann N Y Acad Sci. 420:74-89 (1983).
Iversen et al. Optimized siRNA-PEG conjugates for extended blood circulation and reduced urine excretion in mice. Theranostics 3(3):201-209 (2013).
Jancik et al. Clinical relevance of KRAS in human cancers. J Biomed Biotechnol 2010:150960 (13 pgs.) (2010).
Jearawiriyapaisarn et al. Sustained Dystrophin Expression Induced by Peptide-conjugated Morpholino Oligomers in the Muscles of mdx Mice. Mol Ther. 16(9): 1624-1629 (2008).
Jones et al. Poly(2-alkylacrylic acid) polymers deliver molecules to the cytosol by pH-sensitive disruption of endosomal vesicles. Biochem J 372:65-75 (2003).
Karpeisky et al. Highly efficient synthesis of 2'-O-amino nucleosides and their incorporation in hammerhead ribozymes. Tetrahedron Lett 39:1131-1134 (1998).
Khormaee et al. Endosomolyticanionic polymer for the cytoplasmic delivery of siRNAs in localized in vivo applications. Adv Funct Mater 23:565-574 (2013).
Kim et al. PEG conjugated VEGF siRNA for anti-angiogenic gene therapy. J Cont Rel 116:123-129 (2006).
Kohler et al. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497 (1975).
Koizumi. Ena oligonucleotides as therapeutics. Curr Opin Mol Ther 8(2):144-149 (2006).
Kontermann et al. Bispecific antibodies. Drug Discov Today 20(7):838-847 (2015).
Kozbor et al. The production of monoclonal antibodies from human lymphocytes. Immunology Today 4:72-79 (1983).
Kutmeier et al. Assembly of humanized antibody genes from synthetic oligonucleotides using a single-round PCR. BioTechniques 17:242 (1994).
Langlois et al. Cytoplasmic and Nuclear Retained DMPK mRNAS Are Targets for RNA Interference in Myotonic Dystrophy Cells. J biol Chem 280(17):16949-16954 (2005).
Lee et al. Antisense PMO cocktails effectively skip dystrophin exons 45-55 in myotubes transdifferentiated from DMD patient fibroblasts. PLoS One 13(5):e0197084 (2018).
Leigh et al. The Human Plasma Proteome: History, Character, and Diagnostic Prospects. Mol Cell Proteomics 1:845-867 (2002).
Leroy et al. Epidermal growth factor receptor down-regulation triggers human myoblast differentiation. PLOS One 8(8):e71770 (2013).
Levin. Targeting Therapeutic Oligonucleotides. N Engl J Med 376:86-88 (2017).
Loakes. Survey and summary: The applications of universal DNA base analogues. Nucleic Acids Research 29:2437-2447 (2001).
Loh et al. A Survey of siRNA Nanoscal Delivery Patents. 11 Nanotechnology Law & Bus. (pp. 29-37) (2014).
Lowy et al., Isolation of transforming DNA: Cloning the hamster aprt gene. Cell 22:817-823 (1980).
Lyon et al. Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates. Nat. Biotechnol. 32(10):1059-1062 (2014).
Martinez et al. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110(5):563-574 (2002).
McEnaney et al. Antibody-recruiting molecules: an emerging paradigm for engaging immune function in treating human disease. ACS Chem Biol. 7(7):1139-1151 (2012).
Mei et al. FBXO32 Targets c-Myc for Proteasomal Degradation and Inhibits c-Myc Activity. J Biol Chem 290:16202-16214 (2015).
Miyata et al. Polymer nanotechnolgoy for nucleic acid delivery. Drug Delivery System 31(1):44-53 (2016) (English Abstract).
Morgan et al. Human gene therapy. Ann Rev Biochem 62:191-217 (1993).
Morrison et al. Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains. PNAS USA 81(21):6851-6855 (1984).
Mulders et al. Molecular therapy in myotonic dystrophy: focus on RNAgain-of-function. Hum Mol Gen 19(R1):R90-R97 (2010).
Mulders et al. Triplet-repeat oligonucleotide-mediated reversal of RNA toxicity in myotonic dystrophy. PNAS 106(33):13915-13920 (2009).
Mulligan et al. Selection for animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. PNAS USA 78(4):2072-2076 (1981).
Mulligan. The basic science of gene therapy. Science 260(5110):926-932 (1993).
Muratovska et al. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. FEBS Lett. 558(1-3):63-8 (2004).
Naisbitt et al. Disposition of amodiaquine and related antimalarial agents in human neutrophils: implications fordrug design. J Pharmacol Exp Ther 280:884-893 (1997).
Neuberger et al. Recombinant antibodies possessing novel effector functions. Nature 312(5995):604-608 (1984).
Nielsen et al. Advances in targeted delivery of small interfering RNA using simple bioconjugates. Expert Opinion on Drug Delivery 11(5):791-822 (2014).
Normand-Sdiqui et al. Oligonucleotide delivery: Uptake of rat transferrin receptor antibody (OX / 26) conjugates into an in vitro immortalised cell line model of the blood, brain barrier. Int J Pharmaceut. Int J Pharmaceuticals 163:63-71 (1998).
Obika et al. Synthesis of 2'-0,4'-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3'-endo sugar puckering. Tetrahedron Lett. 38(50):8735-8738 (1997).
O'Hare et al. Transformation of mouse fibroblasts to methotrexate resistance by a recombinant plasmid expressing a prokaryotic dihydrofolate reductase. PNAS USA 78:1527-1531 (1981).
Parmar et al. 5'-(E)-Vinylphosphonate: A Stable Phosphate Mimic Can Improve the RNAi Activity of siRNA-GalNAc Conjugates. Chembiochem 17(11):985-989 (2016).
PCT International Application No. PCT/JP2017/022509, Filed Jun. 19, 2017 (English counterpart US2019240346).
PCT/US2017/025608 International Search Report and Written Opinion dated Jul. 10, 2017.
PCT/US2018/012672 International Search Report and Written Opinion dated May 24, 2018.
PCT/US2018/012672 Invitation to Pay Additional Fees dated Mar. 20, 2018.
PCT/US2018/052289 International Search Report and Written Opinion dated Jan. 11, 2019.
PCT/US2018/054444 International Search Report and Written Opinion dated Feb. 15, 2019.
PCT/US2018/064359 International Invitation to Pay Additional Fees dated Feb. 14, 2019.

(56) **References Cited**

OTHER PUBLICATIONS

PCT/US2018/064359 International Search Report and Written Opinion dated Apr. 11, 2019.
PCT/US2018/54444 Invitation to Pay Additional Fees dated Dec. 27, 2018.
Pei et al. Quantitative evaluation of siRNA delivery in vivo. RNA 16:2553-2563 (2010).
Perrault et al. Mixed deoxyribo- and ribo-oligonucleotides with catalytic activity. Nature 344:565-568 (1990).
Pieken et al. Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes. Science 253:314-317 (1991).
Rozema et al. Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. PNAS USA 104(32):12982-12987 (2007).
Sacheck et al. Rapid disuse and denervation atrophy involve transcriptional changes similar to those of muscle wasting during systemic diseases. The FASEB Journal 21:140-155 (2007).
Santerre et al. Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells. Gene 30(1-3):147-156 (1984).
Sartori et al. Smad2 and 3 transcription factors control muscle mass in adulthood. Am J Physiol Cell Physiol 296:C1248-C1257 (2009).
Schnyder et al. Targeting of skeletal muscle in vitro using biotinylated immunoliposomes. Biochem J 377(Pt.1):61-67 (2004).
Schwarz et al. Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. Molecular Cell 10:537-548 (2002).
Sekyere et al. Examination of the distribution of the transferrin homologue, melanotransferrin (tumour antigen p97), in mouse and human. Biochimica et Biophysica Acta 1722(2):131-142 (2005).
Singh et al. Recent developments in oligonucleotide conjugation. Chem Soc Rev 39(6):2054-2070 (2010).
Skerra et al. Assembly of a functional Immunoglobulin Fv fragment in *Escherichia coli*. Science 240(4855):1038-1041 (1988).
Strop et al. Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates. Chem Biol 20(2):161-167 (2013).
Sugo et al. Development of antibody-siRNA conjugate targeted to cardiac and skeletal muscles. J Control release 237:1-13 (2016).
Summerton, et al. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev. Jun. 1997;7(3):187-95.
Suñé-Pou et al. Targeting Splicing in the Treatment of Human Disease. Genes 8:E87 (2017).
Suriano et al. Beta-catenin (CTNNB1) gene amplification: a new mechanism of protein overexpression in cancer. Genes Chromosomes Cancer 42(3):238-246 (2005).
Szybalska et al. Genetics of human cell line. IV. DNA-mediated heritable transformation of a biochemical trait. PNAS USA 48:2026-2034 (1962).
Takeda et al. Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences. Nature 314(6010):452-454 (1985).
Talasila et al. EGFR Wild-type Amplification and Activation Promote Invasion and Development of Glioblastoma Independent of Angiogenesis. Acta Neuropathol. 125(5):683-698 (2013).
Tolstoshev. Gene Therapy, Concepts, Current Trials and Future Directions. Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993).
Turner et al. The myotonic dystrophies: diagnosis and management. J Neurol Neurosurg Psychiatry 81:358-367 (2010).
U.S. Appl. No. 15/473,849 1st Action Interview dated Nov. 24, 2017.
U.S. Appl. No. 15/476,849 1st Action Interview dated Sep. 11, 2017.
U.S. Appl. No. 15/476,849 Office Action dated Apr. 17, 2018.
U.S. Appl. No. 15/476,849 Office Action dated May 2, 2018.
U.S. Appl. No. 16/128,393 Office Action dated Aug. 26, 2019.
U.S. Appl. No. 16/128,417 Office Action dated Mar. 27, 2020.
U.S. Appl. No. 16/128,417 Office Action dated Sep. 30, 2019.
U.S. Appl. No. 16/128,428 Office Action dated Mar. 27, 2020.
U.S. Appl. No. 16/128,428 Office Action dated Oct. 1, 2019.
U.S. Appl. No. 16/128,440 Office Action dated May 30, 2019.
U.S. Appl. No. 16/128,440 Office Action dated Nov. 9, 2018.
U.S. Appl. No. 16/128,450 Miscellaneous Communication re: Third Party Submission dated Jul. 1, 2019.
U.S. Appl. No. 16/128,450 Office Action dated Apr. 19, 2019.
U.S. Appl. No. 16/128,450 Office Action dated Apr. 30, 2020.
U.S. Appl. No. 16/128,450 Office Action dated Dec. 16, 2020.
U.S. Appl. No. 16/128,450 Office Action dated Sep. 19, 2019.
U.S. Appl. No. 16/129,694 Office Action dated May 30, 2019.
U.S. Appl. No. 16/129,694 Office Action dated Nov. 19, 2018.
U.S. Appl. No. 16/129,696 Miscellaneous Communication re: Third Party Submission dated Jul. 3, 2019.
U.S. Appl. No. 16/129,696 Office Action dated Apr. 13, 2020.
U.S. Appl. No. 16/129,696 Office Action dated Apr. 17, 2019.
U.S. Appl. No. 16/129,696 Office Action dated Dec. 14, 2020.
U.S. Appl. No. 16/129,696 Office Action dated Sep. 19, 2019.
U.S. Appl. No. 16/152,324 Office Action dated Oct. 16, 2020.
U.S. Appl. No. 16/435,422 Miscellaneous Communication re: Third Party Submission dated Apr. 1, 2020.
U.S. Appl. No. 16/435,422 Office Action dated Jul. 2, 2020.
U.S. Appl. No. 16/435,422 Office Action dated Oct. 31, 2019.
U.S. Appl. No. 16/718,092 Miscellaneous Communication re: Third Party Submission dated Oct. 27, 2020.
U.S. Appl. No. 16/435,422 Office Action dated Apr. 15, 2020.
Usman et al. Exploiting the chemical synthesis of RNA. Trends Biochem Sci 17:334-339 (1992).
Valtorta et al. KRAS gene amplification in colorectal cancer and impact on response to EGFR-targeted therapy. Int J Cancer 133:1259-1266 (2013).
Van Deutekom et al. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum Mol Genet. 10(15):1547-54 (2001).
Van Vliet et al. Assessment of the feasibility of exon 45-55 multiexon skipping for duchenne muscular dystrophy. BMC Medical Genetics 9:105 (2008).
Verma et al. Modified oligonucleotides: synthesis and strategy for users. Annu Rev Biochem 67:99-134 (1998).
Vickers et al. Efficient Reduction of Target RNAs by Small Interfering RNA and RNase H-dependent Antisense Agents. J. Biol. Chem 278:7108-7118 (2003).
Walker et al. Improved cellular delivery of antisense oligonucleotides using transferrin receptor antibody-oligonucleotide conjugates. Pharmaceutical research 12(10):1548-1553 (1995).
Ward et al. Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. Nature 341(6242):544-546 (1989).
Watts et al. Chemically modified siRNA: tools and applications. Drug Discov Today 13(19-20):842-855 (2008).
Wigler et al. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11:223-232 (1977).
Wigler et al. Transformation of mammalian cells with an amplifiable dominant-acting gene. PNAS USA 77:3567-3570 (1980).
Winkler. Oligonucleotide conjugates for therapeutic applications. Ther Del 4(7):791-809 (2013).
Wong et al. Co-injection of a targeted, reversibly masked endosomolytic polymer dramatically improves the efficacy of cholesterol-conjugated small interfering RNAs in vivo. Nucleic Acid Ther 22(6):380-390 (2012).
Wu et al. Building complex glycopeptides: Development of a cysteine-free native chemical ligation protocol. Angew. Chem. Int. Ed. 45:4116-4125 (2006).
Wu et al. Cell-penetrating peptides as transporters for morpholino oligomers: effects of amino acid composition on intracellular delivery and cytotoxicity. Nucleic Acids Res 35(15):5182-5191 (2007).
Wu et al. Delivery systems for gene therapy. Biotherapy 3:87-95 (1991).
Wu et al. Site-specific chemical modification of recombinant proteins produced in mammalian cells by using the genetically encoded aldehyde tag. PNAS USA 106(9):3000-3005 (2009).
Xu et al. Delivery systems for siRNA drug development in cancer therapy. Asian Journal of Pharmaceutical Sciences 10(1):1-12 (2015).

(56) **References Cited**

OTHER PUBLICATIONS

Yazdi et al. Influence of cellular trafficking on protein synthesis inhibition of immunotoxins directed against the transferrin receptor. Cancer Res 55:3763-3771 (1995).
Yessine et al. Characterization of the membrane-destabilizing properties of different pH-sensitive methacrylic acid copolymers. Biochimica et Biophysica Acta 1613:28-38 (2003).
Yuan et al. Development of siRNA payloads to target KRAS-mutant cancer. Cancer Discov 4(10):1182-1197 (2014).
Zhang et al. A remote arene-binding site on prostate specific membrane antigen revealed by antibody-recruiting small molecules. J Am Chem Soc. 132(36):12711-12716 (2010).
Co-pending U.S. Appl. No. 17/214,525, inventors Doppalapudi; Venkata Ramana et al., filed Mar. 26, 2021.
Homo sapiens DM1 protein kinase (DMPK), transcript variant 7, mRNA. NCBI reference sequence NM_001288766 (Mar. 12, 2019).
U.S. Appl. No. 17/024,624 Office Action dated Jul. 6, 2021.
U.S. Appl. No. 17/024,624 3rd Party Submission filed Feb. 23, 2021.
U.S. Appl. No. 17/024,624 Office Action dated Mar. 19, 2021.
Xia et al. Intravenous siRNA of brain cancer with receptor targeting and avidin-biotin technology. Pharm Res 24(12):2309-16 (2007).

* cited by examiner

FIG. 1

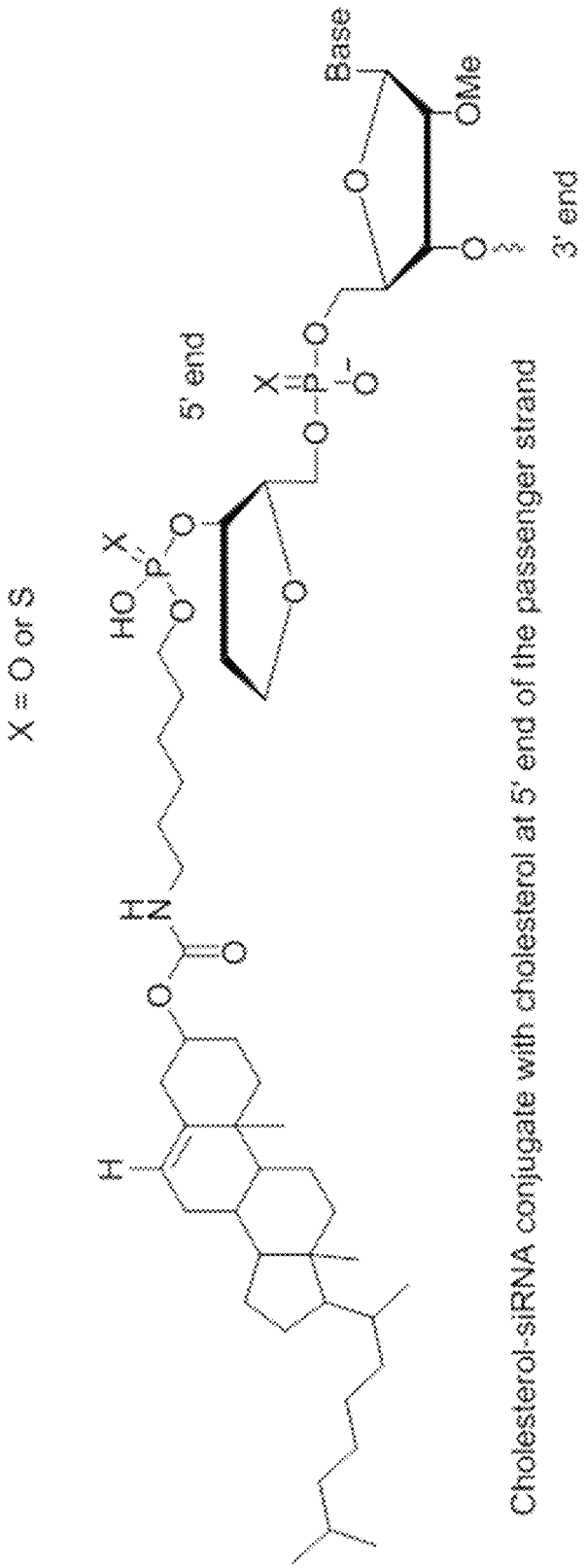

X = O or S
HO
X
P
O
5' end
H
N
O
Base
OMe
3' end
Cholesterol-siRNA conjugate with cholesterol at 5' end of the passenger strand

FIG. 2

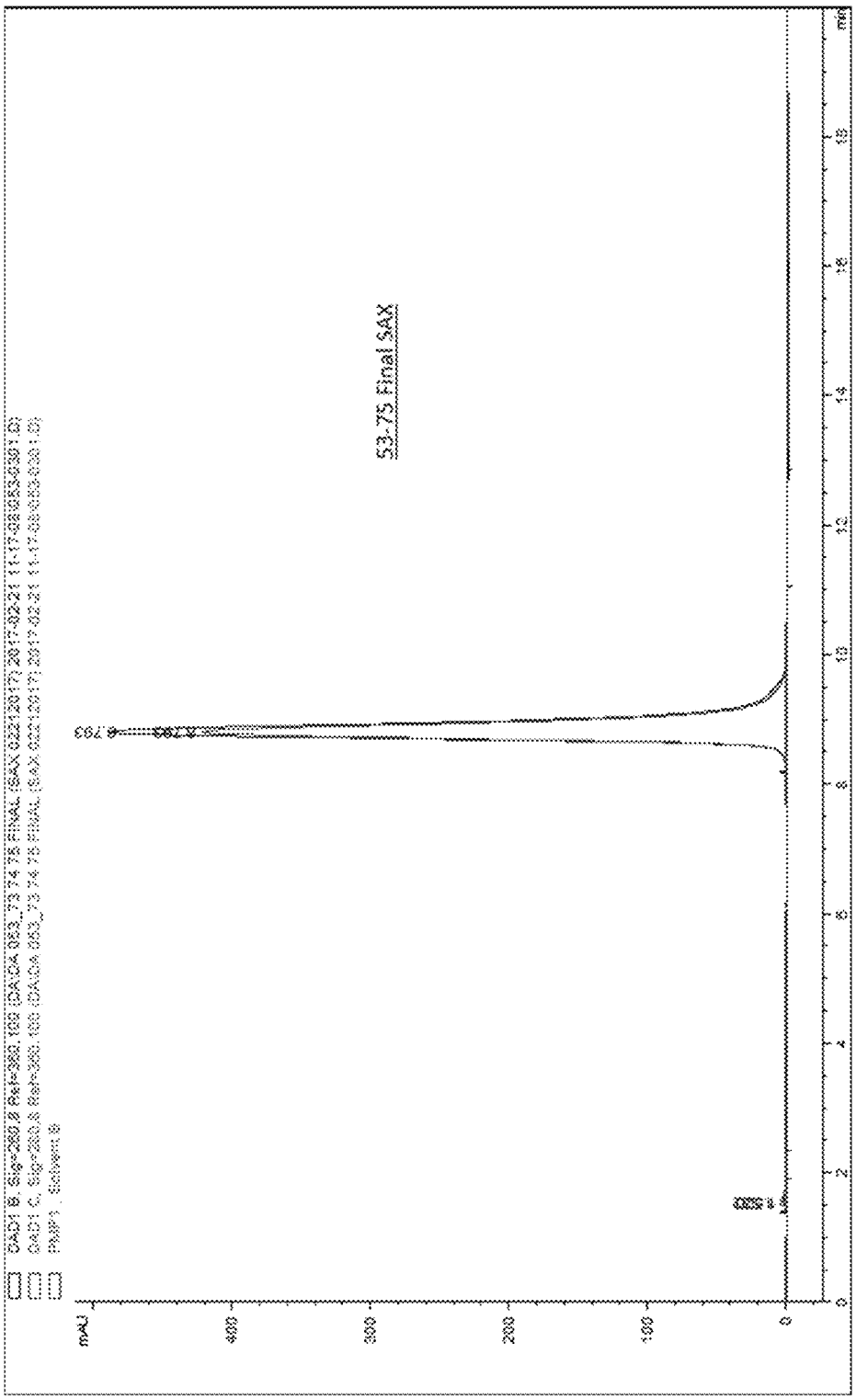


FIG. 3

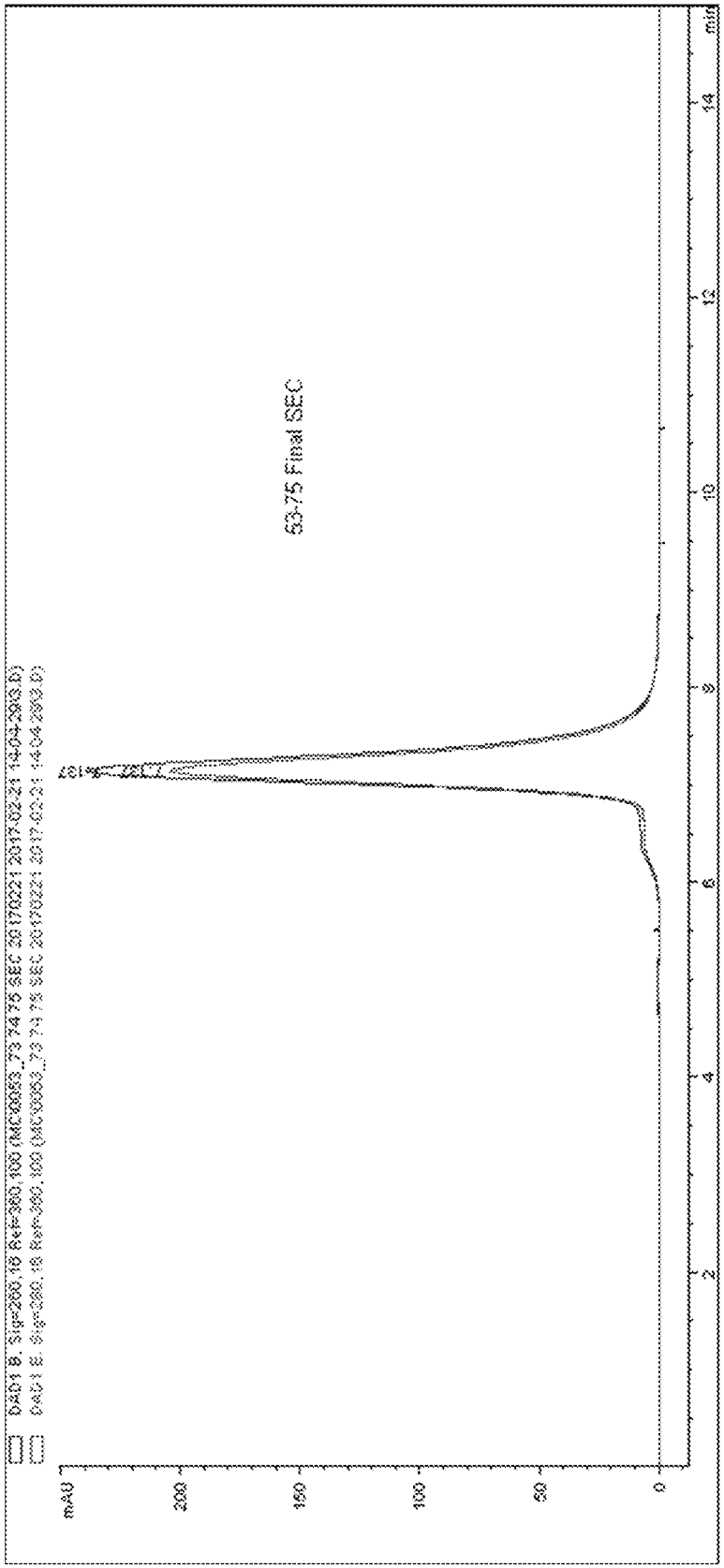


FIG. 4

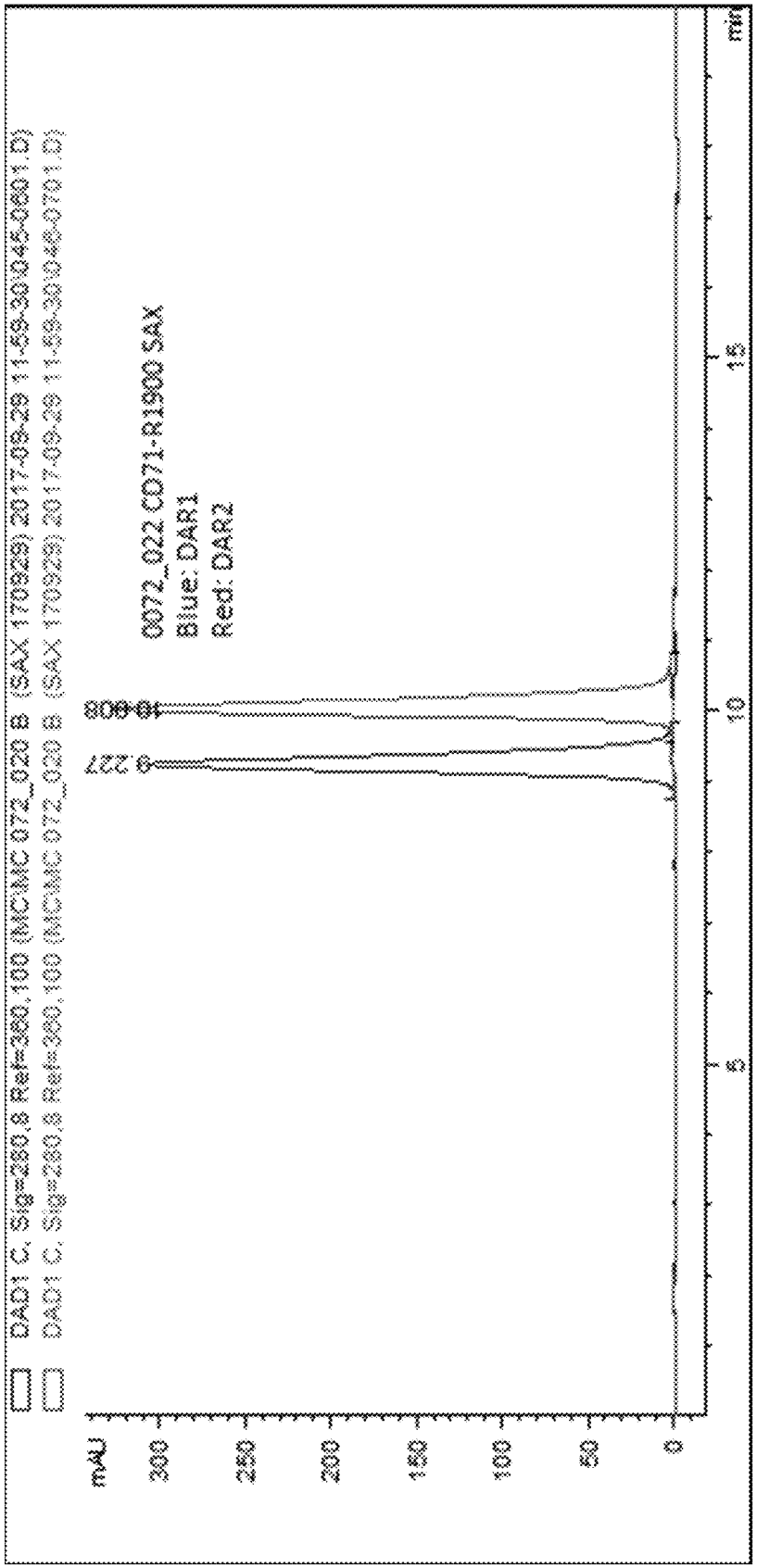

DAD1 C, Sig=280,8 Ref=360,100 (MCMC 072_020 B (SAX 170929) 2017-09-29 11-49-30\045-0501.D)
DAD1 C, Sig=280,8 Ref=360,100 (MCMC 072_020 B (SAX 170929) 2017-09-29 11-49-30\046-0701.D)
0072_022 CO71-R1500 SAX
Blue: DAR1
Red: DAR2
9.227
mAU
200
250
200
150
100
50
0
5
10
15
min

FIG. 5

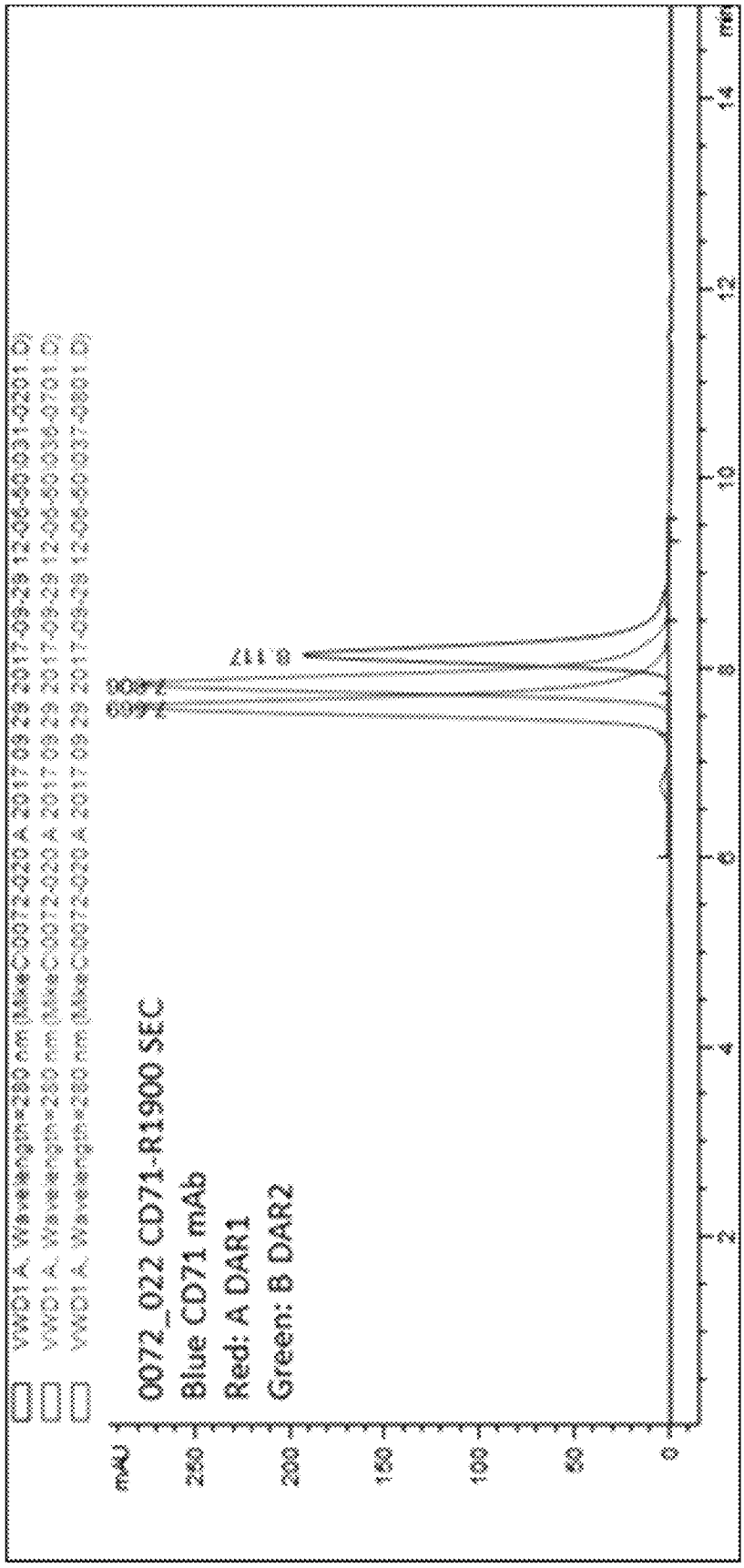

0072_022 CD71-R1900 SEC
Blue CD71 mAb
Red: A DAR1
Green: B DAR2
mAU
250
200
150
100
50
0
2
4
6
8
10
12
14
min

FIG. 6

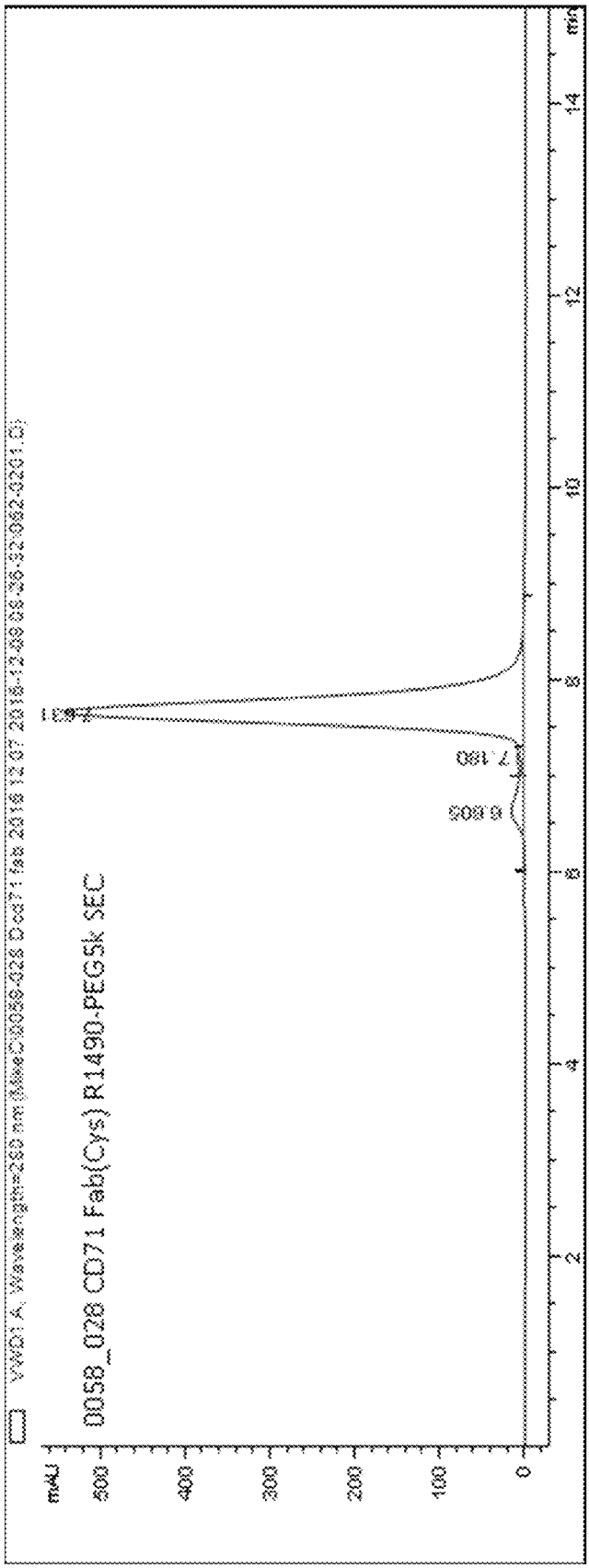

0058_028 CD71 Fab(Cys) R149D-PEG5k SEC
7.180
6.805
mAU
600
400
300
200
100
0
2
4
6
8
10
12
14
min

FIG. 7

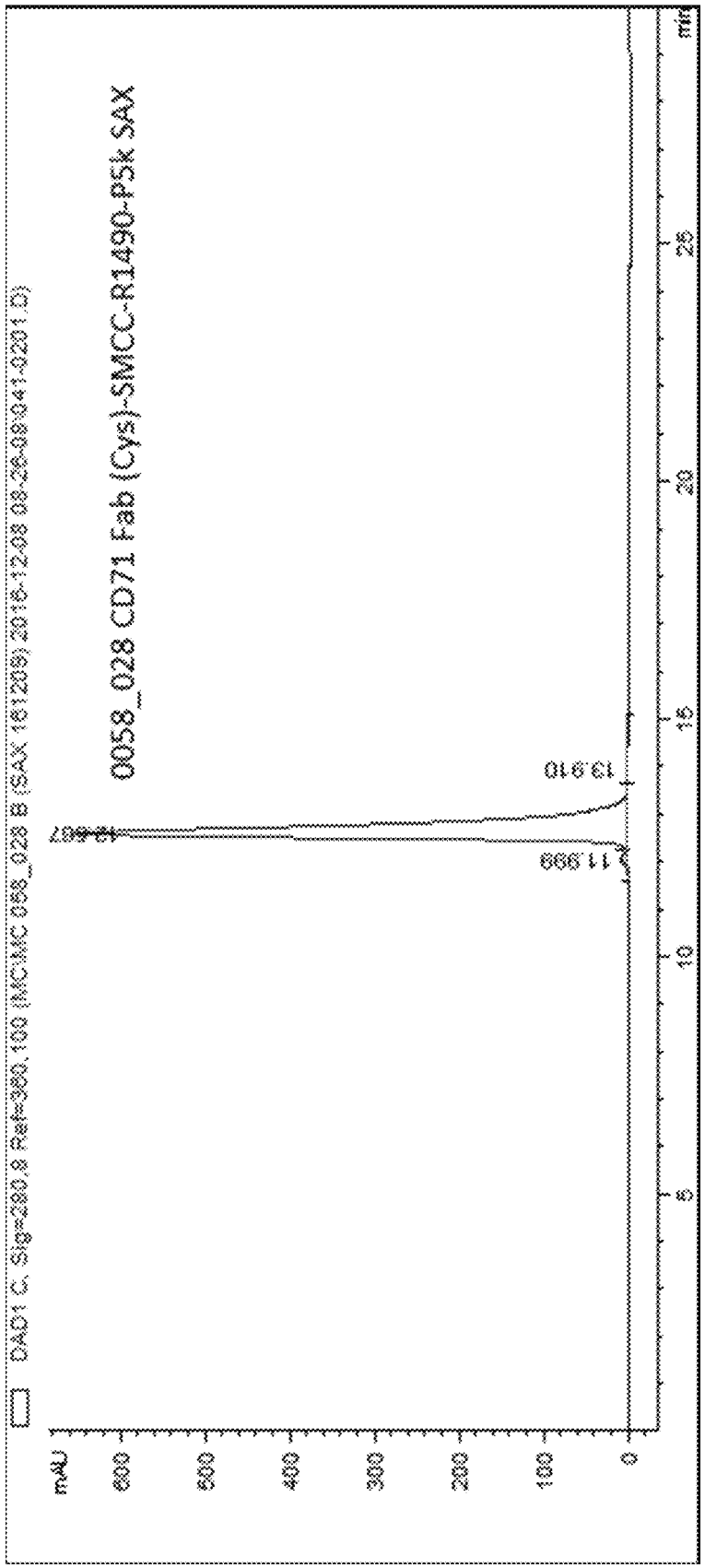

0058_028 CD71 Fab (Cys)-SMCC-R1490-P5k SAX
DAD1 C, Sig=280,8 Ref=360,100 (MC\MC 058_028 B (SAX 161209) 2016-12-08 08-26-08-041-0201.D)
mAU
min
11.999
13.910

FIG. 8

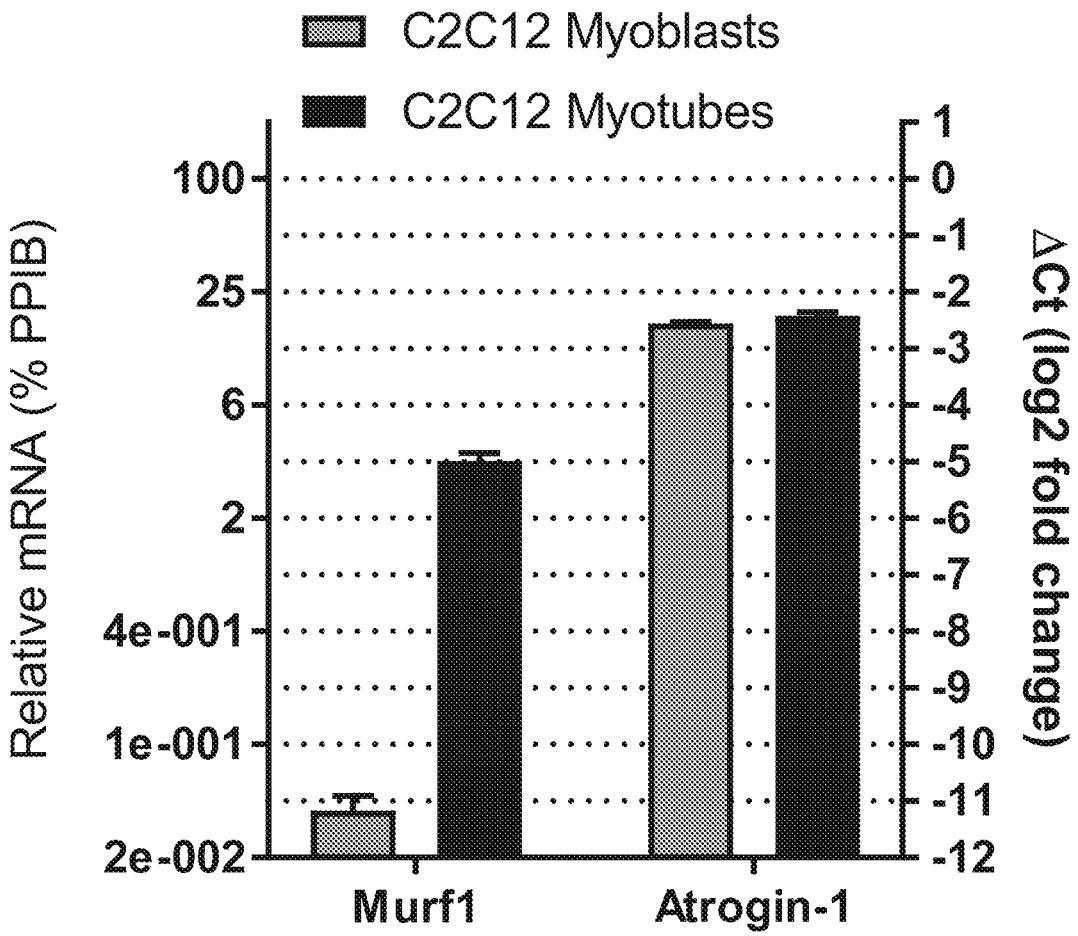

C2C12 Myoblasts
C2C12 Myotubes
Relative mRNA (% PPIB)
100
25
6
2
4e-001
1e-001
2e-002
ΔCt (log2 fold change)
1
0
-1
-2
-3
-4
-5
-6
-7
-8
-9
-10
-11
-12
Murf1
Atrogin-1

FIG. 9A

Group	Test Article	N	siRNA Dose (mg/kg)	Dose Volume (mL/kg)	# of Doses	Harvest Time (h)
1	TfR-mAb-HPRT-PEG5k (n=1)	4	3	**5.0**	1	96
2	TfR-mAb-HPRT-PEG5k (n=1)	4	1	**5.0**	1	96
3	TfR-mAb-HPRT-PEG5k (n=1)	4	0.3	**5.0**	1	96
4	TfR-mAb-HPRT-PEG5k (n=1)	4	0.1	**5.0**	1	96
5	IgG2a-mAb-HPRT-PEG5k (n=1)	4	3	**5.0**	1	96
6	IgG2a-mAb-HPRT-PEG5k (n=1)	4	1	**5.0**	1	96
7	IgG2a-mAb-HPRT-PEG5k (n=1)	4	0.3	**5.0**	1	96
8	IgG2a-mAb-HPRT-PEG5k (n=1)	4	0.1	**5.0**	1	96
9	TfR-mAb-MSTN-PEG5k (n=1)	4	3	**5.0**	1	96
10	TfR-mAb-MSTN-PEG5k (n=1)	4	1	**5.0**	1	96
11	TfR-mAb-MSTN-PEG5k (n=1)	4	0.3	**5.0**	1	96
12	TfR-mAb-MSTN-PEG5k (n=1)	4	0.1	**5.0**	1	96
13	IgG2a-mAb-MSTN-PEG5k (n=1)	4	3	**5.0**	1	96
14	IgG2a-mAb-MSTN-PEG5k (n=1)	4	1	**5.0**	1	96
15	IgG2a-mAb-MSTN-PEG5k (n=1)	4	0.3	**5.0**	1	96
16	IgG2a-mAb-MSTN-PEG5k (n=1)	4	0.1	**5.0**	1	96
17	TfR-mAb-scramble-PEG5k (n=1)	4	3	**5.0**	1	96
18	TfR-mAb-scramble-PEG5k (n=1)	4	1	**5.0**	1	96
19	TfR-mAb-scramble-PEG5k (n=1)	4	0.3	**5.0**	1	96
20	TfR-mAb-scramble-PEG5k (n=1)=1)	4	0.1	**5.0**	1	96
21	PBS Control	5	-	**5.0**	1	96

85 WT mice (CD-1)

FIG. 9B

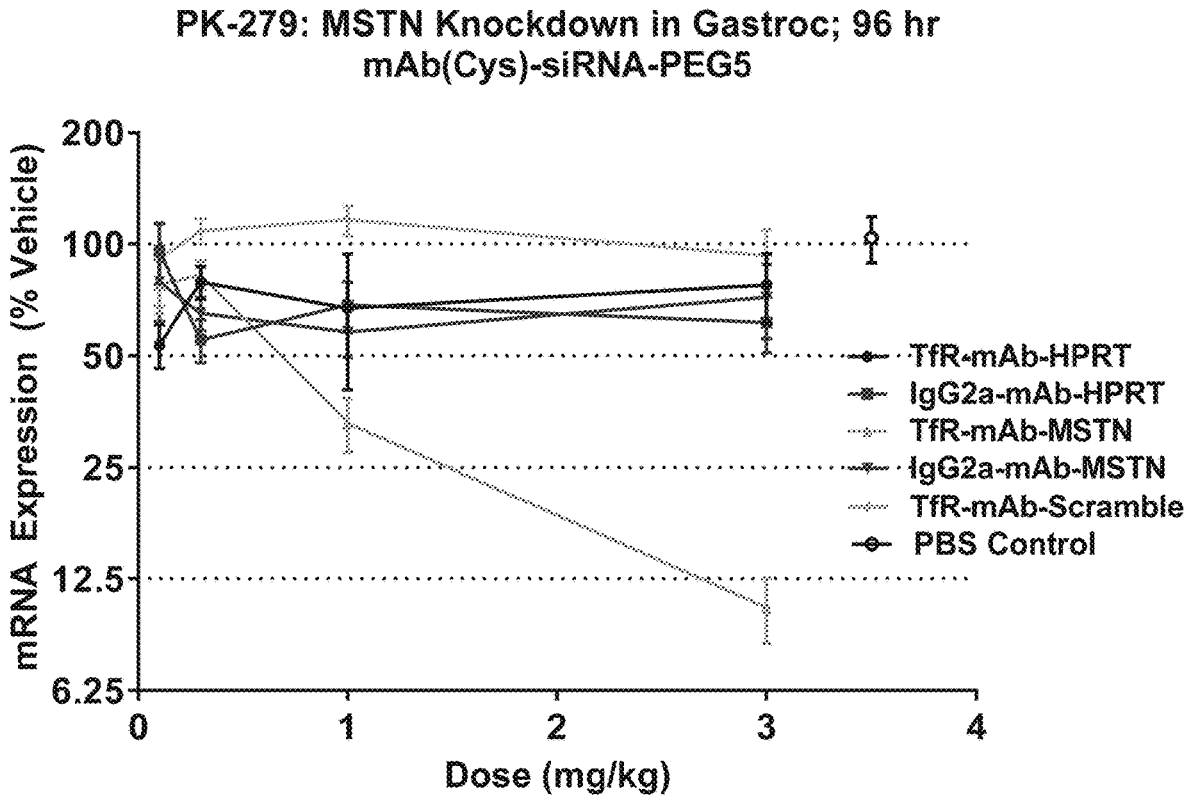

PK-279: MSTN Knockdown in Gastroc; 96 hr
mAb(Cys)-siRNA-PEG5
mRNA Expression (% Vehicle)
200
100
50
25
12.5
6.25
0
1
2
3
4
Dose (mg/kg)
TfR-mAb-HPRT
IgG2a-mAb-HPRT
TfR-mAb-MSTN
IgG2a-mAb-MSTN
TfR-mAb-Scramble
PBS Control

FIG. 10A

Group	Test Article	N	siRNA Dose (mg/kg)	# of Doses	Survival Bleed (h)	Terminal Bleed (h)	Harvest Time (h)
1	TfR-mAb-MSTN (n=1)	4	3	1	0.25	24	24
2	TfR-mAb-MSTN (n=1)	4	3	1	1	96	96
3	TfR-mAb-MSTN (n=1)	4	3	1	2	168	168
4	TfR-mAb-MSTN (n=1)	4	3	1	4	336	336
5	TfR-mAb-scramble (n=1)	4	3	1	0.25	24	24
6	TfR-mAb-scramble (n=1)	4	3	1	1	96	96
7	TfR-mAb-scramble (n=1)	4	3	1	2	168	168
8	TfR-mAb-scramble (n=1)	4	3	1	4	336	336
9	PBS Control	4	-	1	-	-	24
10	PBS Control	4	-	1	-	-	96
11	PBS Control	4	-	1	-	-	168
12	PBS Control	4	-	1	-	-	336
13	TfR-mAb-MSTN (n=1)	5	3	1	504	840	840
14	TfR-mAb-MSTN (n=1)	5	3	1	672	1,008	1,008
15	PBS Control	5	-	1	504	840	840
16	PBS control	5	-	1	672	1,008	1,008

FIG. 10B

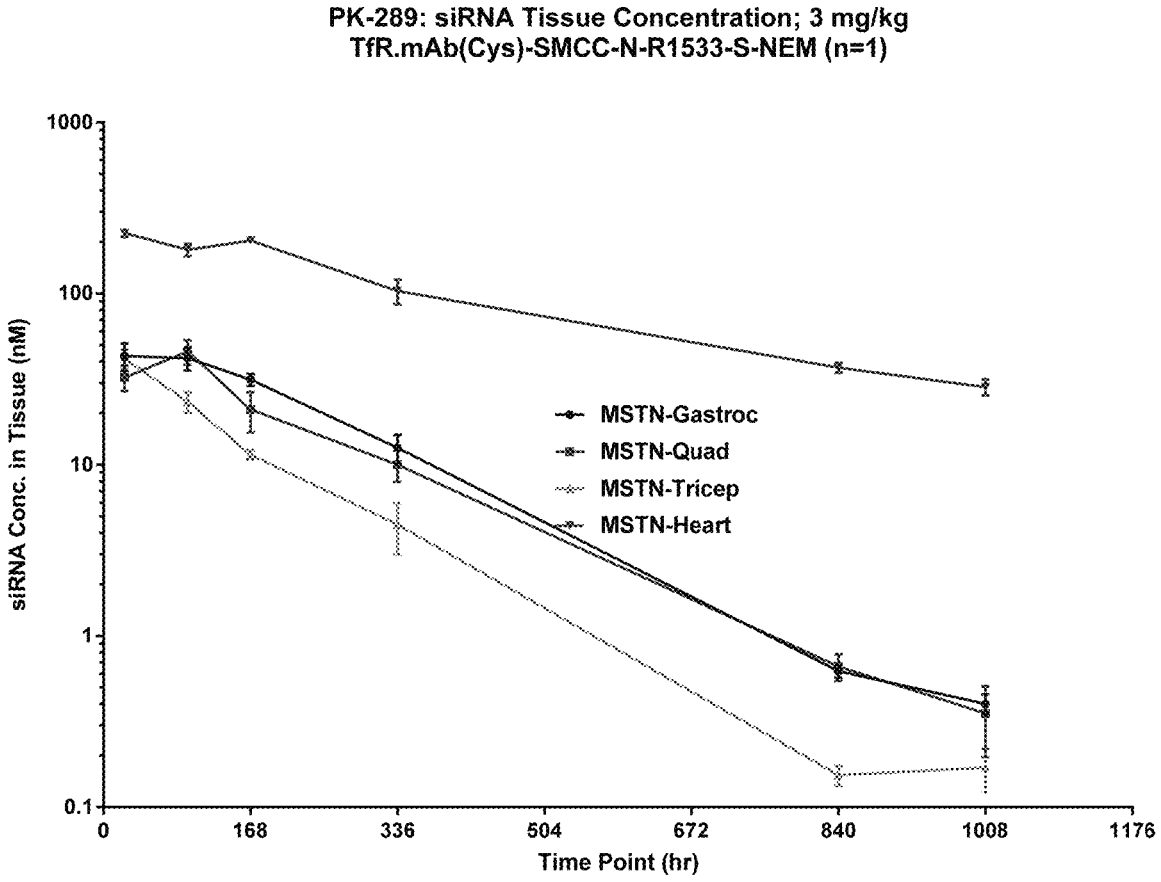

PK-289: siRNA Tissue Concentration; 3 mg/kg
TfR.mAb(Cys)-SMCC-N-R1533-S-NEM (n=1)
siRNA Conc. in Tissue (nM)
1000
100
10
1
0.1
0
168
336
504
672
840
1008
1176
Time Point (hr)
MSTN-Gastroc
MSTN-Quad
MSTN-Tricep
MSTN-Heart

FIG. 10C

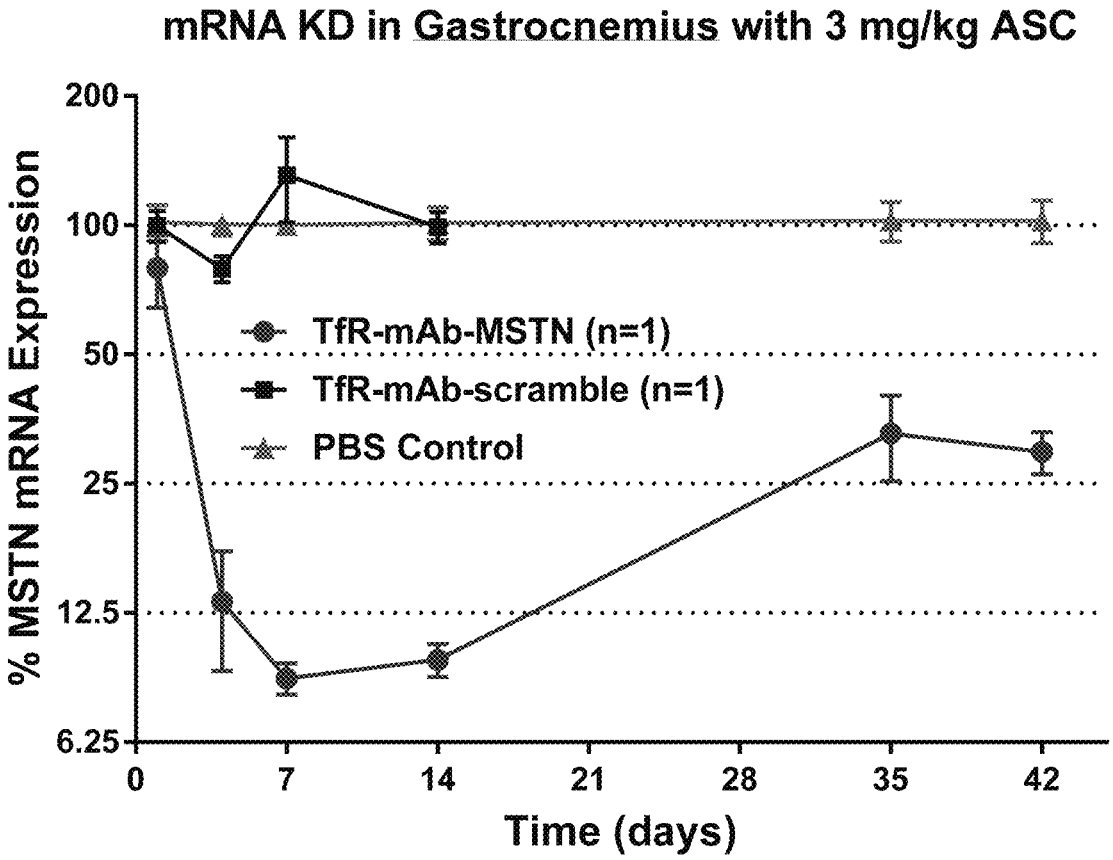

mRNA KD in Gastrocnemius with 3 mg/kg ASC
% MSTN mRNA Expression
200
100
50
25
12.5
6.25
TfR-mAb-MSTN (n=1)
TfR-mAb-scramble (n=1)
PBS Control
0
7
14
21
28
35
42
Time (days)

FIG. 10D

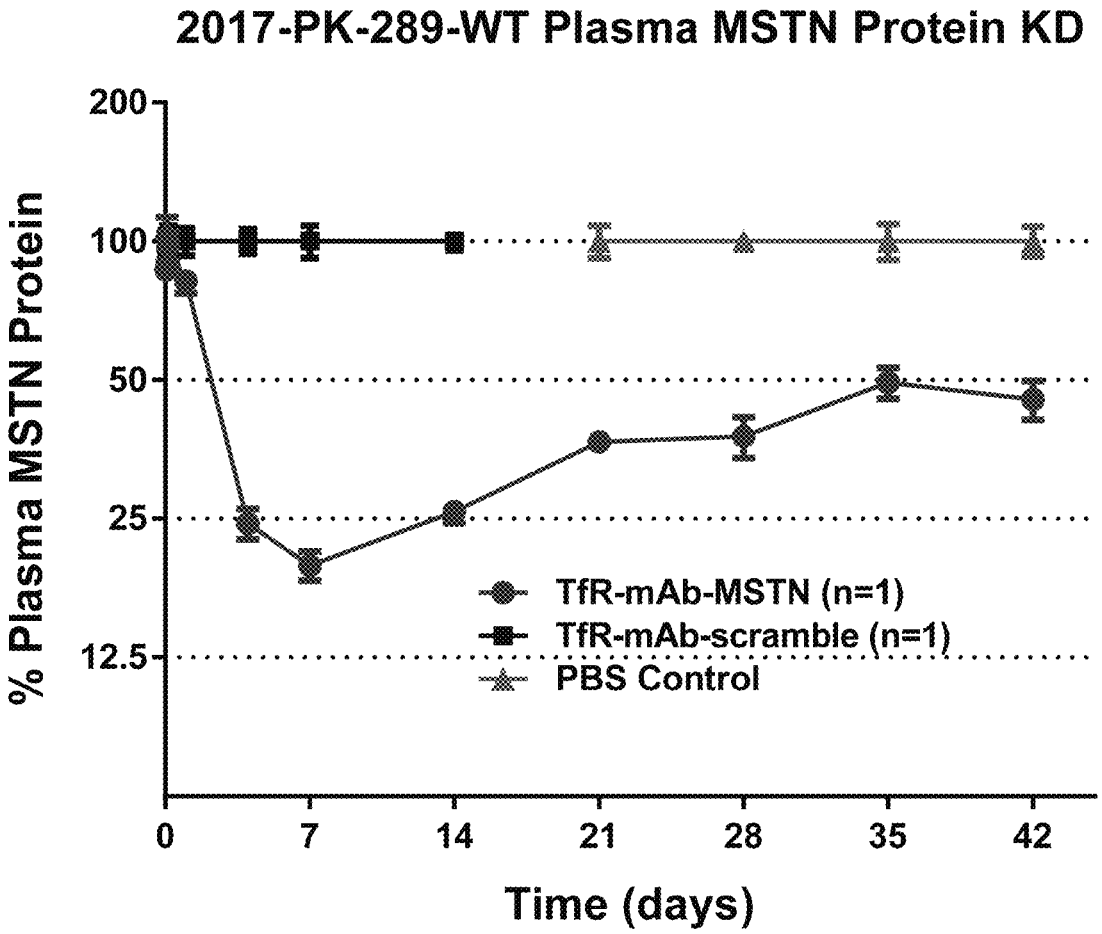

2017-PK-289-WT Plasma MSTN Protein KD
% Plasma MSTN Protein
200
100
50
25
12.5
0
7
14
21
28
35
42
Time (days)
TfR-mAb-MSTN (n=1)
TfR-mAb-scramble (n=1)
PBS Control

FIG. 10E

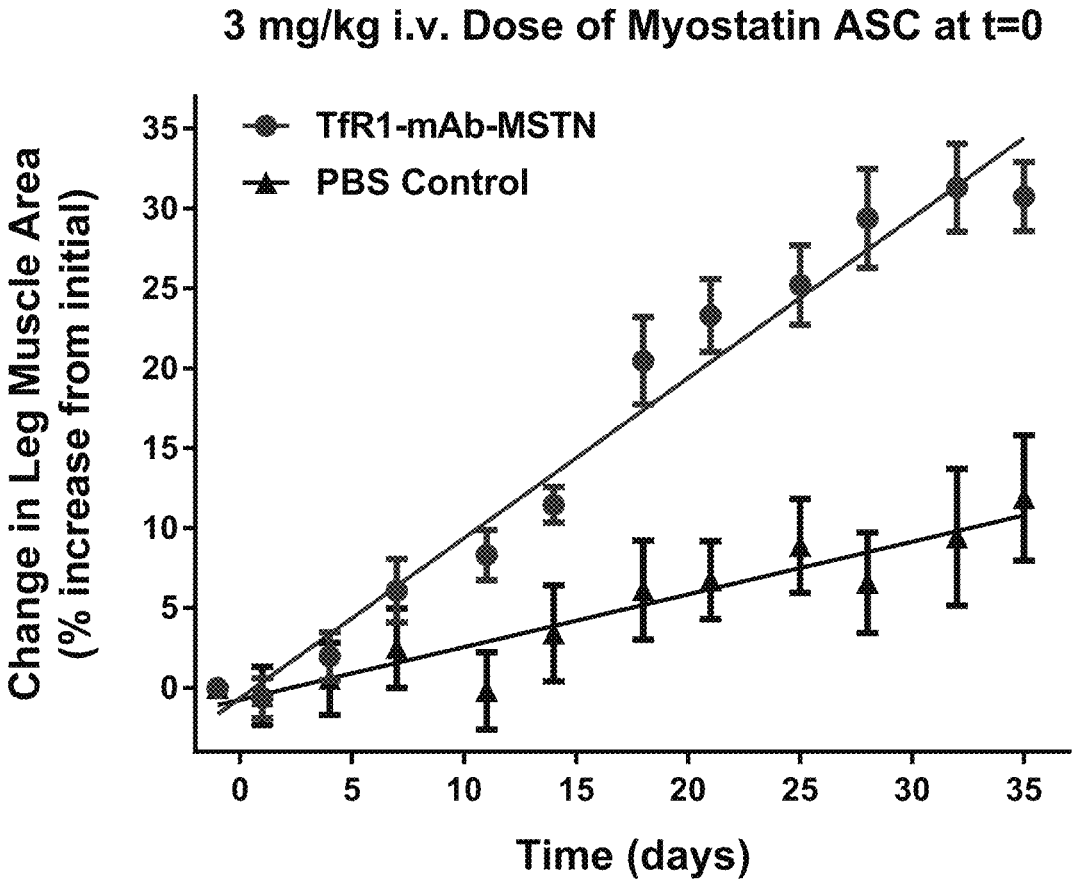

3 mg/kg i.v. Dose of Myostatin ASC at t=0
TfR1-mAb-MSTN
PBS Control
Change in Leg Muscle Area
(% increase from initial)
Time (days)
0
5
10
15
20
25
30
35

FIG. 10F

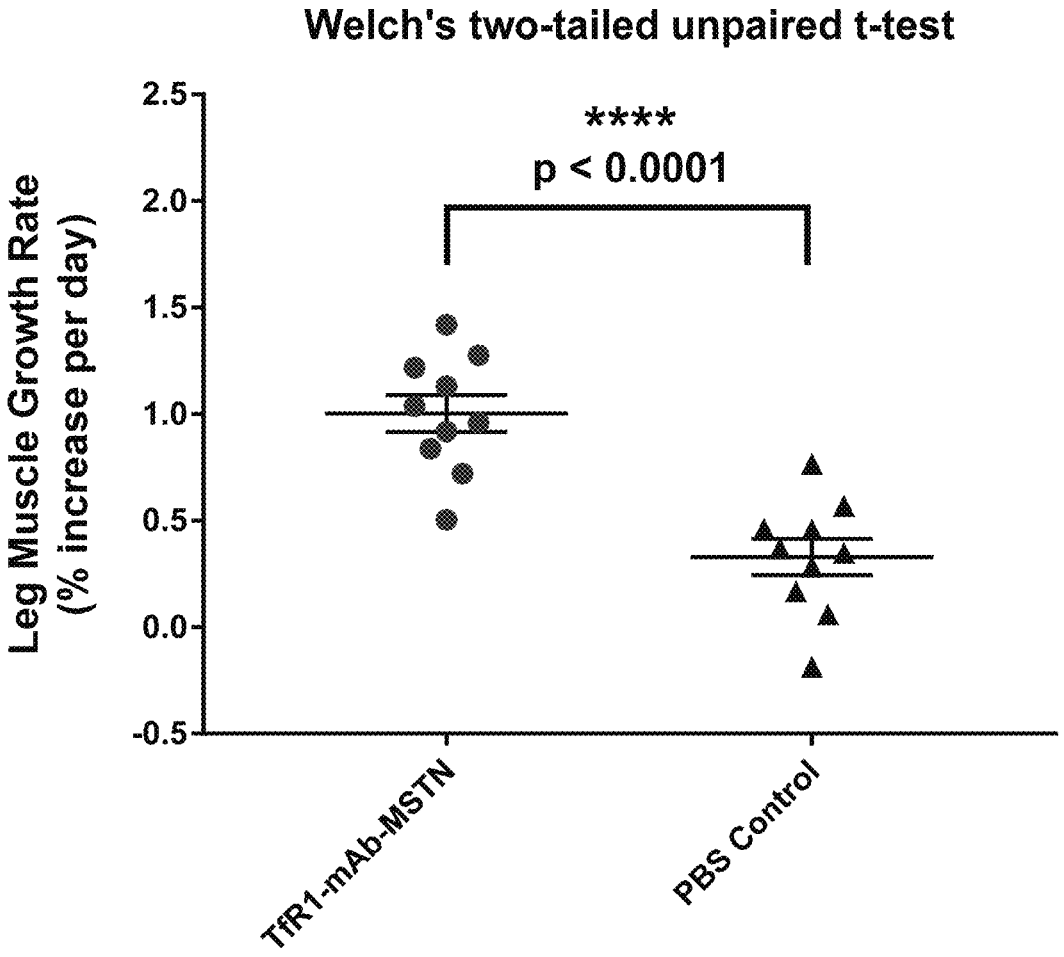

Welch's two-tailed unpaired t-test
****
p < 0.0001
Leg Muscle Growth Rate
(% increase per day)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
TfR1-mAb-MSTN
PBS Control

FIG. 11A

Group	Test Article	N	siRNA Dose (mg/kg)	Dose Volume (mL/kg)	# of Doses	Dose Schedule	Harvest Time (h)
1	TfR-Fab-MSTN* (n=1)	4	6	**5.0**	1	t=0	96
2	TfR-Fab-MSTN* (n=1)	4	2	**5.0**	1	t=0	96
3	TfR-Fab-MSTN* (n=1)	4	0.6	**5.0**	1	t=0	96
4	TfR-Fab-MSTN* (n=1)	4	0.2	**5.0**	1	t=0	96
5	EGFR-Fab-MSTN* (n=1)	4	6	**5.0**	1	t=0	96
6	EGFR-Fab-MSTN* (n=1)	4	2	**5.0**	1	t=0	96
7	EGFR-Fab-MSTN* (n=1)	4	0.6	**5.0**	1	t=0	96
8	EGFR-Fab-MSTN* (n=1)	4	0.2	**5.0**	1	t=0	96
9	TfR-mAb-MSTN (n=1)	4	3	**5.0**	1	t=0	96
10	TfR-mAb-MSTN (n=1)	4	1	**5.0**	1	t=0	96
11	TfR-mAb-MSTN (n=1)	4	0.3	**5.0**	1	t=0	96
12	TfR-mAb-MSTN (n=1)	4	0.1	**5.0**	1	t=0	96
13	TfR-mAb-MSTN (n=2)	4	6	**5.0**	1	t=0	96
14	TfR-mAb-MSTN (n=2)	4	2	**5.0**	1	t=0	96
15	TfR-mAb-MSTN (n=2)	4	0.6	**5.0**	1	t=0	96
16	TfR-mAb-MSTN (n=2)	4	0.2	**5.0**	1	t=0	96
17	EGFR-mAb-MSTN* (n=1)	4	2.6	**5.0**	1	t=0	96
18	EGFR-mAb-MSTN* (n=1)	4	1	**5.0**	1	t=0	96
19	EGFR-mAb-MSTN* (n=2)	4	3	**5.0**	1	t=0	96
20	EGFR-mAb-MSTN* (n=2)	4	1	**5.0**	1	t=0	96
21	PBS Control	5	-	**5.0**	1	t=0	96

85 WT mice (CD-1)

FIG. 11B

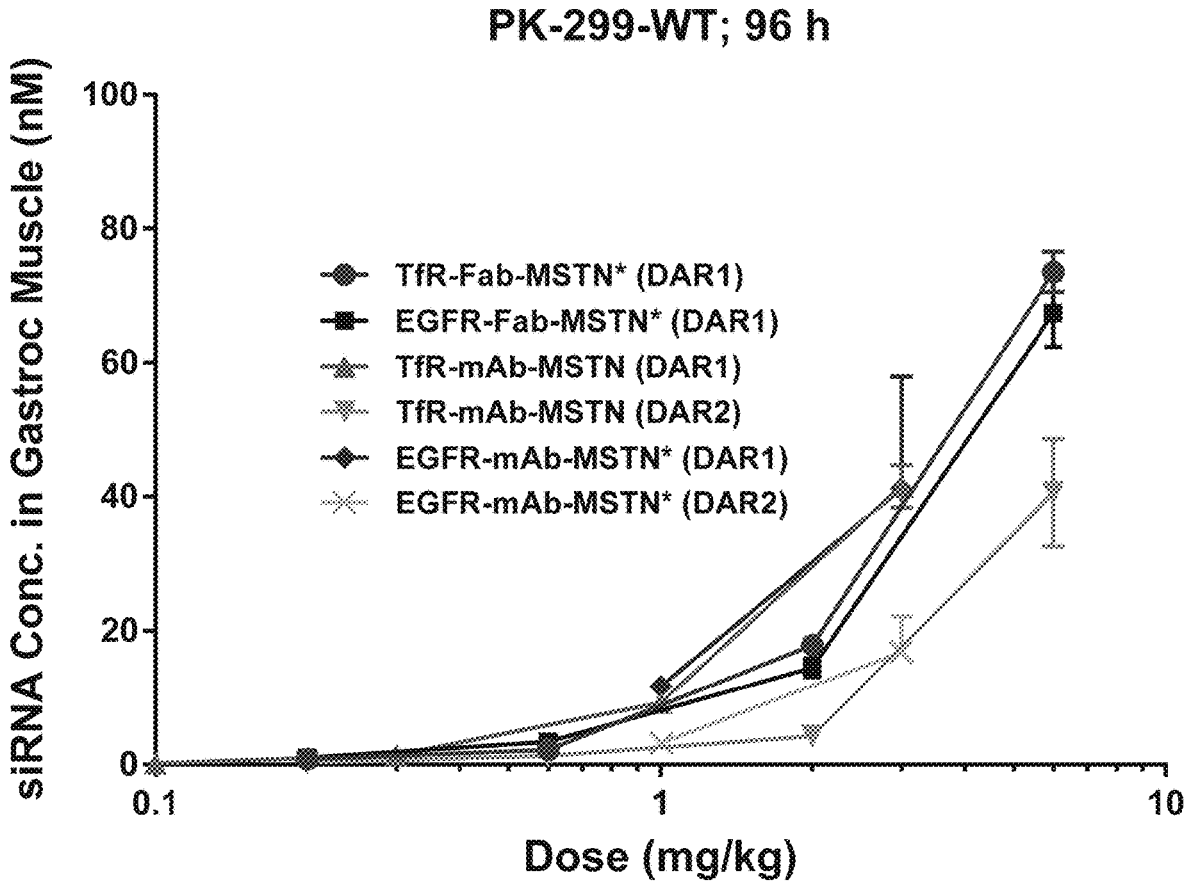

PK-299-WT; 96 h
siRNA Conc. in Gastroc Muscle (nM)
100
80
60
40
20
0
0.1
1
10
Dose (mg/kg)
TfR-Fab-MSTN* (DAR1)
EGFR-Fab-MSTN* (DAR1)
TfR-mAb-MSTN (DAR1)
TfR-mAb-MSTN (DAR2)
EGFR-mAb-MSTN* (DAR1)
EGFR-mAb-MSTN* (DAR2)

FIG. 11C

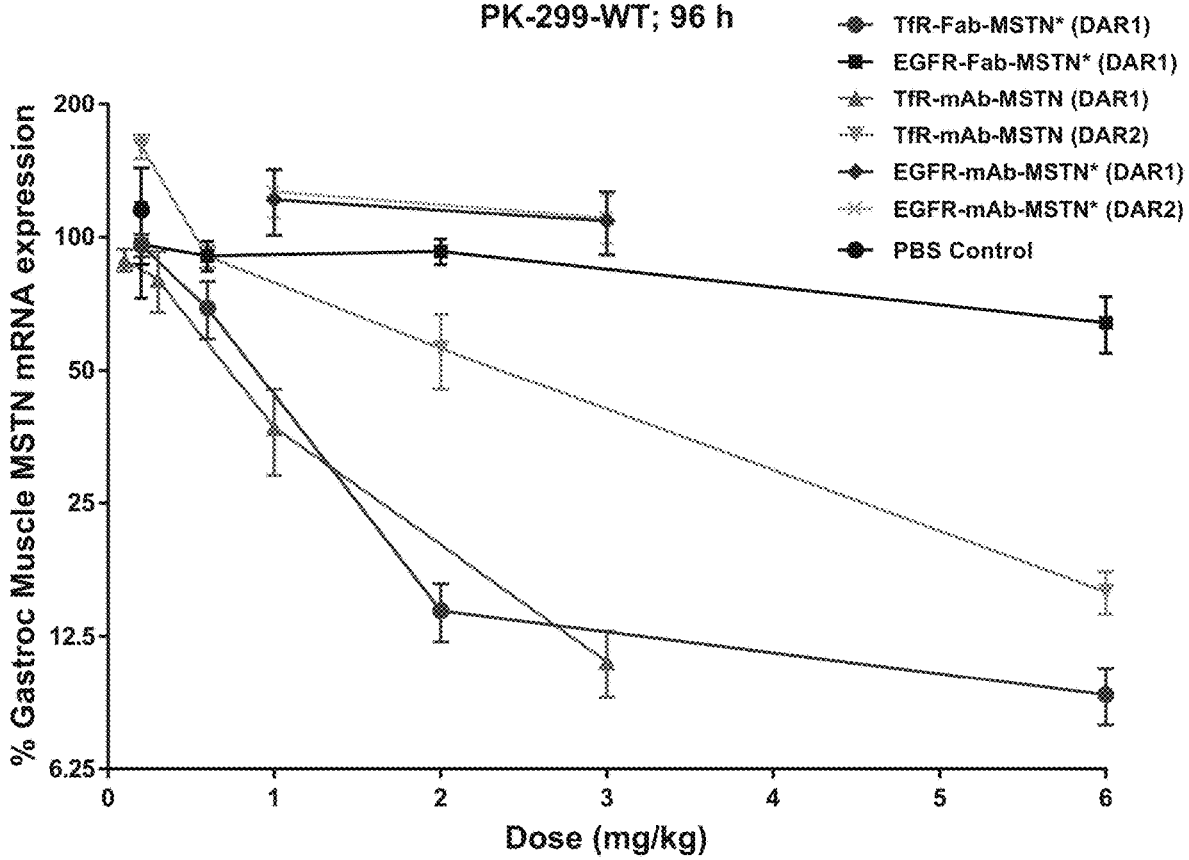

PK-299-WT; 96 h
TfR-Fab-MSTN* (DAR1)
EGFR-Fab-MSTN* (DAR1)
TfR-mAb-MSTN (DAR1)
TfR-mAb-MSTN (DAR2)
EGFR-mAb-MSTN* (DAR1)
EGFR-mAb-MSTN* (DAR2)
PBS Control
% Gastroc Muscle MSTN mRNA expression
200
100
50
25
12.5
6.25
0
1
2
3
4
5
6
Dose (mg/kg)

FIG. 12A

Group	Test Article	N	siRNA Dose (mg/kg)	Dose Volume (mL/kg)	Dose Schedule	Harvest Time (h)
1	Chol-MSTN Control	4	60	**5.0**	t=0	96
2	Chol-MSTN Control	4	20	**5.0**	t=0	96
3	Chol-MSTN Control	4	6	**5.0**	t=0	96
4	Chol-MSTN Control	4	2	**5.0**	t=0	96
5	TfR-Fab-MSTN* (n=1)	4	6	**5.0**	t=0	96
6	TfR-Fab-MSTN* (n=1)	4	2	**5.0**	t=0	96
7	TfR-Fab-MSTN* (n=1)	4	0.6	**5.0**	t=0	96
8	TfR-Fab-MSTN* (n=1)	4	0.2	**5.0**	t=0	96
9	TfR-mAb-MSTN* (n=1)	4	3	**5.0**	t=0	96
10	TfR-mAb-MSTN* (n=1)	4	1	**5.0**	t=0	96
11	TfR-mAb-MSTN* (n=1)	4	0.3	**5.0**	t=0	96
12	TfR-mAb-MSTN* (n=1)	4	0.1	**5.0**	t=0	96
13	TfR-mAb-MSTN (n=1)	4	3	**5.0**	t=0	96
14	TfR-mAb-MSTN (n=1)	4	1	**5.0**	t=0	96
15	TfR-mAb-MSTN (n=1)	4	0.3	**5.0**	t=0	96
16	TfR-mAb-MSTN (n=1)	4	0.1	**5.0**	t=0	96
17	TfR-mAb-scramble (n=1)	4	3	**5.0**	t=0	96
18	TfR-mAb-scramble (n=1)	4	1	**5.0**	t=0	96
19	TfR-mAb-scramble (n=1)	4	0.3	**5.0**	t=0	96
20	TfR-mAb-scramble (n=1)	4	0.1	**5.0**	t=0	96
21	PBS Control	5	-	**5.0**	t=0	96

85 WT mice (CD-1)

FIG. 12B

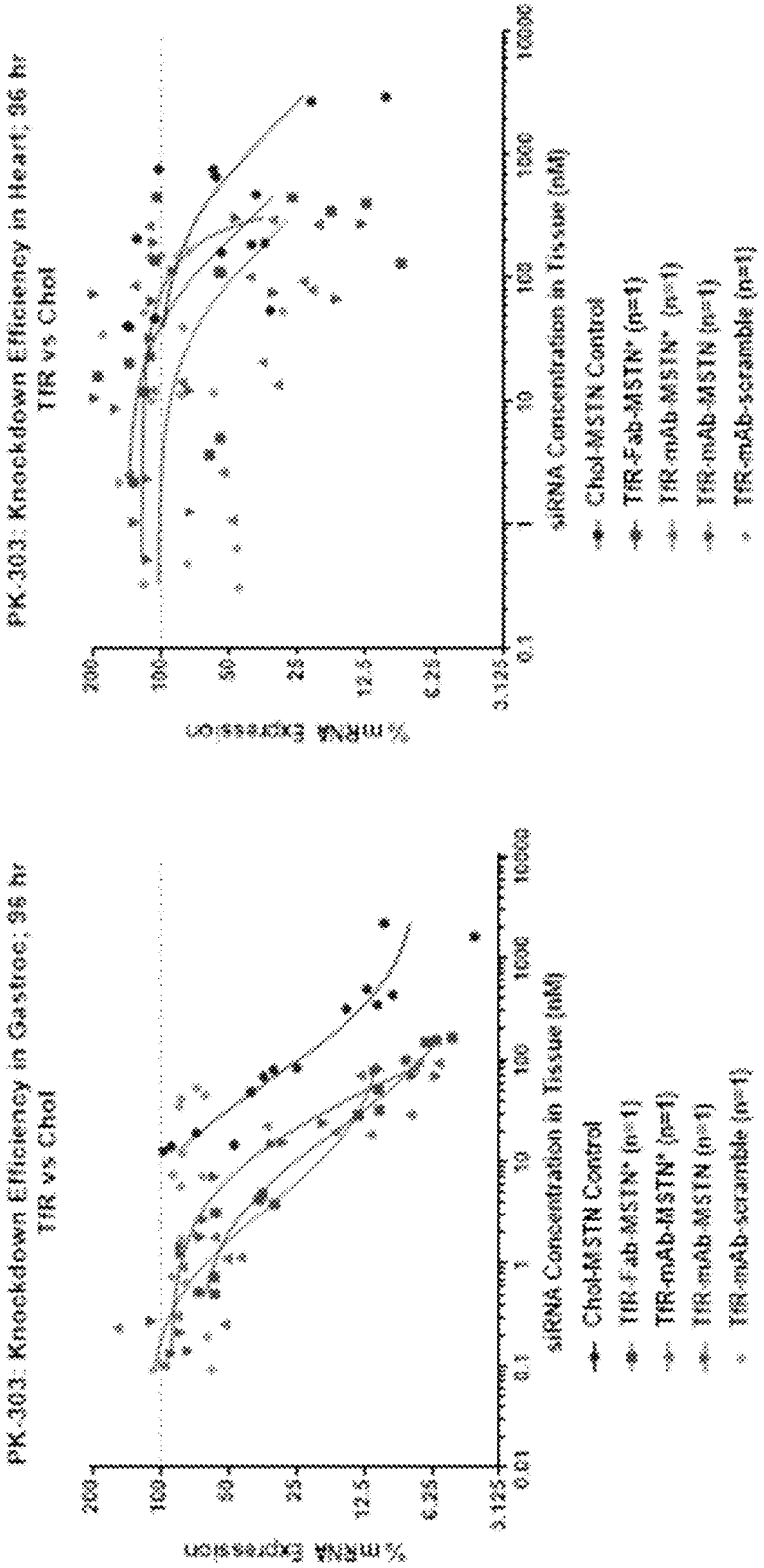

PK-303: Knockdown Efficiency in Heart; 96 hr
TfR vs Chol
% mRNA Expression
200
100
50
25
12.5
6.25
3.125
0.01
0.1
1
10
100
1000
10000
siRNA Concentration in Tissue (nM)
Chol-MSTN Control
TfR-Fab-MSTN* (n=1)
TfR-mAb-MSTN* (n=1)
TfR-mAb-MSTN (n=1)
TfR-mAb-scramble (n=1)
PK-303: Knockdown Efficiency in Gastroc; 96 hr
TfR vs Chol
% mRNA Expression
200
100
50
25
12.5
6.25
3.125
0.01
0.1
1
10
100
1000
10000
siRNA Concentration in Tissue (nM)
Chol-MSTN Control
TfR-Fab-MSTN* (n=1)
TfR-mAb-MSTN* (n=1)
TfR-mAb-MSTN (n=1)
TfR-mAb-scramble (n=1)

FIG. 12C

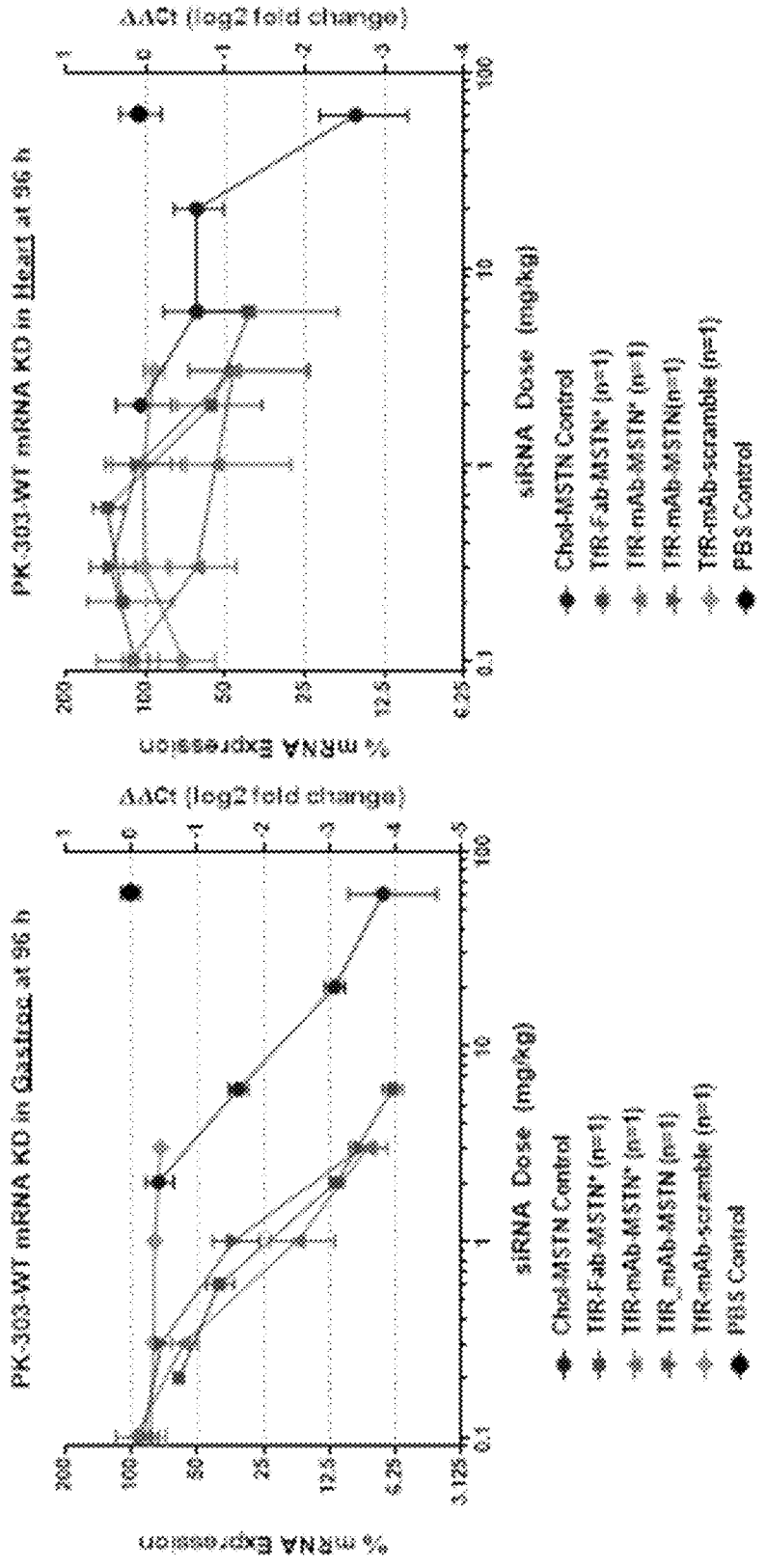

PK-303-WT mRNA KD in Heart at 96 h
ΔΔCt (log2 fold change)
% mRNA Expression
siRNA Dose (mg/kg)
Chol-MSTN Control
TfR-Fab-MSTN* (n=1)
TfR-mAb-MSTN* (n=1)
TfR-mAb-MSTN(n=1)
TfR-mAb-scramble (n=1)
PBS Control
PK-303-WT mRNA KD in Gastroc at 96 h
ΔΔCt (log2 fold change)
% mRNA Expression
siRNA Dose (mg/kg)
Chol-MSTN Control
TfR-Fab-MSTN* (n=1)
TfR-mAb-MSTN* (n=1)
TfR_mAb-MSTN (n=1)
TfR-mAb-scramble (n=1)
PBS Control

FIG. 12D

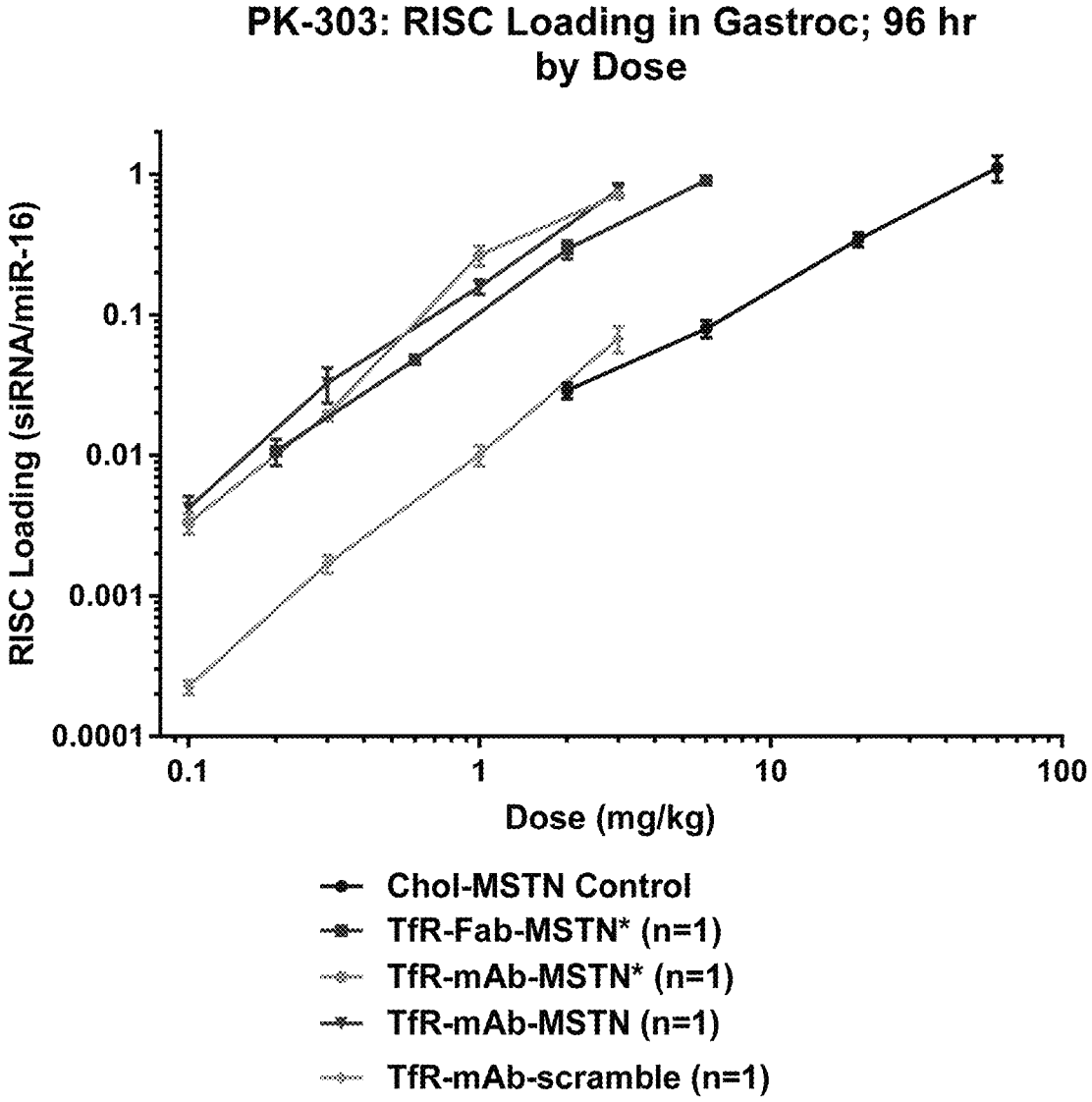

PK-303: RISC Loading in Gastroc; 96 hr
by Dose
RISC Loading (siRNA/miR-16)
1
0.1
0.01
0.001
0.0001
0.1
1
10
100
Dose (mg/kg)
Chol-MSTN Control
TfR-Fab-MSTN* (n=1)
TfR-mAb-MSTN* (n=1)
TfR-mAb-MSTN (n=1)
TfR-mAb-scramble (n=1)

FIG. 13A

Group	Test Article	N	siRNA Dose (mg/kg)	Survival Bleed (day)	Terminal Bleed (day)	Harvest Time (day)
1	Chol-MSTN	5	50	7	14	14
2	Chol-MSTN	5	50	21	28	28
3	Chol-MSTN	5	50	35	42	42
4	Chol-MSTN	5	50	49	56	56
5	TfR-mAb-MSTN (n=1)	5	3	7	14	14
6	TfR-mAb-MSTN (n=1)	5	3	21	28	28
7	TfR-mAb-MSTN (n=1)	5	3	35	42	42
8	TfR-mAb-MSTN (n=1)	5	3	49	56	56
9	TfR-mAb-MSTN (n=2)	5	3	7	14	14
10	TfR-mAb-MSTN (n=2)	5	3	21	28	28
11	TfR-mAb-MSTN (n=2)	5	3	35	42	42
12	TfR-mAb-MSTN (n=2)	5	3	49	56	56
13	TfR-mAb-scramble (n=1)	5	3	7	14	14
14	TfR-mAb-scramble (n=1)	5	3	21	28	28
15	TfR-mAb-scramble (n=1)	5	3	35	42	42
16	TfR-mAb-scramble (n=1)	5	3	49	56	56
17	PBS Control	5	-	7	14	14
18	PBS Control	5	-	21	28	28
19	PBS Control	5	-	35	42	42
20	PBS Control	5	-	49	56	56

FIG. 13B

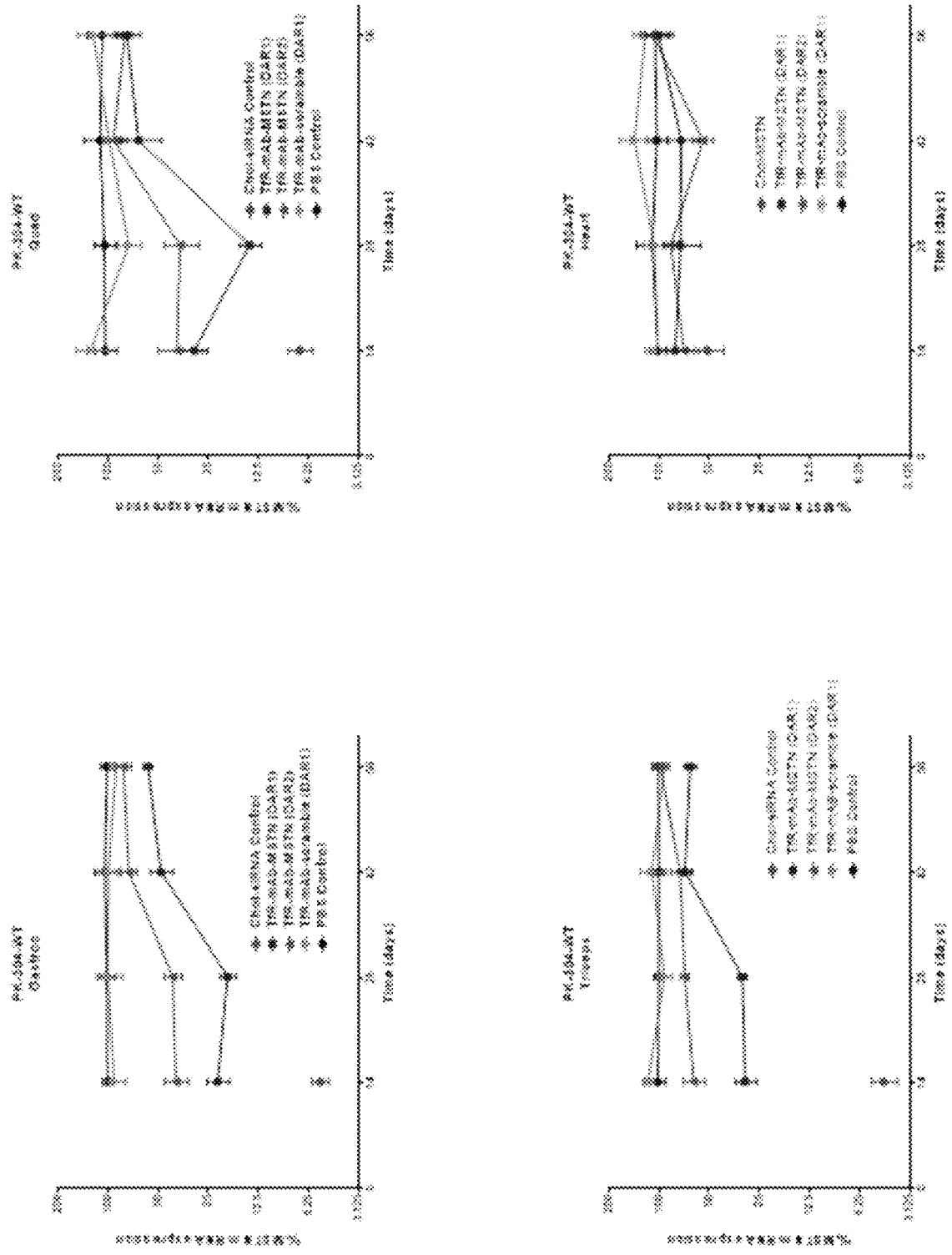


FIG. 13C

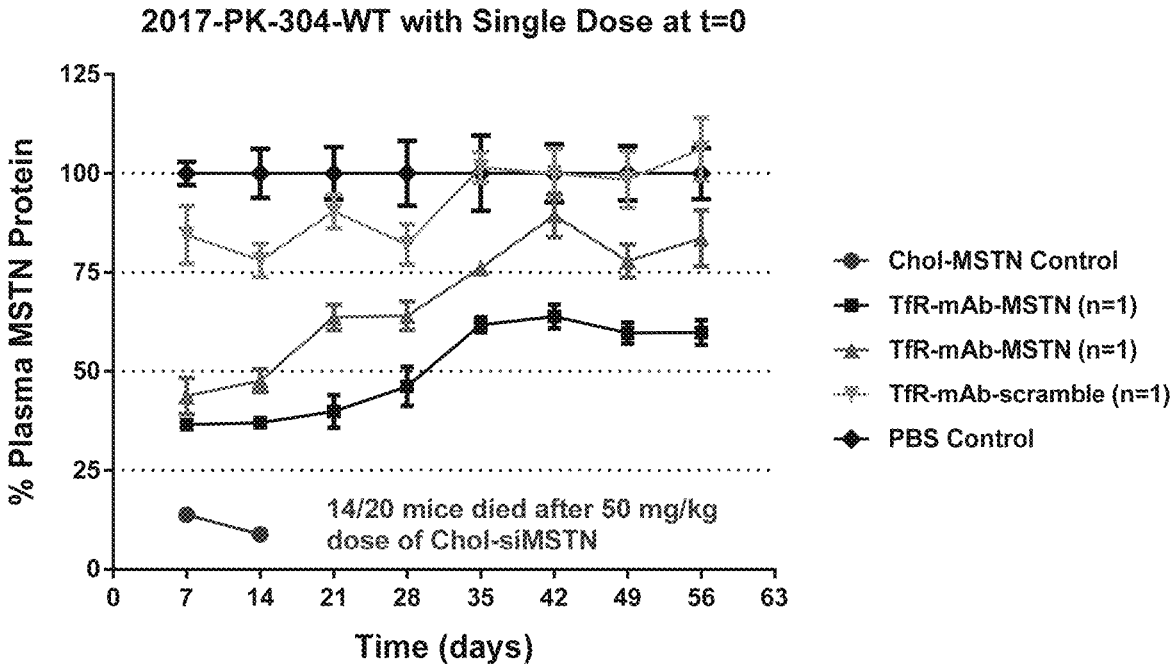

2017-PK-304-WT with Single Dose at t=0
% Plasma MSTN Protein
125
100
75
50
25
0
0
7
14
21
28
35
42
49
56
63
Time (days)
14/20 mice died after 50 mg/kg dose of Chol-siMSTN
Chol-MSTN Control
TfR-mAb-MSTN (n=1)
TfR-mAb-MSTN (n=1)
TfR-mAb-scramble (n=1)
PBS Control

FIG. 13D

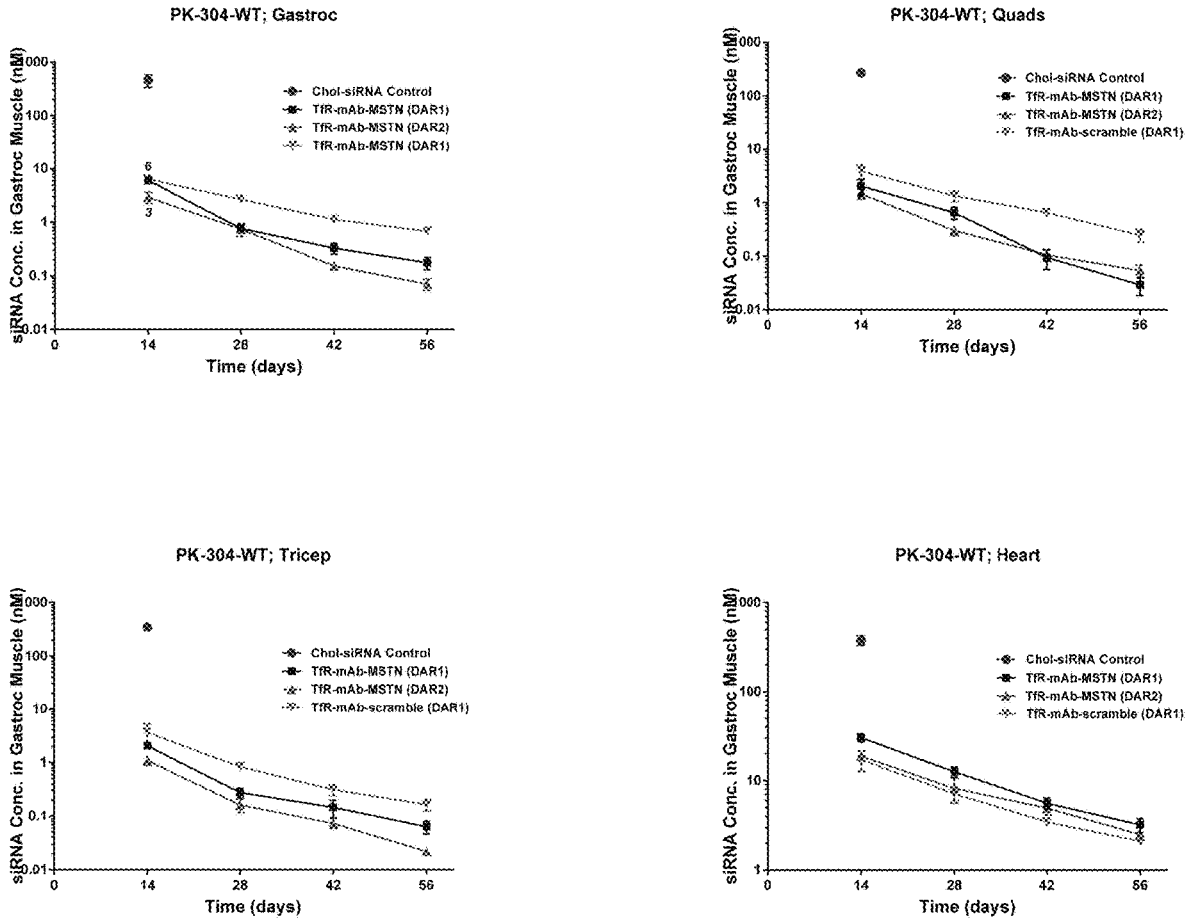

PK-304-WT; Gastroc
PK-304-WT; Quads
PK-304-WT; Tricep
PK-304-WT; Heart
siRNA Conc. in Gastroc Muscle (nM)
Time (days)
Chol-siRNA Control
TfR-mAb-MSTN (DAR1)
TfR-mAb-MSTN (DAR2)
TfR-mAb-scramble (DAR1)

FIG. 13E

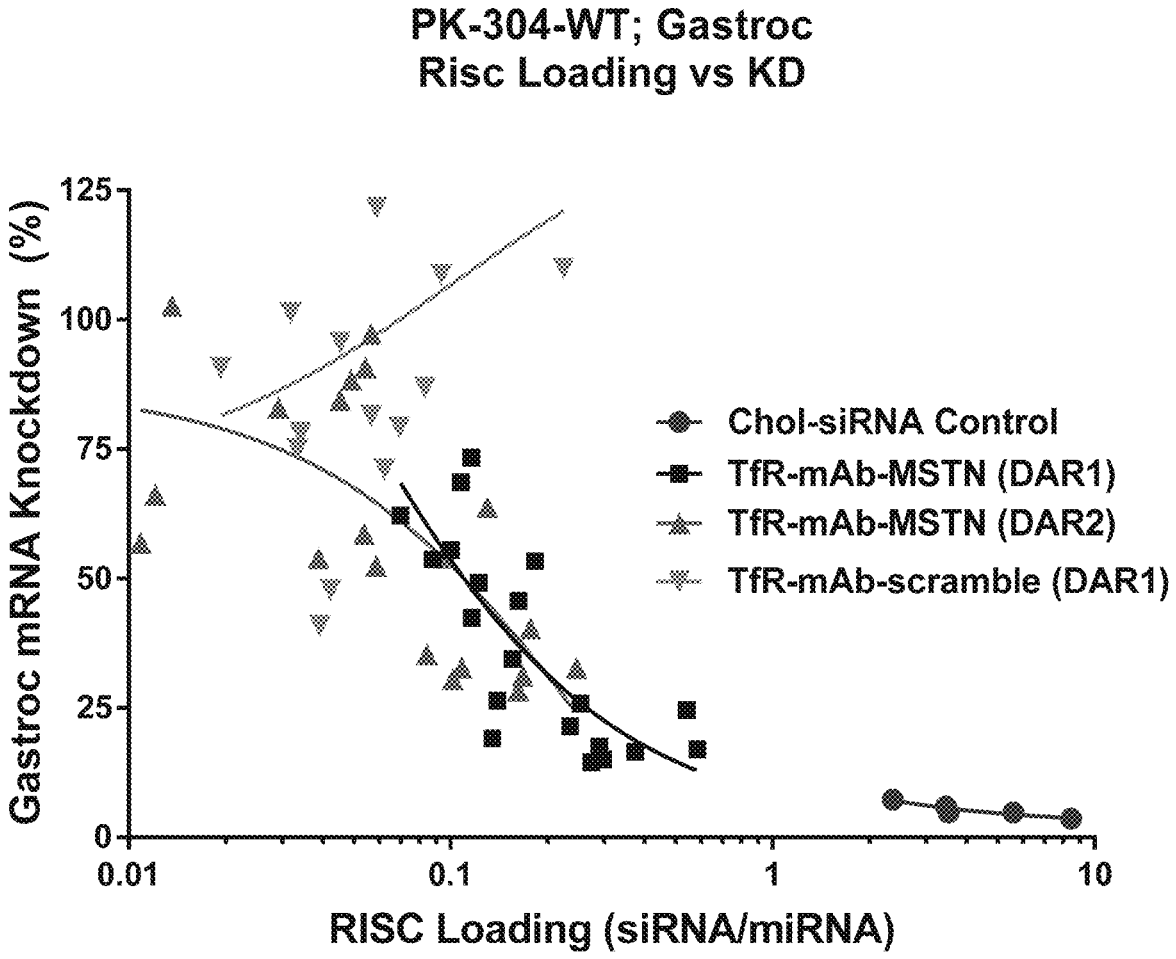

PK-304-WT; Gastroc
Risc Loading vs KD
Gastroc mRNA Knockdown (%)
125
100
75
50
25
0
0.01
0.1
1
10
RISC Loading (siRNA/miRNA)
Chol-siRNA Control
TfR-mAb-MSTN (DAR1)
TfR-mAb-MSTN (DAR2)
TfR-mAb-scramble (DAR1)

FIG. 13F

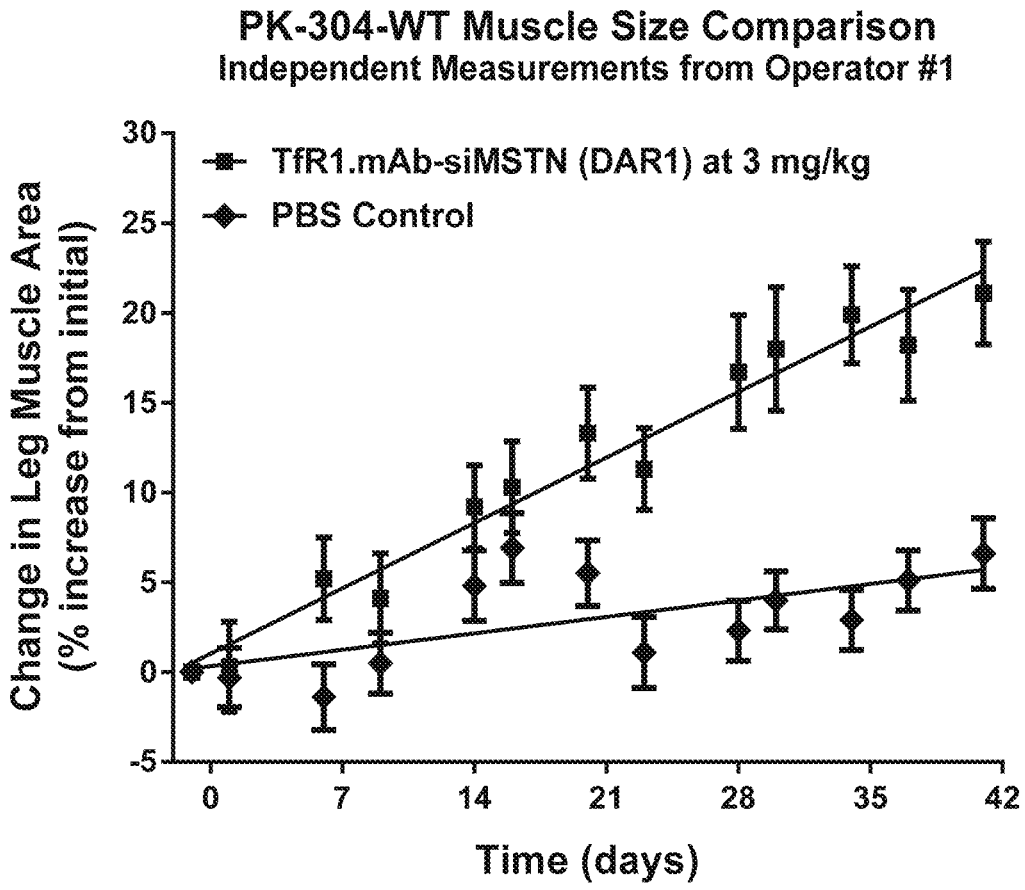

PK-304-WT Muscle Size Comparison
Independent Measurements from Operator #1
TfR1.mAb-siMSTN (DAR1) at 3 mg/kg
PBS Control
Change in Leg Muscle Area
(% increase from initial)
30
25
20
15
10
5
0
-5
0
7
14
21
28
35
42
Time (days)

FIG. 13G

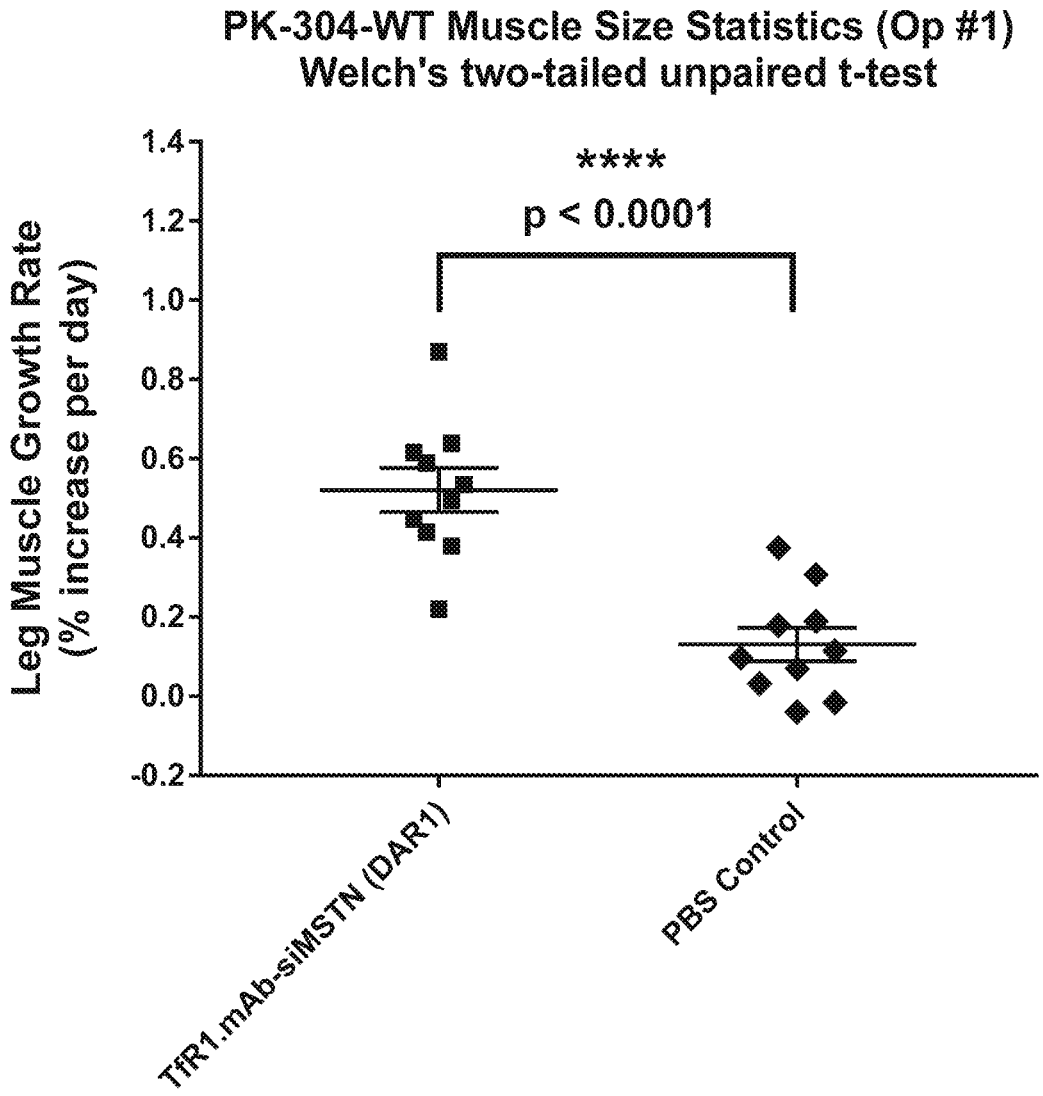

PK-304-WT Muscle Size Statistics (Op #1)
Welch's two-tailed unpaired t-test
****
p < 0.0001
Leg Muscle Growth Rate
(% increase per day)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
TfR1.mAb-siMSTN (DAR1)
PBS Control

FIG. 14A

Group	Test Article	N	each siRNA Dose (mg/kg)	siRNA Mixture (mg/kg)	Harvest Time (h)	Final siRNA dose (mg/kg)
1	TfR-mAb-HPRT(n=1)	4	3		96	3
2	TfR-mAb-HPRT(n=1)	4	1		96	1
3	TfR-mAb-HPRT(n=1)	4	0.3		96	0.3
4	TfR-mAb-HPRT(n=1)	4	0.1		96	0.1
5	TfR-mAb-SSB (n=1)	4	3		96	3
6	TfR-mAb-SSB (n=1)	4	1		96	1
7	m TfR-mAb-SSB (n=1)	4	0.3		96	0.3
8	TfR-mAb-SSB (n=1)	4	0.1		96	0.1
9	TfR-mAb-HPRT (n=1) + TfR-mAb-SSB (n=1)	4	3		96	6
10	TfR-mAb-HPRT (n=1) + TfR-mAb-SSB (n=1)	4	1		96	2
11	TfR-mAb-HPRT (n=1) + TfR-mAb-SSB (n=1)	4	0.3		96	0.6
12	TfR-mAb-HPRT (n=1) + TfR-mAb-SSB (n=1)	4	0.1		96	0.2
13	TfR-mAb-HPRT/SSB(1:1) (n=2)	4		3	96	6
14	TfR-mAb-HPRT/SSB(1:1) (n=2)	4		1	96	2
15	TfR-mAb-HPRT/SSB(1:1) (n=2)	4		0.3	96	0.6
16	TfR-mAb-HPRT/SSB(1:1) (n=2)	4		0.1	96	0.2
	PBS Control	5	-		96	

FIG. 14B

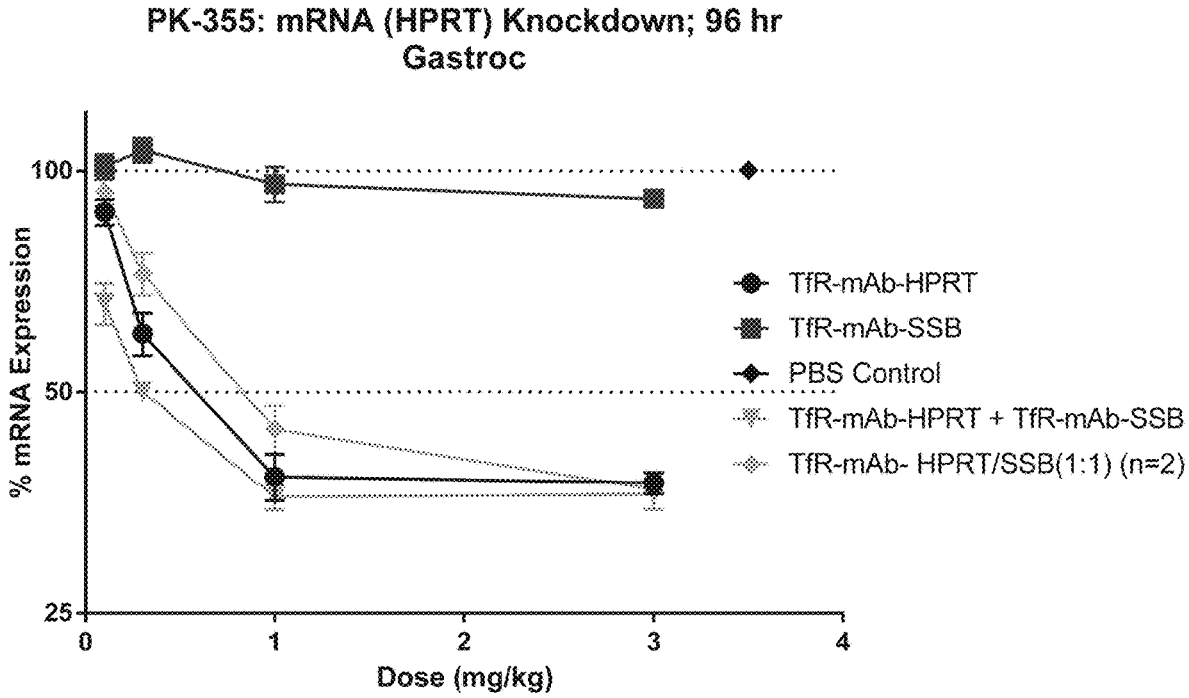

PK-355: mRNA (HPRT) Knockdown; 96 hr
Gastroc
% mRNA Expression
100
50
25
0
1
2
3
4
Dose (mg/kg)
TfR-mAb-HPRT
TfR-mAb-SSB
PBS Control
TfR-mAb-HPRT + TfR-mAb-SSB
TfR-mAb- HPRT/SSB(1:1) (n=2)

FIG. 14C

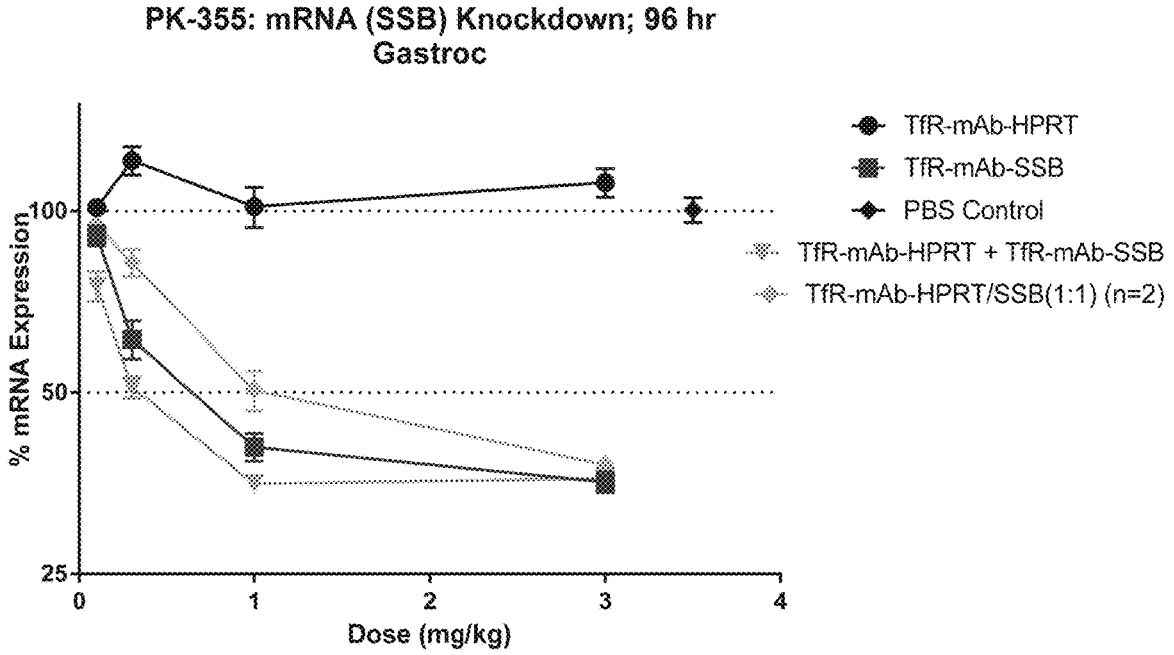


FIG. 14D

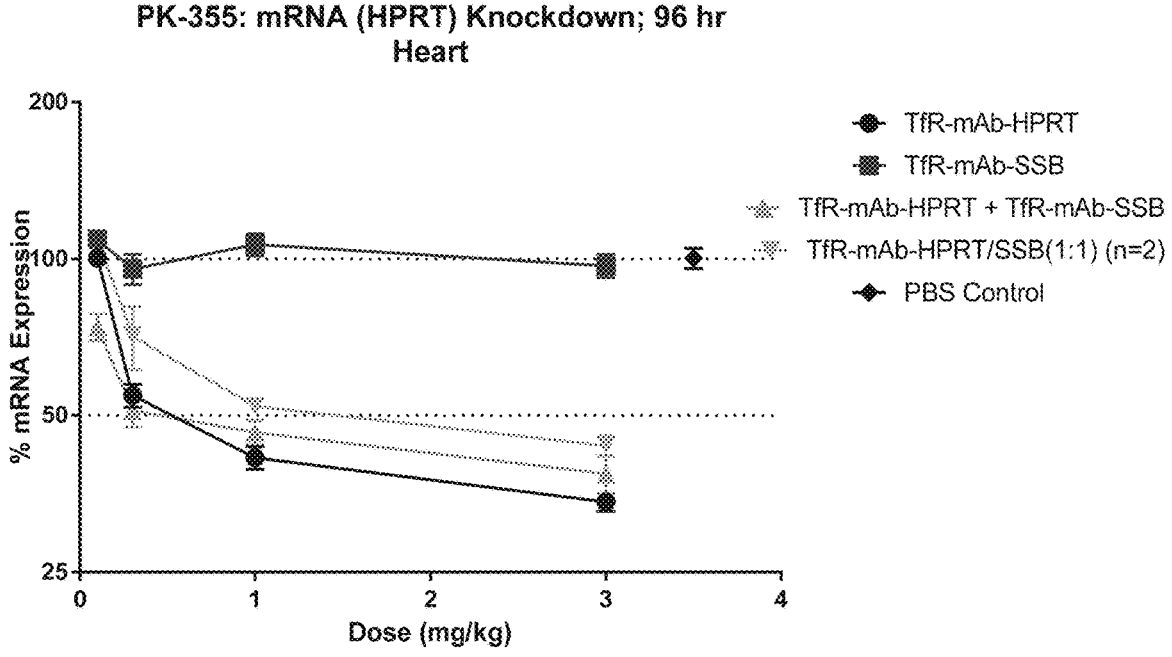


FIG. 14E

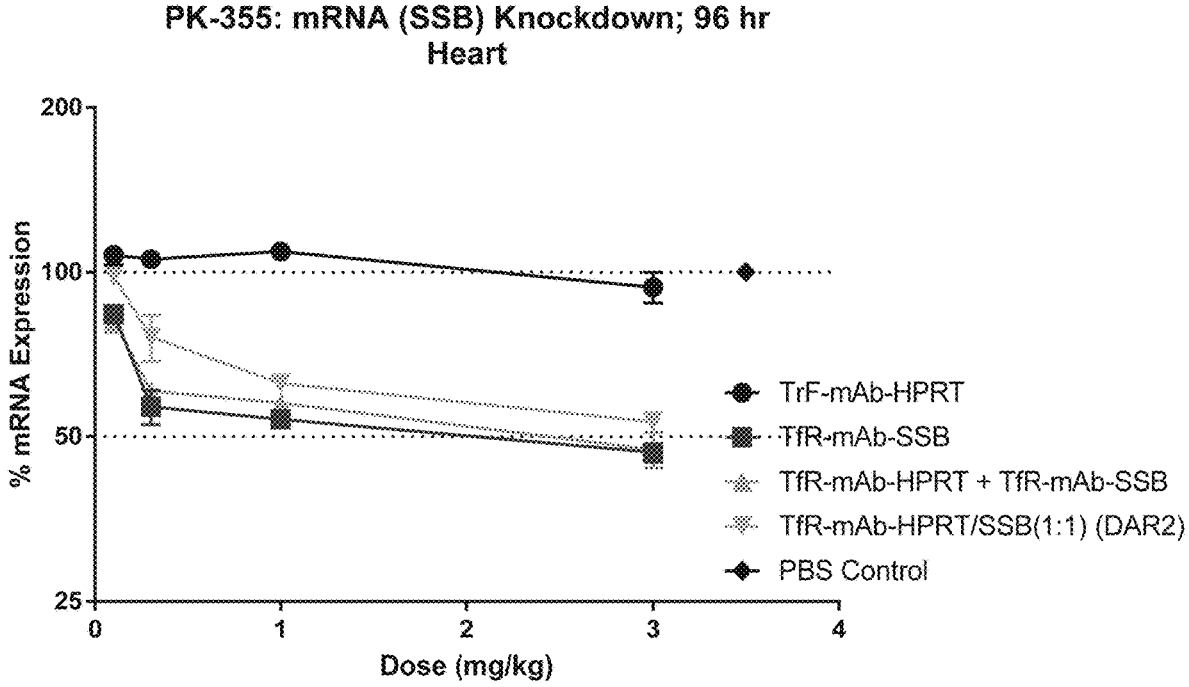

PK-355: mRNA (SSB) Knockdown; 96 hr
Heart
% mRNA Expression
200
100
50
25
0
1
2
3
4
Dose (mg/kg)
TrF-mAb-HPRT
TfR-mAb-SSB
TfR-mAb-HPRT + TfR-mAb-SSB
TfR-mAb-HPRT/SSB(1:1) (DAR2)
PBS Control

FIG. 14F

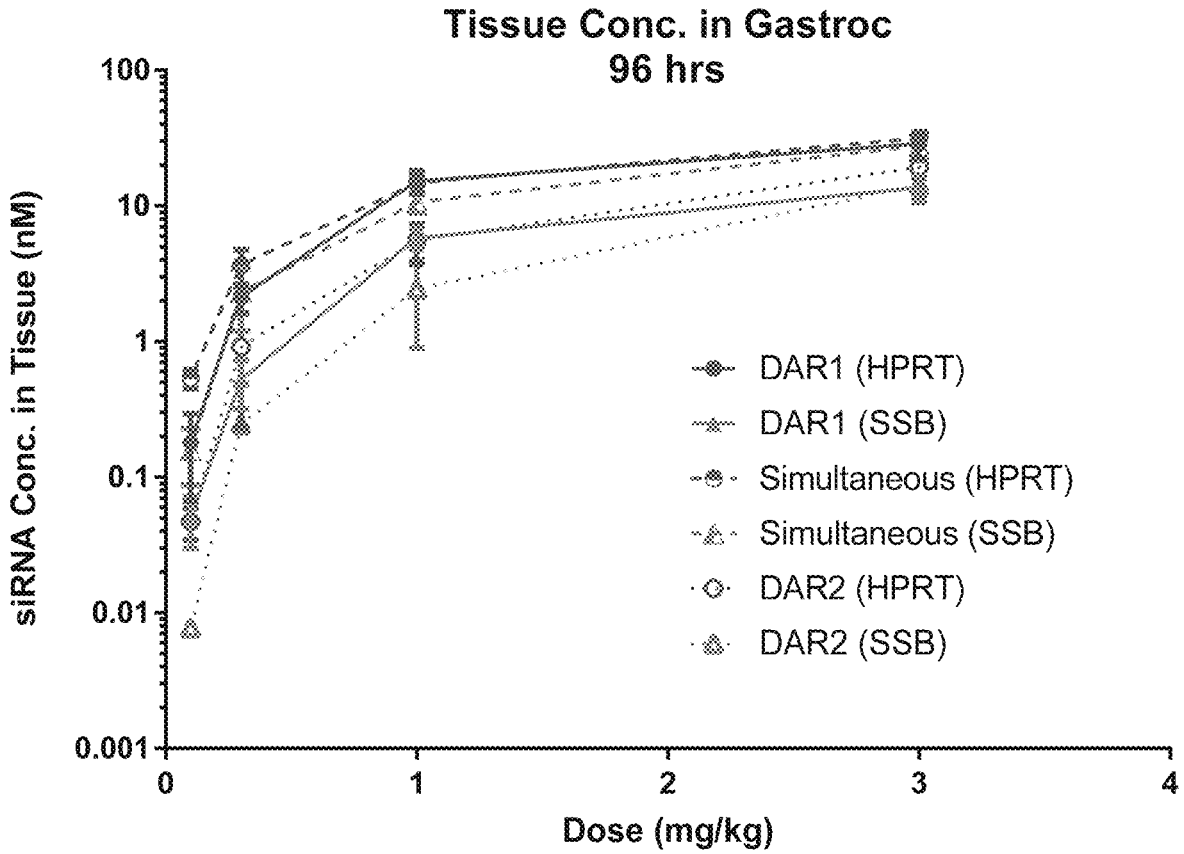

Tissue Conc. in Gastroc
96 hrs
siRNA Conc. in Tissue (nM)
100
10
1
0.1
0.01
0.001
0
1
2
3
4
Dose (mg/kg)
DAR1 (HPRT)
DAR1 (SSB)
Simultaneous (HPRT)
Simultaneous (SSB)
DAR2 (HPRT)
DAR2 (SSB)

FIG. 15A

Group	Test Article	N	siRNA Dose (mg/kg)	Harvest Time (h)
1	TfR-mAb-mAtrogin#1179(n=1)	4	3	96
2	TfR-mAb-mAtrogin#1179(n=1)	4	1	96
3	TfR-mAb-mAtrogin#1179(n=1)	4	0.3	96
4	TfR-mAb-mAtrogin#1179(n=1)	4	0.1	96
5	TfR-mAb-mAtrogin#1504 (n=1)	4	3	96
6	TfR-mAb-mAtrogin#1504 (n=1)	4	1	96
7	TfR-mAb-mAtrogin#1504 (n=1)	4	0.3	96
8	TfR-mAb-mAtrogin#1504 (n=1)	4	0.1	96
9	TfR-mAb-m/hAtrogin#631 (n=1)	4	3	96
10	TfR-mAb-m/hAtrogin#631 (n=1)	4	1	96
11	TfR-mAb-m/hAtrogin#631 (n=1)	4	0.3	96
12	TfR-mAb-m/hAtrogin#631 (n=1)	4	0.1	96
13	TfR-mAb-m/hAtrogin#586 (n=1)	4	3	96
14	TfR-mAb-m/hAtrogin#586 (n=1)	4	1	96
15	TfR-mAb-m/hAtrogin#586 (n=1)	4	0.3	96
16	TfR-mAb-m/hAtrogin#586 (n=1)	4	0.1	96
17	PBS Control	5	-	96

FIG. 15B

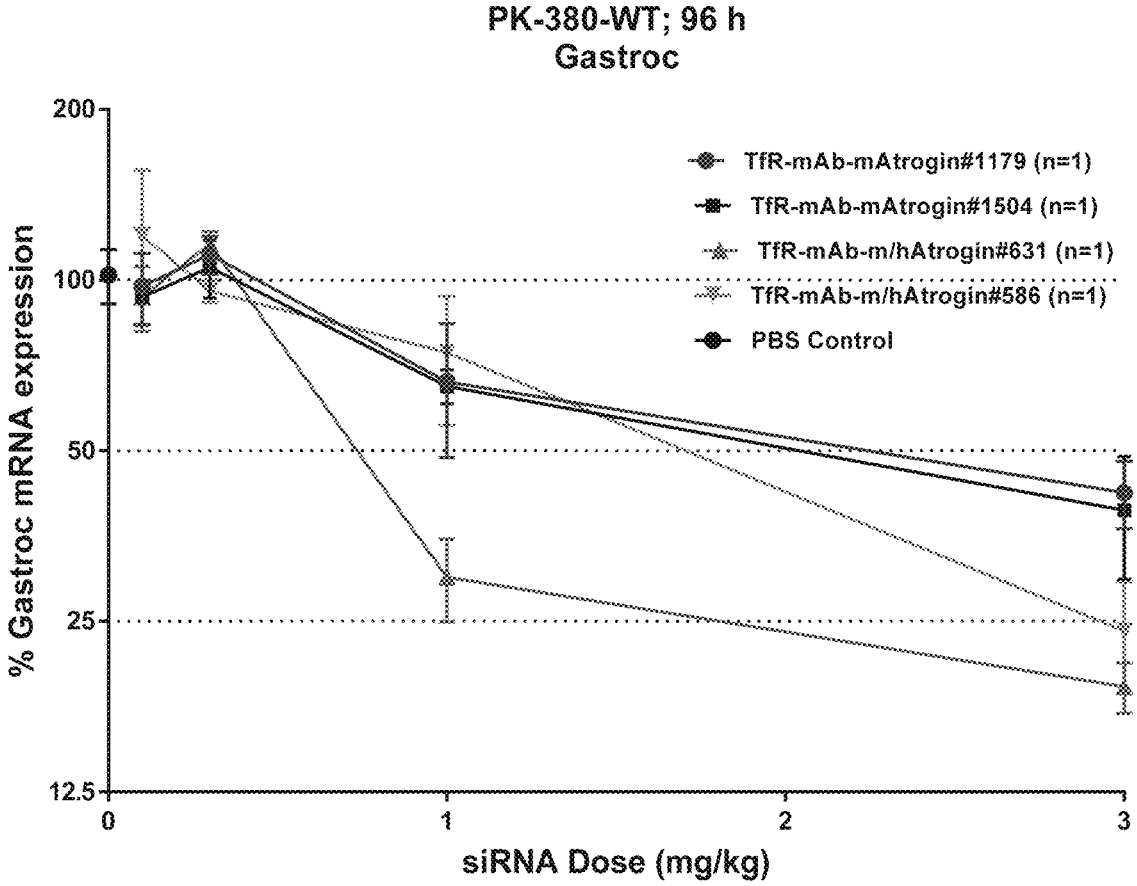

PK-380-WT; 96 h
Gastroc
200
100
50
25
12.5
% Gastroc mRNA expression
0
1
2
3
siRNA Dose (mg/kg)
TfR-mAb-mAtrogin#1179 (n=1)
TfR-mAb-mAtrogin#1504 (n=1)
TfR-mAb-m/hAtrogin#631 (n=1)
TfR-mAb-m/hAtrogin#586 (n=1)
PBS Control

FIG. 15C

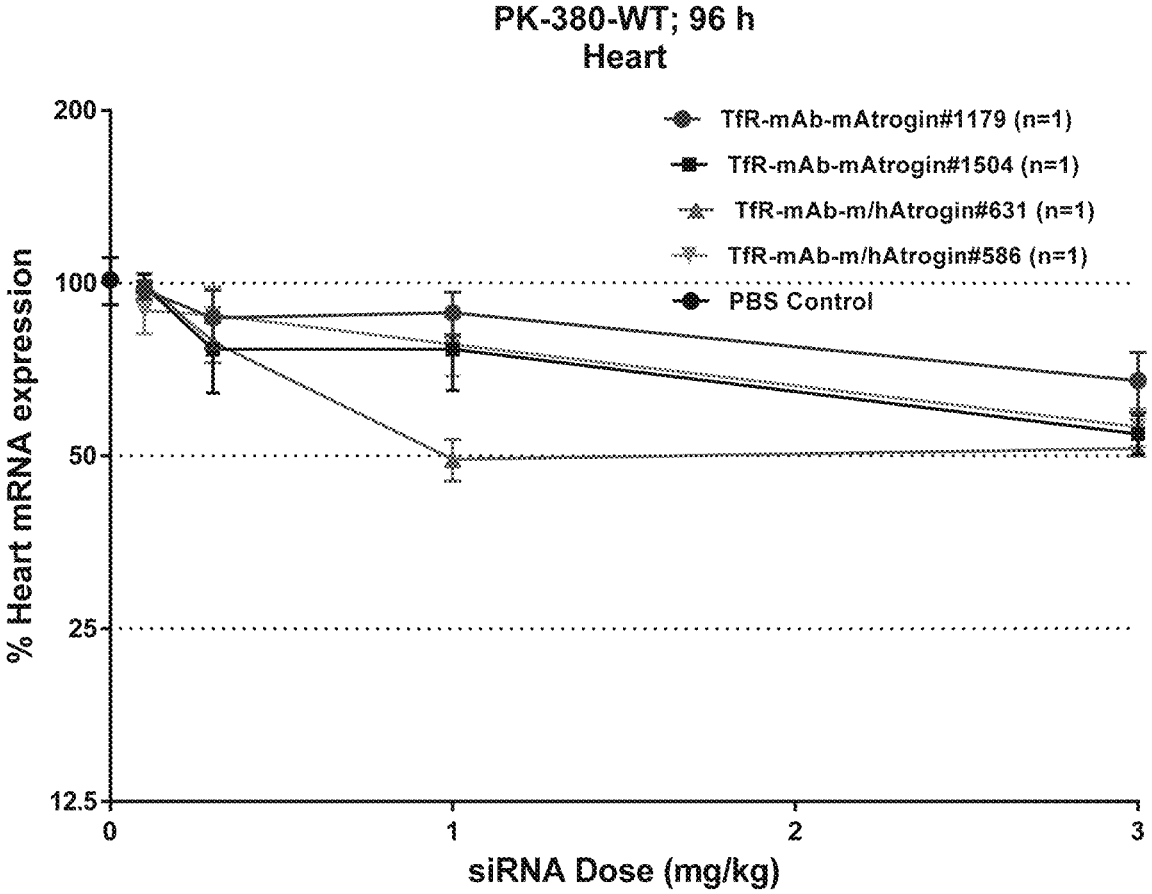

PK-380-WT; 96 h
Heart
TfR-mAb-mAtrogin#1179 (n=1)
TfR-mAb-mAtrogin#1504 (n=1)
TfR-mAb-m/hAtrogin#631 (n=1)
TfR-mAb-m/hAtrogin#586 (n=1)
PBS Control
% Heart mRNA expression
200
100
50
25
12.5
0
1
2
3
siRNA Dose (mg/kg)

FIG. 16A

Group	Test Article	N	siRNA Dose (mg/kg)	Harvest Time (h)
1	TfR-mAb-MuRF1#651(n=1)	4	3	96
2	TfR-mAb-MuRF1#651(n=1)	4	1	96
3	TfR-mAb-MuRF1#651(n=1)	4	0.3	96
4	TfR-mAb-MuRF1#651(n=1)	4	0.1	96
5	TfR-mAb-MuRF1#1387 (n=1)	4	3	96
6	TfR-mAb-MuRF1#1387 (n=1)	4	1	96
7	TfR-mAb-MuRF1#1387 (n=1)	4	0.3	96
8	TfR-mAb-MuRF1#1387 (n=1)	4	0.1	96
9	TfR-mAb-MuRF1#1454 (n=1)	4	3	96
10	TfR-mAb-MuRF1#1454 (n=1)	4	1	96
11	TfR-mAb-MuRF1#1454 (n=1)	4	0.3	96
12	TfR-mAb-MuRF1#1454 (n=1)	4	0.1	96
13	TfR-mAb-MuRF1#1660 (n=1)	4	3	96
14	TfR-mAb-MuRF1#1660 (n=1)	4	1	96
15	TfR-mAb-MuRF1#1660 (n=1)	4	0.3	96
16	TfR-mAb-MuRF1#1660 (n=1)	4	0.1	96
17	PBS Control	5	-	96

FIG. 16B

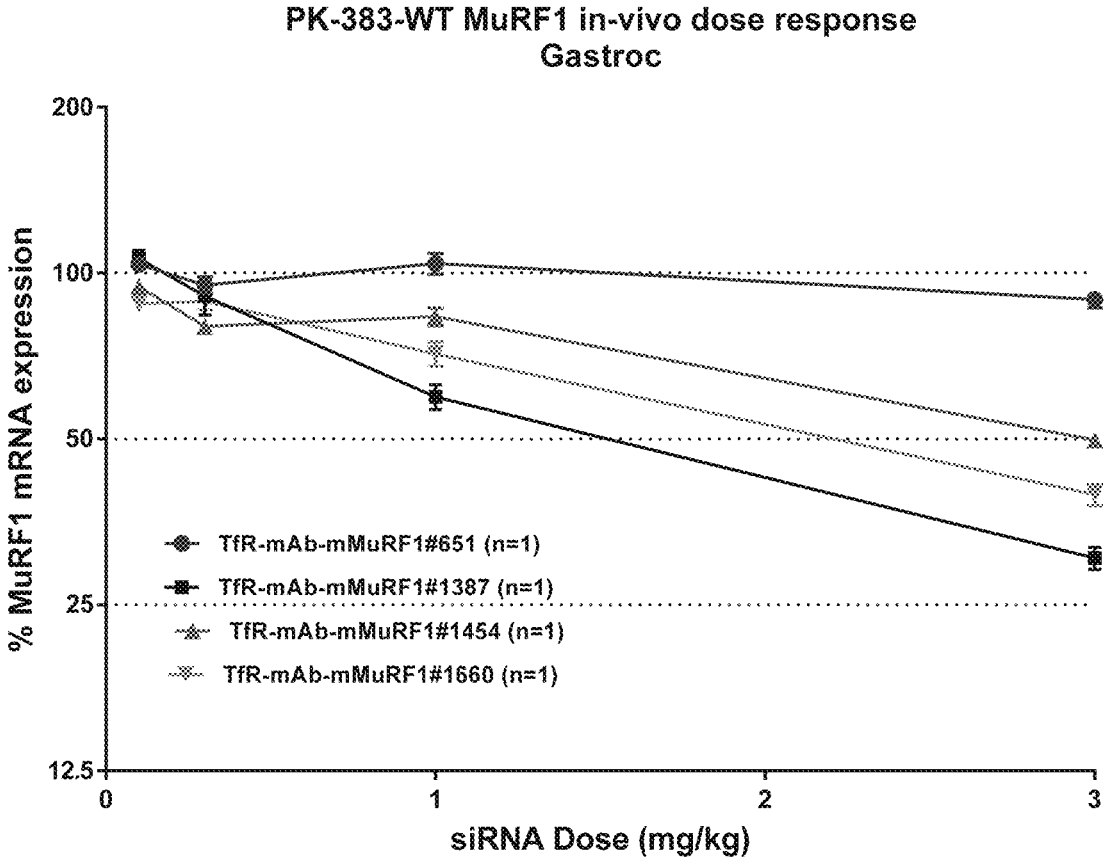

PK-383-WT MuRF1 in-vivo dose response
Gastroc
% MuRF1 mRNA expression
200
100
50
25
12.5
0
1
2
3
siRNA Dose (mg/kg)
TfR-mAb-mMuRF1#651 (n=1)
TfR-mAb-mMuRF1#1387 (n=1)
TfR-mAb-mMuRF1#1454 (n=1)
TfR-mAb-mMuRF1#1660 (n=1)

FIG. 16C

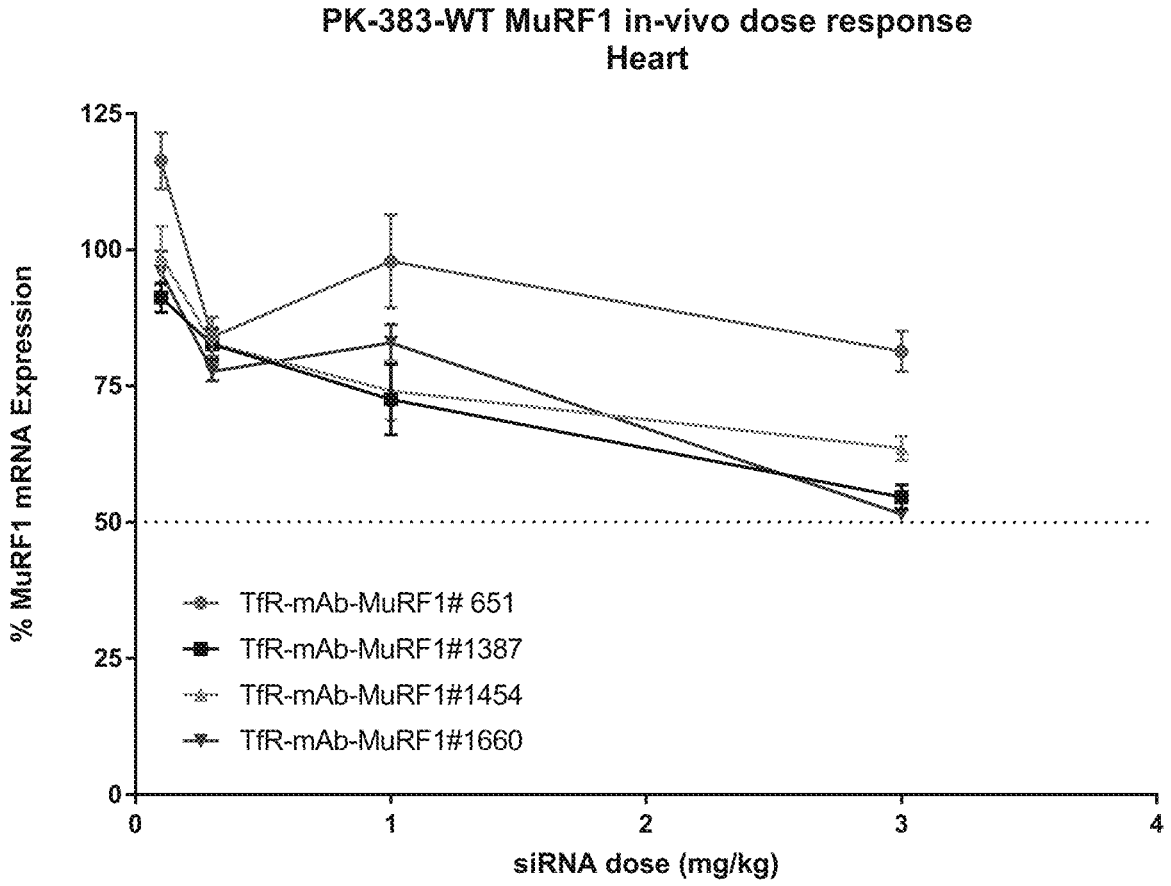

PK-383-WT MuRF1 in-vivo dose response
Heart
% MuRF1 mRNA Expression
125
100
75
50
25
0
0
1
2
3
4
siRNA dose (mg/kg)
TfR-mAb-MuRF1# 651
TfR-mAb-MuRF1#1387
TfR-mAb-MuRF1#1454
TfR-mAb-MuRF1#1660

FIG. 17

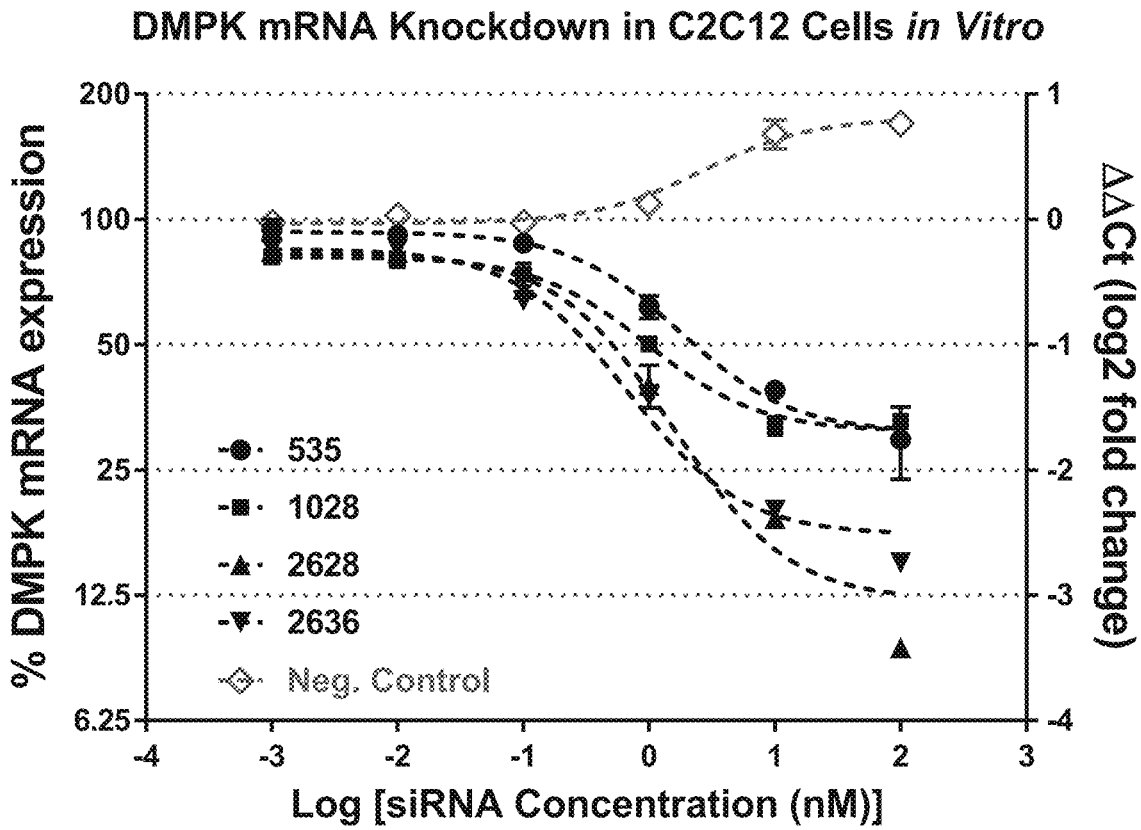

DMPK mRNA Knockdown in C2C12 Cells *in Vitro*
% DMPK mRNA expression
ΔΔCt (log2 fold change)
Log [siRNA Concentration (nM)]
200
100
50
25
12.5
6.25
1
0
-1
-2
-3
-4
-4
-3
-2
-1
0
1
2
3
535
1028
2628
2636
Neg. Control

Fig. 18A

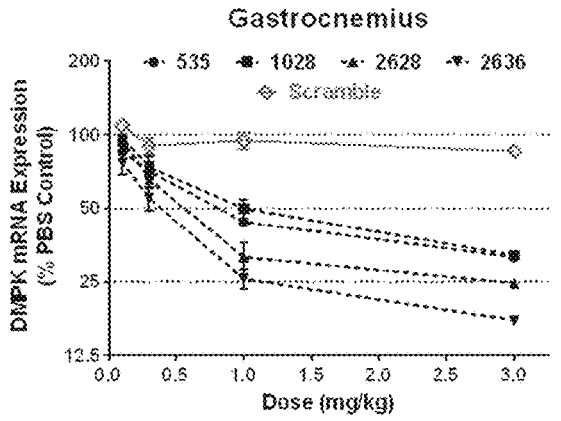


Fig. 18B

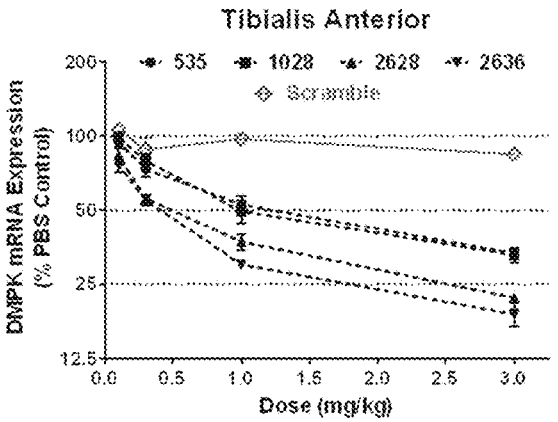


Fig. 18C

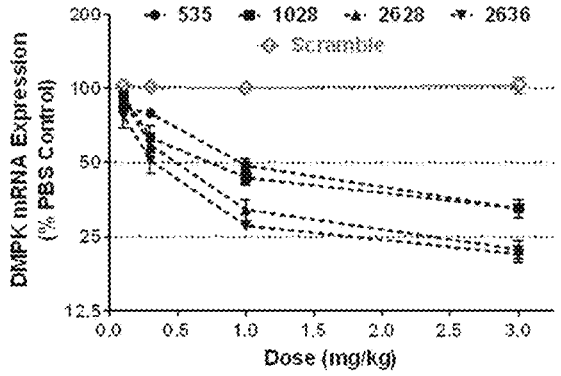


Fig. 18D

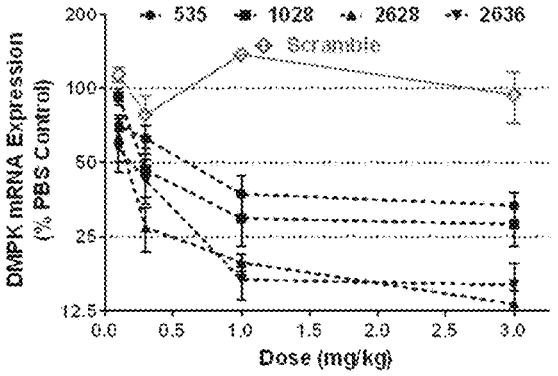


Fig. 18E

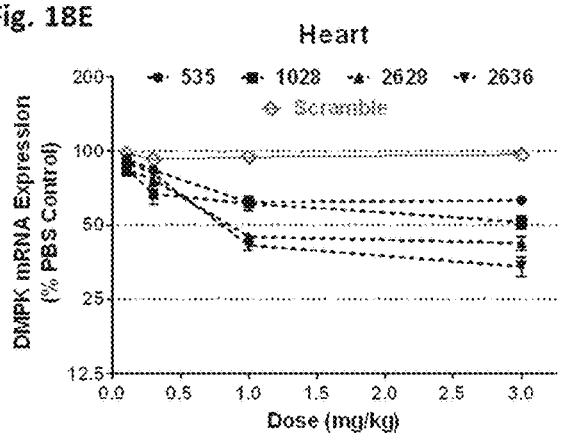


Fig. 18F

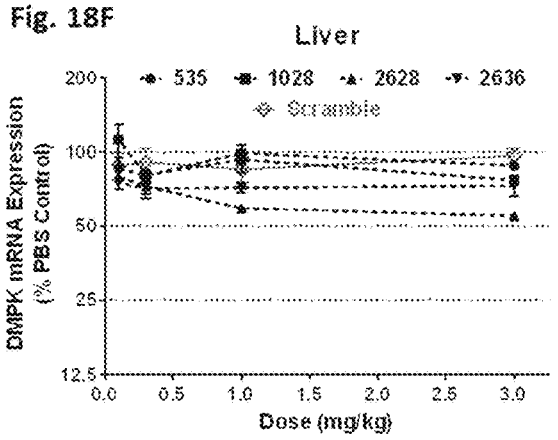


FIG. 19A

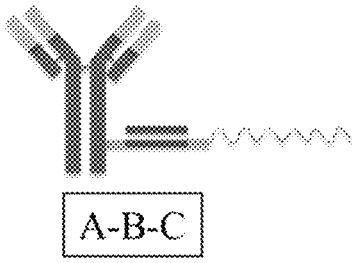


FIG. 19B

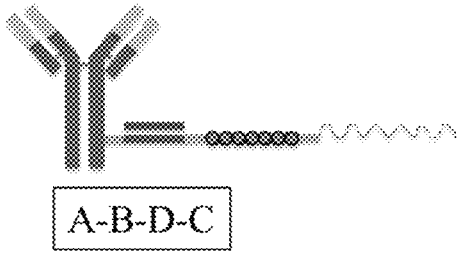


FIG. 19C

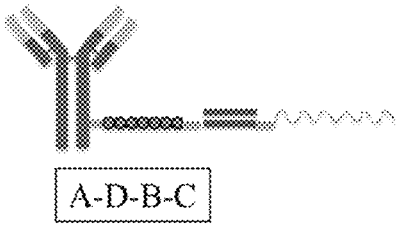


FIG. 19D

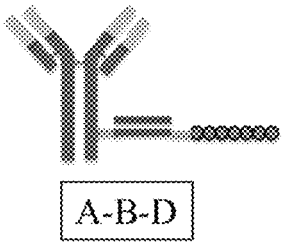


FIG. 19E

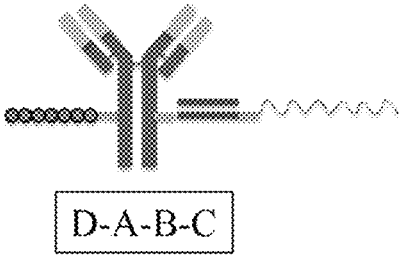


FIG. 19F

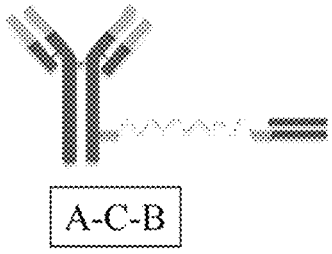


FIG. 19G

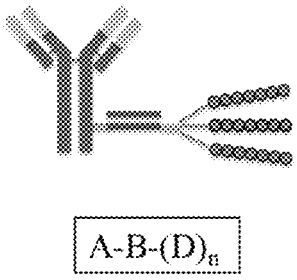


FIG. 19H

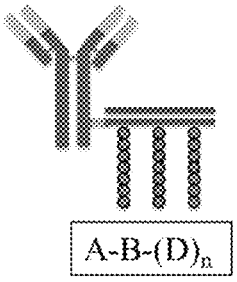


FIG. 19I

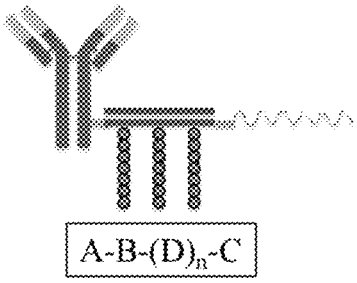


FIG. 19J

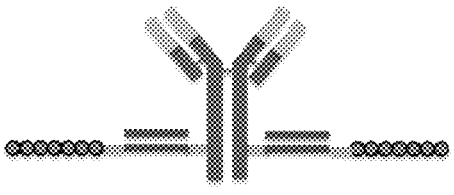


FIG. 19K

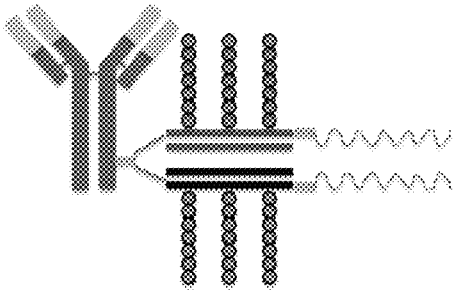


FIG. 19L

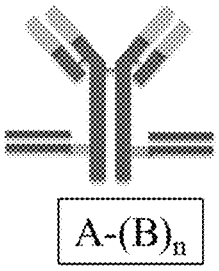


FIG. 19M

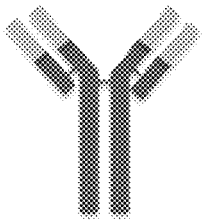


FIG. 20A

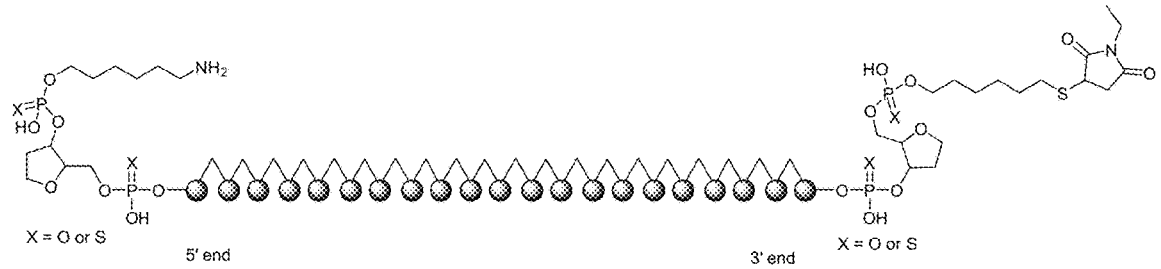


FIG. 20B

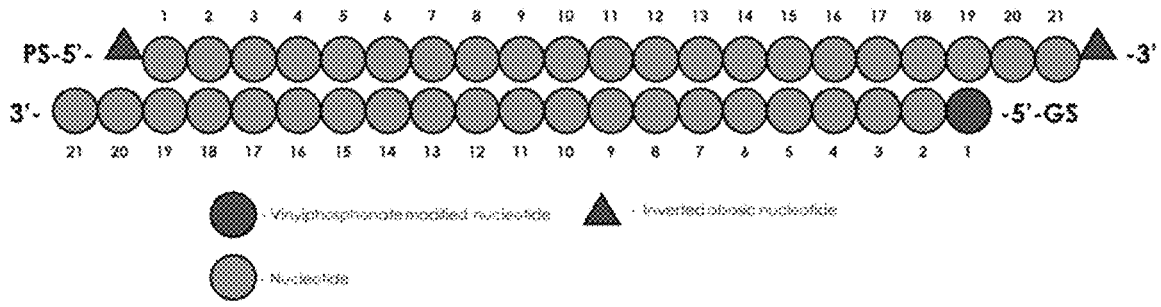


FIG. 21A

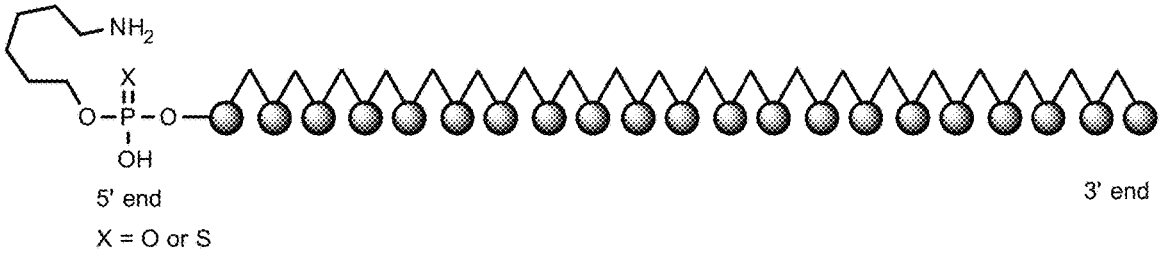


FIG. 21B

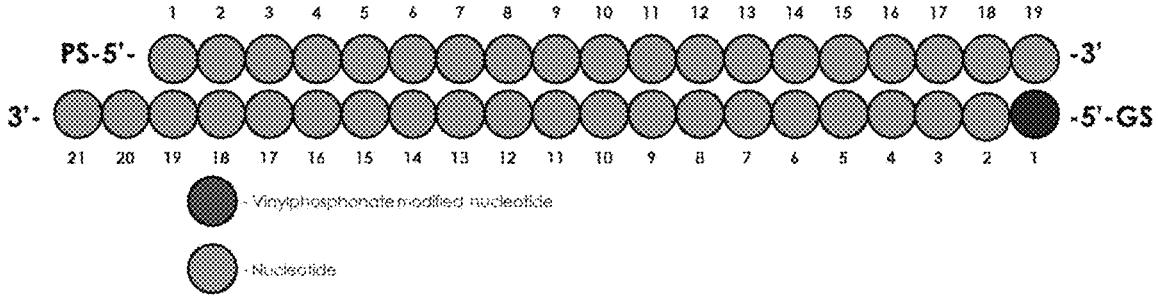


FIG. 22

Group	Test Article	N	siRNA Dose (mg/kg)	Dose Volume (mL/kg)	Harvest Time (h)
1	mTfR1-mAb-Atrogin-1 (DAR1)	4	3	**5.0**	96
2	mTfR1-mAb-Atrogin-1 (DAR1)	4	3	**5.0**	7 days
3	mTfR1-mAb-Atrogin-1 (DAR1)	4	3	**5.0**	14 days
4	mTfR1-mAb-Atrogin-1 (DAR1)	4	3	**5.0**	21 days
5	PBS Control	4	-	**5.0**	96
6	PBS Control	4	-	**5.0**	7 days
7	PBS Control	4	-	**5.0**	14 days
8	PBS Control	4	-	**5.0**	21 days

FIG. 23

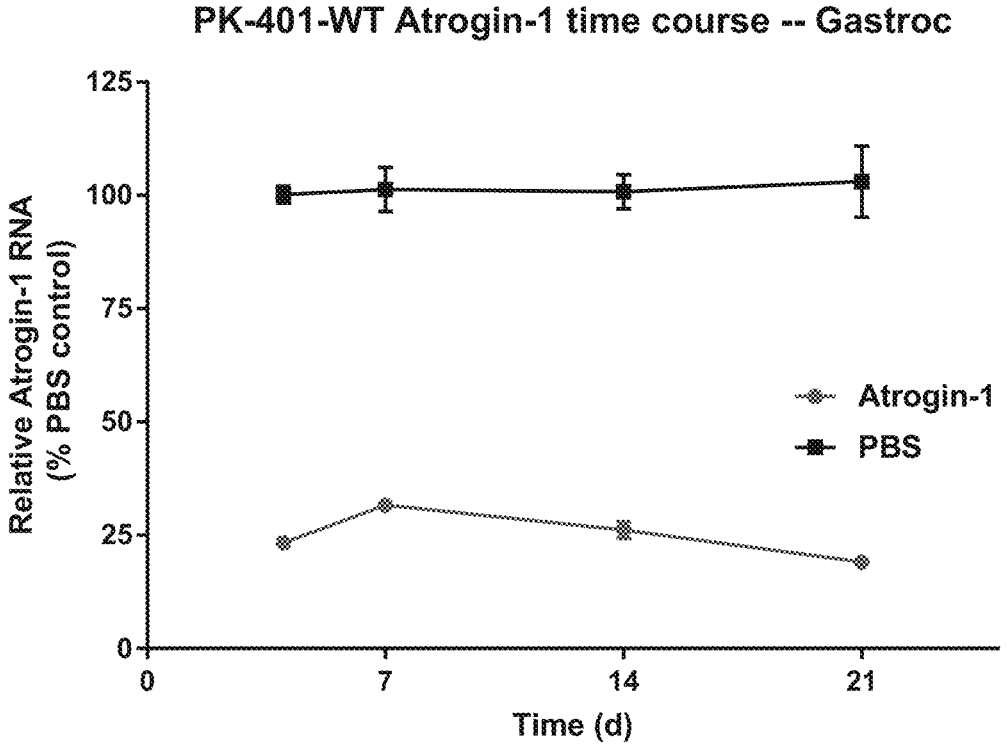

PK-401-WT Atrogin-1 time course -- Gastroc
Relative Atrogin-1 RNA (% PBS control)
125
100
75
50
25
0
0
7
14
21
Time (d)
Atrogin-1
PBS

FIG. 24

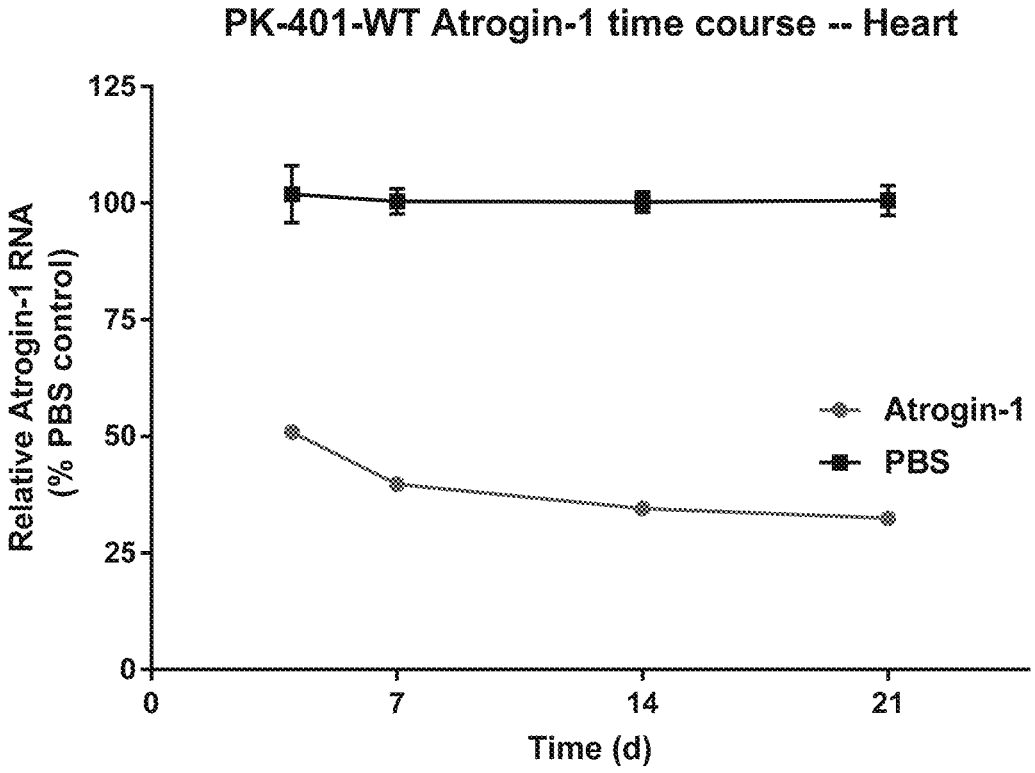

PK-401-WT Atrogin-1 time course -- Heart
Relative Atrogin-1 RNA (% PBS control)
125
100
75
50
25
0
0
7
14
21
Time (d)
Atrogin-1
PBS

FIG. 25

Group	Test Article	N	siRNA Dose (mg/kg)	Dose Volume (mL/kg)	Harvest Time (h)
1	mTfR1-mAb-MuRF1(R2089) (DAR1)	4	3	**5.0**	96
2	mTfR1-mAb-MuRF1(R2089) (DAR1)	4	1	**5.0**	96
3	mTfR1-mAb-MuRF1(R2265) (DAR1)	4	3	**5.0**	96
4	mTfR1-mAb-MuRF1(R2265) (DAR1)	4	1	**5.0**	96
5	mTfR1-mAb-MuRF1(R2265) (DAR1)	4	0.3	**5.0**	96
6	mTfR1-mAb-MuRF1(R2265) (DAR1)	4	0.1	**5.0**	96
7	mTfR1-mAb-MuRF1(R2266) (DAR1)	4	3	**5.0**	96
8	mTfR1-mAb-MuRF1(R2266) (DAR1)	4	1	**5.0**	96
9	mTfR1-mAb-MuRF1(R2266) (DAR1)	4	0.3	**5.0**	96
10	mTfR1-mAb-MuRF1(R2266) (DAR1)	4	0.1	**5.0**	96
11	mTfR1-mAb-MuRF1(R2267) (DAR1)	4	3	**5.0**	96
12	mTfR1-mAb-MuRF1(R2267) (DAR1)	4	1	**5.0**	96
13	mTfR1-mAb-MuRF1(R2267 (DAR1)	4	0.3	**5.0**	96
14	mTfR1-mAb-MuRF1(R2267) (DAR1)	4	0.1	**5.0**	96
15	mTfR1-mAb-MuRF1(R2268) (DAR1)	4	3	**5.0**	96
16	mTfR1-mAb-MuRF1(R2268) (DAR1)	4	1	**5.0**	96
27	mTfR1-mAb-MuRF1(R2268) (DAR1)	4	0.3	**5.0**	96
18	mTfR1-mAb-MuRF1(R2268) (DAR1)	4	0.1	**5.0**	96
19	mTfR1-mAb-MuRF1(R2269) (DAR1)	4	3	**5.0**	96
20	mTfR1-mAb-MuRF1(R2269) (DAR1)	4	1	**5.0**	96
21	mTfR1-mAb-MuRF1(R2269) (DAR1)	4	0.3	**5.0**	96
22	mTfR1-mAb-MuRF1(R2269) (DAR1)	4	0.1	**5.0**	96
23	PBS Control	5	-	**5.0**	96

FIG. 26

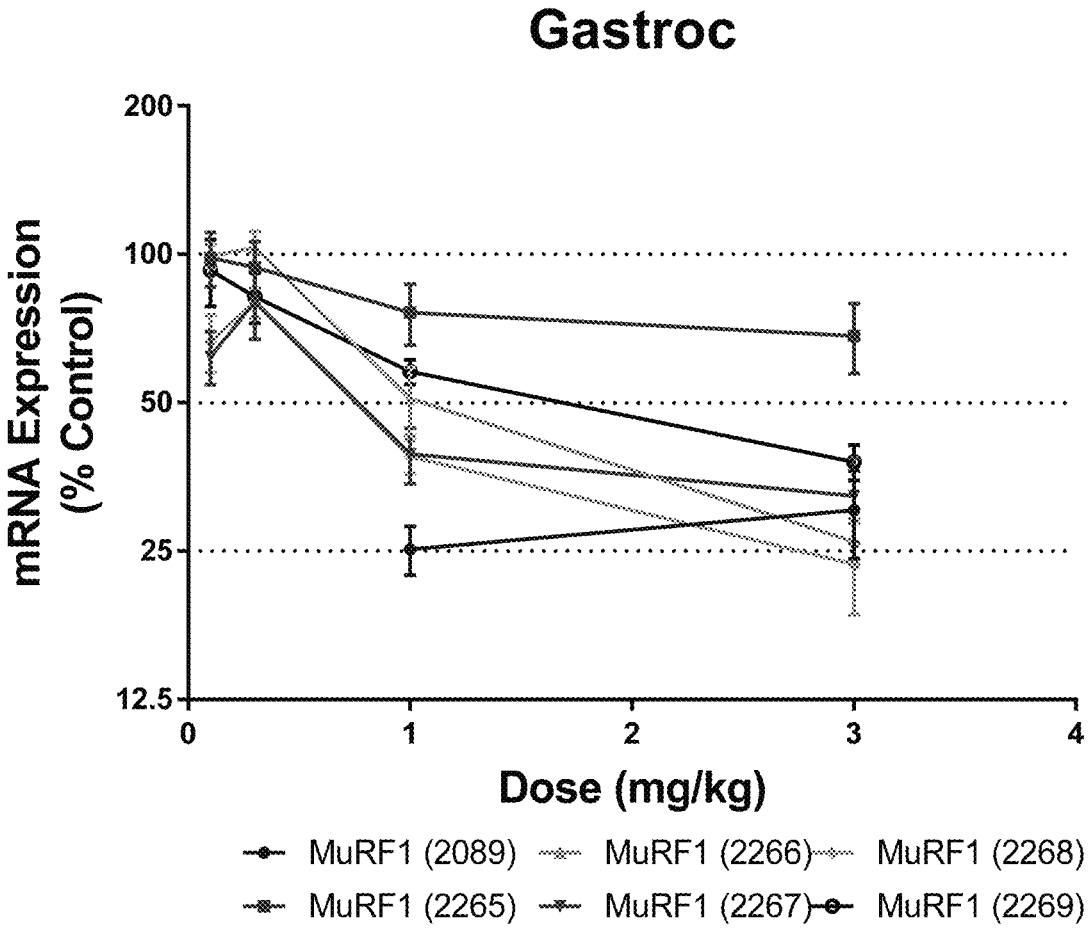

Gastroc
mRNA Expression (% Control)
200
100
50
25
12.5
0
1
2
3
4
Dose (mg/kg)
MuRF1 (2089)
MuRF1 (2266)
MuRF1 (2268)
MuRF1 (2265)
MuRF1 (2267)
MuRF1 (2269)

FIG. 27

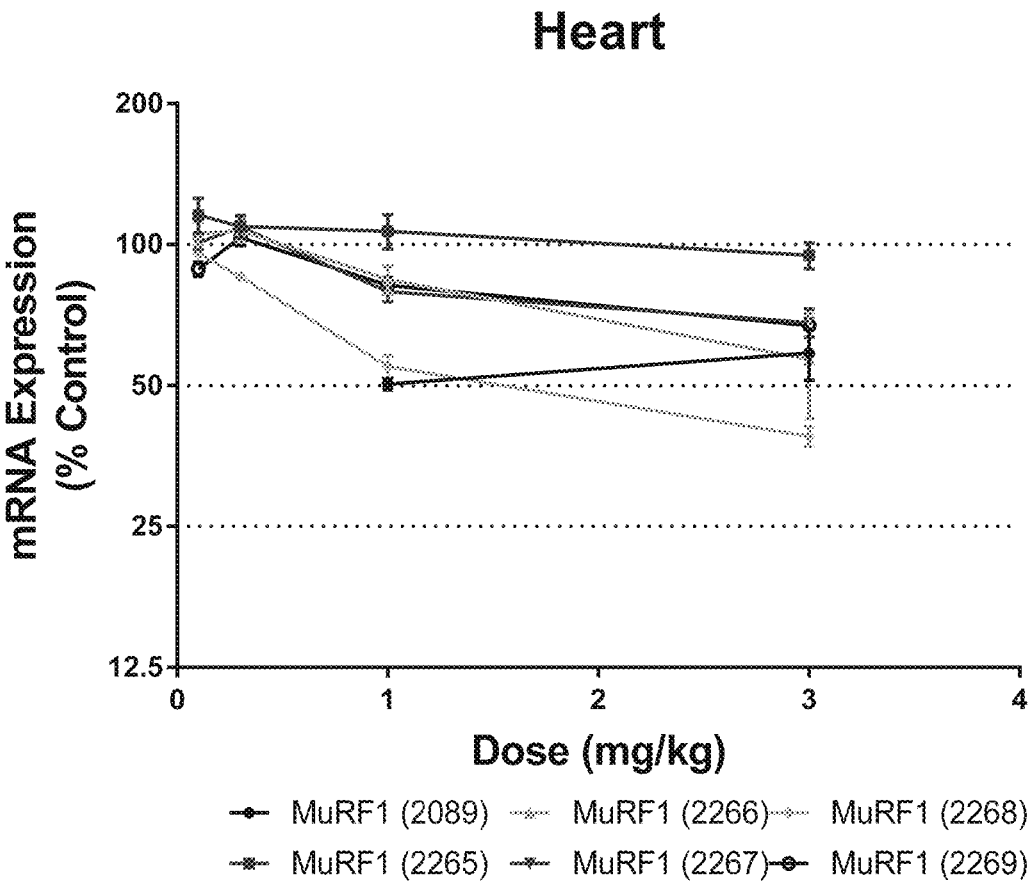

Heart
mRNA Expression (% Control)
200
100
50
25
12.5
0
1
2
3
4
Dose (mg/kg)
MuRF1 (2089)
MuRF1 (2266)
MuRF1 (2268)
MuRF1 (2265)
MuRF1 (2267)
MuRF1 (2269)

FIG. 28

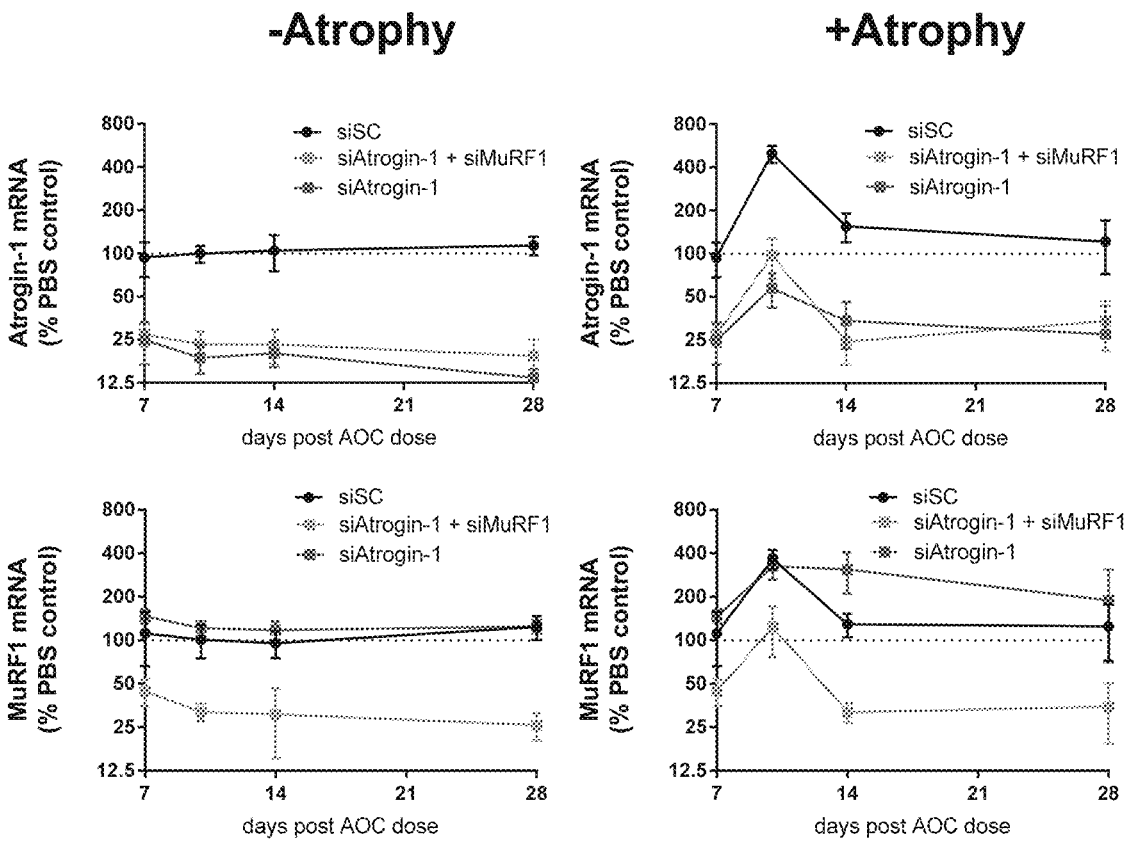

-Atrophy
+Atrophy
Atrogin-1 mRNA (% PBS control)
MuRF1 mRNA (% PBS control)
siSC
siAtrogin-1 + siMuRF1
siAtrogin-1
800
400
200
100
50
25
12.5
7
14
21
28
days post AOC dose

FIG. 29

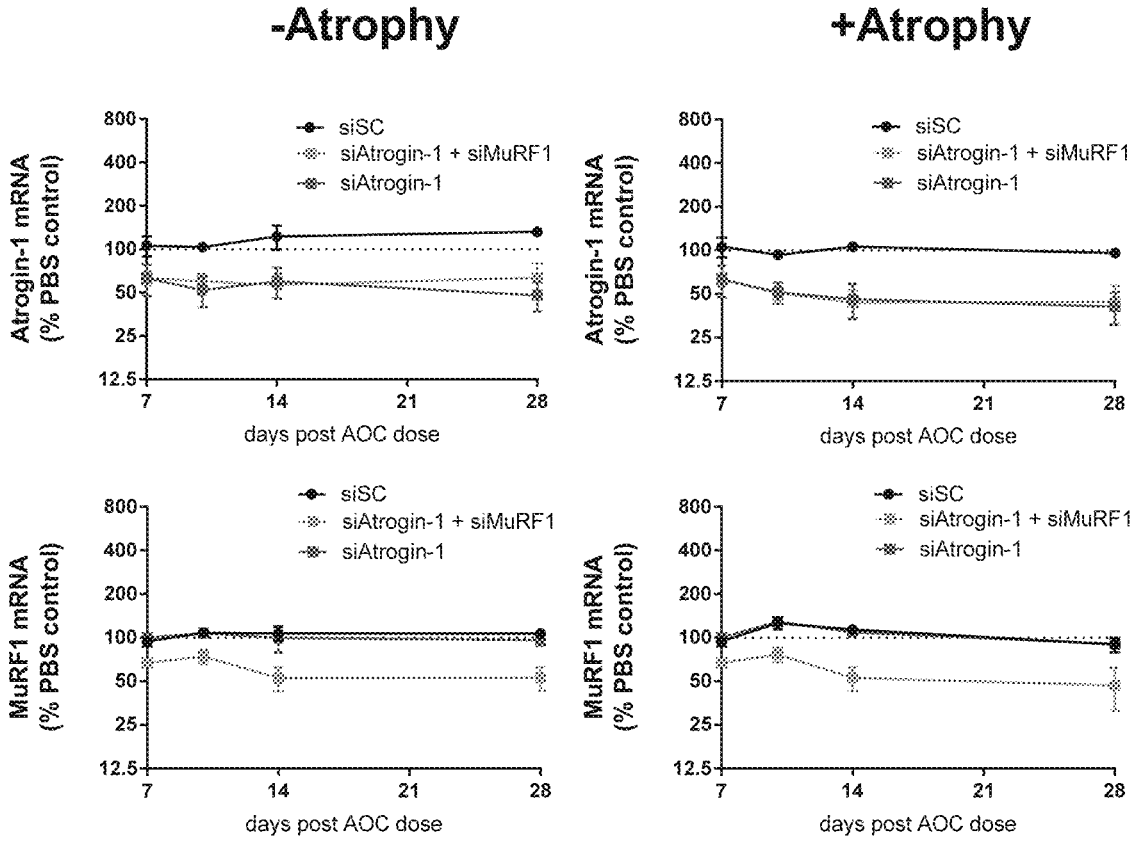

-Atrophy
+Atrophy
Atrogin-1 mRNA (% PBS control)
MuRF1 mRNA (% PBS control)
siSC
siAtrogin-1 + siMuRF1
siAtrogin-1
800
400
200
100
50
25
12.5
7
14
21
28
days post AOC dose

FIG. 30

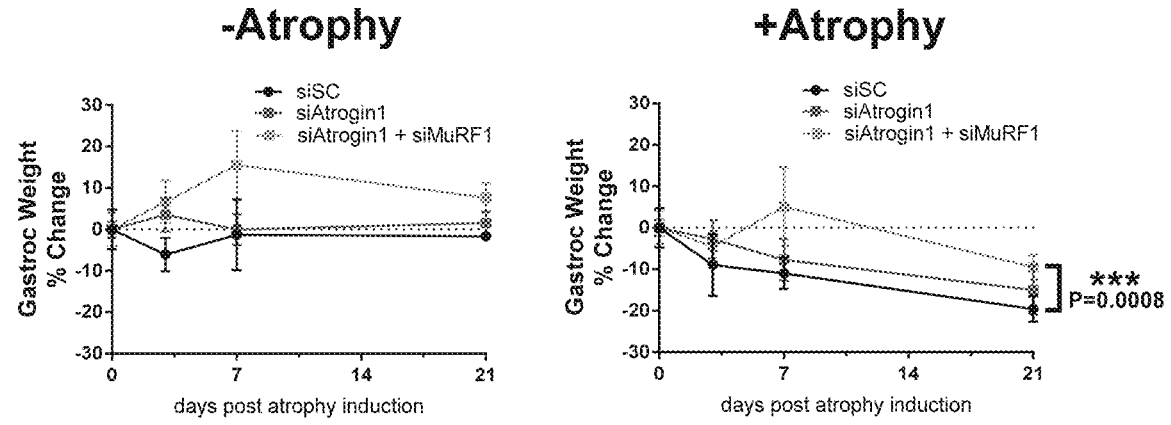

-Atrophy
+Atrophy
Gastroc Weight % Change
siSC
siAtrogin1
siAtrogin1 + siMuRF1
30
20
10
0
-10
-20
-30
0
7
14
21
days post atrophy induction
***
P=0.0008

FIG. 31

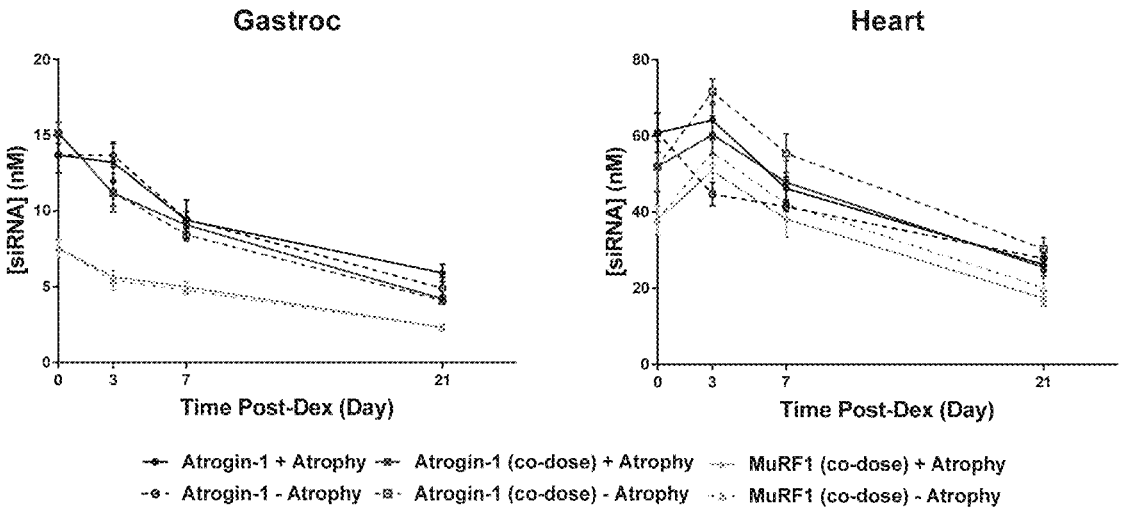

Gastroc
Heart
[siRNA] (nM)
Time Post-Dex (Day)
0
3
7
21
20
15
10
5
80
60
40
20
Atrogin-1 + Atrophy
Atrogin-1 (co-dose) + Atrophy
MuRF1 (co-dose) + Atrophy
Atrogin-1 - Atrophy
Atrogin-1 (co-dose) - Atrophy
MuRF1 (co-dose) - Atrophy

FIG. 32

Group	ASC	Atrophy	N	siRNA Dose (mg/kg)	siRNA: mAb Ratio (mol/mol)	Dose Volume (mL/kg)	# of Doses	Harvest Time (day post Dex)
1	TfR1-Atrogin-1 DAR1		4	9	1.0	5.0	1	0
2	TfR1-Atrogin-1 DAR1	+ PBS	4	9	1.0	5.0	1	3
3	TfR1-Atrogin-1 DAR1	+ Dex	4	9	1.0	5.0	1	3
4	TfR1-Atrogin-1 DAR1		4	3	1.0	5.0	1	0
5	TfR1-Atrogin-1 DAR1	+ PBS	4	3	1.0	5.0	1	3
6	TfR1-Atrogin-1 DAR1	+ Dex	4	3	1.0	5.0	1	3
7	TfR1-Atrogin-1 DAR1		4	1	1.0	5.0	1	0
8	TfR1-Atrogin-1 DAR1	+ PBS	4	1	1.0	5.0	1	3
9	TfR1-Atrogin-1 DAR1	+ Dex	4	1	1.0	5.0	1	3
10	TfR1-Scramble DAR1		4	9	1.0	5.0	1	0
11	TfR1-Scramble DAR1	+ PBS	4	9	1.0	5.0	1	3
12	TfR1-Scramble DAR1	+ Dex	4	9	1.0	5.0	1	3
13	TfR1-Scramble DAR1		4	3	1.0	5.0	1	0
14	TfR1-Scramble DAR1	+ PBS	4	3	1.0	5.0	1	3
15	TfR1-Scramble DAR1	+ Dex	4	3	1.0	5.0	1	3
16	PBS Control		5	-	-	5.0	1	0

FIG. 33

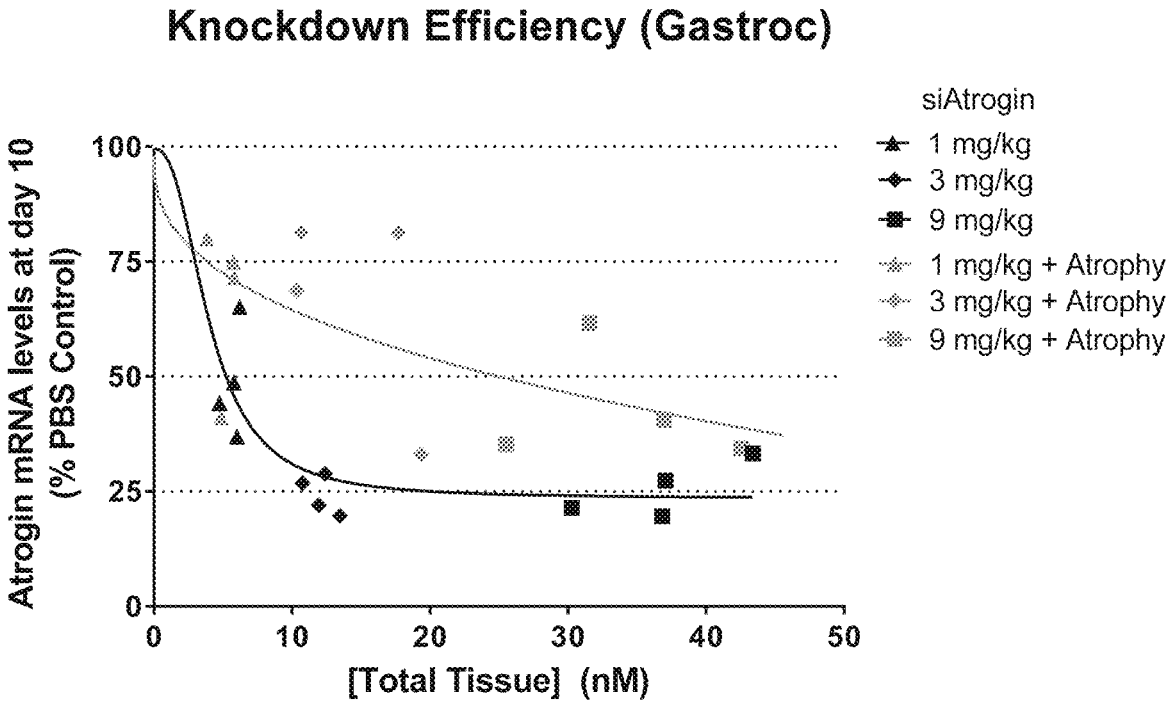

Knockdown Efficiency (Gastroc)
siAtrogin
1 mg/kg
3 mg/kg
9 mg/kg
1 mg/kg + Atrophy
3 mg/kg + Atrophy
9 mg/kg + Atrophy
Atrogin mRNA levels at day 10 (% PBS Control)
100
75
50
25
0
0
10
20
30
40
50
[Total Tissue] (nM)

FIG. 34

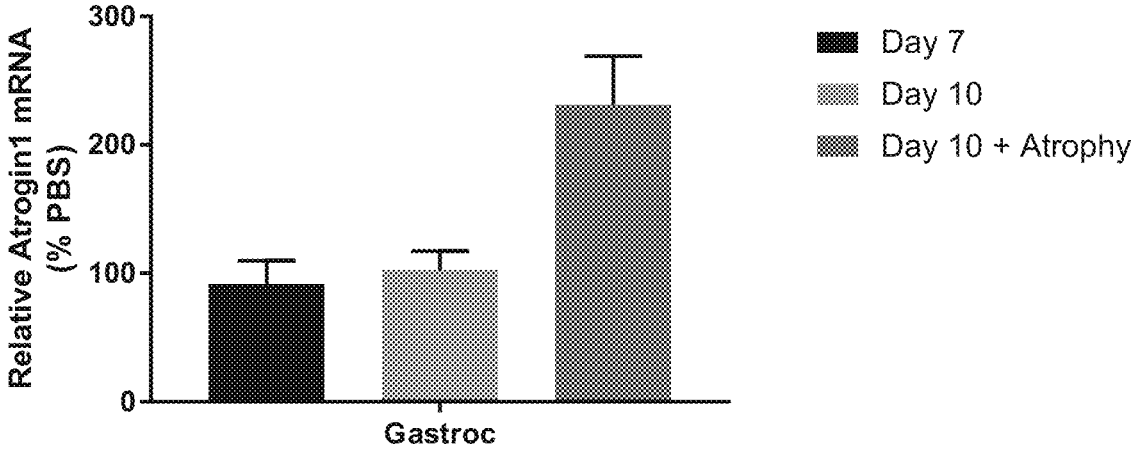

Relative Atrogin1 mRNA (% PBS)
300
200
100
0
Gastroc
Day 7
Day 10
Day 10 + Atrophy

FIG. 35

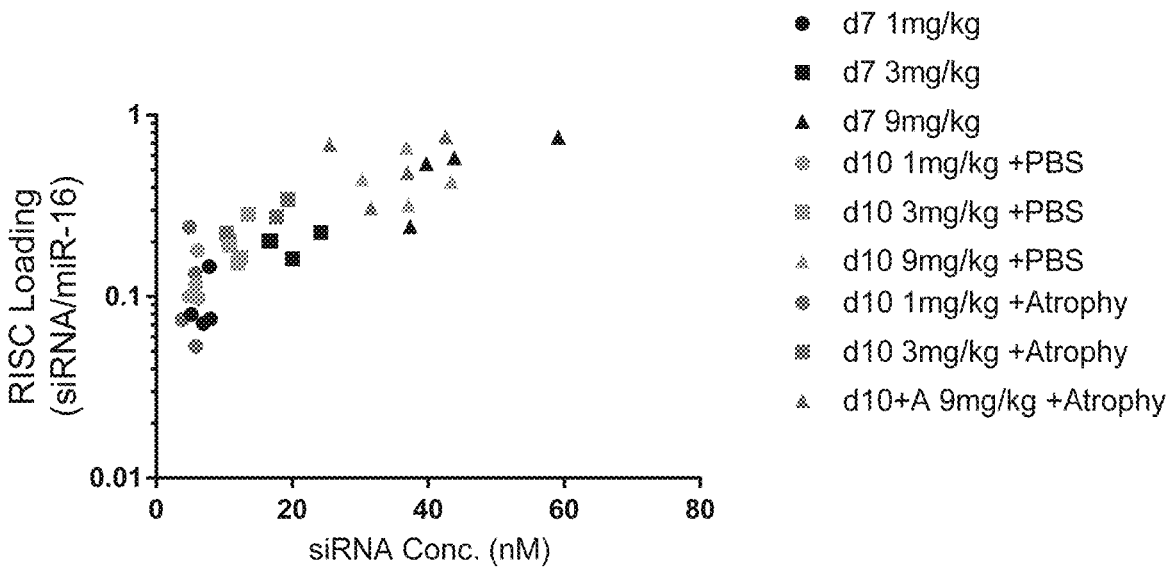

d7 1mg/kg
d7 3mg/kg
d7 9mg/kg
d10 1mg/kg +PBS
d10 3mg/kg +PBS
d10 9mg/kg +PBS
d10 1mg/kg +Atrophy
d10 3mg/kg +Atrophy
d10+A 9mg/kg +Atrophy
RISC Loading (siRNA/miR-16)
1
0.1
0.01
0
20
40
60
80
siRNA Conc. (nM)

FIG. 36

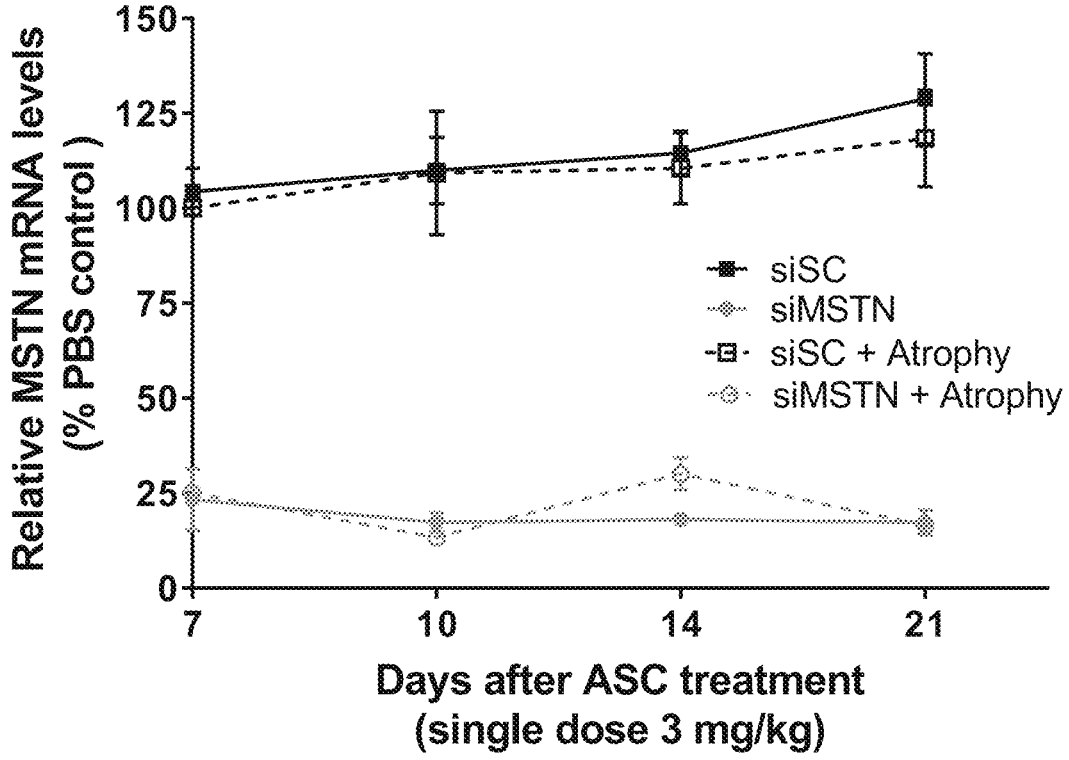

Relative MSTN mRNA levels (% PBS control)
150
125
100
75
50
25
0
7
10
14
21
siSC
siMSTN
siSC + Atrophy
siMSTN + Atrophy
Days after ASC treatment
(single dose 3 mg/kg)

FIG. 37

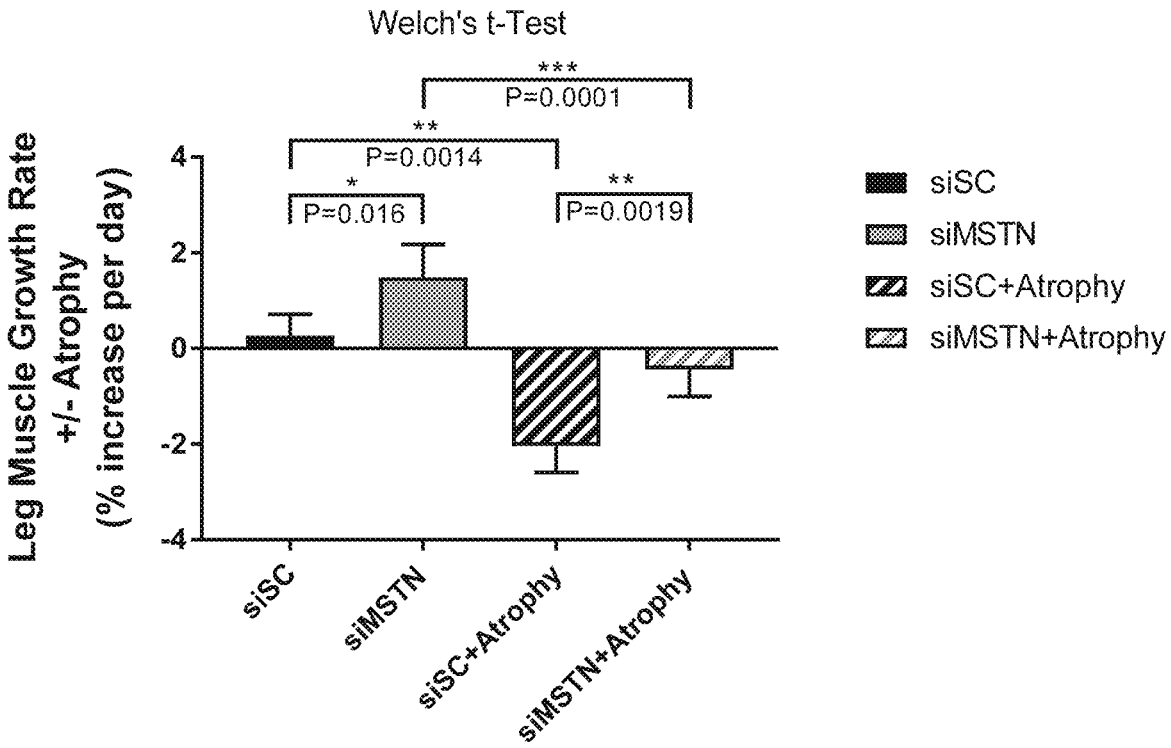

Welch's t-Test
***
P=0.0001
**
P=0.0014
*
P=0.016
**
P=0.0019
Leg Muscle Growth Rate
+/- Atrophy
(% increase per day)
4
2
0
-2
-4
siSC
siMSTN
siSC+Atrophy
siMSTN+Atrophy
siSC
siMSTN
siSC+Atrophy
siMSTN+Atrophy

FIG. 38

Group	ASC	N	Total siRNA Dose (mg/kg)	Dose Volume (mL/kg)	Harvest Time (day post ASC)	Harvest Time (day post DEN)
1	TfR1-Atrogin-1 DAR1	4	4.5	**5.0**	7	0
2	TfR1-Atrogin-1 DAR1	4	4.5	**5.0**	10	3
3	TfR1-Atrogin-1 DAR1	4	4.5	**5.0**	17	10
4	TfR1-Atrogin-1 DAR1	8	4.5	**5.0**	28	21
5	TfR1-MuRF1 DAR1	4	4.5	**5.0**	7	0
6	TfR1-MuRF1 DAR1	4	4.5	**5.0**	10	3
7	TfR1-MuRF1 DAR1	4	4.5	**5.0**	17	10
8	TfR1-MuRF1 DAR1	8	4.5	**5.0**	28	21
9	TfR1-Atrogin-1 + TfR1-MuRF1 DAR1	4	9	**5.0**	7	0
10	TfR1-Atrogin-1 + TfR1-MuRF1 DAR1	4	9	**5.0**	10	3
11	TfR1-Atrogin-1 + TfR1-MuRF1 DAR1	4	9	**5.0**	17	10
12	TfR1-Atrogin-1 + TfR1-MuRF1 DAR1	8	9	**5.0**	28	21
13	TfR1-SC DAR1	4	4.5	**5.0**	7	0
14	TfR1-SC DAR1	4	4.5	**5.0**	10	3
15	TfR1-SC DAR1	4	4.5	**5.0**	17	10
16	TfR1-SC DAR1	8	4.5	**5.0**	28	21
17	PBS Control	5	-	**5.0**	7	0

FIG. 39A

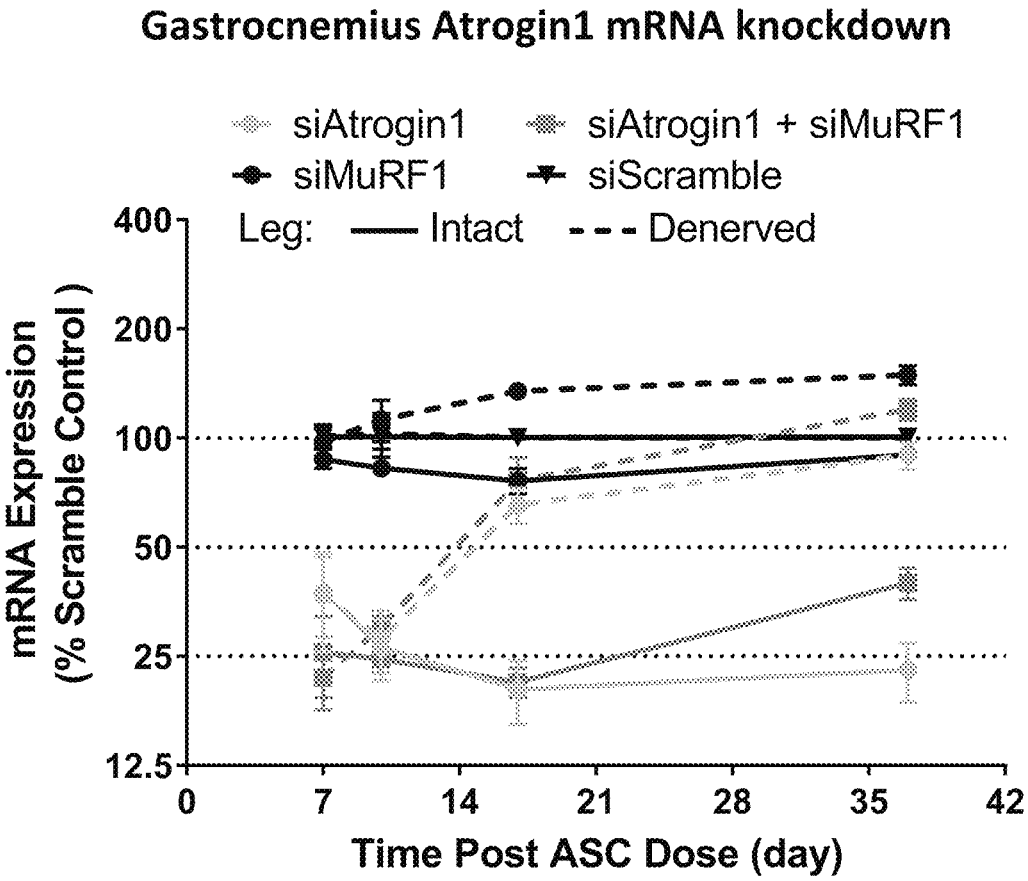

Gastrocnemius Atrogin1 mRNA knockdown
siAtrogin1
siAtrogin1 + siMuRF1
siMuRF1
siScramble
Leg:
Intact
Denerved
mRNA Expression (% Scramble Control)
400
200
100
50
25
12.5
0
7
14
21
28
35
42
Time Post ASC Dose (day)

FIG. 39B

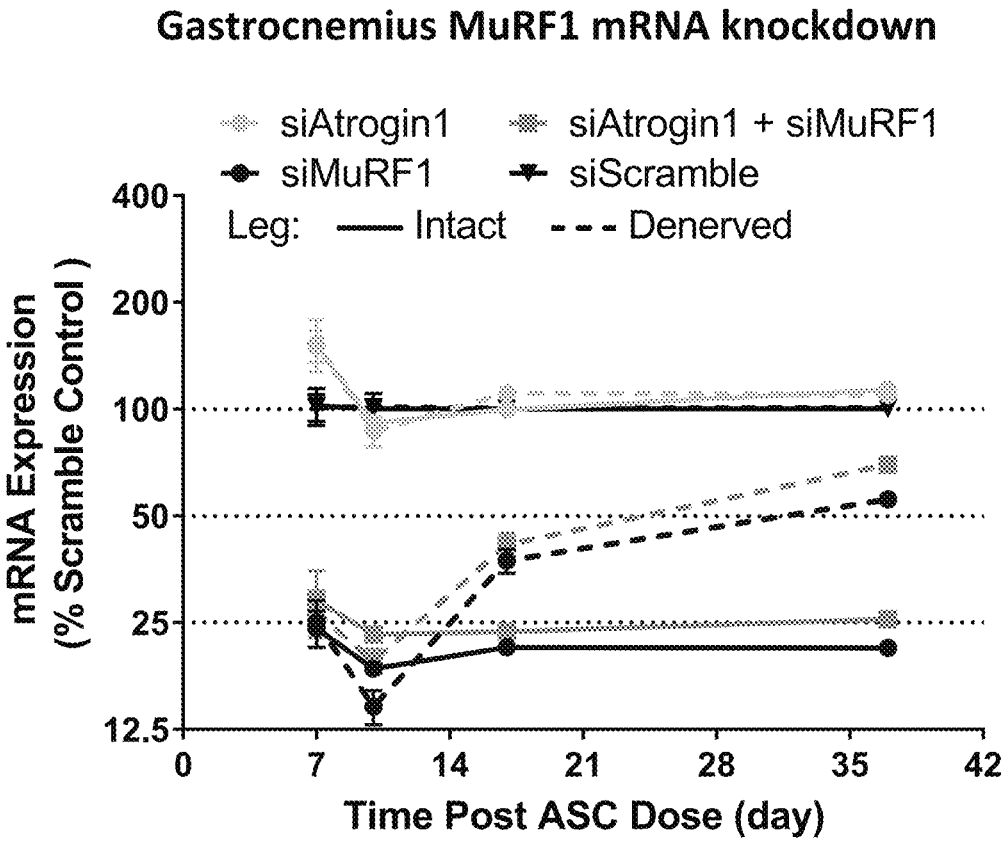

Gastrocnemius MuRF1 mRNA knockdown
siAtrogin1
siAtrogin1 + siMuRF1
siMuRF1
siScramble
Leg:
Intact
Denerved
mRNA Expression
(% Scramble Control)
400
200
100
50
25
12.5
0
7
14
21
28
35
42
Time Post ASC Dose (day)

FIG. 39C

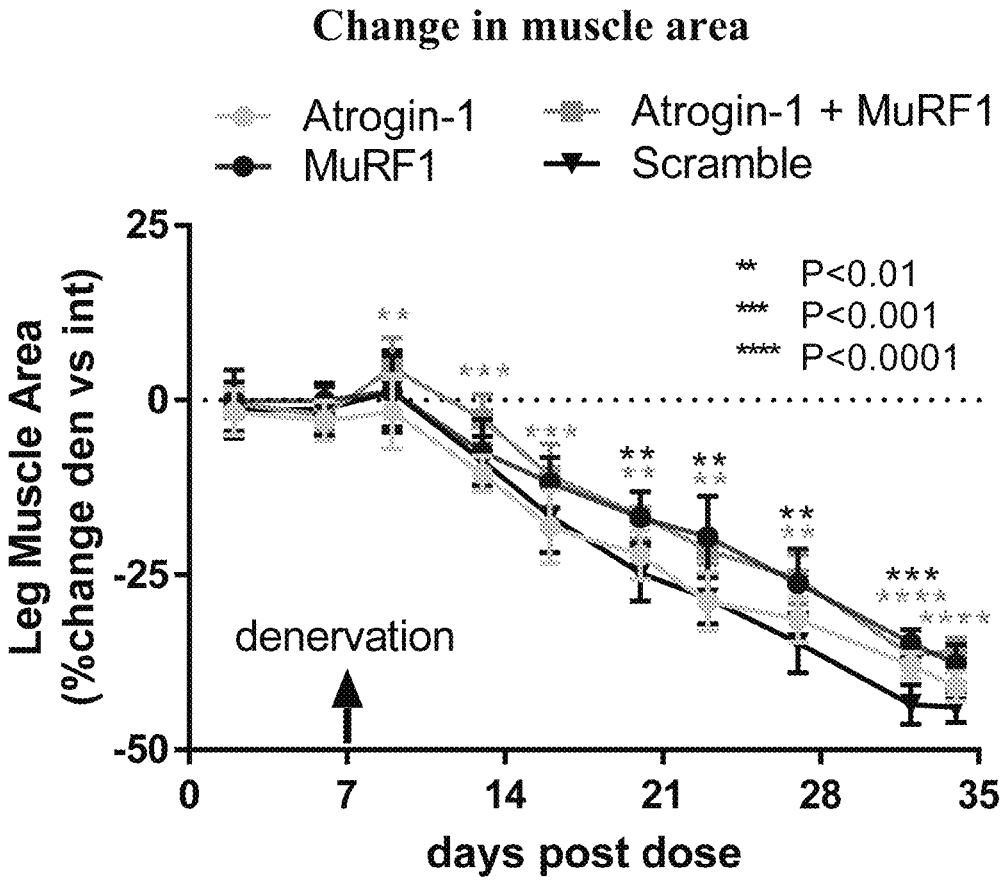

Change in muscle area
Atrogin-1
MuRF1
Atrogin-1 + MuRF1
Scramble
** P<0.01
*** P<0.001
**** P<0.0001
Leg Muscle Area
(%change den vs int)
25
0
-25
-50
denervation
0
7
14
21
28
35
days post dose

FIG. 39D

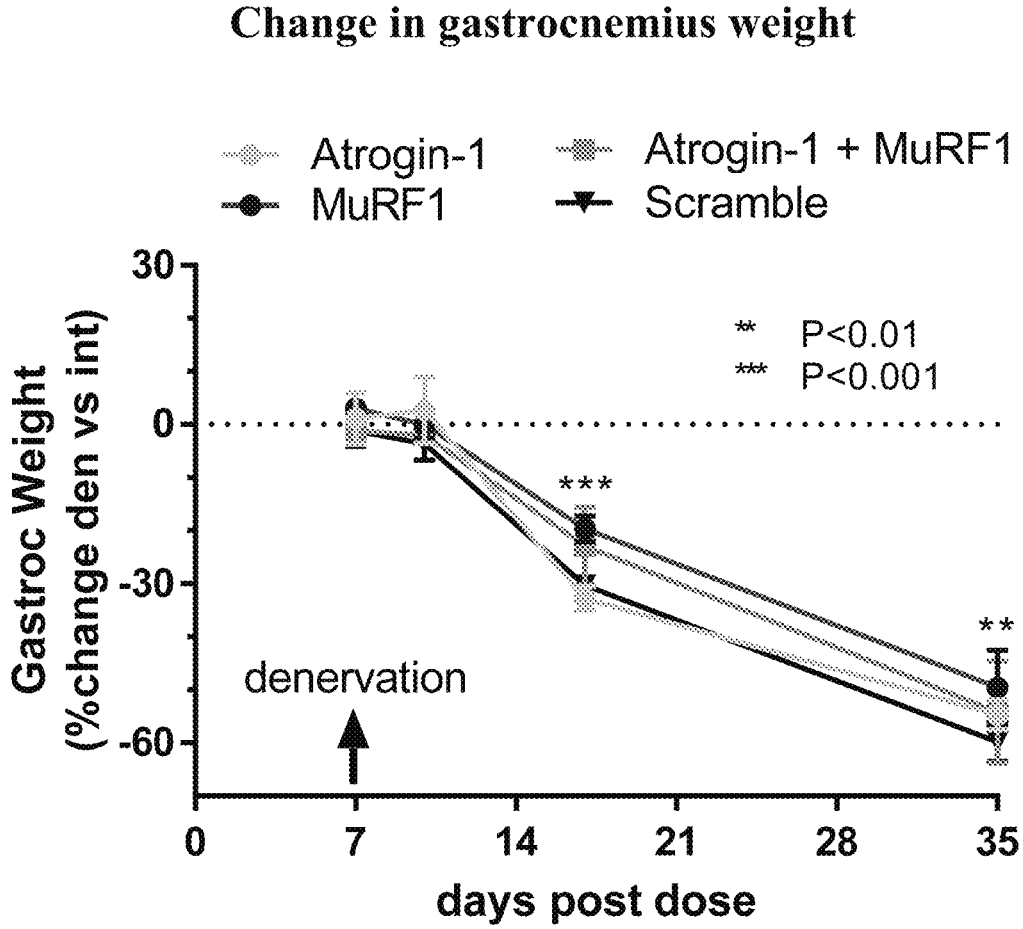

Change in gastrocnemius weight
Atrogin-1
MuRF1
Atrogin-1 + MuRF1
Scramble
Gastroc Weight
(%change den vs int)
30
0
-30
-60
** P<0.01
*** P<0.001
***
**
denervation
0
7
14
21
28
35
days post dose

FIG. 39E

Treatment-induced % Sparing of Muscle Wasting (Leg muscle area)

days post dose	days post denervation	MuRF1	Atrogin-1 + MuRF1
16	9	29.0	34.7
20	13	32.0	33.4
23	16	31.0	24.5
27	20	24.5	26.3
32	25	20.2	16.2
34	27	14.2	18.2

FIG. 39F

Treatment-induced % Sparing of Muscle Wasting (Gastrocnemius weight)

days post dose	days post denervation	MuRF1
17	10	35.2
35	28	17.1

FIG. 40A

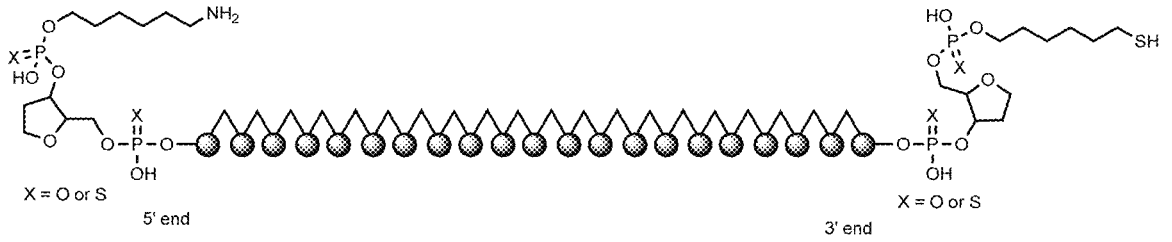


FIG. 40B

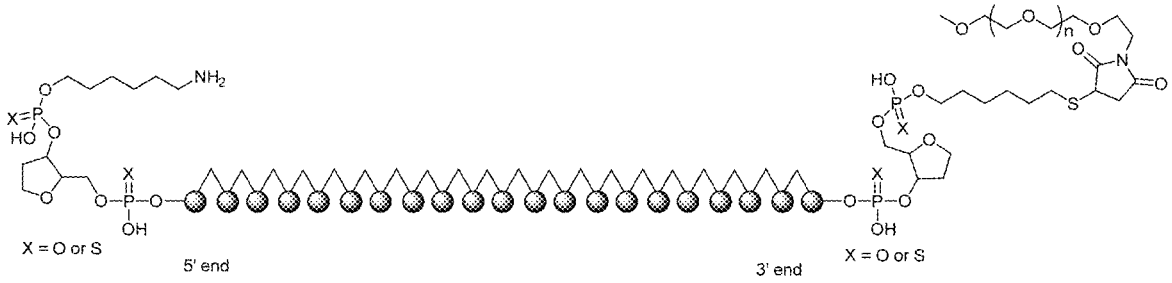


FIG. 40C

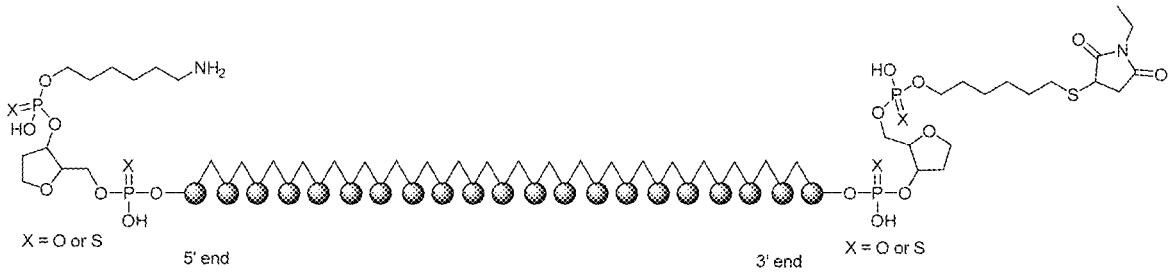


FIG. 40D

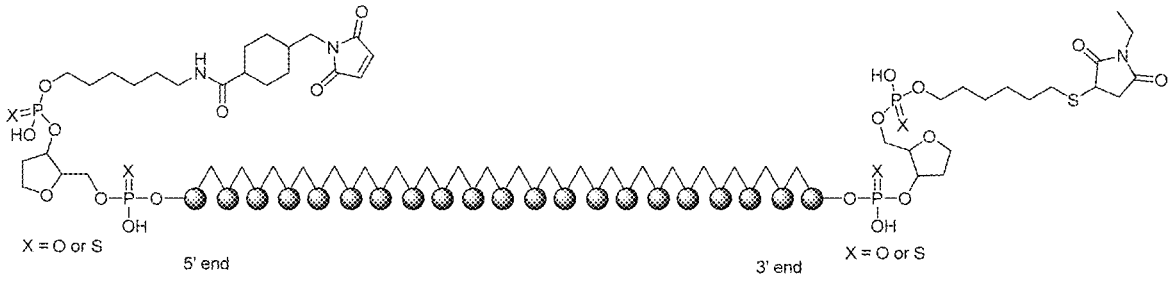


FIG. 40E

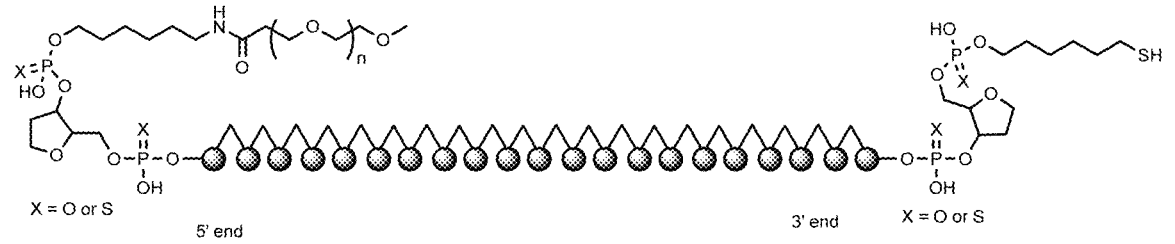


FIG. 40F

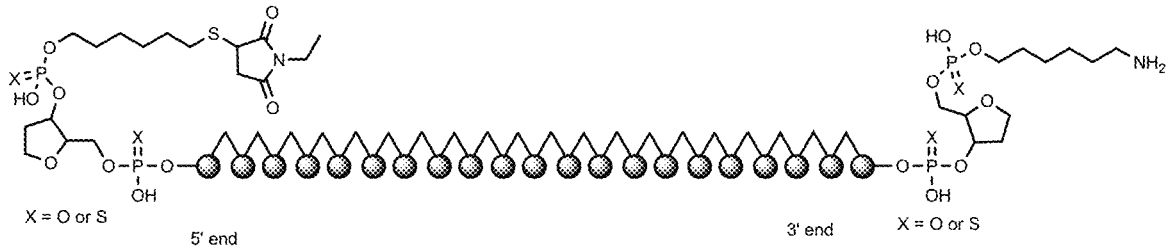


FIG. 41A

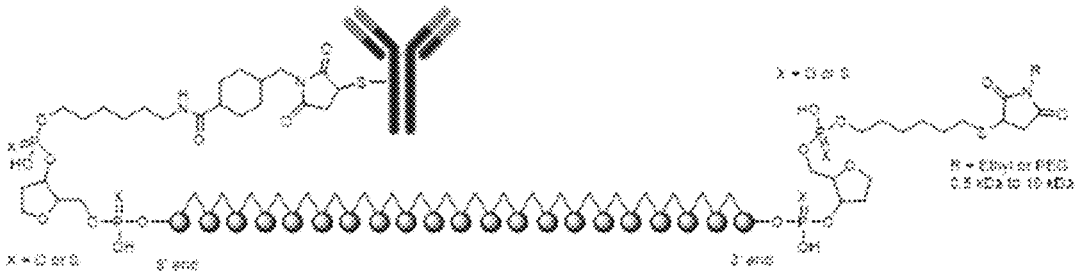


FIG. 41B

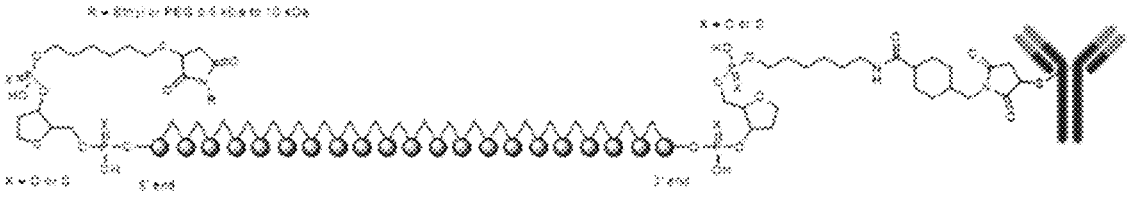


FIG. 41C

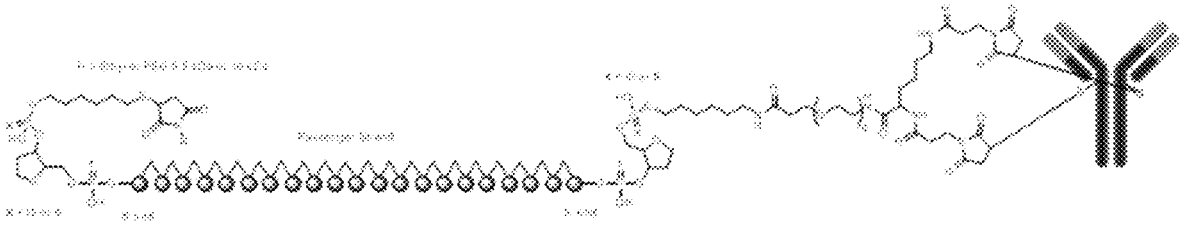


FIG. 41D

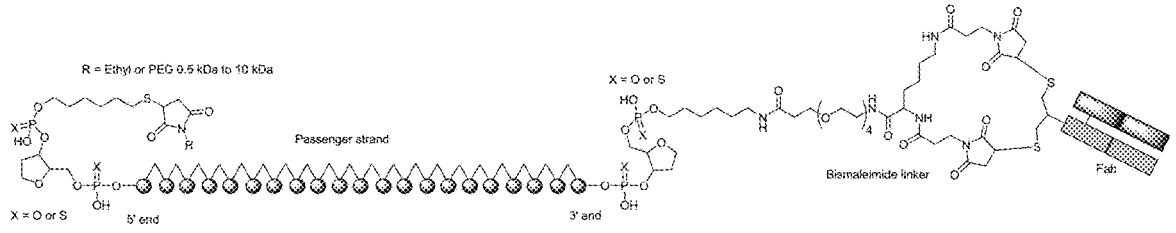


FIG. 41E

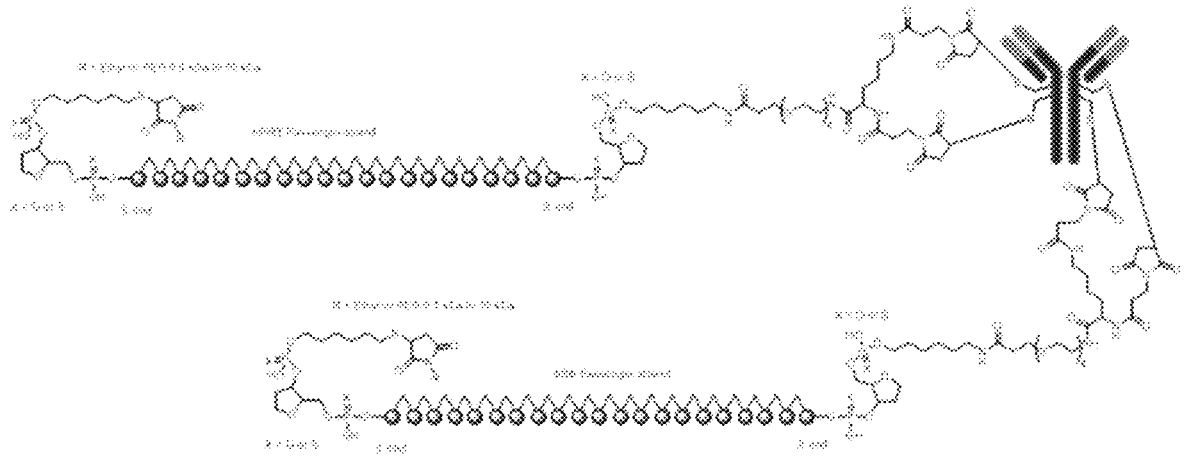


FIG. 41F

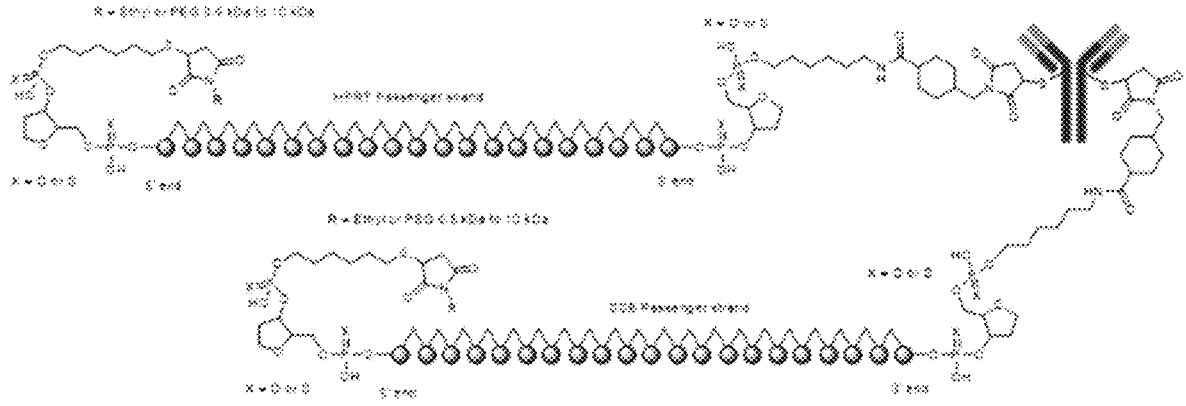


FIG. 42

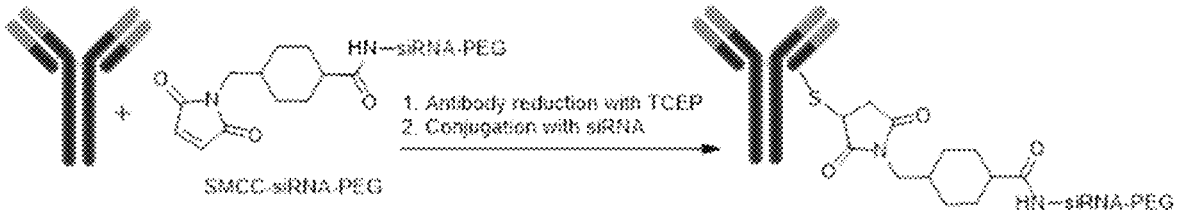

HN—siRNA-PEG
1. Antibody reduction with TCEP
2. Conjugation with siRNA
SMCC-siRNA-PEG
HN—siRNA-PEG

FIG. 43

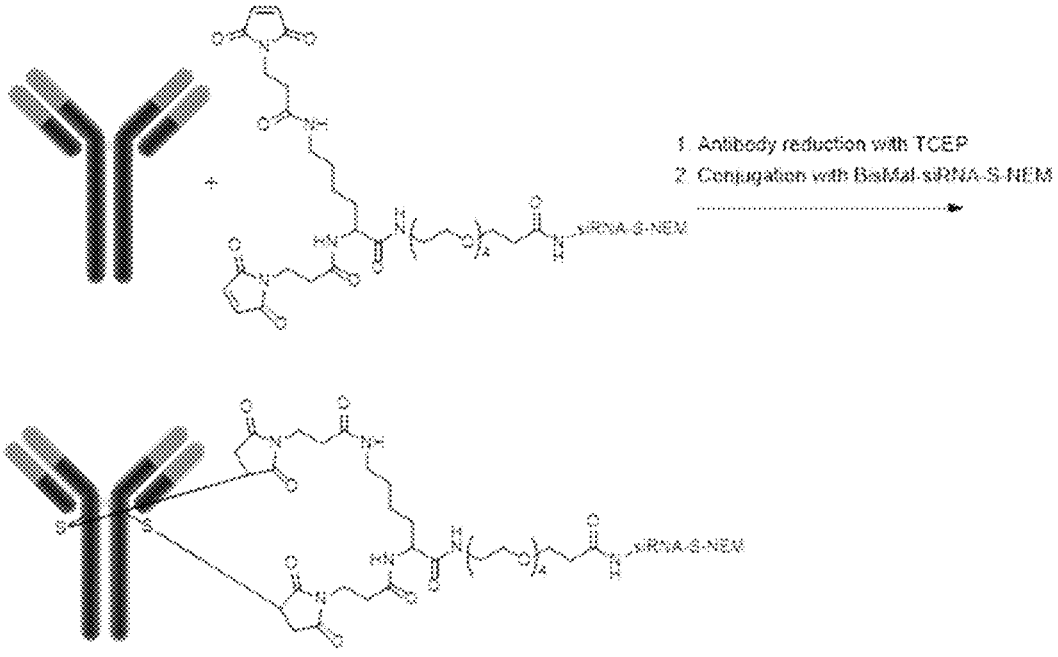
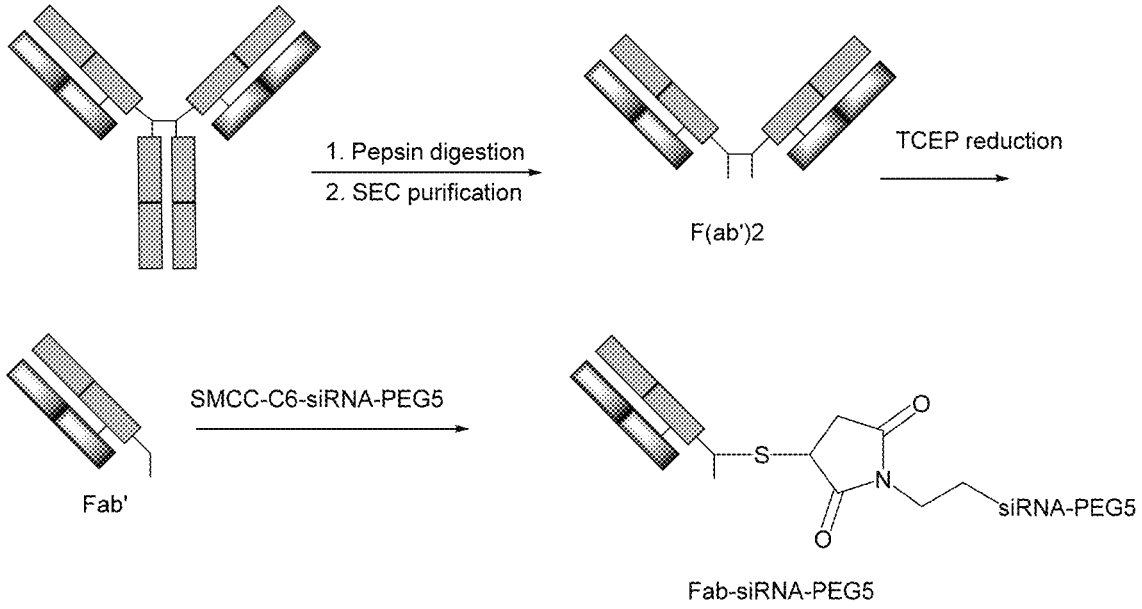

1. Antibody reduction with TCEP
2. Conjugation with BisMal-siRNA-S-NEM
siRNA-S-NEM
siRNA-S-NEM

FIG. 44


1. Pepsin digestion
2. SEC purification
TCEP reduction
F(ab')2
SMCC-C6-siRNA-PEG5
Fab'
S
N
O
O
siRNA-PEG5
Fab-siRNA-PEG5

COMPOSITIONS AND METHODS OF TREATING MUSCLE ATROPHY AND MYOTONIC DYSTROPHY

CROSS-REFERENCE

This application is a divisional of U.S. application Ser. No. 17/024,624, filed on Sep. 17, 2020, which is a continuation of U.S. application Ser. No. 16/435,422, filed Jun. 7, 2019, which is a continuation of PCT Application No. PCT/US2018/064359, filed Dec. 6, 2018, which claims priority to U.S. Provisional Application No. 62/595,545, filed Dec. 6, 2017, and U.S. Provisional Application No. 62/725,883, filed Aug. 31, 2018, which each of the applications is incorporated herein by reference in its entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Jun. 7, 2019, is named 45532-722_401_SL.txt and is 3,143,531 bytes in size.

BACKGROUND OF THE DISCLOSURE

Gene suppression by RNA-induced gene silencing provides several levels of control: transcription inactivation, small interfering RNA (siRNA)-induced mRNA degradation, and siRNA-induced transcriptional attenuation. In some instances, RNA interference (RNAi) provides long lasting effect over multiple cell divisions. As such, RNAi represents a viable method useful for drug target validation, gene function analysis, pathway analysis, and disease therapeutics.

SUMMARY OF THE DISCLOSURE

Disclosed herein, in certain embodiments, are polynucleic acid molecules and pharmaceutical compositions for modulating a gene associated with muscle atrophy (or an atrogene). In some embodiments, also described herein are methods of treating muscle atrophy with a polynucleic acid molecule or a polynucleic acid molecule conjugate disclosed herein.

Disclosed herein, in certain embodiments, is a molecule of Formula (I): A-X_1—B—X_2—C (Formula I) wherein, A is a binding moiety; B is a polynucleotide that hybridizes to a target sequence of an atrogene; C is a polymer; and X_1 and X_2 are each independently selected from a bond or a non-polymeric linker; wherein the polynucleotide comprises at least one 2' modified nucleotide, at least one modified internucleotide linkage, or at least one inverted abasic moiety; and wherein A and C are not attached to B at the same terminus. In some embodiments, the atrogene comprises a differentially regulated (e.g., an upregulated or downregulated) gene within the IGF1-Akt-FoxO pathway, the glucocorticoids-GR pathway, the PGC1α-FoxO pathway, the TNFα-NFκB pathway, or the myostatin-ActRIIb-Smad2/3 pathway. In some embodiments, the atrogene encodes an E3 ligase. In some embodiments, the atrogene encodes a Forkhead box transcription factor. In some embodiments, the atrogene comprises atrogin-1 gene (FBXO32), MuRF1 gene (TRIM63), FOXO1, FOXO3, or MSTN. In some embodiments, the atrogene comprises DMPK. In some embodiments, B consists of a polynucleotide that hybridizes to a target sequence of an atrogene. In some embodiments, C consists of a polymer. In some embodiments, the at least one 2' modified nucleotide comprises 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified nucleotide. In some embodiments, the at least one 2' modified nucleotide comprises locked nucleic acid (LNA) or ethylene nucleic acid (ENA). In some embodiments, the at least one modified internucleotide linkage comprises a phosphorothioate linkage or a phosphorodithioate linkage. In some embodiments, the at least one inverted abasic moiety is at at least one terminus. In some embodiments, the polynucleotide comprises a single strand which hybridizes to the target sequence of an atrogene. In some embodiments, the polynucleotide comprises a first polynucleotide and a second polynucleotide hybridized to the first polynucleotide to form a double-stranded polynucleic acid molecule, wherein either the first polynucleotide or the second polynucleotide also hybridizes to the target sequence of an atrogene. In some embodiments, the second polynucleotide comprises at least one modification. In some embodiments, the first polynucleotide and the second polynucleotide are RNA molecules. In some embodiments, the polynucleotide hybridizes to at least 8 contiguous bases of the target sequence of an atrogene. In some embodiments, the polynucleotide comprises a sequence that is at least 60%, 70%, 80%, 85%, 90%, 95%, or 99% complementary to a sequence as set forth in SEQ ID NOs: 28-141, 370-480, and 703-3406. In some embodiments, the polynucleotide is between about 8 and about 50 nucleotides in length. In some embodiments, the polynucleotide is between about 10 and about 30 nucleotides in length. In some embodiments, the first polynucleotide comprises a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some embodiments, the second polynucleotide comprises a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence as set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some embodiments, X_1 and X_2 are independently a C_1-C_6 alkyl group. In some embodiments, X_1 and X_2 are independently a homobifunctional linker or a heterobifunctional linker, optionally conjugated to a C_1-C_6 alkyl group. In some embodiments, A is an antibody or binding fragment thereof. In some embodiments, A comprises a humanized antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab2, single-chain variable fragment (scFv), diabody, minibody, nanobody, single-domain antibody (sdAb), or camelid antibody or binding fragment thereof. In some embodiments, A is an anti-transferrin receptor antibody or binding fragment thereof. In some embodiments, C is polyethylene glycol. In some embodiments, A-X_1 is conjugated to the 5' end of B and X_2—C is conjugated to the 3' end of B. In some embodiments, X_2—C is conjugated to the 5' end of B and A-X_1 is conjugated to the 3' end of B. In some embodiments, A is directly conjugated to X_1. In some embodiments, C is directly conjugated to X_2. In some embodiments, B is directly conjugated to X_1 and X_2. In some embodiments, the molecule further comprises D. In some embodiments, D is conjugated to C or to A. In some embodiments, D is an endosomolytic polymer.

Disclosed herein, in certain embodiments, is a polynucleic acid molecule conjugate comprising a binding moiety conjugated to a polynucleotide that hybridizes to a target sequence of an atrogene; wherein the polynucleotide optionally comprises at least one 2' modified nucleotide, at least one modified internucleotide linkage, or at least one inverted abasic moiety; and wherein the polynucleic acid molecule conjugate mediates RNA interference against the atrogene, thereby treating muscle atrophy in a subject. In some embodiments, the atrogene comprises a differentially regulated (e.g., an upregulated or downregulated) gene within the IGF1-Akt-FoxO pathway, the glucocorticoids-GR pathway, the PGC1α-FoxO pathway, the TNFα-NFκB pathway, or the myostatin-ActRIIb-Smad2/3 pathway. In some embodiments, the atrogene encodes an E3 ligase. In some embodiments, the atrogene encodes a Forkhead box transcription factor. In some embodiments, the atrogene comprises ligand of the TGF-beta (transforming growth factor-beta) superfamily of proteins. In some embodiments, the atrogene comprises DMPK. In some embodiments, the binding moiety is an antibody or binding fragment thereof. In some embodiments, the binding moiety comprises a humanized antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab2, single-chain variable fragment (scFv), diabody, minibody, nanobody, single-domain antibody (sdAb), or camelid antibody or binding fragment thereof. In some embodiments, the binding moiety is an anti-transferrin receptor antibody or binding fragment thereof. In some embodiments, the binding moiety is cholesterol. In some embodiments, the polynucleotide comprises a single strand which hybridizes to the target sequence of an atrogene. In some embodiments, the polynucleotide comprises a first polynucleotide and a second polynucleotide hybridized to the first polynucleotide to form a double-stranded polynucleic acid molecule, wherein either the first polynucleotide or the second polynucleotide also hybridizes to the target sequence of an atrogene. In some embodiments, the second polynucleotide comprises at least one modification. In some embodiments, the first polynucleotide and the second polynucleotide are RNA molecules. In some embodiments, the polynucleotide hybridizes to at least 8 contiguous bases of the target sequence of an atrogene. In some embodiments, the polynucleotide comprises a sequence that is at least 60%, 70%, 80%, 85%, 90%, 95%, or 99% complementary to a sequence as set forth in SEQ ID NOs: 28-141, 370-480, and 703-3406. In some embodiments, the polynucleotide is between about 8 and about 50 nucleotides in length. In some embodiments, the polynucleotide is between about 10 and about 30 nucleotides in length. In some embodiments, the first polynucleotide comprises a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence as set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some embodiments, the second polynucleotide comprises a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence as set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some embodiments, the polynucleic acid molecule conjugate optionally comprises a linker connecting the binding moiety to the polynucleotide. In some embodiments, the polynucleic acid molecule conjugate further comprises a polymer, optionally indirectly conjugated to the polynucleotide by an additional linker. In some embodiments, the linker and the additional linker are each independently a bond or a non-polymeric linker. In some embodiments, the polynucleic acid molecule conjugate comprises a molecule of Formula (I): A-X_1—B—X_2—C(Formula I) wherein, A is a binding moiety; B is a polynucleotide that hybridizes to a target sequence of an atrogene; C is a polymer; and X_1 and X_2 are each independently selected from a bond or a non-polymeric linker; wherein the polynucleotide comprises at least one 2' modified nucleotide, at least one modified internucleotide linkage, or at least one inverted abasic moiety; and wherein A and C are not attached to B at the same terminus. In some embodiments, the at least one 2' modified nucleotide comprises 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified nucleotide. In some embodiments, the at least one 2' modified nucleotide comprises locked nucleic acid (LNA) or ethylene nucleic acid (ENA). In some embodiments, the at least one modified internucleotide linkage comprises a phosphorothioate linkage or a phosphorodithioate linkage. In some embodiments, the at least one inverted abasic moiety is at at least one terminus. In some embodiments, the muscle atrophy is a diabetes-associated muscle atrophy. In some embodiments, the muscle atrophy is a cancer cachexia-associated muscle atrophy. In some embodiments, the muscle atrophy is associated with insulin deficiency. In some embodiments, the muscle atrophy is associated with chronic renal failure. In some embodiments, the muscle atrophy is associated with congestive heart failure. In some embodiments, the muscle atrophy is associated with chronic respiratory disease. In some embodiments, the muscle atrophy is associated with a chronic infection. In some embodiments, the muscle atrophy is associated with fasting. In some embodiments, the muscle atrophy is associated with denervation. In some embodiments, the muscle atrophy is associated with sarcopenia, glucocorticoid treatment, stroke, and/or heart attack. In some cases, myotonic dystrophy type 1 (DM1) is associated with an expansion of CTG repeats in the 3' UTR of the DMPK gene.

Disclosed herein, in certain embodiments, is a pharmaceutical composition comprising: a molecule described above or a polynucleic acid molecule conjugate described above; and a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition is formulated as a nanoparticle formulation. In some embodiments, the pharmaceutical composition is formulated for parenteral, oral, intranasal, buccal, rectal, or transdermal administration.

Disclosed herein, in certain embodiments, is a method of treating muscle atrophy or myotonic dystrophy in a subject in need thereof, comprising: administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate comprising a binding moiety conjugated to a polynucleotide that hybridizes to a target sequence of an atrogene; wherein the polynucleotide optionally comprises at least one 2' modified nucleotide, at least one modified internucleotide linkage, or at least one inverted abasic moiety; and wherein the polynucleic acid molecule conjugate mediates RNA interference against the atrogene, thereby treating muscle atrophy or myotonic dystrophy in the subject. In some embodiments, the muscle atrophy is a diabetes-associated muscle atrophy. In some embodiments, the muscle atrophy is a cancer cachexia-associated muscle atrophy. In some embodiments, the muscle atrophy is associated with insulin deficiency. In some embodiments, the muscle

atrophy is associated with chronic renal failure. In some embodiments, the muscle atrophy is associated with congestive heart failure. In some embodiments, the muscle atrophy is associated with chronic respiratory disease. In some embodiments, the muscle atrophy is associated with a chronic infection. In some embodiments, the muscle atrophy is associated with fasting. In some embodiments, the muscle atrophy is associated with denervation. In some embodiments, the muscle atrophy is associated with sarcopenia. In some embodiments, the myotonic dystrophy is DM1. In some embodiments, the atrogene comprises a differently regulated (e.g., an upregulated or downregulated) gene within the IGF1-Akt-FoxO pathway, the glucocorticoids-GR pathway, the PGC1α-FoxO pathway, the TNFα-NFκB pathway, or the myostatin-ActRIIb-Smad2/3 pathway. In some embodiments, the atrogene encodes an E3 ligase. In some embodiments, the atrogene encodes a Forkhead box transcription factor. In some embodiments, the atrogene comprises atrogin-1 gene (FBXO32), MuRF1 gene (TRIM63), FOXO1, FOXO3, or MSTN. In some embodiments, the atrogene comprises DMPK. In some embodiments, the polynucleic acid molecule conjugate comprises a molecule of Formula (I): A-X_1—B—X_2—C(Formula I) wherein, A is a binding moiety; B is a polynucleotide that hybridizes to the target sequence of an atrogene; C is a polymer; and X_1 and X_2 are each independently selected from a bond or a non-polymeric linker; wherein the polynucleotide comprises at least one 2' modified nucleotide, at least one modified internucleotide linkage, or at least one inverted abasic moiety; and wherein A and C are not attached to B at the same terminus. In some embodiments, B consists of a polynucleotide that hybridizes to the target sequence of an atrogene. In some embodiments, C consists of a polymer. In some embodiments, the at least one 2' modified nucleotide comprises 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified nucleotide. In some embodiments, the at least one 2' modified nucleotide comprises locked nucleic acid (LNA) or ethylene nucleic acid (ENA). In some embodiments, the at least one modified internucleotide linkage comprises a phosphorothioate linkage or a phosphorodithioate linkage. In some embodiments, the at least one inverted abasic moiety is at at least one terminus. In some embodiments, the polynucleotide comprises a single strand which hybridizes to the target sequence of an atrogene. In some embodiments, the polynucleotide comprises a first polynucleotide and a second polynucleotide hybridized to the first polynucleotide to form a double-stranded polynucleic acid molecule, wherein either the first polynucleotide or the second polynucleotide also hybridizes to the target sequence of an atrogene. In some embodiments, the second polynucleotide comprises at least one modification. In some embodiments, the first polynucleotide and the second polynucleotide are RNA molecules. In some embodiments, the polynucleotide hybridizes to at least 8 contiguous bases of the target sequence of an atrogene. In some embodiments, the polynucleotide comprises a sequence that is at least 60%, 70%, 80%, 85%, 90%, 95%, or 99% complementary to a sequence as set forth in SEQ ID NOs: 28-141, 370-480, and 703-3406. In some embodiments, the polynucleotide is between about 8 and about 50 nucleotides in length. In some embodiments, the polynucleotide is between about 10 and about 30 nucleotides in length. In some embodiments, the first polynucleotide comprises a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence as set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some embodiments, the second polynucleotide comprises a sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a sequence as set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some embodiments, X_1 and X_2 are independently a C_1-C_6 alkyl group. In some embodiments, X_1 and X_2 are independently a homobifunctional linker or a heterobifunctional linker, optionally conjugated to a C_1-C_6 alkyl group. In some embodiments, A is an antibody or binding fragment thereof. In some embodiments, A comprises a humanized antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab2, single-chain variable fragment (scFv), diabody, minibody, nanobody, single-domain antibody (sdAb), or camelid antibody or binding fragment thereof. In some embodiments, A is an anti-transferrin receptor antibody or binding fragment thereof. In some embodiments, C is polyethylene glycol. In some embodiments, A-X_i is conjugated to the 5' end of B and X_2—C is conjugated to the 3' end of B. In some embodiments, X_2—C is conjugated to the 5' end of B and A-X_1 is conjugated to the 3' end of B. In some embodiments, A is directly conjugated to X_1. In some embodiments, C is directly conjugated to X_2. In some embodiments, B is directly conjugated to X_1 and X_2. In some embodiments, the method further comprises D. In some embodiments, D is conjugated to C or to A. In some embodiments, D is an endosomolytic polymer. In some embodiments, the polynucleic acid molecule conjugate is formulated for parenteral, oral, intranasal, buccal, rectal, or transdermal administration. In some embodiments, the subject is a human.

Disclosed herein, in certain embodiments, is a kit comprising a molecule described above or a polynucleic acid molecule conjugate described above.

BRIEF DESCRIPTION OF THE DRAWINGS

Various aspects of the disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings below.

FIG. **1** illustrates an exemplary structure of cholesterol-myostatin siRNA conjugate.

FIG. **2** illustrates SAX HPLC chromatogram of TfR mAb-(Cys)-HPRT-PEG5k, DAR1.

FIG. **3** illustrates SEC HPLC chromatogram of TfR mAb-(Cys)-HPRT-PEG5k, DAR1.

FIG. **4** illustrates an overlay of DAR1 and DAR2 SAX HPLC chromatograms of TfR1mAb-Cys-BisMal-siRNA conjugates.

FIG. **5** illustrates an overlay of DAR1 and DAR2 SEC HPLC chromatograms of TfR1mAb-Cys-BisMal-siRNA conjugates.

FIG. **6** illustrates SEC chromatogram of CD71 Fab-Cys-HPRT-PEG5.

FIG. **7** illustrates SAX chromatogram of CD71 Fab-Cys-HPRT-PEG5.

FIG. **8** illustrates relative expression levels of Murf1 and atrogin-1 in C2C12 myoblasts and myotubes C2C12 myo-

blasts and myotubes were generated as described in Example 4. mRNA levels were determined as described in Example 4.

FIG. **9**A illustrates in vivo study design to assess the ability of exemplary conjugates for their ability to mediate mRNA downregulation of myostatin (MSTN) in skeletal muscle.

FIG. **9**B shows siRNA-mediated mRNA knockdown of mouse MSTN in mouse gastrocnemius (gastroc) muscle.

FIG. **10**A illustrates in vivo study design to assess the ability of exemplary conjugates for their ability to mediate mRNA downregulation of myostatin (MSTN) in skeletal muscle.

FIG. **10**B shows tissue concentration-time profiles out to 1008 h post-dose of an exemplary molecule of Formula (I).

FIG. **10**C shows siRNA-mediated mRNA knockdown of mouse MSTN in mouse gastrocnemius (gastroc) muscle.

FIG. **10**D shows plasma MSTN protein reduction after siRNA-mediated mRNA knockdown of mouse MSTN in mouse gastrocnemius (gastroc) muscle.

FIG. **10**E shows changes in muscle size after siRNA-mediated mRNA knockdown of mouse MSTN in mouse gastrocnemius (gastroc) muscle.

FIG. **10**F shows Welch's two-tailed unpaired t-test of FIG. **10**E.

FIG. **11**A illustrates an exemplary in vivo study design.

FIG. **11**B shows tissue accumulation of siRNA in mouse gastrocnemius (gastroc) muscle after a single i.v. administration of an exemplary molecule of Formula (I) at the doses indicated.

FIG. **11**C shows siRNA-mediated mRNA knockdown of mouse MSTN in mouse gastrocnemius (gastroc) muscle.

FIG. **12**A illustrates an exemplary in vivo study design.

FIG. **12**B shows accumulation of siRNA in various muscle tissue.

FIG. **12**C shows siRNA-mediated mRNA knockdown of mouse MSTN in mouse gastrocnemius (gastroc) and heart muscle.

FIG. **12**D shows RISC loading of the MSTN guide strand in mouse gastrocnemius (gastroc) muscle.

FIG. **13**A illustrates an exemplary in vivo study design.

FIG. **13**B shows siRNA-mediated mRNA knockdown of mouse MSTN in mouse gastrocnemius (gastroc), quadriceps, triceps, and heart.

FIG. **13**C illustrates plasma myostatin levels.

FIG. **13**D illustrates siRNA accumulation in different tissue types: gastrocnemius, triceps, quadriceps, and heart tissues.

FIG. **13**E shows RISC loading of the MSTN guide strand in mouse gastrocnemius (gastroc) muscle.

FIG. **13**F shows change in muscle area.

FIG. **13**G shows Welch's two-tailed unpaired t-test of FIG. **13**F.

FIG. **14**A illustrates an exemplary in vivo study design.

FIG. **14**B shows HPRT mRNA expression of gastrocnemius muscle by exemplary conjugates described herein.

FIG. **14**C shows SSB mRNA expression of gastrocnemius muscle by exemplary conjugates described herein.

FIG. **14**D shows HPRT mRNA expression of heart tissue by exemplary conjugates described herein.

FIG. **14**E shows SSB mRNA expression of heart tissue by exemplary conjugates described herein.

FIG. **14**F shows accumulation of siRNA in gastrocnemius muscle.

FIG. **15**A illustrates an exemplary in vivo study design.

FIG. **15**B shows Atrogin-1 downregulation in gastrocnemius (gastroc) muscle.

FIG. **15**C shows Atrogin-1 downregulation in heart tissue.

FIG. **16**A illustrates an exemplary in vivo study design.

FIG. **16**B shows MuRF-1 downregulation in gastrocnemius muscle.

FIG. **16**C shows MuRF-1 downregulation in heart tissue.

FIG. **17** illustrates siRNAs that were transfected into mouse C2C12 myoblasts in vitro. The four DMPK siRNAs assessed all showed DMPK mRNA knockdown, while the negative control siRNA did not. The dotted lines are three-parameter curves fit by non-linear regression.

FIG. **18**A-FIG. **18**F show in vivo results demonstrating robust dose-responses for DMPK mRNA knockdown 7 days after a single i.v. administration of DMPK siRNA-antibody conjugates. FIG. **18**A: gastrocnemius; FIG. **18**B: Tibialis anterior; FIG. **18**C: quadriceps; FIG. **18**D: diaphragm; FIG. **18**E: heart; and FIG. **18**F: liver.

FIG. **19**A-FIG. **19**L show exemplary antibody-nucleic acid conjugates described herein.

FIG. **19**M presents an antibody cartoon utilized in FIG. **19**A-FIG. **19**L.

FIG. **20**A-FIG. **20**B illustrate an exemplary 21 mer duplex utilized in Example 20. FIG. **20**A shows a representative structure of siRNA passenger strand with C6-NH_2 conjugation handle at the 5' end and C6-S-NEM at 3' end. FIG. **20**B shows a representative structure of a 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs.

FIG. **21**A-FIG. **21**B illustrate a second exemplary 21 mer duplex utilized in Example 20. FIG. **21**A shows a representative structure of siRNA passenger strand with a 5' conjugation handle. FIG. **21**B shows a representative structure of a blunt ended duplex with 19 bases of complementarity and one 3' dinucleotide overhang.

FIG. **22** shows an illustrative in vivo study design.

FIG. **23** illustrates a time course of Atrogin-1 mRNA downregulation in gastroc muscle mediated by a TfR1 antibody siRNA conjugate after IV delivery at a dose of a single dose of 3 mg/kg.

FIG. **24** illustrates a time course of Atrogin-1 mRNA downregulation in heart muscle mediate by a TfR1 antibody siRNA conjugate after IV delivery at a dose of a single dose of 3 mg/kg.

FIG. **25** shows an illustrative in vivo study design.

FIG. **26** shows MuRF1 mRNA downregulation at 96 hours in gastroc muscle mediated by a TfR1 antibody siRNA conjugate after IV delivery at the doses indicated.

FIG. **27** shows MuRF1 mRNA downregulation at 96 hours in heart muscle mediated by a TfR1 antibody siRNA conjugate after IV delivery at the doses indicated.

FIG. **28** shows a time course of MuRF1 and Atrogin-1 mRNA downregulation in gastroc muscle mediated by a TfR1 antibody siRNA conjugate (IV delivery at 3 mg/kg siRNA), in the absence and presence of dexamethasone induce muscle atrophy.

FIG. **29** shows a time course of MuRF1 and Agtrogin1 mRNA downregulation in heart muscle mediated by a TfR1 antibody siRNA conjugate (IV delivery at 3 mg/kg siRNA), in the absence and presence of dexamethasone induce muscle atrophy.

FIG. **30** shows a time course of gastroc weight changes mediated by a TfR1 antibody siRNA conjugate (IV delivery at 3 mg/kg siRNA), in the absence and presence of muscle atrophy.

FIG. **31** shows a time course of siRNA tissue concentrations in gastroc and heart muscle mediated by a TfR1 antibody siRNA conjugate (IV delivery at 3 mg/kg siRNA), in the absence and presence of muscle atrophy.

FIG. **32** shows an illustrative in vivo study design.

FIG. **33** shows Atrogin-1 mRNA downregulation in gastroc muscle, 10 days after TfR1 antibody siRNA conjugate, in the absence a presence of dexamethasone induced atrophy (initiated at day 7), relative to the measure concentration of siRNA in the tissue.

FIG. **34** shows relative Atrogin-1 mRNA levels in gastroc muscle for the scrambled control groups in the absence (groups 10&13, and groups 11&14)) and presence of dexamethasone induced atrophy (groups 12&15).

FIG. **35** shows relative RISC loading of the Atrogin-1 guide strand in mouse gastroc muscle after TfR1-mAb conjugate delivery in the absence and presence of dexamethasone induced atrophy.

FIG. **36** shows a time course of MSTN mRNA downregulation in gastroc muscle after TfR1 antibody siRNA conjugate delivery, in the absence (solid lines) and presence (dotted lines) of dexamethasone induced atrophy (initiated at day 7), relative to the PBS control.

FIG. **37** shows leg muscle growth rate in gastroc muscle, after TfR1-mAb conjugate delivery in the absence and presence of dexamethasone induced atrophy.

FIG. **38** shows an illustrative in vivo study design.

FIG. **39**A shows a single treatment of 4.5 mg/kg (siRNA) of either Atrogin-1 siRNA or MuRF1 siRNA or a single dose of both siRNAs combined resulted in up to 75% downregulation of each target in the gastrocnemius.

FIG. **39**B shows mRNA knockdown of both targets in gastrocnemius is maintained at 75% in the intact leg out to 37 days post ASC dose.

FIG. **39**C shows changes in muscle area.

FIG. **39**D shows changes in gastrocnemius weight.

FIG. **39**E shows treatment-induced percentage sparing of muscle wasting in term of leg muscle area. The statistical analysis compared the treatment groups to the scramble siRNA control group using a Welch's TTest.

FIG. **39**F shows the treatment-induced percentage sparing of muscle wasting in term of gastrocnemius weight.

FIG. **40**A shows a representative structure of siRNA with C6-NH_2 conjugation handle at the 5' end and C6-SH at 3'end of the passenger strand.

FIG. **40**B shows a representative structure of siRNA passenger strand with C6-NH_2 conjugation handle at the 5' end and C6-S-PEG at 3' end.

FIG. **40**C shows a representative structure of siRNA passenger strand with C6-NH_2 conjugation handle at the 5' end and C6-S-NEM at 3' end.

FIG. **40**D shows a representative structure of siRNA passenger strand with C6-N-SMCC conjugation handle at the 5' end and C6-S-NEM at 3' end.

FIG. **40**E shows a representative structure of siRNA passenger strand with PEG at the 5' end and C6-SH at 3' end.

FIG. **40**F shows a representative structure of siRNA passenger strand with C6-S-NEM at the 5' end and C6-NH_2 conjugation handle at 3' end.

FIG. **41**A shows Architecture-1: Antibody-Cys-SMCC-5'-passenger strand. This conjugate was generated by antibody inter-chain cysteine conjugation to maleimide (SMCC) at the 5' end of passenger strand.

FIG. **41**B shows Architecture-2: Antibody-Cys-SMCC-3'-Passenger strand. This conjugate was generated by antibody inter-chain cysteine conjugation to maleimide (SMCC) at the 3' end of passenger strand.

FIG. **41**C shows ASC Architecture-3: Antibody-Cys-bis-Mal-3'-Passenger strand. This conjugate was generated by antibody inter-chain cysteine conjugation to bismaleimide (bisMal)linker at the 3' end of passenger strand.

FIG. **41**D shows ASC Architecture-4: A model structure of the Fab-Cys-bisMal-3'-Passenger strand. This conjugate was generated by Fab inter-chain cysteine conjugation to bismaleimide (bisMal) linker at the 3' end of passenger strand.

FIG. **41**E shows ASC Architecture-5: A model structure of the antibody siRNA conjugate with two different siRNAs attached to one antibody molecule. This conjugate was generated by conjugating a mixture of SSB and HPRT siRNAs to the reduced mAb inter-chain cysteines to bismaleimide (bisMal) linker at the 3' end of passenger strand of each siRNA.

FIG. **41**F shows ASC Architecture-6: A model structure of the antibody siRNA conjugate with two different siRNAs attached. This conjugate was generated by conjugating a mixture of SSB and HPRT siRNAs to the reduced mAb inter-chain cysteines to maleimide (SMCC) linker at the 3' end of passenger strand of each siRNA.

FIG. **42** shows Synthesis scheme-1: Antibody-Cys-SMCC-siRNA-PEG conjugates via antibody cysteine conjugation.

FIG. **43** shows Synthesis scheme-2: Antibody-Cys-Bis-Mal-siRNA-PEG conjugates.

FIG. **44** shows Scheme-3: Fab-siRNA conjugate generation.

DETAILED DESCRIPTION OF THE DISCLOSURE

Muscle atrophy is the loss of muscle mass or the progressive weakening and degeneration of muscles, such as skeletal or voluntary muscles that controls movement, cardiac muscles, and smooth muscles. Various pathophysiological conditions including disuse, starvation, cancer, diabetes, and renal failure, or treatment with glucocorticoids result in muscle atrophy and loss of strength. The phenotypical effects of muscle atrophy are induced by various molecular events, including inhibition of muscle protein synthesis, enhanced turnover of muscle proteins, abnormal regulation of satellite cells differentiation, and abnormal conversion of muscle fibers types.

Extensive research has identified that muscle atrophy is an active process controlled by specific signaling pathways and transcriptional programs. Exemplary pathways involved in this process include, but are not limited to, IGF1-Akt-FoxO, glucocorticoids-GR, PGC1α-FoxO, TNFα-NFκB, and myostatin-ActRIIb-Smad2/3.

In some instances, therapeutic manipulation of mechanisms regulating muscle atrophy has focused on IGF1-Akt, TNFα-NfκB, and myostatin. While IGF1 analogs were shown to be effective in treating muscle atrophy, the involvement of the IGF1-Akt pathway in promoting tumorigenesis and hypertrophy prevents these therapies. Similar risks are involved in the use of β-adrenergic agonists for the regulation of the Akt-mTOR pathway. Inhibition of myostatin by using soluble ActRIIB or ligand blocking ActRIIb antibodies prevented and reversed skeletal muscle loss, and prolonged the survival of tumor-bearing animals. However the mechanism of the anti-atrophic effects of myostatin blockade remains uncertain as neither expression of a dominant-negative ActRIIb, nor knockdown of Smad2/3 prevented muscle loss following denervation (Satori et al., "Smad2 and 3 transcription factors control muscle mass in adulthood", *Am J Physiol Cell Physiol* 296: C1248-C1257, 2009).

Comparing gene expression in different models of muscle atrophy (including diabetes, cancer cachexia, chronic renal

failure, fasting and denervation) has led to the identification of atrophy-related genes, named atrogenes (Sacheck et al., "Rapid disuse and denervation atrophy involve transcriptional changes similar to those of muscle wasting during systemic diseases", *The FASEB Journal,* 21(1): 140-155, 2007), that are commonly up- or downregulated in atrophying muscle. Among genes that are strongly upregulated under atrophy conditions are muscle-specific ubiquitin-protein (E3) ligases (e.g. atrogin-1, MuRF1), Forkhead box transcription factors, and proteins mediating stress responses. In some cases, many of these effector proteins are difficult to regulate using traditional drugs.

Nucleic acid (e.g., RNAi) therapy is a targeted therapy with high selectivity and specificity. However, in some instances, nucleic acid therapy is also hindered by poor intracellular uptake, limited blood stability and non-specific immune stimulation. To address these issues, various modifications of the nucleic acid composition are explored, such as for example, novel linkers for better stabilizing and/or lower toxicity, optimization of binding moiety for increased target specificity and/or target delivery, and nucleic acid polymer modifications for increased stability and/or reduced off-target effect.

In some embodiments, the arrangement or order of the different components that make-up the nucleic acid composition further effects intracellular uptake, stability, toxicity, efficacy, and/or non-specific immune stimulation. For example, if the nucleic acid component includes a binding moiety, a polymer, and a polynucleic acid molecule (or polynucleotide), the order or arrangement of the binding moiety, the polymer, and/or the polynucleic acid molecule (or polynucleotide) (e.g., binding moiety-polynucleic acid molecule-polymer, binding moiety-polymer-polynucleic acid molecule, or polymer-binding moiety-polynucleic acid molecule) further effects intracellular uptake, stability, toxicity, efficacy, and/or non-specific immune stimulation.

In some embodiments, described herein include polynucleic acid molecules and polynucleic acid molecule conjugates for the treatment of muscle atrophy or myotonic dystrophy. In some instances, the polynucleic acid molecule conjugates described herein enhance intracellular uptake, stability, and/or efficacy. In some cases, the polynucleic acid molecule conjugates comprise a molecule of Formula (I): $A\text{-}X_1\text{—}B\text{—}X_2\text{—}C$. In some cases, the polynucleic acid molecules that hybridize to target sequences of one or more atrogenes.

Additional embodiments described herein include methods of treating muscle atrophy or myotonic dystrophy, comprising administering to a subject a polynucleic acid molecule or a polynucleic acid molecule conjugate described herein.

Atrogenes

Atrogenes, or atrophy-related genes, are genes that are upregulated or downregulated in atrophying muscle. In some instances, upregulated atrogenes include genes that encode ubiquitin ligases, Forkhead box transcription factors, growth factors, deubiquitinating enzymes, or proteins that are involved in glucocorticoid-induced atrophy.

Ubiquitin Ligases

In some embodiments, an atrogene described herein encodes an E3 ubiquitin ligase. Exemplary E3 ubiquitin ligases include, but are not limited to, Atrogin-1/MAFbx, muscle RING finger 1 (MuRF1), TNF receptor adaptor protein 6 (TRAF6), F-Box protein 30 (Fbxo30), F-Box protein 40 (Fbxo40), neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4-1), and tripartite motif-containing protein 32 (Trim32). Exemplary mitochondrial ubiquitin ligases include, but are not limited to, Mitochondrial E3 ubiquitin protein ligase 1 (Mul1) and Carboxy terminus of Hsc70 interacting protein (CHIP).

In some embodiments, an atrogene described herein encodes Atrogin-1, also named Muscle Atrophy F-box (MAFbx), a member of the F-box protein family. Atrogin-1/MAFbx is one of the four subunits of the ubiquitin ligase complex SKP1-cullin-F-box (SCF) that promotes degradation of MyoD, a muscle transcription factor, and eukaryotic translation initiation factor 3 subunit F (eIF3-f). Atrogin-1/MAFbx is encoded by FBXO32.

In some embodiments, an atrogene described herein encodes muscle RING finger 1 (MuRF1). MuRF1 is a member of the muscle-specific RING finger proteins and along with family members MuRF2 and MuRF3 are found at the M-line and Z-line lattices of myofibrils. Further, several studies have shown that MuRF1 interacts with and/or modulates the half-life of muscle structural proteins such as troponin I, myosin heavy chains, actin, myosin binding protein C, and myosin light chains 1 and 2. MuRF1 is encoded by TRIM63.

In some embodiments, an atrogene described herein encodes TNF receptor adaptor protein 6 (TRAF6) (also known as interleukin-1 signal transducer, RING finger protein 85, or RNF85). TRAF6 is a member of the E3 ligase that mediates conjugation of Lys63-linked polyubiquitin chains to target proteins. The Lys63-linked polyubiquitin chains signal autophagy-dependent cargo recognition by scaffold protein p62 (SQSTM1). TRAF6 is encoded by the TRAF6 gene.

In some embodiments, an atrogene described herein encodes F-Box protein 30 (Fbxo30) (also known as F-Box only protein, helicase, 18; muscle ubiquitin ligase of SCF complex in atrophy-1; or MUSA1). Fbxo30 is a member of the SCF complex family of E3 ubiquitin ligases. In one study, Fbox30 is proposed to be inhibited by the bone morphogenetic protein (BMP) pathway and upon atrophy-inducing conditions, are upregulated and subsequently undergoes autoubiquitination. Fbxo30 is encoded by the FBXO30 gene.

In some embodiments, an atrogene described herein encodes F-Box protein 40 (Fbxo40) (also known as F-Box only protein 40 or muscle disease-related protein). A second member of the SCF complex family of E3 ubiquitin ligases, Fbxo40 regulates anabolic signals. In some instances, Fbxo40 ubiquitinates and affects the degradation of insulin receptor substrate 1, a downstream effector of insulin receptor-mediated signaling. Fbxo40 is encoded by the FBXO40 gene.

In some embodiments, an atrogene described herein encodes neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4-1), a HECT domain E3 ubiquitin ligase which has been shown to be upregulated in muscle cells during disuse. Nedd4-1 is encoded by the NEDD4 gene.

In some embodiments, an atrogene described herein encodes tripartite motif-containing protein 32 (Trim32). Trim32 is a member of the E3 ubiquitin ligase that is involved in degradation of thin filaments such as actin, tropomyosin, and troponins; α-actinin; and desmin. Trim32 is encoded by the TRIM32 gene.

In some embodiments, an atrogene described herein encodes Mitochondrial E3 ubiquitin protein ligase 1 (Mul1) (also known as mitochondrial-anchored protein ligase, RING finger protein 218, RNF218, MAPL, MULAN, and GIDE). Mul1 is involved in the mitochondrial network remodeling and is up-regulated by the FoxO family of

transcription factors under catabolic conditions, such as for example, denervation or fasting, and subsequently causes mitochondrial fragmentation and removal via autophagy (mitophagy). Furthermore, Mul1 ubiquitinates the mitochondrial pro-fusion protein mitofusin 2, a GTPase that is involved in mitochondrial fusion, leading to the degradation of mitofusin 2. Mul1 is encoded by the MUL1 gene.

In some embodiments, an atrogene described herein encodes Carboxy terminus of Hsc70 interacting protein (CHIP) (also known as STIP1 homology and U-Box containing protein 1, STUB1, CLL-associated antigen KW-8, antigen NY-CO-7, SCAR16, SDCCAG7, or UBOX1). CHIP is a mitochondrial ubiquitin ligase that regulates ubiquitination and lysosomal-ependent degradation of filamin C, a muscle protein found in the Z-line. Z-line or Z-disc is the structure formed between adjacent sarcomeres, and sarcomere is the basic unit of muscle. Alterations of filamin structure triggers binding of the co-chaperone BAG3, a complex that comprises chaperones Hsc70 and HspB8 with CHIP. Subsequent ubiquitination of BAG3 and filamin by CHIP activates the autophagy system, leading to degradation of filamin C. CHIP is encoded by the STUB1 gene.

Forkhead Box Transcription Factors

In some embodiments, an atrogene described herein encodes a Forkhead box transcription factor. Exemplary Forkhead box transcription factors include, but are not limited to, isoforms Forkhead box protein O1 (FoxO1) and Forkhead box protein O3 (FoxO3).

In some embodiments, an atrogene described herein encodes Forkhead box protein O1 (FoxO1) (also known as Forkhead homolog in Rhabdomyoscarcoma, FKHR, or FKH1). FoxO1 is involved in regulation of gluconeogenesis and glycogenolysis by insulin signaling, and the initiation of adipogenesis by preadipocytes. FoxO1 is encoded by the FOXO1 gene.

In some embodiments, an atrogene described herein encodes Forkhead box protein 03 (FoxO3) (also known as Forkhead in Rhabdomyosarcoma-like 1, FKHRL1, or FOXO3A). FoxO3 is activated by AMP-activated protein kinase AMPK, which in term induces expression of atrogin-1 and MuRF1. FoxO3 is encoded by the FOXO3 gene.

Growth Factors

In some embodiments, an atrogene described herein encodes a growth factor. An exemplary growth factor includes myostatin.

In some instances, an atrogene described herein encodes myostatin (Mstn), also known as growth/differentiation factor 8 (GDF-8). Myostatin is intracellularly converted into an activator, and stimulates muscle degradation and suppresses muscle synthesis by inhibiting Akt through the phosphorylation/activation of Smad (small mothers against decapentaplegic). In some instances, myostatin has been found to be regulated by the Akt-FoxO signaling pathway. In additional instances, myostatin has been shown to suppress differentiation of satellite cells, stimulate muscle degradation through the inhibition of the Akt pathway, and suppress muscle synthesis via the mTOR pathway.

Deubiquitinating Enzymes

In some embodiments, an atrogene described herein encodes a deubiquitinating enzyme. Exemplary deubiquitinating enzymes include, but are not limited to, Ubiquitin specific peptidase 14 (USP14) and Ubiquitin specific peptidase 19 (USP19). In some instances, an atrogene described herein encodes USP14 (also known as deubiquitinating enzyme 14 or TGT). In other instances, an atrogene described herein encodes USP19 (also known as zinc finger MYND domain-containing protein 9, deubiquitinating enzyme 19, or ZMYND9). USP14 is encoded by the USP14 gene. USP19 is encoded by the USP19 gene.

Additional Atrogenes

In some embodiments, an atrogene described herein encodes regulated in development and DNA damage response 1 (Redd1), also known as DNA-damage-inducible transcript 4 (DDIT4) and HIF-1 responsive protein RTP801. Redd1 represses mTOR function by sequestering 14-3-3 and increases TSC1/2 activity. Furthermore, Redd1 decreases phosphorylation of 4E-BP1 and S6K1, which are involved in muscle protein synthesis. Redd1 is encoded by the DDIT4 gene.

In some embodiments, an atrogene described herein encodes cathepsin L2, also known as cathepsin V. Cathepsin L2 is a lysosomal cysteine proteinase. It is encoded by the CTSL2 gene.

In some embodiments, an atrogene described herein encodes TG interacting factor, or homeobox protein TGIF1. TG interacting factor is a transcription factor which regulates signaling pathways involved in embryonic development. This protein is encoded by the TGIF gene.

In some embodiments, an atrogene described herein encodes myogenin, also known as myogenic factor 4. Myogenin is a member of the MyoD family of muscle-specific basic-helix-loop-helix (bHLH) transcription factor involved in the coordination of skeletal muscle development and repair. Myogenin is encoded by the MYOG gene.

In some embodiments, an atrogene described herein encodes myotonin-protein kinase (MT-PK), also known as myotonic dystrophy protein kinase (MDPK) or dystrophia myotonica protein kinase (DMK). MT-PK is a Serine/Threonine kinase and further interacts with members of the Rho family of GTPases. In human, MT-PK is encoded by the DMPK gene.

In some embodiments, an atrogene described herein encodes histone deacetylase 2, a member of the histone deacetylase family. Histone deacetylase 2 is encoded by the HDAC2 gene.

In some embodiments, an atrogene described herein encodes histone deacetylase 3, another member of the histone deacetylase family. Histone deacetylase 3 is encoded by the HDAC3 gene.

In some embodiments, an atrogene described herein encodes metallothionein 1L, a member of the metallothionein family. Metallothioneins (MT) are cysteine-rish, low molecular weight proteins that is capable of binding heavy metals, thereby providing protection against metal toxicity and/or oxidative stress. Metallothionein 1L is encoded by the MT1L gene.

In some embodiments, an atrogene described herein encodes metallothionein 1B, a second member of the metallothionein family. Metallothionein 1B is encoded by the MT1B gene.

In some embodiments, an atrogene described herein is an atrogene listed in Table 14.

Polynucleic Acid Molecules

In certain embodiments, a polynucleic acid molecule hybridizes to a target sequence of an atrophy-related gene (also referred to as an atrogene). In some instances, a polynucleic acid molecule described herein hybridizes to a target sequence of an ubiquitin ligase (e.g., an E3 ubiquitin ligase or a mitochondrial ubiquitin ligase). In some instances, a polynucleic acid molecule described herein hybridizes to a target sequence of a Forkhead box transcription factor. In some instances, a polynucleic acid molecule described herein hybridizes to a target sequence of a growth

factor. In some instances, a polynucleic acid molecule described herein hybridizes to a target sequence of a deubiquitinating enzyme.

In some embodiments, a polynucleic acid molecule described herein hybridizes to a target sequence of FBXO32, TRIM63, TRAF6, FBXO30, FBXO40, NEDD4, TRIM32, MUL1, STUB1, FOXO1, FOXO3, MSTN, USP14, USP19, DDIT4, CTSL2, TGIF, MYOG, HDAC2, HDAC3, MT1L, MT1B, or DMPK. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of FBXO32, TRIM63, FOXO1, FOXO3, or MSTN. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of FBXO32. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of TRIM63. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of TRAF6. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of FBXO30. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of FBXO40. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of NEDD4. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of TRIM32. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of MULL. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of STUB1. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of FOXO1. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of FOXO3. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of MSTN. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of USP14. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of USP19. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of DDIT4. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of CTSL2. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of TGIF. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of MYOG. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of HDAC2. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of HDAC3. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of MT1L. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of MT1B. In some cases, a polynucleic acid molecule described herein hybridizes to a target sequence of DMPK.

In some embodiments, the polynucleic acid molecule comprises a sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 50% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 60% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 70% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 75% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 80% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 85% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 90% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 95% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 96% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 97% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 98% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 99% sequence identity to a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480. In some embodiments, the polynucleic acid molecule consists of a target sequence as set forth in SEQ ID NOs: 28-141 and 370-480.

In some embodiments, the polynucleic acid molecule comprises a sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 50% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 60% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 70% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 75% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 80% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 85% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 90% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 95% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 96% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 97% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 98% sequence identity to a target sequence as set forth in

SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule comprises a sequence having at least 99% sequence identity to a target sequence as set forth in SEQ ID NOs: 703-3406. In some embodiments, the polynucleic acid molecule consists of a target sequence as set forth in SEQ ID NOs: 703-3406.

In some embodiments, the polynucleic acid molecule comprises a first polynucleotide and a second polynucleotide. In some instances, the first polynucleotide comprises a sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some cases, the second polynucleotide comprises a sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 142-255, 256-369, 481-591, 592-702, and 3407-14222. In some cases, the polynucleic acid molecule comprises a first polynucleotide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 142-255, 481-591, 3407-6110, and 8815-11518, and a second polynucleotide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 256-369, 592-702, 6111-8814, and 11519-14222.

In some embodiments, the polynucleic acid molecule comprises a sense strand (e.g., a passenger strand) and an antisense strand (e.g., a guide strand). In some instances, the sense strand (e.g., the passenger strand) comprises a sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 142-255, 481-591, 3407-6110, and 8815-11518. In some instances, the antisense strand (e.g., the guide strand) comprises a sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to a target sequence as set forth in SEQ ID NOs: 256-369, 592-702, 6111-8814, and 11519-14222.

In some embodiments, the polynucleic acid molecule described herein comprises RNA or DNA. In some cases, the polynucleic acid molecule comprises RNA. In some instances, RNA comprises short interfering RNA (siRNA), short hairpin RNA (shRNA), microRNA (miRNA), double-stranded RNA (dsRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), or heterogeneous nuclear RNA (hnRNA). In some instances, RNA comprises shRNA. In some instances, RNA comprises miRNA. In some instances, RNA comprises dsRNA. In some instances, RNA comprises tRNA. In some instances, RNA comprises rRNA. In some instances, RNA comprises hnRNA. In some instances, the RNA comprises siRNA. In some instances, the polynucleic acid molecule comprises siRNA.

In some embodiments, the polynucleic acid molecule is from about 10 to about 50 nucleotides in length. In some instances, the polynucleic acid molecule is from about 10 to about 30, from about 15 to about 30, from about 18 to about 25, form about 18 to about 24, from about 19 to about 23, or from about 20 to about 22 nucleotides in length.

In some embodiments, the polynucleic acid molecule is about 50 nucleotides in length. In some instances, the polynucleic acid molecule is about 45 nucleotides in length. In some instances, the polynucleic acid molecule is about 40 nucleotides in length. In some instances, the polynucleic acid molecule is about 35 nucleotides in length. In some instances, the polynucleic acid molecule is about 30 nucleotides in length. In some instances, the polynucleic acid molecule is about 25 nucleotides in length. In some instances, the polynucleic acid molecule is about 20 nucleotides in length. In some instances, the polynucleic acid molecule is about 19 nucleotides in length. In some instances, the polynucleic acid molecule is about 18 nucleotides in length. In some instances, the polynucleic acid molecule is about 17 nucleotides in length. In some instances, the polynucleic acid molecule is about 16 nucleotides in length. In some instances, the polynucleic acid molecule is about 15 nucleotides in length. In some instances, the polynucleic acid molecule is about 14 nucleotides in length. In some instances, the polynucleic acid molecule is about 13 nucleotides in length. In some instances, the polynucleic acid molecule is about 12 nucleotides in length. In some instances, the polynucleic acid molecule is about 11 nucleotides in length. In some instances, the polynucleic acid molecule is about 10 nucleotides in length. In some instances, the polynucleic acid molecule is between about 10 and about 50 nucleotides in length. In some instances, the polynucleic acid molecule is between about 10 and about 45 nucleotides in length. In some instances, the polynucleic acid molecule is between about 10 and about 40 nucleotides in length. In some instances, the polynucleic acid molecule is between about 10 and about 35 nucleotides in length. In some instances, the polynucleic acid molecule is between about 10 and about 30 nucleotides in length. In some instances, the polynucleic acid molecule is between about 10 and about 25 nucleotides in length. In some instances, the polynucleic acid molecule is between about 10 and about 20 nucleotides in length. In some instances, the polynucleic acid molecule is between about 15 and about 25 nucleotides in length. In some instances, the polynucleic acid molecule is between about 15 and about 30 nucleotides in length. In some instances, the polynucleic acid molecule is between about 12 and about 30 nucleotides in length.

In some embodiments, the polynucleic acid molecule comprises a first polynucleotide. In some instances, the polynucleic acid molecule comprises a second polynucleotide. In some instances, the polynucleic acid molecule comprises a first polynucleotide and a second polynucleotide. In some instances, the first polynucleotide is a sense strand or passenger strand. In some instances, the second polynucleotide is an antisense strand or guide strand.

In some embodiments, the polynucleic acid molecule is a first polynucleotide. In some embodiments, the first polynucleotide is from about 10 to about 50 nucleotides in length. In some instances, the first polynucleotide is from about 10 to about 30, from about 15 to about 30, from about 18 to about 25, form about 18 to about 24, from about 19 to about 23, or from about 20 to about 22 nucleotides in length.

In some instances, the first polynucleotide is about 50 nucleotides in length. In some instances, the first polynucleotide is about 45 nucleotides in length. In some instances, the first polynucleotide is about 40 nucleotides in length. In some instances, the first polynucleotide is about 35 nucleotides in length. In some instances, the first polynucleotide is about 30 nucleotides in length. In some instances, the first polynucleotide is about 25 nucleotides in length. In some instances, the first polynucleotide is about 20 nucleotides in length. In some instances, the first polynucleotide is about 19 nucleotides in length. In some instances, the first polynucleotide is about 18 nucleotides in length. In some instances, the first polynucleotide is about 17 nucleotides in

length. In some instances, the first polynucleotide is about 16 nucleotides in length. In some instances, the first polynucleotide is about 15 nucleotides in length. In some instances, the first polynucleotide is about 14 nucleotides in length. In some instances, the first polynucleotide is about 13 nucleotides in length. In some instances, the first polynucleotide is about 12 nucleotides in length. In some instances, the first polynucleotide is about 11 nucleotides in length. In some instances, the first polynucleotide is about 10 nucleotides in length. In some instances, the first polynucleotide is between about 10 and about 50 nucleotides in length. In some instances, the first polynucleotide is between about 10 and about 45 nucleotides in length. In some instances, the first polynucleotide is between about 10 and about 40 nucleotides in length. In some instances, the first polynucleotide is between about 10 and about 35 nucleotides in length. In some instances, the first polynucleotide is between about 10 and about 30 nucleotides in length. In some instances, the first polynucleotide is between about 10 and about 25 nucleotides in length. In some instances, the first polynucleotide is between about 10 and about 20 nucleotides in length. In some instances, the first polynucleotide is between about 15 and about 25 nucleotides in length. In some instances, the first polynucleotide is between about 15 and about 30 nucleotides in length. In some instances, the first polynucleotide is between about 12 and about 30 nucleotides in length.

In some embodiments, the polynucleic acid molecule is a second polynucleotide. In some embodiments, the second polynucleotide is from about 10 to about 50 nucleotides in length. In some instances, the second polynucleotide is from about 10 to about 30, from about 15 to about 30, from about 18 to about 25, form about 18 to about 24, from about 19 to about 23, or from about 20 to about 22 nucleotides in length.

In some instances, the second polynucleotide is about 50 nucleotides in length. In some instances, the second polynucleotide is about 45 nucleotides in length. In some instances, the second polynucleotide is about 40 nucleotides in length. In some instances, the second polynucleotide is about 35 nucleotides in length. In some instances, the second polynucleotide is about 30 nucleotides in length. In some instances, the second polynucleotide is about 25 nucleotides in length. In some instances, the second polynucleotide is about 20 nucleotides in length. In some instances, the second polynucleotide is about 19 nucleotides in length. In some instances, the second polynucleotide is about 18 nucleotides in length. In some instances, the second polynucleotide is about 17 nucleotides in length. In some instances, the second polynucleotide is about 16 nucleotides in length. In some instances, the second polynucleotide is about 15 nucleotides in length. In some instances, the second polynucleotide is about 14 nucleotides in length. In some instances, the second polynucleotide is about 13 nucleotides in length. In some instances, the second polynucleotide is about 12 nucleotides in length. In some instances, the second polynucleotide is about 11 nucleotides in length. In some instances, the second polynucleotide is about 10 nucleotides in length. In some instances, the second polynucleotide is between about 10 and about 50 nucleotides in length. In some instances, the second polynucleotide is between about 10 and about 45 nucleotides in length. In some instances, the second polynucleotide is between about 10 and about 40 nucleotides in length. In some instances, the second polynucleotide is between about 10 and about 35 nucleotides in length. In some instances, the second polynucleotide is between about 10 and about 30 nucleotides in length. In some instances, the second polynucleotide is between about 10 and about 25 nucleotides in length. In some instances, the second polynucleotide is between about 10 and about 20 nucleotides in length. In some instances, the second polynucleotide is between about 15 and about 25 nucleotides in length. In some instances, the second polynucleotide is between about 15 and about 30 nucleotides in length. In some instances, the second polynucleotide is between about 12 and about 30 nucleotides in length.

In some embodiments, the polynucleic acid molecule comprises a first polynucleotide and a second polynucleotide. In some instances, the polynucleic acid molecule further comprises a blunt terminus, an overhang, or a combination thereof. In some instances, the blunt terminus is a 5' blunt terminus, a 3' blunt terminus, or both. In some cases, the overhang is a 5' overhang, 3' overhang, or both. In some cases, the overhang comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 non-base pairing nucleotides. In some cases, the overhang comprises 1, 2, 3, 4, 5, or 6 non-base pairing nucleotides. In some cases, the overhang comprises 1, 2, 3, or 4 non-base pairing nucleotides. In some cases, the overhang comprises 1 non-base pairing nucleotide. In some cases, the overhang comprises 2 non-base pairing nucleotides. In some cases, the overhang comprises 3 non-base pairing nucleotides. In some cases, the overhang comprises 4 non-base pairing nucleotides.

In some embodiments, the sequence of the polynucleic acid molecule is at least 40%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or 99.5% complementary to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule is at least 50% complementary to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule is at least 60% complementary to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule is at least 70% complementary to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule is at least 80% complementary to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule is at least 90% complementary to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule is at least 95% complementary to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule is at least 99% complementary to a target sequence described herein. In some instances, the sequence of the polynucleic acid molecule is 100% complementary to a target sequence described herein.

In some embodiments, the sequence of the polynucleic acid molecule has 5 or less mismatches to a target sequence described herein. In some embodiments, the sequence of the polynucleic acid molecule has 4 or less mismatches to a target sequence described herein. In some instances, the sequence of the polynucleic acid molecule has 3 or less mismatches to a target sequence described herein. In some cases, the sequence of the polynucleic acid molecule has 2 or less mismatches to a target sequence described herein. In some cases, the sequence of the polynucleic acid molecule has 1 or less mismatches to a target sequence described herein.

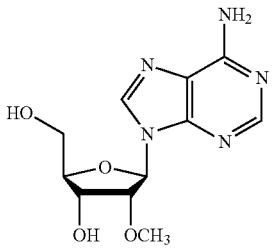
In some embodiments, the specificity of the polynucleic acid molecule that hybridizes to a target sequence described herein is a 95%, 98%, 99%, 99.5% or 100% sequence complementarity of the polynucleic acid molecule to a target sequence. In some instances, the hybridization is a high stringent hybridization condition.

In some embodiments, the polynucleic acid molecule has reduced off-target effect. In some instances, "off-target" or "off-target effects" refer to any instance in which a polynucleic acid polymer directed against a given target causes an unintended effect by interacting either directly or indirectly with another mRNA sequence, a DNA sequence or a cellular protein or other moiety. In some instances, an "off-target effect" occurs when there is a simultaneous degradation of other transcripts due to partial homology or complementarity between that other transcript and the sense and/or antisense strand of the polynucleic acid molecule.

In some embodiments, the polynucleic acid molecule comprises natural or synthetic or artificial nucleotide analogues or bases. In some cases, the polynucleic acid molecule comprises combinations of DNA, RNA and/or nucleotide analogues. In some instances, the synthetic or artificial nucleotide analogues or bases comprise modifications at one or more of ribose moiety, phosphate moiety, nucleoside moiety, or a combination thereof.

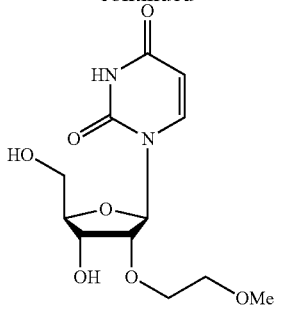
In some embodiments, nucleotide analogues or artificial nucleotide base comprise a nucleic acid with a modification at a 2' hydroxyl group of the ribose moiety. In some instances, the modification includes an H, OR, R, halo, SH, SR, NH2, NHR, NR2, or CN, wherein R is an alkyl moiety. Exemplary alkyl moiety includes, but is not limited to, halogens, sulfurs, thiols, thioethers, thioesters, amines (primary, secondary, or tertiary), amides, ethers, esters, alcohols and oxygen. In some instances, the alkyl moiety further comprises a modification. In some instances, the modification comprises an azo group, a keto group, an aldehyde group, a carboxyl group, a nitro group, a nitroso, group, a nitrile group, a heterocycle (e.g., imidazole, hydrazino or hydroxylamino) group, an isocyanate or cyanate group, or a sulfur containing group (e.g., sulfoxide, sulfone, sulfide, and disulfide). In some instances, the alkyl moiety further comprises a hetero substitution. In some instances, the carbon of the heterocyclic group is substituted by a nitrogen, oxygen or sulfur. In some instances, the heterocyclic substitution includes but is not limited to, morpholino, imidazole, and pyrrolidino.

In some instances, the modification at the 2' hydroxyl group is a 2'-O-methyl modification or a 2'-O-methoxyethyl (2'-O-MOE) modification. In some cases, the 2'-O-methyl modification adds a methyl group to the 2' hydroxyl group of the ribose moiety whereas the 2'O-methoxyethyl modification adds a methoxyethyl group to the 2' hydroxyl group of the ribose moiety. Exemplary chemical structures of a 2'-O-methyl modification of an adenosine molecule and 2'O-methoxyethyl modification of an uridine are illustrated below.



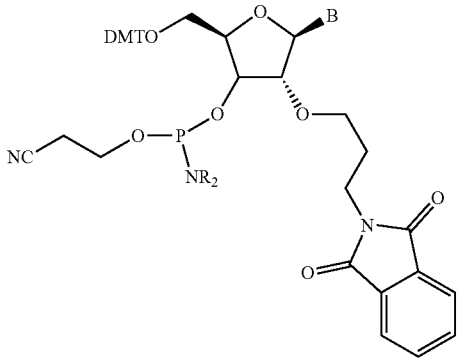
2'-O-methyl-adenosine

-continued



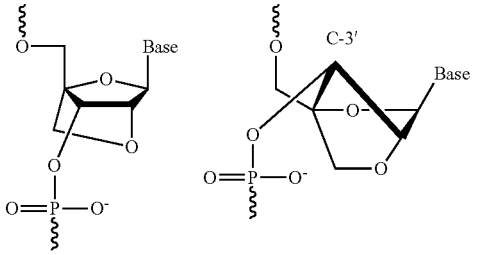
2'-O-methoxyethyl uridine

In some instances, the modification at the 2' hydroxyl group is a 2'-O-aminopropyl modification in which an extended amine group comprising a propyl linker binds the amine group to the 2' oxygen. In some instances, this modification neutralizes the phosphate derived overall negative charge of the oligonucleotide molecule by introducing one positive charge from the amine group per sugar and thereby improves cellular uptake properties due to its zwitterionic properties. An exemplary chemical structure of a 2'-O-aminopropyl nucleoside phosphoramidite is illustrated below.



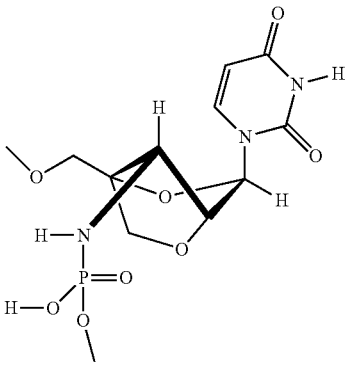
2'-O-aminopropyl nucleoside phosphoramidite

In some instances, the modification at the 2' hydroxyl group is a locked or bridged ribose modification (e.g., locked nucleic acid or LNA) in which the oxygen molecule bound at the 2' carbon is linked to the 4' carbon by a methylene group, thus forming a 2'-C,4'-C-oxy-methylene-linked bicyclic ribonucleotide monomer. Exemplary representations of the chemical structure of LNA are illustrated below. The representation shown to the left highlights the chemical connectivities of an LNA monomer. The representation shown to the right highlights the locked 3'-endo (^{3}E) conformation of the furanose ring of an LNA monomer.

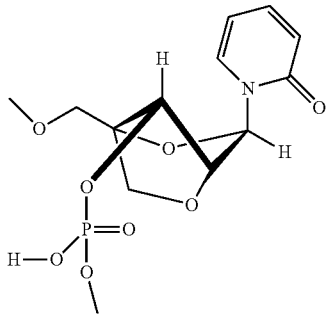


LNA (Locked Nucleic Acids)

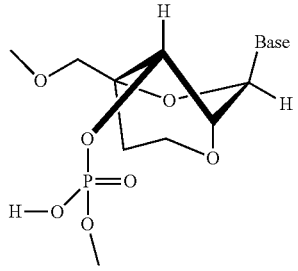
In some instances, the modification at the 2' hydroxyl group comprises ethylene nucleic acids (ENA) such as for example 2'-4'-ethylene-bridged nucleic acid, which locks the sugar conformation into a C_3'-endo sugar puckering conformation. ENA are part of the bridged nucleic acids class of modified nucleic acids that also comprises LNA. Exemplary chemical structures of the ENA and bridged nucleic acids are illustrated below.



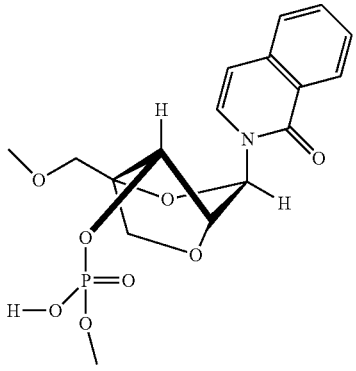
3'-amino-2',4'-BNA



2',4'-BNA-2-pyridone



2',4'-ENA

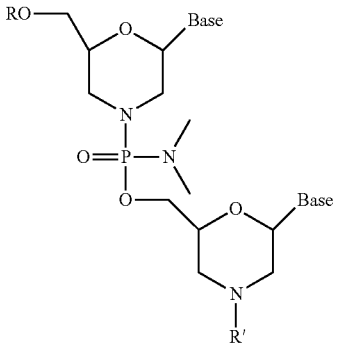


2',4'-BNA-1-isoquinolone

In some embodiments, additional modifications at the 2' hydroxyl group include 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA).

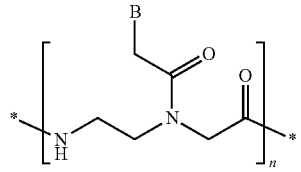
In some embodiments, nucleotide analogues comprise modified bases such as, but not limited to, 5-propynyluridine, 5-propynylcytidine, 6-methyladenine, 6-methylguanine, N, N, -dimethyladenine, 2-propyladenine, 2propylguanine, 2-aminoadenine, 1-methylinosine, 3-methyluridine, 5-methylcytidine, 5-methyluridine and other nucleotides having a modification at the 5 position, 5-(2-amino) propyl uridine, 5-halocytidine, 5-halouridine, 4-acetylcytidine, 1-methyladenosine, 2-methyladenosine, 3-methylcytidine, 6-methyluridine, 2-methylguanosine, 7-methylguanosine, 2,2-dimethylguanosine, 5-methylaminoethyluridine, 5-methyloxyuridine, deazanucleotides such as 7-deaza-adenosine, 6-azouridine, 6-azocytidine, 6-azothymidine, 5-methyl-2-thiouridine, other thio bases such as 2-thiouridine and 4-thiouridine and 2-thiocytidine, dihydrouridine, pseudouridine, queuosine, archaeosine, naphthyl and substituted naphthyl groups, any O- and N-alkylated purines and pyrimidines such as N6-methyladenosine, 5-methylcarbonylmethyluridine, uridine 5-oxyacetic acid, pyridine-4-one, pyridine-2-one, phenyl and modified phenyl groups such as aminophenol or 2,4,6-trimethoxy benzene, modified cytosines that act as G-clamp nucleotides, 8-substituted adenines and guanines, 5-substituted uracils and thymines, azapyrimidines, carboxyhydroxyalkyl nucleotides, carboxyalkylaminoalkyl nucleotides, and alkylcarbonylalkylated nucleotides. Modified nucleotides also include those nucleotides that are modified with respect to the sugar moiety, as well as nucleotides having sugars or analogs thereof that are not ribosyl. For example, the sugar moieties, in some cases are or be based on, mannoses, arabinoses, glucopyranoses, galactopyranoses, 4'-thioribose, and other sugars, heterocycles, or carbocycles. The term nucleotide also includes what are known in the art as universal bases. By way of example, universal bases include but are not limited to 3-nitropyrrole, 5-nitroindole, or nebularine.

In some embodiments, nucleotide analogues further comprise morpholinos, peptide nucleic acids (PNAs), methylphosphonate nucleotides, thiolphosphonate nucleotides, 2'-fluoro N3-P5'-phosphoramidites, 1',5'-anhydrohexitol nucleic acids (HNAs), or a combination thereof. Morpholino or phosphorodiamidate morpholino oligo (PMO) comprises synthetic molecules whose structure mimics natural nucleic acid structure by deviates from the normal sugar and phosphate structures. In some instances, the five member ribose ring is substituted with a six member morpholino ring containing four carbons, one nitrogen and one oxygen. In some cases, the ribose monomers are linked by a phosphordiamidate group instead of a phosphate group. In such cases, the backbone alterations remove all positive and negative charges making morpholinos neutral molecules capable of crossing cellular membranes without the aid of cellular delivery agents such as those used by charged oligonucleotides.

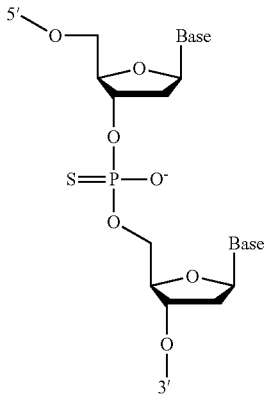


Morpholino

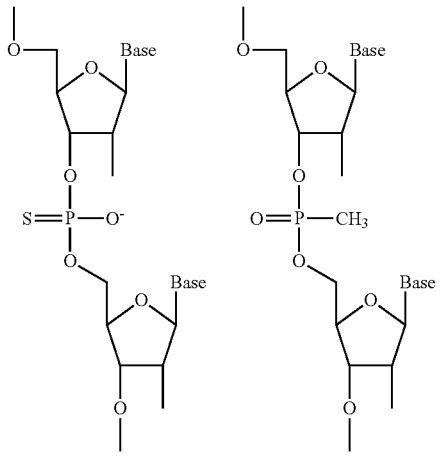
In some embodiments, peptide nucleic acid (PNA) does not contain sugar ring or phosphate linkage and the bases are attached and appropriately spaced by oligoglycine-like molecules, therefore, eliminating a backbone charge.



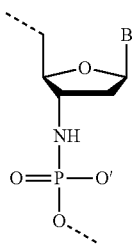
In some embodiments, one or more modifications optionally occur at the internucleotide linkage. In some instances, modified internucleotide linkage include, but is not limited to, phosphorothioates, phosphorodithioates, methylphosphonates, 5'-alkylenephosphonates, 5'-methylphosphonate, 3'-alkylene phosphonates, borontrifluoridates, borano phosphate esters and selenophosphates of 3'-5'linkage or 2'-5'linkage, phosphotriesters, thionoalkylphosphotriesters, hydrogen phosphonate linkages, alkyl phosphonates, alkylphosphonothioates, arylphosphonothioates, phosphoroselenoates, phosphorodiselenoates, phosphinates, phosphoramidates, 3'-alkylphosphoramidates, aminoalkylphosphoramidates, thionophosphoramidates, phosphoropiperazidates, phosphoroanilothioates, phosphoroanilidates, ketones, sulfones, sulfonamides, carbonates, carbamates, methylenehydrazos, methylenedimethylhydrazos, formacetals, thioformacetals, oximes, methyleneiminos, methylenemethyliminos, thioamidates, linkages with riboacetyl groups, aminoethyl glycine, silyl or siloxane linkages, alkyl or cycloalkyl linkages with or without heteroatoms of, for example, 1 to 10 carbons that are saturated or unsaturated and/or substituted and/or contain heteroatoms, linkages with morpholino structures, amides, polyamides wherein the bases are attached to the aza nitrogens of the backbone directly or indirectly, and combinations thereof. Phosphorothioate antisene oligonucleotides (PS ASO) are antisense oligonucleotides comprising a phosphorothioate linkage. An exemplary PS ASO is illustrated below.



In some instances, the modification is a methyl or thiol modification such as methylphosphonate or thiolphosphonate modification. Exemplary thiolphosphonate nucleotide (left) and methylphosphonate nucleotide (right) are illustrated below.

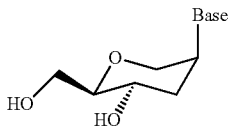


In some instances, a modified nucleotide includes, but is not limited to, 2'-fluoro N3-P5'-phosphoramidites illustrated as:



N3'-P5' Phosphoroamidate

In some instances, a modified nucleotide includes, but is not limited to, hexitol nucleic acid (or 1',5'-anhydrohexitol nucleic acids (HNA)) illustrated as:



HNA

In some embodiments, one or more modifications further optionally include modifications of the ribose moiety, phosphate backbone and the nucleoside, or modifications of the nucleotide analogues at the 3' or the 5' terminus. For example, the 3' terminus optionally include a 3' cationic group, or by inverting the nucleoside at the 3'-terminus with a 3'-3' linkage. In another alternative, the 3'-terminus is optionally conjugated with an aminoalkyl group, e.g., a 3' C5-aminoalkyl dT. In an additional alternative, the 3'-terminus is optionally conjugated with an abasic site, e.g., with an apurinic or apyrimidinic site. In some instances, the 5-terminus is conjugated with an aminoalkyl group, e.g., a 5'-O-alkylamino substituent. In some cases, the 5'-terminus is conjugated with an abasic site, e.g., with an apurinic or apyrimidinic site.

In some embodiments, the polynucleic acid molecule comprises one or more of the artificial nucleotide analogues described herein. In some instances, the polynucleic acid molecule comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 25, or more of the artificial nucleotide analogues described herein. In some embodiments, the artificial nucleotide analogues include 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified, LNA, ENA, PNA, HNA, morpholino, methylphosphonate nucleotides, thiolphosphonate nucleotides, 2'-fluoro N3-P5'-phosphoramidites, or a combination thereof. In some instances, the polynucleic acid molecule comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 25, or more of the artificial nucleotide analogues selected from 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified, LNA, ENA, PNA, HNA, morpholino, methylphosphonate nucleotides, thiolphosphonate nucleotides, 2'-fluoro N3-P5'-phosphoramidites, or a combination thereof. In some instances, the polynucleic acid molecule comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 25, or more of 2'-O-methyl modified nucleotides. In some instances, the polynucleic acid molecule comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 25, or more of 2'-O-methoxyethyl (2'-O-MOE) modified nucleotides. In some instances, the polynucleic acid molecule comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 25, or more of thiolphosphonate nucleotides.

In some instances, the polynucleic acid molecule comprises at least one of: from about 5% to about 100% modification, from about 10% to about 100% modification, from about 20% to about 100% modification, from about 30% to about 100% modification, from about 40% to about 100% modification, from about 50% to about 100% modification, from about 60% to about 100% modification, from about 70% to about 100% modification, from about 80% to about 100% modification, and from about 90% to about 100% modification.

In some cases, the polynucleic acid molecule comprises at least one of: from about 10% to about 90% modification, from about 20% to about 90% modification, from about 30% to about 90% modification, from about 40% to about 90% modification, from about 50% to about 90% modification, from about 60% to about 90% modification, from about 70% to about 90% modification, and from about 80% to about 100% modification.

In some cases, the polynucleic acid molecule comprises at least one of: from about 10% to about 80% modification, from about 20% to about 80% modification, from about 30% to about 800 modification, from about 40% to about 80% modification, from about 50% to about 80% modification, from about 60% to about 80% modification, and from about 70% to about 80% modification.

In some instances, the polynucleic acid molecule comprises at least one of: from about 10% to about 70% modification, from about 20% to about 70% modification, from about 30% to about 70% modification, from about 40% to about 70% modification, from about 50% to about 70% modification, and from about 60% to about 70% modification.

In some instances, the polynucleic acid molecule comprises at least one of: from about 10% to about 60% modification, from about 20% to about 60% modification, from about 30% to about 60% modification, from about 40% to about 60% modification, and from about 50% to about 60% modification.

In some cases, the polynucleic acid molecule comprises at least one of: from about 10% to about 50% modification, from about 20% to about 50% modification, from about 30% to about 50% modification, and from about 40% to about 50% modification.

In some cases, the polynucleic acid molecule comprises at least one of: from about 10% to about 40% modification, from about 20% to about 40% modification, and from about 30% to about 40% modification.

In some cases, the polynucleic acid molecule comprises at least one of: from about 10% to about 30% modification, and from about 20% to about 30% modification.

In some cases, the polynucleic acid molecule comprises from about 10% to about 20% modification.

In some cases, the polynucleic acid molecule comprises from about 15% to about 90%, from about 20% to about 80%, from about 30% to about 70%, or from about 40% to about 60% modifications.

In additional cases, the polynucleic acid molecule comprises at least about 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 99% modification.

In some embodiments, the polynucleic acid molecule comprises at least about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22 or more modifications.

In some instances, the polynucleic acid molecule comprises at least about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22 or more modified nucleotides.

In some instances, from about 5 to about 100% of the polynucleic acid molecule comprise the artificial nucleotide analogues described herein. In some instances, about 5%,

10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of the polynucleic acid molecule comprise the artificial nucleotide analogues described herein. In some instances, about 5% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 10% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 15% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 20% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 25% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 30% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 35% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 40% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 45% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 50% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 55% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 60% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 65% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 70% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 75% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 80% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 85% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 90% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 95% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 96% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 97% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 98% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 99% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some instances, about 100% of the polynucleic acid molecule comprises the artificial nucleotide analogues described herein. In some embodiments, the artificial nucleotide analogues include 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified, LNA, ENA, PNA, HNA, morpholino, methylphosphonate nucleotides, thiolphosphonate nucleotides, 2'-fluoro N3-P5'-phosphoramidites, or a combination thereof.

In some embodiments, the polynucleic acid molecule comprises from about 1 to about 25 modifications in which the modification comprises an artificial nucleotide analogues described herein. In some embodiments, the polynucleic acid molecule comprises about 1 modification in which the modification comprises an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 2 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 3 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 4 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 5 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 6 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 7 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 8 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 9 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 10 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 11 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 12 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 13 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 14 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 15 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 16 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 17 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 18 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 19 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 20 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 21 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic

acid molecule comprises about 22 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 23 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 24 modifications in which the modifications comprise an artificial nucleotide analogue described herein. In some embodiments, the polynucleic acid molecule comprises about 25 modifications in which the modifications comprise an artificial nucleotide analogue described herein.

In some embodiments, a polynucleic acid molecule is assembled from two separate polynucleotides wherein one polynucleotide comprises the sense strand and the second polynucleotide comprises the antisense strand of the polynucleic acid molecule. In other embodiments, the sense strand is connected to the antisense strand via a linker molecule, which in some instances is a polynucleotide linker or a non-nucleotide linker.

In some embodiments, a polynucleic acid molecule comprises a sense strand and antisense strand, wherein pyrimidine nucleotides in the sense strand comprises 2'-O-methylpyrimidine nucleotides and purine nucleotides in the sense strand comprise 2'-deoxy purine nucleotides. In some embodiments, a polynucleic acid molecule comprises a sense strand and antisense strand, wherein pyrimidine nucleotides present in the sense strand comprise 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein purine nucleotides present in the sense strand comprise 2'-deoxy purine nucleotides.

In some embodiments, a polynucleic acid molecule comprises a sense strand and antisense strand, wherein the pyrimidine nucleotides when present in said antisense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides when present in said antisense strand are 2'-O-methyl purine nucleotides.

In some embodiments, a polynucleic acid molecule comprises a sense strand and antisense strand, wherein the pyrimidine nucleotides when present in said antisense strand are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides when present in said antisense strand comprise 2'-deoxy-purine nucleotides.

In some embodiments, a polynucleic acid molecule comprises a sense strand and antisense strand, wherein the sense strand includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense strand. In other embodiments, the terminal cap moiety is an inverted deoxy abasic moiety.

In some embodiments, a polynucleic acid molecule comprises a sense strand and an antisense strand, wherein the antisense strand comprises a phosphate backbone modification at the 3' end of the antisense strand. In some instances, the phosphate backbone modification is a phosphorothioate.

In some embodiments, a polynucleic acid molecule comprises a sense strand and an antisense strand, wherein the antisense strand comprises a glyceryl modification at the 3' end of the antisense strand.

In some embodiments, a polynucleic acid molecule comprises a sense strand and an antisense strand, in which the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or about one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and in which the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In other embodiments, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In some embodiments, a polynucleic acid molecule comprises a sense strand and an antisense strand, in which the sense strand comprises about 1 to about 25, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and in which the antisense strand comprises about 1 to about 25 or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In other embodiments, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 25 or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In some embodiments, a polynucleic acid molecule comprises a sense strand and an antisense strand, in which the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages, and/or about one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the

5'-end, or both of the 3'- and 5'-ends of the antisense strand. In other embodiments, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more pyrimidine nucleotides of the sense and/or antisense strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

In some embodiments, a polynucleic acid molecule comprises a sense strand and an antisense strand, in which the antisense strand comprises about 1 to about 25 or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 25 or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more phosphorothioate internucleotide linkages, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In other embodiments, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In some embodiments, a polynucleic acid molecule described herein is a chemically-modified short interfering nucleic acid molecule having about 1 to about 25, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more phosphorothioate internucleotide linkages in each strand of the polynucleic acid molecule.

In another embodiment, a polynucleic acid molecule described herein comprises 2'-5' internucleotide linkages. In some instances, the 2'-5' internucleotide linkage(s) is at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both sequence strands. In addition instances, the 2'-5' internucleotide linkage(s) is present at various other positions within one or both sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the polynucleic acid molecule comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the polynucleic acid molecule comprise a 2'-5' internucleotide linkage.

In some embodiments, a polynucleic acid molecule is a single stranded polynucleic acid molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the polynucleic acid molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence, and wherein one or more pyrimidine nucleotides present in the polynucleic acid are 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any purine nucleotides present in the polynucleic acid are 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides), and a terminal cap modification, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence, the polynucleic acid molecule optionally further comprising about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the polynucleic acid molecule, wherein the terminal nucleotides further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages, and wherein the polynucleic acid molecule optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In some cases, one or more of the artificial nucleotide analogues described herein are resistant toward nucleases such as for example ribonuclease such as RNase H, deoxyribonuclease such as DNase, or exonuclease such as 5'-3' exonuclease and 3'-5' exonuclease when compared to natural polynucleic acid molecules. In some instances, artificial nucleotide analogues comprising 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified, LNA, ENA, PNA, HNA, morpholino, methylphosphonate nucleotides, thiolphosphonate nucleotides, 2'-fluoro N3-P5'-phosphoramidites, or combinations thereof are resistant toward nucleases such as for example ribonuclease such as RNase H, deoxyribonuclease such as DNase, or exonuclease such as 5'-3' exonuclease and 3'-5' exonuclease. In some instances, 2'-O-methyl modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, 2'O-methoxyethyl (2'-O-MOE) modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, 2'-O-aminopropyl modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, 2'-deoxy modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, T-deoxy-2'-fluoro modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, 2'-O-aminopropyl (2'-O-AP) modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, 2'-O-dimethylaminoethyl (2'-O-DMAOE) modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, 2'-O-dimethylaminopropyl (2'-O-DMAP) modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE) modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, 2'-O-N-methylacetamido (2'-O-NMA) modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, LNA modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease

or 3'-5' exonuclease resistance). In some instances, ENA modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, HNA modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, morpholinos is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, PNA modified polynucleic acid molecule is resistant to nucleases (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, methylphosphonate nucleotides modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, thiolphosphonate nucleotides modified polynucleic acid molecule is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, polynucleic acid molecule comprising 2'-fluoro N3-P5'-phosphoramidites is nuclease resistance (e.g., RNase H, DNase, 5'-3' exonuclease or 3'-5' exonuclease resistance). In some instances, the 5' conjugates described herein inhibit 5'-3' exonucleolytic cleavage. In some instances, the 3' conjugates described herein inhibit 3'-5' exonucleolytic cleavage.

In some embodiments, one or more of the artificial nucleotide analogues described herein have increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. The one or more of the artificial nucleotide analogues comprising 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified, LNA, ENA, PNA, HNA, morpholino, methylphosphonate nucleotides, thiolphosphonate nucleotides, or 2'-fluoro N3-P5'-phosphoramidites have increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-O-methyl modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-O-methoxyethyl (2'-O-MOE) modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-O-aminopropyl modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-deoxy modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, T-deoxy-2'-fluoro modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-O-aminopropyl (2'-O-AP) modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-O-dimethylaminoethyl (2'-O-DMAOE) modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-O-dimethylaminopropyl (2'-O-DMAP) modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE) modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, 2'-O-N-methylacetamido (2'-O-NMA) modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, LNA modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, ENA modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, PNA modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, HNA modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, morpholino modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, methylphosphonate nucleotides modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, thiolphosphonate nucleotides modified polynucleic acid molecule has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some instances, polynucleic acid molecule comprising 2'-fluoro N3-P5'-phosphoramidites has increased binding affinity toward their mRNA target relative to an equivalent natural polynucleic acid molecule. In some cases, the increased affinity is illustrated with a lower Kd, a higher melt temperature (Tm), or a combination thereof.

In some embodiments, a polynucleic acid molecule described herein is a chirally pure (or stereo pure) polynucleic acid molecule, or a polynucleic acid molecule comprising a single enantiomer. In some instances, the polynucleic acid molecule comprises L-nucleotide. In some instances, the polynucleic acid molecule comprises D-nucleotides. In some instance, a polynucleic acid molecule composition comprises less than 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1%, or less of its mirror enantiomer. In some cases, a polynucleic acid molecule composition comprises less than 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1%, or less of a racemic mixture. In some instances, the polynucleic acid molecule is a polynucleic acid molecule described in: U.S. Patent Publication Nos: 2014/194610 and 2015/211006; and PCT Publication No.: WO2015107425.

In some embodiments, a polynucleic acid molecule described herein is further modified to include an aptamer conjugating moiety. In some instances, the aptamer conjugating moiety is a DNA aptamer conjugating moiety. In some instances, the aptamer conjugating moiety is Alphamer (Centauri Therapeutics), which comprises an aptamer portion that recognizes a specific cell-surface target and a portion that presents a specific epitopes for attaching to circulating antibodies. In some instance, a polynucleic acid molecule described herein is further modified to include an aptamer conjugating moiety as described in: U.S. Pat. Nos. 8,604,184, 8,591,910, and 7,850,975.

In additional embodiments, a polynucleic acid molecule described herein is modified to increase its stability. In some embodiment, the polynucleic acid molecule is RNA (e.g., siRNA). In some instances, the polynucleic acid molecule is

modified by one or more of the modifications described above to increase its stability. In some cases, the polynucleic acid molecule is modified at the 2' hydroxyl position, such as by 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, T-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), T-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modification or by a locked or bridged ribose conformation (e.g., LNA or ENA). In some cases, the polynucleic acid molecule is modified by 2'-O-methyl and/or 2'-O-methoxyethyl ribose. In some cases, the polynucleic acid molecule also includes morpholinos, PNAs, HNA, methylphosphonate nucleotides, thiolphosphonate nucleotides, and/or 2'-fluoro N3-P5'-phosphoramidites to increase its stability. In some instances, the polynucleic acid molecule is a chirally pure (or stereo pure) polynucleic acid molecule. In some instances, the chirally pure (or stereo pure) polynucleic acid molecule is modified to increase its stability. Suitable modifications to the RNA to increase stability for delivery will be apparent to the skilled person.

In some instances, the polynucleic acid molecule is a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. In some instances, the polynucleic acid molecule is assembled from two separate polynucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (e.g., each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 19, 20, 21, 22, 23, or more base pairs); the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the polynucleic acid molecule is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the polynucleic acid molecule are linked by means of a nucleic acid based or non-nucleic acid-based linker(s).

In some cases, the polynucleic acid molecule is a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. In other cases, the polynucleic acid molecule is a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide is processed either in vivo or in vitro to generate an active polynucleic acid molecule capable of mediating RNAi. In additional cases, the polynucleic acid molecule also comprises a single-stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such polynucleic acid molecule does not require the presence within the polynucleic acid molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide further comprises a terminal phosphate group, such as a 5'-phosphate (see for example Martinez et al., 2002, *Cell.*, 110, 563-574 and Schwarz et al., 2002, *Molecular Cell,* 10, 537-568), or 5',3'-diphosphate.

In some instances, an asymmetric hairpin is a linear polynucleic acid molecule comprising an antisense region, a loop portion that comprises nucleotides or non-nucleotides, and a sense region that comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complimentary nucleotides to base pair with the antisense region and form a duplex with loop. For example, an asymmetric hairpin polynucleic acid molecule comprises an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 19 to about 22 nucleotides) and a loop region comprising about 4 to about 8 nucleotides, and a sense region having about 3 to about 18 nucleotides that are complementary to the antisense region. In some cases, the asymmetric hairpin polynucleic acid molecule also comprises a 5'-terminal phosphate group that is chemically modified. In additional cases, the loop portion of the asymmetric hairpin polynucleic acid molecule comprises nucleotides, non-nucleotides, linker molecules, or conjugate molecules.

In some embodiments, an asymmetric duplex is a polynucleic acid molecule having two separate strands comprising a sense region and an antisense region, wherein the sense region comprises fewer nucleotides than the antisense region to the extent that the sense region has enough complimentary nucleotides to base pair with the antisense region and form a duplex. For example, an asymmetric duplex polynucleic acid molecule comprises an antisense region having length sufficient to mediate RNAi in a cell or in vitro system (e.g. about 19 to about 22 nucleotides) and a sense region having about 3 to about 18 nucleotides that are complementary to the antisense region.

In some cases, a universal base refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, *Nucleic Acids Research,* 29, 2437-2447).

Polynucleic Acid Molecule Synthesis

In some embodiments, a polynucleic acid molecule described herein is constructed using chemical synthesis and/or enzymatic ligation reactions using procedures known in the art. For example, a polynucleic acid molecule is chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the polynucleic acid molecule and target nucleic acids. Exemplary methods include those described in: U.S. Pat. Nos. 5,142,047; 5,185,444; 5,889,136; 6,008,400; and 6,111,086; PCT Publication No. WO2009099942; or European Publication No. 1579015. Additional exemplary methods include those

described in: Griffey et al., "2'-O-aminopropyl ribonucleotides: a zwitterionic modification that enhances the exonuclease resistance and biological activity of antisense oligonucleotides," *J. Med Chem.* 39(26):5100-5109 (1997)); Obika, et al. "Synthesis of 2'-O,4'-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3, -endo sugar puckering". *Tetrahedron Letters* 38 (50): 8735 (1997); Koizumi, M. "ENA oligonucleotides as therapeutics". *Current opinion in molecular therapeutics* 8 (2): 144-149 (2006); and Abramova et al., "Novel oligonucleotide analogues based on morpholino nucleoside subunits-antisense technologies: new chemical possibilities," Indian Journal of Chemistry 48B:1721-1726 (2009). Alternatively, the polynucleic acid molecule is produced biologically using an expression vector into which a polynucleic acid molecule has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted polynucleic acid molecule will be of an antisense orientation to a target polynucleic acid molecule of interest).

In some embodiments, a polynucleic acid molecule is synthesized via a tandem synthesis methodology, wherein both strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate fragments or strands that hybridize and permit purification of the duplex.

In some instances, a polynucleic acid molecule is also assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the molecule.

Additional modification methods for incorporating, for example, sugar, base and phosphate modifications include: Eckstein et al., International Publication PCT No. WO 92/07065; Perrault et al. Nature, 1990, 344, 565-568; Pieken et al. *Science,* 1991, 253, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.,* 1992, 17, 334-339; Usman et al. International Publication PCT No. WO 93/15187; Sproat, U.S. Pat. No. 5,334,711 and Beigelman et al., 1995, *J. Biol. Chem.,* 270, 25702; Beigelman et al., International PCT publication No. WO 97/26270; Beigelman et al., U.S. Pat. No. 5,716,824; Usman et al., U.S. Pat. No. 5,627,053; Woolf et al., International PCT Publication No. WO 98/13526; Thompson et al., U.S. Ser. No. 60/082,404 which was filed on Apr. 20, 1998; Karpeisky et al., 1998, *Tetrahedron Lett.,* 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences)*, 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina et al., 1997, *Bioorg. Med. Chem.,* 5, 1999-2010. Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis.

In some instances, while chemical modification of the polynucleic acid molecule internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications sometimes cause toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages in some cases is minimized. In such cases, the reduction in the concentration of these linkages lowers toxicity, increases efficacy and higher specificity of these molecules.

Polynucleic Acid Molecule Conjugates

In some embodiments, a polynucleic acid molecule is further conjugated to a polypeptide A for delivery to a site of interest. In some cases, a polynucleic acid molecule is conjugated to a polypeptide A and optionally a polymeric moiety.

In some instances, at least one polypeptide A is conjugated to at least one B. In some instances, the at least one polypeptide A is conjugated to the at least one B to form an A-B conjugate. In some embodiments, at least one A is conjugated to the 5' terminus of B, the 3' terminus of B, an internal site on B, or in any combinations thereof. In some instances, the at least one polypeptide A is conjugated to at least two B. In some instances, the at least one polypeptide A is conjugated to at least 2, 3, 4, 5, 6, 7, 8, or more B.

In some embodiments, at least one polypeptide A is conjugated at one terminus of at least one B while at least one C is conjugated at the opposite terminus of the at least one B to form an A-B-C conjugate. In some instances, at least one polypeptide A is conjugated at one terminus of the at least one B while at least one of C is conjugated at an internal site on the at least one B. In some instances, at least one polypeptide A is conjugated directly to the at least one C. In some instances, the at least one B is conjugated indirectly to the at least one polypeptide A via the at least one C to form an A-C-B conjugate.

In some instances, at least one B and/or at least one C, and optionally at least one D are conjugated to at least one polypeptide A. In some instances, the at least one B is conjugated at a terminus (e.g., a 5' terminus or a 3' terminus) to the at least one polypeptide A or are conjugated via an internal site to the at least one polypeptide A. In some cases, the at least one C is conjugated either directly to the at least one polypeptide A or indirectly via the at least one B. If indirectly via the at least one B, the at least one C is conjugated either at the same terminus as the at least one polypeptide A on B, at opposing terminus from the at least one polypeptide A, or independently at an internal site. In some instances, at least one additional polypeptide A is further conjugated to the at least one polypeptide A, to B, or to C. In additional instances, the at least one D is optionally conjugated either directly or indirectly to the at least one polypeptide A, to the at least one B, or to the at least one C. If directly to the at least one polypeptide A, the at least one D is also optionally conjugated to the at least one B to form an A-D-B conjugate or is optionally conjugated to the at least one B and the at least one C to form an A-D-B-C conjugate. In some instances, the at least one D is directly conjugated to the at least one polypeptide A and indirectly to the at least one B and the at least one C to form a D-A-B-C conjugate. If indirectly to the at least one polypeptide A, the at least one D is also optionally conjugated to the at least one B to form an A-B-D conjugate or is optionally conjugated to the at least one B and the at least one C to form an A-B-D-C conjugate. In some instances, at least one additional D is further conjugated to the at least one polypeptide A, to B, or to C.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**A.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**B.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**C.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**D.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**E.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**F.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**G.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**H.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**I.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**J.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**K.

In some embodiments, a polynucleic acid molecule conjugate comprises a construct as illustrated in FIG. **19**L

The antibody cartoon as illustrated in FIG. **19**M is for representation purposes only and encompasses a humanized antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab2, single-chain variable fragment (scFv), diabody, minibody, nanobody, single-domain antibody (sdAb), or camelid antibody or binding fragment thereof.

Binding Moiety

In some embodiments, the binding moiety A is a polypeptide. In some instances, the polypeptide is an antibody or its fragment thereof. In some cases, the fragment is a binding fragment. In some instances, the antibody or binding fragment thereof comprises a humanized antibody or binding fragment thereof, murine antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab_2, $F(ab)'_3$ fragments, single-chain variable fragment (scFv), bis-scFv, $(scFv)_2$, diabody, minibody, nanobody, triabody, tetrabody, disulfide stabilized Fv protein (dsFv), single-domain antibody (sdAb), Ig NAR, camelid antibody or binding fragment thereof, bispecific antibody or biding fragment thereof, or a chemically modified derivative thereof.

In some instances, A is an antibody or binding fragment thereof. In some instances, A is a humanized antibody or binding fragment thereof, murine antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab_2, $F(ab)'_3$ fragments, single-chain variable fragment (scFv), bis-scFv, $(scFv)_2$, diabody, minibody, nanobody, triabody, tetrabody, disulfide stabilized Fv protein ("dsFv"), single-domain antibody (sdAb), Ig NAR, camelid antibody or binding fragment thereof, bispecific antibody or biding fragment thereof, or a chemically modified derivative thereof. In some instances, A is a humanized antibody or binding fragment thereof. In some instances, A is a murine antibody or binding fragment thereof. In some instances, A is a chimeric antibody or binding fragment thereof. In some instances, A is a monoclonal antibody or binding fragment thereof. In some instances, A is a monovalent Fab'. In some instances, A is a diavalent Fab_2. In some instances, A is a single-chain variable fragment (scFv).

In some embodiments, the binding moiety A is a bispecific antibody or binding fragment thereof. In some instances, the bispecific antibody is a trifunctional antibody or a bispecific mini-antibody. In some cases, the bispecific antibody is a trifunctional antibody. In some instances, the trifunctional antibody is a full length monoclonal antibody comprising binding sites for two different antigens.

In some cases, the bispecific antibody is a bispecific mini-antibody. In some instances, the bispecific mini-antibody comprises divalent Fab_2, $F(ab)'_3$ fragments, bis-scFv, $(scFv)_2$, diabody, minibody, triabody, tetrabody or a bi-specific T-cell engager (BiTE). In some embodiments, the bi-specific T-cell engager is a fusion protein that contains two single-chain variable fragments (scFvs) in which the two scFvs target epitopes of two different antigens.

In some embodiments, the binding moiety A is a bispecific mini-antibody. In some instances, A is a bispecific Fab_2. In some instances, A is a bispecific $F(ab)'_3$ fragment. In some cases, A is a bispecific bis-scFv. In some cases, A is a bispecific $(scFv)_2$. In some embodiments, A is a bispecific diabody. In some embodiments, A is a bispecific minibody. In some embodiments, A is a bispecific triabody. In other embodiments, A is a bispecific tetrabody. In other embodiments, A is a bi-specific T-cell engager (BiTE).

In some embodiments, the binding moiety A is a trispecific antibody. In some instances, the trispecific antibody comprises $F(ab)'_3$ fragments or a triabody. In some instances, A is a trispecific $F(ab)'_3$ fragment. In some cases, A is a triabody. In some embodiments, A is a trispecific antibody as described in Dimas, et al., "Development of a trispecific antibody designed to simultaneously and efficiently target three different antigens on tumor cells," *Mol. Pharmaceutics,* 12(9): 3490-3501 (2015).

In some embodiments, the binding moiety A is an antibody or binding fragment thereof that recognizes a cell surface protein. In some instances, the binding moiety A is an antibody or binding fragment thereof that recognizes a cell surface protein on a muscle cell. In some cases, the binding moiety A is an antibody or binding fragment thereof that recognizes a cell surface protein on a skeletal muscle cell.

In some embodiments, exemplary antibodies include, but are not limited to, an anti-myosin antibody, an anti-transferrin antibody, and an antibody that recognizes Muscle-Specific kinase (MuSK). In some instances, the antibody is an anti-transferrin (anti-CD71) antibody.

In some embodiments, the binding moiety A is conjugated to a polynucleic acid molecule (B) non-specifically. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) via a lysine residue or a cysteine residue, in a non-site specific manner. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) via a lysine residue in a non-site specific manner. In some cases, the binding moiety A is conjugated to a polynucleic acid molecule (B) via a cysteine residue in a non-site specific manner.

In some embodiments, the binding moiety A is conjugated to a polynucleic acid molecule (B) in a site-specific manner. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) through a lysine residue, a cysteine residue, at the 5'-terminus, at the 3'-terminus, an unnatural amino acid, or an enzyme-modified or enzyme-catalyzed residue, via a site-specific manner. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) through a lysine residue via a site-specific manner. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) through a cysteine residue via a site-specific manner. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) at the 5'-terminus via a site-specific manner. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) at the 3'-terminus via a site-specific manner. In some instances, the binding moiety A is conjugated to a polynucleic acid molecule (B) through an unnatural amino acid via a site-specific manner. In some instances, the binding moiety A is conju-

gated to a polynucleic acid molecule (B) through an enzyme-modified or enzyme-catalyzed residue via a site-specific manner.

In some embodiments, one or more polynucleic acid molecule (B) is conjugated to a binding moiety A. In some instances, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or more polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 1 polynucleic acid molecule is conjugated to one binding moiety A. In some instances, about 2 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 3 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 4 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 5 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 6 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 7 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 8 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 9 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 10 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 11 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 12 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 13 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 14 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 15 polynucleic acid molecules are conjugated to one binding moiety A. In some instances, about 16 polynucleic acid molecules are conjugated to one binding moiety A. In some cases, the one or more polynucleic acid molecules are the same. In other cases, the one or more polynucleic acid molecules are different.

In some embodiments, the number of polynucleic acid molecule (B) conjugated to a binding moiety A forms a ratio. In some instances, the ratio is referred to as a DAR (drug-to-antibody) ratio, in which the drug as referred to herein is the polynucleic acid molecule (B). In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 1 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 2 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 3 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 4 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 5 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 6 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 7 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 8 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 9 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 10 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 11 or greater. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 12 or greater.

In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 1. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 2. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 3. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 4. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 5. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 6. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 7. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 8. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 9. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 10. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 11. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 12. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 13. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 14. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 15. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is about 16.

In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is 1. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is 2. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is 4. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is 6. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is 8. In some instances, the DAR ratio of the polynucleic acid molecule (B) to binding moiety A is 12.

In some instances, a conjugate comprising polynucleic acid molecule (B) and binding moiety A has improved activity as compared to a conjugate comprising polynucleic acid molecule (B) without a binding moiety A. In some instances, improved activity results in enhanced biologically relevant functions, e.g., improved stability, affinity, binding, functional activity, and efficacy in treatment or prevention of a disease state. In some instances, the disease state is a result of one or more mutated exons of a gene. In some instances, the conjugate comprising polynucleic acid molecule (B) and binding moiety A results in increased exon skipping of the one or more mutated exons as compared to the conjugate comprising polynucleic acid molecule (B) without a binding moiety A. In some instances, exon skipping is increased by at least or about 5%, 10%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or more than 95% in the conjugate comprising polynucleic acid molecule (B) and binding moiety A as compared to the conjugate comprising polynucleic acid molecule (B) without a binding moiety A.

In some embodiments, an antibody or its binding fragment is further modified using conventional techniques known in the art, for example, by using amino acid deletion, insertion, substitution, addition, and/or by recombination and/or any other modification (e.g. posttranslational and chemical modifications, such as glycosylation and phosphorylation) known in the art either alone or in combination. In some instances, the modification further comprises a modification for modulating interaction with Fc receptors. In some instances, the one or more modifications include those described in, for example, International Publication No. WO97/34631, which discloses amino acid residues involved in the interaction between the Fc domain and the FcRn receptor. Methods for introducing such modifications in the nucleic acid sequence underlying the amino acid sequence of an antibody or its binding fragment is well known to the person skilled in the art.

In some instances, an antibody binding fragment further encompasses its derivatives and includes polypeptide sequences containing at least one CDR.

In some instances, the term "single-chain" as used herein means that the first and second domains of a bi-specific single chain construct are covalently linked, preferably in the form of a co-linear amino acid sequence encodable by a single nucleic acid molecule.

In some instances, a bispecific single chain antibody construct relates to a construct comprising two antibody derived binding domains. In such embodiments, bi-specific single chain antibody construct is tandem bi-scFv or diabody. In some instances, a scFv contains a VH and VL domain connected by a linker peptide. In some instances, linkers are of a length and sequence sufficient to ensure that each of the first and second domains can, independently from one another, retain their differential binding specificities.

In some embodiments, binding to or interacting with as used herein defines a binding/interaction of at least two antigen-interaction-sites with each other. In some instances, antigen-interaction-site defines a motif of a polypeptide that shows the capacity of specific interaction with a specific antigen or a specific group of antigens. In some cases, the binding/interaction is also understood to define a specific recognition. In such cases, specific recognition refers to that the antibody or its binding fragment is capable of specifically interacting with and/or binding to at least two amino acids of each of a target molecule. For example, specific recognition relates to the specificity of the antibody molecule, or to its ability to discriminate between the specific regions of a target molecule. In additional instances, the specific interaction of the antigen-interaction-site with its specific antigen results in an initiation of a signal, e.g. due to the induction of a change of the conformation of the antigen, an oligomerization of the antigen, etc. In further embodiments, the binding is exemplified by the specificity of a "key-lock-principle". Thus in some instances, specific motifs in the amino acid sequence of the antigen-interaction-site and the antigen bind to each other as a result of their primary, secondary or tertiary structure as well as the result of secondary modifications of said structure. In such cases, the specific interaction of the antigen-interaction-site with its specific antigen results as well in a simple binding of the site to the antigen.

In some instances, specific interaction further refers to a reduced cross-reactivity of the antibody or its binding fragment or a reduced off-target effect. For example, the antibody or its binding fragment that bind to the polypeptide/protein of interest but do not or do not essentially bind to any of the other polypeptides are considered as specific for the polypeptide/protein of interest. Examples for the specific interaction of an antigen-interaction-site with a specific antigen comprise the specificity of a ligand for its receptor, for example, the interaction of an antigenic determinant (epitope) with the antigenic binding site of an antibody.

Additional Binding Moieties

In some embodiments, the binding moiety is a plasma protein. In some instances, the plasma protein comprises albumin. In some instances, the binding moiety A is albumin. In some instances, albumin is conjugated by one or more of a conjugation chemistry described herein to a polynucleic acid molecule. In some instances, albumin is conjugated by native ligation chemistry to a polynucleic acid molecule. In some instances, albumin is conjugated by lysine conjugation to a polynucleic acid molecule.

In some instances, the binding moiety is a steroid. Exemplary steroids include cholesterol, phospholipids, di- and triacylglycerols, fatty acids, hydrocarbons that are saturated, unsaturated, comprise substitutions, or combinations thereof. In some instances, the steroid is cholesterol. In some instances, the binding moiety is cholesterol. In some instances, cholesterol is conjugated by one or more of a conjugation chemistry described herein to a polynucleic acid molecule. In some instances, cholesterol is conjugated by native ligation chemistry to a polynucleic acid molecule. In some instances, cholesterol is conjugated by lysine conjugation to a polynucleic acid molecule.

In some instances, the binding moiety is a polymer, including but not limited to polynucleic acid molecule aptamers that bind to specific surface markers on cells. In this instance the binding moiety is a polynucleic acid that does not hybridize to a target gene or mRNA, but instead is capable of selectively binding to a cell surface marker similarly to an antibody binding to its specific epitope of a cell surface marker.

In some cases, the binding moiety is a peptide. In some cases, the peptide comprises between about 1 and about 3 kDa. In some cases, the peptide comprises between about 1.2 and about 2.8 kDa, about 1.5 and about 2.5 kDa, or about 1.5 and about 2 kDa. In some instances, the peptide is a bicyclic peptide. In some cases, the bicyclic peptide is a constrained bicyclic peptide. In some instances, the binding moiety is a bicyclic peptide (e.g., bicycles from Bicycle Therapeutics).

In additional cases, the binding moiety is a small molecule. In some instances, the small molecule is an antibody-recruiting small molecule. In some cases, the antibody-recruiting small molecule comprises a target-binding terminus and an antibody-binding terminus, in which the target-binding terminus is capable of recognizing and interacting with a cell surface receptor. For example, in some instances, the target-binding terminus comprising a glutamate urea compound enables interaction with PSMA, thereby, enhances an antibody interaction with a cell that expresses PSMA. In some instances, a binding moiety is a small molecule described in Zhang et al., "A remote arene-binding site on prostate specific membrane antigen revealed by antibody-recruiting small molecules," J Am Chem Soc. 132(36): 12711-12716 (2010); or McEnaney, et al., "Antibody-recruiting molecules: an emerging paradigm for engaging immune function in treating human disease," ACS Chem Biol. 7(7): 1139-1151 (2012).

Production of Antibodies or Binding Fragments Thereof

In some embodiments, polypeptides described herein (e.g., antibodies and its binding fragments) are produced using any method known in the art to be useful for the

synthesis of polypeptides (e.g., antibodies), in particular, by chemical synthesis or by recombinant expression, and are preferably produced by recombinant expression techniques.

In some instances, an antibody or its binding fragment thereof is expressed recombinantly, and the nucleic acid encoding the antibody or its binding fragment is assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., 1994, *BioTechniques* 17:242), which involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, a nucleic acid molecule encoding an antibody is optionally generated from a suitable source (e.g., an antibody cDNA library, or cDNA library generated from any tissue or cells expressing the immunoglobulin) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence.

In some instances, an antibody or its binding is optionally generated by immunizing an animal, such as a rabbit, to generate polyclonal antibodies or, more preferably, by generating monoclonal antibodies, e.g., as described by Kohler and Milstein (1975, *Nature* 256:495-497) or, as described by Kozbor et al. (1983, *Immunology Today* 4:72) or Cole et al. (1985 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Alternatively, a clone encoding at least the Fab portion of the antibody is optionally obtained by screening Fab expression libraries (e.g., as described in Huse et al., 1989, Science 246:1275-1281) for clones of Fab fragments that bind the specific antigen or by screening antibody libraries (See, e.g., Clackson et al., 1991, *Nature* 352:624; Hane et al., 1997 *Proc. Natl. Acad. Sci. USA* 94:4937).

In some embodiments, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, *Proc. Natl. Acad. Sci.* 81:851-855; Neuberger et al., 1984, *Nature* 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity are used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region, e.g., humanized antibodies.

In some embodiments, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,694,778; Bird, 1988, *Science* 242:42342; Huston et al., 1988, *Proc. Natl. Acad. Sci. USA* 85:5879-5883; and Ward et al., 1989, *Nature* 334:544-54) are adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in *E. coli* are also optionally used (Skerra et al., 1988, *Science* 242:1038-1041).

In some embodiments, an expression vector comprising the nucleotide sequence of an antibody or the nucleotide sequence of an antibody is transferred to a host cell by conventional techniques (e.g., electroporation, liposomal transfection, and calcium phosphate precipitation), and the transfected cells are then cultured by conventional techniques to produce the antibody. In specific embodiments, the expression of the antibody is regulated by a constitutive, an inducible or a tissue, specific promoter.

In some embodiments, a variety of host-expression vector systems is utilized to express an antibody or its binding fragment described herein. Such host-expression systems represent vehicles by which the coding sequences of the antibody is produced and subsequently purified, but also represent cells that are, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody or its binding fragment in situ. These include, but are not limited to, microorganisms such as bacteria (e.g., *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing an antibody or its binding fragment coding sequences; yeast (e.g., *Saccharomyces Pichia*) transformed with recombinant yeast expression vectors containing an antibody or its binding fragment coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing an antibody or its binding fragment coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus (CaMV) and tobacco mosaic virus (TMV)) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing an antibody or its binding fragment coding sequences; or mammalian cell systems (e.g., COS, CHO, BH, 293, 293T, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g. the adenovirus late promoter; the vaccinia virus 7.5K promoter).

For long-term, high-yield production of recombinant proteins, stable expression is preferred. In some instances, cell lines that stably express an antibody are optionally engineered. Rather than using expression vectors that contain viral origins of replication, host cells are transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells are then allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn are cloned and expanded into cell lines. This method can advantageously be used to engineer cell lines which express the antibody or its binding fragments.

In some instances, a number of selection systems are used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 192, *Proc. Natl. Acad Sci. USA* 48:202), and adenine phosphoribosyltransferase (Lowy et al., 1980, *Cell* 22:817) genes are employed in tk-, hgprt- or aprt-cells, respectively. Also, antimetabolite resistance are used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., 1980, *Proc. Natl. Acad Sci. USA* 77:357; O'Hare et al., 1981, *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (*Clinical Pharmacy* 12:488-505; Wu and Wu, 1991, *Biotherapy* 3:87-95; Tolstoshev, 1993, *Ann. Rev. Pharmacol. Toxicol.* 32:573-596; Mulligan, 1993, *Science* 260:926-932; and Morgan and Anderson, 1993, *Ann. Rev. Biochem.* 62:191-217; May, 1993, *TIB TECH* 11(5):155-215) and hygro, which confers resistance to hygromycin (Santerre et al., 1984, *Gene* 30:147). Methods

commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds., 1993, *Current Protocols in Molecular Biology*, John Wiley & Sons, NY; Kriegler, 1990, *Gene Transfer and Expression, A Laboratory Manual*, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli et al. (eds), 1994, *Current Protocols in Human Genetics*, John Wiley & Sons, NY.; Colberre-Garapin et al., 1981, *J. Mol. Biol.* 150:1).

In some instances, the expression levels of an antibody are increased by vector amplification (for a review, see Bebbington and Hentschel, *The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning*, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing an antibody is amplifiable, an increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the nucleotide sequence of the antibody, production of the antibody will also increase (Crouse et al., 1983, *Mol. Cell Biol.* 3:257).

In some instances, any method known in the art for purification or analysis of an antibody or antibody conjugates is used, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. Exemplary chromatography methods included, but are not limited to, strong anion exchange chromatography, hydrophobic interaction chromatography, size exclusion chromatography, and fast protein liquid chromatography.

Conjugation Chemistry

In some embodiments, a polynucleic acid molecule B is conjugated to a binding moiety. In some instances, the binding moiety comprises amino acids, peptides, polypeptides, proteins, antibodies, antigens, toxins, hormones, lipids, nucleotides, nucleosides, sugars, carbohydrates, polymers such as polyethylene glycol and polypropylene glycol, as well as analogs or derivatives of all of these classes of substances. Additional examples of binding moiety also include steroids, such as cholesterol, phospholipids, di- and triacylglycerols, fatty acids, hydrocarbons (e.g., saturated, unsaturated, or contains substitutions), enzyme substrates, biotin, digoxigenin, and polysaccharides. In some instances, the binding moiety is an antibody or binding fragment thereof. In some instances, the polynucleic acid molecule is further conjugated to a polymer, and optionally an endosomolytic moiety.

In some embodiments, the polynucleic acid molecule is conjugated to the binding moiety by a chemical ligation process. In some instances, the polynucleic acid molecule is conjugated to the binding moiety by a native ligation. In some instances, the conjugation is as described in: Dawson, et al. "Synthesis of proteins by native chemical ligation," *Science* 1994, 266, 776-779; Dawson, et al. "Modulation of Reactivity in Native Chemical Ligation through the Use of Thiol Additives," *J. Am. Chem. Soc.* 1997, 119, 4325-4329; Hackeng, et al. "Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology.," *Proc. Natl. Acad. Sci. USA* 1999, 96, 10068-10073; or Wu, et al. "Building complex glycopeptides: Development of a cysteine-free native chemical ligation protocol," *Angew. Chem. Int. Ed.* 2006, 45, 4116-4125. In some instances, the conjugation is as described in U.S. Pat. No. 8,936,910. In some embodiments, the polynucleic acid molecule is conjugated to the binding moiety either site-specifically or non-specifically via native ligation chemistry.

In some instances, the polynucleic acid molecule is conjugated to the binding moiety by a site-directed method utilizing a "traceless" coupling technology (Philochem). In some instances, the "traceless" coupling technology utilizes an N-terminal 1,2-aminothiol group on the binding moiety which is then conjugate with a polynucleic acid molecule containing an aldehyde group. (see Casi et al., "Site-specific traceless coupling of potent cytotoxic drugs to recombinant antibodies for pharmacodelivery," *JACS* 134(13): 5887-5892 (2012))

In some instances, the polynucleic acid molecule is conjugated to the binding moiety by a site-directed method utilizing an unnatural amino acid incorporated into the binding moiety. In some instances, the unnatural amino acid comprises p-acetylphenylalanine (pAcPhe). In some instances, the keto group of pAcPhe is selectively coupled to an alkoxy-amine derivatived conjugating moiety to form an oxime bond. (see Axup et al., "Synthesis of site-specific antibody-drug conjugates using unnatural amino acids," *PNAS* 109(40): 16101-16106 (2012)).

In some instances, the polynucleic acid molecule is conjugated to the binding moiety by a site-directed method utilizing an enzyme-catalyzed process. In some instances, the site-directed method utilizes SMARTag™ technology (Catalent, Inc.). In some instances, the SMARTag™ technology comprises generation of a formylglycine (FGly) residue from cysteine by formylglycine-generating enzyme (FGE) through an oxidation process under the presence of an aldehyde tag and the subsequent conjugation of FGly to an alkylhydraine-functionalized polynucleic acid molecule via hydrazino-Pictet-Spengler (HIPS) ligation. (see Wu et al., "Site-specific chemical modification of recombinant proteins produced in mammalian cells by using the genetically encoded aldehyde tag," *PNAS* 106(9): 3000-3005 (2009); Agarwal, et al., "A Pictet-Spengler ligation for protein chemical modification," *PNAS* 110(1): 46-51 (2013))

In some instances, the enzyme-catalyzed process comprises microbial transglutaminase (mTG). In some cases, the polynucleic acid molecule is conjugated to the binding moiety utilizing a microbial transglutaminase-catalyzed process. In some instances, mTG catalyzes the formation of a covalent bond between the amide side chain of a glutamine within the recognition sequence and a primary amine of a functionalized polynucleic acid molecule. In some instances, mTG is produced from *Streptomyces mobarensis*. (see Strop et al., "Location matters: site of conjugation modulates stability and pharmacokinetics of antibody drug conjugates," *Chemistry and Biology* 20(2) 161-167 (2013))

In some instances, the polynucleic acid molecule is conjugated to the binding moiety by a method as described in PCT Publication No. WO2014/140317, which utilizes a sequence-specific transpeptidase.

In some instances, the polynucleic acid molecule is conjugated to the binding moiety by a method as described in U.S. Patent Publication Nos. 2015/0105539 and 2015/0105540.

Polymer Conjugating Moiety

In some embodiments, a polymer moiety C is further conjugated to a polynucleic acid molecule described herein, a binding moiety described herein, or in combinations thereof. In some instances, a polymer moiety C is conjugated a polynucleic acid molecule. In some cases, a polymer moiety C is conjugated to a binding moiety. In other cases, a polymer moiety C is conjugated to a polynucleic acid molecule-binding moiety molecule. In additional cases, a polymer moiety C is conjugated, as illustrated supra.

In some instances, the polymer moiety C is a natural or synthetic polymer, consisting of long chains of branched or unbranched monomers, and/or cross-linked network of monomers in two or three dimensions. In some instances, the polymer moiety C includes a polysaccharide, lignin, rubber, or polyalkylen oxide (e.g., polyethylene glycol). In some instances, the at least one polymer moiety C includes, but is not limited to, alpha-, omega-dihydroxylpolyethyleneglycol, biodegradable lactone-based polymer, e.g. polyacrylic acid, polylactide acid (PLA), poly(glycolic acid) (PGA), polypropylene, polystyrene, polyolefin, polyamide, polycyanoacrylate, polyimide, polyethylene terephthalate (also known as poly(ethylene terephthalate), PET, PETG, or PETE), polytetramethylene glycol (PTG), or polyurethane as well as mixtures thereof. As used herein, a mixture refers to the use of different polymers within the same compound as well as in reference to block copolymers. In some cases, block copolymers are polymers wherein at least one section of a polymer is build up from monomers of another polymer. In some instances, the polymer moiety C comprises polyalkylene oxide. In some instances, the polymer moiety C comprises PEG. In some instances, the polymer moiety C comprises polyethylene imide (PEI) or hydroxy ethyl starch (HES).

In some instances, C is a PEG moiety. In some instances, the PEG moiety is conjugated at the 5' terminus of the polynucleic acid molecule while the binding moiety is conjugated at the 3' terminus of the polynucleic acid molecule. In some instances, the PEG moiety is conjugated at the 3' terminus of the polynucleic acid molecule while the binding moiety is conjugated at the 5' terminus of the polynucleic acid molecule. In some instances, the PEG moiety is conjugated to an internal site of the polynucleic acid molecule. In some instances, the PEG moiety, the binding moiety, or a combination thereof, are conjugated to an internal site of the polynucleic acid molecule. In some instances, the conjugation is a direct conjugation. In some instances, the conjugation is via native ligation.

In some embodiments, the polyalkylene oxide (e.g., PEG) is a polydisperse or monodisperse compound. In some instances, polydisperse material comprises disperse distribution of different molecular weight of the material, characterized by mean weight (weight average) size and dispersity. In some instances, the monodisperse PEG comprises one size of molecules. In some embodiments, C is poly- or monodispersed polyalkylene oxide (e.g., PEG) and the indicated molecular weight represents an average of the molecular weight of the polyalkylene oxide, e.g., PEG, molecules.

In some embodiments, the molecular weight of the polyalkylene oxide (e.g., PEG) is about 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1450, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3250, 3350, 3500, 3750, 4000, 4250, 4500, 4600, 4750, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 10,000, 12,000, 20,000, 35,000, 40,000, 50,000, 60,000, or 100,000 Da.

In some embodiments, C is polyalkylene oxide (e.g., PEG) and has a molecular weight of about 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1450, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3250, 3350, 3500, 3750, 4000, 4250, 4500, 4600, 4750, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 10,000, 12,000, 20,000, 35,000, 40,000, 50,000, 60,000, or 100,000 Da. In some embodiments, C is PEG and has a molecular weight of about 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1450, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000, 3250, 3350, 3500, 3750, 4000, 4250, 4500, 4600, 4750, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 10,000, 12,000, 20,000, 35,000, 40,000, 50,000, 60,000, or 100,000 Da. In some instances, the molecular weight of C is about 200 Da. In some instances, the molecular weight of C is about 300 Da. In some instances, the molecular weight of C is about 400 Da. In some instances, the molecular weight of C is about 500 Da. In some instances, the molecular weight of C is about 600 Da. In some instances, the molecular weight of C is about 700 Da. In some instances, the molecular weight of C is about 800 Da. In some instances, the molecular weight of C is about 900 Da. In some instances, the molecular weight of C is about 1000 Da. In some instances, the molecular weight of C is about 1100 Da. In some instances, the molecular weight of C is about 1200 Da. In some instances, the molecular weight of C is about 1300 Da. In some instances, the molecular weight of C is about 1400 Da. In some instances, the molecular weight of C is about 1450 Da. In some instances, the molecular weight of C is about 1500 Da. In some instances, the molecular weight of C is about 1600 Da. In some instances, the molecular weight of C is about 1700 Da. In some instances, the molecular weight of C is about 1800 Da. In some instances, the molecular weight of C is about 1900 Da. In some instances, the molecular weight of C is about 2000 Da. In some instances, the molecular weight of C is about 2100 Da. In some instances, the molecular weight of C is about 2200 Da. In some instances, the molecular weight of C is about 2300 Da. In some instances, the molecular weight of C is about 2400 Da. In some instances, the molecular weight of C is about 2500 Da. In some instances, the molecular weight of C is about 2600 Da. In some instances, the molecular weight of C is about 2700 Da. In some instances, the molecular weight of C is about 2800 Da. In some instances, the molecular weight of C is about 2900 Da. In some instances, the molecular weight of C is about 3000 Da. In some instances, the molecular weight of C is about 3250 Da. In some instances, the molecular weight of C is about 3350 Da. In some instances, the molecular weight of C is about 3500 Da. In some instances, the molecular weight of C is about 3750 Da. In some instances, the molecular weight of C is about 4000 Da. In some instances, the molecular weight of C is about 4250 Da. In some instances, the molecular weight of C is about 4500 Da. In some instances, the molecular weight of C is about 4600 Da. In some instances, the molecular weight of C is about 4750 Da. In some instances, the molecular weight of C is about 5000 Da. In some instances, the molecular weight of C is about 5500 Da. In some instances, the molecular weight of C is about 6000 Da. In some instances, the molecular weight of C is about 6500 Da. In some instances, the molecular weight of C is about 7000 Da. In some instances, the molecular weight of C is about 7500 Da. In some instances, the molecular weight of C is about 8000 Da. In some instances, the molecular weight of C is about 10,000 Da. In some instances, the molecular weight of C is about 12,000 Da. In some instances, the molecular weight of C is about 20,000 Da. In some instances, the molecular weight of C is about 35,000 Da. In some instances, the molecular weight of C is about 40,000 Da. In some instances, the molecular weight of C is about 50,000 Da. In some instances, the molecular weight of C is about 60,000 Da. In some instances, the molecular weight of C is about 100,000 Da.

In some embodiments, the polyalkylene oxide (e.g., PEG) comprises discrete ethylene oxide units (e.g., four to about 48 ethylene oxide units). In some instances, the polyalkylene

oxide comprising the discrete ethylene oxide units is a linear chain. In other cases, the polyalkylene oxide comprising the discrete ethylene oxide units is a branched chain.

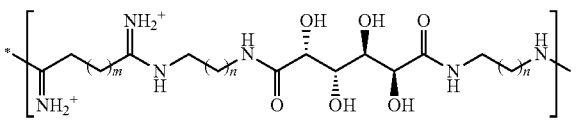
In some instances, the polymer moiety C is a polyalkylene oxide (e.g., PEG) comprising discrete ethylene oxide units. In some cases, the polymer moiety C comprises between about 4 and about 48 ethylene oxide units. In some cases, the polymer moiety C comprises about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, or about 48 ethylene oxide units.

In some instances, the polymer moiety C is a discrete PEG comprising, e.g., between about 4 and about 48 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, or about 48 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 4 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 5 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 6 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 7 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 8 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 9 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 10 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 11 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 12 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 13 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 14 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 15 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 16 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 17 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 18 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 19 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 20 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 21 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 22 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 23 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 24 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 25 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 26 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 27 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 28 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 29 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 30 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 31 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 32 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 33 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 34 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 35 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 36 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 37 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 38 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 39 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 40 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 41 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 42 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 43 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 44 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 45 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 46 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 47 ethylene oxide units. In some cases, the polymer moiety C is a discrete PEG comprising, e.g., about 48 ethylene oxide units.

In some cases, the polymer moiety C is dPEG® (Quanta Biodesign Ltd).

In some embodiments, the polymer moiety C comprises a cationic mucic acid-based polymer (cMAP). In some instances, cMAP comprises one or more subunit of at least one repeating subunit, and the subunit structure is represented as Formula (V):

Formula V



wherein m is independently at each occurrence 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10, preferably 4-6 or 5; and n is independently at each occurrence 1, 2, 3, 4, or 5. In some embodiments, m and n are, for example, about 10.

In some instances, cMAP is further conjugated to a PEG moiety, generating a cMAP-PEG copolymer, an mPEG-cMAP-PEGm triblock polymer, or a cMAP-PEG-cMAP triblock polymer. In some instances, the PEG moiety is in a

range of from about 500 Da to about 50,000 Da. In some instances, the PEG moiety is in a range of from about 500 Da to about 1000 Da, greater than 1000 Da to about 5000 Da, greater than 5000 Da to about 10,000 Da, greater than 10,000 to about 25,000 Da, greater than 25,000 Da to about 50,000 Da, or any combination of two or more of these ranges.

In some instances, the polymer moiety C is cMAP-PEG copolymer, an mPEG-cMAP-PEGm triblock polymer, or a cMAP-PEG-cMAP triblock polymer. In some cases, the polymer moiety C is cMAP-PEG copolymer. In other cases, the polymer moiety C is an mPEG-cMAP-PEGm triblock polymer. In additional cases, the polymer moiety C is a cMAP-PEG-cMAP triblock polymer.

In some embodiments, the polymer moiety C is conjugated to the polynucleic acid molecule, the binding moiety, and optionally to the endosomolytic moiety as illustrated supra.

Endosomolytic Moiety

In some embodiments, a molecule of Formula (I): A-X_1—B—X_2—C, further comprises an additional conjugating moiety. In some instances, the additional conjugating moiety is an endosomolytic moiety. In some cases, the endosomolytic moiety is a cellular compartmental release component, such as a compound capable of releasing from any of the cellular compartments known in the art, such as the endosome, lysosome, endoplasmic reticulum (ER), golgi apparatus, microtubule, peroxisome, or other vesicular bodies with the cell. In some cases, the endosomolytic moiety comprises an endosomolytic polypeptide, an endosomolytic polymer, an endosomolytic lipid, or an endosomolytic small molecule. In some cases, the endosomolytic moiety comprises an endosomolytic polypeptide. In other cases, the endosomolytic moiety comprises an endosomolytic polymer.

Endosomolytic Polypeptides

In some embodiments, a molecule of Formula (I): A-X_1—B—X_2—C, is further conjugated with an endosomolytic polypeptide. In some cases, the endosomolytic polypeptide is a pH-dependent membrane active peptide. In some cases, the endosomolytic polypeptide is an amphipathic polypeptide. In additional cases, the endosomolytic polypeptide is a peptidomimetic. In some instances, the endosomolytic polypeptide comprises INF, melittin, meucin, or their respective derivatives thereof. In some instances, the endosomolytic polypeptide comprises INF or its derivatives thereof. In other cases, the endosomolytic polypeptide comprises melittin or its derivatives thereof. In additional cases, the endosomolytic polypeptide comprises meucin or its derivatives thereof.

In some instances, INF7 is a 24 residue polypeptide those sequence comprises CGIFGEIEELIEEGLENLIDWGNA (SEQ ID NO: 1), or GLFEAIEGFIENGWEGMIDGWYGC (SEQ ID NO: 2). In some instances, INF7 or its derivatives comprise a sequence of:

(SEQ ID NO: 3)
GLFEAIEGFIENGWEGMIWDYGSGSCG,

(SEQ ID NO: 4)
GLFEAIEGFIENGWEGMIDG WYG-(PEG)6-NH2,
or

(SEQ ID NO: 5)
GLFEAIEGFIENGWEGMIWDYG-SGSC-K(GalNAc)2.

In some cases, melittin is a 26 residue polypeptide those sequence comprises CLIGAILKVLATGLPTLISWIKNKRKQ (SEQ ID NO: 6), or GIGAVLKVLTTGLPALISWIKRKRQQ (SEQ ID NO: 7). In some instances, melittin comprises a polypeptide sequence as described in U.S. Pat. No. 8,501,930.

In some instances, meucin is an antimicrobial peptide (AMP) derived from the venom gland of the scorpion *Mesobuthus eupeus*. In some instances, meucin comprises of meucin-13 those sequence comprises IFGAIAGLLKNIF-NH_2 (SEQ ID NO: 8) and meucin-18 those sequence comprises FFGHLFKLATKIIPSLFQ (SEQ ID NO: 9).

In some instances, the endosomolytic polypeptide comprises a polypeptide in which its sequence is at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% sequence identity to INF7 or its derivatives thereof, melittin or its derivatives thereof, or meucin or its derivatives thereof. In some instances, the endosomolytic moiety comprises INF7 or its derivatives thereof, melittin or its derivatives thereof, or meucin or its derivatives thereof.

In some instances, the endosomolytic moiety is INF7 or its derivatives thereof. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NOs: 1-5. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 1. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 2-5. In some cases, the endosomolytic moiety comprises SEQ ID NO: 1. In some cases, the endosomolytic moiety comprises SEQ ID NO: 2-5. In some cases, the endosomolytic moiety consists of SEQ ID NO: 1. In some cases, the endosomolytic moiety consists of SEQ ID NO: 2-5.

In some instances, the endosomolytic moiety is melittin or its derivatives thereof. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NOs: 6 or 7. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 6. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 7. In some cases, the endosomolytic moiety comprises SEQ ID NO: 6. In some cases, the endosomolytic moiety comprises SEQ ID NO: 7. In some cases, the endosomolytic moiety consists of SEQ ID NO: 6. In some cases, the endosomolytic moiety consists of SEQ ID NO: 7.

In some instances, the endosomolytic moiety is meucin or its derivatives thereof. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NOs: 8 or 9. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 8. In some cases, the endosomolytic moiety comprises a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to SEQ ID NO: 9. In some cases, the endosomolytic moiety comprises SEQ ID NO: 8. In some cases, the endosomolytic moiety comprises SEQ ID NO: 9. In some cases, the endosomolytic moiety consists of SEQ ID NO: 8. In some cases, the endosomolytic moiety consists of SEQ ID NO: 9.

In some instances, the endosomolytic moiety comprises a sequence as illustrated in Table 1.

NAME	ORIGIN	AMINO ACID SEQUENCE	SEQ ID NO:	TYPE
Pep-1	NLS from Simian Virus 40 large antigen and Reverse transcriptase of HIV	KETWWETWWTEWSQPKKKRKV	10	Primary amphipathic
pVEC	VE-cadherin	LLIILRRRRIRKQAHAHSK	11	Primary amphipathic
VT5	Synthetic peptide	DPKGDPKGVTVTVTVTVTGKGDP KPD	12	β-sheet amphipathic
C105Y	1-antitrypsin	CSIPPEVKFNKPFVYLI	13	—
Transportan	Galanin and mastoparan	GWTLNSAGYLLGKINLKALAALA KKIL	14	Primary amphipathic
TP10	Galanin and mastoparan	AGYLLGKINLKALAALAKKIL	15	Primary amphipathic
MPG	A hydrofobic domain from the fusion sequence of HIV gp41 and NLS of SV40 T antigen	GALFLGFLGAAGSTMGA	16	β-sheet amphipathic
gH625	Glycoprotein gH of HSV type I	HGLASTLTRWAHYNALIRAF	17	Secondary amphipathic α-helical
CADY	PPTG1 peptide	GLWRALWRLLRSLWRLLWRA	18	Secondary amphipathic α-helical
GALA	Synthetic peptide	WEAALAEALAEALAEHLAEALAE ALEALAA	19	Secondary amphipathic α-helical
INF	Influenza HA2 fusion peptide	GLFEAIEGFIENGWEGMIDGWYGC	20	Secondary amphipathic α-helical/ pH-dependent membrane active peptide
HA2E5-TAT	Influenza HA2 subunit of influenza virus X31 strain fusion peptide	GLFGAIAGFIENGWEGMIDGWYG	21	Secondary amphipathic α-helical/ PH-dependent membrane active peptide
HA2-penetratin	Influenza HA2 subunit of influenza virus X31 strain fusion peptide	GLFGAIAGFIENGWEGMIDGRQIKI WFQNRRMKW KK-amide	22	pH-dependent membrane active peptide
HA-K4	Influenza HA2 subunit of influenza virus X31 strain fusion peptide	GLFGAIAGFIENGWEGMIDG-SSKKKK	23	pH-dependent membrane active peptide
HA2E4	Influenza HA2 subunit of influenza virus X31 strain fusion peptide	GLFEAIAGFIENGWEGMIDGGGYC	24	pH-dependent membrane active peptide

-continued

NAME	ORIGIN	AMINO ACID SEQUENCE	SEQ ID NO:	TYPE
H5WYG	HA2 analogue	GLFHAIAHFIHGGWH GLIHGWYG	25	pH-dependent membrane active peptide
GALA-INF3-(PEG)6-NH	INF3 fusion peptide	GLFEAIEGFIENGWEGLAEALAEAL EALAA-(PEG)6-NH2	26	pH-dependent membrane active peptide
CM18-TAT11	Cecropin-A-Melittin$_{2-12}$ (CM_{18}) fusion peptide	KWKLFKKIGAVLKVLTTG-YGRKKRRQRRR	27	pH-dependent membrane active peptide

In some cases, the endosomolytic moiety comprises a Bak BH3 polypeptide which induces apoptosis through antagonization of suppressor targets such as Bcl-2 and/or Bcl-x_L. In some instances, the endosomolytic moiety comprises a Bak BH3 polypeptide described in Albarran, et al., "Efficient intracellular delivery of a pro-apoptotic peptide with a pH-responsive carrier," *Reactive & Functional Polymers* 71: 261-265 (2011).

In some instances, the endosomolytic moiety comprises a polypeptide (e.g., a cell-penetrating polypeptide) as described in PCT Publication Nos. WO2013/166155 or WO2015/069587.

Endosomolytic Lipids

In some embodiments, the endosomolytic moiety is a lipid (e.g., a fusogenic lipid). In some embodiments, a molecule of Formula (I): A-X_1—B—X_2—C, is further conjugated with an endosomolytic lipid (e.g., fusogenic lipid). Exemplary fusogenic lipids include 1,2-dileoyl-sn-3-phosphoethanolamine (DOPE), phosphatidylethanolamine (POPE), palmitoyloleoylphosphatidylcholine (POPC), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-ol (Di-Lin), N-methyl(2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-1,3-dioxolan-4-yl)methanamine (DLin-k-DMA) and N-methyl-2-(2,2-di((9Z,12Z)-octadeca-9,12-dienyl)-1,3-dioxolan-4-yl)ethanamine (XTC).

In some instances, an endosomolytic moiety is a lipid (e.g., a fusogenic lipid) described in PCT Publication No. WO09/126,933.

Endosomolytic Small Molecules

In some embodiments, the endosomolytic moiety is a small molecule. In some embodiments, a molecule of Formula (I): A-X_1—B—X_2—C, is further conjugated with an endosomolytic small molecule. Exemplary small molecules suitable as endosomolytic moieties include, but are not limited to, quinine, chloroquine, hydroxychloroquines, amodiaquins (carnoquines), amopyroquines, primaquines, mefloquines, nivaquines, halofantrines, quinone imines, or a combination thereof. In some instances, quinoline endosomolytic moieties include, but are not limited to, 7-chloro-4-(4-diethylamino-1-methylbutyl-amino)quinoline (chloroquine); 7-chloro-4-(4-ethyl-(2-hydroxyethyl)-amino-1-methylbutyl-amino)quinoline (hydroxychloroquine); 7-fluoro-4-(4-diethylamino-1-methylbutyl-amino)quinoline; 4-(4-diethylamino-1-methylbutylamino) quinoline; 7-hydroxy-4-(4-diethyl-amino-1-methylbutylamino)quinoline; 7-chloro-4-(4-diethylamino-1-butylamino)quinoline (desmethylchloroquine); 7-fluoro-4-(4-diethylamino-1-butylamino)quinoline); 4-(4-diethyl-amino-1-butylamino) quinoline; 7-hydroxy-4-(4-diethylamino-1-butylamino)quinoline; 7-chloro-4-(1-carboxy-4-diethylamino-1-butylamino)quinoline; 7-fluoro-4-(1-carboxy-4-diethyl-amino-1-butylamino)quinoline; 4-(1-carboxy-4-diethylamino-1-butylamino) quinoline; 7-hydroxy-4-(1-carboxy-4-diethylamino-1-butylamino)quinoline; 7-chloro-4-(1-carboxy-4-diethylamino-1-methylbutylamino) quinoline; 7-fluoro-4-(1-carboxy-4-diethyl-amino-1-methylbutylamino)quinoline; 4-(1-carboxy-4-diethylamino-1-methylbutylamino)quinoline; 7-hydroxy-4-(1-carboxy-4-diethylamino-1-methylbutylamino)quinoline; 7-fluoro-4-(4-ethyl-(2-hydroxyethyl)-amino-1-methylbutylamino) quinoline; 4-(4-ethyl-(2-hydroxy-ethyl)-amino-1-methylbutylamino-)quinoline; 7-hydroxy-4-(4-ethyl-(2-hydroxyethyl)-amino-1-methylbutylamino)quinoline; hydroxychloroquine phosphate; 7-chloro-4-(4-ethyl-(2-hydroxyethyl-1)-amino-1-butylamino)quinoline (desmethylhydroxychloroquine); 7-fluoro-4-(4-ethyl-(2-hydroxyethyl)-amino-1-butylamino)quinoline; 4-(4-ethyl-(2-hydroxyethyl)-amino-1-butylamino)quinoline; 7-hydroxy-4-(4-ethyl-(2-hydroxyethyl)-amino-1-butylamino) quinoline; 7-chloro-4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-butylamino)quinoline; 7-fluoro-4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-butylamino)quinoline; 4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-butylamino)quinoline; 7-hydroxy-4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-butylamino)quinoline; 7-chloro-4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-methylbutylamino)quinoline; 7-fluoro-4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-methylbutylamino) quinoline; 4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-methylbutylamino)quinoline; 7-hydroxy-4-(1-carboxy-4-ethyl-(2-hydroxyethyl)-amino-1-methylbutylamino) quinoline; 8-[(4-aminopentyl)amino-6-methoxydihydrochloride quinoline; 1-acetyl-1,2,3,4-tetrahydroquinoline; 8-[(4-aminopentyl)amino]-6-methoxyquinoline dihydrochloride; 1-butyryl-1,2,3,4-tetrahydroquinoline; 3-chloro-4-(4-hydroxy-alpha,alpha'-bis(2-methyl-1-pyrrolidinyl)-2,5-xylidinoquinoline, 4-[(4-diethyl-amino)-1-methylbutyl-amino]-6-methoxyquinoline; 3-fluoro-4-(4-hydroxy-alpha,alpha'-bis(2-methyl-1-pyrrolidinyl)-2,5-xylidinoquinoline, 4-[(4-diethylamino)-1-methylbutyl-amino]-6-methoxyquinoline; 4-(4-hydroxy-alpha, alpha'-bis(2-methyl-1-pyrrolidinyl)-2,5-xylidinoquinoline;

4-[(4-diethylamino)-1-methylbutyl-amino]-6-methoxyquinoline; 3,4-dihydro-1-(2H)-quinolinecarboxyaldehyde; 1,1'-pentamethylene diquinoleinium diiodide; 8-quinolinol sulfate and amino, aldehyde, carboxylic, hydroxyl, halogen, keto, sulfhydryl and vinyl derivatives or analogs thereof. In some instances, an endosomolytic moiety is a small molecule described in Naisbitt et al (1997, J Pharmacol Exp Therapy 280:884-893) and in U.S. Pat. No. 5,736,557.

Linkers

In some embodiments, a linker described herein is a cleavable linker or a non-cleavable linker. In some instances, the linker is a cleavable linker. In other instances, the linker is a non-cleavable linker.

In some cases, the linker is a non-polymeric linker. A non-polymeric linker refers to a linker that does not contain a repeating unit of monomers generated by a polymerization process. Exemplary non-polymeric linkers include, but are not limited to, C_1-C_6 alkyl group (e.g., a C_5, C_4, C_3, C_2, or C_1 alkyl group), homobifunctional cross linkers, heterobifunctional cross linkers, peptide linkers, traceless linkers, self-immolative linkers, maleimide-based linkers, or combinations thereof. In some cases, the non-polymeric linker comprises a C_1-C_6 alkyl group (e.g., a C_5, C_4, C_3, C_2, or C, alkyl group), a homobifunctional cross linker, a heterobifunctional cross linker, a peptide linker, a traceless linker, a self-immolative linker, a maleimide-based linker, or a combination thereof. In additional cases, the non-polymeric linker does not comprise more than two of the same type of linkers, e.g., more than two homobifunctional cross linkers, or more than two peptide linkers. In further cases, the non-polymeric linker optionally comprises one or more reactive functional groups.

In some instances, the non-polymeric linker does not encompass a polymer that is described above. In some instances, the non-polymeric linker does not encompass a polymer encompassed by the polymer moiety C. In some cases, the non-polymeric linker does not encompass a polyalkylene oxide (e.g., PEG). In some cases, the non-polymeric linker does not encompass a PEG.

In some instances, the linker comprises a homobifunctional linker. Exemplary homobifunctional linkers include, but are not limited to, Lomant's reagent dithiobis (succinimidylpropionate) DSP, 3'3'-dithiobis(sulfosuccinimidyl proprionate (DTSSP), disuccinimidyl suberate (DSS), bis (sulfosuccinimidyl)suberate (BS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo DST), ethylene glycobis(succinimidylsuccinate) (EGS), disuccinimidyl glutarate (DSG), N,N'-disuccinimidyl carbonate (DSC), dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), dimethyl suberimidate (DMS), dimethyl-3,3'-dithiobispropionimidate (DTBP), 1,4-di-3'-(2'-pyridyldithio)propionamido)butane (DPDPB), bismaleimidohexane (BMH), aryl halide-containing compound (DFDNB), such as e.g. 1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene, 4,4'-difluoro-3,3'-dinitrophenylsulfone (DFDNPS), bis-[β-(4-azidosalicylamido)ethyl]disulfide (BASED), formaldehyde, glutaraldehyde, 1,4-butanediol diglycidyl ether, adipic acid dihydrazide, carbohydrazide, o-toluidine, 3,3'-dimethylbenzidine, benzidine, α,α'-p-diaminodiphenyl, diiodo-p-xylene sulfonic acid, N,N'-ethylene-bis(iodoacetamide), or N,N'-hexamethylene-bis(iodoacetamide).

In some embodiments, the linker comprises a heterobifunctional linker. Exemplary heterobifunctional linker include, but are not limited to, amine-reactive and sulfhydryl cross-linkers such as N-succinimidyl 3-(2-pyridyldithio) propionate (sPDP), long-chain N-succinimidyl 3-(2-pyridyldithio)propionate (LC-sPDP), water-soluble-long-chain N-succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-sPDP), succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (sMPT), sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-sMPT), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBs), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBs), N-succinimidyl(4-iodoacteyl)aminobenzoate (sIAB), sulfosuccinimidyl(4-iodoacteyl)aminobenzoate (sulfo-sIAB), succinimidyl-4-(p-maleimidophenyl)butyrate (sMPB), sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate (sulfo-sMPB), N-(γ-maleimidobutyryloxy)succinimide ester (GMBs), N-(γ-maleimidobutyryloxy)sulfosuccinimide ester (sulfo-GMBs), succinimidyl 6-((iodoacetyl)amino)hexanoate (sIAX), succinimidyl 6-[6-(((iodoacetyl)amino) hexanoyl)amino]hexanoate (sIAXX), succinimidyl 4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate (sIAC), succinimidyl 6-((((4-iodoacetyl)amino)methyl)cyclohexane-1-carbonyl)amino) hexanoate (sIACX), p-nitrophenyl iodoacetate (NPIA), carbonyl-reactive and sulfhydryl-reactive cross-linkers such as 4-(4-N-maleimidophenyl) butyric acid hydrazide (MPBH), 4-(N-maleimidomethyl) cyclohexane-1-carboxyl-hydrazide-8 (M_2C2H), 3-(2-pyridyldithio)propionyl hydrazide (PDPH), amine-reactive and photoreactive cross-linkers such as N-hydroxysuccinimidyl-4-azidosalicylic acid (NHs-AsA), N-hydroxysulfosuccinimidyl-4-azidosalicylic acid (sulfo-NHs-AsA), sulfosuccinimidyl-(4-azidosalicylamido)hexanoate (sulfo-NHs-LC-AsA), sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (sAsD), N-hydroxysuccinimidyl-4-azidobenzoate (HsAB), N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HsAB), N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sANPAH), sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-sANPAH), N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOs), sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate (sAND), N-succinimidyl-4(4-azidophenyl)1,3'-dithiopropionate (sADP), N-sulfosuccinimidyl(4-azidophenyl)-1,3'-dithiopropionate (sulfo-sADP), sulfosuccinimidyl 4-(ρ-azidophenyl)butyrate (sulfo-sAPB), sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-1,3'-dithiopropionate (sAED), sulfosuccinimidyl 7-azido-4-methylcoumain-3-acetate (sulfo-sAMCA), ρ-nitrophenyl diazopyruvate (ρNPDP), ρ-nitrophenyl-2-diazo-3,3,3-trifluoropropionate (PNP-DTP), sulfhydryl-reactive and photoreactive cross-linkers such as1-(ρ-Azidosalicylamido)-4-(iodoacetamido) butane (AsIB), N-[4-(ρ-azidosalicylamido)butyl]-3'-(2'-pyridyldithio)propionamide (APDP), benzophenone-4-iodoacetamide, benzophenone-4-maleimide carbonyl-reactive and photoreactive cross-linkers such as ρ-azidobenzoyl hydrazide (ABH), carboxylate-reactive and photoreactive cross-linkers such as 4-(ρ-azidosalicylamido) butylamine (AsBA), and arginine-reactive and photoreactive cross-linkers such as ρ-azidophenyl glyoxal (APG).

In some instances, the linker comprises a reactive functional group. In some cases, the reactive functional group comprises a nucleophilic group that is reactive to an electrophilic group present on a binding moiety. Exemplary electrophilic groups include carbonyl groups-such as aldehyde, ketone, carboxylic acid, ester, amide, enone, acyl halide or acid anhydride. In some embodiments, the reactive functional group is aldehyde. Exemplary nucleophilic groups include hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide.

In some embodiments, the linker comprises a maleimide group. In some instances, the maleimide group is also referred to as a maleimide spacer. In some instances, the maleimide group further encompasses a caproic acid, forming maleimidocaproyl (mc). In some cases, the linker comprises maleimidocaproyl (mc). In some cases, the linker is maleimidocaproyl (mc). In other instances, the maleimide group comprises a maleimidomethyl group, such as succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC) or sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC) described above.

In some embodiments, the maleimide group is a self-stabilizing maleimide. In some instances, the self-stabilizing maleimide utilizes diaminopropionic acid (DPR) to incorporate a basic amino group adjacent to the maleimide to provide intramolecular catalysis of tiosuccinimide ring hydrolysis, thereby eliminating maleimide from undergoing an elimination reaction through a retro-Michael reaction. In some instances, the self-stabilizing maleimide is a maleimide group described in Lyon, et al., "Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates," *Nat. Biotechnol.* 32(10): 1059-1062 (2014). In some instances, the linker comprises a self-stabilizing maleimide. In some instances, the linker is a self-stabilizing maleimide.

In some embodiments, the linker comprises a peptide moiety. In some instances, the peptide moiety comprises at least 2, 3, 4, 5, or 6 more amino acid residues. In some instances, the peptide moiety comprises at most 2, 3, 4, 5, 6, 7, or 8 amino acid residues. In some instances, the peptide moiety comprises about 2, about 3, about 4, about 5, or about 6 amino acid residues. In some instances, the peptide moiety is a cleavable peptide moiety (e.g., either enzymatically or chemically). In some instances, the peptide moiety is a non-cleavable peptide moiety. In some instances, the peptide moiety comprises Val-Cit (valine-citrulline), Gly-Gly-Phe-Gly (SEQ ID NO: 14223), Phe-Lys, Val-Lys, Gly-Phe-Lys, Phe-Phe-Lys, Ala-Lys, Val-Arg, Phe-Cit, Phe-Arg, Leu-Cit, Ile-Cit, Trp-Cit, Phe-Ala, Ala-Leu-Ala-Leu (SEQ ID NO: 14224), or Gly-Phe-Leu-Gly (SEQ ID NO: 14225). In some instances, the linker comprises a peptide moiety such as: Val-Cit (valine-citrulline), Gly-Gly-Phe-Gly (SEQ ID NO: 14223), Phe-Lys, Val-Lys, Gly-Phe-Lys, Phe-Phe-Lys, Ala-Lys, Val-Arg, Phe-Cit, Phe-Arg, Leu-Cit, Ile-Cit, Trp-Cit, Phe-Ala, Ala-Leu-Ala-Leu (SEQ ID NO: 14224), or Gly-Phe-Leu-Gly (SEQ ID NO: 14225). In some cases, the linker comprises Val-Cit. In some cases, the linker is Val-Cit.

In some embodiments, the linker comprises a benzoic acid group, or its derivatives thereof. In some instances, the benzoic acid group or its derivatives thereof comprise paraaminobenzoic acid (PABA). In some instances, the benzoic acid group or its derivatives thereof comprise gamma-aminobutyric acid (GABA).

In some embodiments, the linker comprises one or more of a maleimide group, a peptide moiety, and/or a benzoic acid group, in any combination. In some embodiments, the linker comprises a combination of a maleimide group, a peptide moiety, and/or a benzoic acid group. In some instances, the maleimide group is maleimidocaproyl (me). In some instances, the peptide group is val-cit. In some instances, the benzoic acid group is PABA. In some instances, the linker comprises a mc-val-cit group. In some cases, the linker comprises a val-cit-PABA group. In additional cases, the linker comprises a mc-val-cit-PABA group.

In some embodiments, the linker is a self-immolative linker or a self-elimination linker. In some cases, the linker is a self-immolative linker. In other cases, the linker is a self-elimination linker (e.g., a cyclization self-elimination linker). In some instances, the linker comprises a linker described in U.S. Pat. No. 9,089,614 or PCT Publication No. WO2015038426.

In some embodiments, the linker is a dendritic type linker. In some instances, the dendritic type linker comprises a branching, multifunctional linker moiety. In some instances, the dendritic type linker is used to increase the molar ratio of polynucleotide B to the binding moiety A. In some instances, the dendritic type linker comprises PAMAM dendrimers.

In some embodiments, the linker is a traceless linker or a linker in which after cleavage does not leave behind a linker moiety (e.g., an atom or a linker group) to a binding moiety A, a polynucleotide B, a polymer C, or an endosomolytic moiety D. Exemplary traceless linkers include, but are not limited to, germanium linkers, silicium linkers, sulfur linkers, selenium linkers, nitrogen linkers, phosphorus linkers, boron linkers, chromium linkers, or phenylhydrazide linker. In some cases, the linker is a traceless aryl-triazene linker as described in Hejesen, et al., "A traceless aryl-triazene linker for DNA-directed chemistry," *Org Biomol Chem* 11(15): 2493-2497 (2013). In some instances, the linker is a traceless linker described in Blaney, et al., "Traceless solid-phase organic synthesis," *Chem. Rev.* 102: 2607-2024 (2002). In some instances, a linker is a traceless linker as described in U.S. Pat. No. 6,821,783.

In some instances, the linker is a linker described in U.S. Pat. Nos. 6,884,869; 7,498,298; 8,288,352; 8,609,105; or 8,697,688; U.S. Patent Publication Nos. 2014/0127239; 2013/028919; 2014/286970; 2013/0309256; 2015/037360; or 2014/0294851; or PCT Publication Nos. WO2015057699; WO2014080251; WO2014197854; WO2014145090; or WO2014177042.

In some embodiments, X_1 and X_2 are each independently a bond or a non-polymeric linker. In some instances, X_1 and X_2 are each independently a bond. In some cases, X_1 and X_2 are each independently a non-polymeric linker.

In some instances, X_1 is a bond or a non-polymeric linker. In some instances, X_1 is a bond. In some instances, X_1 is a non-polymeric linker. In some instances, the linker is a C_1-C_6 alkyl group. In some cases, X_1 is a C_1-C_6 alkyl group, such as for example, a C_5, C_4, C_3, C_2, or C_1 alkyl group. In some cases, the C_1-C_6 alkyl group is an unsubstituted C_1-C_6 alkyl group. As used in the context of a linker, and in particular in the context of X_1, alkyl means a saturated straight or branched hydrocarbon radical containing up to six carbon atoms. In some instances, X_i includes a homobifunctional linker or a heterobifunctional linker described supra. In some cases, X_1 includes a heterobifunctional linker. In some cases, X_1 includes sMCC. In other instances, X_1 includes a heterobifunctional linker optionally conjugated to a C_1-C_6 alkyl group. In other instances, X_1 includes sMCC optionally conjugated to a C_1-C_6 alkyl group. In additional instances, X_1 does not include a homobifunctional linker or a heterobifunctional linker described supra.

In some instances, X_2 is a bond or a linker. In some instances, X_2 is a bond. In other cases, X_2 is a linker. In additional cases, X_2 is a non-polymeric linker. In some embodiments, X_2 is a C_1-C_6 alkyl group. In some instances, X_2 is a homobifunctional linker or a heterobifunctional linker described supra. In some instances, X_2 is a homobifunctional linker described supra. In some instances, X_2 is a heterobifunctional linker described supra. In some instances, X_2 comprises a maleimide group, such as maleimidocaproyl (mc) or a self-stabilizing maleimide group described above. In some instances, X_2 comprises a peptide moiety, such as

Val-Cit. In some instances, X_2 comprises a benzoic acid group, such as PABA. In additional instances, X_2 comprises a combination of a maleimide group, a peptide moiety, and/or a benzoic acid group. In additional instances, X_2 comprises a mc group. In additional instances, X_2 comprises a mc-val-cit group. In additional instances, X_2 comprises a val-cit-PABA group. In additional instances, X_2 comprises a mc-val-cit-PABA group.

Methods of Use

Muscle atrophy refers to a loss of muscle mass and/or to a progressive weakening and degeneration of muscles. In some cases, the loss of muscle mass and/or the progressive weakening and degeneration of muscles occurs due to a high rate of protein degradation, a low rate of protein synthesis, or a combination of both. In some cases, a high rate of muscle protein degradation is due to muscle protein catabolism (i.e., the breakdown of muscle protein in order to use amino acids as substrates for gluconeogenesis).

In one embodiment, muscle atrophy refers to a significant loss in muscle strength. By significant loss in muscle strength is meant a reduction of strength in diseased, injured, or unused muscle tissue in a subject relative to the same muscle tissue in a control subject. In an embodiment, a significant loss in muscle strength is a reduction in strength of at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, or more relative to the same muscle tissue in a control subject. In another embodiment, by significant loss in muscle strength is meant a reduction of strength in unused muscle tissue relative to the muscle strength of the same muscle tissue in the same subject prior to a period of nonuse. In an embodiment, a significant loss in muscle strength is a reduction of at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, or more relative to the muscle strength of the same muscle tissue in the same subject prior to a period of nonuse.

In another embodiment, muscle atrophy refers to a significant loss in muscle mass. By significant loss in muscle mass is meant a reduction of muscle volume in diseased, injured, or unused muscle tissue in a subject relative to the same muscle tissue in a control subject. In an embodiment, a significant loss of muscle volume is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, or more relative to the same muscle tissue in a control subject. In another embodiment, by significant loss in muscle mass is meant a reduction of muscle volume in unused muscle tissue relative to the muscle volume of the same muscle tissue in the same subject prior to a period of nonuse. In an embodiment, a significant loss in muscle tissue is at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, or more relative to the muscle volume of the same muscle tissue in the same subject prior to a period of nonuse. Muscle volume is optionally measured by evaluating the cross-section area of a muscle such as by Magnetic Resonance Imaging (e.g., by a muscle volume/cross-section area (CSA) MRI method).

Myotonic dystrophy is a multisystemic neuromuscular disease comprising two main types: myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2). DM1 is caused by a dominantly inherited "CTG" repeat expansion in the gene DM protein kinase (DMPK), which when transcribed into mRNA, forms hairpins that bind with high affinity to the Muscleblind-like (MBNL) family of proteins. MBNL proteins are involved in post-transcriptional splicing and polyadenylatin site regulation and loss of the MBNL protein functions lead to downstream accumulation of nuclear foci and increase in mis-splicing events and subsequently to myotonia and other clinical symptoms.

In some embodiments, described herein is a method of treating muscle atrophy or myotonic dystrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein or a polynucleic acid molecule conjugate described herein. In some instances, the muscle atrophy is associated and/or induced by cachexia (e.g., cancer cachexia), denervation, myopathy, motor neuron diseases, diabetes, chronic obstructive pulmonary disease, liver disease, congestive heart failure, chronic renal failure, chronic infection, sepsis, fasting, sarcopenia, glucocorticoid-induced atrophy, disuse, or space flight. In some cases, myotonic dystrophy is DM1.

Cachexia

Cachexia is an acquired, accelerated loss of muscle caused by an underlying disease. In some instances, cachexia refers to a loss of body mass that cannot be reversed nutritionally, and is generally associated with an underlying disease, such as cancer, COPD, AIDS, heart failure, and the like. When cachexia is seen in a patient with end-stage cancer, it is called "cancer cachexia". Cancer cachexia affects the majority of patients with advanced cancer and is associated with a reduction in treatment tolerance, response to therapy, quality of life and duration of survival. It some instances, cancer cachexia is defined as a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass, with or without loss of fat mass, which cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment. In some cases, skeletal muscle loss appears to be the most significant event in cancer cachexia. In addition, the classification of cancer cachexia suggests that the diagnostic criteria takes into account not only that weight loss is a signal event of the cachectic process but that the initial reserve of the patient should also be considered, such as low BMI or low level of muscularity.

In some embodiments, described herein is a method of treating cachexia-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein or a polynucleic acid molecule conjugate described herein. In additional embodiments, described herein is a method of treating cancer cachexia-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein or a polynucleic acid molecule conjugate described herein.

Denervation

Denervation is an injury to the peripheral motoneurons with a partial or complete interruption of the nerve fibers between an organ and the central nervous system, resulting in an interruption of nerve conduction and motoneuron firing which, in turn, prevents the contractability of skeletal muscles. This loss of nerve function is either localized or generalized due to the loss of an entire motor neuron unit. The resulting inability of skeletal muscles to contract leads to muscle atrophy. In some instances, denervation is associated with or as a result of degenerative, metabolic, or inflammatory neuropathy (e.g., Guillain-Barre syndrome, peripheral neuropathy, or exposure to environmental toxins or drugs). In additional instances, denervation is associated with a physical injury, e.g., a surgical procedure.

In some embodiments, described herein is a method of treating muscle atrophy associated with or induced by

denervation in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by denervation in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Myopathy

Myopathy is an umbrella term that describes a disease of the muscle. In some instances, myopathy includes myotonia; congenital myopathy such as nemaline myopathy, multi/minicore myopathy and myotubular (centronuclear) myopathy; mitochondrial myopathy; familial periodic paralysis; inflammatory myopathy; metabolic myopathy, for example, caused by a glycogen or lipid storage disease; dermatomyositis; polymyositis; inclusion body myositis; myositis ossificans; rhabdomyolysis; and myoglobinurias. In some instances, myopathy is caused by a muscular dystrophy syndrome, such as Duchenne, Becker, myotonic, fascioscapulohumeral, Emery-Dreifuss, oculopharyngeal, scapulohumeral, limb girdle, Fukuyama, a congenital muscular dystrophy, or hereditary distal myopathy. In some instances, myopathy is caused by myotonic dystrophy (e.g., myotonic dystrophy type 1 or DM1). In some instances, myopathy is caused by DM1.

In some embodiments, described herein is a method of treating muscle atrophy associated with or induced by myopathy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by myopathy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Motor Neuron Diseases

Motor neuron disease (MND) encompasses a neurological disorder that affects motor neurons, cells that control voluntary muscles of the body. Exemplary motor neuron diseases include, but are not limited to, adult motor neuron diseases, infantile spinal muscular atrophy, amyotrophic lateral sclerosis, juvenile spinal muscular atrophy, autoimmune motor neuropathy with multifocal conductor block, paralysis due to stroke or spinal cord injury, or skeletal immobilization due to trauma.

In some embodiments, described herein is a method of treating muscle atrophy associated with or induced by a motor neuron disease in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by a motor neuron disease in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Diabetes

Diabetes (diabetes mellitus, DM) comprises type 1 diabetes, type 2 diabetes, type 3 diabetes, type 4 diabetes, double diabetes, latent autoimmune diabetes (LAD), gestational diabetes, neonatal diabetes mellitus (NDM), maturity onset diabetes of the young (MODY), Wolfram syndrome, Alström syndrome, prediabetes, or diabetes insipidus. Type 2 diabetes, also called non-insulin dependent diabetes, is the most common type of diabetes accounting for 95% of all diabetes cases. In some instances, type 2 diabetes is caused by a combination of factors, including insulin resistance due to pancreatic beta cell dysfunction, which in turn leads to high blood glucose levels. In some cases, increased glucagon levels stimulate the liver to produce an abnormal amount of unneeded glucose, which contributes to high blood glucose levels.

Type 1 diabetes, also called insulin-dependent diabetes, comprises about 5% to 10% of all diabetes cases. Type 1 diabetes is an autoimmune disease where T cells attack and destroy insulin-producing beta cells in the pancreas. In some embodiments, Type 1 diabetes is caused by genetic and environmental factors.

Type 4 diabetes is a recently discovered type of diabetes affecting about 20% of diabetic patients age 65 and over. In some embodiments, type 4 diabetes is characterized by age-associated insulin resistance.

In some embodiments, type 3 diabetes is used as a term for Alzheimer's disease resulting in insulin resistance in the brain.

In some embodiments, described herein is a method of treating diabetes-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein or a polynucleic acid molecule conjugate described herein. In additional embodiments, described herein is a method of treating cancer diabetes-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein or a polynucleic acid molecule conjugate described herein.

Chronic Obstructive Pulmonary Disease

Chronic obstructive pulmonary disease (COPD) is a type of obstructive lung disease characterized by long-term breathing problems and poor airflow. Chronic bronchitis and emphysema are two different types of COPD. In some instances, described herein is a method of treating muscle atrophy associated with or induced by COPD (e.g., chronic bronchitis or emphysema) in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by COPD (e.g., chronic bronchitis or emphysema) in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Liver Diseases

Liver disease (or hepatic disease) comprises fibrosis, cirrhosis, hepatitis, alcoholic liver disease, hepatic steatosis, a hereditary disease, or primary liver cancer. In some instances, described herein is a method of treating muscle atrophy associated with or induced by a liver disease in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by a liver disease in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Congestive Heart Failure

Congestive heart failure is a condition in which the heart is unable to pump enough blood and oxygen to the body's tissues. In some instances, described herein is a method of treating muscle atrophy associated with or induced by congestive heart failure in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In

other embodiments, described herein is a method of treating muscle atrophy associated with or induced by congestive heart failure in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Chronic Renal Failure

Chronic renal failure or chronic kidney disease is a condition characterized by a gradual loss of kidney function over time. In some instances, described herein is a method of treating muscle atrophy associated with or induced by a chronic renal failure in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by a chronic renal failure in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Chronic Infections

In some embodiments, chronic infection such as AIDS further leads to muscle atrophy. In some instances, described herein is a method of treating muscle atrophy associated with or induced by a chronic infection (e.g., AIDS) in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by a chronic infection (e.g., AIDS) in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Sepsis

Sepsis is an immune response to an infection leading to tissue damage, organ failure, and/or death. In some embodiments, described herein is a method of treating muscle atrophy associated with or induced by sepsis in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by sepsis in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Fasting

Fasting is a willing abstinence or reduction from some or all food, drinks, or both, for a period of time. In some embodiments, described herein is a method of treating muscle atrophy associated with or induced by fasting in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by fasting in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Sarcopenia

Sarcopenia is the continuous process of muscle atrophy in the course of regular aging that is characterized by a gradual loss of muscle mass and muscle strength over a span of months and years. A regular aging process means herein an aging process that is not influenced or accelerated by the presence of disorders and diseases which promote skeletomuscular neurodegeneration.

In some embodiments, described herein is a method of treating muscle atrophy associated with or induced by sarcopenia in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating muscle atrophy associated with or induced by sarcopenia in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Glucocorticoid-Associated Muscle Atrophy

In some embodiments, treatment with a glucocorticoid further results in muscle atrophy. Exemplary glucocorticoids include, but are not limited to, cortisol, dexamethasone, betamethasone, prednisone, methylprednisolone, and prednisolone.

In some embodiments, described herein is a method of treating glucocorticoid-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating glucocorticoid-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Disuse-Associated Muscle Atrophy

Disuse-associated muscle atrophy results when a limb is immobilized (e.g., due to a limb or joint fracture or an orthopedic surgery such as a hip or knee replacement surgery). As used herein, "immobilization" or "immobilized" refers to the partial or complete restriction of movement of limbs, muscles, bones, tendons, joints, or any other body parts for an extended period of time (e.g., for 2 days, 3 days, 4 days, 5 days, 6 days, a week, two weeks, or more). In some instances, a period of immobilization includes short periods or instances of unrestrained movement, such as to bathe, to replace an external device, or to adjust an external device. Limb immobilization is optionally carried out by any variety of external devices including, but are not limited to, braces, slings, casts, bandages, and splints (any of which is optionally composed of hard or soft material including but not limited to cloth, gauze, fiberglass, plastic, plaster, or metal), as well as any variety of internal devices including surgically implanted splints, plates, braces, and the like. In the context of limb immobilization, the restriction of movement involves a single joint or multiple joints (e.g., simple joints such as the shoulder joint or hip joint, compound joints such as the radiocarpal joint, and complex joints such as the knee joint, including but not limited to one or more of the following: articulations of the hand, shoulder joints, elbow joints, wrist joints, auxiliary articulations, sternoclavicular joints, vertebral articulations, temporomandibular joints, sacroiliac joints, hip joints, knee joints, and articulations of the foot), a single tendon or ligament or multiple tendons or ligaments (e.g., including but not limited to one or more of the following: the anterior cruciate ligament, the posterior cruciate ligament, rotator cuff tendons, medial collateral ligaments of the elbow and knee, flexor tendons of the hand, lateral ligaments of the ankle, and tendons and ligaments of the jaw or temporomandibular joint), a single bone or multiple bones (e.g., including but not limited to one or more of the Wowing: the skull, mandible, clavicle, ribs, radius, ulna, humorous, pelvis, sacrum, femur, patella, phalanges, carpals, metacarpals, tarsals, metatarsals, fibula, tibia, scapula, and vertebrae), a single muscle or multiple muscles (e.g., including but not limited to one or more of the following: latissimus dorsi, trapezius, deltoid, pectorals, biceps, triceps, external obliques, abdominals, gluteus maximus, hamstrings, quadriceps, gastrocnemius, and diaphragm); a single limb or multiple limbs one or more of the

arms and legs), or the entire skeletal muscle system or portions thereof (e.g., in the case of a full body cast or spica cast).

In some embodiments, described herein is a method of treating disuse-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule described herein. In other embodiments, described herein is a method of treating disuse-associated muscle atrophy in a subject, which comprises administering to the subject a therapeutically effective amount of a polynucleic acid molecule conjugate described herein.

Pharmaceutical Formulation

In some embodiments, the pharmaceutical formulations described herein are administered to a subject by multiple administration routes, including but not limited to, parenteral (e.g., intravenous, subcutaneous, intramuscular), oral, intranasal, buccal, rectal, or transdermal administration routes. In some instances, the pharmaceutical composition describe herein is formulated for parenteral (e.g., intravenous, subcutaneous, intramuscular, intra-arterial, intraperitoneal, intrathecal, intracerebral, intracerebroventricular, or intracranial) administration. In other instances, the pharmaceutical composition describe herein is formulated for oral administration. In still other instances, the pharmaceutical composition describe herein is formulated for intranasal administration.

In some embodiments, the pharmaceutical formulations include, but are not limited to, aqueous liquid dispersions, self-emulsifying dispersions, solid solutions, liposomal dispersions, aerosols, solid dosage forms, powders, immediate release formulations, controlled release formulations, fast melt formulations, tablets, capsules, pills, delayed release formulations, extended release formulations, pulsatile release formulations, multiparticulate formulations (e.g., nanoparticle formulations), and mixed immediate and controlled release formulations.

In some instances, the pharmaceutical formulation includes multiparticulate formulations. In some instances, the pharmaceutical formulation includes nanoparticle formulations. In some instances, nanoparticles comprise cMAP, cyclodextrin, or lipids. In some cases, nanoparticles comprise solid lipid nanoparticles, polymeric nanoparticles, self-emulsifying nanoparticles, liposomes, microemulsions, or micellar solutions. Additional exemplary nanoparticles include, but are not limited to, paramagnetic nanoparticles, superparamagnetic nanoparticles, metal nanoparticles, fullerene-like materials, inorganic nanotubes, dendrimers (such as with covalently attached metal chelates), nanofibers, nanohorns, nano-onions, nanorods, nanoropes and quantum dots. In some instances, a nanoparticle is a metal nanoparticle, e.g., a nanoparticle of scandium, titanium, vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, yttrium, zirconium, niobium, molybdenum, ruthenium, rhodium, palladium, silver, cadmium, hafnium, tantalum, tungsten, rhenium, osmium, iridium, platinum, gold, gadolinium, aluminum, gallium, indium, tin, thallium, lead, bismuth, magnesium, calcium, strontium, barium, lithium, sodium, potassium, boron, silicon, phosphorus, germanium, arsenic, antimony, and combinations, alloys or oxides thereof.

In some instances, a nanoparticle includes a core or a core and a shell, as in a core-shell nanoparticle.

In some instances, a nanoparticle is further coated with molecules for attachment of functional elements (e.g., with one or more of a polynucleic acid molecule or binding moiety described herein). In some instances, a coating comprises chondroitin sulfate, dextran sulfate, carboxymethyl dextran, alginic acid, pectin, carragheenan, fucoidan, agaropectin, porphyran, karaya gum, gellan gum, xanthan gum, hyaluronic acids, glucosamine, galactosamine, chitin (or chitosan), polyglutamic acid, polyaspartic acid, lysozyme, cytochrome C, ribonuclease, trypsinogen, chymotrypsinogen, a-chymotrypsin, polylysine, polyarginine, histone, protamine, ovalbumin or dextrin or cyclodextrin. In some instances, a nanoparticle comprises a graphene-coated nanoparticle.

In some cases, a nanoparticle has at least one dimension of less than about 500 nm, 400 nm, 300 nm, 200 nm, or 100 nm.

In some instances, the nanoparticle formulation comprises paramagnetic nanoparticles, superparamagnetic nanoparticles, metal nanoparticles, fullerene-like materials, inorganic nanotubes, dendrimers (such as with covalently attached metal chelates), nanofibers, nanohorns, nano-onions, nanorods, nanoropes or quantum dots. In some instances, a polynucleic acid molecule or a binding moiety described herein is conjugated either directly or indirectly to the nanoparticle. In some instances, at least 1, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more polynucleic acid molecules or binding moieties described herein are conjugated either directly or indirectly to a nanoparticle.

In some embodiments, the pharmaceutical formulation comprises a delivery vector, e.g., a recombinant vector, the delivery of the polynucleic acid molecule into cells. In some instances, the recombinant vector is DNA plasmid. In other instances, the recombinant vector is a viral vector. Exemplary viral vectors include vectors derived from adeno-associated virus, retrovirus, adenovirus, or alphavirus. In some instances, the recombinant vectors capable of expressing the polynucleic acid molecules provide stable expression in target cells. In additional instances, viral vectors are used that provide for transient expression of polynucleic acid molecules.

In some embodiments, the pharmaceutical formulation includes a carrier or carrier materials selected on the basis of compatibility with the composition disclosed herein, and the release profile properties of the desired dosage form. Exemplary carrier materials include, e.g., binders, suspending agents, disintegration agents, filling agents, surfactants, solubilizers, stabilizers, lubricants, wetting agents, diluents, and the like. Pharmaceutically compatible carrier materials include, but are not limited to, acacia, gelatin, colloidal silicon dioxide, calcium glycerophosphate, calcium lactate, maltodextrin, glycerine, magnesium silicate, polyvinylpyrrollidone (PVP), cholesterol, cholesterol esters, sodium caseinate, soy lecithin, taurocholic acid, phosphotidylcholine, sodium chloride, tricalcium phosphate, dipotassium phosphate, cellulose and cellulose conjugates, sugars sodium stearoyl lactylate, carrageenan, monoglyceride, diglyceride, pregelatinized starch, and the like. See, e.g., *Remington: The Science and Practice of Pharmacy*, Nineteenth Ed (Easton, Pa.: Mack Publishing Company, 1995); Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pa. 1975; Liberman, H. A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y., 1980; and *Pharmaceutical Dosage Forms and* Drug Delivery Systems, Seventh Ed. (Lippincott Williams & Wilkins 1999).

In some instances, the pharmaceutical formulation further includes pH adjusting agents or buffering agents which include acids such as acetic, boric, citric, lactic, phosphoric and hydrochloric acids; bases such as sodium hydroxide, sodium phosphate, sodium borate, sodium citrate, sodium

acetate, sodium lactate and tris-hydroxymethylaminomethane; and buffers such as citrate/dextrose, sodium bicarbonate and ammonium chloride. Such acids, bases and buffers are included in an amount required to maintain pH of the composition in an acceptable range.

In some instances, the pharmaceutical formulation includes one or more salts in an amount required to bring osmolality of the composition into an acceptable range. Such salts include those having sodium, potassium or ammonium cations and chloride, citrate, ascorbate, borate, phosphate, bicarbonate, sulfate, thiosulfate or bisulfite anions; suitable salts include sodium chloride, potassium chloride, sodium thiosulfate, sodium bisulfite and ammonium sulfate.

In some instances, the pharmaceutical formulation further includes diluent which are used to stabilize compounds because they provide a more stable environment. Salts dissolved in buffered solutions (which also provide pH control or maintenance) are utilized as diluents in the art, including, but not limited to a phosphate buffered saline solution. In certain instances, diluents increase bulk of the composition to facilitate compression or create sufficient bulk for homogenous blend for capsule filling. Such compounds include e.g., lactose, starch, mannitol, sorbitol, dextrose, microcrystalline cellulose such as Avicel®; dibasic calcium phosphate, dicalcium phosphate dihydrate; tricalcium phosphate, calcium phosphate; anhydrous lactose, spray-dried lactose; pregelatinized starch, compressible sugar, such as Di-Pac® (Amstar); mannitol, hydroxypropylmethylcellulose, hydroxypropylmethylcellulose acetate stearate, sucrose-based diluents, confectioner's sugar; monobasic calcium sulfate monohydrate, calcium sulfate dihydrate; calcium lactate trihydrate, dextrates; hydrolyzed cereal solids, amylose; powdered cellulose, calcium carbonate; glycine, kaolin; mannitol, sodium chloride; inositol, bentonite, and the like.

In some cases, the pharmaceutical formulation includes disintegration agents or disintegrants to facilitate the breakup or disintegration of a substance. The term "disintegrate" include both the dissolution and dispersion of the dosage form when contacted with gastrointestinal fluid. Examples of disintegration agents include a starch, e.g., a natural starch such as corn starch or potato starch, a pregelatinized starch such as National 1551 or Amijel®, or sodium starch glycolate such as Promogel® or Explotab®, a cellulose such as a wood product, methylcrystalline cellulose, e.g., Avicel®, Avicel® PH101, Avicel® PH102, Avicel® PH105, Elcema® P100, Emcocel®, Vivacel®, Ming Tia®, and Solka-Floc®, methylcellulose, croscarmellose, or a cross-linked cellulose, such as cross-linked sodium carboxymethylcellulose (Ac-Di-Sol®), cross-linked carboxymethylcellulose, or cross-linked croscarmellose, a cross-linked starch such as sodium starch glycolate, a cross-linked polymer such as crospovidone, a cross-linked polyvinylpyrrolidone, alginate such as alginic acid or a salt of alginic acid such as sodium alginate, a clay such as Veegum® HV (magnesium aluminum silicate), a gum such as agar, guar, locust bean, Karaya, pectin, or tragacanth, sodium starch glycolate, bentonite, a natural sponge, a surfactant, a resin such as a cation-exchange resin, citrus pulp, sodium lauryl sulfate, sodium lauryl sulfate in combination starch, and the like.

In some instances, the pharmaceutical formulation includes filling agents such as lactose, calcium carbonate, calcium phosphate, dibasic calcium phosphate, calcium sulfate, microcrystalline cellulose, cellulose powder, dextrose, dextrates, dextran, starches, pregelatinized starch, sucrose, xylitol, lactitol, mannitol, sorbitol, sodium chloride, polyethylene glycol, and the like.

Lubricants and glidants are also optionally included in the pharmaceutical formulations described herein for preventing, reducing or inhibiting adhesion or friction of materials. Exemplary lubricants include, e.g., stearic acid, calcium hydroxide, talc, sodium stearyl fumerate, a hydrocarbon such as mineral oil, or hydrogenated vegetable oil such as hydrogenated soybean oil (Sterotex®), higher fatty acids and their alkali-metal and alkaline earth metal salts, such as aluminum, calcium, magnesium, zinc, stearic acid, sodium stearates, glycerol, talc, waxes, Stearowet®, boric acid, sodium benzoate, sodium acetate, sodium chloride, leucine, a polyethylene glycol (e.g., PEG-4000) or a methoxypolyethylene glycol such as Carbowax™, sodium oleate, sodium benzoate, glyceryl behenate, polyethylene glycol, magnesium or sodium lauryl sulfate, colloidal silica such as Syloid™, Cab-O-Silo, a starch such as corn starch, silicone oil, a surfactant, and the like.

Plasticizers include compounds used to soften the microencapsulation material or film coatings to make them less brittle. Suitable plasticizers include, e.g., polyethylene glycols such as PEG 300, PEG 400, PEG 600, PEG 1450, PEG 3350, and PEG 800, stearic acid, propylene glycol, oleic acid, triethyl cellulose and triacetin. Plasticizers also function as dispersing agents or wetting agents.

Solubilizers include compounds such as triacetin, triethylcitrate, ethyl oleate, ethyl caprylate, sodium lauryl sulfate, sodium doccusate, vitamin E TPGS, dimethylacetamide, N-methylpyrrolidone, N-hydroxyethylpyrrolidone, polyvinylpyrrolidone, hydroxypropylmethyl cellulose, hydroxypropyl cyclodextrins, ethanol, n-butanol, isopropyl alcohol, cholesterol, bile salts, polyethylene glycol 200-600, glycofurol, transcutol, propylene glycol, and dimethyl isosorbide and the like.

Stabilizers include compounds such as any antioxidation agents, buffers, acids, preservatives and the like.

Suspending agents include compounds such as polyvinylpyrrolidone, e.g., polyvinylpyrrolidone K12, polyvinylpyrrolidone K17, polyvinylpyrrolidone K25, or polyvinylpyrrolidone K30, vinyl pyrrolidone/vinyl acetate copolymer (S630), polyethylene glycol, e.g., the polyethylene glycol has a molecular weight of about 300 to about 6000, or about 3350 to about 4000, or about 7000 to about 5400, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, hydroxymethylcellulose acetate stearate, polysorbate-80, hydroxyethylcellulose, sodium alginate, gums, such as, e.g., gum tragacanth and gum acacia, guar gum, xanthans, including xanthan gum, sugars, cellulosics, such as, e.g., sodium carboxymethylcellulose, methylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose, polysorbate-80, sodium alginate, polyethoxylated sorbitan monolaurate, polyethoxylated sorbitan monolaurate, povidone and the like.

Surfactants include compounds such as sodium lauryl sulfate, sodium docusate, Tween 60 or 80, triacetin, vitamin E TPGS, sorbitan monooleate, polyoxyethylene sorbitan monooleate, polysorbates, polaxomers, bile salts, glyceryl monostearate, copolymers of ethylene oxide and propylene oxide, e.g., Pluronic® (BASF), and the like. Additional surfactants include polyoxyethylene fatty acid glycerides and vegetable oils, e.g., polyoxyethylene (60) hydrogenated castor oil; and polyoxyethylene alkylethers and alkylphenyl

ethers, e.g., octoxynol 10, octoxynol 40. Sometimes, surfactants is included to enhance physical stability or for other purposes.

Viscosity enhancing agents include, e.g., methyl cellulose, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, hydroxypropylmethyl cellulose acetate stearate, hydroxypropylmethyl cellulose phthalate, carbomer, polyvinyl alcohol, alginates, acacia, chitosans and combinations thereof.

Wetting agents include compounds such as oleic acid, glyceryl monostearate, sorbitan monooleate, sorbitan monolaurate, triethanolamine oleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monolaurate, sodium docusate, sodium oleate, sodium lauryl sulfate, sodium doccusate, triacetin, Tween 80, vitamin E TPGS, ammonium salts and the like.

Therapeutic Regimens

In some embodiments, the pharmaceutical compositions described herein are administered for therapeutic applications. In some embodiments, the pharmaceutical composition is administered once per day, twice per day, three times per day or more. The pharmaceutical composition is administered daily, every day, every alternate day, five days a week, once a week, every other week, two weeks per month, three weeks per month, once a month, twice a month, three times per month, or more. The pharmaceutical composition is administered for at least 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 18 months, 2 years, 3 years, or more.

In some embodiments, one or more pharmaceutical compositions are administered simultaneously, sequentially, or at an interval period of time. In some embodiments, one or more pharmaceutical compositions are administered simultaneously. In some cases, one or more pharmaceutical compositions are administered sequentially. In additional cases, one or more pharmaceutical compositions are administered at an interval period of time (e.g., the first administration of a first pharmaceutical composition is on day one followed by an interval of at least 1, 2, 3, 4, 5, or more days prior to the administration of at least a second pharmaceutical composition).

In some embodiments, two or more different pharmaceutical compositions are coadministered. In some instances, the two or more different pharmaceutical compositions are coadministered simultaneously. In some cases, the two or more different pharmaceutical compositions are coadministered sequentially without a gap of time between administrations. In other cases, the two or more different pharmaceutical compositions are coadministered sequentially with a gap of about 0.5 hour, 1 hour, 2 hour, 3 hour, 12 hours, 1 day, 2 days, or more between administrations.

In the case wherein the patient's status does improve, upon the doctor's discretion the administration of the composition is given continuously; alternatively, the dose of the composition being administered is temporarily reduced or temporarily suspended for a certain length of time (i.e., a "drug holiday"). In some instances, the length of the drug holiday varies between 2 days and 1 year, including by way of example only, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, 15 days, 20 days, 28 days, 35 days, 50 days, 70 days, 100 days, 120 days, 150 days, 180 days, 200 days, 250 days, 280 days, 300 days, 320 days, 350 days, or 365 days. The dose reduction during a drug holiday is from 10%-100%, including, by way of example only, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

Once improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, can be reduced, as a function of the symptoms, to a level at which the improved disease, disorder or condition is retained.

In some embodiments, the amount of a given agent that correspond to such an amount varies depending upon factors such as the particular compound, the severity of the disease, the identity (e.g., weight) of the subject or host in need of treatment, but nevertheless is routinely determined in a manner known in the art according to the particular circumstances surrounding the case, including, e.g., the specific agent being administered, the route of administration, and the subject or host being treated. In some instances, the desired dose is conveniently presented in a single dose or as divided doses administered simultaneously (or over a short period of time) or at appropriate intervals, for example as two, three, four or more sub-doses per day.

The foregoing ranges are merely suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are not uncommon. Such dosages is altered depending on a number of variables, not limited to the activity of the compound used, the disease or condition to be treated, the mode of administration, the requirements of the individual subject, the severity of the disease or condition being treated, and the judgment of the practitioner.

In some embodiments, toxicity and therapeutic efficacy of such therapeutic regimens are determined by standard pharmaceutical procedures in cell cultures or experimental animals, including, but not limited to, the determination of the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index and it is expressed as the ratio between LD50 and ED50. Compounds exhibiting high therapeutic indices are preferred. The data obtained from cell culture assays and animal studies are used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with minimal toxicity. The dosage varies within this range depending upon the dosage form employed and the route of administration utilized.

Kits/Article of Manufacture

Disclosed herein, in certain embodiments, are kits and articles of manufacture for use with one or more of the compositions and methods described herein. Such kits include a carrier, package, or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the container(s) comprising one of the separate elements to be used in a method described herein. Suitable containers include, for example, bottles, vials, syringes, and test tubes. In one embodiment, the containers are formed from a variety of materials such as glass or plastic.

The articles of manufacture provided herein contain packaging materials. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, bags, containers, bottles, and any packaging material suitable for a selected formulation and intended mode of administration and treatment.

For example, the container(s) include target nucleic acid molecule described herein. Such kits optionally include an identifying description or label or instructions relating to its use in the methods described herein.

A kit typically includes labels listing contents and/or instructions for use, and package inserts with instructions for use. A set of instructions will also typically be included.

In one embodiment, a label is on or associated with the container. In one embodiment, a label is on a container when letters, numbers or other characters forming the label are attached, molded or etched into the container itself; a label is associated with a container when it is present within a receptacle or carrier that also holds the container, e.g., as a package insert. In one embodiment, a label is used to indicate that the contents are to be used for a specific therapeutic application. The label also indicates directions for use of the contents, such as in the methods described herein.

In certain embodiments, the pharmaceutical compositions are presented in a pack or dispenser device which contains one or more unit dosage forms containing a compound provided herein. The pack, for example, contains metal or plastic foil, such as a blister pack. In one embodiment, the pack or dispenser device is accompanied by instructions for administration. In one embodiment, the pack or dispenser is also accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the drug for human or veterinary administration. Such notice, for example, is the labeling approved by the U.S. Food and Drug Administration for prescription drugs, or the approved product insert. In one embodiment, compositions containing a compound provided herein formulated in a compatible pharmaceutical carrier are also prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Certain Terminology

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the claimed subject matter belongs. It is to be understood that the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of any subject matter claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. In this application, the use of "or" means "and/or" unless stated otherwise. Furthermore, use of the term "including" as well as other forms, such as "include", "includes," and "included," is not limiting.

As used herein, ranges and amounts can be expressed as "about" a particular value or range. About also includes the exact amount. Hence "about 5 μL" means "about 5 μL" and also "5 μL." Generally, the term "about" includes an amount that would be expected to be within experimental error.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

As used herein, the terms "individual(s)", "subject(s)" and "patient(s)" mean any mammal. In some embodiments, the mammal is a human. In some embodiments, the mammal is a non-human. None of the terms require or are limited to situations characterized by the supervision (e.g. constant or intermittent) of a health care worker (e.g. a doctor, a registered nurse, a nurse practitioner, a physician's assistant, an orderly or a hospice worker).

The term "therapeutically effective amount" relates to an amount of a polynucleic acid molecule conjugate that is sufficient to provide a desired therapeutic effect in a mammalian subject. In some cases, the amount is single or multiple dose administration to a patient (such as a human) for treating, preventing, preventing the onset of, curing, delaying, reducing the severity of, ameliorating at least one symptom of a disorder or recurring disorder, or prolonging the survival of the patient beyond that expected in the absence of such treatment. Naturally, dosage levels of the particular polynucleic acid molecule conjugate employed to provide a therapeutically effective amount vary in dependence of the type of injury, the age, the weight, the gender, the medical condition of the subject, the severity of the condition, the route of administration, and the particular inhibitor employed. In some instances, therapeutically effective amounts of polynucleic acid molecule conjugate, as described herein, is estimated initially from cell culture and animal models. For example, IC_{50} values determined in cell culture methods optionally serve as a starting point in animal models, while IC_{50} values determined in animal models are optionally used to find a therapeutically effective dose in humans.

Skeletal muscle, or voluntary muscle, is generally anchored by tendons to bone and is generally used to effect skeletal movement such as locomotion or in maintaining posture. Although some control of skeletal muscle is generally maintained as an unconscious reflex (e.g., postural muscles or the diaphragm), skeletal muscles react to conscious control. Smooth muscle, or involuntary muscle, is found within the walls of organs and structures such as the esophagus, stomach, intestines, uterus, urethra, and blood vessels.

Skeletal muscle is further divided into two broad types: Type I (or "slow twitch") and Type II (or "fast twitch"). Type I muscle fibers are dense with capillaries and are rich in mitochondria and myoglobin, which gives Type I muscle tissue a characteristic red color. In some cases, Type I muscle fibers carries more oxygen and sustain aerobic activity using fats or carbohydrates for fuel. Type I muscle fibers contract for long periods of time but with little force. Type II muscle fibers are further subdivided into three major subtypes (IIa, IIx, and IIb) that vary in both contractile speed and force generated. Type II muscle fibers contract quickly and powerfully but fatigue very rapidly, and therefore produce only short, anaerobic bursts of activity before muscle contraction becomes painful.

Unlike skeletal muscle, smooth muscle is not under conscious control.

Cardiac muscle is also an involuntary muscle but more closely resembles skeletal muscle in structure and is found only in the heart. Cardiac and skeletal muscles are striated in that they contain sarcomeres that are packed into highly regular arrangements of bundles. By contrast, the myofibrils of smooth muscle cells are not arranged in sarcomeres and therefore are not striated.

Muscle cells encompass any cells that contribute to muscle tissue. Exemplary muscle cells include myoblasts, satellite cells, myotubes, and myofibril tissues.

As used here, muscle force is proportional to the cross-sectional area (CSA), and muscle velocity is proportional to muscle fiber length. Thus, comparing the cross-sectional areas and muscle fibers between various kinds of muscles is capable of providing an indication of muscle atrophy. Various methods are known in the art to measure muscle strength and muscle weight, see, for example, "Musculoskeletal assessment: Joint range of motion and manual muscle

strength" by Hazel M. Clarkson, published by Lippincott Williams & Wilkins, 2000. The production of tomographic images from selected muscle tissues by computed axial tomography and sonographic evaluation are additional methods of measuring muscle mass.

EXAMPLES

These examples are provided for illustrative purposes only and not to limit the scope of the claims provided herein.

Example 1. siRNA Sequences and Synthesis

All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. All the siRNA passenger strand contains conjugation handles in different formats, C6-NH_2 and/or C6-SH, one at each end of the strand. The conjugation handle or handles were connected to siRNA passenger strand via inverted abasic phosphodiester or phosphorothioate. Below FIGS. **40**A-F are representative structures of the formats used in the in vivo experiments.

Cholesterol-Myostatin siRNA Conjugate

The sequence of the guide/antisense strand was complementary to the gene sequence starting a base position 1169 for the mouse mRNA transcript for MSTN (UUAUUAUUUGUUCUUUGCCUU; SEQ ID NO: 14226). Base, sugar and phosphate modifications were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained a 5' cholesterol which was conjugated as described below in FIG. **1**.

Example 2. General Experimental Protocol and Materials

Animals

All animal studies were conducted following protocols in accordance with the Institutional Animal Care and Use Committee (IACUC) at Explora BioLabs, which adhere to the regulations outlined in the USDA Animal Welfare Act as well as the "Guide for the Care and Use of Laboratory Animals" (National Research Council publication, 8th Ed., revised in 2011). All mice were obtained from either Charles River Laboratories or Harlan Laboratories.

Wild type CD-1 mice (4-6 week old) were dosed via intravenous (iv) injection with the indicated ASCs (or antibody-nucleic acid conjugate) and doses.

Anti-Transferrin Receptor Antibody

Anti-mouse transferrin receptor antibody or CD71 mAb is a rat IgG2a subclass monoclonal antibody that binds mouse CD71 or mouse transferrin receptor 1 (mTfR1). The antibody was produced by BioXcell and it is commercially available (Catalog # BE0175).

IgG2a Isotype Control Antibody

Rat IgG2a isotype control antibody was purchased from BioXcell (Clone 2A3, Catalog #BE0089) and this antibody is specific to trinitrophenol and does not have any known antigens in mouse.

Anti-EGFR Antibody

Anti-EGFR antibody is a fully human IgG1κ monoclonal antibody directed against the human epidermal growth factor receptor (EGFR). It is produced in the Chinese Hamster Ovary cell line DJT33, which has been derived from the CHO cell line CHO-K1SV by transfection with a GS vector carrying the antibody genes derived from a human anti-EGFR antibody producing hybridoma cell line (2F8). Standard mammalian cell culture and purification technologies are employed in the manufacturing of anti-EGFR antibody.

The theoretical molecular weight (MW) of anti-EGFR antibody without glycans is 146.6 kDa. The experimental MW of the major glycosylated isoform of the antibody is 149 kDa as determined by mass spectrometry. Using SDS-PAGE under reducing conditions the MW of the light chain was found to be approximately 25 kDa and the MW of the heavy chain to be approximately 50 kDa. The heavy chains are connected to each other by two inter-chain disulfide bonds, and one light chain is attached to each heavy chain by a single inter-chain disulfide bond. The light chain has two intra-chain disulfide bonds and the heavy chain has four intra-chain disulfide bonds. The antibody is N-linked glycosylated at Asn305 of the heavy chain with glycans composed of N-acetyl-glucosamine, mannose, fucose and galactose. The predominant glycans present are fucosylated bi-antennary structures containing zero or one terminal galactose residue.

The charged isoform pattern of the IgG1κ antibody has been investigated using imaged capillary IEF, agarose IEF and analytical cation exchange HPLC. Multiple charged isoforms are found, with the main isoform having an isoelectric point of approximately 8.7.

The major mechanism of action of anti-EGFR antibody is a concentration dependent inhibition of EGF-induced EGFR phosphorylation in A431 cancer cells. Additionally, induction of antibody-dependent cell-mediated cytotoxicity (ADCC) at low antibody concentrations has been observed in pre-clinical cellular in vitro studies.

In Vitro Evaluation of siRNA Potency and Efficacy

C2C12 myoblasts (ATCC) were grown in DMEM supplemented with 10% v/v FBS. For transfection, cells were plated at a density of 10.000 cells/well in 24-well plates, and transfected within 24 hours. C2C12 myotubes were generated by incubating confluent C2C12 myoblast cultures in DMEM supplemented with 2% v/v horse serum for 3-4 days. During and after differentiation the medium was changed daily. Pre-differentiated primary human skeletal muscle cells were obtained from ThermoFisher and plated in DMEM with 2% v/v horse serum according to recommendations by the manufacturer. Human SJCRH30 rhabdomyosarcoma myoblasts (ATCC) were grown in DMEM supplemented with 10% v/v heat-inactivated fetal calf serum, 4.5 mg/mL glucose, 4 mM L-glutamine, 10 mM HEPES, and 1 mM sodium pyruvate. For transfections cells were plated in a density of 10.000-20.000 cells/well in 24-well plates and transfected within 24 hours. All cells were transfected with various concentrations of the siRNAs (0.0001-100 nM; 10-fold dilutions) using RNAiMax (ThermoFisher) according to the recommendation by the manufacturer. Transfected cells were incubated in 5% CO2 at 37° C. for 2 days, then washed with PBS, and harvested in 300 ul TRIzol (ThermoFisher) and stored at −80° C. RNA was prepared using a ZYMO 96-well RNA kit (ThermoFisher) and relative RNA expression levels quantified by RT-qPCR using commercially available TaqMan probes (LifeTechnology). Expression data were analyzed using the □□CT method normalized to Ppib expression, and are presented as % KD relative to mock-transfected cells. Data were analyzed by nonlinear regression using a 3 parameter dose response inhibition function (GraphPad Prism 7.02). All knock down results present the maximal observed KD under these experimental conditions.

Myostatin ELISA

Myostatin protein in plasma was quantified using the GDF-8 (Myostatin) Quantikine ELISA Immunoassay (part# DGDF80) from R&D Systems according to the manufacturer's instructions.

RISC Loading Assay

Specific immunoprecipitation of the RISC from tissue lysates and quantification of small RNAs in the immunoprecipitates were determine by stem-loop PCR, using an adaptation of the assay described by Pei et al. Quantitative evaluation of siRNA delivery in vivo. RNA (2010), 16:2553-2563.

Example 3. Conjugate Synthesis

The structures in FIGS. **41**A-F illustrate exemplary A-X_1—B—X_2—Y (Formula I) architectures described herein.

Example 3.1 Antibody siRNA Conjugate Synthesis Using SMCC Linker

FIG. **42** shows Synthesis scheme-1: Antibody-Cys-SMCC-siRNA-PEG conjugates via antibody cysteine conjugation.

Step 1: Antibody Interchain Disulfide Reduction with TCEP

Antibody was buffer exchanged with borax buffer (pH 8) and made up to 10 mg/ml concentration. To this solution, 2 equivalents of TCEP in water was added and rotated for 2 hours at RT. The resultant reaction mixture was buffer exchanged with pH 7.4 PBS containing 5 mM EDTA and added to a solution of SMCC-C6-siRNA or SMCC-C6-siRNA-C6-NHCO-PEG-XkDa (2 equivalents) (X=0.5 kDa to 10 kDa) in pH 7.4 PBS containing 5 mM EDTA at RT and rotated overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA.

Step 2: Purification

The crude reaction mixture was purified by AKTA explorer FPLC using anion exchange chromatography method-1 as described in Example 3.4. Fractions containing DAR1 and DAR>2 antibody-siRNA-PEG conjugates were separated, concentrated and buffer exchanged with pH 7.4 PBS.

Step-3: Analysis of the Purified Conjugate

The isolated conjugates were characterized by SEC, SAX chromatography and SDS-PAGE. The purity of the conjugate was assessed by analytical HPLC using either anion exchange chromatography method-2 or anion exchange chromatography method-3. Both methods are described in Example 3.4. Isolated DAR1 conjugates are typically eluted at 9.0±0.3 min on analytical SAX method and are greater than 90% pure. The typical DAR>2 cysteine conjugate contains more than 85% DAR2 and less than 15% DAR3.

FIG. **2** illustrates SAX HPLC chromatogram of TfR mAb-(Cys)-HPRT-PEG5k, DAR1.

FIG. **3** illustrates SEC HPLC chromatogram of TfR mAb-(Cys)-HPRT-PEG5k, DAR1.

Example 3.2. Antibody siRNA Conjugate Synthesis Using Bis-Maleimide (BisMal) Linker

FIG. **43** shows Synthesis scheme-2: Antibody-Cys-BisMal-siRNA-PEG conjugates.

Step 1: Antibody Reduction with TCEP

Antibody was buffer exchanged with borax buffer (pH 8) and made up to 5 mg/ml concentration. To this solution, 2 equivalents of TCEP in water was added and rotated for 2 hours at RT. The resultant reaction mixture was exchanged with pH 7.4 PBS containing 5 mM EDTA and added to a solution of BisMal-C6-siRNA-C6-S-NEM (2 equivalents) in pH 7.4 PBS containing 5 mM EDTA at RT and kept at 4° C. overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA.

Step 2: Purification

The crude reaction mixture was purified by AKTA explorer FPLC using anion exchange chromatography method-1. Fractions containing DAR1 and DAR2 antibody-siRNA conjugates were separated, concentrated and buffer exchanged with pH 7.4 PBS.

Step-3: Analysis of the Purified Conjugate

The isolated conjugates were characterized by either mass spec or SDS-PAGE. The purity of the conjugate was assessed by analytical HPLC using either anion exchange chromatography method-2 or 3 as well as size exclusion chromatography method-1.

FIG. **4** illustrates an overlay of DAR1 and DAR2 SAX HPLC chromatograms of TfR1mAb-Cys-BisMal-siRNA conjugates.

FIG. **5** illustrates an overlay of DAR1 and DAR2 SEC HPLC chromatograms of TfR1mAb-Cys-BisMal-siRNA conjugates.

Example 3.3. Fab' Generation from mAb and Conjugation to siRNA

FIG. **44** shows Scheme-3: Fab-siRNA conjugate generation.

Step 1: Antibody Digestion with Pepsin

Antibody was buffer exchanged with pH 4.0, 20 mM sodium acetate/acetic acid buffer and made up to 5 mg/ml concentration. Immobilized pepsin (Thermo Scientific, Prod#20343) was added and incubated for 3 hours at 37° C. The reaction mixture was filtered using 30 kDa MWCO Amicon spin filters and pH 7.4 PBS. The retentate was collected and purified using size exclusion chromatography to isolate F(ab')2. The collected F(ab')2 was then reduced by 10 equivalents of TCEP and conjugated with SMCC-C6-siRNA-PEG5 at room temperature in pH 7.4 PBS. Analysis of reaction mixture on SAX chromatography showed Fab-siRNA conjugate along with unreacted Fab and siRNA-PEG.

Step 2: Purification

The crude reaction mixture was purified by AKTA explorer FPLC using anion exchange chromatography method-1. Fractions containing DAR1 and DAR2 Fab-siRNA conjugates were separated, concentrated and buffer exchanged with pH 7.4 PBS.

Step-3: Analysis of the Purified Conjugate

The characterization and purity of the isolated conjugate was assessed by analytical HPLC using anion exchange chromatography method-2 or 3 as well as by SEC method-1.

FIG. **6** illustrates SEC chromatogram of CD71 Fab-Cys-HPRT-PEG5.

FIG. **7** illustrates SAX chromatogram of CD71 Fab-Cys-HPRT-PEG5.

Example 3.4. Purification and Analytical Methods

Anion Exchange Chromatography Method (SAX)-1.

1. Column: Tosoh Bioscience, TSKGel SuperQ-5PW, 21.5 mm ID×15 cm, 13 um
2. Solvent A: 20 mM TRIS buffer, pH 8.0; Solvent B: 20 mM TRIS, 1.5 M NaCl, pH 8.0; Flow Rate: 6.0 ml/min

3. Gradient:

a.	% A	% B	Column Volume
b.	100	0	1.00
c.	60	40	18.00
d.	40	60	2.00
e.	40	60	5.00
f.	0	100	2.00
g.	100	0	2.00

Anion Exchange Chromatography (SAX) Method-2

1. Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm
2. Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl; Flow Rate: 0.75 ml/min
3. Gradient:

a.	Time	% A	% B	
b.	0.0	90	10	
c.	3.00		90	10
d.	11.00	40	60	
e.	13.00	40	60	
f.	15.00	90	10	
g.	20.00	90	10	

Anion Exchange Chromatography (SAX) Method-3

1. Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm
2. Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl
3. Flow Rate: 0.75 ml/min
4. Gradient:

a.	Time	% A	% B
b.	0.0	90	10
c.	3.00	90	10
d.	11.00	40	60
e.	23.00	40	60
f.	25.00	90	10
g.	30.00	90	10

Size Exclusion Chromatography (SEC) Method-1

1. Column: TOSOH Biosciences, TSKgelG3000SW XL, 7.8×300 mm, 5 μM
2. Mobile phase: 150 mM phosphate buffer
3. Flow Rate: 1.0 ml/min for 15 mins

Example 4. In Vitro Screen: Atrogin-1

Identification of siRNAs for the Regulation of Mouse and Human/NHP Atrogin-1

A bioinformatics screen conducted identified 56 siRNAs (19 mers) that bind specifically to mouse atrogin-1 (Fbxo32 NM_026346.3). In addition, 6 siRNAs were identified that target mouse atrogin-1 and human atrogin-1 (FBXO32; NM_058229.3). A screen for siRNAs (19 mers) targeting specifically human/NHP atrogin-1 (FBXO32; NM_058229.3) yielded 52 candidates (Table 2A-Table 2B3). All selected siRNA target sites do not harbor SNPs (pos. 2-18).

Tables 2A and 2B illustrate identified siRNA candidates for the regulation of mouse and human/NHP atrogin-1.

TABLE 2A

19mer pos. in NM_0263 46.3	exon #	mouse	mouse x human	sequence of total 23mer target site in NM_026346.3	SEQ ID NO:
7	1	X		GGGCAGCGGCCCGGGAUAAAUAC	28
8	1	X		GGCAGCGGCCCGGGAUAAAUACU	29
499	3-Feb	X		AACCAAAACUCAGUACUUCCAUC	30
553	4-Mar	X		UACGAAGGAGCGCCAUGGAUACU	31
590	4	X	X	GCUUUCAACAGACUGGACUUCUC	32
631	4	X		CAGAAGAUUCAACUACGUAGUAA	33
694	5	X		GAGUGGCAUCGCCCAAAAGAACU	34
772	6	X		AAGACUUAUACGGGAACUUCUCC	35
1178	8	X		AAGCUUGUACGAUGUUACCCAAG	36
1179	8	X		AGCUUGUACGAUGUUACCCAAGA	37
1256	9-Aug	X	X	UGGAAGGGCACUGACCAUCCGUG	38
1258	9-Aug	X	X	GAAGGGCACUGACCAUCCGUGCA	39
1260	9	X	X	AGGGCACUGACCAUCCGUGCACG	40
1323	9	X		AAGACUUUAUCAAUUUGUUCAAG	41
1401	9	X		GGGAGUCGGGACACUUCAUUUGU	42
1459	9	X		CGGGGGAUACGUCAUUGAGGAGA	43
1504	9	X		UUGCCGAUGGAAAUUUACAAAUG	44
1880	9	X	X	CCACACAAUGGUCUACCUCUAAA	45
1884	9	X	X	ACAAUGGUCUACCUCUAAAAGCA	46
2455	9	X		CUGAUAGAUGUGUUCGUCUUAAA	47
2570	9	X		AUCUCAGGGCUUAAGGAGUUAAU	48
2572	9	X		CUCAGGGCUUAAGGAGUUAAUUC	49
2936	9	X		CUGAUUUGCAGGGUCUUACAUCU	50
3006	9	X		CUGGUGGCCAAAUUAAGUUGAAU	51
3007	9	X		UGGUGGCCAAAUUAAGUUGAAUU	52
3115	9	X		GAGAUUACAAACAUUGUAACAGA	53
3668	9	X		CAGCGCAAAACUAGUUAGCCAGU	54
3676	9	X		AACUAGUUAGCCAGUCUUACAGA	55
3715	9	X		AAGUCAUAUAGCAUCCAUACACC	56
3800	9	X		UAGUAGGUGCUUGCAGGUUCUCC	57
3845	9	X		AUGGUAUGUGACACAACCGAAGA	58
3856	9	X		CACAACCGAAGAAUCGUUUGACG	59
4026	9	X		GGCAAGCAAGAUACCCAUAUUAG	60
4095	9	X		AGCUCUUAGGACAUUAAUAGUCU	61
4139	9	X		UGCAGGACUCCCAGACUUAAAAC	62
4183	9	X		CCCAGAACUGCUAGUACAAAAGC	63
4203	9	X		AGCAAGAGGGGUGUGGCUAUAGA	64

TABLE 2A-continued

4208	9	X	GAGGGGUGUGGCUAUAGAAGUUG	65
4548	9	X	GACCAUGUCGCUACUACCAUUGC	66
4554	9	X	GUCGCUACUACCAUUGCUUCAAG	67
4563	9	X	ACCAUUGCUUCAAGUGGGUAUCU	68
4567	9	X	UUGCUUCAAGUGGGUAUCUCAGU	69
4673	9	X	CUGGUUAGUGAUGAUCAACUUCA	70
4858	9	X	UGCCGCUUCAUACGGGAGAAAAA	71
4970	9	X	UCGGCUUCAACGCAUUGUUUAUU	72
5022	9	X	CUGCCUGGUUAUAAAGCAAUAAC	73
5235	9	X	ACCUGUUAGUGCUUAAACAGACU	74
5237	9	X	CUGUUAGUGCUUAAACAGACUCA	75
5279	9	X	GGGGCAAACGCAGGGGUGUUACU	76
5292	9	X	GGGUGUUACUCUUUGAUAUAUCA	77
5443	9	X	AUCCCAGACUUUAGACCAAAAGG	78
5640	9	X	UUGUGGACGUGUGUAAAUUCAUG	79
6000	9	X	UUCAUUGACCAACCAGUCUUAAG	80
6105	9	X	UGCCGCAACCUCCCAAGUCAUAU	81
6530	9	X	GAGUAUAGACAUGCGUGUUAACU	82
6537	9	X	GACAUGCGUGUUAACUAUGCACA	83
6608	9	X	UUGGUUCCAUCUUUAUACCAAAU	84
6668	9	X	GUGUCUAAGCUUAGAAGCUUUAA	85
6720	9	X	UGGGUUGAACACUUUAACUAAAC	86
6797	9	X	AUCUGAAUCCUGUAUAACUUAUU	87
6799	9	X	CUGAAUCCUGUAUAACUUAUUUG	88
6803	9	X	AUCCUGUAUAACUUAUUUGCACA	89

19mer pos. in NM_058229.3	human + NHP	sequence of total 23mer target site in NM_058229.3	SEQ ID NO:
586	x	UUCCAGAAGAUUUAACUACGUGG	90
589	x	CAGAAGAUUUAACUACGUGGUCC	91
1068	x	AGCGGCAGAUCCGCAAACGAUUA	92
1071	x	GGCAGAUCCGCAAACGAUUAAUU	93
1073	x	CAGAUCCGCAAACGAUUAAUUCU	94
1075	x	GAUCCGCAAACGAUUAAUUCUGU	95
1076	x	AUCCGCAAACGAUUAAUUCUGUC	96
1077	x	UCCGCAAACGAUUAAUUCUGUCA	97
1079	x	CGCAAACGAUUAAUUCUGUCAGA	98
1083	x	AACGAUUAAUUCUGUCAGACAAA	99
1127	x	AUGUAUUUCAAACUUGUCCGAUG	100
1142	x	GUCCGAUGUUACCCAAGGAAAGA	101

TABLE 2A-continued

1164	x	AGCAGUAUGGAGAUACCCUUCAG 102
1228	x	CCAUCCGUGCACUGCCAAUAACC 103
1254	x	AGAGCUGCUCCGUUUCACUUUCA 104
1361	x	UGGGAAUAUGGCAUUUGGACACU 105
1492	x	UGUGAACUUCUCACUAGAAUUGG 106
1500	x	UCUCACUAGAAUUGGUAUGGAAA 107
1563	x	CAGCAAGACUAUAAGGGCAAUAA 108
1566	x	CAAGACUAUAAGGGCAAUAAUUC 109
1635	x	UCUUAUAGUUCCCUAGGAAGAAA 110
1679	x	AUAGGACGCUUUGUUUACAAUGU 111
2487	x	UUUUCUUUAGGUCCAACAUCAAA 112
2488	x	UUUCUUUAGGUCCAACAUCAAAA 113
2582	x	AGGAGAGGUACCACAAGUUCAUC 114
2661	x	GAGGCAAAUAUCAGCAGGUAACU 115
2663	x	GGCAAAUAUCAGCAGGUAACUGU 116
2790	x	UUUCCUACAACAAUGUACAUAUA 117
2999	x	AAGAGACAAGCUAUGAUACAACA 118
3875	x	GAAAUCAACCUUUAUGGUUCUCU 119
4036	x	GUGCCACGUGGUAUCUGUUAAGU 120
4039	x	CCACGUGGUAUCUGUUAAGUAUG 121
4059	x	AUGGCCAGAGCCUCACAUAUAAG 122
4062	x	GCCAGAGCCUCACAUAUAAGUGA 123
4065	x	AGAGCCUCACAUAUAAGUGAAGA 124
4117	x	AUAAUAGUCUAUAGAAUUUCUAU 125
4444	x	GCCUAGAGUCUCUUGAGAGUAAA 126
4653	x	GAAAGCAUCCCCAAUGUAUCAGU 127
4665	x	AAUGUAUCAGUUGUGAGAUGAUU 128
4787	x	CUACUAGCACUUGGGCAGUAAGG 129
5162	x	UUAACUAAACUCUAUCAUCAUUU 130
5261	x	CUGGCCUAAAAUCCUAUUAGUGC 131
5270	x	AAUCCUAUUAGUGCUUAAACAGA 132
5272	x	UCCUAUUAGUGCUUAAACAGACC 133
5338	x	UUUGAUAUAUCUUGGGUCCUUGA 134
5737	x	UGGCUGUUAACGUUUCCAUUUCA 135
5739	x	GCUGUUAACGUUUCCAUUUCAAG 136
6019	x	CUCAGAGGUACAUUUAAUCCAUC 137
6059	x	CAGGACCAGCUAUGAGAUUCAGU 138
6140	x	GGGGGAUUAUUCCAUGAGGCAGC 139
6431	x	GGCUCCAAGCUGUAUUCUAUACU 140
6720	x	UUUGUACCAGACGGUGGCAUAUU 141

TABLE 2B

19mer pos. in NM_026346.3	exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
7	1	GCAGCGGCCCGGGAUAAAAU	142	AUUUAUCCCGGGCCGCUGC	256
8	1	CAGCGGCCCGGGAUAAAUA	143	UAUUUAUCCCGGGCCGCUG	257
499	3-Feb	CCAAAACUCAGUACUUCCA	144	UGGAAGUACUGAGUUUUGG	258
553	4-Mar	CGAAGGAGCGCCAUGGAUA	145	UAUCCAUGGCGCUCCUUCG	259
590	4	UUUCAACAGACUGGACUUC	146	GAAGUCCAGUCUGUUGAAA	260
631	4	GAAGAUUCAACUACGUAGU	147	ACUACGUAGUUGAAUCUUC	261
694	5	GUGGCAUCGCCCAAAAGAA	148	UUCUUUUGGGCGAUGCCAC	262
772	6	GACUUAUACGGGAACUUCU	149	AGAAGUUCCCGUAUAAGUC	263
1178	8	GCUUGUACGAUGUUACCCA	150	UGGGUAACAUCGUACAAGC	264
1179	8	CUUGUACGAUGUUACCCAA	151	UUGGGUAACAUCGUACAAG	265
1256	9-Aug	GAAGGGCACUGACCAUCCG	152	CGGAUGGUCAGUGCCCUUC	266
1258	9-Aug	AGGGCACUGACCAUCCGUG	153	CACGGAUGGUCAGUGCCCU	267
1260	9	GGCACUGACCAUCCGUGCA	154	UGCACGGAUGGUCAGUGCC	268
1323	9	GACUUUAUCAAUUUGUUCA	155	UGAACAAAUUGAUAAAGUC	269
1401	9	GAGUCGGGACACUUCAUUU	156	AAAUGAAGUGUCCCGACUC	270
1459	9	GGGGAUACGUCAUUGAGGA	157	UCCUCAAUGACGUAUCCCC	271
1504	9	GCCGAUGGAAAUUUACAAA	158	UUUGUAAAUUUCCAUCGGC	272
1880	9	ACACAAUGGUCUACCUCUA	159	UAGAGGUAGACCAUUGUGU	273
1884	9	AAUGGUCUACCUCUAAAAG	160	CUUUUAGAGGUAGACCAUU	274
2455	9	GAUAGAUGUGUUCGUCUUA	161	UAAGACGAACACAUCUAUC	275
2570	9	CUCAGGGCUUAAGGAGUUA	162	UAACUCCUUAAGCCCUGAG	276
2572	9	CAGGGCUUAAGGAGUUAAU	163	AUUAACUCCUUAAGCCCUG	277
2936	9	GAUUUGCAGGGUCUUACAU	164	AUGUAAGACCCUGCAAAUC	278

TABLE 2B-continued

exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
3006	9 GGUGGCCAAAUUAAGUUGA	165	UCAACUUAAUUUGGCCACC	279
3007	9 GUGGCCAAAUUAAGUUGAA	166	UUCAACUUAAUUUGGCCAC	280
3115	9 GAUUACAAACAUUGUAACA	167	UGUUACAAUGUUUGUAAUC	281
3668	9 GCGCAAAACUAGUUAGCCA	168	UGGCUAACUAGUUUUGCGC	282
3676	9 CUAGUUAGCCAGUCUUACA	169	UGUAAGACUGGCUAACUAG	283
3715	9 GUCAUAUAGCAUCCAUACA	170	UGUAUGGAUGCUAUAUGAC	284
3800	9 GUAGGUGCUUGCAGGUUCU	171	AGAACCUGCAAGCACCUAC	285
3845	9 GGUAUGUGACACAACCGAA	172	UUCGGUUGUGUCACAUACC	286
3856	9 CAACCGAAGAAUCGUUUGA	173	UCAAACGAUUCUUCGGUUG	287
4026	9 CAAGCAAGAUACCCAUAUU	174	AAUAUGGGUAUCUUGCUUG	288
4095	9 CUCUUAGGACAUUAAUAGU	175	ACUAUUAAUGUCCUAAGAG	289
4139	9 CAGGACUCCCAGACUUAAA	176	UUUAAGUCUGGGAGUCCUG	290
4183	9 CAGAACUGCUAGUACAAAA	177	UUUUGUACUAGCAGUUCUG	291
4203	9 CAAGAGGGGUGUGGCUAUA	178	UAUAGCCACACCCCUCUUG	292
4208	9 GGGGUGUGGCUAUAGAAGU	179	ACUUCUAUAGCCACACCCC	293
4548	9 CCAUGUCGCUACUACCAUU	180	AAUGGUAGUAGCGACAUGG	294
4554	9 CGCUACUACCAUUGCUUCA	181	UGAAGCAAUGGUAGUAGCG	295
4563	9 CAUUGCUUCAAGUGGGUAU	182	AUACCCACUUGAAGCAAUG	296
4567	9 GCUUCAAGUGGGUAUCUCA	183	UGAGAUACCCACUUGAAGC	297
4673	9 GGUUAGUGAUGAUCAACUU	184	AAGUUGAUCAUCACUAACC	298
4858	9 CCGCUUCAUACGGGAGAAA	185	UUUCUCCCGUAUGAAGCGG	299
4970	9 GGCUUCAACGCAUUGUUUA	186	UAAACAAUGCGUUGAAGCC	300
5022	9 GCCUGGUUAUAAAGCAAUA	187	UAUUGCUUUAUAACCAGGC	301
5235	9 CUGUUAGUGCUUAAACAGA	188	UCUGUUUAAGCACUAACAG	302
5237	9 GUUAGUGCUUAAACAGACU	189	AGUCUGUUUAAGCACUAAC	303

TABLE 2B-continued

	exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
5279	9	GGCAAACGCAGGGGUGUUA	190	UAACACCCCUGCGUUUGCC	304
5292	9	GUGUUACUCUUUGAUAUAU	191	AUAUAUCAAAGAGUAACAC	305
5443	9	CCCAGACUUUAGACCAAAA	192	UUUUGGUCUAAAGUCUGGG	306
5640	9	GUGGACGUGUGUAAAUUCA	193	UGAAUUUACACACGUCCAC	307
6000	9	CAUUGACCAACCAGUCUUA	194	UAAGACUGGUUGGUCAAUG	308
6105	9	CCGCAACCUCCCAAGUCAU	195	AUGACUUGGGAGGUUGCGG	309
6530	9	GUAUAGACAUGCGUGUUAA	196	UUAACACGCAUGUCUAUAC	310
6537	9	CAUGCGUGUUAACUAUGCA	197	UGCAUAGUUAACACGCAUG	311
6608	9	GGUUCCAUCUUUAUACCAA	198	UUGGUAUAAAGAUGGAACC	312
6668	9	GUCUAAGCUUAGAAGCUUU	199	AAAGCUUCUAAGCUUAGAC	313
6720	9	GGUUGAACACUUUAACUAA	200	UUAGUUAAAGUGUUCAACC	314
6797	9	CUGAAUCCUGUAUAACUUA	201	UAAGUUAUACAGGAUUCAG	315
6799	9	GAAUCCUGUAUAACUUAUU	202	AAUAAGUUAUACAGGAUUC	316
6803	9	CCUGUAUAACUUAUUUGCA	203	UGCAAAUAAGUUAUACAGG	317
19mer pos. in NM_05822 9.3					
586		CCAGAAGAUUUAACCUAGU	204	ACGUAGUUAAAUCUUCUGG	318
589		GAAGAUUUAACUACGUGGU	205	ACCACGUAGUUAAAUCUUC	319
1068		CGGCAGAUCCGCAAACGAU	206	AUCGUUUGCGGAUCUGCCG	320
1071		CAGAUCCGCAAACGAUUAA	207	UUAAUCGUUUGCGGAUCUG	321
1073		GAUCCGCAAACGAUUAAUU	208	AAUUAAUCGUUUGCGGAUC	322
1075		UCCGCAAACGAUUAAUUCU	209	AGAAUUAAUCGUUUGCGGA	323
1076		CCGCAAACGAUUAAUUCUG	210	CAGAAUUAAUCGUUUGCGG	324
1077		CGCAAACGAUUAAUUCUGU	211	ACAGAAUUAAUCGUUUGCG	325
1079		CAAACGAUUAAUUCUGUCA	212	UGACAGAAUUAAUCGUUUG	326

TABLE 2B-continued

	exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
1083		CGAUUAAUUCUGUCAGACA	213	UGUCUGACAGAAUUAAUCG	327
1127		GUAUUUCAAACUUGUCCGA	214	UCGGACAAGUUUGAAAUAC	328
1142		CCGAUGUUACCCAAGGAAA	215	UUUCCUUGGGUAACAUCGG	329
1164		CAGUAUGGAGAUACCCUUC	216	GAAGGGUAUCUCCAUACUG	330
1228		AUCCGUGCACUGCCAAUAA	217	UUAUUGGCAGUGCACGGAU	331
1254		AGCUGCUCCGUUUCACUUU	218	AAAGUGAAACGGAGCAGCU	332
1361		GGAAUAUGGCAUUUGGACA	219	UGUCCAAAUGCCAUAUUCC	333
1492		UGAACUUCUCACUAGAAUU	220	AAUUCUAGUGAGAAGUUCA	334
1500		UCACUAGAAUUGGUAUGGA	221	UCCAUACCAAUUCUAGUGA	335
1563		GCAAGACUAUAAGGGCAAU	222	AUUGCCCUUAUAGUCUUGC	336
1566		AGACUAUAAGGGCAAUAAU	223	AUUAUUGCCCUUAUAGUCU	337
1635		UUAUAGUUCCCUAGGGAAGA	224	UCUUCCUAGGGAACUAUAA	338
1679		AGGACGCUUUGUUUACAAU	225	AUUGUAAACAAAGCGUCCU	339
2487		UUCUUUAGGUCCAACACAUCA	226	UGAUGUUGGACCUAAAGAA	340
2488		UCUUUAGGUCCAACAUCAA	227	UUGAUGUUGGACCUAAAGA	341
2582		GAGAGGUACCACAAGUUCA	228	UGAACUUGUGGUACCUCUC	342
2661		GGCAAAUAUCAGCAGGUAA	229	UUACCUGCUGAUAUUUGCC	343
2663		CAAAUAUCAGCAGGUAACU	230	AGUUACCUGCUGAUAUUUG	344
2790		UCCUACAACAAUGUACAUA	231	UAUGUACAUUGUUGUAGGA	345
2999		GAGACAAGCUAUGAUACAA	232	UUGUAUCAUAGCUUGUCUC	346
3875		AAUCAACCUUUAUGGUUCU	233	AGAACCAUAAAGGUUGAUU	347
4036		GCCACGUGGUAUCUGUUAA	234	UUAACAGAUACCACGUGGC	348
4039		ACGUGGUAUCUGUUAAGUA	235	UACUUAACAGAUACCACGU	349
4059		GGCCAGAGCCUCACAUAUA	236	UAUAUGUGAGGCUCUGGCC	350
4062		CAGAGCCUCACAUAUAAGU	237	ACUUAUAUGUGAGGCUCUG	351

TABLE 2B-continued

exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
4065	AGCCUCACAUAUA AGUGAA	238	UUCACUUAUAUGUGAG GCU	352
4117	AAUAGUCUAUAGA AUUUCU	239	AGAAAUUCUAUAGACU AUU	353
4444	CUAGAGUCUCUUG AGAGUA	240	UACUCUCAAGAGACUCU AG	354
4653	AAGCAUCCCCAAU GUAUCA	241	UGAUACAUUGGGGAUG CUU	355
4665	UGUAUCAGUUGUG AGAUGA	242	UCAUCUCACAACUGAUA CA	356
4787	ACUAGCACUUGGG CAGUAA	243	UUACUGCCCAAGUGCUA GU	357
5162	AACUAAACUCUAU CAUCAU	244	AUGAUGAUAGAGUUUA GUU	358
5261	GGCCUAAAAUCCU AUUAGU	245	ACUAAUAGGAUUUUAG GCC	359
5270	UCCUAUUAGUGCU UAAACA	246	UGUUUAAGCACUAAUA GGA	360
5272	CUAUUAGUGCUUA AACAGA	247	UCUGUUUAAGCACUAAU AG	361
5338	UGAUAUAUCUUGG GUCCUU	248	AAGGACCCAAGAUAUAU CA	362
5737	GCUGUUAACGUUU CCAUUU	249	AAAUGGAAACGUUAAC AGC	363
5739	UGUUAACGUUUCC AUUUCA	250	UGAAAUGGAAACGUUA ACA	364
6019	CAGAGGUACAUUU AAUCCA	251	UGGAUUAAAUGUACCUC UG	365
6059	GGACCAGCUAUGA GAUUCA	252	UGAAUCUCAUAGCUGGU CC	366
6140	GGGAUUAUUCCAU GAGGCA	253	UGCCUCAUGGAAUAAUC CC	367

TABLE 2B-continued

exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
6431	CUCCAAGCUGUAU UCUAUA	254	UAUAGAAUACAGCUUG GAG	368
6720	UGUACCAGACGGU GGCAUA	255	UAUGCCACCGUCUGGUA CA	369

Evaluation of Selected Atrogin-1 siRNAs in Transfected Mouse C2C12 Myoblasts, Mouse C2C12 Myotubes, Pre-Differentiated Myotubes of Primary Human Skeletal Muscle Cells, and Human SJCRH30 Rhabdomyosarcoma Myoblasts.

From the 62 identified siRNAs targeting mouse atrogin-1 and 52 targeting human atrogin-1, 30 and 20 siRNAs were selected for synthesis and functional analysis, respectively. The activity of these siRNAs was analyzed in transfected mouse C2C12 myoblasts, mouse C2C12 myotubes, pre-differentiated myotubes of primary human skeletal muscle cells, and human SJCRH30 rhabdomyosarcoma myoblasts.

None of the tested siRNAs targeting mouse atrogin-1 showed significant activity in mouse C2C12 myotubes (at 10 nM), however 3 siRNAs downregulated mouse atrogin-1 mRNA by >75% in C2C12 myoblasts (Table 3). In contrast, siRNAs targeting Murf1, which is exclusively expressed in C2C12 myotubes (FIG. **8**), were active in C2C12 myotubes, demonstrating that siRNAs can be transfected into C2C12 myotubes. To determine whether atrogin-1 might be alternatively spliced in C2C12 myoblasts and myotubes, various positions in the atrogin-1 mRNA were probed by RT-qPCR, but yielded similar results. Among the 20 tested siRNAs targeting human atrogin-1 only four yielded >75% KD. For both, mouse and human atrogin-1, active siRNAs localized either within or close to the coding region. One of the siRNAs targeting mouse atrogin-1 (1179) was strongly cross-reactive with human atrogin-1. While this siRNA failed to show significant activity in mouse C2C12 myotubes, it effectively downregulated human atrogin-1 in myotubes of primary human skeletal muscle cells. All efficacious siRNAs downregulated their respective targets with sub-nanomolar potency.

TABLE 3

illustrates activity of selected atrogin-1 siRNAs in transfected mouse C2C12 myoblasts, mouse C2C12 myotubes, pre-differentiated myotubes of primary human skeletal muscle cells, and human SJCRH30 rhabdomyosarcoma myoblasts. For experimental procedures see Example 2.

mu atrogin-1 NM_026346. 3 ID#	muC2C12 myotubes % KD (10 nM)	muC2C12 myoblasts % KD	muC2C12 myoblasts IC50(nM)	huSkMC myotubes % KD	huSkMC myotubes IC50(nM)	huSJCRH30 myoblasts % KD	huSJCRH30 myoblasts IC50 (nM)
8	no KD	no KD					
499	8.5	34.4					
553	9.7	10.6					
590	10.1	30.5	0.628	62.8			
631	14.4	83.7	0.159	81.5	0.129	54.5	0.614
694	10.2	50.5	1.228	72.4	0.011	64.6	0.004
772	8.8	64.7	0.872				0.084
1179	7.2	76.6	0.160	88.5	0.010	86.0	0.015
1256	2.7	32.5					
1260	no KD	13.6					
1459	16.1	60.8	0.258	24.4	1.714		
1504	12.8	76.8	0.092	29.1	0.433	17.3	0.423
1880	14.3	58.6	0.192	66.5			
1884	7.7	54.6	0.135	54.8			0.002
2572	13.7	61.5	0.928	16.0	2.664	18.4	0.027
3007	14.4	32.7					

TABLE 3-continued

illustrates activity of selected atrogin-1 siRNAs in transfected mouse C2C12 myoblasts, mouse C2C12 myotubes, pre-differentiated myotubes of primary human skeletal muscle cells, and human SJCRH30 rhabdomyosarcoma myoblasts. For experimental procedures see Example 2.

mu atrogin-1 NM_026346. 3 ID#	muC2C12 myotubes % KD (10 nM)	muC2C12 myoblasts % KD	muC2C12 myoblasts IC50(nM)	huSkMC myotubes % KD	huSkMC myotubes IC50(nM)	huSJCRH30 myoblasts % KD	huSJCRH30 myoblasts IC50 (nM)
3668	1.4	6.9					
3715	0.7	17.2					
3856	no KD	5.9					
4139	2.7	10.5					
4567	12.8	56.1	1.589	0.0		37.2	1.028
4673	11.6	34.9					
4970	15.6	35.7					
5292	20.3	49.6	0.106	19.3	0.441	11.2	0.02
5640	13.6	40.4					
6000	19.7	21.2					
6530	3.5	no KD					
6608	7.4	no KD					
6720	17.5	no KD					
6799	15.4	no KD					
586		66.1	0.326	89.9	0.008	90.6	0.011
1071		0.0	no KD	55.9			
1077		14.4	1.774	93.9	0.009	93.9	0.016
1083		no KD	no KD	92.8	0.047	92.2	0.056
1361		no KD		80.1	0.003	81.4	0.118
1566				49.9			
1679		15.1	1.471	55.8			
2582				46.2			
2663		no KD	no KD	64.2			
2999		no KD	no KD	55.0			
4036		no KD	0.200	64.7			
4059				3.2			
4117		24.2	1.541	68.0			
5162				15.7			
5261				44.4			
5272				47.4			
5737		no KD	no KD	60.8			
6019				44.4			
6059		no KD	no KD	57.6			
6431		no KD	no KD	65.0			

Example 5. In Vitro Screen: MuRF-1

Identification of siRNAs targeting mouse Murf1 (Trim63) and/or human/NHP MuRF1 (TRIM63)

A bioinformatics screen was conducted and identified 51 siRNAs (19 mers) that bind specifically to mouse Murf1 sequences that show >3 sequence derivations from mouse Murf2 (Trim55; NM_001039048.2) or Murf3 (Trim54). In addition, 9 siRINAs were identified that target mouse Murf1 and human MuRF1 (TRIM63; NM_032588.3). A screen for siRNAs (19 mers) targeting specifically human and NHP MuRF1 (NM_032588.3) yielded 52 candidates (Table 4A-Table 41B). All selected siRNA target sites do not harbor SNPs (pos. 2-18).

Tables 4A and 4B illustrate identified siRNA candidates for the regulation of mouse and human/NHP MuRF1.

TABLE 4A

19mer pos. in NM_ 001039048.2	exon #	mouse X	mousesequence of total 23mer target site humanin NM_001039048.2	SEQ ID NO:
33	1	x	GAGGAUCCGAGUGGGUUUGGAGA	370
82	1	x	CGAGACAGUCGCAUUUCAAAGCA	371

TABLE 4A-continued

109	1	x		GGAUUAUAAAUCUAGCCUGAUUC 372
130	1	x		UCCUGAUGGAAACGCUAUGGAGA 373
264	2	x		AGGCUGCGAAUCCCUACUGGACC 374
318	2	x		GUCGUUUCCGUUGCCCCUCGUGC 375
328	2	x		UUGCCCCUCGUGCCGCCAUGAAG 376
329	2	x		UGCCCCUCGUGCCGCCAUGAAGU 377
330	2	x		GCCCCUCGUGCCGCCAUGAAGUG 378
337	2	x		GUGCCGCCAUGAAGUGAUCAUGG 379
346	2	x		UGAAGUGAUCAUGGACCGGCACG 380
423	3-Febx		X	AGCAGGAGUGCUCCAGUCGGCCC 381
457	3	x		CAGCCACCCGAUGUGCAAGGAAC 382
460	3	x		CCACCCGAUGUGCAAGGAACACG 383
495	3	x	X	UCAACAUCUACUGUCUCACGUGU 384
497	3	x		AACAUCUACUGUCUCACGUGUGA 385
499	3	x	X	CAUCUACUGUCUCACGUGUGAGG 386
500	3	x	X	AUCUACUGUCUCACGUGUGAGGU 387

TABLE 4A-continued

502	3	x	X	CUACUGUCUCACGUGUGAGGUGC	388
505	3	x	X	CUGUCUCACGUGUGAGGUGCCUA	389
507	3	x		GUCUCACGUGUGAGGUGCCUACU	390
511	3	x		CACGUGUGAGGUGCCUACUUGCU	391
538	3	x		GUGCAAGGUGUUUGGGGCUCACC	392
609	4	x		CUGAGCUGAGUAACUGCAUCUCC	393
616	4	x		GAGUAACUGCAUCUCCAUGCUGG	394
646	4	x		CAACGACCGAGUGCAGACGAUCA	395
651	4	x		ACCGAGUGCAGACGAUCAUCUCU	396
787	5	x	X	GCUGCAGCGGAUCACGCAGGAGC	397
790	5	x		GCAGCGGAUCACGCAGGAGCAGG	398
911	5	x		GAGCCCGGAGGGGCUACCUUCCU	399
1012	7	x		UGAGAACAUGGACUACUUUACUC	400
1016	7	x		AACAUGGACUACUUUACUCUGGA	401
1018	7	x		CAUGGACUACUUUACUCUGGACU	402
1022	7	x		GACUACUUUACUCUGGACUUAGA	403
1130	8	x		GAGGAAGAGGGCGUGACCACAGA	404
1266	9	x		UACAAUAGGGAAGUGUGUCUUCU	405
1351	9	x		ACACAAUUGGAAAUGUAUCCAAA	406
1364	9	x		UGUAUCCAAAACGUCACAGGACA	407
1366	9	x		UAUCCAAAACGUCACAGGACACU	408
1369	9	x		CCAAAACGUCACAGGACACUUUU	409
1380	9	x		CAGGACACUUUUCUACGUUGGUG	410
1386	9	x		ACUUUUCUACGUUGGUGCGAAAU	411
1387	9	x		CUUUUCUACGUUGGUGCGAAAUG	412
1390	9	x		UUCUACGUUGGUGCGAAAUGAAA	413
1391	9	x		UCUACGUUGGUGCGAAAUGAAAU	414
1393	9	x		UACGUUGGUGCGAAAUGAAAUAU	415
1397	9	x		UUGGUGCGAAAUGAAAUAUUUUG	416
1454	9	x	X	UAUAUGUAUGCCAAUUUGGUGCU	417
1458	9	x	X	UGUAUGCCAAUUUGGUGCUUUUU	418
1460	9	x		UAUGCCAAUUUGGUGCUUUUUGU	419
1462	9	x		UGCCAAUUUGGUGCUUUUUGUAC	420
1466	9	x		AAUUUGGUGCUUUUUGUACGAGA	421
1478	9	x		UUUGUACGAGAACUUUUGUAUGA	422
1480	9	x		UGUACGAGAACUUUUGUAUGAUC	423
1481	9	x		GUACGAGAACUUUUGUAUGAUCA	424
1483	9	x		ACGAGAACUUUUGUAUGAUCACG	425
1520	9	x		GACUGGCGAUUGUCACAAAGUGG	426
1658	9	x		GGAUAGGACUGAAUUUGUGUUAU	427
1660	9	x		AUAGGACUGAAUUUGUGUUAUAU	428

19mer pos. in NM_032588.3	hu man + NHP	sequence of total 23mer target site in NM_032588.3	SEQ ID NO:
28	X	GGAAGCCAACAGGAUCCGACCCG	429
75	X	CCCAGGUCUACUUAGAGCAAAGU	430
77	X	CAGGUCUACUUAGAGCAAAGUUA	431
153	X	AGUCGAGCCUGAUCCAGGAUGGG	432
239	X	CCAGUGGUCAUCUUGCCGUGCCA	433
245	X	GUCAUCUUGCCGUGCCAGCACAA	434
248	X	AUCUUGCCGUGCCAGCACAACCU	435
249	X	UCUUGCCGUGCCAGCACAACCUG	436
259	X	CCAGCACAACCUGUGCCGGAAGU	437
339	X	UGUCCAUGUCUGGAGGCCGUUUC	438
367	X	CCCCACCUGCCGCCACGAGGUGA	439
368	X	CCCACCUGCCGCCACGAGGUGAU	440
370	X	CACCUGCCGCCACGAGGUGAUCA	441
371	X	ACCUGCCGCCACGAGGUGAUCAU	442
372	X	CCUGCCGCCACGAGGUGAUCAUG	443
373	X	CUGCCGCCACGAGGUGAUCAUGG	444
374	X	UGCCGCCACGAGGUGAUCAUGGA	445
375	X	GCCGCCACGAGGUGAUCAUGGAU	446
379	X	CCACGAGGUGAUCAUGGAUCGUC	447
380	X	CACGAGGUGAUCAUGGAUCGUCA	448
381	X	ACGAGGUGAUCAUGGAUCGUCAC	449
384	X	AGGUGAUCAUGGAUCGUCACGGA	450
385	X	GGUGAUCAUGGAUCGUCACGGAG	451
386	X	GUGAUCAUGGAUCGUCACGGAGU	452
387	X	UGAUCAUGGAUCGUCACGGAGUG	453
451	X	CAUCUACAAACAGGAGUGCUCCA	454
458	X	AAACAGGAGUGCUCCAGUCGGCC	455
459	X	AACAGGAGUGCUCCAGUCGGCCG	456
461	X	CAGGAGUGCUCCAGUCGGCCGCU	457
491	X	GGCAGUCACCCCAUGUGCAAGGA	458
499	X	CCCCAUGUGCAAGGAGCACGAAG	459
503	X	AUGUGCAAGGAGCACGAAGAUGA	460
531	X	UCAACAUCUACUGUCUCACGUGU	461
535	X	CAUCUACUGUCUCACGUGUGAGG	462
539	X	UACUGUCUCACGUGUGAGGUGCC	463

TABLE 4A-continued

564	X	CCUGCUCCAUGUGCAAGGUGUUU	464
568	X	CUCCAUGUGCAAGGUGUUUGGGA	465
610	X	GGCCCCAUUGCAGAGUGUCUUCC	466
612	X	CCCCAUUGCAGAGUGUCUUCCAG	467
645	X	CUGAACUGAAUAACUGUAUCUCC	468
647	X	GAACUGAAUAACUGUAUCUCCAU	469
670	X	GCUGGUGGCGGGGAAUGACCGUG	470
671	X	CUGGUGGCGGGGAAUGACCGUGU	471
672	X	UGGUGGCGGGGAAUGACCGUGUG	472
673	X	GGUGGCGGGGAAUGACCGUGUGC	473
812	X	AAAAGUGAGUUGCUGCAGCGGAU	474
860	X	AGCUUCAUCGAGGCCCUCAUCCA	475
968	X	CUCUUGACUGCCAAGCAACUCAU	476
970	X	CUUGACUGCCAAGCAACUCAUCA	477
977	X	GCCAAGCAACUCAUCAAAAGCAU	478
979	X	CAAGCAACUCAUCAAAAGCAUUG	479
980	X	AAGCAACUCAUCAAAAGCAUUGU	480

TABLE 4B

19mer pos. in NM_001039048.2	exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
33	1	GGAUCCGAGUGGGUUUGGA	481	UCCAAACCCACUCGGAUCC	592
82	1	AGACAGUCGCAUUUCAAAG	482	CUUUGAAAUGCGACUGUCU	593
109	1	AUUAUAAAUCUAGCCUGAU	483	AUCAGGCUAGAUUUAUAAU	594
130	1	CUGAUGGAAACGCUAUGGA	484	UCCAUAGCGUUUCCAUCAG	595
264	2	GCUGCGAAUCCCUACUGGA	485	UCCAGUAGGGAUUCGCAGC	596
318	2	CGUUUCCGUUGCCCCUCGU	486	ACGAGGGGCAACGGAAACG	597
328	2	GCCCCUCGUGCCGCCAUGA	487	UCAUGGCGGCACGAGGGGC	598
329	2	CCCCUCGUGCCGCCAUGAA	488	UUCAUGGCGGCACGAGGGG	599
330	2	CCCUCGUGCCGCCAUGAAG	489	CUUCAUGGCGGCACGAGGG	600
337	2	GCCGCCAUGAAGUGAUCAU	490	AUGAUCACUUCAUGGCGGC	601

TABLE 4B-continued

	exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
346	2	AAGUGAUCAUGGACCGGCA	491	UGCCGGUCCAUGAUCACUU	602
423	3-Feb	CAGGAGUGCUCCAGUCGGC	492	GCCGACUGGAGCACUCCUG	603
457	3	GCCACCCGAUGUGCAAGGA	493	UCCUUGCACAUCGGGUGGC	604
460	3	ACCCGAUGUGCAAGGAACA	494	UGUUCCUUGCACAUCGGGU	605
495	3	AACAUCUACUGUCUCACGU	495	ACGUGAGACAGUAGAUGUU	606
497	3	CAUCUACUGUCUCACGUGU	496	ACACGUGAGACAGUAGAUG	607
499	3	UCUACUGUCUCACGUGUGA	497	UCACACGUGAGACAGUAGA	608
500	3	CUACUGUCUCACGUGUGAG	498	CUCACACGUGAGACAGUAG	609
502	3	ACUGUCUCACGUGUGAGGU	499	ACCUCACACGUGAGACAGU	610
505	3	GUCUCACGUGUGAGGUGCC	500	GGCACCUCACACGUGAGAC	611
507	3	CUCACGUGUGAGGUGCCUA	501	UAGGCACCUCACACGUGAG	612
511	3	CGUGUGAGGUGCCUACUUG	502	CAAGUAGGCACCUCACACG	613
538	3	GCAAGGUGUUUGGGGCUCA	503	UGAGCCCCAAACACCUUGC	614
609	4	GAGCUGAGUAACUGCAUCU	504	AGAUGCAGUUACUCAGCUC	615
616	4	GUAACUGCAUCUCCAUGCU	505	AGCAUGGAGAUGCAGUUAC	616
646	4	ACGACCGAGUGCAGACGAU	506	AUCGUCUGCACUCGGUCGU	617
651	4	CGAGUGCAGACGAUCAUCU	507	AGAUGAUCGUCUGCACUCG	618
787	5	UGCAGCGGAUCACGCAGGA	508	UCCUGCGUGAUCCGCUGCA	619
790	5	AGCGGAUCACGCAGGAGCA	509	UGCUCCUGCGUGAUCCGCU	620
911	5	GCCCGGAGGGGCUACCUUC	510	GAAGGUAGCCCCUCCGGGC	621
1012	7	AGAACAUGGACUACUUUAC	511	GUAAAGUAGUCCAUGUUCU	622
1016	7	CAUGGACUACUUUACUCUG	512	CAGAGUAAAGUAGUCCAUG	623
1018	7	UGGACUACUUUACUCUGGA	513	UCCAGAGUAAAGUAGUCCA	624
1022	7	CUACUUUACUCUGGACUUA	514	UAAGUCCAGAGUAAAGUAG	625
1130	8	GGAAGAGGGCGUGACCACA	515	UGUGGUCACGCCCUCUUCC	626

TABLE 4B-continued

	exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
1266	9	CAAUAGGGAAGUG UGUCUU	516	AAGACACACUUCCCUAU UG	627
1351	9	ACAAUUGGAAAUG UAUCCA	517	UGGAUACAUUUCCAAUU GU	628
1364	9	UAUCCAAAACGUC ACAGGA	518	UCCUGUGACGUUUUGGA UA	629
1366	9	UCCAAAACGUCAC AGGACA	519	UGUCCUGUGACGUUUUG GA	630
1369	9	AAAACGUCACAGG ACACUU	520	AAGUGUCCUGUGACGUU UU	631
1380	9	GGACACUUUUCUA CGUUGG	521	CCAACGUAGAAAAGUGU CC	632
1386	9	UUUUCUACGUUGG UGCGAA	522	UUCGCACCAACGUAGAA AA	633
1387	9	UUUCUACGUUGGU GCGAAA	523	UUUCGCACCAACGUAGA AA	634
1390	9	CUACGUUGGUGCG AAAUGA	524	UCAUUUCGCACCAACGU AG	635
1391	9	UACGUUGGUGCGA AAUGAA	525	UUCAUUUCGCACCAACG UA	636
1393	9	CGUUGGUGCGAAA UGAAAU	526	AUUUCAUUUCGCACCAA CG	637
1397	9	GGUGCGAAAUGAA AUAUUU	527	AAAUAUUUCAUUUCGCA CC	638
1454	9	UAUGUAUGCCAAU UUGGUG	528	CACCAAAUUGGCAUACA UA	639
1458	9	UAUGCCAAUUUGG UGCUUU	529	AAAGCACCAAAUUGGCA UA	640
1460	9	UGCCAAUUUGGUG CUUUUU	530	AAAAAGCACCAAAUUGG CA	641
1462	9	CCAAUUUGGUGCU UUUUGU	531	ACAAAAAGCACCAAAUU GG	642
1466	9	UUUGGUGCUUUUU GUACGA	532	UCGUACAAAAAGCACCA AA	643
1478	9	UGUACGAGAACUU UUGUAU	533	AUACAAAAGUUCUCGUA CA	644
1480	9	UACGAGAACUUUU GUAUGA	534	UCAUACAAAAGUUCUCG UA	645
1481	9	ACGAGAACUUUU GUAUGAU	535	AUCAUACAAAAGUUCUC GU	646
1483	9	GAGAACUUUUGU AUGAUCA	536	UGAUCAUACAAAAGUUC UC	647
1520	9	CUGGCGAUUGUC ACAAAGU	537	ACUUUGUGACAAUCGCC AG	648
1658	9	AUAGGACUGAAU UUGUGUU	538	AACACAAAUUCAGUCCU AU	649
1660	9	AGGACUGAAUUU GUGUUAU	539	AUAACACAAAUUCAGUC CU	650

TABLE 4B-continued

19mer pos. in NM_032588.3	exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
28		AAGCCAACAGGA UCCGACC	540	GGUCGGAUCCUGUUGGC UU	651
75		CAGGUCUACUUA GAGCAAA	541	UUUGCUCUAAGUAGACC UG	652
77		GGUCUACUUAGA GCAAAGU	542	ACUUUGCUCUAAGUAGA CC	653
153		UCGAGCCUGAUC CAGGAUG	543	CAUCCUGGAUCAGGCUC GA	654
239		AGUGGUCAUCUU GCCGUGC	544	GCACGGCAAGAUGACCA CU	655
245		CAUCUUGCCGUG CCAGCAC	545	GUGCUGGCACGGCAAGA UG	656
248		CUUGCCGUGCCA GCACAAC	546	GUUGUGCUGGCACGGCA AG	657
249		UUGCCGUGCCAG CACAACC	547	GGUUGUGCUGGCACGGC AA	658
259		AGCACAACCUGU GCCGGAA	548	UUCCGGCACAGGUUGUG CU	659
339		UCCAUGUCUGGA GGCCGUU	549	AACGGCCUCCAGACAUG GA	660
367		CCACCUGCCGCC ACGAGGU	550	ACCUCGUGGCGGCAGGU GG	661
368		CACCUGCCGCCA CGAGGUG	551	CACCUCGUGGCGGCAGG UG	662
370		CCUGCCGCCACG AGGUGAU	552	AUCACCUCGUGGCGGCA GG	663
371		CUGCCGCCACGA GGUGAUC	553	GAUCACCUCGUGGCGGC AG	664
372		UGCCGCCACGAG GUGAUCA	554	UGAUCACCUCGUGGCGG CA	665
373		GCCGCCACGAGG UGAUCAU	555	AUGAUCACCUCGUGGCG GC	666
374		CCGCCACGAGGU GAUCAUG	556	CAUGAUCACCUCGUGGC GG	667
375		CGCCACGAGGUG AUCAUGG	557	CCAUGAUCACCUCGUGG CG	668
379		ACGAGGUGAUCA UGGAUCG	558	CGAUCCAUGAUCACCUC GU	669
380		CGAGGUGAUCAU GGAUCGU	559	ACGAUCCAUGAUCACCU CG	670
381		GAGGUGAUCAUG GAUCGUC	560	GACGAUCCAUGAUCACC UC	671
384		GUGAUCAUGGAU CGUCACG	561	CGUGACGAUCCAUGAUC AC	672
385		UGAUCAUGGAUC GUCACGG	562	CCGUGACGAUCCAUGAU CA	673

TABLE 4B-continued

exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
386	GAUCAUGGAUCG UCACGGA	563	UCCGUGACGAUCCAUGA UC	674
387	AUCAUGGAUCGU CACGGAG	564	CUCCGUGACGAUCCAUG AU	675
451	UCUACAAACAGG AGUGCUC	565	GAGCACUCCUGUUUGUA GA	676
458	ACAGGAGUGCUC CAGUCGG	566	CCGACUGGAGCACUCCU GU	677
459	CAGGAGUGCUCC AGUCGGC	567	GCCGACUGGAGCACUCC UG	678
461	GGAGUGCUCCAG UCGGCCG	568	CGGCCGACUGGAGCACU CC	679
491	CAGUCACCCCAU GUGCAAG	569	CUUGCACAUGGGGUGAC UG	680
499	CCAUGUGCAAGG AGCACGA	570	UCGUGCUCCUUGCACAU GG	681
503	GUGCAAGGAGCA CGAAGAU	571	AUCUUCGUGCUCCUUGC AC	682
531	AACAUCUACUGU CUCACGU	572	ACGUGAGACAGUAGAUG UU	683
535	UCUACUGUCUCA CGUGUGA	573	UCACACGUGAGACAGUA GA	684
539	CUGUCUCACGUG UGAGGUG	574	CACCUCACACGUGAGAC AG	685
564	UGCUCCAUGUGC AAGGUGU	575	ACACCUUGCACAUGGAG CA	686
568	CCAUGUGCAAGG UGUUUGG	576	CCAAACACCUUGCACAU GG	687
610	CCCCAUUGCAGA GUGUCUU	577	AAGACACUCUGCAAUGG GG	688
612	CCAUUGCAGAGU GUCUUCC	578	GGAAGACACUCUGCAAU GG	689
645	GAACUGAAUAAC UGUAUCU	579	AGAUACAGUUAUUCAGU UC	690
647	ACUGAAUAACUG UAUCUCC	580	GGAGAUACAGUUAUUCA GU	691
670	UGGUGGCGGGGA AUGACCG	581	CGGUCAUUCCCCGCCAC CA	692

TABLE 4B-continued

exon #	sense strand sequence (5'-3')	SEQ ID NO:	antisense strand sequence (5'-3')	SEQ ID NO:
671	GGUGGCGGGGAA UGACCGU	582	ACGGUCAUUCCCCGCCA CC	693
672	GUGGCGGGGAAU GACCGUG	583	CACGGUCAUUCCCCGCC AC	694
673	UGGCGGGGAAUG ACCGUGU	584	ACACGGUCAUUCCCCGC CA	695
812	AAGUGAGUUGCU GCAGCGG	585	CCGCUGCAGCAACUCAC UU	696
860	CUUCAUCGAGGC CCUCAUC	586	GAUGAGGGCCUCGAUGA AG	697
968	CUUGACUGCCAA GCAACUC	587	GAGUUGCUUGGCAGUCA AG	698
970	UGACUGCCAAGC AACUCAU	588	AUGAGUUGCUUGGCAGU CA	699
977	CAAGCAACUCAU CAAAAGC	589	GCUUUUGAUGAGUUGCU UG	700
979	AGCAACUCAUCA AAAGCAU	590	AUGCUUUUGAUGAGUUG CU	701
980	GCAACUCAUCAA AAGCAUU	591	AAUGCUUUUGAUGAGUU GC	702

Activity of Selected MuRF1 siRNAs in Transfected Mouse C2C12 Myotubes and Pre-Differentiated Myotubes of Primary Human Skeletal Muscle Cells

From the 60 identified siRINAs targeting mouse Murf1 and 25 siRNAs targeting human MuRF1, 35 and 25 siRNAs were selected for synthesis, respectively. The activity of these siRNAs was analyzed in transfected mouse C2C12 myotubes and pre-differentiated primary human skeletal muscle cells (Table 5). Among the siRNAs targeting mouse MurF1, 14 displayed >70% knock down of Murf1, but <20% knock down of Murf2 and Murf3 in C2C 12 myotubes. At least 6 of these 14 siRNAs were cross reactive with human MuRF1. Among the tested siRNAs targeting human MuRF1, 8 displayed >70% knock down of MuRF1, but <20% knock down of MuRF2 and MuRF3 in pre-differentiated myotubes of primary human skeletal muscle cells. Only 1 of these 8 siRNAs showed significant cross-reactivity with mouse Muf1. All efficacious siRNAs downregulated their respective targets with subnanomolar potency.

TABLE 5

illustrates activity of selected MuRF1 siRNAs in transfected mouse C2C12 myotubes and pre-differentiated myotubes of primary human skeletal muscle cells. Cells were grown and transfected and RNAs isolated and analyzed as described in Example 5.

19mer position in NM_ 026346.3	muC2C12 myotubes mMuRF1 KD(%)	muC2C12 myotubes mMuRF2 KD(%)	muC2C12 myotubes mMuRF3 KD(%)	muC2C12 myotubes mMuRF1 IC50(nM)	huSkMC myotubes hMuRF1 KD(%)	huSkMC myotubes hMuRF2 KD(%)	huSkMC myotubes hMuRF3 KD(%)	huSkMC myotubes hMuRf1 IC50(nM)
33	24.3	0.0	5.5					
109	73.4	0.0	6.0	0.073				
130	45.8	10.4	16.0					
264	79.6	3.7	22.0	0.172	80.9	5.6	0.0	0.018
337	42.9	10.4	7.9					
423	65.9	15.5	8.9	0.288	64.5			

TABLE 5-continued

illustrates activity of selected MuRF1 siRNAs in transfected mouse C2C12 myotubes and pre-differentiated myotubes of primary human skeletal muscle cells. Cells were grown and transfected and RNAs isolated and analyzed as described in Example 5.

19mer position in NM_026346.3	muC2C12 myotubes mMuRF1 KD(%)	muC2C12 myotubes mMuRF2 KD(%)	muC2C12 myotubes mMuRF3 KD(%)	muC2C12 myotubes mMuRF1 IC50(nM)	huSkMC myotubes hMuRF1 KD(%)	huSkMC myotubes hMuRF2 KD(%)	huSkMC myotubes hMuRF3 KD(%)	huSkMC myotubes hMuRf1 IC50(nM)
460	56.4	16.6	17.8					
495	70.7	9.6	31.9	0.495	76.1	49.0	4.4	0.105
499	73.8	12.7	7.0	0.116	76.0	11.3	24.0	0.150
500	69.0	20.7	11.5	0.167	92.5	23.3	50.0	
538	48.6	10.7	16.5					
651	78.3	5.8	0.0	0.082	53.9	0.0	4.2	0.150
787	26.0	13.5	7.9					
911	43.5	0.0	10.0					
1012	74.1	0.0	27.5	0.014	83.1	6.6	0.0	0.019
1018	72.6	12.3	6.3	0.121				
1022	70.9	26.5	22.9	0.042				
1130	60.6	22.0	16.3	0.206				
1266	73.5	27.1	19.6	0.007	83.8	7.5	42.3	0.184
1351	77.5	44.2	0.0	0.008	79.0	6.6	52.4	0.509
1364	71.6	0.8	2.8	0.012	27.9			
1387	79.1	9.5	14.9	0.007	66.7	15.0	13.3	0.059
1390	73.6	33.1	10.0	0.012				
1393	75.2	28.4	4.5	0.044	73.3	34.0	12.1	0.141
1397	78.8	5.2	16.4	0.008	74.9	23.0	12.3	0.009
1454	73.8	6.5	8.7	0.019	68.2	7.0	1.7	0.004
1458	73.1	0.0	6.4	0.016	85.8	13.7	42.7	0.008
1462	69.9	17.2	19.8	0.017	63.8	3.8	0.0	0.005
1466	75.0	17.8	19.1	0.012	68.4	6.2	0.0	0.045
1480	71.1	11.9	6.9	0.022				
1481	65.8	12.2	0.4	0.266				
1483	74.6	1.1	4.0	0.030				
1520	72.8	1.6	8.8	0.012	30.6			
1658	73.7	21.6	9.8	0.028	26.0	15.6	6.6	0.005
1660	76.2	0.0	0.0	0.017				
75	25.8				69.6	0.0	0.0	
77	14.5	17.4	13.8		83.7	7.1	13.9	0.152
245	60.3			0.905	70.9	33.1	14.3	
259	49.7			2.759	75.1	84.5	71.1	0.053
339					52.7	1.2	14.3	
367					0.0	0.0	0.0	
370	8.8			0.033	21.2	25.0	4.1	
373					64.6	21.8	14.1	
374	33.7	7.0	14.8	0.659	89.7	10.0	19.7	0.110
380	19.5			0.013	70.6	8.5	0.4	
386					69.9	19.9	16.4	0.002
459	66.7			0.148	78.1	25.2	7.1	
491					57.1	16.3	1.3	
503	56.4			0.101	52.0	89.9	86.1	
535	70.8	32.4	13.4	0.074	82.1	24.0	9.5	0.008
564	8.2			0.002	72.4	26.1	0.6	
610	6.5	18.7	17.0		89.6	9.4	15.2	0.107
645	46.2	13.9	2.3	3.475	85.2	0.0	0.0	0.007
647	77.5	20.1	0.0	0.211	94.6	4.4	19.4	0.006
673					35.5	13.4	4.5	
860					77.1	22.5	0.0	
970	8.8	29.3	10.6		84.9	12.9	7.3	0.056
977	19.9	5.9	0.0	2.838	93.6	51.0	12.6	0.117
979					87.4	29.6	17.4	0.058
980	0.0	36.0	2.1		93.6	4.7	20.5	0.118

Example 6. 2017-PK-279-WT—CD71 vs IgG2A isotype, HPRT vs MSTN siRNA Design and Synthesis

MSTN: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAUUAUUUGUUCUUUGCCUU (SEQ ID NO: 14226). Base, sugar and phosphate modifications were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker.

HPRT: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAAAAUCUACAGUCAUAGUU (SEQ ID NO: 14227). Base, sugar and phosphate modifications were used

to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 3' end and a C6-SH at the 5' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker.

Negative control siRNA sequence (scramble): A published (Burke et al. (2014) Pharm. Res., 31(12):3445-60) 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGC-UAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via a phosphodiester -inverted abasic-phosphodiester linker.

ASC Synthesis and Characterization

The CD71 mAb-siRNA DAR1 conjugates were made, purified and characterized as described in Example 3. All conjugates were made through cysteine conjugation, a SMCC linker and the PEG was attached at the thiol using architecture 1 for MSTN and the scrambled siRNA and architecture 2 for the HPRT siRNA, see Example 3. Conjugates were characterized chromatographically as described in Table 6.

TABLE 6

HPLC retention time (RT) in minutes

Groups	Conjugate	RT, SAX Method-2	RT, SEC Method-1
1-4	TfR-mAb-HPRT-PEG5k; DAR1	8.8	7.1
5-8	IgG2a-mAb-HPRT-PEG5k; DAR1	8.9	7.7
9-12	TfR-mAb-MSTN-PEG5k; DAR1	8.7	7.2
13-16	IgG2a-mAb-MSTN-PEG5k; DAR1	8.9	7.7
17-20	TfR-mAb-scrambled-PEG5k; DAR1	8.4	7.2

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of myostatin (MSTN) in skeletal muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs and doses, see FIG. **9**A. After 96 hours, gastrocnemius (gastroc) muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Results

For gastrocnemius muscle harvested 96 hours post-dose, maximum MSTN mRNA downregulation of greater than 90% was observed after a single intravenous dose of 3 mg/kg of siRNA, see FIG. **9**B. In addition, a dose response was also observed (dose range: 0.3 to 3.0 mg/kg siRNA) and no significant mRNA downregulation was observed for the control groups.

Conclusions

In gastrocnemius muscle, it was demonstrated that an ASC is able to downregulate a muscle specific gene. The ASC was made with an anti-transferrin antibody conjugated to an siRNA designed to down regulate MSTN mRNA. Mouse gastroc muscle expresses the transferrin receptor and the conjugate has a mouse specific anti-transferrin antibody to target the siRNA, resulting in accumulation of the conjugates in gastroc muscle. Receptor mediate uptake resulted in siRNA mediated knockdown of the MSTN mRNA.

Example 7. 2017-PK-289-WT—CD71 mAb MSTN Time Course for Phenotype siRNA Design and Synthesis

MSTN: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3") of the guide/antisense strand was UUAUUAUUUGUUCUUUGCCUU (SEQ ID NO: 14226). Base, sugar and phosphate modifications were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

Negative control siRNA sequence (scramble): A published (Burke et al. (2014) Pharm. Res., 31(12):3445-60) 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGC-UAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

ASC Synthesis and Characterization

The CD71 mAb-siRNA DAR1 conjugates were made and characterized as described in Example 3. All conjugates were made through cysteine conjugation, a SMCC linker and the thiol was end capped with NEM using architecture 1. Conjugates were characterized chromatographically as described in Table 7.

TABLE 7

HPLC retention time (RT) in minutes			
Groups	Conjugate	RT, SAX Method-2	RT, SEC Method-1
1-4, 13 & 14	TfR-mAb-MSTN-NEM; DAR1	8.7	10.0
5-8	TfR-mAb-scrambled-NEM; DAR1	8.9	10.0

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of myostatin (MSTN) in skeletal muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs at the doses indicated in FIG. **10**A. Plasma and tissue samples were also taken as indicated in FIG. **10**A. Muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt). Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

Plasma myostatin levels were determined using an ELISA, see Example 2 for full experimental details. Changes in leg muscle area were determined: The leg-to-be-measured were shaved and a line was drawn using indelible ink to mark region of measurement. Mice were restrained in a cone restraint and the right leg was held by hand. Digital calipers were used to take one measurement on the sagittal plane and another on the coronal plane. The procedure was repeated twice per week.

Results

Quantifiable levels of siRNA accumulated in muscle tissue after a single intravenous dose of the antibody siRNA conjugates, see FIG. **10**B. Robust MSTN mRNA downregulation was observed in gastrocnemius muscle, which resulted in a reduction in the levels of MSTN protein in the plasma, after a single intravenous dose of 3 mg/kg of siRNA, see FIG. **10**C and FIG. **10**D. Maximum mRNA downregulation of ~90% was observed between 7-14 days post-dose. At 6 weeks post-dose gastroc muscle had approximately 75% mRNA downregulation, which corresponded to about a 50% reduction in plasma protein levels relative to the PBS or anti-transferrin antibody conjugated scrambled controls. Downregulation of MSTN resulted in statistically significant increases in muscle size, see FIG. **10**E and FIG. **10**F.

Conclusions

In this example it was demonstrated that accumulation of siRNA in various muscle tissues after a single dose of an anti-transferrin antibody targeted siRNA conjugate. In Gastroc muscle, significant and long-lasting siRNA mediated MSTN mRNA downregulation was observed. Mouse gastroc muscle expresses transferrin receptor and the conjugate has a mouse specific anti-transferrin antibody to target the siRNA, resulting in accumulation of the conjugates in gastroc muscle. Receptor mediate uptake resulted in siRNA mediated knockdown of the MSTN gene.

Example 8: 2017-PK-299-WT—MSTN Zalu Vs TfR, mAb Vs Fab, DAR1 vs DAR2 siRNA Design and Synthesis

MSTN: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAUUAUUUGUUCUUUGCCUU (SEQ ID NO: 14226). Base, sugar and phosphate modifications were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

MSTN*: MSTN: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAUUAUUUGUUCUUUGCCUU (SEQ ID NO: 14226). Base, sugar and phosphate modifications were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained one conjugation handle, a C6-NH_2 at the 5' end, which was connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker.

ASC Synthesis and Characterization

The CD71 mAb-siRNA DAR1 and DAR2 conjugates were made and characterized as described in Example 3. Groups 1-8 and 17-20 were made through cysteine conjugation and a BisMal linker using architecture 3. Groups 9-16 were made through cysteine conjugation, a SMCC linker and the free thiol was end capped with NEM PEG using architecture 1. Conjugates were characterized chromatographically as described in Table 8.

TABLE 8

HPLC retention time (RT) in minutes			
Groups	Conjugate	RT, SAX Method-2	RT, SEC Method-1
1-4	TfR-Fab-MSTN; DAR1	8.7	10.0
5-8	EGFR-Fab-MSTN; DAR1	8.9	10.0
9-12	TfR-mAb-MSTN-NEM; DAR1	9.5	7.9
13-16	TfR-mAb-MSTN-nEM; DAR2	10.3	8.1
17-18	EGFR-mAb-MSTN; DAR1	9.3	NT
19-20	EGFR-mAb-MSTN; DAR2	10.2	NT

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of myostatin (MSTN) in skeletal muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs at the doses indicated in FIG. **11**A. Plasma

and tissue samples were also taken as indicated in FIG. **11**A. Gastrocnemius (gastroc) muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt). Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

Results

Quantifiable levels of siRNA accumulated in muscle tissue after a single intravenous dose of the antibody and Fab siRNA conjugates, see FIG. **11**B. Robust MSTN mRNA downregulation was observed in gastroc muscle, when the anti-transferrin antibody conjugate was administered as a DAR1 or DAR2, or as a Fab DAR1 conjugate, see FIG. **11**C.

Conclusions

In this example it was demonstrated that accumulation of siRNA in gastroc muscle tissue after a single dose of an anti-transferrin antibody and Fab targeted siRNA conjugates. In Gastroc muscle, siRNA mediated MSTN mRNA downregulation with DAR1 and DAR2 antibody conjugates were observed, in addition to the DAR1 Fab conjugate. Mouse gastroc muscle expresses transferrin receptor and the conjugate has a mouse specific anti-transferrin antibody or Fab to target the siRNA, resulting in accumulation of the conjugates in gastroc muscle. Receptor mediate uptake resulted in siRNA mediated knockdown of the MSTN gene.

Example 9: 2017-PK-303-WT—Dose Response MSTN mAb Vs Fab Vs Chol siRNA Design and Synthesis

MSTN: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAUUAUUUGUUCUUUGCCUU (SEQ ID NO: 14226). Base, sugar and phosphate modifications were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

MSTN*: MSTN: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAUUAUUUGUUCUUUGCCUU (SEQ ID NO: 14226). Base, sugar and phosphate modifications were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained one conjugation handle, a C6-NH_2 at the 5' end, which was connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker.

Negative control siRNA sequence (scramble): A published (Burke et al. (2014) Pharm. Res., 31(12):3445-60) 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGCUAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

ASC Synthesis and Characterization

The CD71 mAb-siRNA DAR1 and DAR2 conjugates were made and characterized as described in Example 3. Groups 5-12 made through cysteine conjugation, a BisMal linker using architecture 3. Groups 13-16 were made through cysteine conjugation, a SMCC linker, the free thiol was end capped with NEM using architecture 1. Groups 17-20 were made through cysteine conjugation, a BisMal linker, the free thiol was end capped with NEM using architecture 3. Conjugates were characterized chromatographically as described Table 9.

TABLE 9

		HPLC retention time (RT) in minutes	
Groups	Conjugate	RT, SAX Method-2	RT, SEC Method-1
5-8	TfR-Fab-MSTN*; DAR1	8.7	10.0
9-12	TfR-mAb-MSTN*; DAR1	9.3	7.8
13-16	TfR-mAb-MSTN; DAR1	9.5	7.9
17-20	TfR-mAb-scramble; DAR1	9.1	7.3

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of myostatin (MSTN) in skeletal muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs at the doses indicated in FIG. **12**A. Tissue samples were also taken as indicated in FIG. **12**A. Gastrocnemius (gastroc) muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value

(ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt). Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

Intracellular RISC loading was determined as described in Example 2.

Results

Quantifiable levels of siRNA accumulated in gastroc and heart tissue, see FIG. **12**B, after a single intravenous dose of the antibody and Fab siRNA conjugates. Robust MSTN mRNA downregulation was observed in gastroc muscle, when the ASC was targeted with either the anti-transferrin receptor antibody or Fab see FIG. **12**B and FIG. **12**C. Much higher concentrations of siRNA were delivered to heart tissue, but this did not result in robust myostatin mRNA downregulation, see FIG. **12**B. Compared to the cholesterol siRNA conjugate, much lower doses of the ASCs were required to achieve equivalent mRNA downregulation. The amount of RISC loading of the MSTN siRNA guide strand correlated with downregulation of the mRNA, see FIG. **12**D.

Conclusions

In this example it was demonstrated that accumulation of siRNA in gastrocnemius muscle tissue after a single dose of an anti-transferrin antibody and Fab targeted siRNA conjugates. In Gastroc muscle, siRNA mediated MSTN mRNA downregulation with the DAR1 anti-transferrin antibody or Fab conjugates was observed. Mouse gastroc muscle expresses transferrin receptor and the conjugate have a mouse specific anti-transferrin antibody or Fab to target the payload, resulting in accumulation of the conjugates in gastroc muscle and loading into the RISC complex. Receptor mediate uptake resulted in siRNA mediated MSTN mRNA downregulation.

Example 10: 2017-PK-304-WT—PK with MSTN Phenotype mAb Vs Chol siRNA Design and Synthesis

MSTN: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAUUAUUUGUUCUUUGCCUU (SEQ ID NO: 14226). Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

Negative control siRNA sequence (scramble): A published (Burke et al. (2014) Pharm. Res., 31(12):3445-60) 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGCUAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

ASC Synthesis and Characterization

The CD71 mAb-siRNA DAR1 and DAR2 conjugates were made and characterized as described in example 3. Groups 5-12 were made through cysteine conjugation, a SMCC linker, the free thiol was end capped with NEM using architecture 1. Groups 13-16 were made through cysteine conjugation, a BisMal linker, the free thiol was end capped with NEM using architecture 3. Conjugates were characterized chromatographically as described in Table 10.

TABLE 10

	HPLC retention time (RT) in minutes		
Groups	Conjugate	RT, SAX Method-2	RT, SEC Method-1
5-8	TfR-mAb-MSTN; DAR1	9.5	7.9
9-12	TfR-mAb-MSTN; DAR2	10.3	7.6
13-16	TfR-mAb-scramble; DAR1	9.1	7.3

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of myostatin (MSTN) in skeletal muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs at the doses indicated in FIG. **13**A. Tissue samples were also taken as indicated in FIG. **13**A. Gastrocnemius (gastroc) muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt). Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

Intracellular RISC loading was determined as described in Example 2. Plasma MSTN protein levels were measured by ELISA as described in Example 2.

Changes in leg muscle area were determined: the leg-to-be measured were shaved and a line was drawn using indelible ink to mark region of measurement. Mice were

restrained in a small decapicone bag. Digital calipers were used to take one measurement on the sagittal plane and another on the coronal plane. The procedure was repeated twice per week.

Results

Quantifiable levels of siRNA accumulated in gastrocnemius, triceps, quadriceps (Quad), and heart tissues, see FIG. **13**D, after a single intravenous dose of the antibody siRNA conjugates at 3 mg/kg. MSTN mRNA downregulation was observed in gastrocnemius, quadriceps, and triceps with the DAR1 and DAR2 conjugates but not in heart tissue, see FIG. **13**B. MSTN mRNA downregulation resulted in a reduction in the plasma concentration of MSTN protein, as measured by ELISA, see FIG. **13**C. The amount of RISC loading of the MSTN siRNA guide strand correlated with downregulation of the mRNA, see FIG. **13**E. Downregulation of MSTN resulted in statistically significant increases in muscle size, see FIG. **13**F and FIG. **13**G.

Conclusions

In this example it was demonstrated that accumulation of siRNA in gastrocnemius, quadriceps, and triceps muscle tissues after a single dose of anti-transferrin antibody siRNA conjugates, DAR1 and DAR2. In all three tissues, measurable siRNA mediated MSTN mRNA downregulation with the DARI and DAR2 anti-transferrin antibody conjugates was observed. mRNA downregulation correlated with a reduced level of plasma MSTN protein and RISC loading of the siRNA guide strand. All three muscle tissues expressed transferrin receptor and the conjugate has a mouse specific anti-transferrin antibody to target the siRNA, resulting in accumulation of the conjugates in muscle. Receptor mediate uptake resulted in siRNA mediated knockdown of the MSTN gene.

Example 11: 2017-PK-355-WT Multiple siRNA Dosing siRNA Design and Synthesis

HPRT: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUAAAAUCUACAGUCAUAGUU (SEQ ID NO: 14227). Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained a single conjugation handles, a C6-NH_2 at the 5', which was connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker.

SSB: A 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was designed against mouse MSTN. The sequence (5' to 3') of the guide/antisense strand was UUACAUUAAAGUCUGUUGUUU (SEQ ID NO: 14229). Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained a single conjugation handles, a C6-NH_2 at the 5', which was connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker.

ASC Synthesis and Characterization

The CD71 mAb-siRNA conjugates were made and characterized as described in Example 3.

Groups 1-4 and 5-8 were made through cysteine conjugation, a BisMal linker, no 3' conjugation handle on the passenger strand using architecture 3. Groups 13-16 were made through cysteine conjugation, a BisMal linker, no 3' conjugation handle on the passenger strand, but were DAR2 conjugates made with a mixture of HPRT and SSB siRNAs using architecture 4. Conjugates were characterized chromatographically as described in Table 11.

TABLE 11

HPLC retention time (RT) in minutes

Conjugate	RT, SAX Method-2	RT, SEC Method-1
TfR-mAb-HPRT; DAR1	9.0	12.5 (0.5 ml flow rate, 25 min run)
TfR-mAb-SSB; DAR1	9.4	No Data
TfR-mAb-HPRT/SSB (1:1) DAR2	10.09	No Data

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of two house keeper genes (HPRT and SSB) in skeletal muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs at the doses indicated in FIG. **14**A. Tissue samples were also taken as indicated in FIG. **14**A. Gastrocnemius (gastroc) muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference ($\Delta\Delta$Ct). Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

The RISC loading assay was conducted as described in Example 2.

Results

After a single intravenous dose of the antibody siRNA conjugates at the indicated doses, mRNA downregulation was observed in gastroc and heart tissue, see FIG. **14**B-FIG. **14**D. Co-administration of a mixture of two ASC's, targeting two different genes (HPRT and SSB) resulted in efficient mRNA downregulation of both targets in gastroc and heart tissue. In addition, administration of a DAR2 conjugate synthesized using a 1:1 mixture of the two different siRNAs (HPRT and SSB) also resulted in efficient mRNA down regulation of both targets in gastroc and heart tissue. All approaches to delivery resulted in measurable amounts of siRNA accumulating in gatroc tissue, see FIG. **14**F.

Conclusions

In this example, it was demonstrated that accumulation of siRNA in gastroc and heart tissue after a single dose of anti-transferrin antibody siRNA conjugates. Two genes were downregulated by co-administration of two ASC produced with the same anti-transferrin antibody but conjugated to two different siRNAs (HPRT and SSB). In addition, two genes were downregulated by an anti-transferrin mAb DAR2 conjugate synthesized using a 1:1 mixture of two different siRNAs (HPRT and SSB). In some instances, simultaneous downregulation of more than one gene is useful in muscle atrophy.

Example 12: 2017-PK-380-WT Activity of Atrogin-1 siRNAs In Vivo (Dose Response) siRNA Design and Synthesis

Atrogin-1 siRNAs: 4 different 21 mer duplexes with 19 bases of complementarity and 3' dinucleotide overhangs were designed against Atrogin-1, see Example 4 for details of the sequence. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. The same design was used for all four siRNAs. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

ASC Synthesis and Characterization

The CD71 mAb-siRNA conjugates were made and characterized as described in Example 3.

Groups 1-16 were made through cysteine conjugation, a BisMal linker, the free thiol was end capped with NEM using architecture 3. Conjugates were characterized chromatographically as described Table 12.

TABLE 12

HPLC retention time (RT) in minutes			
Groups	Conjugate	RT, SAX Method-2	RT, SEC Method-1
1-4	mTfR1(Cys)-BisMal-N-mAtrogin#1179; DAR1	9.2	7.7
5-8	mTfR1(Cys)-BisMal-N-mAtrogin#1504; DAR1	9.3	7.8
9-12	mTfR1(Cys)-BisMal-N-mAtrogin#631; DAR1	9.3	7.8
13-16	mTfR1(Cys)-BisMal-N-mAtrogin#586; DAR1	9.2	7.8

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of Atrogin-1 in skeletal muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs at the doses indicated in FIG. **15**A. Tissue samples were taken as indicated in FIG. **15**A. Gastrocnemius (gastroc) muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Results

After a single intravenous dose of the antibody siRNA conjugates at the indicated doses, up to 80% atrogin-1 mRNA downregulation was observed in gastroc muscle and up to 50% in heart tissue, see FIG. **15**B and FIG. **15**C.

Conclusions

As illustrated in this example, antibody siRNA conjugates differentially downregulate Atrogin-1 in muscle and heart.

Example 13: 2017-PK-383-WT Activity of MuRF1 siRNA In Vivo (Dose Response) siRNA Design and Synthesis

MuRF1 siRNAs: 4 different 21 mer duplexes with 19 bases of complementarity and 3' dinucleotide overhangs were designed against Atrogin-1, see Example 5 for details of the sequence. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. The same design was used for all four siRNAs. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphorothioate-inverted abasic-phosphorothioate linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

ASC Synthesis and Characterization

The CD71 mAb-siRNA conjugates were made and characterized as described in Example 3.

Groups 1-16 were made through cysteine conjugation, a BisMal linker, the free thiol was end capped with NEM using architecture 3. Conjugates were characterized chromatographically as described Table 13.

TABLE 13

HPLC retention time (RT) in minutes			
Groups	Conjugate	RT, SAX Method-2	RT, SEC Method-1
1-4	mTfR1(Cys)-BisMal-N-MuRF#651-S-NEM; DAR1	9.2	7.8
5-8	mTfR1(Cys)-BisMal-N-MuRF#1387-S-NEM; DAR1	9.3	7.8
9-13	mTfR1(Cys)-BisMal-N-MuRF#1454-S-NEM; DAR1	9.1	7.8
14-18	mTfR1(Cys)-BisMal-N-MuRF#1660-S-NEM; DAR1	9.1	7.8

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of MuRF-1 in skeletal and heart muscle in vivo in wild type CD-1 mice. Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs at the doses indicated in FIG. **16**A. Tissue samples were taken as indicated in FIG. **16**A. Gastrocne-

mius (gastroc) muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Results

After a single intravenous dose of the antibody siRNA conjugates at the indicated doses, MuRF1 mRNA in gastroc muscle was downregulated to up to 70% and up to 50% in heart tissue, see FIG. **16**B and FIG. **16**C.

Conclusions

As illustrated in this example, antibody siRNA conjugates differentially downregulate MuRF1 in muscle and heart.

Example 14

Table 14 illustrates exemplary siRNA (or atrogene) targets to regulate muscle atrophy. In some instances, a polynucleic acid molecule hybridizes to a target region of an atrogene described in Table 14.

Function	Gene	Name
Protein Degradation	FBXO32	Atrogin-1
	Trim63	MuRF1
	TRAF6	TNF receptor-associated factor 6
	USP14	Ubiquitin specific protease 14
	CTSL2	Cathepsin L2
Transcription	Foxo1	Forkhead box O1
	Foxo3	Forkhead box O3
	TGIF	TG interacting factor
	MYOG	myogenin
	HDAC2	Histone deacetylase 2
	HDAC3	Histone deacetylase 3
Stress Response	MT1L	Metallothionein 1L
	MT1B	Metallothionein 1B

Example 15: Sequences

23-mer target sequences within one DMPK transcript variant (NM_001288766) are assigned with SEQ ID NOs: 703-3406. The set of 23-mer target sequences for this transcript variant was generated by walking down the length of the transcript one base at a time, and a similar set of target sequences could be generated for the other DMPK transcript variants using the same procedure. One common siRNA structure that can be used to target these sites in the DMPK transcript is a 19-mer fully complimentary duplex with 2 overhanging (not base-paired) nucleotides on the 3' end of each strand. Thus, adding the 19-mer with both of the 2 nucleotide overhangs results in a total of 23 bases for the target site. Since the overhangs can be comprised of a sequence reflecting that of the target transcript or other nucleotides (for example a non-related dinucleotide sequence such as "UU"), the 19-mer fully complimentary sequence can be used to describe the siRNA for each 23-mer target site.

19-mer sense and antisense sequences for siRNA duplexes targeting each site within the DMPK transcript are assigned with SEQ ID NOs: 3407-8814 (with the first sense and antisense pairing as SEQ ID NO: 3407 and SEQ ID NO: 6111). SEQ ID NOs: 3407-6110 illustrate the sense strand. SEQ ID NOs: 6111-8814 illustrate the antisense strand. The DMPK transcript variant NM_001288766 has been used for illustration but a similar set of siRNA duplexes can be generated by walking through the other DMPK transcript variants. When the antisense strand of the siRNA loads into Ago2, the first base associates within the Ago2 binding pocket while the other bases (starting at position 2 of the antisense strand) are displayed for complimentary mRNA binding. Since "U" is the thermodynamically preferred first base for binding to Ago2 and does not bind the target mRNA, all of the antisense sequences can have "U" substituted into the first base without affecting the target complementarity and specificity. Correspondingly, the last base of the sense strand 19-mer (position 19) is switched to "A" to ensure base pairing with the "U" at the first position of the antisense strand.

SEQ ID NOs: 8815-11518 are similar to SEQ ID NOs: 3407-6110 except the last position of the 19-mer sense strand substituted with base "A".

SEQ ID NOs: 11519-14222 are similar to SEQ ID NOs: 6111-8814 except the first position of the 19-mer antisense strand substituted with base "U".

SEQ ID NO: 8815 and SEQ ID NO: 11519 for the first respective sense and antisense pairing.

Example 16: Initial Screening of a Selected Set of DMPK siRNAs for In Vitro Activity

The initial set of DMPK siRNAs from SEQ ID NOs: 8815-14222 was narrowed down to a list of 81 siRNA sequences using a bioinformatic analysis aimed at selecting the sequences with the highest probability of on-target activity and the lowest probability of off-target activity. The bioinformatic methods for selecting active and specific siRNAs are well described in the field of RNAi and a person skilled in the arts would be able to generate a similar list of DMPK siRNA sequences against any of the other DMPK transcript variants. The DMPK siRNAs in the set of 81 sequences were synthesized on small scale using standard solid phase synthesis methods that are described in the oligonucleotide synthesis literature. Both unmodified and chemically modified siRNAs are known to produce effective knockdown following in vitro transfection. The DMPK siRNA sequences were synthesized using base, sugar and phosphate modifications that are described in the field of RNAi to optimize the potency of the duplex and reduce immunogenicity. Two human cell lines were used to assess the in vitro activity of the DMPK siRNAs: first, SJCRH30 human rhabdomyosarcoma cell line (ATCC® CRL-2061™); and second, Myotonic Dystrophy Type 1 (DM1) patient-derived immortalized human skeletal myoblasts. For the initial screening of the DMPK siRNA library, each DMPK siRNA was transfected into SJCRH30 cells at 1 nM and 0.01 nM final concentration, as well as into DM1 myoblasts at 10 nM and 1 nM final concentration. The siRNAs were formulated with transfection reagent Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's "forward transfection" instructions. Cells were plated 24 h prior to transfection in triplicate on 96-well tissue culture plates, with 8500 cells per well for SJCRH30 and 400 1 cells per well for DM1 myoblasts. At 48 h (SJCRH30) or 72 h (DM1 myoblasts) post-transfection cells were washed with PBS and harvested with TRIzol® reagent (Life Technologies). RNA was isolated using the Direct-zol-96 RNA Kit (Zymo Research) according to the manufactur-

er's instructions. 10 μl of RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA samples were evaluated by qPCR with DMPK-specific and PPIB-specific TaqMan human gene expression probes (Thermo Fisher) using TaqMan® Fast Advanced Master Mix (Applied Biosystems). DMPK values were normalized within each sample to PPIB gene expression. The quantification of DMPK downregulation was performed using the standard $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate, with Table 15A and Table 15B) presenting the mean values of the triplicates.

TABLE 15A

ID #[1]	sense strand sequence (5'-3') Passenger Strand (PS)	SEQ ID NO:	antisense strand sequence (5'-3') Guide Strand (GS)	SEQ ID NO:
385	GCUUAAGGAGGUCCGACUA	9199	UAGUCGGACCUCCUUAAGC	11903
443	GGGGCGUUCAGCGAGGUAA	9257	UUACCUCGCUGAACGCCCC	11961
444	GGGCGUUCAGCGAGGUAGA	9258	UCUACCUCGCUGAACGCCC	11962
445	GGCGUUCAGCGAGGUAGCA	9259	UGCUACCUCGCUGAACGCC	11963
533	AGGGGCGAGGUGUCGUGCA	9347	UGCACGACACCUCGCCCCU	12051
534	GGGGCGAGGUGUCGUGCUA	9348	UAGCACGACACCUCGCCCC	12052
535	GGGCGAGGUGUCGUGCUUA	9349	UAAGCACGACACCUCGCCC	12053
539	GAGGUGUCGUGCUUCCGUA	9353	UACGGAAGCACGACACCUC	12057
540	AGGUGUCGUGCUUCCGUGA	9354	UCACGGAAGCACGACACCU	12058
541	GGUGUCGUGCUUCCGUGAA	9355	UUCACGGAAGCACGACACC	12059
543	UGUCGUGCUUCCGUGAGGA	9357	UCCUCACGGAAGCACGACA	12061
544	GUCGUGCUUCCGUGAGGAA	9358	UUCCUCACGGAAGCACGAC	12062
576	UGAAUGGGGACCGGCGGUA	9390	UACCGCCGGUCCCCAUUCA	12094
577	GAAUGGGGACCGGCGGUGA	9391	UCACCGCCGGUCCCCAUUC	12095
581	GGGGACCGGCGGUGGAUCA	9395	UGAUCCACCGCCGGUCCCC	12099
583	GGACCGGCGGUGGAUCACA	9397	UGUGAUCCACCGCCGGUCC	12101
584	GACCGGCGGUGGAUCACGA	9398	UCGUGAUCCACCGCCGGUC	12102
690	AGUUUGGGGAGCGGAUUCA	9504	UGAAUCCGCUCCCCAAACU	12208
716	AUGGCGCGCUUCUACCUGA	9530	UCAGGUAGAAGCGCGCCAU	12234
717	UGGCGCGCUUCUACCUGGA	9531	UCCAGGUAGAAGCGCGCCA	12235

TABLE 15A-continued

ID #[1]	sense strand sequence (5'-3') Passenger Strand (PS)	SEQ ID NO:	antisense strand sequence (5'-3') Guide Strand (GS)	SEQ ID NO:
785	AGGGACAUCAAACCCGACA	9599	UGUCGGGUUUGAUGUCCCU	12303
786	GGGACAUCAAACCCGACAA	9600	UUGUCGGGUUUGAUGUCCC	12304
789	ACAUCAAACCCGACAACAA	9603	UUGUUGUCGGGUUUGAUGU	12307
1026	GGCAGACGCCCUUCUACGA	9840	UCGUAGAAGGGCGUCUGCC	12544
1027	GCAGACGCCCUUCUACGCA	9841	UGCGUAGAAGGGCGUCUGC	12545
1028	CAGACGCCCUUCUACGCGA	9842	UCGCGUAGAAGGGCGUCUG	12546
1029	AGACGCCCUUCUACGCGGA	9843	UCCGCGUAGAAGGGCGUCU	12547
1037	UUCUACGCGGAUUCCACGA	9851	UCGUGGAAUCCGCGUAGAA	12555
1039	CUACGCGGAUUCCACGGCA	9853	UGCCGUGGAAUCCGCGUAG	12557
1041	ACGCGGAUUCCACGGCGGA	9855	UCCGCCGUGGAAUCCGCGU	12559
1043	GCGGAUUCCACGGCGGAGA	9857	UCUCCGCCGUGGAAUCCGC	12561
1044	CGGAUUCCACGGCGGAGAA	9858	UUCUCCGCCGUGGAAUCCG	12562
1047	AUUCCACGGCGGAGACCUA	9861	UAGGUCUCCGCCGUGGAAU	12565
1071	AGAUCGUCCACUACAAGGA	9885	UCCUUGUAGUGGACGAUCU	12589
1073	AUCGUCCACUACAAGGAGA	9887	UCUCCUUGUAGUGGACGAU	12591
1262	CCCUUUACACCGGAUUUCA	10076	UGAAAUCCGGUGUAAAGGG	12780
1263	CCUUUACACCGGAUUUCGA	10077	UCGAAAUCCGGUGUAAAGG	12781
1264	CUUUACACCGGAUUUCGAA	10078	UUCGAAAUCCGGUGUAAAG	12782
1265	UUUACACCGGAUUUCGAAA	10079	UUUCGAAAUCCGGUGUAAA	12783
1267	UACACCGGAUUUCGAAGGA	10081	UCCUUCGAAAUCCGGUGUA	12785
1268	ACACCGGAUUUCGAAGGUA	10082	UACCUUCGAAAUCCGGUGU	12786
1269	CACCGGAUUUCGAAGGUGA	10083	UCACCUUCGAAAUCCGGUG	12787
1274	GAUUUCGAAGGUGCCACCA	10088	UGGUGGCACCUUCGAAAUC	12792
1276	UUUCGAAGGUGCCACCGAA	10090	UUCGGUGGCACCUUCGAAA	12794
1283	GGUGCCACCGACACAUGCA	10097	UGCAUGUGUCGGUGGCACC	12801

TABLE 15A-continued

ID #[1]	sense strand sequence (5'-3') Passenger Strand (PS)	SEQ ID NO:	antisense strand sequence (5'-3') Guide Strand (GS)	SEQ ID NO:
1297	AUGCAACUUCGACUUG GUA	10111	UACCAAGUCGAAGUUGCAU	12815
1342	ACUGUCGGACAUUCGG GAA	10156	UUCCCGAAUGUCCGACAGU	12860
1343	CUGUCGGACAUUCGGG AAA	10157	UUUCCCGAAUGUCCGACAG	12861
1344	UGUCGGACAUUCGGGA AGA	10158	UCUUCCCGAAUGUCCGACA	12862
1346	UCGGACAUUCGGGAAG GUA	10160	UACCUUCCCGAAUGUCCGA	12864
1825	UGCUCCUGUUCGCCGU UGA	10639	UCAACGGCGAACAGGAGCA	13343
1886	CCACGCCGGCCAACUC ACA	10700	UGUGAGUUGGCCGGCGUGG	13404
1890	GCCGGCCAACUCACCG CAA	10704	UUGCGGUGAGUUGGCCGGC	13408
1898	ACUCACCGCAGUCUGG CGA	10712	UCGCCAGACUGCGGUGAGU	13416
1945	CCCUAGAACUGUCUUC GAA	10759	UUCGAAGACAGUUCUAGGG	13463
1960	CGACUCCGGGGCCCCG UUA	10774	UAACGGGGCCCCGGAGUCG	13478
2126	GCCGGCGAACGGGGCU CGA	10940	UCGAGCCCCGUUCGCCGGC	13644
2127	CCGGCGAACGGGGCUC GAA	10941	UUCGAGCCCCGUUCGCCGG	13645
2149	UCCUUGUAGCCGGGAA UGA	10963	UCAUUCCCGGCUACAAGGA	13667
2150	CCUUGUAGCCGGGAAU GCA	10964	UGCAUUCCCGGCUACAAGG	13668
2268	CCCUGACGUGGAUGGG CAA	11082	UUGCCCAUCCACGUCAGGG	13786
2272	GACGUGGAUGGGCAAA CUA	11086	UAGUUUGCCCAUCCACGUC	13790
2528	GCUUCGGCGGUUUGGA UAA	11342	UUAUCCAAACCGCCGAAGC	14046
2529	CUUCGGCGGUUUGGAU AUA	11343	UAUAUCCAAACCGCCGAAG	14047
2530	UUCGGCGGUUUGGAUA UUA	11344	UAAUAUCCAAACCGCCGAA	14048
2531	UCGGCGGUUUGGAUAU UUA	11345	UAAAUAUCCAAACCGCCGA	14049
2532	CGGCGGUUUGGAUAUU UAA	11346	UUAAAUAUCCAAACCGCCG	14050
2554	CCUCGUCCUCCGACUC GCA	11368	UGCGAGUCGGAGGACGAGG	14072
2558	GUCCUCCGACUCGCUG ACA	11372	UGUCAGCGAGUCGGAGGAC	14076
2600	CAAUCCACGUUUUGGA UGA	11414	UCAUCCAAAACGUGGAUUG	14118

TABLE 15A-continued

ID #[1]	sense strand sequence (5'-3') Passenger Strand (PS)	SEQ ID NO:	antisense strand sequence (5'-3') Guide Strand (GS)	SEQ ID NO:
2628	CCGACAUUCCUCGGUA UUA	11442	UAAUACCGAGGAAUGUCGG	14146
2629	CGACAUUCCUCGGUAU UUA	11443	UAAAUACCGAGGAAUGUCG	14147
2631	ACAUUCCUCGGUAUUU AUA	11445	UAUAAAUACCGAGGAAUGU	14149
2636	CCUCGGUAUUUAUUGU CUA	11450	UAGACAAUAAAUACCGAGG	14154
2639	CGGUAUUUAUUGUCUG UCA	11453	UGACAGACAAUAAAUACCG	14157
2675	CCCCGACCCUCGCGAA UAA	11489	UUAUUCGCGAGGGUCGGGG	14193
2676	CCCGACCCUCGCGAAU AAA	11490	UUUAUUCGCGAGGGUCGGG	14194
2679	GACCCUCGCGAAUAAA AGA	11493	UCUUUUAUUCGCGAGGGUC	14197
2680	ACCCUCGCGAAUAAAA GGA	11494	UCCUUUUAUUCGCGAGGGU	14198
2681	CCCUCGCGAAUAAAAG GCA	11495	UGCCUUUUAUUCGCGAGGG	14199
2682	CCUCGCGAAUAAAAGG CCA	11496	UGGCCUUUUAUUCGCGAGG	14200
Neg. Con-trol	n/a	n/a	n/a	n/a

[1]19mer position in NM_001288766.1

TABLE 15B

ID #[1]	qPCR[2]	qPCR[3]	qPCR[4]	qPCR[5]
385	150.8	153.8	64.1	142.5
443	112.7	95.8	56.7	127.8
444	76.5	66.2	36.7	113.6
445	61.4	107.7	29.4	110.8
533	168.8	119.8	85.7	118.1
534	91.4	44.8	26.7	94.2
535	101.0	65.9	33.1	109.9
539	81.7	70.2	34.1	102.4
540	68.3	56.8	40.0	114.6
541	112.1	107.3	73.8	120.6
543	42.6	59.9	41.9	117.8
544	42.4	107.5	66.9	154.7
576	107.4	119.0	85.0	127.5
577	101.6	90.1	72.1	106.6
581	199.3	97.7	69.9	103.5
583	66.6	77.5	66.4	100.3
584	26.3	37.3	31.0	88.3
690	163.6	84.1	58.0	92.7
716	29.0	39.6	29.4	86.0
717	44.4	45.7	52.8	102.5
785	79.9	93.2	71.3	101.0
786	85.5	63.8	54.3	92.2
789	45.4	51.3	43.8	96.9
1026	55.6	77.3	32.0	110.4
1027	98.9	94.7	35.2	108.3
1028	132.1	104.9	27.3	87.3
1029	62.2	95.5	45.9	94.2
1037	68.2	80.2	65.3	97.0
1039	42.3	79.3	53.6	97.0
1041	67.2	64.4	73.2	98.6

TABLE 15B-continued

ID #[1]	qPCR[2]	qPCR[3]	qPCR[4]	qPCR[5]
1043	342.8	86.6	61.5	96.5
1044	109.5	84.8	42.7	94.0
1047	101.3	72.1	35.2	90.7
1071	88.5	99.6	91.6	101.3
1073	134.3	63.0	36.3	93.6
1262	36.5	59.6	27.3	117.5
1263	47.6	79.7	33.9	104.3
1264	64.2	54.5	43.7	95.4
1265	19.8	57.6	30.9	91.6
1267	61.3	85.9	73.4	97.1
1268	32.0	28.3	38.0	92.3
1269	42.6	49.1	42.4	96.7
1274	63.6	55.4	78.0	98.5
1276	52.2	36.9	35.2	82.5
1283	35.2	62.8	56.6	95.9
1297	20.3	55.7	32.2	91.0
1342	44.6	46.7	41.5	94.5
1343	65.8	80.0	56.1	119.2
1344	30.9	63.7	51.7	116.7
1346	133.8	102.9	98.0	104.0
1825	54.1	69.2	28.6	86.7
1886	786.9	282.0	130.5	98.4
1890	28.8	30.3	51.5	94.4
1898	125.5	57.5	67.7	97.6
1945	23.5	22.6	21.8	57.6
1960	28.4	33.7	35.7	87.9
2126	147.9	87.2	86.8	98.1
2127	46.5	51.9	52.7	96.2
2149	44.7	41.5	62.0	99.6
2150	110.4	89.1	63.4	114.1
2268	53.5	48.6	60.8	113.1
2272	56.5	54.7	46.9	92.5
2528	32.5	32.8	32.7	76.9
2529	19.6	25.8	21.4	59.5
2530	29.5	25.9	32.8	68.1
2531	22.2	31.6	25.4	64.3
2532	44.4	35.6	29.2	74.0
2554	13.7	22.6	26.8	60.9
2558	54.6	47.4	28.0	72.0
2600	205.4	209.6	n.d.	n.d.
2628	12.6	28.5	20.1	56.2
2629	12.8	39.5	20.6	63.8
2631	97.4	68.6	39.7	104.4
2636	62.0	68.6	16.8	58.7
2639	33.2	46.1	22.2	81.0
2675	57.7	82.5	n.d.	n.d.
2676	31.1	53.0	n.d.	n.d.
2679	44.7	75.7	n.d.	n.d.
2680	89.2	61.5	n.d.	n.d.
2681	19.0	28.6	n.d.	n.d.
2682	98.2	61.8	n.d.	n.d.
Neg. Control	101.2	100.6	101.1	106.4

[2]DM1 myoblasts; 10 nM; % DMPK mRNA
[3]DM1 myoblasts; 1 nM; % DMPK mRNA
[4]SJCRH30; 1 nM; % DMPK mRNA
[5]SJCRH30; 0.01 nM; % DMPK mRNA

Example 17: In Vitro Dose Response Curves for a Selected Set of DMPK siRNAs

To further validate the activity of the DMPK siRNAs, many of the sequences that showed the best activity in the initial screen were selected for a follow-up evaluation in dose response format. Once again, two human cell lines were used to assess the in vitro activity of the DMPK siRNAs: first, SJCRH30 human rhabdomyosarcoma cell line; and second, Myotonic Dystrophy Type 1 (DM1) patient-derived immortalized human skeletal myoblasts. The selected siRNAs were transfected in a 10-fold dose response at 100, 10, 1, 0.1, 0.01, 0,001, and 0.0001 nM final concentrations or in a 9-fold dose response at 50, 5.55556, 0.617284, 0.068587, 0.007621, 0.000847, and 0.000094 nM final concentrations. The siRNAs were formulated with transfection reagent Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's "forward transfection" instructions. Cells were plated 24 h prior to transfection in triplicate on 96-well tissue culture plates, with 8500 cells per well for SJCRH30 and 4000 cells per well for DM1 myoblasts. At 48 h (SJCRH30) or 72 h (DM1 myoblasts) post-transfection cells were washed with PBS and harvested with TRIzol® reagent (Life Technologies). RNA was isolated using the Direct-zol-96 RNA Kit (Zymo Research) according to the manufacturer's instructions. 10 µl of RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA samples were evaluated by qPCR with DMPK-specific and PPIB-specific TaqMan human gene expression probes (Thermo Fisher) using TaqMan® Fast Advanced Master Mix (Applied Biosystems). DMPK values were normalized within each sample to PPIB gene expression. The quantification of DMPK downregulation was performed using the standard $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate, with Tables 16A-B, 17A-B, and 18A-B presenting the mean values of the triplicates as well as the calculated IC_{50} values determined from fitting curves to the dose-response data by non-linear regression.

TABLE 16A

ID #[1]	sense strand sequence (5'-3') Passenger Strand (PS)	SEQ ID NO:	antisense strand sequence (5'-3') Guide Strand (GS)	SEQ ID NO:
535	GGGCGAGGUGU CGUGCUUA	9349	UAAGCACGACACCUCGCCC	12053
584	GACCGGCGGUG GAUCACGA	9398	UCGUGAUCCACCGCCGGUC	12102
716	AUGGCGCGCUU CUACCUGA	9530	UCAGGUAGAAGCGCGCCAU	12234
1028	CAGACGCCCUU CUACGCGA	9842	UCGCGUAGAAGGGCGUCUG	12546
1276	UUUCGAAGGUG CCACCGAA	10090	UUCGGUGGCACCUUCGAAA	12794
1825	UGCUCCUGUUC GCCGUUGA	10639	UCAACGGCGAACAGGAGCA	13343
1945	CCCUAGAACUG UCUUCGAA	10759	UUCGAAGACAGUUCUAGGG	13463
2529	CUUCGGCGGUU UGGAUAUA	11343	UAUAUCCAAACCGCCGAAG	14047
2558	GUCCUCCGACU CGCUGACA	11372	UGUCAGCGAGUCGGAGGAC	14076
2628	CCGACAUUCCU CGGUAUUA	11442	UAAUACCGAGGAAUGUCGG	14146
2636	CCUCGGUAUUU AUUGUCUA	11450	UAGACAAUAAAUACCGAGG	14154

[1]19mer position in NM_001288766.1

TABLE 16B

ID #[1]	qPCR[2]	qPCR[3]	qPCR[4]	qPCR[5]	qPCR[6]	qPCR[7]	qPCR[8]	IC50 (nM)
535	111.9	105.4	106.3	82.4	36.7	29.5	35.7	0.165
584	90.5	90.2	84.7	67.8	38.0	25.8	28.3	0.190
716	88.9	85.2	81.9	62.0	32.6	19.3	20.3	0.181
1028	88.5	81.8	83.0	61.3	32.7	27.3	31.5	0.127
1276	87.0	85.0	84.0	66.1	40.5	34.0	36.4	0.150
1825	85.1	85.9	83.7	69.1	36.2	25.2	25.0	0.259
1945	85.0	81.7	74.4	44.9	22.9	17.7	17.2	0.070
2529	83.3	81.8	75.3	50.6	24.6	17.5	17.7	0.103
2558	84.3	81.1	74.3	45.4	23.4	13.3	11.8	0.088
2628	85.3	84.0	79.5	59.8	30.3	23.5	25.1	0.140
2636	86.3	86.9	74.3	44.0	19.8	12.4	13.0	0.070

[2]SJCRH30; 0.0001 nM; % DMPK mRNA
[3]SJCRH30; 0.001 nM; % DMPK mRNA
[4]SJCRH30; 0.01 nM; % DMPK mRNA
[5]SJCRH30; 0.1 nM; % DMPK mRNA
[6]SJCRH30; 1 nM; % DMPK mRNA
[7]SJCRH30; 10 nM; % DMPK mRNA
[8]SJCRH30; 100 nM; % DMPK mRNA

TABLE 17A

ID #[1]	sense strand sequence (5'-3') Passenger Strand (PS)	SEQ ID NO:	antisense strand sequence (5'-3') Guide Strand (GS)	SEQ ID NO:
2600	CAAUCCACGUU UUGGAUGA	11414	UCAUCCAAAACGUGGAUUG	14118
2636	CCUCGGUAUUU AUUGUCUA	11450	UAGACAAUAAAUACCGAGG	14154
2675	CCCCGACCCUC GCGAAUAA	11489	UUAUUCGCGAGGGUCGGGG	14193
2676	CCCGACCCUCG CGAAUAAA	11490	UUUAUUCGCGAGGGUCGGG	14194
2679	GACCCUCGCGA AUAAAAGA	11493	UCUUUUAUUCGCGAGGGUC	14197
2680	ACCCUCGCGAA UAAAAGGA	11494	UCCUUUUAUUCGCGAGGGU	14198
2681	CCCUCGCGAAU AAAAGGCA	11495	UGCCUUUUAUUCGCGAGGG	14199
2682	CCUCGCGAAUA AAAGGCCA	11496	UGGCCUUUUAUUCGCGAGG	14200

[1]19mer position in NM_001288766.1

TABLE 17B

ID #[1]	qPCR[2]	qPCR[3]	qPCR[4]	qPCR[5]	qPCR[6]	qPCR[7]	IC50 (nM)
2600	107.5	107.6	108.1	106.3	103.1	72.7	31.31
2636	81.1	81.1	74.0	47.2	25.7	11.5	0.073
2675	88.1	88.3	84.3	64.6	38.1	20.7	0.151
2676	88.9	78.9	84.4	72.7	44.9	35.6	0.204
2679	84.0	87.3	82.7	53.3	31.4	13.5	0.091
2680	87.4	85.3	85.1	68.5	44.5	39.6	0.110
2681	87.0	85.4	77.6	49.6	26.5	16.0	0.061
2682	82.4	83.9	77.1	50.8	27.3	31.1	0.047

[2]SJCRH30; 0.000094 nM; % DMPK mRNA
[3]SJCRH30; 0.000847 nM; % DMPK mRNA
[4]SJCRH30; 0.007621 nM; % DMPK mRNA
[5]SJCRH30; 0.068587 nM; % DMPK mRNA
[6]SJCRH30; 0.617284 nM; % DMPK mRNA
[7]SJCRH30; 5.55556 nM; % DMPK mRNA

TABLE 18A

ID #[1]	sense strand sequence (5'-3') Passenger Strand (PS)	SEQ ID NO:	antisense strand sequence (5'-3') Guide Strand (GS)	SEQ ID NO:
584	GACCGGCGGUG GAUCACGA	9398	UCGUGAUCCACCGCCGGUC	12102
716	AUGGCGCGCUU CUACCUGA	9530	UCAGGUAGAAGCGCGCCAU	12234
1265	UUUACACCGGA UUUCGAAA	10079	UUUCGAAAUCCGGUGUAAA	12783
1297	AUGCAACUUCG ACUUGGUA	10111	UACCAAGUCGAAGUUGCAU	12815
1945	CCCUAGAACUG UCUUCGAA	10759	UUCGAAGACAGUUCUAGGG	13463
1960	CGACUCCGGGG CCCCGUUA	10774	UAACGGGGCCCCGGAGUCG	13478
2529	CUUCGGCGGUU UGGAUAUA	11343	UAUAUCCAAACCGCCGAAG	14047
2530	UUCGGCGGUUU GGAUAUUA	11344	UAAUAUCCAAACCGCCGAA	14048
2531	UCGGCGGUUUG GAUAUUUA	11345	UAAAUAUCCAAACCGCCGA	14049
2554	CCUCGUCCUCC GACUCGCA	11368	UGCGAGUCGGAGGACGAGG	14072
2628	CCGACAUUCCU CGGUAUUA	11442	UAAUACCGAGGAAUGUCGG	14146
2629	CGACAUUCCUC GGUAUUUA	11443	UAAAUACCGAGGAAUGUCG	14147
2681	CCCUCGCGAAU AAAAGGCA	11495	UGCCUUUUAUUCGCGAGGG	14199

[1]19mer position in NM_001288766.1

TABLE 18B

ID #[1]	qPCR[2]	qPCR[3]	qPCR[4]	qPCR[5]	qPCR[6]	qPCR[7]	IC50 (nM)
584	90.8	77.0	97.7	71.9	45.0	29.7	0.228
716	96.5	82.5	77.0	64.6	43.3	33.9	0.080
1265	68.5	80.9	68.0	57.1	37.5	25.7	0.146
1297	71.4	67.2	69.4	53.5	40.5	25.4	0.171
1945	71.8	62.3	41.7	29.8	22.4	15.3	0.006
1960	63.0	65.4	62.1	45.8	31.1	28.3	0.068
2529	63.5	58.7	49.2	31.1	22.9	21.9	0.017
2530	69.3	66.7	53.1	43.2	38.8	24.5	0.016
2531	69.9	72.4	57.3	40.2	35.4	25.6	0.018
2554	68.2	70.1	51.2	43.0	32.1	17.3	0.043
2628	69.7	67.9	62.5	38.4	31.6	17.1	0.042
2629	72.1	65.6	69.0	42.1	34.4	13.7	0.078
2681	82.4	91.5	87.6	55.5	29.3	19.6	0.084

[2]DM1 myoblasts; 0.000094 nM; % DMPK mRNA
[3]DM1 myoblasts; 0.000847 nM; % DMPK mRNA
[4]DM1 myoblasts; 0.007621 nM; % DMPK mRNA
[5]DM1 myoblasts; 0.068587 nM; % DMPK mRNA
[6]DM1 myoblasts; 0.617284 nM; % DMPK mRNA
[7]DM1 myoblasts; 5.55556 nM; % DMPK mRNA

Example 18: In Vitro Experiments to Determine Species Cross-Reactivity in Mouse

The selected siRNAs were transfected at 100, 10, 1, 0.1, 0.01, 0,001, and 0.0001 nM final concentrations into C2C12 mouse muscle myoblasts (ATCC® CRL-1772™). The siRNAs were formulated with transfection reagent Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's "forward transfection" instructions. Cells were plated 24 h prior to transfection in triplicate on 96-well tissue culture plates, with 4000 cells per well for C2C12 seeding. At 48 h post-transfection cells were washed with PBS and harvested with TRIzol® reagent (Life Technologies). RNA was isolated using the Direct-zol-96 RNA Kit (Zymo Research) according to the manufacturer's instructions. 10 μl of RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA samples were evaluated by qPCR with DMPK-specific and PPIB-specific TaqMan mouse gene expression probes (Thermo Fisher) using TaqMan® Fast Advanced Master Mix (Applied Biosystems). DMPK values were normalized within each sample to PPIB gene expression. The quantification of DMPK downregulation was performed using the standard $2^{-\Delta\Delta Ct}$ method. All experiments were performed in triplicate, with the results shown in FIG. **17**. Four DMPK siRNAs (the numbers indicated in the FIG. **17** legend correspond to the ID # that is listed in Table 19 (Tables 19A-19B)) were shown to effectively cross-react with mouse DMPK mRNA, producing robust mRNA knockdown in the mouse C2C12 myoblast cell line. Two of the siRNAs (ID #s 535 and 1028) were slightly less effective and only produced approximately 70% maximum mRNA knockdown. Two of the siRNAs (ID #s 2628 and 2636) were more effective and produced approximately 90% maximum mRNA knockdown.

Example 19: In Vivo Experiments to Determine Species Cross-Reactivity in Mouse

Animals

All animal studies were conducted following protocols in accordance with the Institutional Animal Care and Use Committee (IACUC) at Explora BioLabs, which adhere to the regulations outlined in the USDA Animal Welfare Act as well as the "Guide for the Care and Use of Laboratory Animals" (National Research Council publication, 8th Ed., revised in 2011). All mice were obtained from either Charles River Laboratories or Harlan Laboratories.

Conjugate Preparation

The in vivo studies used a total of five siRNAs: four DMPK siRNAs that were shown to be cross-reactive with mouse in vitro (FIG. **17**) and one siRNA with a scrambled sequence that does not produce DMPK knockdown and can be used as a negative control. All siRNAs were synthesized using standard solid phase synthesis methods that are described in the oligonucleotide synthesis literature. The single strands were purified by HPLC using standard methods and then pure single strands were mixed at equimolar ratios to generate pure duplexes. All siRNAs were synthesized with a hexylamine linker on the 5' end of the passenger (sense) strand that can act as a conjugation handle for linkage to the antibody. The siRNAs were synthesized using optimal base, sugar, and phosphate modifications that are well described in the field of RNAi to maximize the potency, maximize the metabolic stability, and minimize the immunogenicity of the duplex.

The anti-mouse transferrin receptor (TfR1, also known as CD71) monoclonal antibody (mAb) is a rat IgG2a subclass monoclonal antibody that binds mouse CD71 protein with high affinity. This CD71 antibody was produced by BioXcell and it is commercially available (Catalog # BE0175). The antibody-siRNA conjugates were synthesized using the CD71 mAb from BioXcell and the respective DMPK or scramble siRNAs. All conjugates were synthesized through cysteine conjugation to the antibody and amine conjugation to the siRNA (through the hexylamine) utilizing a bismaleimide-TFP ester linker as previously described. All conjugates were purified by strong cation exchange (SAX) to isolate only the conjugate with a drug-antibody ratio (DAR) equal to 1 (i.e. a molar ratio of 1 siRNA per mAb), as previously described. All antibody-siRNA conjugates were formulated by dilution in PBS for in vivo dosing.

In Vivo Dosing and Analysis

Purified DAR1 antibody-siRNA conjugates were dosed into groups (n=4) of female wild-type CO-1 mice (4-6 weeks old) at 0.1, 0.3, 1, and 3 mg/kg (based on the weight of siRNA) by a single i.v. bolus injection into the tail vein at a dosing volume of 5 mL/kg. A single sham dose of PBS vehicle was injected at matched dose volumes into a control group (n=5) of female wild-type CD-1 mice (also 4-6 weeks old). The mice were sacrificed by CO_2 asphyxiation 7 days post-dose and 20-30 mg pieces of multiple tissues (gastrocnemius, tibialis anterior, quadriceps, diaphragm, heart, and liver) were harvested from each mouse and snap-frozen in liquid nitrogen. TRIzol® reagent (Life Technologies) was added and then each tissue piece was homogenized using a TissueLyser II (Qiagen). RNA was isolated using the Direct-zol-96 RNA Kit (Zymo Research) according to the manufacturer's instructions. 10 μl of RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA samples were evaluated by qPCR with DMPK-specific and PPIB-specific TaqMan mouse gene expression probes (Thermo Fisher) using TaqMan® Fast Advanced Master Mix (Applied Biosystems). DMPK values were normalized within each sample to PPIB gene expression. The quantification of DMPK downregulation was performed using the standard $2^{-\Delta\Delta Ct}$ method by comparing the treated animals to the PBS control group. The in vivo DMPK mRNA knockdown results are presented in FIG. **18**A-FIG. **18**F. All four DMPK siRNAs (the numbers indi-

cated in the FIG. **18**A-FIG. **18**F legends correspond to the ID # that is listed in Table 19 (Tables 19A-19B)) were shown to effectively reduce levels of DMPK mRNA in all skeletal muscles that were analyzed (gastrocnemius, tibialis anterior, quadriceps, and diaphragm) in a dose-dependent manner. The most active siRNA achieved greater than 75% DMPK mRNA knockdown in all skeletal muscles at the highest dose (3 mg/kg). The in vivo DMPK knockdown observed in skeletal muscles of mice (FIG. **18**A-FIG. **18**F) correlated well with the in vitro DMPK knockdown observed in the mouse C2C12 myoblast cell line (FIG. **17**), with siRNA ID #s 2628 and 2636 demonstrating higher mRNA knockdown than siRNA ID #s 535 and 1028. In addition to DMPK mRNA knockdown in skeletal muscle, strong activity (greater than 50% mRNA knockdown) was observed in mouse cardiac muscle (heart) as well. Finally, poor activity (less than 50% mRNA knockdown) was observed in mouse liver. These results demonstrate that it is possible to achieve robust DMPK mRNA knockdown in multiple mouse muscle groups (including both skeletal and cardiac), while minimizing the knockdown in off-target tissues such as the liver.

Example 20: siRNA Synthesis

All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified using HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. All the siRNA passenger strands contained a C6-NH_2 conjugation handle on the 5' end, see FIG. **20**A-FIG. **21**B. For the 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs, the conjugation handle was connected to siRNA passenger strand via an inverted abasic phosphodiester, see FIG. **20**A-FIG. **20**B for the structures. For the blunt ended duplex with 19 bases of complementarity and one 3' dinucleotide overhang the conjugation handle was connected to siRNA passenger strand via a phosphodiester on the terminal base, see FIG. **21**A-FIG. **21**B for the structures.

Purified single strands were duplexed to get the double stranded siRNA.

Example 21: 2017-PK-401-C57BL6: In Vivo Transferrin mAb Conjugate Delivery of Various Atrogin-1 siRNAs

For groups 1-4, see study design in FIG. **22**, the 21 mer Atrogin-1 guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UCUACGUAGUUGAAUCUUCUU (SEQ ID NO: 14230). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

Antibody siRNA Conjugate Synthesis Using Bis-Maleimide (BisMal) Linker

Step 1: Antibody Reduction with TCEP

Antibody was buffer exchanged with 25 mM borate buffer (pH 8) with 1 mM DTPA and made up to 10 mg/ml concentration. To this solution, 4 equivalents of TCEP in the same borate buffer were added and incubated for 2 hours at 37° C. The resultant reaction mixture was combined with a solution of BisMal-siRNA (1.25 equivalents) in pH 6.0 10 mM acetate buffer at RT and kept at 4° C. overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA. The reaction mixture was treated with 10 EQ of N-ethylmaleimide (in DMSO at 10 mg/mL) to cap any remaining free cysteine residues.

Step 2: Purification

The crude reaction mixture was purified by AKTA Pure FPLC using anion exchange chromatography (SAX) method-1. Fractions containing DAR1 and DAR2 antibody-siRNA conjugates were isolated, concentrated, and buffer exchanged with pH 7.4 PBS.

Anion Exchange Chromatography Method (SAX)-1.

Column: Tosoh Bioscience, TSKGel SuperQ-5PW, 21.5 mm ID×15 cm, 13 um

Solvent A: 20 mM TRIS buffer, pH 8.0; Solvent B: 20 mM TRIS, 1.5 M NaCl, pH 8.0; Flow Rate: 6.0 ml/min

Gradient:

a.	% A	% B	Column	Volume
b.	100	0	1	
c.	81	19		0.5
d.	50	50		13
e.	40	60		0.5
f.	0	100		0.5
g.	100	0	2	

Anion Exchange Chromatography (SAX) Method-2

Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm

Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl; Flow Rate: 0.75 ml/min

Gradient:

a.	Time	% A	% B
b.	0.0	90	10
c.	3.00	90	10
d.	11.00	40	60
e.	14.00	40	60
f.	15.00	20	80
g.	16.00	90	10
h.	20.00	90	10

Step-3: Analysis of the Purified Conjugate

The purity of the conjugate was assessed by analytical HPLC using anion exchange chromatography method-2. For conjugate mTfR1-mAb-Atrogin-1 (DAR1), the SAX retention time was 9.1 min and % purity (by chromatographic peak area) was 99.

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of Atrogin-1 in skeletal muscle, in an in vivo experiment (C57BL6 mice). Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs and doses, see FIG. **22**. After the indicated time points, gastrocnemius (gastroc) and heart muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse

transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Results

The Atrogin-1 siRNA guide strands was able to mediate downregulation of the target gene in gastroc and heart muscle when conjugated to an anti-TfR mAb targeting the transferrin receptor, see FIG. **23** and FIG. **24**.

Conclusions

In this example, it was demonstrated that a TfR1-siA-trogin-1 conjugate, after in vivo delivery, mediated specific down regulation of the target gene in gastroc and heart muscle. The ASC was made with an anti-transferrin antibody, mouse gastroc and heart muscle expresses the transferrin receptor and the conjugate has a mouse specific anti-transferrin antibody to target the siRNA, resulting in accumulation of the conjugates in gastroc and heart muscle. Receptor mediate uptake resulted in siRNA mediated knockdown of the target mRNA.

Example 22: 2017-PK-413-C57BL6: In Vivo Transferrin mAb Conjugate Delivery of Various MuRF1 Sequence

For groups 1-2, see study design in FIG. **25**, the 21 mer MuRF1 (2089) guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UUUCGCAC-CAACGUAGAAAUU (SEQ ID NO: 14231). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linkers. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

For groups 3-6, see study design in figure G, the 21 mer MuRF1 (2265) guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UCGUGAGAC-AGUAGAUGUUUU (SEQ ID NO: 14232). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained a single conjugation handle, a C6-NH_2 at the 5' end connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker.

For groups 7-10, see study design in figure G, the 21 mer MuRF1 (2266) guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UCACACGUGA-GACAGUAGAUU (SEQ ID NO: 14233). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained a single conjugation handle, a C6-NH_2 at the 5' end connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker.

For groups 11-14, see study design in figure G, the 21 mer MuRF1 (2267) guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UUCACACGUGA-GACAGUAGUU (SEQ ID NO: 14234). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained a single conjugation handle, a C6-NH_2 at the 5' end connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker.

For groups 15-18, see study design in figure G, the 21 mer MuRF1 (2268) guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UAAUAUUU-CAUUUCGCACCUU (SEQ ID NO: 14235). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained a single conjugation handle, a C6-NH_2 at the 5' end connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker.

For groups 19-22, see study design in figure G, the 21 mer MuRF1 (2269) guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UAAGCAC-CAAAUUGGCAUAUU (SEQ ID NO: 14236). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained a single conjugation handle, a C6-NH_2 at the 5' end connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker.

Antibody siRNA Conjugate Synthesis Using Bis-Maleimide (BisMal) Linker

Step 1: Antibody reduction with TCEP

Antibody was buffer exchanged with 25 mM borate buffer (pH 8) with 1 mM DTPA and made up to 10 mg/ml concentration. To this solution, 4 equivalents of TCEP in the same borate buffer were added and incubated for 2 hours at 37° C. The resultant reaction mixture was combined with a solution of BisMal-siRNA (1.25 equivalents) in pH 6.0 10 mM acetate buffer at RT and kept at 4° C. overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA. The reaction mixture was treated with 10 EQ of N-ethylmaleimide (in DMSO at 10 mg/mL) to cap any remaining free cysteine residues.

Step 2: Purification

The crude reaction mixture was purified by AKTA Pure FPLC using anion exchange chromatography (SAX) method-1. Fractions containing DAR1 and DAR2 antibody-siRNA conjugates were isolated, concentrated and buffer exchanged with pH 7.4 PBS.

Anion Exchange Chromatography Method (SAX)-1.

Column: Tosoh Bioscience, TSKGel SuperQ-5PW, 21.5 mm ID×15 cm, 13 um

Solvent A: 20 mM TRIS buffer, pH 8.0; Solvent B: 20 mM TRIS, 1.5 M NaCl, pH 8.0; Flow Rate: 6.0 ml/min

Gradient:

a.	% A	% B	Column Volume
b.	100	0	1
c.	81	19	0.5
d.	50	50	13
e.	40	60	0.5
f	0	100	0.5
g.	100	0	2

Anion Exchange Chromatography (SAX) Method-2

Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm

Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl; Flow Rate: 0.75 ml/min

Gradient:

a.	Time	% A	% B
b.	0.0	90	10
c.	3.00	90	10
d.	11.00	40	60
e.	14.00	40	60
f.	15.00	20	80
g.	16.00	90	10
h.	20.00	90	10

Step-3: Analysis of the Purified Conjugate

The purity of the conjugate was assessed by analytical HPLC using anion exchange chromatography method-2 (Table 19).

TABLE 19

Conjugate	SAX retention time (mm)	% purity (by peak area)
mTfR1-mAb-MuRF1(R2089) (DAR1)	9.3	99
mTfR1-mAb-MuRF1(R2265) (DAR1)	9.1	95
mTfR1-mAb-MuRF1(R2266) (DAR1)	9.1	98
mTfR1-mAb-MuRF1(R2267) (DAR1)	9.1	98
mTfR1-mAb-MuRF1(R2268) (DAR1)	9.1	97
mTfR1-mAb-MuRF1(R2269) (DAR1)	9.2	97

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of MuRF1 in muscle (gastroc and heart), in an in vivo experiment (C57BL6 mice). Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs and doses, see FIG. **25**. After 96 hours, gastrocnemius (gastroc) and heart muscle tissues were harvested and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Results

The MuRF1 siRNA guide strands was able to mediate downregulation of the target gene in gastroc and heart muscle when conjugated to an anti-TfR1 mAb targeting the transferrin receptor 1, see FIG. **26** and FIG. **27**.

Conclusions

In this example, it was demonstrated that TfR1-MuRF1 conjugates, after in vivo delivery, mediated specific down regulation of the target gene in gastroc and heart muscle. The ASC was made with an anti-transferrin1 antibody, mouse gastroc and heart muscle expresses the transferrin receptor1 and the conjugate has a mouse specific anti-transferrin antibody to target the siRNA, resulting in accumulation of the conjugates in gastroc muscle. Receptor mediate uptake resulted in siRNA mediated knockdown of the target mRNA.

Example 23: 2017-PK-412-C57BL6: Prevention of Dexamethasone Induce Muscle Atrophy with Atrogin-1 and MuRF1 TfR1-mAb Conjugates

For this experiment three different siRNAs were used:

(1): A 21 mer Atrogin-1 guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UCUACGUAGUUGAAUCUUCUU (SEQ ID NO: 14230). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linkers. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

(2): A 21 mer MuRF1 guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UUUCGCACCAACGUAGAAAUU (SEQ ID NO: 14231). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linkers. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

(3): Negative control siRNA sequence (scramble): A published (Burke et al. (2014) Pharm. Res., 31(12):3445-60) 21 mer duplex with 19 bases of complementarity and 3' dinucleotide overhangs was used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGCUAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the

double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

Antibody siRNA Conjugate Synthesis Using Bis-Maleimide (BisMal) Linker

Step 1: Antibody Reduction with TCEP

Antibody was buffer exchanged with 25 mM borate buffer (pH 8) with 1 mM DTPA and made up to 10 mg/ml concentration. To this solution, 4 equivalents of TCEP in the same borate buffer were added and incubated for 2 hours at 37° C. The resultant reaction mixture was combined with a solution of BisMal-siRNA (1.25 equivalents) in pH 6.0 10 mM acetate buffer at RT and kept at 4° C. overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA. The reaction mixture was treated with 10 EQ of N-ethylmaleimide (in DMSO at 10 mg/mL) to cap any remaining free cysteine residues.

Step 2: Purification

The crude reaction mixture was purified by AKTA Pure FPLC using anion exchange chromatography (SAX) method-1. Fractions containing DAR1 and DAR2 antibody-siRNA conjugates were isolated, concentrated and buffer exchanged with pH 7.4 PBS.

Anion Exchange Chromatography Method (SAX)-1.

Column: Tosoh Bioscience, TSKGel SuperQ-5PW, 21.5 mm ID×15 cm, 13 um

Solvent A: 20 mM TRIS buffer, pH 8.0; Solvent B: 20 mM TRIS, 1.5 M NaCl, pH 8.0; Flow Rate: 6.0 ml/min

Gradient:

a.	% A	% B	Column Volume
b.	100	0	1
c.	81	19	0.5
d.	50	50	13
e.	40	60	0.5
f	0	100	0.5
g.	100	0	2

Anion Exchange Chromatography (SAX) Method-2

Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm

Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl; Flow Rate: 0.75 ml/min

Gradient:

a.	Time	% A	% B
b.	0.0	90	10
c.	3.00	90	10
d.	11.00	40	60
e.	14.00	40	60
f.	15.00	20	80
g.	16.00	90	10
h.	20.00	90	10

Step-3: Analysis of the Purified Conjugate

The purity of the conjugate was assessed by analytical HPLC using anion exchange chromatography method-2 (Table 20).

TABLE 20

Conjugate	SAX retention time (min)	% purity (by peak area)
mTfR1-Atrogin-1 (DAR1)	9.3	97
mTfR1-MuRF1 (DAR1)	9.5	98
mTfR1-SC (DAR1)	9.0	99

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of MuRF1 and Atrogin-1 in muscle (gastroc) in the presence and absence of muscle atrophy, in an in vivo experiment (C57BL6 mice). Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs and doses, see Table 21. Seven days post conjugate delivery, for groups 2-4, 9-11, and 16-18, muscle atrophy was induced by the daily administration, via intraperitoneal injection (10 mg/kg) of dexamethasone for 21 days. For the control groups 5-7, 12-14 and 19-21 (no induction of muscle atrophy) PBS was administered by the daily intraperitoneal injection. Groups 1, 8, 15 and 22 were harvested at day 7 to establish the baseline measurements of mRNA expression and muscle weighted, prior to induction of muscle atrophy. At the time points indicated, gastrocnemius (gastroc) and heart muscle tissues were harvested, weighed and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Quantitation of tissue siRNA concentrations was determined using a stem-loop PCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

TABLE 21

Animal and Group Info			Dex/PBS Dosing				Compound Info		
				Dose Volume	# of	Dose	siRNA Dose	# of	Harvest Time
Group	Test Article	N	ROA	(mL/kg)	Doses	Schedule	(mg/kg)	Doses	(d)
1	mTfR1-Atrogin-1 (DAR1)	5	—	—	—	—	3	1	7

TABLE 21-continued

Animal and Group Info			Dex/PBS Dosing					Compound Info		
Group	Test Article	N	ROA	Dose Volume (mL/kg)	# of Doses	Dose Schedule	siRNA Dose (mg/kg)	# of Doses	Harvest Time (d)
2	mTfR1-Atrogin-1 (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	10
3	mTfR1-Atrogin-1 (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	17
4	mTfR1-Atrogin-1 (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	28
5	mTfR1-Atrogin-1 (DAR1), PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	10
6	mTfR1-Atrogin-1 (DAR1), PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	17
7	mTfR1-Atrogin-1 (DAR1), PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	28
8	mTfR1-Atrogin-1 (DAR1) + mTfR1-MuRF1 (DAR1)	5	—	—	—	—	3 + 3	1	7
9	mTfR1-Atrogin-1 (DAR1) + mTfR1-MuRF1 (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3 + 3	1	10
10	mTfR1-Atrogin-1 (DAR1) + mTfR1-MuRF1 (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3 + 3	1	17
11	mTfR1-Atrogin-1 (DAR1) + mTfR1-MuRF1 (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3 + 3	1	28
12	mTfR1-Atrogin-1 (DAR1) + mTfR1-MuRF1 (DAR1), + PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3 + 3	1	10
13	mTfR1-Atrogin-1 (DAR1) + mTfR1-MuRF1 (DAR1), +PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3 + 3	1	17
14	mTfR1-Atrogin-1 (DAR1) + mTfR1-MuRF1 (DAR1), +PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3 + 3	1	28
15	mTfR1-SC (DAR1)	5	—	—	—	—	3	1	7
16	mTfR1-SC DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	10
17	mTfR1-SC (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	17
18	mTfR1-SC (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	28
19	mTfR1-SC (DAR1), PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	10
20	mTfR1-SC (DAR1), PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	17
21	mTfR1-SC (DAR1), PBS	5	IP	6.25	Daily; 21 Days	192 h Post ASC	3	1	28
22	PBS Control	5	—	—	—	—	—	1	7

Results

The data are summarized in FIG. **28**-FIG. **31**. Co-delivery of Atrogin-1 and MuRF1 siRNAs efficiently downregulated Atrogin-1 and MuRF1 mRNA expression in normal and atrophic muscles, when delivered using a TfR1 mAb conjugate. Induction of atrophy transiently induces Atrogin-1 and MuRF1 expression about 4-fold. A single dose of mTfR1-Atrogin-1+TfR1.mAb-siMuRF1 (3 mg/kg, each and dose as a mixture) reduced Atrogin-1 and MuRF1 mRNA levels by >70% in normal and atrophic gastrocnemius muscle. Downregulation of MuRF1 and Atrogin-1 mRNA increases gastrocnemius weight by 5-10% and reduces DEX-induced gastrocnemius weight loss by 50%. Downregulation of Atrogin-1 alone has no significant effect on gastrocnemius weight changes. In the absence of muscle atrophy treatment with Atrogin-1/MuRF1 siRINAs induces muscle hypertrophy.

Conclusions

In this example, it was demonstrated that co-delivery of Atrogin-1 and MuRF1 siRNAs efficiently downregulated Atrogin-1 and MuRF1 mRNA expression in normal and atrophic gastroc muscles, when delivered using a TfR1 mAb conjugate. The conjugates had little effect on heart muscle, where downregulation of Atrogin-1 could be detrimental. Downregulation of MuRF1 and Atrogin-1 mRNA increased gastroc muscle weight by 5-10% and reduced DEX-induced gastroc muscle weight loss by 50%. Downregulation of Atrogin-1 alone has no significant effect on gastrocnemius weight changes. The ASC were made with an anti-transferrin antibody, mouse gastroc muscle expresses the transferrin receptor and the conjugate has a mouse specific anti-transferrin antibody to target the siRNA, resulting in accumulation of the conjugates in gastroc muscle. Receptor mediate uptake resulted in siRNA mediated knockdown of the target mRNA.

Example 24: 2017-PK-435-C57BL6: In Vivo Dose Response Experiment for Transferrin mAb Conjugate Delivery of Atrogin-1

For groups 1-12, see study design in FIG. **32**, the 21 mer Atrogin-1 guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UCGUAGUUAAAUCUUCUGGUU (SEQ ID NO: 14237). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in figure A. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linkers. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

For groups 13-18 see study design in FIG. **32**, a 21 mer negative control siRNA sequence (scramble) (published by Burke et al. (2014) Pharm. Res., 31(12):3445-60) with 19 bases of complementarity and 3' dinucleotide overhangs was used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGCUAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

Antibody siRNA Conjugate Synthesis Using Bis-Maleimide (BisMal) Linker

Step 1: Antibody Reduction with TCEP

Antibody was buffer exchanged with 25 mM borate buffer (pH 8) with 1 mM DTPA and made up to 10 mg/ml concentration. To this solution, 4 equivalents of TCEP in the same borate buffer were added and incubated for 2 hours at 37° C. The resultant reaction mixture was combined with a solution of BisMal-siRNA (1.25 equivalents) in pH 6.0 10 mM acetate buffer at RT and kept at 4° C. overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA. The reaction mixture was treated with 10 EQ of N-ethylmaleimide (in DMSO at 10 mg/mL) to cap any remaining free cysteine residues.

Step 2: Purification

The crude reaction mixture was purified by AKTA Pure FPLC using anion exchange chromatography (SAX) method-1. Fractions containing DAR1 and DAR2 antibody-siRNA conjugates were isolated, concentrated and buffer exchanged with pH 7.4 PBS.

Anion Exchange Chromatography Method (SAX)-1.

Column: Tosoh Bioscience, TSKGel SuperQ-5PW, 21.5 mm ID×15 cm, 13 um

Solvent A: 20 mM TRIS buffer, pH 8.0; Solvent B: 20 mM TRIS, 1.5 M NaCl, pH 8.0; Flow Rate: 6.0 ml/min

Gradient:

a.	% A	% B	Column	Volume
b.	100	0	1	
c.	81		19	0.5
d.	50		50	13
e.	40		60	0.5
f	0		100	0.5
g.	100	0	2	

Anion Exchange Chromatography (SAX) Method-2

Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm

Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl; Flow Rate: 0.75 ml/min

Gradient:

a.	Time	% A	% B	
b.	0.0		90	10
c.	3.00	90	10	
d.	11.00	40	60	
e.	14.00	40	60	
f.	15.00	20	80	
g.	16.00	90	10	
h.	20.00	90	10	

Step-3: Analysis of the Purified Conjugate

The purity of the conjugate was assessed by analytical HPLC using anion exchange chromatography method-2 (Table 22).

TABLE 22

Conjugate	SAX retention time (min)	% purity (by peak area)
TfR1-Atrogin-1 DAR1	9.2	99
TfR1-Scramble DAR1	8.9	93

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of Atrogin-1 in muscle (gastroc) in the presence and absence of muscle atrophy, in an in vivo experiment (C57BL6 mice). Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs and doses, see FIG. **32**. Seven days post conjugate delivery, for groups 3, 6, 9, 12, and 15, muscle atrophy was induced by the daily administration via intraperitoneal injection (10 mg/kg) of dexamethasone for 3 days. For the control groups 2, 5, 8, 11, and 14 (no induction of muscle atrophy) PBS was administered by the daily intraperitoneal injection. Groups 1, 4, 7, 10, and 13 were harvested at day 7 to establish the baseline measurements of mRNA expression and muscle weighted, prior to induction of muscle atrophy. At three days post-atrophy induction (or 10 days post conjugate delivery), gastrocnemius (gastroc) muscle tissues were harvested, weighed and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

Results

The data are summarized in FIG. **33**-FIG. **35**. The Atrogin-1 siRNA guide strands were able to mediate downregulation of the target gene in gastroc muscle when conjugated to an anti-TfR mAb targeting the transferrin receptor, see FIG. **33**. Increasing the dose from 3 to 9 mg/kg reduced atrophy-induced Atrogin-1 mRNA levels 2-3 fold. The maximal KD achievable with this siRNA was 80% and a tissue concentration of 40 nM was needed to achieve maximal KD in atrophic muscles. This highlights the conjugate delivery approach is able to change disease induce mRNA expression levels of Atrogin-1 (see FIG. **34**), by increasing the increasing the dose. FIG. **35** highlights that mRNA down regulation is mediated by RISC loading of the Atrogin-1 guide strands and is concentration dependent.

Conclusions

In this example, it was demonstrated that a TfR1-Atrogin-1 conjugates, after in vivo delivery, mediated specific down regulation of the target gene in gastroc muscle in a dose dependent manner. After induction of atrophy the conjugate was able to mediate disease induce mRNA expression levels of Atrogin-1 at the higher doses. Higher RISC loading of the Atrogin-1 guide strand correlated with increased mRNA downregulation.

Example 25: 2017-PK-381-C57BL6: Myostatin (MSTN) Downregulation Reduces Muscle Loss in Dexamethasone-Treated Mice

For groups 1-12, see study design in Table 24, the 21 mer Atrogin-1 guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UCGUAGUUAAAUCUUCUGGUU (SEQ ID NO: 14237). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linkers. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

For groups 13-18 see study design in Table 24, a 21 mer negative control siRNA sequence (scramble) (published by Burke et al. (2014) Pharm. Res., 31(12):3445-60) with 19 bases of complementarity and 3' dinucleotide overhangs were used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGCUAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phospharamidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

Antibody siRNA Conjugate Synthesis Using Bis-Maleimide (BisMal) Linker

Step 1: Antibody Reduction with TCEP

Antibody was buffer exchanged with 25 mM borate buffer (pH 8) with 1 mM DTPA and made up to 10 mg/ml concentration. To this solution, 4 equivalents of TCEP in the same borate buffer were added and incubated for 2 hours at 37° C. The resultant reaction mixture was combined with a solution of BisMal-siRNA (1.25 equivalents) in pH 6.0 10 mM acetate buffer at RT and kept at 4° C. overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA. The reaction mixture was treated with 10 EQ of N-ethylmaleimide (in DMSO at 10 mg/mL) to cap any remaining free cysteine residues.

Step 2: Purification

The crude reaction mixture was purified by AKTA Pure FPLC using anion exchange chromatography (SAX) method-1. Fractions containing DAR1 and DAR2 antibody-siRNA conjugates were isolated, concentrated and buffer exchanged with pH 7.4 PBS.

Anion Exchange Chromatography Method (SAX)-1.

Column: Tosoh Bioscience, TSKGel SuperQ-5PW, 21.5 mm ID×15 cm, 13 um

Solvent A: 20 mM TRIS buffer, pH 8.0; Solvent B: 20 mM TRIS, 1.5 M NaCl, pH 8.0; Flow Rate: 6.0 ml/min

Gradient:

a.	% A	% B	Column	Volume
b.	100	0	1	
c.	81	19	0.5	
d.	50	50	13	
e.	40	60	0.5	
f	0	100	0.5	
g.	100	0	2	

Anion Exchange Chromatography (SAX) Method-2

Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm

Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl; Flow Rate: 0.75 ml/min

Gradient:

a.	Time	% A	% B
b.	0.0	90	10
c.	3.00	90	10
d.	11.00	40	60
e.	14.00	40	60
f.	15.00	20	80
g.	16.00	90	10
h.	20.00	90	10

Step-3: Analysis of the Purified Conjugate

The purity of the conjugate was assessed by analytical HPLC using anion exchange chromatography method-2 (Table 23).

TABLE 23

Conjugate	SAX retention time (min)	% purity (by peak area)
mTfR1-MSTN (DAR1)	9.2	98
mTfR1-SC (DAR1)	8.9	98

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of MSTN in muscle (gastroc) in the presence and absence of muscle atrophy, in an in vivo experiment (C57BL6 mice). Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs and doses, see Table 24. Seven days post conjugate delivery, for groups 2, 3, 4, 9, 10 and 11, muscle atrophy was induced by the daily administration via intraperitoneal injection (10 mg/kg) of dexamethasone for 13 days. For the control groups 5, 6, 7, 12, 13 and 14 (no induction of muscle atrophy), PBS was administered by the daily intraperitoneal injection. Groups 1 and 8 were harvested at day 7 to establish the baseline measurements of mRNA expression and muscle weighted, prior to induction of muscle atrophy. At 3, 7, and 14 days post-atrophy induction (or 10, 14, and 21 days post conjugate delivery), gastrocnemius (gastroc) muscle tissues were harvested, weighed and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

TABLE 24

			Dex/PBS Dosing						
Animal and Group Info			Dose				Compound Info		
Group	Test Article	N	ROA	Volume (mL/kg)	# of Doses	Dose Schedule	siRNA Dose (mg/kg)	ROA	Harvest Time (d)
1	mTfR1-MSTN (DAR1)	5	—	—	—	—	3	IV	7
2	mTfR1-MSTN (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	10
3	mTfR1-MSTN (DAR = 1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	14
4	mTfR1-MSTN (DAR = 1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	21

TABLE 24-continued

Animal and Group Info			Dex/PBS Dosing Dose				Compound Info		
Group	Test Article	N	ROA	Volume (mL/kg)	# of Doses	Dose Schedule	siRNA Dose (mg/kg)	ROA	Harvest Time (d)
5	mTfR1-MSTN (DAR1), PBS	5	IP	6 .25	Daily; 13 Days	192 h Post ASC	3	IV	10
6	mTfR1-MSTN (DAR1), PBS	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	14
7	mTfR1-MSTN (DAR1), PBS	5	IP	6 .25	Daily; 13 Days	192 h Post ASC	3	IV	21
8	mTfR1-SC (DAR1)	5	—	—	—	—	3	IV	7
9	mTfR1-SC (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	10
10	mTfR1-SC (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	14
11	mTfR1-SC (DAR1), +DEX (10 mg/kg)	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	21
12	mTfR1-SC (DAR1), PBS	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	10
13	mTfR1-SC (DAR1), PBS	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	14
14	mTfR1-SC (DAR1), PBS	5	IP	6.25	Daily; 13 Days	192 h Post ASC	3	IV	21
15	PBS Control	5	—	—	—	—	—	IV	7

Results

The data are summarized in FIG. **36** and FIG. **37**. The MSTN siRNA guide strands were able to mediate downregulation of the target gene in gastroc muscle when conjugated to an anti-TfR mAb targeting the transferrin receptor, see FIG. **36**, in the presence and absence of dexamethasone induced atrophy. A single of 3 mg/kg siRNA downregulated MSTN mRNA levels by >75%. In the presence of dexamethasone induced atrophy, MSTN downregulation increased muscle mass and attenuates Dex-induced muscle loss, see FIG. **37**.

Conclusions

In this example, it was demonstrated that a TfR1-MSTN conjugate, after in vivo delivery, mediated specific down regulation of the target gene in gastroc muscle. After induction of atrophy the conjugate was able to increase muscle mass and attenuate Dex-induced muscle loss.

Example 26: 2017-PK-496-C57BL6: Atrogin-1 and MuRF1 Downregulation Reduces Leg Muscle Loss Upon Sciatic Nerve Denervation in Mice

For groups 1-4, see study design in FIG. **38**, the 21 mer Atrogin-1 guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UUGGGUAACAUCGUACAAGUU (SEQ ID NO: 14238). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linkers. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

For groups 5-6, see study design in figure V, the 21 mer MuRF1 guide strand was designed. The sequence (5' to 3') of the guide/antisense strand was UUUCGCACCAACGUAGAAAUU (SEQ ID NO: 14231). The guide and fully complementary RNA passenger strands were assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Base, sugar and phosphate modifications that are well described in the field of RNAi were used to optimize the potency of the duplex and reduce immunogenicity. Purified single strands were duplexed to get the double stranded siRNA described in FIG. **20**B. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodies-

ter linkers. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

For groups 7-12, the Atrogin-1 and MuRF1 were design as above. After conjugation to the TfR1 mAb and after purification and isolation of the individual DAR1 species, were mixed and co-administered.

For group 13, see study design in FIG. **38**, a 21 mer negative control siRNA sequence (scramble) (published by Burke et al. (2014) Pharm. Res., 31(12):3445-60) with 19 bases of complementarity and 3' dinucleotide overhangs was used. The sequence (5' to 3') of the guide/antisense strand was UAUCGACGUGUCCAGCUAGUU (SEQ ID NO: 14228). The same base, sugar and phosphate modifications that were used for the active MSTN siRNA duplex were used in the negative control siRNA. All siRNA single strands were fully assembled on solid phase using standard phosphoramidite chemistry and purified over HPLC. Purified single strands were duplexed to get the double stranded siRNA. The passenger strand contained two conjugation handles, a C6-NH_2 at the 5' end and a C6-SH at the 3' end. Both conjugation handles were connected to siRNA passenger strand via phosphodiester-inverted abasic-phosphodiester linker. Because the free thiol was not being used for conjugation, it was end capped with N-ethylmaleimide.

Antibody siRNA Conjugate Synthesis Using Bis-Maleimide (BisMal) Linker

Step 1: Antibody Reduction with TCEP

Antibody was buffer exchanged with 25 mM borate buffer (pH 8) with 1 mM DTPA and made up to 10 mg/ml concentration. To this solution, 4 equivalents of TCEP in the same borate buffer were added and incubated for 2 hours at 37° C. The resultant reaction mixture was combined with a solution of BisMal-siRNA (1.25 equivalents) in pH 6.0 10 mM acetate buffer at RT and kept at 4° C. overnight. Analysis of the reaction mixture by analytical SAX column chromatography showed antibody siRNA conjugate along with unreacted antibody and siRNA. The reaction mixture was treated with 10 EQ of N-ethylmaleimide (in DMSO at 10 mg/mL) to cap any remaining free cysteine residues.

Step 2: Purification

The crude reaction mixture was purified by AKTA Pure FPLC using anion exchange chromatography (SAX) method-1. Fractions containing DAR1 and DAR2 antibody-siRNA conjugates were isolated, concentrated and buffer exchanged with pH 7.4 PBS.

Anion Exchange Chromatography Method (SAX)-1.

Column: Tosoh Bioscience, TSKGel SuperQ-5PW, 21.5 mm ID×15 cm, 13 um

Solvent A: 20 mM TRIS buffer, pH 8.0; Solvent B: 20 mM TRIS, 1.5 M NaCl, pH 8.0; Flow Rate: 6.0 ml/min

Gradient:

a.	% A	% B	Column	Volume
b.	100	0	1	
c.	81	19	0.5	
d.	50	50	13	
e.	40	60	0.5	
f	0	100	0.5	
g.	100	0	2	

Anion Exchange Chromatography (SAX) Method-2

Column: Thermo Scientific, ProPac™ SAX-10, Bio LC™, 4×250 mm

Solvent A: 80% 10 mM TRIS pH 8, 20% ethanol; Solvent B: 80% 10 mM TRIS pH 8, 20% ethanol, 1.5 M NaCl; Flow Rate: 0.75 ml/min

Gradient:

a.	Time	% A	% B
b.	0.0	90	10
c.	3.00	90	10
d.	11.00	40	60
e.	14.00	40	60
f.	15.00	20	80
g.	16.00	90	10
h.	20.00	90	10

Step-3: Analysis of the Purified Conjugate

The purity of the conjugate was assessed by analytical HPLC using anion exchange chromatography method-2 (Table 25).

TABLE 25

Conjugate	SAX retention time (min)	% purity (by peak area)
TfR1-Atrogin-1 DAR1	9.2	95
TfR1-MuRF1 DAR1	9.3	92
mTfR1-SC (DAR1)	8.9	76

In Vivo Study Design

The conjugates were assessed for their ability to mediate mRNA downregulation of MuRF1 and Atrogin-1 in muscle (gastroc) in the presence and absence of sciatic nerve denervation, in an in vivo experiment (C57BL6 mice). Mice were dosed via intravenous (iv) injection with PBS vehicle control and the indicated ASCs and doses, see FIG. **38**. Seven days post conjugate delivery, for groups 2-4, 6-8, 10-12 and 14-16, leg muscle atrophy was induced by sciatic nerve denervation. Denervation was not induced for the control groups 1, 5, 9, 13, and 17.

For the Denervation procedure at day 7, mice were anesthesed (5% isoflurane) and administer a subcutaneous dose of 0.1 mg/kg Buprenorphine. The right dorsal pelvic region was shaved from the sciatic notch to the knee. The area was disinfected with alternating alcohol and povidone-iodine. The sciatic notch was identified by palpation and an incision made from the sciatic notch towards the knee, approximately 1 cm. The bicep femoris muscle was split to expose the sciatic nerve and about a 1 cm fragment was removed by cauterizing both ends. The muscle and skin were then sutured to close the incision. The operative limb was then inspected daily to observe the condition of the surgical wound and observe the animal for overall health.

For groups 4, 8, 12, and 16 changes in leg muscle area were determined: The leg-to-be-measured were shaved and a line was drawn using indelible ink to mark region of measurement. Mice were restrained in a cone restraint and the right leg was held by hand. Digital calipers were used to take one measurement on the sagittal plane and another on the coronal plane. The procedure was repeated twice per week. For all groups at the time points indicated, gastrocnemius (gastroc) and heart muscle tissues were harvested, weighed and snap-frozen in liquid nitrogen. mRNA knockdown in target tissue was determined using a comparative qPCR assay as described in the methods section. Total RNA was extracted from the tissue, reverse transcribed and mRNA levels were quantified using TaqMan qPCR, using the appropriately designed primers and probes. PPIB (housekeeping gene) was used as an internal RNA loading control, results were calculated by the comparative Ct method, where the difference between the target gene Ct

value and the PPIB Ct value (ΔCt) is calculated and then further normalized relative to the PBS control group by taking a second difference (ΔΔCt).

Quantitation of tissue siRNA concentrations was determined using a stem-loop qPCR assay as described in the methods section. The antisense strand of the siRNA was reverse transcribed using a TaqMan MicroRNA reverse transcription kit using a sequence-specific stem-loop RT primer. The cDNA from the RT step was then utilized for real-time PCR and Ct values were transformed into plasma or tissue concentrations using the linear equations derived from the standard curves.

FIG. **39**A shows a single treatment of 4.5 mg/kg (siRNA) of either Atrogin-1 siRNA or MuRF1 siRNA or a single dose of both siRNAs combined resulted in up to 75% downregulation of each target in the gastrocnemius.

FIG. **39**B shows mRNA knockdown of both targets in gastrocnemius is maintained at 75% in the intact leg out to 37 days post ASC dose.

In the denerved leg, Atrogin1 mRNA knockdown is maintained 3 days post denervation, but is reduced to 20% by 10 days post denervation and to 0% by 30 days post denervation. MuRF1 mRNA knockdown in the denerved leg is enhanced to 80-85% 3 days post denervation, but is reduced to 50% by 10 days post denervation and to 40% by 30 days post denervation (FIG. **39**C).

The mRNA knockdown of each target was not impacted by the knockdown of the other target when treated with the combination of both siRNAs (FIG. **39**D).

Based on leg muscle area measurements, siRNA-mediated downregulation of MuRF1 and the combination of MuRF1 and Atrogin-1 reduced denervation-induced muscle wasting by up to 30%. Treatment with MuRF1 siRNA alone showed similar responses than treatment with the combination of MuRF1 and Atrogin-1. Downregulation of Atrogin-1 alone had no significant effect on leg muscle area. The statistical analysis compared the treatment groups to the scramble siRNA control group using a Welch's TTest. See FIG. **39**E.

Based on the Gastrocnemius weight only MuRF1 showed statistically significant differences from the scramble siRNA control group. Similar to the results obtained by measuring leg muscle area, downregulation of MuRF1 showed an up to 35% reduction in denervation-induced muscle wasting. These results agree with effects of MuRF1 knock out in mice (Bodine et al., Science 291, 2001). See FIG. **39**F.

While preferred embodiments of the present disclosure have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the disclosure. It should be understood that various alternatives to the embodiments of the disclosure described herein may be employed in practicing the disclosure. It is intended that the following claims define the scope of the disclosure and that methods and structures within the scope of these claims and their equivalents be covered thereby.

SEQUENCE LISTING

The patent contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (https://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US11246941B2). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What is claimed is:

1. A method of treating myotonic dystrophy in a subject in need thereof, comprising administering to said subject a therapeutically effective amount of a single-stranded antisense oligonucleotide (ASO) conjugate comprising an anti-transferrin receptor antibody or antigen binding fragment thereof conjugated to an ASO sequence that hybridizes to a target sequence in exons 1-13, excluding CUG repeats, of human DMPK mRNA, wherein the ASO sequence is 8 to 30 nucleotides in length, thereby treating myotonic dystrophy in said subject.

2. The method of claim **1**, wherein the ASO sequence comprises at least one 2' modified nucleotide, at least one modified internucleotide linkage, or at least one inverted abasic moiety.

3. The method of claim **1**, wherein the ASO sequence mediates RNA interference against the human DMPK mRNA and modulates myotonic dystrophy in said subject.

4. The method of claim **3**, wherein the RNA interference against the human DMPK mRNA is mediated via RNase H activity in a muscle cell.

5. The method of claim **1**, wherein the anti-transferrin receptor antibody or antigen binding fragment thereof binds to a transferrin receptor on cell surface of a muscle cell.

6. The method of claim **1**, wherein the ASO sequence hybridizes to at least 8 contiguous bases of the target sequence of the human DMPK mRNA.

7. The method of claim **1**, wherein the ASO conjugate comprises a linker connecting the anti-transferrin receptor antibody or the antigen binding fragment thereof to the ASO sequence.

8. The method of claim **2**, wherein the at least one 2' modified nucleotide:

comprises 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl, 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O-N-methylacetamido (2'-O-NMA) modified nucleotide;

comprises locked nucleic acid (LNA) or ethylene nucleic acid (ENA); or

comprises a combination thereof.

9. The method of claim **2**, wherein the at least one modified internucleotide linkage comprises a phosphorothioate linkage or a phosphorodithioate linkage.

10. The method of claim **1**, wherein the ASO sequence comprises 3 or more 2' modified nucleotides selected from 2'-O-methyl and 2'-deoxy-2'-fluoro.

11. The method of claim 1, wherein the ASO conjugate has an ASO/drug to antibody ratio (DAR) from about 1 to about 4.

12. The method of claim 1, wherein the ASO sequence comprises a 5'-terminal vinylphosphonate modified nucleotide.

13. The method of claim 1, wherein the myotonic dystrophy is DM1.

14. The method of claim 1, wherein the myotonic dystrophy is associated with muscle atrophy.

15. The method of claim 1, wherein the ASO conjugate is administered parenterally.

16. The method of claim 1, wherein the ASO conjugate preferentially mediates RNA interference against the human DMPK mRNA in a muscle cell of said subject.

17. The method of claim 1, wherein the ASO sequence that hybridizes to the target sequence of the human DMPK mRNA is selected from a group consisting of SEQ ID NOs: 6111-8814.

18. The method of claim 1, wherein the ASO sequence that hybridizes to the target sequence of the human DMPK mRNA is selected from a group consisting of SEQ ID NOs: 11519-14222.

19. The method of claim 1, wherein the ASO sequence that hybridizes to the target sequence in exons 2-13 of the human DMPK mRNA is selected from SEQ ID NOs: 11903, 11961, 11962, 11963, 12051, 12052, 12053, 12057, 12058, 12059, 12061, 12062, 12094, 12095, 12099, 12101, 12102, 12208, 12234, 12235, 12303, 12304, 12307, 12544, 12545, 12546, 12547, 12555, 12557, 12559, 12561, 12562, 12565, 12589, 12591, 12780, 12781, 12782, 12783, 12785, 12786, 12787, 12792, 12794, 12801, 12815, 12860, 12861, 12862, 12864, 13343, 13404, 13408, 13416, 13463, 13478, 13644, 13645, 13667, 13668, 13786, 13790, 14046, 14047, 14048, 14049, 14050, 14072, 14076, 14118, 14146, 14147, 14149, 14154, 14157, 14193, 14194, 14198, 14199, and 14200.

20. The method of claim 19, wherein the ASO sequence that hybridizes to the target sequence in exons 4, 5, or 6 of the human DMPK mRNA is selected from SEQ ID NOs: 12102, 12234, 12303, 12304, and 12307.

21. The method of claim 19, wherein the ASO sequence that hybridizes to the target sequence in exons 7 or 8 of the human DMPK mRNA is selected from SEQ ID NOs: 12544, 12545, 12546, 12547, 12555, 12557, 12559, 12561, 12562, 12565, 12589, 12783, and 12815.

22. The method of claim 19, wherein the ASO sequence that hybridizes to the target sequence in exon 9 of the human DMPK mRNA is selected from SEQ ID NOs: 12860, 12861, 12862, and 12864.

23. The method of claim 19, wherein the ASO sequence that hybridizes to the target sequence in exon 13 of the human DMPK mRNA is selected from SEQ ID NOs: 13343, 13463, 13478, 14047, 14048, 14049, 14072, 14146, 14147, 14199, and 14200.

* * * * *