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(54) Title: FOLLISTATIN IN TREATING DUCHENNE MUSCULAR DYSTROPHY

(57) Abstract: [0001] The present invention provides, among other things, methods and compositions for treating muscular dystrophy, in particular, Duchenne muscular dystrophy (DMD). In some embodiments, a method according to the present invention includes administering to an individual who is suffering from or susceptible to DMD an effective amount of a recombinant follistatin protein such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset.



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FOLLISTATIN IN TREATING DUCHENNE MUSCULAR DYSTROPHY**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority from U.S. provisional patent application serial number 61/756,996, filed January 25, 2013, and U.S. provisional patent application serial number 61/915,733, filed December 13, 2013, the disclosures of which are hereby incorporated in their entirety.

BACKGROUND

[0002] Duchenne muscular dystrophy (DMD) is a recessive X-linked form of muscular dystrophy, which results in muscle degeneration and eventual death. The disorder is caused by a mutation in the dystrophin gene, located on the human X chromosome, which codes for the protein dystrophin, an important structural component within muscle tissue that provides structural stability to the dystroglycan complex (DGC) of the cell membrane. Dystrophin links the internal cytoplasmic actin filament network and extracellular matrix, providing physical strength to muscle fibers. Accordingly, alteration or absence of dystrophin results in abnormal sarcolemmal membrane function. While persons of both sexes can carry the mutation, boys typically have a severe phenotype with early disability and mortality, whereas females carrying a mutation typically exhibit a much milder phenotype.

[0003] Presently, there is no known cure for DMD. Many therapeutic avenues have been investigated including gene therapy and various administration protocols of corticosteroids. While some of these treatments may delay certain signs and symptoms, there is presently no satisfactory therapeutic option for DMD patients.

SUMMARY OF THE INVENTION

[0004] The present invention provides, among other things, improved methods and compositions for treating muscular dystrophy, in particular, Duchenne muscular dystrophy (DMD) and/or Becker Muscular Dystrophy, based on follistatin protein therapy. As described

herein, including in the Examples below, the present inventors demonstrated, *for the first time*, that systemic administration of a recombinant follistatin protein (e.g., a follistatin-Fc recombinant fusion protein) into a DMD animal model resulted in effective muscle growth in various tissues throughout the body and reduced muscle fibrosis and/or necrosis, characteristic symptoms of DMD. In addition, the present inventors have also demonstrated that follistatin-Fc fusion proteins according to the present invention have extended serum half-life of up to about 5 days. Without wishing to be bound by any theory, it is contemplated that the unexpectedly long serum half-life may have contributed to the superior *in vivo* efficacy. Indeed, prior to the present invention, follistatin was known to be a modulator of myostatin and activin, both of which are important negative regulators of muscle growth. However, prior to the present invention, it was reported that follistatin has a particularly short serum half-life, which constituted a significant hurdle for developing follistatin as a protein therapeutic. For example, a typical commercially available wild-type follistatin (FS315) protein has a serum half-life of about an hour. Fc-fusion protein had been used to extend the serum half-life of follistatin. However, due to the large size of the Fc domain and the relatively smaller size of the follistatin protein, it was thought that a direct fusion of the Fc domain to the follistatin protein may interfere with the normal structure and function of a wild-type follistatin protein. The reported poor pharmacokinetic/pharmacodynamic (PK/PD) properties of follistatin and uncertainty associated with follistatin-Fc fusion protein had discouraged scientists and clinicians from further developing follistatin as a protein therapy for DMD or other muscular dystrophy. Indeed, prior to the present invention, gene therapy has been the focus of follistatin based therapy for DMD. The unexpectedly superior *in vivo* efficacy and half-life shown by the present inventors establishes for the first time that follistatin can be an effective protein therapeutic for treatment of DMD.

[0005] In one aspect, the present invention provides methods of treating Duchenne Muscular Dystrophy (DMD) including administering to an individual who is suffering from or susceptible to DMD an effective amount of a recombinant follistatin protein such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset. In some embodiments, at least one symptom or feature of DMD is selected from the group consisting of muscle wasting, muscle weakness, muscle fragility, joint contracture,

skeletal deformation, fatty infiltration of muscle, replacement of muscle with non-contractile tissue (e.g., muscle fibrosis), muscle necrosis, cardiomyopathy, impaired swallowing, impaired bowel and bladder function, muscle ischemia, cognitive impairment function (e.g., learning difficulties, higher risk of neurobehavioral disorders, cognitive defects), behavioral dysfunction, socialization impairment, scoliosis, and impaired respiratory function.

[0006] In some embodiments, a recombinant follistatin protein suitable for the present invention includes an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the wild-type human Follistatin protein

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKG P V C G L D G K T Y R N E C
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSCNSIS
EDTEEEEEDEDQDYSFPISILEW (SEQ ID NO:1).

[0007] In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 70% identical to the wild-type human Follistatin protein SEQ ID NO:1. In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 80% identical to the wild-type human Follistatin protein SEQ ID NO:1. In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 90% identical to the wild-type human Follistatin protein SEQ ID NO:1. In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 95% identical to the wild-type human Follistatin protein SEQ ID NO:1. In some embodiments, the recombinant follistatin protein includes an amino acid sequence identical to the wild-type human Follistatin protein SEQ ID NO:1.

[0008] In some embodiments, the recombinant follistatin protein comprises one or more deletions, mutations or insertions as compared to the wild-type human Follistatin protein. In some embodiments, the recombinant follistatin protein comprises a deletion of amino acids

residues 212-288 of SEQ ID NO:1 (which corresponds to domain 3). In some embodiments, the recombinant follistatin protein comprises the heparin binding site.

[0009] In some embodiments, the present invention provides methods of treating Duchenne Muscular Dystrophy (DMD) including administering to an individual who is suffering from or susceptible to DMD an effective amount of a recombinant follistatin protein such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset, wherein the recombinant follistatin protein comprises an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCISISEDTEEEEEDEDQDYSFPISSI
LEW (SEQ ID NO:2).

[0010] In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 70% identical to SEQ ID NO:2. In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 80% identical to SEQ ID NO:2. In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 90% identical to SEQ ID NO:2. In some embodiments, the recombinant follistatin protein includes an amino acid sequence at least 95% identical to SEQ ID NO:2. In some embodiments, the recombinant follistatin protein includes an amino acid sequence identical to SEQ ID NO:2.

[0011] In some embodiments, the at least one symptom or feature of DMD is selected from the group consisting of muscle wasting, muscle weakness, muscle fragility, muscle hypertrophy, muscle pseudohypertrophy, joint contracture, skeletal deformation, cardiomyopathy, impaired swallowing, impaired bowel and bladder function, muscle ischemia, cognitive impairment, behavioral dysfunction, socialization impairment, scoliosis, and impaired respiratory function.

[0012] In some embodiments, the recombinant follistatin protein is fused to an Fc domain. In some embodiments, an Fc domain suitable for the present invention comprises an

amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO:3); or

KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPV
LDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:
4); or

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP
VLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID
NO: 14).

[0013] In some embodiments, the Fc domain comprises an amino acid sequence at least 80% identical to SEQ ID NO: 3, 4, or 14. In some embodiments, the Fc domain comprises an amino acid sequence at least 90% identical to SEQ ID NO: 3, 4, or 14. In some embodiments, the Fc domain comprises an amino acid sequence at least 95% identical to SEQ ID NO:3, 4, or 14.

[0014] In some embodiments, a suitable Fc domain comprises one or more mutations that improve binding between the Fc domain and the FcRn receptor resulting in prolonged serum half-life. In some embodiments, a suitable Fc domain comprises one or more mutations at one or more positions corresponding to Thr 250, Met 252, Ser 254, Thr 256, Thr 307, Glu 380, Met 428, His 433 and/or Asn 434 of human IgG1. In particular embodiments, a suitable Fc domain contains mutations H433K (His433Lys) and/or N434F (Asn434Phe). In particular embodiments,

a suitable Fc domain comprises a sequence shown below which incorporates the mutations of H433K (His433Lys) and N434F (Asn434Phe):

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSGDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALKFHYTQKSLSLSPGK (SEQ ID
NO:15).

[0015] In some embodiments, a recombinant follistatin protein is fused to the Fc domain via a linker. In some embodiments, the linker is a peptide comprising 3-100 amino acids. In some embodiments, the linker is not a linker consisting of ALEVLFGQP. In some embodiments, the linker comprises between 10-100, 10-90, 10-80, 10-70, 10-60, 10-50, 10-40, 10-30, 10-20, 10-15 amino acids. In some embodiments, the linker comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95 amino acids. In some embodiments, the linker comprises a sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to GAPGGGGGAAAAAGGGGGGAP (GAG linker, SEQ ID NO: 5). In some embodiments, the linker comprises a sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to GAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAP (GAG2 linker, SEQ ID NO: 6). In some embodiments, the linker comprises a sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) identical to GAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAP (GAG3 linker, SEQ ID NO:7). In some embodiments, the linker comprises a sequence identical to SEQ ID NO:5, SEQ ID NO:6, or SEQ ID NO:7.

[0016] In some embodiments, the present invention provides a recombinant follistatin fusion protein including a follistatin polypeptide, an Fc domain, and a linker with a length of at least 10 (e.g., at least 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95) amino acids that associates the follistatin polypeptide with the Fc domain. In some embodiments, the present invention provides a recombinant follistatin fusion protein including a follistatin polypeptide, an Fc domain, and a linker that associates the follistatin polypeptide with the Fc domain, wherein the linker is not a linker consisting of ALEVLFGQP. In some embodiments, a

suitable follistatin polypeptide comprises an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the wild-type human Follistatin protein (SEQ ID NO: 1). In some embodiments, the recombinant follistatin fusion protein is capable of binding to activin, myostatin and/or GDF-11 and has an in vivo half-life ranging from about 0.5-10 days.

[0017] In particular embodiments, a recombinant follistatin protein suitable for the present invention comprises an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:8

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLLEVKHSGSCNSIS
EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
GGGGAPGGGGGAAAAAGGGGGGAPKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRT
PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW
LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY
PSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEA
LHNHYTQKSLSLSPGK (SEQ ID NO:8),

or

SEQ ID NO: 9

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLLEVKHSGSCNSIS
EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
GGGGAPGGGGGAAAAAGGGGGGAPEPKSCDKTHTCPPCPAPPELLGGPSVFLFPPKPKD
TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT

VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 9).

[0018] In particular embodiments, a recombinant follistatin protein suitable for the present invention comprises an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:10

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCISISEDTEEEEEDEDEDQDYSFPIS
LEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
GGGGAPKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO:10),

or

SEQ ID NO: 11

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCISISEDTEEEEEDEDEDQDYSFPIS
LEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
GGGGAPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK (SEQ ID NO: 11).

[0019] In particular embodiments, a recombinant follistatin protein suitable for the present invention comprises an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLLEVKHSGSCNSIS
EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGGAPGGGGGAAAAAGG
GGGGGAPGGGGGAAAAAGGGGGGGAPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALHNHYTQKSLSLSPGK (SEQ ID NO:16)

Or

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLLEVKHSGSCNSIS
EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGGAPGGGGGAAAAAGG
GGGGGAPGGGGGAAAAAGGGGGGGAPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALKFHYTQKSLSLSPGK (SEQ ID NO:17)

[0020] In some embodiments, a recombinant follistatin protein suitable for the present invention is produced from mammalian cells. In some embodiments, the mammalian cells are

human cells. In some embodiments, the mammalian cells are Chinese Hamster Ovary (CHO) cells or HT1080 cells.

[0021] It will be appreciated that embodiments of the invention may be delivered via a variety of routes. In some embodiments, the recombinant follistatin protein is administered systemically. In some embodiments, the systemic administration is selected from intravenous, intradermal, inhalation, transdermal (topical), intraocular, intramuscular, subcutaneous, intramuscular, oral, and/or transmucosal administration.

[0022] Embodiments may be administered via a multiplicity of dosing regimens. In some embodiments, the recombinant follistatin protein is administered bimonthly, monthly, triweekly, biweekly, weekly, daily, or at variable intervals.

[0023] In some embodiments, the recombinant follistatin protein is delivered to one or more target tissues selected from striated muscle (e.g., skeletal muscle, cardiac muscle). In some embodiments, the recombinant follistatin protein is delivered to the heart. In some embodiments, the recombinant follistatin protein is delivered to skeletal muscle. In some embodiments, the recombinant follistatin protein is delivered to one or more skeletal muscles selected from Table 1. In some embodiments, the striated muscle (e.g., skeletal muscle) is selected from the group consisting of triceps, tibialis anterior, soleus, gastrocnemius, biceps, trapezius, deltoids, quadriceps, and diaphragm.

[0024] In some embodiments, the administration of the recombinant follistatin protein results in muscle regeneration, fibrosis reduction, increased muscle strength, increased flexibility, increased range of motion, increased stamina, reduced fatigability, increased blood flow, improved cognition, improved pulmonary function, inflammation inhibition, reduced muscle fibrosis and/or necrosis.

[0025] In another aspect, the present invention provides compositions used in various methods described herein. In some embodiments, the present invention provides recombinant follistatin fusion proteins including a follistatin polypeptide, an Fc domain, and a linker that associates the follistatin polypeptide with the Fc domain, wherein the follistatin polypeptide comprises an amino acid sequence at least at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to the wild-type human

follistatin protein (SEQ ID NO:1). In some embodiments, the recombinant follistatin fusion protein is capable of binding to activins, myostatin and/or GDF-11. In some embodiments, the recombinant follistatin fusion protein has an *in vivo* half-life greater than about 2 days (e.g., greater than about 2.5 days, about 3 days, about 3.5 days, about 4 days, about 4.5 days, about 5 days, about 5.5 days, about 6 days). In some embodiments, the recombinant follistatin fusion protein has an *in vivo* half-life ranging from about 2-10 days (e.g., ranging from about 2.5-10 days, from about 3-10 days, from about 3.5-10 days, from about 4-10 days, from about 4.5-10 days, from about 5-10 days, from about 3-8 days, from about 3.5-8 days, from about 4-8 days, from about 4.5-8 days, from about 5-8 days, from about 3-6 days, from about 3.5-6 days, from about 4-6 days, from about 4.5-6 days, from about 5-6 days). In some embodiments, the *in vivo* half-life is measured in one or more of mice, rats, non-human primates, and/or humans. In some embodiments, the follistatin polypeptide has an amino acid sequence at least 70% identical to the wild-type human follistatin protein (SEQ ID NO:1). In some embodiments, the follistatin polypeptide has an amino acid sequence at least 80% identical to the wild-type human follistatin protein (SEQ ID NO:1). In some embodiments, the follistatin polypeptide has an amino acid sequence at least 90% identical to the wild-type human follistatin protein (SEQ ID NO:1). In some embodiments, the follistatin polypeptide has an amino acid sequence at least 95% identical to the wild-type human follistatin protein (SEQ ID NO:1). In some embodiments, the follistatin polypeptide has an amino acid sequence identical to the wild-type human follistatin protein (SEQ ID NO:1). In some embodiments, the follistatin polypeptide contains a deletion of amino acids residues 212-288 of SEQ ID NO:1 (which corresponds to domain 3). In various embodiments, the follistatin polypeptide contains the heparin sulfate binding site.

[0026] In some embodiments, the present invention provides recombinant follistatin fusion proteins including a follistatin polypeptide, an Fc domain, and a linker that associates the follistatin polypeptide with the Fc domain, wherein the follistatin polypeptide comprises an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS

EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCISEDTEEEEEDEDEDQDYSFPISSI
LEW (SEQ ID NO:2).

[0027] In some embodiments, the recombinant follistatin fusion protein is capable of binding to activins, myostatin and/or GDF-11. In some embodiments, the recombinant follistatin fusion protein has an *in vivo* half-life greater than about 2 days (e.g., greater than about 2.5 days, about 3 days, about 3.5 days, about 4 days, about 4.5 days, about 5 days, about 5.5 days, about 6 days). In some embodiments, the recombinant follistatin fusion protein has an *in vivo* half-life ranging from about 2-10 days (e.g., ranging from about 2.5-10 days, from about 3-10 days, from about 3.5-10 days, from about 4-10 days, from about 4.5-10 days, from about 5-10 days, from about 3-8 days, from about 3.5-8 days, from about 4-8 days, from about 4.5-8 days, from about 5-8 days, from about 3-6 days, from about 3.5-6 days, from about 4-6 days, from about 4.5-6 days, from about 5-6 days). In some embodiments, the *in vivo* half-life is measured in one or more of mice, rats, non-human primates, and/or humans.

[0028] In some embodiments, the Fc domain is an IgG1 Fc domain. In some embodiments, the Fc domain has an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK
(SEQ ID NO:3); or

KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPV
LDSGDSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK (SEQ ID NO:
4); or

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTP

VLDSGDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 14).

[0029] In some embodiments, a suitable Fc domain comprises one or more mutations that improve binding between the Fc domain and the FcRn receptor resulting in prolonged serum half-life. In some embodiments, the Fc domain comprises one or more mutations at one or more positions corresponding to Thr 250, Met 252, Ser 254, Thr 256, Thr 307, Glu 380, Met 428, His 433, and/or Asn 434 of human IgG1. In particular embodiments, a suitable Fc domain contains mutations H433K (His433Lys) and/or N434F (Asn434Phe). In particular embodiments, a suitable Fc domain comprises a sequence shown below which incorporates the mutations of H433K (His433Lys) and N434F (Asn434Phe):

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
KAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPP
VLDSGDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL**KF**HYTQKSLSLSPGK (SEQ ID NO:15).

[0030] In some embodiments, a recombinant follistatin fusion protein according to the present invention includes a linker such that the Fc fusion via the linker does not substantially change the binding properties of follistatin to cognate ligands, including maintaining the lack of binding to heparin. In some embodiments, a suitable linker is a peptide comprising 3-60 amino acids. In some embodiments, a suitable linker is a peptide comprising at least 10 (e.g., at least 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95) amino acids. In some embodiments, a suitable linker comprises a sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to

GAPGGGGGAAAAAGGGGGGGAP (GAG linker, SEQ ID NO: 5). In some embodiments, a suitable linker comprises a sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to

GAPGGGGGAAAAAGGGGGGGAPGGGGGAAAAAGGGGGGGAP (GAG2 linker, SEQ ID NO: 6). In some embodiments, a suitable linker comprises a sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to

GAPGGGGGAAAAAGGGGGGGAPGGGGGAAAAAGGGGGGGAPGGGGGAAAAAGGGGGG
GAP (GAG3 linker, SEQ ID NO:7).

[0031] In particular embodiments, a recombinant follistatin fusion protein provided by the present invention comprises an amino acid sequence at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:8, 9, 10, 11, 16 or 17:

[0032] In some embodiments, the present invention provides nucleic acids comprising a nucleotide sequence encoding a recombinant follistatin fusion protein described herein. In some embodiments, the present invention provides a cell comprising a nucleic acid comprising a nucleotide sequence encoding a recombinant follistatin fusion protein described herein. In some embodiments, the present invention provides pharmaceutical compositions comprising a recombinant follistatin fusion protein described herein and a pharmaceutically acceptable carrier.

[0033] As used in this application, the terms “about” and “approximately” are used as equivalents. Any numerals used in this application with or without about/approximately are meant to cover any normal fluctuations appreciated by one of ordinary skill in the relevant art.

[0034] Other features, objects, and advantages of the present invention are apparent in the detailed description that follows. It should be understood, however, that the detailed description, while indicating embodiments of the present invention, is given by way of illustration only, not limitation. Various changes and modifications within the scope of the invention will become apparent to those skilled in the art from the detailed description.

BRIEF DESCRIPTION OF THE DRAWING

[0035] The drawings are for illustration purposes only not for limitation.

[0036] FIG. 1 shows exemplary results illustrating that FS315-Fc does not inhibit BMP-9 or BMP-10 signaling through the Smad 1/5/8 pathway as compared to a commercially available soluble activin receptor (sActRIIB). FIG. 1A shows exemplary results of the BMP-9 inhibition assay and FIG. 1B shows exemplary results of the BMP-10 inhibition assay.

[0037] FIG. 2 shows exemplary results illustrating that FS315-Fc inhibits myostatin-and activin-mediated Smad 2/3 signaling. FIG. 2A shows exemplary results of the myostatin inhibition assay and FIG.2B shows exemplary results of an activin A inhibition assay.

[0038] FIG. 3 shows exemplary results illustrating PK profile across tissues. An exemplary follistatin-Fc protein has a serum half-life of ~ 5 days in mouse serum (FIG.3A) and a tissue half-life of 2 – 5 days (FIG.3B).

[0039] FIG. 4 shows exemplary results illustrating the effect of an exemplary follistatin-Fc protein on muscle weight of quadriceps (FIG.4A), gastrocnemius (FIG.4B), tibialis anterior (FIG.4C), and triceps (FIG.4D) after 4 and 10 weeks of exposure to 1 mg/kg FS315-mFc and 6 weeks exposure to 8 mg/kg. The muscle weights are corrected to baseline body weight.

[0040] FIG. 5 shows exemplary results illustrating the effect of exemplary follistatin-Fc protein on serum follistatin levels over time. FIG.5A. shows the levels in the serum after treatment with 1 mg/kg FS315-mFc over 10 weeks, and FIG.5B. shows the levels in the serum after treatment with 8 mg/kg FS315-mFc over 6 weeks.

[0041] FIG. 6 shows exemplary results illustrating the effect of exemplary follistatin-Fc protein on muscle weight of the gastrocnemius after exposure to FS315-mFc, sActRIIB-mFc, or PBS control.

[0042] FIG. 7 shows exemplary results illustrating the effects of follistatin wild-type and variants on body weight. Panel A shows exemplary average body weight of animals in each group over time, and panel B shows exemplary average body weight of animals in each group at week 6 post-injection.

[0043] FIG. 8 shows exemplary results illustrating the effect of follistatin variants on the weight of injected muscle at week 2 post-injection. Panel A shows the mass of the injected muscle gastrocnemius while panel B shows the mass of the injected muscle quadriceps.

[0044] FIG. 9 shows exemplary results illustrating the effect of domain 3 deletion on injected muscle and muscle remote from the injection site two weeks post-injection. The left quadriceps was a site of injection while the right quadriceps is the contralateral intra-animal control muscle.

[0045] FIG. 10 shows exemplary results illustrating the effect of follistatin variants on the weight of specific muscles in injection site and distal from the injection site at week 4 post-injection. Panel A (gastrocnemius) and panel B (quadriceps) are injected muscle. Panel C (tibialis anterior), Panel D (triceps) and Panel E (diaphragm) are muscle distal from injection site.

[0046] FIG. 11 shows exemplary results illustrating the effect of domain 3 deletion on injected muscle and muscle remote from the injection site four weeks post-injection. The left quadriceps was a site of injection while the right quadriceps is the contralateral intra-animal control muscle.

[0047] FIG. 12 shows exemplary results illustrating the effect of follistatin variants on fiber size in both injected muscles and distal muscles at week 2 post-injection in the (A) quadriceps, (B) gastrocnemius, (C) tibialis anterior, (D) triceps, and (E) diaphragm

[0048] FIG. 13 shows exemplary results illustrating the effect of follistatin variants on fiber size in both injected muscles and distal muscles at week 4 post-injection, in the (A) quadriceps, (B) gastrocnemius, (C) tibialis anterior, (D) triceps, and (E) diaphragm

[0049] FIG. 14 shows exemplary results illustrating the effect of follistatin variants on fiber size in both injected muscles and distal muscles at week 6 post-injection, in the (A) quadriceps, (B) gastrocnemius, (C) tibialis anterior, (D) triceps, and (E) diaphragm

[0050] FIG. 15 shows exemplary results demonstrating the effect of an exemplary follistatin-Fc protein on the diameter of myofibers in the gastrocnemius of A) C57 mice and B) mdx mice after 4 weeks of exposure.

[0051] FIG. 16 shows exemplary results demonstrating the effect of an exemplary follistatin-Fc protein on the body weight of treated C57 mice after 2, 4, 6, or 8 weeks of exposure, as compared to vehicle control animals.

[0052] FIG. 17 shows exemplary results demonstrating the effect of an exemplary follistatin-Fc protein on the weight of the triceps and quadriceps of treated animals as a percent increase over vehicle control animals, after 4 and 8 weeks of exposure.

[0053] FIG. 18 shows exemplary results demonstrating the effect of an exemplary follistatin-Fc protein on the diameter of myofibers in the triceps and quadriceps of treated animals as percent increase over vehicle control animals after 4 and 8 weeks of exposure.

[0054] FIG. 19 shows exemplary levels of follistatin-Fc protein in the serum of animals administered the fusion protein via twice weekly subcutaneous injection after 1, 3, 4, 6, or 8 weeks of exposure. Panel A) shows the results from animals euthanized after 4 weeks and panel B) shows the results from animals euthanized after 8 weeks of exposure.

[0055] FIG. 20 shows exemplary results demonstrating the effect of follistatin-Fc protein on the mRNA expression of three markers of fibrosis: alpha-smooth muscle actin, collagen triple helix repeat containing 1 protein (cthrcl), and collagen I, in the quadriceps of treated animals as compared to vehicle control animals after 6 or 12 weeks of exposure.

[0056] FIG. 21 shows exemplary H&E stained sections of quadriceps and triceps tissue of mdx mice treated with vehicle or follistatin-Fc protein for six weeks. Also shown are exemplary H&E stains from the quadriceps and triceps of C57 control mice.

[0057] FIG. 22 shows exemplary collagen I stained sections of quadriceps, triceps, and diaphragm tissue of mdx mice treated with vehicle or follistatin-Fc protein for twelve weeks. Also shown are exemplary collagen I stains from the quadriceps, triceps and diaphragm of C57 control mice

[0058] FIG. 23 shows exemplary results demonstrating the effects of twice weekly intramuscular injections of one of two follistatin variants, a FS315-mFc fusion protein and a dFSD3-mFc variant fusion protein, on the muscle weights of C57BL/10 mice treated for four weeks as compared to the contra-lateral vehicle control muscle.

[0059] FIG. 24 shows exemplary results demonstrating that the FS315-GAG3-mFc and FS315-GAG3-hFc fusion proteins inhibit (A) myostatin and (B) activin signaling in the CAGA-luciferase assay to the same extent as native FS315. In comparison, Sino Biological FS315-hFc (manufactured by Sino Biological Inc. Catalog Number 10685-H02H) which contains a 9 amino acid linker is significantly less potent.

[0060] FIG. 25 shows levels of FS315-GAG3-hFc protein in the serum of rats dosed with a single SC injection of 10 mg/kg protein. The calculated serum half-life was 3.5 days.

DEFINITIONS

[0061] In order for the present invention to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth throughout the specification.

[0062] *Animal*: As used herein, the term “animal” refers to any member of the animal kingdom. In some embodiments, “animal” refers to humans, at any stage of development. In some embodiments, “animal” refers to non-human animals, at any stage of development. In certain embodiments, the non-human animal is a mammal (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a sheep, cattle, a primate, and/or a pig). In some embodiments, animals include, but are not limited to, mammals, birds, reptiles, amphibians, fish, insects, and/or worms. In some embodiments, an animal may be a transgenic animal, genetically-engineered animal, and/or a clone.

[0063] *Approximately or about*: As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0064] *Bioavailability*: As used herein, the term “bioavailability” generally refers to the percentage of the administered dose that reaches the blood stream of a subject.

[0065] *Biologically active*: As used herein, the phrase “biologically active” refers to a characteristic of any agent that has activity in a biological system, and particularly in an organism. For instance, an agent that, when administered to an organism, has a biological effect on that organism, is considered to be biologically active. In particular embodiments, where a peptide is biologically active, a portion of that peptide that shares at least one biological activity of the peptide is typically referred to as a “biologically active” portion.

[0066] *Cardiac Muscle:* As used herein, the term “cardiac muscle” refers to a type of involuntary striated muscle found in the walls of the heart, and particularly the myocardium.

[0067] *Carrier or diluent:* As used herein, the terms “carrier” and “diluent” refers to a pharmaceutically acceptable (e.g., safe and non-toxic for administration to a human) carrier or diluting substance useful for the preparation of a pharmaceutical formulation. Exemplary diluents include sterile water, bacteriostatic water for injection (BWFI), a pH buffered solution (e.g. phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

[0068] *Follistatin or recombinant follistatin:* As used herein, the term “follistatin (FS)” or “recombinant follistatin” refers to any wild-type and modified follistatin proteins (e.g., follistatin proteins with amino acid mutations, deletions, insertions, and/or fusion proteins) that retain substantial follistatin biological activity unless otherwise specified. A non-limiting example of deletions is a domain 3 deletion ($\Delta D3$ or dFSD3). A non-limiting example of fusion proteins is an Fc-fusion protein.

[0069] *Functional equivalent or derivative:* As used herein, the term “functional equivalent” or “functional derivative” denotes, in the context of a functional derivative of an amino acid sequence, a molecule that retains a biological activity (either function or structural) that is substantially similar to that of the original sequence. A functional derivative or equivalent may be a natural derivative or is prepared synthetically. Exemplary functional derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The substituting amino acid desirably has chemico-physical properties which are similar to that of the substituted amino acid. Desirable similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophilicity, and the like.

[0070] *Fusion protein:* As used herein, the term “fusion protein” or “chimeric protein” refers to a protein created through the joining of two or more originally separate proteins, or portions thereof. In some embodiments, a linker or spacer will be present between each protein.

[0071] *Half-Life:* As used herein, the term “half-life” is the time required for a quantity such as protein concentration or activity to fall to half of its value as measured at the beginning of a time period.

[0072] *Hypertrophy*: As used herein the term “hypertrophy” refers to the increase in volume of an organ or tissue due to the enlargement of its component cells.

[0073] *Improve, increase, or reduce*: As used herein, the terms “improve,” “increase” or “reduce,” or grammatical equivalents, indicate values that are relative to a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control subject (or multiple control subject) in the absence of the treatment described herein. A “control subject” is a subject afflicted with the same form of disease as the subject being treated, who is about the same age as the subject being treated.

[0074] *In Vitro*: As used herein, the term “*in vitro*” refers to events that occur in an artificial environment, *e.g.*, in a test tube or reaction vessel, in cell culture, *etc.*, rather than within a multi-cellular organism.

[0075] *In Vivo*: As used herein, the term “*in vivo*” refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, *in vitro* systems).

[0076] *Linker*: As used herein, the term “linker” refers to, in a fusion protein, an amino acid sequence other than that appearing at a particular position in the natural protein and is generally designed to be flexible or to interpose a structure, such as an α -helix, between two protein moieties. A linker is also referred to as a spacer. A linker or a spacer typically does not have biological function on its own.

[0077] *Polypeptide*: The term “polypeptide” as used herein refers to a sequential chain of amino acids linked together via peptide bonds. The term is used to refer to an amino acid chain of any length, but one of ordinary skill in the art will understand that the term is not limited to lengthy chains and can refer to a minimal chain comprising two amino acids linked together via a peptide bond. As is known to those skilled in the art, polypeptides may be processed and/or modified. As used herein, the terms “polypeptide” and “peptide” are used inter-changeably.

[0078] *Prevent*: As used herein, the term “prevent” or “prevention”, when used in connection with the occurrence of a disease, disorder, and/or condition, refers to reducing the risk of developing the disease, disorder and/or condition. See the definition of “risk.”

[0079] *Protein*: The term “protein” as used herein refers to one or more polypeptides that function as a discrete unit. If a single polypeptide is the discrete functioning unit and does not require permanent or temporary physical association with other polypeptides in order to form the discrete functioning unit, the terms “polypeptide” and “protein” may be used interchangeably. If the discrete functional unit is comprised of more than one polypeptide that physically associate with one another, the term “protein” refers to the multiple polypeptides that are physically coupled and function together as the discrete unit.

[0080] *Risk*: As will be understood from context, a “risk” of a disease, disorder, and/or condition comprises a likelihood that a particular individual will develop a disease, disorder, and/or condition (e.g., muscular dystrophy). In some embodiments, risk is expressed as a percentage. In some embodiments, risk is from 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 up to 100%. In some embodiments risk is expressed as a risk relative to a risk associated with a reference sample or group of reference samples. In some embodiments, a reference sample or group of reference samples have a known risk of a disease, disorder, condition and/or event (e.g., muscular dystrophy). In some embodiments a reference sample or group of reference samples are from individuals comparable to a particular individual. In some embodiments, relative risk is 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more.

[0081] *Striated muscle*: As used herein, the term “striated muscle” refers to multinucleated muscle tissue with regular arrangement of their intracellular contractile units, sarcomeres, leading to the appearance of striations using microscopy and under voluntary control. Typically, striated muscle can be cardiac muscle, skeletal muscle, and Branchiomeric muscles.

[0082] *Smooth muscle*: As used herein, the term “smooth muscle” refers to involuntarily controlled, non-striated muscle, including unitary and multi-unit muscle.

[0083] *Subject*: As used herein, the term “subject” refers to a human or any non-human animal (e.g., mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate). A human includes pre- and post-natal forms. In many embodiments, a subject is a human being. A subject can be a patient, which refers to a human presenting to a medical provider for diagnosis or treatment of a disease. The term “subject” is used herein interchangeably with “individual” or

“patient.” A subject can be afflicted with or is susceptible to a disease or disorder but may or may not display symptoms of the disease or disorder.

[0084] *Substantially:* As used herein, the term “substantially” refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term “substantially” is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0085] *Substantial homology:* The phrase “substantial homology” is used herein to refer to a comparison between amino acid or nucleic acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be “substantially homologous” if they contain homologous residues in corresponding positions. Homologous residues may be identical residues. Alternatively, homologous residues may be non-identical residues will appropriately similar structural and/or functional characteristics. For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as “hydrophobic” or “hydrophilic” amino acids., and/or as having “polar” or “non-polar” side chains. Substitution of one amino acid for another of the same type may often be considered a “homologous” substitution.

[0086] As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul, et al., Basic local alignment search tool, *J. Mol. Biol.*, 215(3): 403-410, 1990; Altschul, et al., *Methods in Enzymology*; Altschul, et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402, 1997; Baxevanis, et al., *Bioinformatics : A Practical Guide to the Analysis of Genes and Proteins*, Wiley, 1998; and Misener, et al., (eds.), *Bioinformatics Methods and Protocols* (Methods in Molecular Biology, Vol. 132), Humana Press, 1999. In addition to identifying homologous sequences, the programs mentioned above typically provide an indication of the degree of homology. In some embodiments, two sequences are considered to be substantially homologous if at least 50%,

55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are homologous over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more residues.

[0087] *Substantial identity*: The phrase “substantial identity” is used herein to refer to a comparison between amino acid or nucleic acid sequences. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be “substantially identical” if they contain identical residues in corresponding positions. As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul, et al., Basic local alignment search tool, *J. Mol. Biol.*, 215(3): 403-410, 1990; Altschul, et al., *Methods in Enzymology*; Altschul et al., *Nucleic Acids Res.* 25:3389-3402, 1997; Baxevanis et al., *Bioinformatics : A Practical Guide to the Analysis of Genes and Proteins*, Wiley, 1998; and Misener, et al., (eds.), *Bioinformatics Methods and Protocols* (Methods in Molecular Biology, Vol. 132), Humana Press, 1999. In addition to identifying identical sequences, the programs mentioned above typically provide an indication of the degree of identity. In some embodiments, two sequences are considered to be substantially identical if at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of their corresponding residues are identical over a relevant stretch of residues. In some embodiments, the relevant stretch is a complete sequence. In some embodiments, the relevant stretch is at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more residues.

[0088] *Suffering from*: An individual who is “suffering from” a disease, disorder, and/or condition has been diagnosed with or displays one or more symptoms of the disease, disorder, and/or condition.

[0089] *Susceptible to:* An individual who is “susceptible to” a disease, disorder, and/or condition has not been diagnosed with the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition may not exhibit symptoms of the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, condition, or event (for example, DMD) may be characterized by one or more of the following: (1) a genetic mutation associated with development of the disease, disorder, and/or condition; (2) a genetic polymorphism associated with development of the disease, disorder, and/or condition; (3) increased and/or decreased expression and/or activity of a protein associated with the disease, disorder, and/or condition; (4) habits and/or lifestyles associated with development of the disease, disorder, condition, and/or event (5) having undergone, planning to undergo, or requiring a transplant. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will develop the disease, disorder, and/or condition. In some embodiments, an individual who is susceptible to a disease, disorder, and/or condition will not develop the disease, disorder, and/or condition.

[0090] *Target tissues:* As used herein, the term “target tissues” refers to any tissue that is affected by a disease to be treated such as Duchenne muscular dystrophy (DMD). In some embodiments, target tissues include those tissues that display disease-associated pathology, symptom, or feature, including but not limited to muscle wasting, skeletal deformation, cardiomyopathy, and impaired respiratory function.

[0091] *Therapeutically effective amount:* As used herein, the term “therapeutically effective amount” of a therapeutic agent means an amount that is sufficient, when administered to a subject suffering from or susceptible to a disease, disorder, and/or condition, to treat, diagnose, prevent, and/or delay the onset of the symptom(s) of the disease, disorder, and/or condition. It will be appreciated by those of ordinary skill in the art that a therapeutically effective amount is typically administered via a dosing regimen comprising at least one unit dose.

[0092] *Treating:* As used herein, the term “treat,” “treatment,” or “treating” refers to any method used to partially or completely alleviate, ameliorate, relieve, inhibit, prevent, delay onset of, reduce severity of and/or reduce incidence of one or more symptoms or features of a

particular disease, disorder, and/or condition. Treatment may be administered to a subject who does not exhibit signs of a disease and/or exhibits only early signs of the disease for the purpose of decreasing the risk of developing pathology associated with the disease.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[0093] The present invention provides, among other things, methods and compositions for treating muscular dystrophy, including Duchenne muscular dystrophy (DMD) and/or Becker Muscular Dystrophy, based on follistatin as a protein therapeutic. In some embodiments, the present invention provides methods of treating DMD including administering to an individual who is suffering from or susceptible to DMD an effective amount of a recombinant follistatin protein such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset.

[0094] Various aspects of the invention are described in detail in the following sections. The use of sections is not meant to limit the invention. Each section can apply to any aspect of the invention. In this application, the use of “or” means “and/or” unless stated otherwise.

Duchenne muscular dystrophy (DMD)

[0095] DMD is a disease characterized by progressive deterioration of muscles and loss of muscle related functions throughout the body. It is contemplated that the present invention provides methods and compositions for regenerating muscle and treating fibrosis, inflammation and other symptoms or features associated with DMD and other muscular dystrophies in various muscle tissues. In some embodiments, use of provided methods and compositions in a subject result in a decrease fibrosis and/or necrosis in that subject.

Muscle tissues

[0096] There are two major types of muscle tissue in an animal – striated muscle and smooth muscle. As used herein, the term “striated muscle” refers to muscle tissues containing repeating sarcomeres. Striated muscle tends to be under voluntary control and attached to the

skeleton, though there are some exceptions, such as cardiac muscle, which has several properties of striated muscle, but is not under voluntary control. Generally, striated muscle allows for voluntary movement of the body and includes the major muscle groups including the quadriceps, gastrocnemius, biceps, triceps, trapezius, deltoids, and many others. Striated muscle tends to be very long and, many striated muscles are able to function independently. Some striated muscle, however, is not attached to the skeleton, including those in the mouth, anus, heart, and upper portion of the esophagus.

[0097] Smooth muscle, on the other hand, has very different structure. Rather than a series of long muscles with separate skeletal attachments, smooth muscle tends to be organized into continuous sheets with mechanical linkages between smooth muscle cells. Smooth muscle is often located in the walls of hollow organs and is usually not under voluntary control. Smooth muscles lining a particular organ must bear the same load and contract concurrently. Smooth muscle functions, at least in part, to handle changes in load on hollow organs caused by movement and/or changes in posture or pressure. This dual role means that smooth muscle must not only be able to contract like striated muscle, but also that it must be able to contract tonically to maintain organ dimensions against sustained loads. Examples of smooth muscles are those lining blood vessels, bladder, gastrointestinal track such as rectum.

[0098] The strength of a muscle depends on the number and sizes of the muscle's cells and on their anatomic arrangement. Increasing the diameter of a muscle fiber either by the increase in size of existing myofibrils (hypertrophy) and/or the formation of more muscle cells (hyperplasia) will increase the force-generating capacity of the muscle.

[0099] Muscles may also be grouped by location or function. In some embodiments, a recombinant follistatin protein is targeted to one or more muscles of the face, one or more muscles for mastication, one or more muscles of the tongue and neck, one or more muscles of the thorax, one or more muscles of the pectoral girdle and arms, one or more muscles of the arm and shoulder, one or more ventral and dorsal forearm muscles, one or more muscles of the hand, one or more muscles of the erector spinae, one or more muscles of the pelvic girdle and legs, and/or one or more muscles of the foreleg and foot.

[0100] In some embodiments, muscles of the face include, but are not limited to, intraocular muscles such as ciliary, iris dilator, iris sphincter; muscles of the ear such as auriculares, temporoparietalis, stapedius, tensor tympani; muscles of the nose such as procerus, nasalis, dilator naris, depressor septi nasi, levator labii superioris alaeque nasi; muscles of the mouth such as levator anguli oris, depressor anguli oris, orbicularis oris, Buccinator, Zygomaticus Major and Minor, Platysma, Levator Labii Superioris, Depressor Labii Inferioris, Risorius, Mentalis, and/or Corrugator Supercilii.

[0101] In some embodiments, muscles of mastication include, but are not limited to, Masseter, Temporalis, Medial Pterygoid, Lateral Pterygoid. In some embodiments, muscles of the tongue and neck include, but are not limited to, Genioglossus, Styloglossus, Palatoglossus, Hyoglossus, Digastric, Stylohyoid, Mylohyoid, Geniohyoid, Omohyoid, Sternohyoid, Sternothyroid, Thyrohyoid, Sternocleidomastoid, Anterior Scalene, Middle Scalene, and/or Posterior Scalene.

[0102] In some embodiments, muscles of the thorax, pectoral girdle, and arms include, but are not limited to, Subclavius Pectoralis major, Pectoralis minor, Rectus abdominis, External abdominal oblique, Internal abdominal oblique, Transversus Abdominis, Diaphragm, External Intercostals, Internal Intercostals, Serratus Anterior, Trapezius, Levator Scapulae, Rhomboideus Major, Rhomboideus Minor, Latissimus dorsi, Deltoid, subscapularis, supraspinatus, infraspinatus, Teres major, Teres minor, and/or Coracobrachialis.

[0103] In some embodiments, muscles of the arm and shoulder include, but are not limited to, Biceps brachii-Long Head, Biceps brachii-Short Head, Triceps brachii-Long Head, Triceps brachii Lateral Head, Triceps brachii-Medial Head, Anconeus, Pronator teres, Supinator, and/or Brachialis.

[0104] In some embodiments, muscles of the ventral and dorsal forearm include, but are not limited to, Brachioradialis, Flexor carpi radialis, Flexor carpi ulnaris, Palmaris longus, Extensor carpi ulnaris, Extensor carpi radialis longus, Extensor carpi radialis brevis, Extensor digitorum, Extensor digiti minimi.

[0105] In some embodiments, muscles of the hand include, but are not limited to intrinsic muscles of the hand such as thenar, abductor pollicis brevis, flexor pollicis brevis,

opponens pollicis, hypothenar, abductor digiti minimi, the flexor digiti minimi brevis, opponens digiti minimi, palmar interossei, dorsal interossei and/or lumbricals.

[0106] In some embodiments, muscles of the erector spinae include, but are not limited to, cervicalis, spinalis, longissimus, and/or iliocostalis.

[0107] In some embodiments, muscles of the pelvic girdle and the legs include, but are not limited to, Psoas Major, Iliacus, quadratus femoris, Adductor longus, Adductor brevis, Adductor magnus, Gracilis, Sartorius, Quadriceps femoris such as, rectus femoris, vastus lateralis, vastus medialis, vastus intermedius, Gastrocnemius, Fibularis (Peroneus) Longus, Soleus, Gluteus maximus, Gluteus medius, Gluteus minimus, Hamstrings: Biceps Femoris: Long Head, Hamstrings: Biceps Femoris: Short Head, Hamstrings: Semitendinosus, Hamstrings: Semimembranosus, Tensor fasciae latae, Pectineus, and/or Tibialis anterior.

[0108] In some embodiments, muscles of the foreleg and foot include, but are not limited to, Extensor digitorum longus, Extensor hallucis longus, peroneus brevis, plantaris, Tibialis posterior, Flexor hallucis longus, extensor digitorum brevis, extensor hallucis brevis, Abductor hallucis, flexor hallucis brevis, Abductor digiti minimi, flexor digiti minimi, opponens digiti minimi, extensor digitorum brevis, lumbricales of the foot, Quadratus plantae or flexor accessorius, flexor digitorum brevis, dorsal interossei, and/or plantar interossei.

[0109] Exemplary muscle targets are summarized in Table 1.

Table 1

ORBICULARIS OCULI			
Intraocular: ciliary, iris dilator, iris sphincter			
Ear: auriculares, temporoparietalis, stapedius, tensor tympani			
Nose: procerus, nasalis, dilator naris, depressor septi nasi, levator labii superioris alaeque nasi			
Mouth: levator anguli oris, depressor anguli oris, orbicularis oris			
Buccinator	Zygomaticus Major and Minor	Platysma	Levator Labii Superioris

Depressor Labii Inferioris	Risorius	Mentalis	Corrugator Supercilii
Anconeus	Pronator teres	Supinator	Brachialis
MUSCLES OF MASTICATION			
Masseter	Temporalis	Medial Pterygoid	Lateral Pterygoid
MUSCLES OF THE TONGUE AND NECK			
Genioglossus	Styloglossus	Palatoglossus	Hyoglossus
Digastric	Stylohyoid	Mylohyoid	Geniohyoid
Omohyoid	Sternohyoid	Sternothyroid	Thyrohyoid
Sternocleidomastoid	Anterior Scalene	Middle Scalene	Posterior Scalene
MUSCLES OF THE THORAX, PECTORAL GIRDLE AND ARMS			
Subclavius	Pectoralis major	Pectoralis minor	Rectus abdominis
External abdominal oblique	Internal abdominal oblique	Transversus Abdominis	Diaphragm
External Intercostals	Internal Intercostals	Serratus Anterior	Trapezius
Levator Scapulae	Rhomboideus Major	Rhomboideus Minor	Latissimus dorsi
Deltoid	subscapularis	supraspinatus	infraspinatus
Teres major	Teres minor	Coracobrachialis	
ARM AND SHOULDER			
Biceps brachii-Long Head	Biceps brachii-Short Head	Triceps brachii-Long Head	Triceps brachii-Lateral Head
Triceps brachii-Medial Head	Anconeus	Pronator teres	Supinator
Brachialis			
FOREARM MUSCLES: Ventral and Dorsal			
Brachioradialis	Flexor carpi	Flexor carpi	Palmaris longus

	radialis	ulnaris	
Extensor carpi ulnaris	Extensor carpi radialis longus	Extensor carpi radialis brevis	Extensor digitorum
Extensor digiti minimi	erector spinae: cervicalis	erector spinae: spinalis	erector spinae: longissimus
erector spinae: iliocostalis			
Intrinsic Muscles of the Hand: thenar, abductor pollicis brevis, flexor pollicis brevis, and the opponens pollicis			
Intrinsic Muscles of the Hand: hypothenar, abductor digiti minimi, the flexor digiti minimi brevis, and the opponens digiti minimi			
Intrinsic Muscles of the Hand: palmar interossei, dorsal interossei and lumbricals			
MUSCLES OF THE PELVIC GIRDLE AND THE LEGS			
Iliopsoas: Psoas Major	Iliopsoas: Iliacus	quadratus femoris	Adductor longus
Adductor brevis	Adductor magnus	Gracilis	Sartorius
Quadriceps femoris: rectus femoris	Quadriceps femoris: vastus lateralis	Quadriceps femoris: vastus medialis	Quadriceps femoris: vastus intermedius
Gastrocnemius	Fibularis (Peroneus) Longus	Soleus	Gluteus maximus
Gluteus medius	Gluteus minimus	Hamstrings: Biceps Femoris: Long Head	Hamstrings: Biceps Femoris: Short Head
Hamstrings: Semitendinosus	Hamstrings: Semimembranosus	Tensor fasciae latae	Pectineus
Tibialis anterior			
MUSCLES OF THE FORELEG AND FOOT			
Extensor digitorum	Extensor hallucis	peroneus brevis	plantaris

longus	longus		
Tibialis posterior	Flexor hallucis longus	extensor digitorum brevis	extensor hallucis brevis
Abductor hallucis	flexor hallucis brevis	Abductor digiti minimi	flexor digiti minimi
opponens digiti minimi	extensor digitorum brevis	lumbricales of the foot	Quadratus plantae or flexor accessorius
Flexor digitorum brevis	dorsal interossei	plantar interossei	

Muscular Dystrophy

[0110] Muscular dystrophies are a group of inherited disorders that cause degeneration of muscle, leading to weak and impaired movements. A central feature of all muscular dystrophies is that they are progressive in nature. Muscular dystrophies include, but are not limited to: Duchenne muscular dystrophy (DMD), Becker muscular dystrophy, Emery-Dreifuss muscular dystrophy, facioscapulohumeral muscular dystrophy, limb-girdle muscular dystrophies, and myotonic dystrophy Types 1 and 2, including the congenital form of Myotonic dystrophy Type 1. Symptoms may vary by type of muscular dystrophy with some or all muscles being affected. Exemplary symptoms of muscular dystrophies include delayed development of muscle motor skills, difficulty using one or more muscle groups, difficulty swallowing, speaking or eating, drooling, eyelid drooping, frequent falling, loss of strength in a muscle or group of muscles as an adult, loss in muscle size, problems walking due to weakness or altered biomechanics of the body, muscle hypertrophy, muscle pseudohypertrophy, fatty infiltration of muscle, replacement of muscle with non-contractile tissue (e.g., muscle fibrosis), muscle necrosis, and/or cognitive or behavioral impairment/mental retardation.

[0111] While there are no known cures for muscular dystrophies, several supportive treatments are used which include both symptomatic and disease modifying therapies. Corticosteroids, physical therapy, orthotic devices, wheelchairs, or other assistive medical devices for ADLs and pulmonary function are commonly used in muscular dystrophies. Cardiac

pacemakers are used to prevent sudden death from cardiac arrhythmias in Myotonic dystrophy. Anti-myotonic agents which improve the symptoms of myotonia (inability to relax) include mexilitine, and in some cases phenytoin, procainamide and quinine.

Duchenne muscular dystrophy

[0112] Duchenne muscular dystrophy (DMD) is a recessive X-linked form of muscular dystrophy which results in muscle degeneration and eventual death. DMD is characterized by weakness in the proximal muscles, abnormal gait, pseudohypertrophy in the gastrocnemius (calf) muscles, and elevated creatine kinase (CK). Many DMD patients are diagnosed around the age of 5, when symptoms/signs typically become more obvious. Affected individuals typically stop walking around age 10-13 and die in or before their mid to late 20's due to cardiorespiratory dysfunction.

[0113] The disorder DMD is caused by a mutation in the dystrophin gene, located on the human X chromosome, which codes for the protein dystrophin, an important structural component within muscle tissue that provides structural stability to the dystroglycan complex (DGC) of the cell membrane. Dystrophin links the internal cytoplasmic actin filament network and extracellular matrix, providing physical strength to muscle fibers. Accordingly, alteration or absence of dystrophin results in abnormal sarcolemmal membrane tearing and necrosis of muscle fibers. While persons of both sexes can carry the mutation, females rarely exhibit severe signs of the disease.

[0114] A main symptom of DMD is muscle weakness associated with muscle wasting with the voluntary muscles being first affected typically, especially affecting the muscles of the hips, pelvic area, thighs, shoulders, and calf muscles. Muscle weakness also occurs in the arms, neck, and other areas. Calves are often enlarged. Signs and symptoms usually appear before age 6 and may appear as early as infancy. Other physical symptoms include, but are not limited to, delayed ability to walk independently, progressive difficulty in walking, stepping, or running, and eventual loss of ability to walk (usually by the age of 15); frequent falls; fatigue; difficulty with motor skills (running, hopping, jumping); increased lumbar lordosis, leading to shortening of the hip-flexor muscles; contractures of achilles tendon and hamstrings impairing functionality

because the muscle fibers shorten and fibrosis occurs in connective tissue; muscle fiber deformities; pseudohypertrophy (enlargement) of tongue and calf muscles caused by replacement of muscle tissue by fat and connective tissue; higher risk of neurobehavioral disorders (e.g., ADHD), learning disorders (dyslexia), and non-progressive weaknesses in specific cognitive skills (in particular short-term verbal memory); skeletal deformities (including scoliosis in some cases).

Recombinant follistatin proteins

[0115] As used herein, recombinant follistatin proteins suitable for the present invention include any wild-type and modified follistatin proteins (e.g., follistatin proteins with amino acid mutations, deletions, insertions, and/or fusion proteins) that retain substantial follistatin biological activity. Typically, a recombinant follistatin protein is produced using recombinant technology. However, follistatin proteins (wild-type or modified) purified from natural resources or synthesized chemically can be used according to the present invention. Typically, a suitable recombinant follistatin protein has an *in vivo* half-life of or greater than about 12 hours, 18 hours, 24 hours, 36 hours, 2 days, 2.5 days, 3 days, 3.5 days, 4 days, 4.5 days, 5 days, 5.5 days, 6 days, 6.5 days, 7 days, 7.5 days, 8 days, 8.5 days, 9 days, 9.5 days, or 10 days. In some embodiments, a recombinant follistatin protein has an *in vivo* half-life of between 0.5 and 10 days, between 1 day and 10 days, between 1 day and 9 days, between 1 day and 8 days, between 1 day and 7 days, between 1 day and 6 days, between 1 day and 5 days, between 1 day and 4 days, between 1 day and 3 days, between 2 days and 10 days, between 2 days and 9 days, between 2 days and 8 days, between 2 days and 7 days, between 2 days and 6 days, between 2 days and 5 days, between 2 days and 4 days, between 2 days and 3 days, between 2.5 days and 10 days, between 2.5 days and 9 days, between 2.5 days and 8 days, between 2.5 days and 7 days, between 2.5 days and 6 days, between 2.5 days and 5 days, between 2.5 days and 4 days, between 3 days and 10 days, between 3 days and 9 days, between 3 days and 8 days, between 3 days and 7 days, between 3 days and 6 days, between 3 days and 5 days, between 3 days and 4 days, between 3.5 days and 10 days, between 3.5 days and 9 days, between 3.5 days and 8 days, between 3.5 days and 7 days, between 3.5 days and 6 days, between 3.5 days and 5 days, between 3.5 days and 4 days, between 4 days and 10 days, between 4 days and 9 days, between 4

days and 8 days, between 4 days and 7 days, between 4 days and 6 days, between 4 days and 5 days, between 4.5 days and 10 days, between 4.5 days and 9 days, between 4.5 days and 8 days, between 4.5 days and 7 days, between 4.5 days and 6 days, between 4.5 days and 5 days, between 5 days and 10 days, between 5 days and 9 days, between 5 days and 8 days, between 5 days and 7 days, between 5 days and 6 days, between 5.5 days and 10 days, between 5.5 days and 9 days, between 5.5 days and 8 days, between 5.5 days and 7 days, between 5.5 days and 6 days, between 6 days and 10 days, between 7 days and 10 days, between 8 days and 10 days, between 9 days and 10 days.

[0116] Follistatin (FS) was first isolated from follicular fluid, as a protein factor capable of suppressing pituitary cell follicle stimulating hormone (FSH) secretion. FS exerts its influence over FSH at least in part through the binding and neutralization of activin.

[0117] There are at least two isoforms of FS: FS288 and FS315, created through alternative splicing at the C-terminus. The 288-amino acid isoform has a distinctive structure comprised of a 63 amino acid N-terminal region containing hydrophobic residues important for activin binding, with the major portion of the protein (residues 64-288) comprising three 10-cysteine FS domains of approximately 73-75 amino acids each. These 10-cysteine domains, from N-terminus to C-terminus, are referred to as domain 1, domain 2 and domain 3, respectively. The FS315 isoform is created through an acidic extension of the C-terminus encoded by an extra exon. FS288 tends to be tissue-bound due to the presence of a heparin binding domain, while FS315 tends to be a circulating form, potentially because the heparin binding domain is masked by the extended C-terminus.

[0118] It has been shown that FS inhibits both myostatin and activin *in vitro* and that this inhibition can lead to hypertrophy *in vivo* in mice (Lee et al., *Regulation of Muscle Mass by Follistatin and Activins*, (2010), MOL. ENDOCRINOL., 24(10): 1998-2008; Gilson et al., *Follistatin Induces Muscle Hypertrophy Through Satellite Cell Proliferation and Inhibition of Both Myostatin and Activin*, (2009), J. PHYSIOL. ENDOCRINOL., 297(1):E157-E164). Without wishing to be held to a particular theory, this observed effect may be at least partially due to FS preventing activation of the Smad2/3 pathway by myostatin and activin. Activation of the Smad2/3 pathway has been shown to result in negative regulation of muscle growth (Zhu et al., *Follistatin Improves Skeletal Muscle Healing After Injury and Disease Through an*

Interaction with Muscle Regeneration, Angiogenesis, and Fibrosis, (2011),
MUSCULOSKELETAL PATHOLOGY, 179(2):915-930).

[0119] The amino acid sequences of a typical wild-type or naturally-occurring human FS315 and FS288 protein are shown in Table 2.

Table 2. Exemplary Human Follistatin Isoforms

FS315	GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNG GAPNCIPCKETCENVDCGPGKKCRMNKKNKPRVCAPDCSNITWKG P V C G L D G K TYRNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAYCVT CNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSC <u>EDIQCTGGKKCLWDFKVGGRGCSLCDELCPDSKSDEPVCASDNATYASECAMKE</u> <u>AACSSGVLLLEVKHSGSCNSISEDTEEEEEDEDQDYSFPISSILEW</u> (SEQ ID NO:1)
FS288	GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNG GAPNCIPCKETCENVDCGPGKKCRMNKKNKPRVCAPDCSNITWKG P V C G L D G K TYRNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAYCVT CNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSC EDIQCTGGKKCLWDFKVGGRGCSLCDELCPDSKSDEPVCASDNATYASECAMKE AACSSGVLLLEVKHSGSCN (SEQ ID NO:12)

[0120] Thus, in some embodiments, a recombinant follistatin protein suitable for the present invention is human FS315 (SEQ ID NO:1). As disclosed herein, SEQ ID NO:1 represents the canonical amino acid sequence for the human follistatin protein. In some embodiments, a follistatin protein may be a splice isoform such as FS 288 (SEQ ID NO:12). In some embodiments, a suitable recombinant follistatin protein may be a homologue or an analogue of a wild-type or naturally-occurring protein. For example, a homologue or an analogue of human wild-type or naturally-occurring follistatin protein may contain one or more amino acid or domain substitutions, deletions, and/or insertions as compared to a wild-type or naturally-occurring follistatin protein (e.g., SEQ ID NO:1), while retaining substantial follistatin protein activity. Thus, in some embodiments, a recombinant follistatin protein suitable for the present invention is substantially homologous to human FS315 follistatin protein (SEQ ID NO:1). In some embodiments, a recombinant follistatin protein suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous to SEQ ID NO:1. In some embodiments, a recombinant follistatin protein suitable for the present invention is substantially identical to human FS315 follistatin protein (SEQ ID NO:1). In some embodiments, a recombinant follistatin protein suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:1.

[0121] Homologues or analogues of human follistatin proteins can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references that compile such methods. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be “substantially homologous” if they contain homologous residues in corresponding positions. Homologous residues may be identical residues. Alternatively, homologous residues may be non-identical residues will appropriately similar structural and/or functional characteristics. For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as “hydrophobic” or “hydrophilic” amino acids., and/or as having “polar” or “non-polar” side chains. Substitution of one amino acid for another of the same type may often be considered a “homologous” substitution. In some embodiments, conservative substitutions of amino acids include substitutions made among amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D. In some embodiments, a “conservative amino acid substitution” refers to an amino acid substitution that does not alter the relative charge or size characteristics of the protein in which the amino acid substitution is made.

[0122] As is well known in this art, amino acid or nucleic acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTN for nucleotide sequences and BLASTP, gapped BLAST, and PSI-BLAST for amino acid sequences. Exemplary such programs are described in Altschul, et al., Basic local alignment search tool, *J. Mol. Biol.*, 215(3): 403-410, 1990; Altschul, et al., *Methods in Enzymology*; Altschul, et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402, 1997; Baxevanis, et al., *Bioinformatics : A Practical Guide to the Analysis of Genes and Proteins*, Wiley, 1998; and Misener, et al., (eds.), *Bioinformatics Methods and Protocols* (Methods in Molecular Biology,

Vol. 132), Humana Press, 1999. In addition to identifying homologous sequences, the programs mentioned above typically provide an indication of the degree of homology.

[0123] In some embodiments, a recombinant follistatin protein suitable for the present invention contains one or more amino acid deletions, insertions or replacement as compared to a wild-type human follistatin protein. For example, a suitable recombinant follistatin protein may contain amino acid substitutions at positions corresponding to Y185 and/or L191, of SEQ ID NO:1.

Domain deletion variants

[0124] In some embodiments, a recombinant follistatin protein suitable for the present invention contains one or more domain deletions, insertions or replacement (e.g., domain swapping) as compared to a wild-type human follistatin protein. For example, a recombinant follistatin protein suitable for the present invention may contain a deletion, insertion and/or replacement of amino acid sequences corresponding to domain 1, 2 and/or 3. In certain embodiments, a recombinant follistatin protein comprises a deletion of amino acids residues 212-288 of SEQ ID NO:1 (which corresponds to domain 3), as shown below:

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIENG
GAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKG
PVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTN
NAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCISISEDTE
EEEEDEDEDQDYSFPISSI
LEW (SEQ ID NO:2).

[0125] It is contemplated that a suitable recombinant follistatin protein may be a homologue or an analogue of a suitable domain deletion variant, containing one or more amino acid substitutions, deletions, and/or insertions as compared to the suitable follistatin domain deletion variant (e.g., SEQ ID NO:2), while retaining substantial follistatin protein activity. Thus, in some embodiments, a recombinant follistatin protein suitable for the present invention has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous or identical to SEQ ID NO:2.

Follistatin fusion proteins

[0126] It is contemplated that a suitable recombinant follistatin protein can be in a fusion protein configuration. For example, a recombinant follistatin protein suitable for the present invention may be a fusion protein between a follistatin domain and another domain or moiety that typically can facilitate a therapeutic effect of follistatin by, for example, enhancing or increasing stability, potency and/or delivery of follistatin protein, or reducing or eliminating immunogenicity, clearance, or toxicity. Such suitable domains or moieties for a follistatin fusion protein include but are not limited to Fc domain, XTEN domain.

Fc Domain

[0127] In some embodiments, a suitable recombinant follistatin protein contains an Fc domain or a portion thereof that binds to the FcRn receptor. As a non-limiting example, a suitable Fc domain may be derived from an immunoglobulin subclass such as IgG. In some embodiments, a suitable Fc domain is derived from IgG1, IgG2, IgG3, or IgG4. In some embodiments, a suitable Fc domain is derived from IgM, IgA, IgD, or IgE. Particularly suitable Fc domains include those derived from human or humanized antibodies. In some embodiments, a suitable Fc domain is a modified Fc portion, such as a modified human Fc portion.

[0128] In some embodiments, a suitable Fc domain comprises an amino acid sequence shown below

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
 NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
 EKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
 KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
 (SEQ ID NO:3), or

KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
 GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
 AKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPV
 LDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:
 4); or

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYV
 DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
 KAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
 VLDSGDSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSLSPGK (SEQ ID
 NO: 14).

[0129] In some embodiments, a suitable Fc domain comprises an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous or identical to SEQ ID NO: 3, SEQ ID NO: 4, or SEQ ID NO: 14.

[0130] It is contemplated that improved binding between Fc domain and the FcRn receptor results in prolonged serum half-life. Thus, in some embodiments, a suitable Fc domain comprises one or more amino acid mutations that lead to improved binding to FcRn. Various mutations within the Fc domain that effect improved binding to FcRn are known in the art and can be adapted to practice the present invention. In some embodiments, a suitable Fc domain comprises one or more mutations at one or more positions corresponding to Thr 250, Met 252, Ser 254, Thr 256, Thr 307, Glu 380, Met 428, His 433 and/or Asn 434 of human IgG1.

[0131] For example, a suitable Fc domain may contain mutations of H433K (His433Lys) and/or N434F (Asn434Phe). As a non-limiting example, a suitable Fc domain may contain mutations H433K (His433Lys) and N434F (Asn434Phe). An exemplary Fc domain sequence incorporating the mutations is shown below:

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYV
 DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS
 KAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
 VLDSGDSFFLYSKLTVDKSRWQQGNVFSVSMHEALKFHYTQKSLSLSPGK (SEQ ID
 NO:15).

[0132] Additional amino acid substitutions that can be included in a Fc domain include those described in, e.g., U.S. Patent Nos. 6,277,375; 8,012,476; and 8,163,881, which are incorporated herein by reference.

Linker or Spacer

[0133] A follistatin domain may be directly or indirectly linked to an Fc domain. In some embodiments, a suitable recombinant follistatin protein contains a linker or spacer that joins a follistatin domain and an Fc domain. An amino acid linker or spacer is generally designed to be flexible or to interpose a structure, such as an alpha-helix, between the two protein moieties. A linker or spacer can be relatively short, or can be longer. Typically, a linker or spacer contains for example 3-100 (e.g., 5-100, 10-100, 20-100, 30-100, 40-100, 50-100, 60-100, 70-100, 80-100, 90-100, 5-55, 10-50, 10-45, 10-40, 10-35, 10-30, 10-25, 10-20) amino acids in length. In some embodiments, a linker or spacer is equal to or longer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 amino acids in length. Typically, a longer linker may decrease steric hindrance. In some embodiments, a linker will comprise a mixture of glycine and serine residues. In some embodiments, the linker may additionally comprise threonine, proline and/or alanine residues. Thus, in some embodiments, the linker comprises between 10-100, 10-90, 10-80, 10-70, 10-60, 10-50, 10-40, 10-30, 10-20, 10-15 amino acids. In some embodiments, the linker comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95 amino acids. In some embodiments, the linker is not a linker consisting of ALEVLFGQP.

[0134] As non-limiting examples, linkers or spacers suitable for the present invention include but are not limited to: GAPGGGGGAAAAAGGGGGGAP (GAG linker, SEQ ID NO:5); GAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAP (GAG2 linker, SEQ ID NO:6); and GAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAP (GAG3 linker, SEQ ID NO:7).

[0135] Suitable linkers or spacers also include those having an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous or identical to the above exemplary linkers, e.g., GAG linker (SEQ ID NO:5), GAG2 linker (SEQ ID NO:6), or GAG3 linker (SEQ ID NO:7). Additional linkers suitable for use with some embodiments may be found in US20120232021, filed on March 2, 2012, the disclosure of which is hereby incorporated by reference in its entirety,

[0136] In some embodiments, a linker is provided that associates the follistatin polypeptide with the Fc domain without substantially affecting the ability of the follistatin polypeptide to bind to any of its cognate ligands (e.g., activin, myostatin, heparin, etc.). In some embodiments, a linker is provided such that the binding of a follistatin peptide to heparin is not altered as compared to the follistatin polypeptide alone. For example, in some embodiments, a follistatin polypeptide is a FS315 polypeptide, which normally does not bind heparin unless it is associated with activin. In some such embodiments, a linker is provided that does not result in increased heparin binding of the FS315 polypeptide as compared to the FS315 polypeptide alone.

Exemplary Follistatin Fusion Proteins

[0137] In particular embodiments, a suitable recombinant follistatin fusion protein includes a follistatin polypeptide, an Fc domain, and a linker that associates the follistatin polypeptide with the Fc domain, wherein the follistatin polypeptide comprises an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the wild-type human FS315 protein (SEQ ID NO:1) or a domain 3 deleted FS315 protein (SEQ ID NO:2). Typically, a suitable recombinant follistatin fusion protein is capable of binding to activin and myostatin. In some embodiments, a suitable recombinant follistatin fusion protein has an *in vivo* half-life ranging from about 0.5-6 days (e.g., about 0.5-5.5 days, about 0.5-5 days, about 1-5 days, about 1.5-5 days, about 1.5-4.5 days, about 1.5-4.0 days, about 1.5-3.5 days, about 1.5-3 days, about 1.5-2.5 days, about 2-6 days, about 2-5.5 days, about 2-5 days, about 2-4.5 days, about 2-4 days, about 2-3.5 days, about 2-3 days). In some embodiments, a suitable recombinant follistatin fusion protein has an *in vivo* half-life ranging from about 2-10 days (e.g., ranging from about 2.5-10 days, from about 3-10 days, from about 3.5-10 days, from about 4-10 days, from about 4.5-10 days, from about 5-10 days, from about 3-8 days, from about 3.5-8 days, from about 4-8 days, from about 4.5-8 days, from about 5-8 days, from about 3-6 days, from about 3.5-6 days, from about 4-6 days, from about 4.5-6 days, from about 5-6 days).

[0138] As non-limiting examples, suitable follistatin Fc fusion proteins may have an amino acid sequence shown below:

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
 ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
 EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
 VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSCNSIS
 EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
 GGGGAPGGGGGAAAAAGGGGGGAPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKD
 TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
 VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLT
 CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
 SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:8),

or

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
 NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
 ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
 EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
 VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSCNSIS
 EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
 GGGGAPGGGGGAAAAAGGGGGGAPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKD
 TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
 VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLT
 CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC
 SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO: 9).

[0139] As other non-limiting examples, suitable follistatin Fc fusion proteins may have an amino acid sequence shown below:

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
 NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
 ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
 EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCISISEDTEEEEEDEDQDYSFPISSI

LEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
 GGGGAPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG
 QPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSL
 SPGK (SEQ ID NO:10),

or

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
 NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
 ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAVCVTCNRICPEPASS
 EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCISEDTEEEEEDEDQDYSFPISSI
 LEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
 GGGGAPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
 DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG
 QPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVSMHEALHNHYTQKSLSL
 SPGK (SEQ ID NO:11).

[0140] As yet other non-limiting examples, suitable follistatin Fc fusion proteins may have an amino acid sequence shown below:

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
 NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
 ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAVCVTCNRICPEPASS
 EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
 VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLLEVKHSGSCNSIS
 EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
 GGGGAPGGGGGAAAAAGGGGGGAPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
 RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
 DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVK

GFYPSDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALHNHYTQKSLSLSPGK (SEQ ID NO:16)

Or

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLLEVKHSGSCNSIS
EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGGAAAAAGGGGGGGAPGGGGGGAAAAAGG
GGGGGAPGGGGGGAAAAAGGGGGGGAPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ
DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVK
GFYPSDIAVEWESNGQPENNYKTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSCSVM
HEALKFHYTQKSLSLSPGK (SEQ ID NO:17)

[0141] In some embodiments, a suitable recombinant follistatin Fc fusion protein has an amino acid sequence at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homologous or identical to SEQ ID NO:8, 9, 10, 11, 16 or 17.

[0142] It is contemplated that a follistatin-Fc fusion protein may be provided in various configurations including homodimeric or monomeric configurations. For example, a suitable homodimeric configuration may be designed to have the C-terminal end of fusion partner (e.g., a follistatin polypeptide plus linker) attached to the N-terminal end of both Fc polypeptide strands. A suitable monomeric configuration may be designed to have the C-terminal end of fusion partner (e.g., a follistatin polypeptide plus linker) fused to one Fc dimer. A monomeric configuration may decrease steric hindrance.

[0143] As used herein, “percent (%) amino acid sequence identity” with respect to a reference protein sequence (e.g., a reference follistatin protein sequence) identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference sequence, after aligning the sequences and introducing

gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Preferably, the WU-BLAST-2 software is used to determine amino acid sequence identity (Altschul *et al.*, Methods in Enzymology 266, 460-480 (1996); <http://blast.wustl.edu/blast/README.html>). WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span=1, overlap fraction=0.125, word threshold (T)=11. HSP score (S) and HSP S2 parameters are dynamic values and are established by the program itself, depending upon the composition of the particular sequence, however, the minimum values may be adjusted and are set as indicated above.

Production of Recombinant Follistatin Proteins

[0144] A recombinant follistatin protein suitable for the present invention may be produced by any available means. For example, a recombinant follistatin protein may be recombinantly produced by utilizing a host cell system engineered to express a recombinant follistatin protein-encoding nucleic acid. Alternatively or additionally, a recombinant follistatin protein may be produced by activating endogenous genes. Alternatively or additionally, a recombinant follistatin protein may be partially or fully prepared by chemical synthesis.

[0145] Where proteins are recombinantly produced, any expression system can be used. To give but a few examples, known expression systems include, for example, *E.coli*, egg, baculovirus, plant, yeast, or mammalian cells.

[0146] In some embodiments, recombinant follistatin proteins suitable for the present invention are produced in mammalian cells. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include BALB/c mouse myeloma line (NSO/I, ECACC No: 85110503); human retinoblasts (PER.C6, CruCell, Leiden, The

Netherlands); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (HEK293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59,1977); human fibrosarcoma cell line (e.g., HT1080); baby hamster kidney cells (BHK21, ATCC CCL 10); Chinese hamster ovary cells +/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216, 1980); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68, 1982); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[0147] In some embodiments, the present invention provides recombinant follistatin proteins produced from human cells. In some embodiments, the present invention provides recombinant follistatin proteins produced from CHO cells or HT1080 cells.

[0148] Typically, cells that are engineered to express a recombinant follistatin protein may comprise a transgene that encodes a recombinant follistatin protein described herein. It should be appreciated that the nucleic acids encoding recombinant follistatin protein may contain regulatory sequences, gene control sequences, promoters, non-coding sequences and/or other appropriate sequences for expressing the recombinant follistatin protein. Typically, the coding region is operably linked with one or more of these nucleic acid components.

[0149] The coding region of a transgene may include one or more silent mutations to optimize codon usage for a particular cell type. For example, the codons of a follistatin transgene may be optimized for expression in a vertebrate cell. In some embodiments, the codons of a follistatin transgene may be optimized for expression in a mammalian cell. In some embodiments, the codons of a follistatin transgene may be optimized for expression in a human cell.

Pharmaceutical composition and administration

[0150] The present invention further provides a pharmaceutical composition containing a recombinant follistatin protein described herein and a physiologically acceptable carrier or excipient.

[0151] Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions (*e.g.*, NaCl), saline, buffered saline, alcohols, glycerol, ethanol, gum arabic, vegetable oils, benzyl alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, sugars such as mannitol, sucrose, or others, dextrose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, *etc.*, as well as combinations thereof. The pharmaceutical preparations can, if desired, be mixed with auxiliary agents (*e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, flavoring and/or aromatic substances and the like) which do not deleteriously react with the active compounds or interference with their activity. In a preferred embodiment, a water-soluble carrier suitable for intravenous administration is used.

[0152] A suitable pharmaceutical composition or medicament, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. A composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. A composition can also be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulations can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, polyvinyl pyrrolidone, sodium saccharine, cellulose, magnesium carbonate, *etc.*

[0153] A pharmaceutical composition or medicament can be formulated in accordance with the routine procedures as a pharmaceutical composition adapted for administration to human beings. For example, in some embodiments, a composition for intravenous administration typically is a solution in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a

hermetically sealed container such as an ampule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water, saline or dextrose/water. Where the composition is administered by injection, an ampule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0154] A recombinant follistatin protein described herein can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, *etc.*

Routes of Administration

[0155] A recombinant follistatin protein described herein (or a composition or medicament containing a recombinant follistatin protein described herein) is administered by any appropriate route. In some embodiments, a recombinant follistatin protein or a pharmaceutical composition containing the same is administered systemically. Systemic administration may be intravenous, intradermal, inhalation, transdermal (topical), intraocular, intramuscular, subcutaneous, intramuscular, oral and/or transmucosal administration. In some embodiments, a recombinant follistatin protein or a pharmaceutical composition containing the same is administered subcutaneously. As used herein, the term “subcutaneous tissue”, is defined as a layer of loose, irregular connective tissue immediately beneath the skin. For example, the subcutaneous administration may be performed by injecting a composition into areas including, but not limited to, the thigh region, abdominal region, gluteal region, or scapular region. In some embodiments, a recombinant follistatin protein or a pharmaceutical composition containing the same is administered intravenously. In some embodiments, a recombinant follistatin protein or a pharmaceutical composition containing the same is administered orally. More than one route can be used concurrently, if desired.

[0156] In some embodiments, administration results only in a localized effect in an individual, while in other embodiments, administration results in effects throughout multiple portions of an individual, for example, systemic effects. Typically, administration results in delivery of a recombinant follistatin protein to one or more target tissues. In some embodiments, the recombinant follistatin protein is delivered to one or more target tissues including, but not limited to, heart, brain, spinal cord, striated muscle (e.g., skeletal muscle), smooth muscle, kidney, liver, lung, and/or spleen. In some embodiments, the recombinant follistatin protein is delivered to the heart. In some embodiments, the recombinant follistatin protein is delivered to striated muscle, in particular, skeletal muscle. In some embodiments, the recombinant follistatin protein is delivered to triceps, tibialis anterior, soleus, gastrocnemius, biceps, trapezius, deltoids, quadriceps, and/or diaphragm.

Dosage Forms and Dosing Regimen

[0157] In some embodiments, a composition is administered in a therapeutically effective amount and/or according to a dosing regimen that is correlated with a particular desired outcome (e.g., with treating or reducing risk for a muscular dystrophy, such as Duchenne muscular dystrophy).

[0158] Particular doses or amounts to be administered in accordance with the present invention may vary, for example, depending on the nature and/or extent of the desired outcome, on particulars of route and/or timing of administration, and/or on one or more characteristics (e.g., weight, age, personal history, genetic characteristic, lifestyle parameter, severity of cardiac defect and/or level of risk of cardiac defect, etc., or combinations thereof). Such doses or amounts can be determined by those of ordinary skill. In some embodiments, an appropriate dose or amount is determined in accordance with standard clinical techniques. Alternatively or additionally, in some embodiments, an appropriate dose or amount is determined through use of one or more *in vitro* or *in vivo* assays to help identify desirable or optimal dosage ranges or amounts to be administered.

[0159] In various embodiments, a recombinant follistatin protein is administered at a therapeutically effective amount. Generally, a therapeutically effective amount is sufficient to

achieve a meaningful benefit to the subject (e.g., treating, modulating, curing, preventing and/or ameliorating the underlying disease or condition). In some particular embodiments, appropriate doses or amounts to be administered may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[0160] In some embodiments, a provided composition is provided as a pharmaceutical formulation. In some embodiments, a pharmaceutical formulation is or comprises a unit dose amount for administration in accordance with a dosing regimen correlated with achievement of the reduced incidence or risk of a muscular dystrophy, such as Duchenne muscular dystrophy.

[0161] In some embodiments, a formulation comprising a recombinant follistatin protein described herein administered as a single dose. In some embodiments, a formulation comprising a recombinant follistatin protein described herein is administered at regular intervals. Administration at an “interval,” as used herein, indicates that the therapeutically effective amount is administered periodically (as distinguished from a one-time dose). The interval can be determined by standard clinical techniques. In some embodiments, a formulation comprising a recombinant follistatin protein described herein is administered bimonthly, monthly, twice monthly, triweekly, biweekly, weekly, twice weekly, thrice weekly, daily, twice daily, or every six hours. The administration interval for a single individual need not be a fixed interval, but can be varied over time, depending on the needs of the individual.

[0162] As used herein, the term “bimonthly” means administration once per two months (*i.e.*, once every two months); the term “monthly” means administration once per month; the term “triweekly” means administration once per three weeks (*i.e.*, once every three weeks); the term “biweekly” means administration once per two weeks (*i.e.*, once every two weeks); the term “weekly” means administration once per week; and the term “daily” means administration once per day.

[0163] In some embodiments, a formulation comprising a recombinant follistatin protein described herein is administered at regular intervals indefinitely. In some embodiments, a formulation comprising a recombinant follistatin protein described herein is administered at regular intervals for a defined period.

Combination Therapy

[0164] In some embodiments, a recombinant follistatin protein is administered in combination with one or more known therapeutic agents (e.g., corticosteroids) currently used for treatment of a muscular dystrophy. In some embodiments, the known therapeutic agent(s) is/are administered according to its standard or approved dosing regimen and/or schedule. In some embodiments, the known therapeutic agent(s) is/are administered according to a regimen that is altered as compared with its standard or approved dosing regimen and/or schedule. In some embodiments, such an altered regimen differs from the standard or approved dosing regimen in that one or more unit doses is altered (e.g., reduced or increased) in amount, and/or in that dosing is altered in frequency (e.g., in that one or more intervals between unit doses is expanded, resulting in lower frequency, or is reduced, resulting in higher frequency).

EXAMPLES***Example 1. Follistatin targets myostatin and activin specifically***

[0165] This example illustrates Follistatin binding to target and non-target ligands to evaluate safety of Follistatin as a protein therapeutic for treating DMD. Without wishing to be bound by theory, it is contemplated that activation of Smad2/3 pathway by myostatin and activin leads to inhibition of myogenic protein expression. As a result, myoblasts can't differentiate into muscle. Therefore, myostatin and activin are considered viable targets for muscle regeneration. However, many myostatin and activin antagonists such as soluble activin receptor type IIB (sActRIIB) also bind bone morphogenetic proteins (BMPs) due to certain structural similarities. BMPs, especially, BMP-9 and BMP-10, are considered pivotal morphogenetic signals, orchestrating tissue architecture throughout the body. Inhibition of such BMPs may lead to undesired pathological conditions. As described in detail below, the experimental data described in this example confirm that Follistatin specifically targets myostatin and activin with high affinity and does not bind to non-target BMPs with meaningful affinity. Thus this example demonstrates that Follistatin can be a safe protein therapeutic with fewer undesired off-target effects as compared to other myostatin modulators such as sActRIIB.

[0166] Specifically, commercially available follistatins (FS315, manufactured by R&D Systems and follistatin-Fc human chimera FS315-hFc, manufactured by Sino Biological), and FS315-GAG3-mFc fusion proteins were used to assess binding affinity and kinetics to activin, myostatin, and BMPs using Biacore assays. Briefly, FS315 was immobilized onto a CM5 chip, and follistatin-Fc fusion proteins were captured using human or mouse antibody capture kits (GE Healthcare). Post amine-coupling, a concentration series of activin, myostatin, or BMPs (e.g., BMP-2, -4, -6, -7, -9, -10, and GDF-11) was added as soluble analyte at 25° C. sActRIIB-hFc was used as a control. Binding affinities (Kd) and kinetics were determined using standard methods. Exemplary results are shown in Table 3.

Table 3. Exemplary Binding Affinity and Kinetics Data

Ligand	KD values (M)			
	FS315	FS315-hFc	FS315-mFc	sActRIIB-hFc
BMP-2	4.4E-07	no binding	no binding	no binding
BMP-4	1.4E-08	NM	8.1E-08	1.3E-07
BMP-6	3.6E-10	9.0E-10	NM	5.7E-11
BMP-7	3.8E-08	NM	NM	1.1E-09
BMP-9	no binding	no binding	no binding	5.4E-11
BMP-10	1.0E-07	1.5E-07	no binding	1.0E-11
GDF-11	N/A	8.2E-10	1.8E-14	3.4E-10
myostatin	1.0E-13	8.4E-14	7.3E-14	1.3E-12
activin	7.3E-10	1.9E-10	3.8E-14	7.0E-11

NM = not measurable due to poor curve fit or high binding to reference chip

[0167] As shown in Table 3, follistatin (e.g., FS315 or FS315-Fc) binds targets myostatin and activin with high affinity but does not bind BMP-9 and -10 (Kd not measurable or greater than 10^{-7} M), while sActRIIB-Fc binds to myostatin, activin and BMPs with similar affinity. Surprisingly and importantly, the Fc fusion increases affinity of follistatin to primary target myostatin by at least 10-fold.

[0168] In addition, luciferase reporter assays were used to further determine if follistatin specifically inhibits myostatin and activin signaling (Smad 2/3 pathway) but not BMP signaling (Smad 1/5/8 pathway). Specifically, a BMP Response Element (BRE)-luciferase assay was used to determine if follistatin can inhibit Smad 1/5/8 pathway by measuring reduction of luciferase

signal (Korchynskiy et al., *Identification and Functional Characterization of Distinct Critically Important Bone Morphogenetic Protein-specific Response Elements in the Id1 Promoter*, (2002), J BIOL CHEM., 277(7):4883-4891). A CAGA-luciferase assay was used to determine if follistatin can inhibit Smad 2/3 pathway by measuring reduction of luciferase signal (Dennler et al., *Direct binding of Smad3 and Smad4 to critical TGF β -inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene*, (1998), EMBO J, 17(11):3091-3100). Briefly, HEK293 cells were co-transfected with either the BRE (BRE-Id1-luc) or CAGA-luciferase (p(CAGA)₁₂-MLP-luc vector) constructs and renilla-luciferase construct (Promega pGL4.74 [hRluc/TK]) overnight. The following day, cells were treated with myostatin and activin (for Smad 2/3 pathway induction, CAGA-luciferase reporter), or BMP-9 and BMP-10 (for Smad 1/5/8 pathway induction, BRE-luciferase reporter) with or without a concentration series of follistatin. After an overnight incubation, the luciferase signal was determined using the Promega Dual-Glo Assay kit, with values normalized to renilla control. In this experiment, native follistatin (R&D Systems) and follistatin Fc fusion proteins (Sino Biological FS315-hFc, FS315-GAG3-mFc) were tested. FS315-GAG3-mFc is shown below.

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
 ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSSTCVVDQTNNAVCVTCNRICPEPASS
 EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
 VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLLEVKHSGSCNSIS
 EDTEEEEEDEDQDYSFPISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
 GGGGAPGGGGGAAAAAGGGGGGAPGCKPCICTVPEVSSVFIFPPKPKDVLITLTPKVT
 CVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEF
 KCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVE
 WQWNGQPAENYKNTQPIMDTDGSYFVYSKLVQKSNWEAGNTFTCSVLHEGLHNHHT
 EKSLSHSPGK (SEQ ID NO:13)

[0169] Exemplary results of the BRE-luciferase assay are shown in FIG. 1. FS315-Fc does not inhibit BMP-9 or -10 signaling through the Smad 1/5/8 pathway.

[0170] Exemplary results of the CAGA-luciferase assay are shown in FIG. 2. Both FS315 and FS315-GAG3-mFc showed potent inhibition of Smad2/3 signaling at doses of 0.1 nM

and above as compared to the amount of Smad 2/3 induction observed after administration of physiologically relevant levels of myostatin (1.2 nM) and activin (0.4 nM), known activators of Smad 2/3. These results indicate that follistatin is a potent and specific inhibitor of myostatin and activin activity. The presence of the Fc fusion did not detrimentally affect the potency of follistatin, as indicated by the similar inhibitory curves between the native FS315 molecule and the FS315-GAG3-mFc fusion protein. Unexpectedly and importantly, the Fc fusion protein according to the present invention significantly increases the binding affinity of follistatin to primary target myostatin (e.g., by at least 10 fold as shown in Table 3).

Example 2. Follistatin fusion protein FS315-GAG3-mFc has extended serum half-life

[0171] Prior to our invention, it was reported that follistatin has a short serum half-life, which is a concern for developing follistatin as a protein therapeutic. For example, typical commercial FS315 protein has a serum half-life of about an hour. In this Example, the *in vivo* half-life of FS315-GAG3-mFc fusion protein was determined and it has a significantly extended serum half-life.

[0172] Specifically, an imprinting control region (ICR) mouse was selected as a model and I¹²⁵-labeled FS315-GAG3-mFc was administered subcutaneously at 1.0mg/kg (~2 µCi/animal). After administration, samples of serum and tissues were taken up to 10 days post-injection. The tissues sampled were: thyroid, liver, kidney, lung, spleen, diaphragm, heart, quadriceps and triceps. Exemplary results of the serum samples are shown in FIG. 3A. As can be seen, the serum half-life of FS315-GAG3-mFc is approximately 5 days, which is surprisingly long as compared to the short follistatin serum half-life (about 1 hour) known in the art. Exemplary results of the PK profile across various tissues are shown in FIG. 3B and Table 4. The half-life of Follistatin-Fc, with the exception of the thyroid, is between two and five days across tissues. Again, the extended tissue half-life profile is unexpected.

[0173] The extended *in vivo* half-life data further confirm that follistatin can be an effective protein therapeutic for treatment of DMD.

Table 4. Exemplary FS315-GAG3-mFc In Vivo PK Data

Tissue	$t_{1/2}$ (h)	Cmax (ng/g tissue or ng/mL serum)	AUC _{0-last} (hr/ng/g tissue or ng/mL serum)	AUC _{0-∞} (hr/ng/g tissue or ng/mL serum)
Serum	134	14.0	557	782
Thyroid	118	467.4	57019	71769
Kidney	77	9.8	221	249
Liver	48	4.4	118	127
Lung	116	3.9	106	136
Spleen	95	5.8	72	83
Heart	105	1.6	46	55
Diaphragm	99	1.0	28	33
Triceps	78	1.7	44	50
Quadriceps	99	0.8	25	31

Example 3. In vivo efficacy of FS315-GAG3-mFc

[0174] This Example demonstrates that administration of follistatin (e.g., FS315-GAG3-mFc) to mdx mouse model of Duchenne muscular dystrophy results in a trend of increased muscle mass even at a low dose of 1 mg/kg. In this example, the terms “FS315-GAG3-mFc”, “FS315-Fc” and “FS315-mFc” used interchangeably.

[0175] Specifically, in this study, 45 mdx mice were treated with empty vehicle, 0 mg/kg, 1.0 mg/kg or 8 mg/kg FS315-GAG3-mFc. Animals in the vehicle or treatment groups received two subcutaneous (interscapular) injections per week for the duration of the study and follistatin fusion protein levels were assessed through retro-orbital sampling.

[0176] Half of the vehicle treated control animals were sacrificed with the 1 mg/kg FS315-Fc group, and the remaining vehicle treated animals along with the untreated control animals, were sacrificed with the 8 mg/kg treatment group. Exemplary treatment schedule was as shown in Table 5A and B:

Table 5. Exemplary injection and sampling schedule in mdx Mice

5A: 1 mg/kg FS315-GAG3-mFc treatment course

Event	Day
Pre-bleed, Injection 1	0
Injection 2	3
Blood sample taken, Injection 3	7
Injection 4	10
Blood sample taken, Injection 5	14
Injection 6	17
Blood sample taken, Injection 7	21
Injection 8	24
Blood sample taken, Injection 9	29
Sacrifice, week 4 time point	30
Injection 10	32
Blood sample taken, Injection 11	35
Injection 12	38
Blood sample taken	44
Injection 13	45
Injection 14	49
Blood sample taken, Injection 15	52
Injection 16	56
Blood sample taken, Injection 17	59
Injection 18	63
Injection 19	66
Blood sample taken	70
Final sacrifice, week 10	71

5B. 8 mg/kg FS315-GAG3-mFc treatment course

Event	Day
Pre-bleed	0
Injection 1	1
Injection 2	5
Blood sample taken, Injection 3	8
Injection 4	12
Blood sample taken, Injection 5	15
Injection 6	19
Injection 7	22
Injection 8	26
Blood sample taken, Injection 9	30
Injection 10	33
Injection 11	37
Injection 12	41
Blood sample taken	43
Final sacrifice, week 6	44

[0177] Exemplary data regarding muscle weights in the vehicle treated versus 1 mg/kg FS315-Fc group are shown in FIG 4. Specifically, FIG. 4 shows the muscle weights for the quadriceps (FIG. 4A), gastrocnemius (FIG. 4B), tibialis anterior (FIG. 4C) and triceps (FIG. 4D) in grams after 4 and 10 weeks of treatment with 1 mg/kg, and 6 weeks of treatment with 8 mg/kg. The muscle weight data is adjusted for baseline body weight.

[0178] Exemplary data for the circulating levels of follistatin after administration is shown in FIG. 5. Specifically, FIG.5A shows the levels of FS315-mFc in the serum of animals treated with twice weekly injections of 1 mg/kg, and FIG.5B shows the levels of FS315-mFc in the serum of animals treated twice weekly with 8 mg/kg.

[0179] As is shown in FIGS. 4-5, there is a clear indication that FS315-Fc increases muscle mass in animal models of DMD.

Example 4. In vivo efficacy of recombinant follistatin-Fc fusion protein

[0180] This example demonstrates that administration of a follistatin-Fc fusion protein results in muscle hypertrophy (e.g., increased muscle mass and myofiber diameters) *in vivo*.

[0181] In this study, both C57BL/10 and mdx mice were injected with FS315-GAG3-mFc directly into the gastrocnemius muscle (intramuscular, IM). Specifically, each mouse received 2 injections, one on each side, twice weekly. The left gastrocnemius received 20 μ L of a 1 mg/mL solution of FS315-GAG3-mFc for a total of 20 μ g protein per injection. The right gastrocnemius received 20 μ L of PBS (vehicle control). Injections occurred twice weekly for a total of 4 weeks. 24h after the final injection, mice were sacrificed and the gastrocnemius muscles were carefully dissected and weighed. A group receiving the soluble activin type IIB receptor-Fc mouse chimera (sActRIIB-mFc, R&D Systems) at the same dose was included as a positive control. In addition, untreated mice were included as a negative control.

[0182] FIG. 6 shows significantly increased muscle mass in both C57 control mice as well as mdx mice after twice weekly treatment with 20 μ g FS315-mFc or sActRIIB-mFc. The study design and numerical data represented in FIG. 6 are shown in Table 6 below:

Table 6. Muscle Weight

Strain	Group	N	Test Gastroc Weight (g)	PBS Gastroc Weight (g)	Test-PBS Gastroc Weight (g)	P-Value**
C57	FS315-mFc*	8	0.17 \pm 0.016 (0.18)	0.15 \pm 0.013 (0.16)	0.02 \pm 0.013 (0.024)	0.02
	sActRIIB-mFc	8	0.18 \pm 0.016 (0.17)	0.16 \pm 0.02 (0.16)	0.019 \pm 0.017 (0.016)	0.09
	Untreated	5	0.16 \pm 0.013 (0.15)	0.16 \pm 0.0074 (0.16)	0.0032 \pm 0.012 (0.003)	>0.99
mdx	FS315-mFc	10	0.19 \pm 0.017 (0.18)	0.17 \pm 0.019 (0.16)	0.021 \pm 0.013 (0.018)	0.005
	sActRIIB-mFc	8	0.21 \pm 0.024 (0.2)	0.19 \pm 0.026 (0.19)	0.024 \pm 0.017 (0.018)	0.04
	Untreated	5	0.16 \pm 0.019 (0.16)	0.17 \pm 0.023 (0.17)	-0.0084 \pm 0.0055 (-0.06)	0.16

*All Follistatin constructs used in this example contain a GAG3 linker.

** P-values obtained from paired t-test and are Bonferroni-corrected (correcting for 6 statistical tests)

[0183] Myofiber diameters were determined through digital whole slide scanning of the injected gastrocnemius muscle. Samples were fixed in 10% neutral buffered saline, processed and embedded in paraffin, cut into 5 μm sections, and stained with Alexa fluor 488 conjugated Wheat Germ Agglutinin (WGA), a method that stains muscle cell membranes. The scanned images were analyzed using image analysis software (ImageScope and ImagePro Plus). For each myofiber, the average diameter was determined by measuring the myofiber cross section length at 2 degree intervals, passing through the myofiber's centroid.

[0184] In accordance with FIG. 6, FIG 15 demonstrates an increase in the myofiber diameters of gastrocnemius muscle treated with FS315-mFc. This increase occurred in both the C57 (WT, FIG. 15A) and mdx mice (FIG. 15B). Demonstration of the shift to larger diameters indicates that the increased muscle weights are a consequence of muscle hypertrophy. Table 7 is a summary of exemplary mean diameter changes and corresponding statistical analysis.

Table 7

MEAN DIAMETER			
Contrast (comparison)	Mean diff	Standard error	p
WT FS315-mFc vs vehicle	12.5	0.8	<0.0001
mdx FS315-mFc vs vehicle	5.3	1.2	<0.0001
No injection WT vs mdx	14.6	5.8	0.04

[0185] The statistical model used was a hierarchical linear model (HLM), which is able to account for the multiple measurements made within each animal. The differences between untreated and treated legs within each strain and treatment group are highly significant ($p < 0.0001$, which corroborate the muscle weight data).

[0186] These data demonstrate that follistatin, in particular, follistatin-Fc fusion protein can effectively induce muscle growth and treat muscle atrophy associated with DMD.

Example 5. In Vivo Efficacy of Exemplary Follistatin Variants

[0187] The *in vivo* half-life and efficacy data based on the wild-type follistatin FS315 protein shown in Examples 2-4 demonstrates that follistatin can be used as an effective protein therapy for DMD. This example demonstrates that protein therapeutics can also be developed based on follistatin variants.

[0188] Specifically, exemplary follistatin domain deletions or point mutations were generated as described in Table 8 below and tested for their muscle regeneration efficacy using a well-established IM/AAV delivery system to facilitate the comparison between the variants and the wild-type follistatin.

[0189] In this study, a total of 35 C57 mice aged 3-4 weeks were used across seven groups. The seven groups included five mice each, with five follistatin variants being tested (Table 8), and wild type FS315 and an empty vector used as controls. The gene encoding for FS315 has an additional 29 amino acids representing the signal peptide that is cleaved upon secretion from the cell. Thus, FS315 and FS344 refer to the same wild type construct and are used interchangeably in the examples below.

Table 8. Efficacy Screening of Exemplary Follistatin Variants

Variant	Mutation
dFSD2	Domain 2 deletion
dFSD3	Domain 3 deletion
FSD1/1/3	Domain 2 deletion, replacement with Domain 1
Y185A	Point mutant/Domain 2
L191D	Point mutant/Domain 2

[0190] The follistatin variants were administered via a single unilateral injection into the left quadriceps and left gastrocnemius using an AAV9 vector at a dose of approximately 1×10^{11} viral particles per animal. The following endpoints were examined at 2, 4 and 6 weeks post injection: Follistatin levels in serum and urine, mouse weight, individual muscle weights (both injected and distal muscle groups), and histology (e.g., fiber counts, size and type, etc.). The contralateral muscle served as an intra-animal comparator for this study.

[0191] As shown in FIG. 7, both the wild type FS315 and the domain 3 deletion mutant significantly increased body weight as compared to empty vector control. In particular, the tested domain 3 deleted follistatin variant increases body weight as early as 3 weeks post-injection.

[0192] FIG. 8 shows exemplary average muscle weights of the A) gastrocnemius and B) quadriceps on both the ipsilateral and contralateral sides two weeks post-injection. Both wild type FS315 and the domain 3 deletion follistatin variants showed significantly increased muscle mass on the ipsilateral side by week 2 post-injection (FIG. 8). In particular, muscles injected with FS315 and dFSD3 were 60% to 70% greater in weight as compared to empty vector (FIG. 8). The dissected quad muscle that was injected with dFSD3 was noticeably larger than the contra-lateral untreated muscle at week 2 (FIG. 9).

[0193] At week 4, domain 3 deleted and wild-type follistatin increased muscle mass in both injected and distal muscle. See FIG. 10. As observed at week 2, dFSD3 caused a significant hypertrophic effect at week 4, with noticeably larger muscle mass in injected muscle compared to the untreated side (FIG. 11). Follistatin levels in serum, determined by ELISA, were similar for the wild-type and dFSD3 treated mice, and averaged 30 ng/mL at weeks 2, 4 and 6 (data not shown).

[0194] Myofiber size was also determined in both injected and distal muscle tissues at week 2, 4 and 6 post-injection using standard histological and immunohistochemical methods. Exemplary week 2, 4 and 6 results are shown in Figure 12, 13 and 14, respectively. All statistics were done using 1 way ANOVA with Dunnett's Multiple Comparison Test in GraphPad Prism. Error bars represent SEM.

[0195] As shown in Figure 12, at week 2, in injected muscles, myofiber hypertrophy was observed in Quad in the FS344 (23%), dFSD3 (30%), Y185A and L191D groups and in Gastroc in the FS344 (17%) and dFSD3 (25%) groups. In distal muscle groups, myofiber hypertrophy was observed in TA in the dFSD2 (12%) group. No hypertrophy was observed in Triceps or Diaphragm.

[0196] As shown in Figure 13, at week 4, in injected muscles, myofiber hypertrophy was observed in Quad in the FS344 (41%), dFSD3 (50%), dFSD113 and L191D groups and in Gastroc in the FS344 (42%), dFSD2, dFSD3 (73%), dFSD113, Y185A and L191D groups. In distal muscle groups, hypertrophy was observed in TA in the dFSD3 (10%) group and in Diaphragm in the FS344 (23%) and dFSD2 (29%) groups. No hypertrophy was observed in Triceps.

[0197] As shown in Figure 14, at week 6, in injected muscles, myofiber hypertrophy was observed in Quad in the FS344 (41%), dFSD3 (30%), Y185A groups and in Gastroc in the FS344 (90%), dFSD3 (49%), Y185A, L191D groups. In distal muscles, myofiber hypertrophy was observed in TA in the FS344 (26%), dFSD3 (41%), dFSD113, Y185A, and L191D groups and in Diaphragm in the dFSD3 (35%) group. Minimal hypertrophy was observed in Triceps.

[0198] Taken together, these results indicate that protein therapeutics may be developed based on follistatin variants. For example, follistatin domain deletions or point mutations may retain or improve muscle regeneration efficacy. As shown above, domain 3 deletion can be a particularly useful follistatin variant for treating DMD.

Example 6. Systemic Efficacy of FS315-GAG3-mFc

[0199] As shown in Example 4, injection of FS315-mFc into the gastrocnemius resulted in increased muscle mass versus control muscles. This example shows that systemic injection of FS315-mFc is also capable of inducing muscle growth at various sites distal to the site of injection. All follistatin constructs used in this example contain a GAG3 linker.

[0200] A total of 20 C57BL/6 mice were used in this study with half of the animals receiving a subcutaneous (interscapular) injection of PBS twice per week for 8 weeks (control)

and the other half receiving a subcutaneous (interscapular) injection of 10 mg/kg FS315-mFc twice per week for 8 weeks. Animals were sacrificed 24 hours after the last injection and the weight of the left and right quadriceps, gastrocnemius, tibialis anterior and triceps were measured, as was total body weight of the animal. Table 9 below outlines the experimental design for this example.

Table 9. Experimental Design

Group	N	Mouse Strain	Test Article	Injection Route	Dose	Dosing Schedule	Sacrifice (24 hours post last injection)
A	10	WT C57BL/6	FS315-mFc	SC (Interscapular)	10 mg/kg	Twice weekly	4 weeks
B	10	WT C57BL/6	FS315-mFc	SC (Interscapular)	10 mg/kg	Twice weekly	8 weeks
C	10	WT C57BL/6	Vehicle (PBS)	SC (Interscapular)	NA	Twice weekly	4 weeks
D	10	WT C57BL/6	Vehicle (PBS)	SC (Interscapular)	NA	Twice weekly	8 weeks

[0201] FIG. 16 shows exemplary body weight data through the 8 week course of the study. As can be seen, body weights for the FS315-mFc treated animals were significantly greater than those vehicle treated control animals beginning at 2 weeks and continuing throughout the 8 week study. FIG. 17 represents exemplary muscle weight data. The triceps muscles from mice treated with FS315-mFc were significantly greater in weight compared to

vehicle control as early as week 4. After 8 weeks of treatment, both triceps and quadriceps muscle groups demonstrated significant increases in weight compare to vehicle (FIG. 17). Myofiber size was determined by the method described in Example 4. FIG. 18 shows the percent increase in myofiber diameter for the triceps and quadriceps muscle groups at weeks 4 and 8. Both muscle groups demonstrated a shift towards greater myofiber size after treatment with FS315-mFc at both 4 and 8 weeks.

[0202] In addition, serum follistatin levels were increased following subcutaneous injection. For example, FS315-mFc levels in the sera of treated mice (by twice weekly subcutaneous injection) are shown in FIG. 19. FS315-mFc levels were highest in the serum collected at weeks 4 and 8 sacrifice time points, consistent with the amount of time between the FS315-mFc injection and the serum collection (24h). At these points, serum levels of FS315-mFc averaged about 200 ng/mL. The biweekly retro-orbital bleeds were collected 3 days after FS315-mFc injection, and serum levels of FS315-mFc averaged between about 30 – 50 ng/mL.

[0203] These data demonstrates that systemic injection of FS315-mFc (e.g., subcutaneous injection) can effectively induce muscle growth in various muscle tissues throughout the body.

Example 7. Systemic efficacy of Follistatin-Fc fusion protein in DMD mouse model

[0204] This example further demonstrates systemic efficacy in DMD disease model. In particular, as shown below, systemic injection of FS315-mFc successfully reduced progression of various characteristic DMD symptoms such as muscle necrosis and/or fibrosis. All follistatin constructs used in this example contain a GAG3 linker.

[0205] The mdx mouse model has been used extensively as the preclinical model for demonstrating proof of concept of candidate therapies for DMD. Both the limb muscle groups and diaphragm of the mdx mouse show extensive pathology that tends to increase with age. Such pathology is characterized by areas of inflammatory infiltrate, necrosis, and fibrosis in muscle. FS315-mFc was tested in this model to evaluate its effect on progression of fibrosis in muscle. A total of 50 mdx mice were used in this Example, with 20 animals receiving a subcutaneous injection of PBS, and 30 animals receiving a subcutaneous injection of 10 mg/kg

FS315-mFc twice per week for 12 weeks. (see Table 10). Animals were sacrificed 24 hours after the last injection and tissues were collected for analysis of necrosis and fibrosis (see Table 11).

Table 10. Experimental Design

Group	N	Test Article	Injection Route	Dose	Dosing Schedule	Sacrifice (24 hours post last injection)
A	15	FS315-mFc	SC	10 mg/kg	Twice weekly	6 weeks
B	10	PBS	SC	N/A	Twice weekly	6 weeks
C	15	FS315-mFc	SC	10 mg/kg	Twice weekly	12 weeks
D	10	PBS	SC	N/A	Twice weekly	12 weeks

Table 11. Tissue collection and processing

Diaphragm	Quadriceps	Gastrocnemius	Triceps
½ snap frozen for protein analysis	1 snap frozen for protein analysis	1 snap frozen for protein analysis	1 snap frozen for protein analysis
½ fixed in formalin for histological analysis	1 fixed in formalin for histological analysis	1 fixed in formalin for histological analysis	1 fixed in formalin for histological analysis

[0206] FIG. 20 shows exemplary effect of FS315-mFc on fibrotic protein expression at the RNA level. Specifically, RT-PCR of collagen type I, alpha-smooth muscle actin, and collagen triple helix repeat containing 1 protein demonstrated a significant reduction in the expression of these fibrosis-related proteins as early as 6 weeks after twice weekly SC treatment.

[0207] Tables 12 and 13 summarize the histopathological evaluation of necrosis (as determined by evaluation of H&E stained sections) and fibrosis (as determined by evaluation of collagen I stained muscle sections in FS315-mFc treated mdx mouse muscle) in muscle tissue sections. For the FS315-mFc and vehicle treated groups, there were 15 and 10 total animals per group, respectively. As indicated in Table 12, FS315-mFc treatment significantly reduced the incidence of necrosis in limb muscles as early as 6 weeks from initiation of twice weekly injections. This reduction in necrosis is illustrated in the images of H&E sections through quadriceps and triceps muscle (FIG. 21). The incidence of fibrosis, demonstrated by collagen I staining of muscle tissue sections, was significantly reduced after 12 weeks of FS315-mFc treatment (also see Table 13). This reduction in collagen deposition is illustrated in the images of collagen I stained muscle sections (FIG. 22).

[0208] The results of this study demonstrates that FS315-mFc can successfully treat DMD by effectively reducing the progression of the diseased muscle pathology in the DMD mouse model including, but not limited to, muscle necrosis and/or fibrosis

Table 12. Incidence of necrosis in FS315-mFc treated mdx mouse muscle (p values indicate degree of significance between vehicle and FS315-mFc, using Fisher's Exact Test)

	Week 6 Quad p=0.001		Week 12 Quad p=0.049	
Score	Vehicle	FS315-mFc	Vehicle	FS315-mFc
Minimal	10%	67%	30%	73%
Mild	40%	33%	70%	27%
Marked	50%	0%	0%	0%
	Week 6 Triceps p<0.001		Week 12 Triceps p=0.02	
Score	Vehicle	FS315-mFc	Vehicle	FS315-mFc
Minimal	0%	53%	20%	73%
Mild	10%	33%	70%	20%
Marked	90%	14%	10%	7%
	Week 6 Gastroc p<0.001		Week 12 Gastroc p=0.007	
Score	Vehicle	FS315-mFc	Vehicle	FS315-mFc
Minimal	0%	73%	20%	80%
Mild	40%	27%	60%	20%
Marked	60%	0%	20%	0%

Minimal: <5%; mild: <30%; marked: >30% in total checked muscle area

Table 13. Incidence of fibrosis, (p values indicate degree of significance between vehicle and FS315-mFc, using Fisher's Exact Test)

	Week 12 Quad p=0.0001	
Score	Vehicle	FS315-mFc
Minimal	0%	80%
Mild	70%	20%
Marked	30%	0%
	Week 12 Triceps p=0.001	
Score	Vehicle	FS315-mFc
Minimal	0%	67%
Mild	80%	20%
Marked	20%	13%
	Week 12 Gastroc p<0.0001	
Score	Vehicle	FS315-mFc
Minimal	0%	73%
Mild	20%	27%
Marked	80%	0%
Minimal: ~1%; mild: <5%; marked: >5% in total checked muscle area		

Example 8. *In vivo* efficacy of recombinant follistatin domain 3 deletion Fc fusion protein

[0209] This Example demonstrates that a follistatin domain 3 deletion (dFSD3) Fc fusion protein effectively induced muscle growth *in vivo*, similar to wild type follistatin-Fc fusion protein. All follistatin constructs used in this example contain a GAG3 linker.

[0210] Specifically, the domain 3 deleted construct described in Example 5 was fused to the same mFc as used for FS315-mFc. In addition, the same GAG3 linker sequence was used to fuse dFSD3 to mFc. C57BL/10 mice were injected with both fusion proteins directly into the gastrocnemius muscle, as described in Example 4. Mice were sacrificed after 4 weeks of twice weekly injections of 20 µg of fusion protein, and the opposite gastrocnemius muscle received the same volume of PBS. 24h after the final injection, the treated mice were sacrificed and the

injected gastrocnemius muscles were carefully injected and weighed. As indicated in FIG 23, the dFSD3-GAG3-mFc fusion protein led to a significant increase in muscle mass over vehicle control, and the increase was similar to that observed with FS315-mFc. This Example indicates that a follistatin domain 3 deletion Fc fusion protein (e.g., dFSD3-GAG3-mFc) is active *in vivo* and another promising therapeutic candidate for DMD treatment.

Example 9. Advantage of longer linker on follistatin function

[0211] This example demonstrates that a longer linker, in particular a linker containing at least 10 amino acids, provides unexpected advantage on follistatin function. Specifically, this example shows that the FS315-GAG3-Fc fusion protein (murine and/or human Fc), containing a 57 amino acid linker, is more potent in its ability to inhibit myostatin and activin compared to a commercial available FS315-hFc fusion protein from Sino Biological (Sino Biological Inc. Catalog Number 10685-H02H), which contains a 9 amino acid linker ALEVLFGQP. The concentration of myostatin and activin used for the signaling assay was 1.2 nM. As indicated in FIG 24 and Table 14, the FS315-GAG3-mFc and FS315-GAG3-hFc fusion proteins inhibit myostatin and activin signaling in the CAGA-luciferase assay to the same extent as native FS315. In comparison, the commercially available FS315-hFc fusion protein (Sino Biological) is significantly less potent. The calculated IC₅₀'s are as follows:

Table 14: Exemplary IC₅₀ values for follistatin inhibition of myostatin and activin in the CAGA-luciferase reporter assay for Smad2/3 signaling

Material	Ligand	IC ₅₀ (nM)
FS315 (R&D Systems)	Myostatin	0.45
FS315-GAG3-mFc	Myostatin	0.46
FS315-GAG3-hFc	Myostatin	0.68
FS315-hFc (Sino Biological)	Myostatin	2.99
FS315 (R&D Systems)	Activin	0.40
FS315-GAG3-mFc	Activin	0.36

FS315-GAG3-hFc	Activin	0.70
FS315-hFc (Sino Biological)	Activin	2.90

[0212] Without wishing to be held to a particular theory, it is possible that a longer linker (e.g., a 57 amino acid linker in this particular construct FS315-GAG3-Fc) between the FS315 protein and the Fc region may permit a more native conformation of FS315 as compared to a fusion protein with a much shorter linker (e.g., 9 amino acids), allowing for binding to target ligands and inhibition of signaling to a similar extent as that observed with native FS315. In comparison, the commercially available FS315-hFc protein has a much shorter linker of 9 amino acids, with significantly less separation between the Fc and the FS315 protein, potentially causing a detrimental effect on FS315 conformation and physiological activity.

Example 10. Follistatin fusion protein FS315-GAG3-hFc has extended serum half-life

[0213] In this example, we demonstrated that provided follistatin fusion proteins, in particular, those with a longer linker (e.g., a linker with at least 10 amino acids) has extended serum half-life. Specifically, for the first time, we demonstrated here a FS315 fused to human Fc with the GAG3 linker (FS315-GAG3-hFc), has an extended serum half-life when administered subcutaneously (SC) into Sprague-Dawley rats at a single dose of 10 mg/kg. After administration, serum was collected at time points ranging from 15 min to 5 days. FS315-GAG3-hFc was measured in rat serum using a Mesoscale Discovery (MSD) assay that captures the human FS315 and detects the human Fc domain of the intact fusion protein in serum samples. Levels of FS315-GAG3-hFc in rat serum are shown in FIG. 25. The PK parameters are summarized in Table 15.

Table 15. FS315-GAG3-hFc In Vivo PK Data

t $\frac{1}{2}$ (h)	C _{max} (ng/mL)	T _{max} (h)	AUC _{0-last} (hr*ng/mL)	AUC _{0-∞} (hr*ng/mL)
84	1372	48	114585	205803

[0214] In sum, the above Examples demonstrate that follistatin, including provided variants, are highly effective in inducing muscle hypertrophy and attenuating muscle necrosis and fibrosis in DMD disease model by, e.g., systemic administration. Thus, follistatin and provided variants can be effective protein therapeutics for the treatment of DMD.

EQUIVALENTS AND SCOPE

[0215] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the following claims.

We claim:

1. A method of treating Duchenne muscular dystrophy (DMD) comprising administering to an individual who is suffering from or susceptible to DMD an effective amount of a recombinant follistatin protein comprising an amino acid sequence of SEQ ID NO: 1 or 2 fused to an Fc domain via a peptide linker comprising 10 or more amino acids such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset, and wherein the peptide linker comprises the amino acid sequence of SEQ ID NO: 5, 6, or 7.
2. The method of claim 1, wherein the recombinant follistatin protein comprises a deletion of amino acids residues 212-288 of SEQ ID NO:1.
3. The method of claim 1, wherein the Fc domain comprises an amino acid sequence of
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTTPVLDSGSEFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPG
K (SEQ ID NO:3).
4. The method of claim 1, wherein the recombinant follistatin protein is produced from mammalian cells.
5. The method of claim 1, wherein the recombinant follistatin protein is administered systemically.
6. The method of claim 1, wherein the administration of the recombinant follistatin protein results in muscle regeneration, increased muscle strength, increased flexibility, increased range of motion, increased stamina, reduced fatigability, increased blood flow, improved cognition, improved pulmonary function, inflammation inhibition, reduced muscle fibrosis, and/or reduced muscle necrosis.

7. The method of claim 1, wherein the at least one symptom or feature of DMD is selected from the group consisting of muscle wasting, muscle weakness, muscle fragility, muscle necrosis, muscle fibrosis, joint contracture, skeletal deformation, cardiomyopathy, impaired swallowing, impaired bowel and bladder function, muscle ischemia, cognitive impairment, behavioral dysfunction, socialization impairment, scoliosis, and impaired respiratory function.
8. A recombinant follistatin fusion protein comprising
a follistatin polypeptide;
an Fc domain; and
a linker that associates the follistatin polypeptide with the Fc domain,
wherein the follistatin polypeptide comprises an amino acid sequence of SEQ ID NO.: 1 or 2; wherein the linker comprises the amino acid sequence of SEQ ID NO: 5, 6, or 7; and further wherein the recombinant follistatin fusion protein is capable of binding to activin, myostatin and/or GDF-11 and has an in vivo half-life ranging from about 0.5-10 days.
9. The recombinant follistatin fusion protein of claim 8, wherein the follistatin polypeptide contains a deletion of amino acids residues 212-288 of SEQ ID NO:1.
10. The recombinant follistatin fusion protein of claim 8, wherein the Fc domain is an IgG1 Fc domain.
11. The recombinant follistatin fusion protein of claim 8, wherein the Fc domain has an amino acid sequence of
EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPG
K (SEQ ID NO:3).
12. The recombinant follistatin fusion protein of claim 8, wherein the recombinant follistatin fusion protein comprises an amino acid sequence of SEQ ID NO:8

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFN
GGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPPVCGLDGKTY
RNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAVCVTCNRICP
EPASSEQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKC
LWDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNSISEDTEEEEEDEDQDYSPFISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAA
AAGGGGGGAPGGGGGAAAAAGGGGGGAPKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCL
VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSSV
MHEALHNHYTQKSLSLSPGK (SEQ ID NO:8), or

SEQ ID NO: 9

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFN
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAVCVTCNRICPEPASS
EQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFK
VGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSCNSIS
EDTEEEEEDEDQDYSPFISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGG
GGGGAPGGGGGAAAAAGGGGGGAPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKD
TLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLT
CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFC
SVMHEALHNHYTQKSLSLSPGK (SEQ ID NO:9).

13. The recombinant follistatin fusion protein of claim 8, wherein the recombinant follistatin fusion protein comprises an amino acid sequence of SEQ ID NO:10

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFN
GGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPPVCGLDGKTY
RNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAVCVTCNRICP
EPASSEQYLCGNDGVITYSSACHLRKATCLLGRSIGLAYEGKCI
SISEDTEEEEEDEDQDY
SPFISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAA

AAAGGGGGGAPKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
KALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSL
SPGK (SEQ ID NO: 10), or

SEQ ID NO: 11

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFN
GGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPPVCGLDGKTY
RNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAVCVTCNRICP
EPASSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCISISEDTEEEEEEDEDQDY
SFPISSILEWGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAA
AAAGGGGGGAPEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVE
WESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFCSCVMHEALHNHYT
QKSLSLSPGK (SEQ ID NO:11).

14. A nucleic acid comprising a nucleotide sequence encoding the recombinant follistatin fusion protein of claim 8.
15. A cell comprising a nucleic acid of claim 14.
16. A pharmaceutical composition comprising a recombinant follistatin fusion protein of claim 8 and a pharmaceutically acceptable carrier.
17. A method of treating Duchenne muscular dystrophy (DMD) comprising administering to an individual who is suffering from or susceptible to DMD an effective amount of a recombinant follistatin protein comprising an amino acid sequence SEQ ID NOs: 8, 9, 10 or 11, such that at least one symptom or feature of DMD is reduced in intensity, severity, or frequency, or has delayed onset.

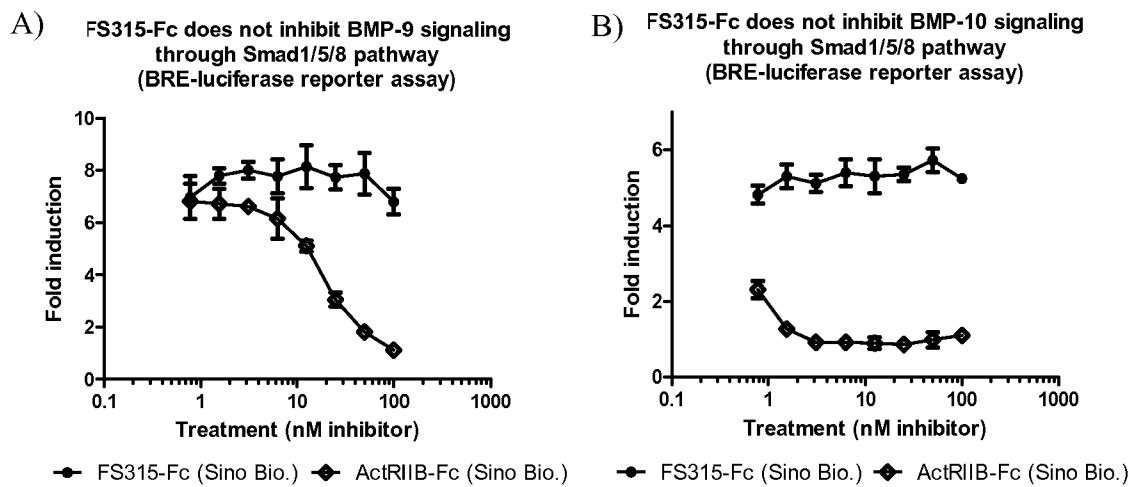
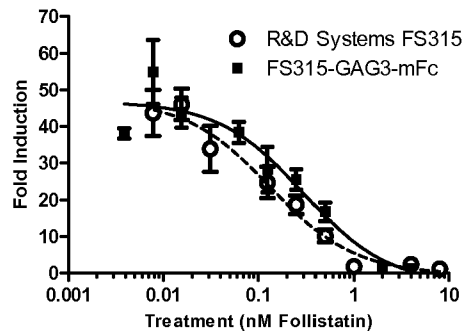


FIG. 1

FS315-Fc does not inhibit BMP-9 or -10 signaling through Smad1/5/8 pathway (BRE-luciferase reporter assay)

A) FS315-GAG3-mFc inhibits myostatin signaling through Smad2/3 pathway (CAGA-luciferase reporter assay)



B) FS315-GAG3-mFc inhibits activinA signaling through Smad2/3 pathway (CAGA-luciferase reporter assay)

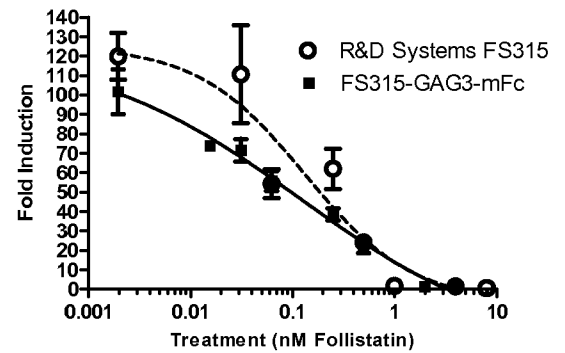


FIG. 2

FS315-GAG3-mFc inhibits myostatin and activin A signaling through Smad2/3 pathway (CAGA-luciferase reporter assay)

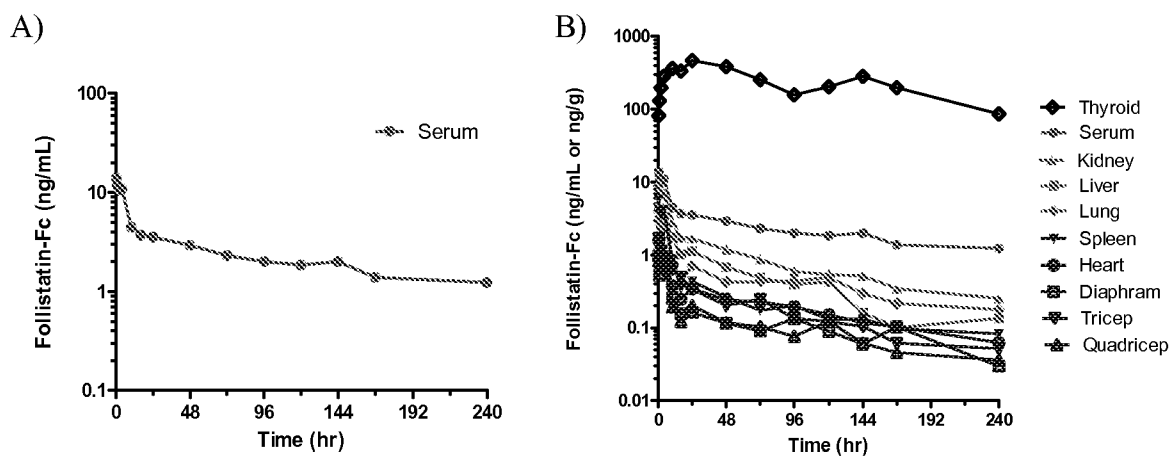


FIG. 3

FS315-mFc PK profile in mouse serum and tissue after SC injection of 1 mg/kg. Estimated tissue half-life is 2-5 days, serum half-life ~ 5 days.

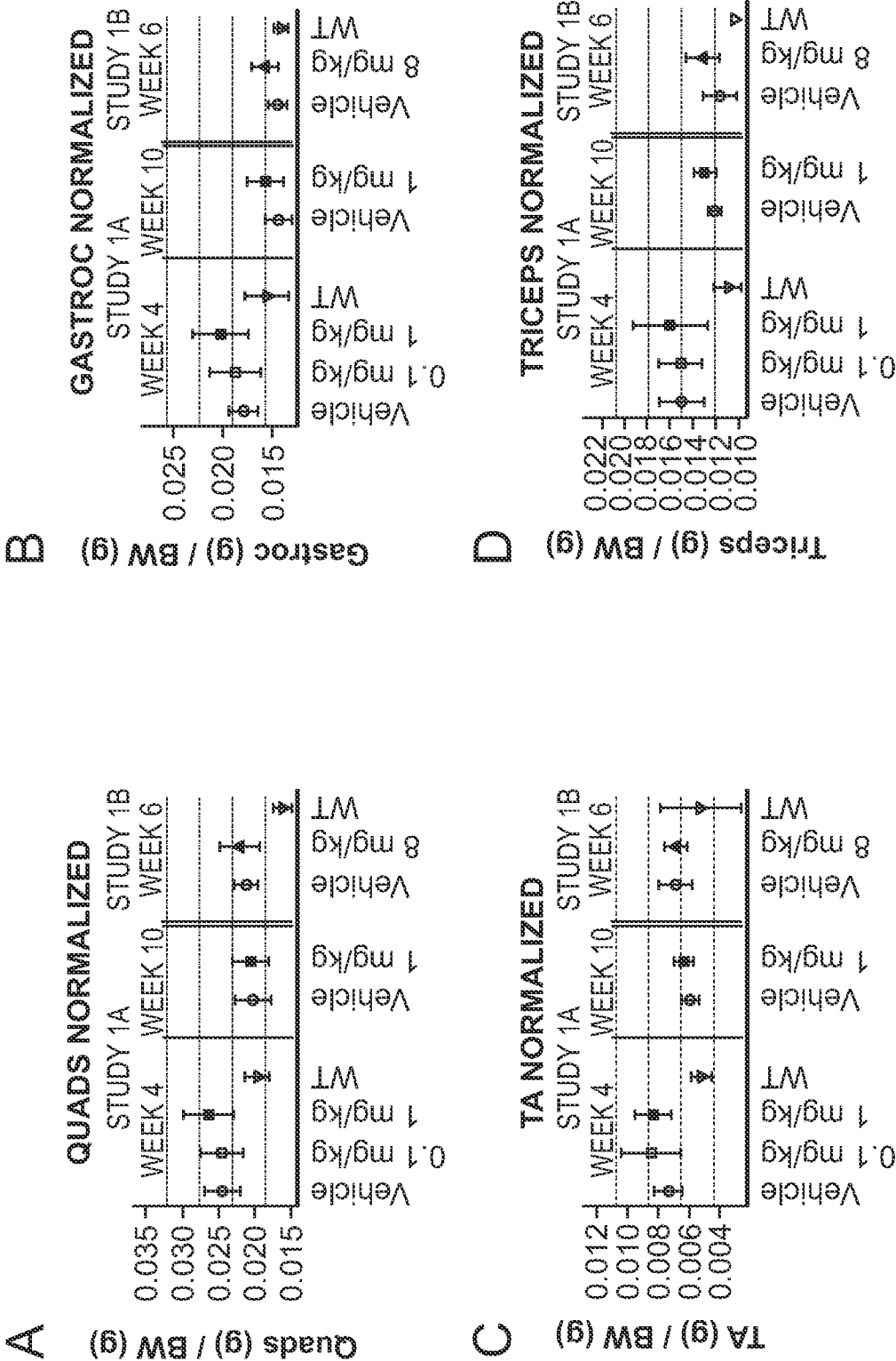
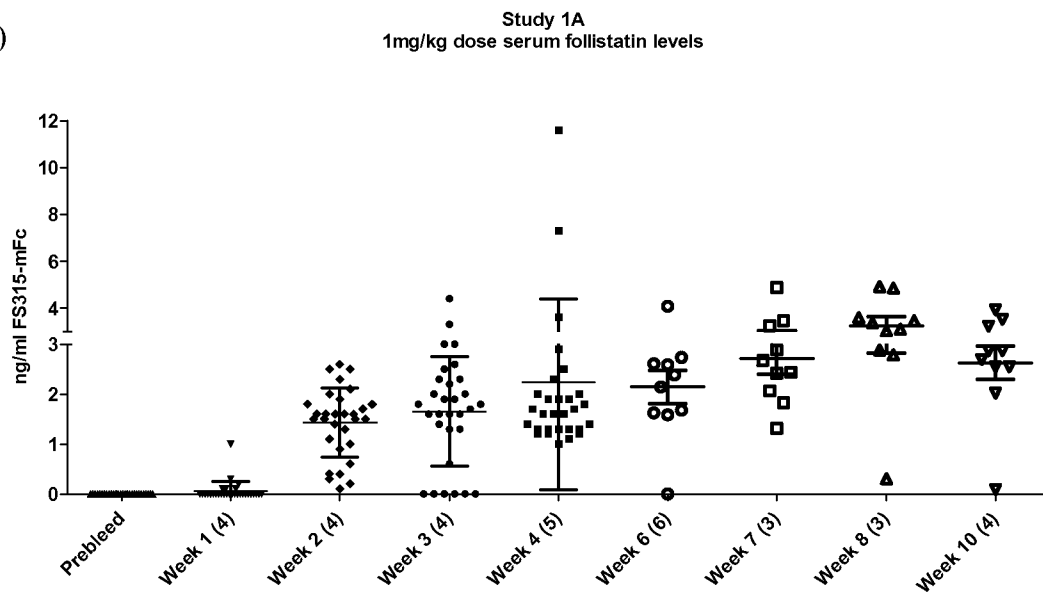


FIG. 4

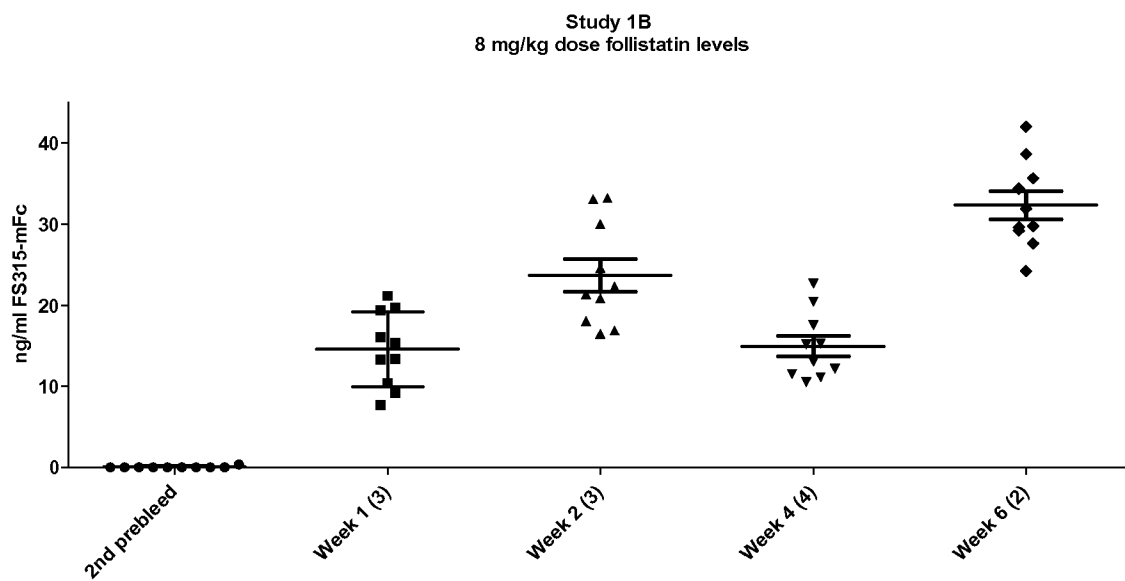
Changes in muscle mass after twice weekly SC administration of FS315-mFc into mdx mice. The muscle weights are normalized to baseline body weight. There is a trend for increased muscle weight in mdx mice treated with 1 mg/kg for 10 weeks or 8 mg/kg for 6 weeks.

A)



Number in parentheses indicates number of days between time of follistatin injection and time of blood sampling for follistatin assay

B)



Number in parentheses indicates number of days between time of follistatin injection and time of blood sampling for follistatin assay

FIG.5

The levels of FS315-mFc in serum of mdx mice treated with 1 mg/kg (A) and 8 mg/kg (B).

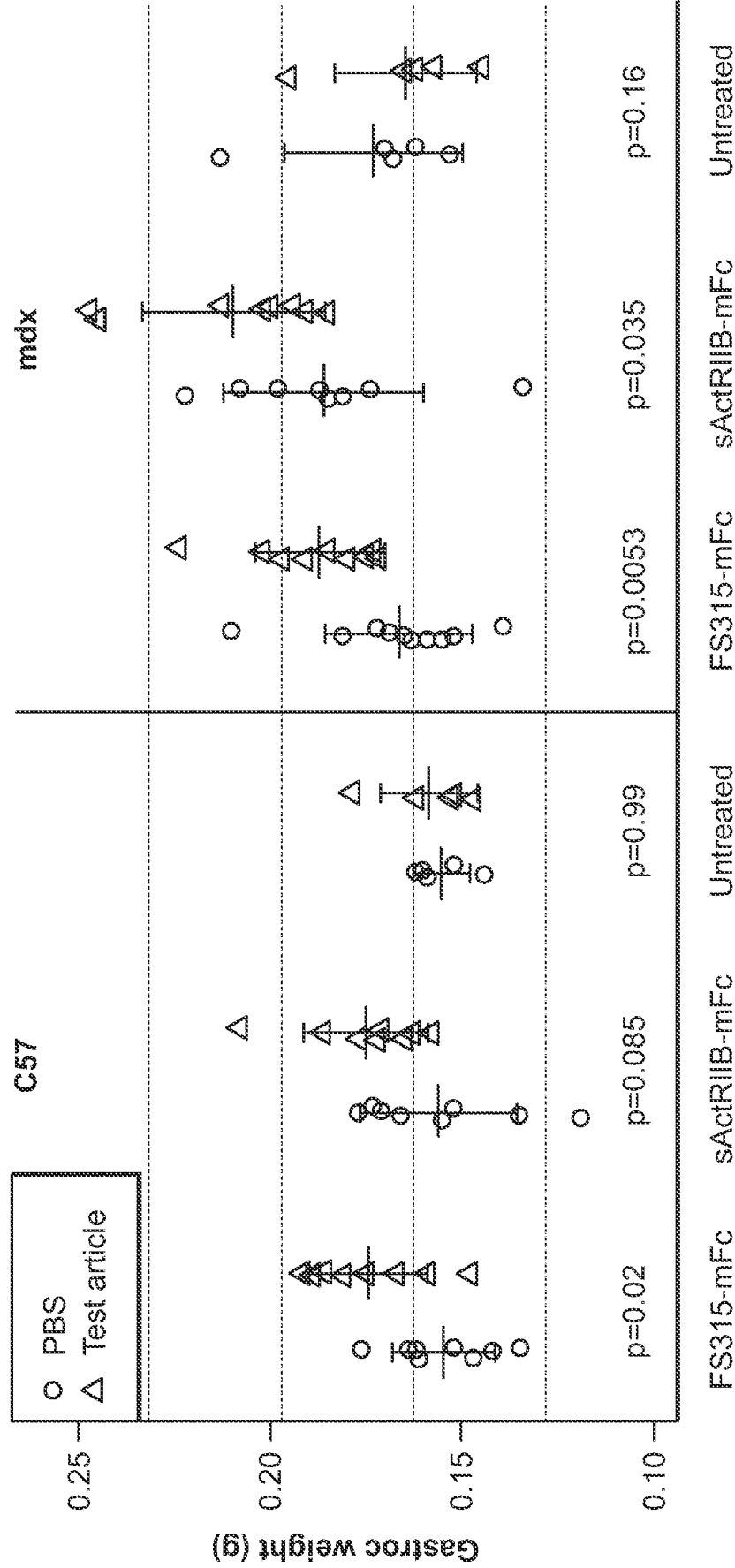


FIG. 6

The effect of intramuscular injection of FS315-mFc on muscle weight after 4 weeks of twice weekly injection of 20 µg directly into the gastrocnemius muscle. The contra-lateral gastrocnemius muscle received an equivalent volume of PBS and acts as the control for each treatment group. The untreated group did not receive injections, and each data set for this group represents right and left gastrocnemius muscles. P-values obtained from paired t-test with Bonferroni correction.

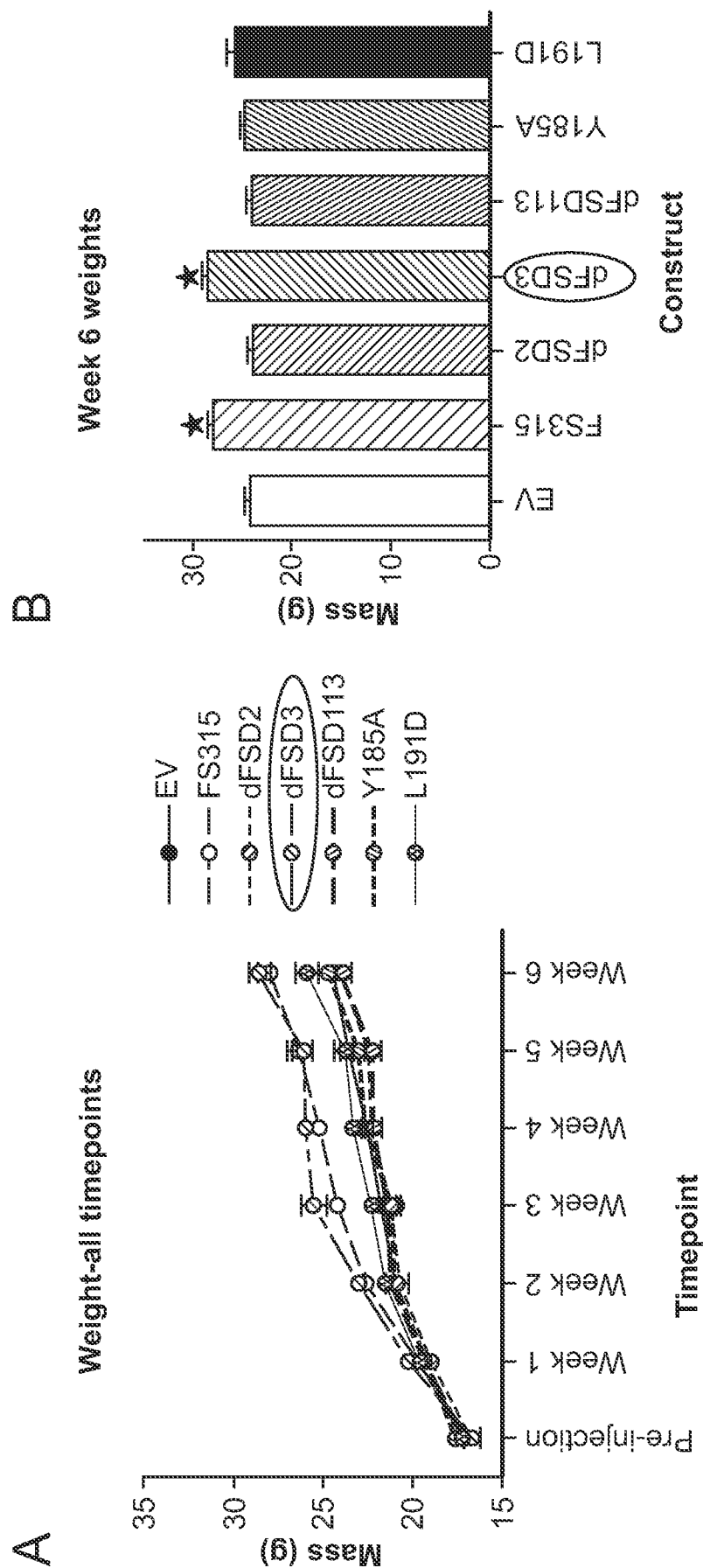


FIG. 7

Weekly body weight after intramuscular gene delivery of follistatin variants to gastrocnemius and quadriceps muscle of C57 mice.

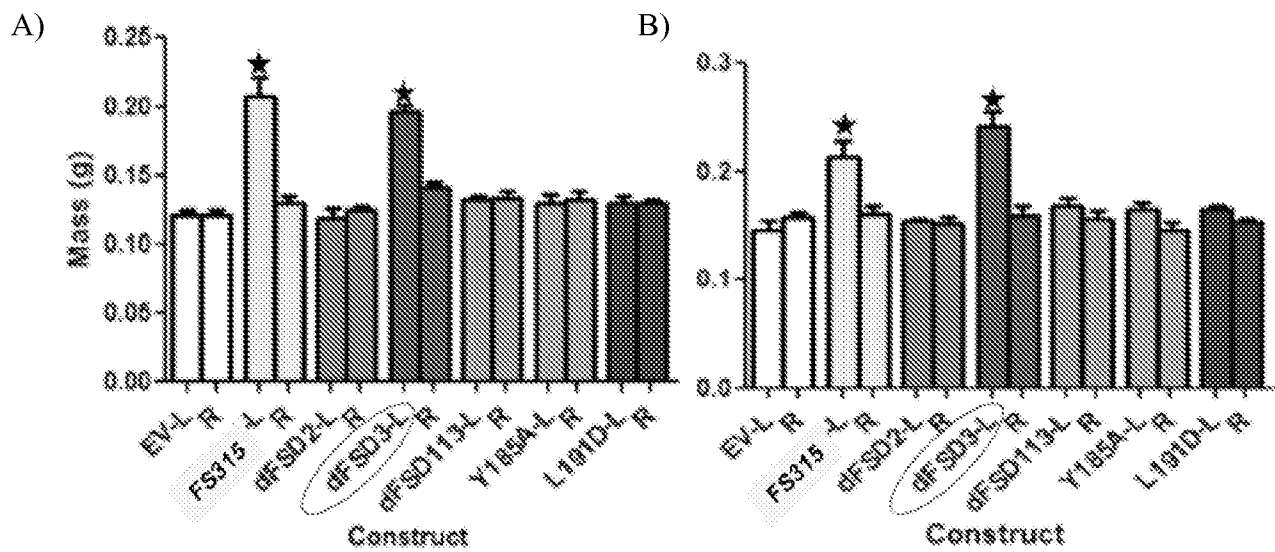


FIG. 8

Week 2 muscle weights after intramuscular gene delivery of follistatin variants to gastrocnemius (A) and quadriceps (B) muscle of C57 mice.

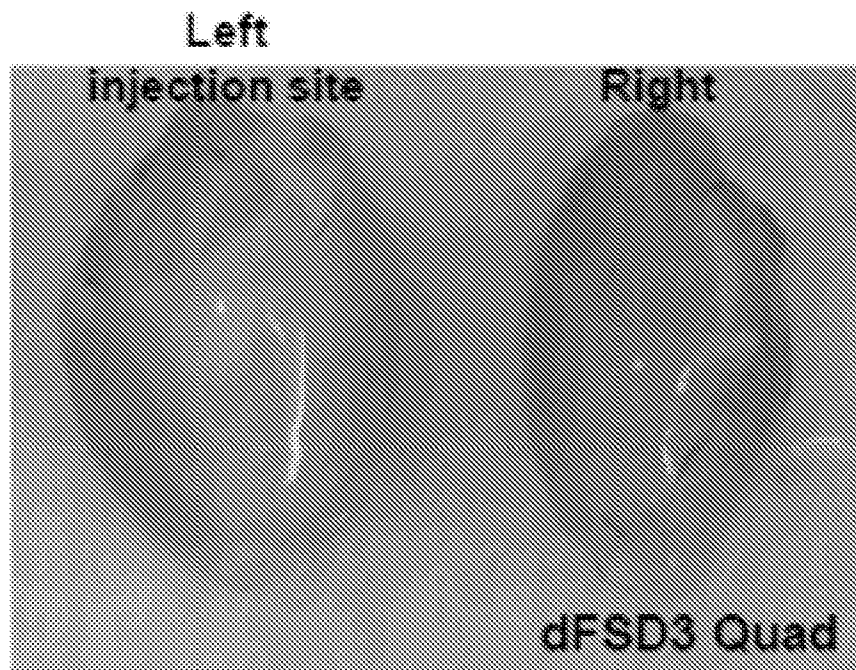
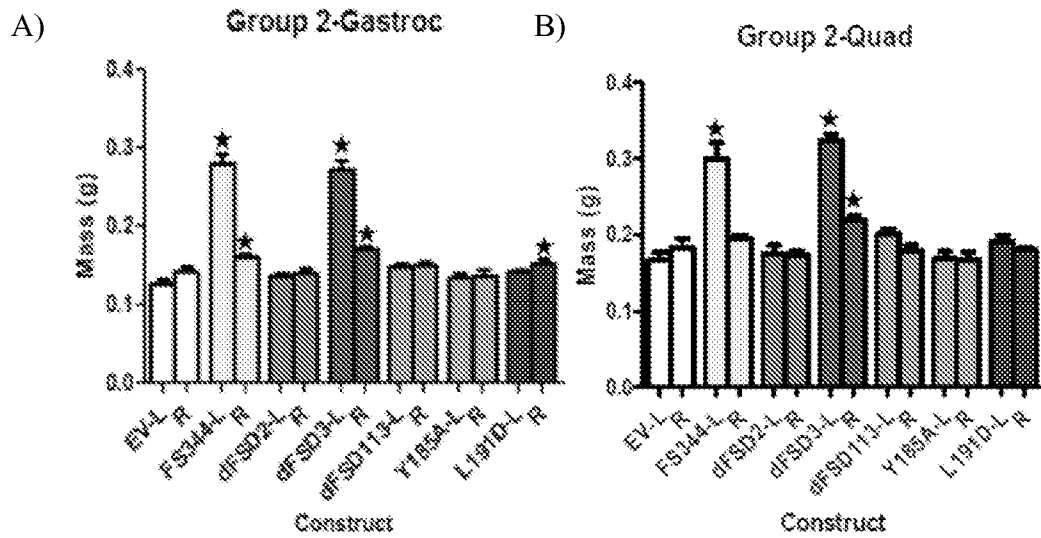


FIG. 9

Week 2 gross morphology of dissected quadriceps muscle receiving dFSD3 via direct gene delivery. The right quad muscle did not receive an injection of dFSD3.

Injected Muscle



Muscle Distal from Injection Site

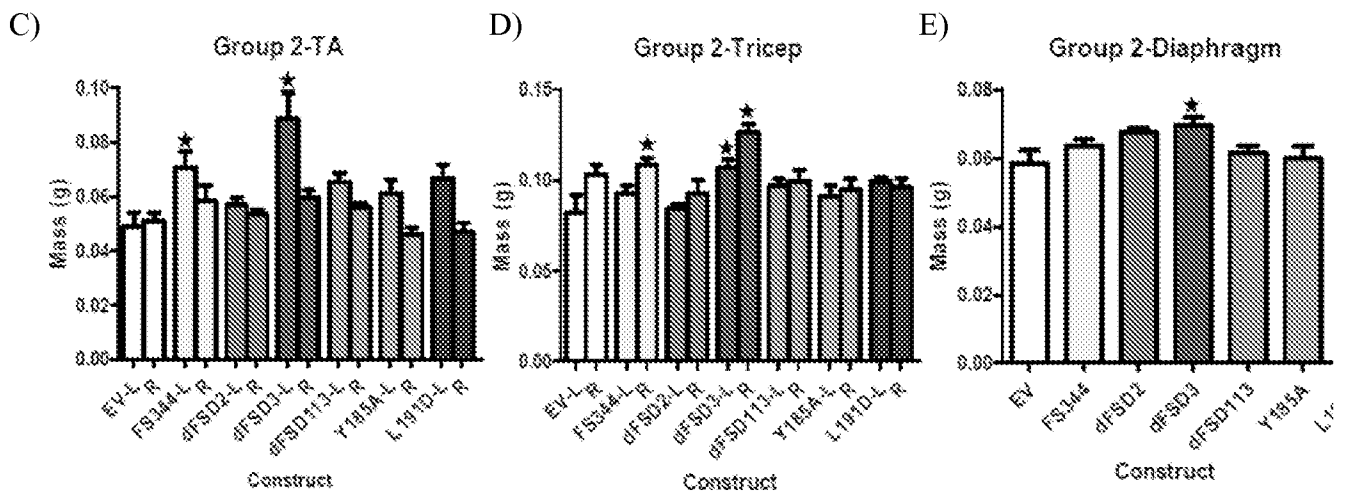


FIG. 10

Week 4 muscle weights after intramuscular gene delivery of follistatin variants to gastrocnemius (A) and quadriceps (B) muscle of C57 mice. The following muscles were remote from the injection site: C) tibialis anterior, D) triceps, E) diaphragm.

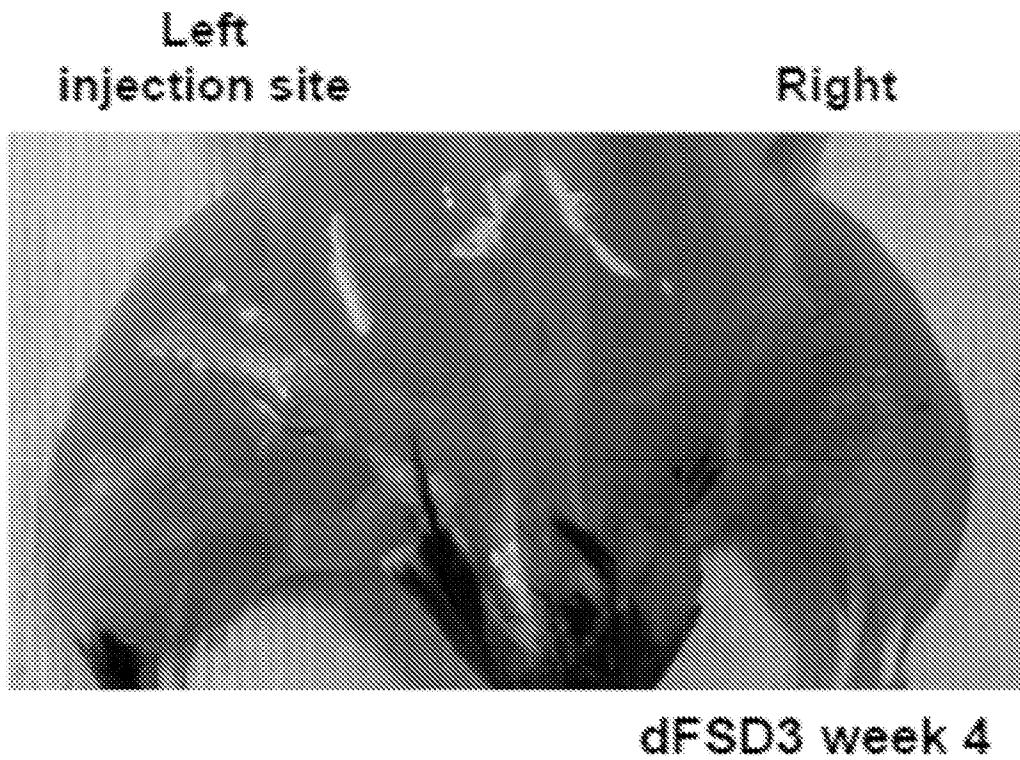
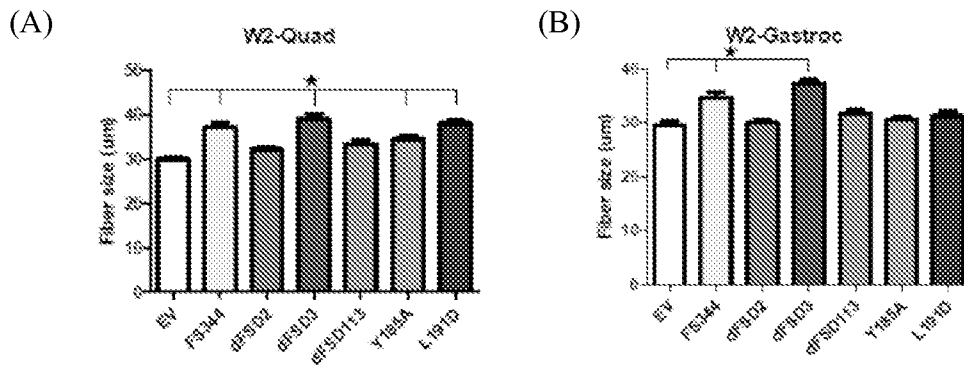


FIG. 11

Week 4 gross morphology of quadriceps muscle receiving dFSD3 via direct gene delivery. The right quad muscle did not receive an injection of dFSD3.

Injected Muscles



Distal Muscles

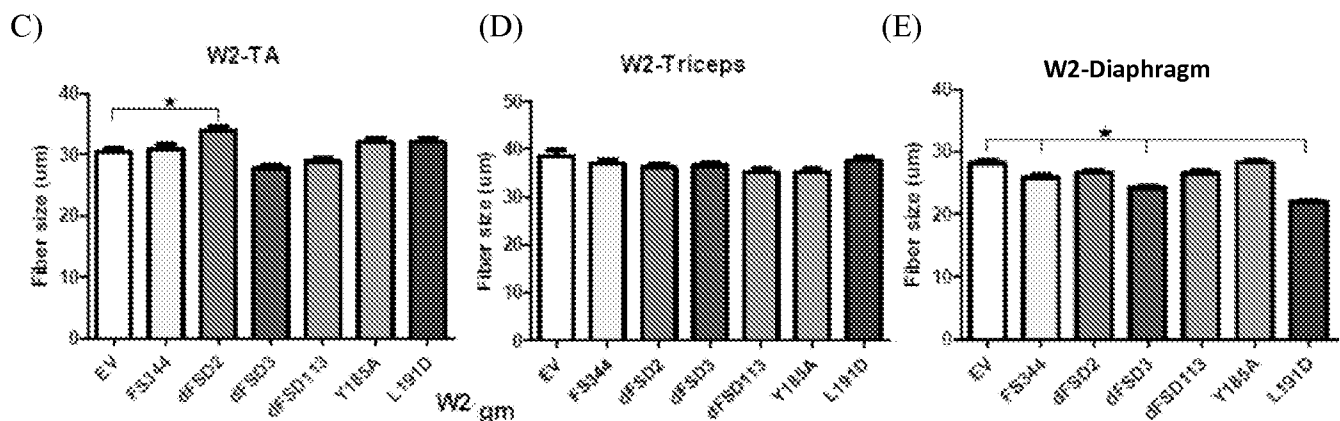


FIG. 12

Week 2 muscle myofiber diameter after intramuscular gene delivery of follistatin variants to quadriceps (A) and gastrocnemius (B) muscle of C57 mice. The following muscles were remote from the injection site: (C) tibialis anterior, (D) triceps, (E) diaphragm.

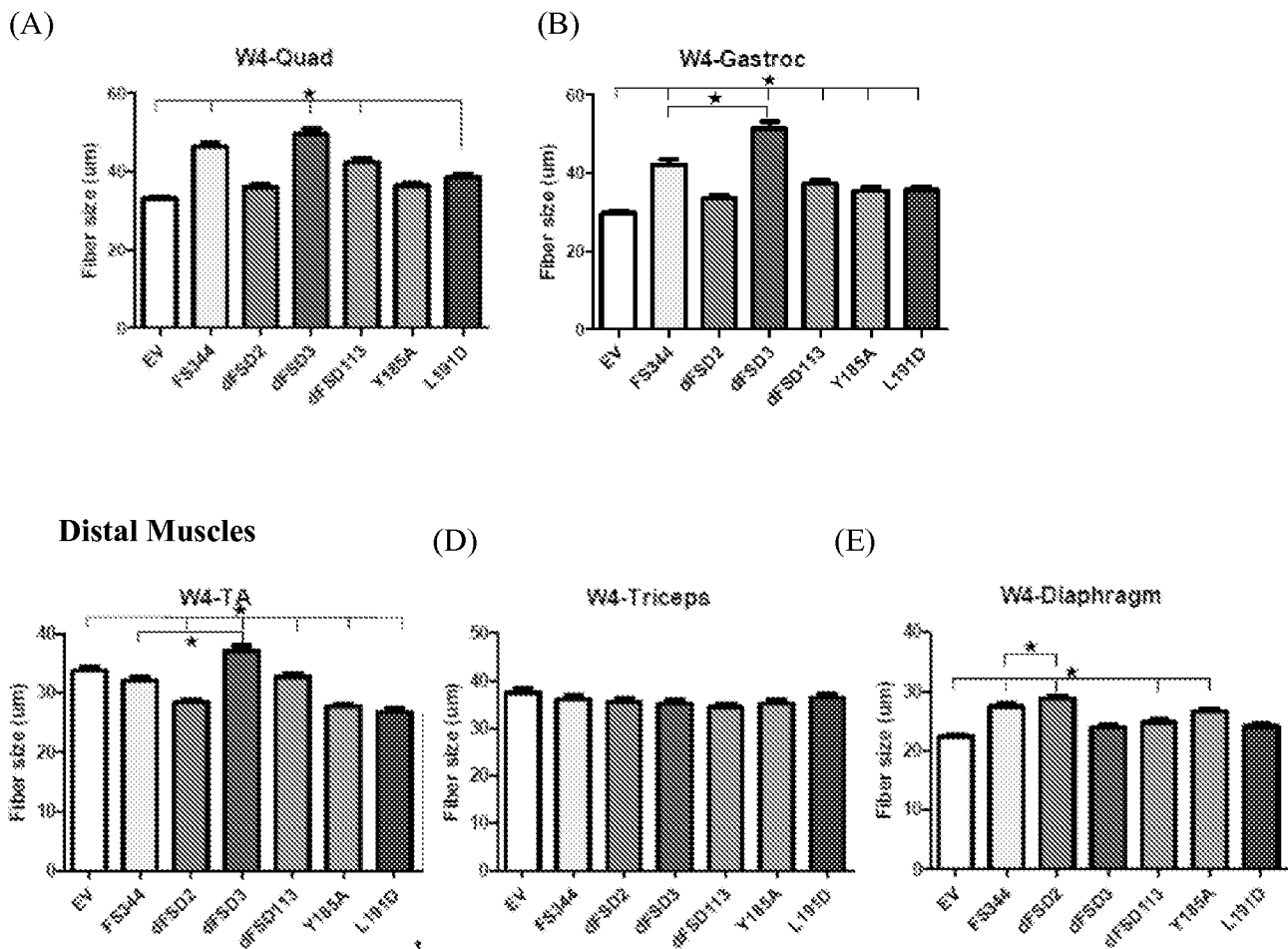
Injected Muscles

FIG. 13

Week 4 muscle myofiber diameter after intramuscular gene delivery of follistatin variants to quadriceps (A) and gastrocnemius (B) muscle of C57 mice. The following muscles were remote from the injection site: C) tibialis anterior, D) triceps, E) diaphragm.

Injected Muscles

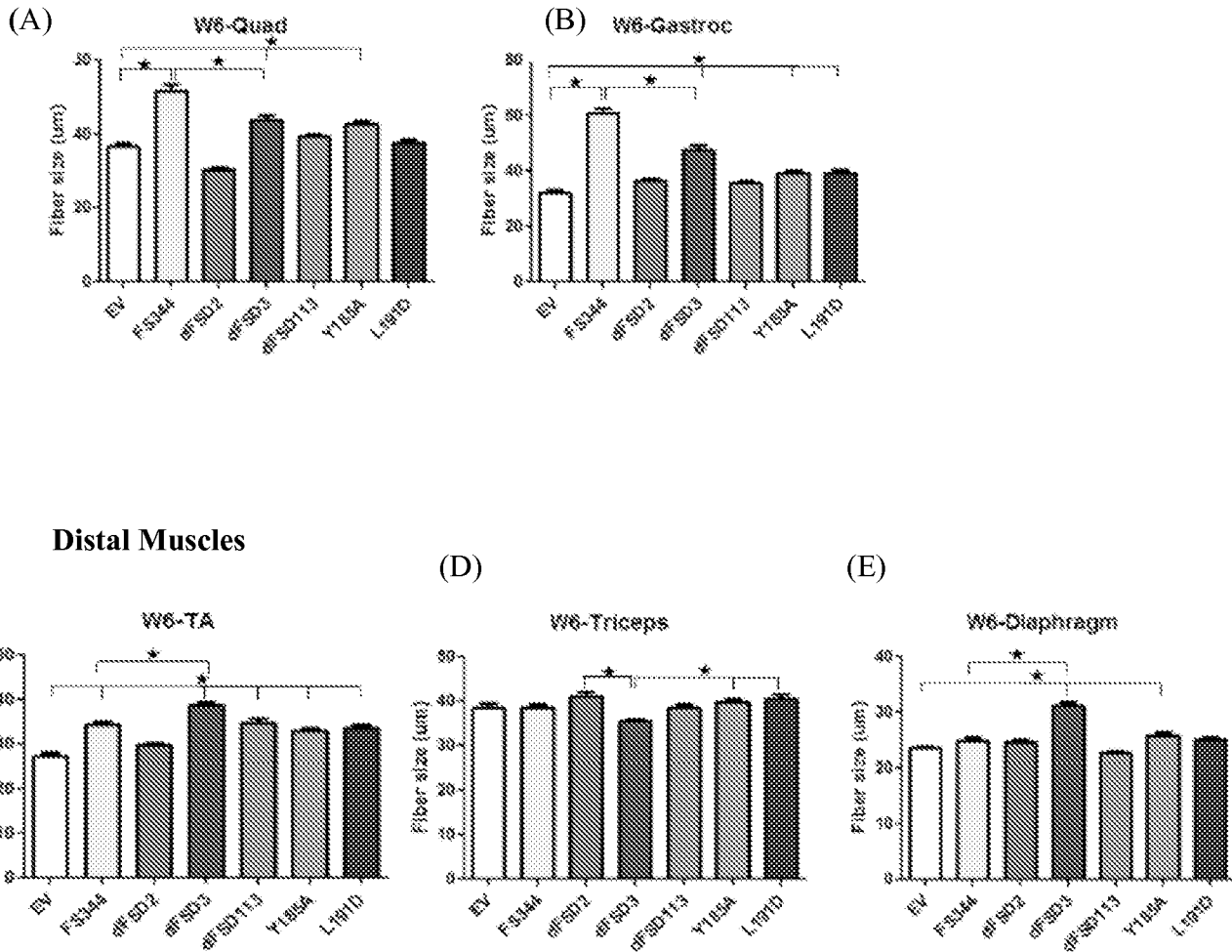
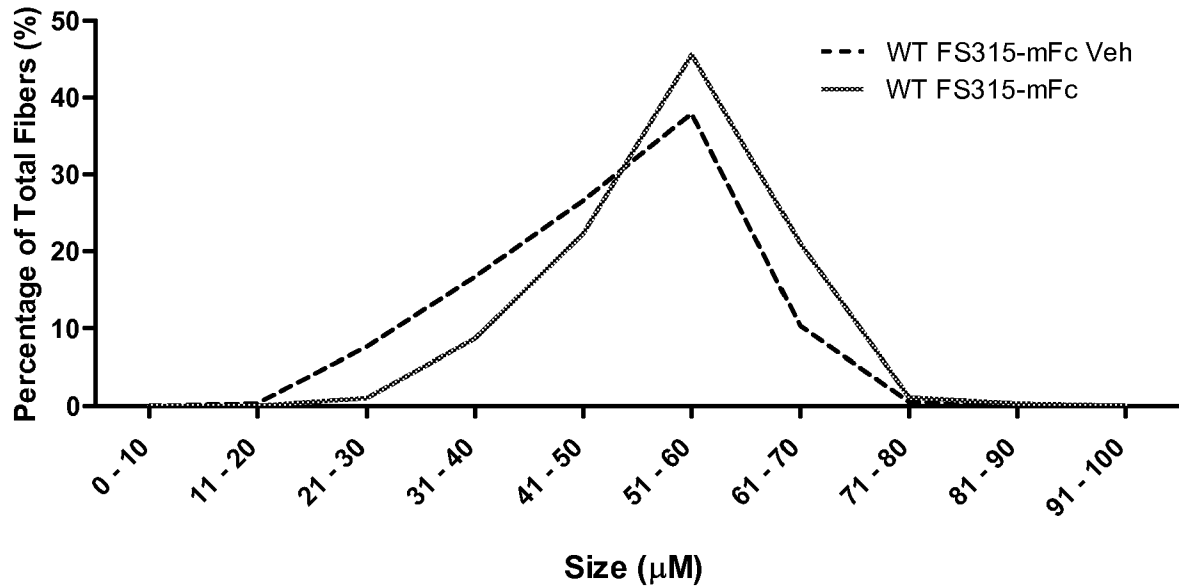


FIG. 14

Week 6 muscle myofiber diameter after intramuscular gene delivery of follistatin variants to quadriceps (A) and gastrocnemius (B) muscle of C57 mice. The following muscles were remote from the injection site: C) tibialis anterior, D) triceps, E) diaphragm.

A)

Myofiber size distribution of gastrocnemius in FS315-mFc treated WT

B)

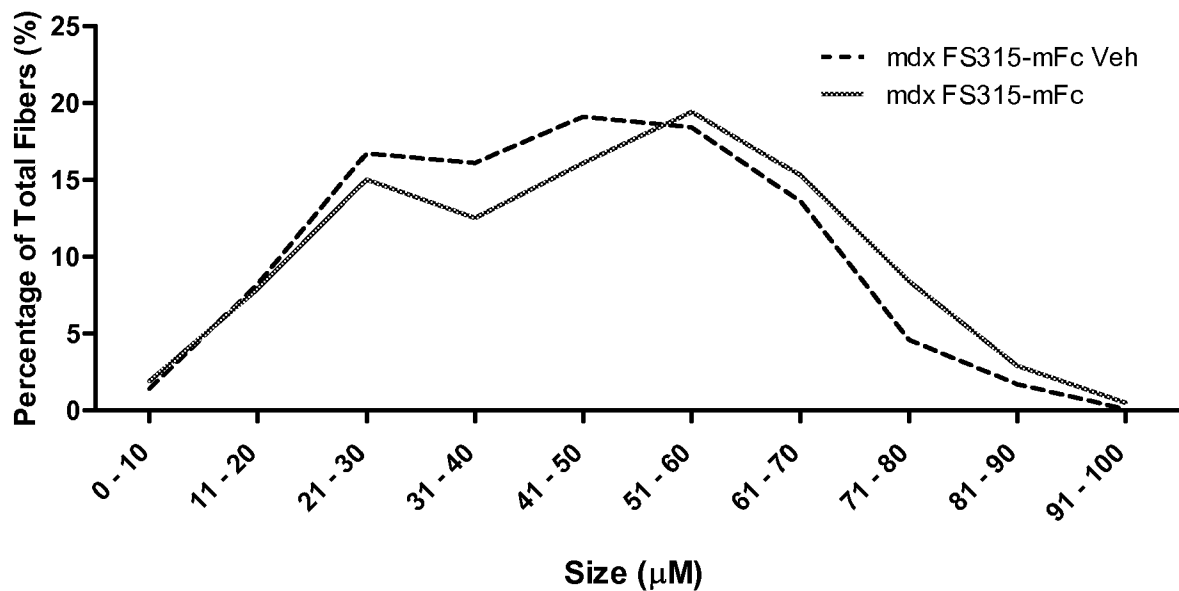
Myofiber size distribution of gastrocnemius in FS315-mFc treated mdx

FIG. 15

Size distribution of myofibers after intra-muscular FS315-mFc injection into the gastrocnemius of C57 (WT) (A) or mdx (B) mice.

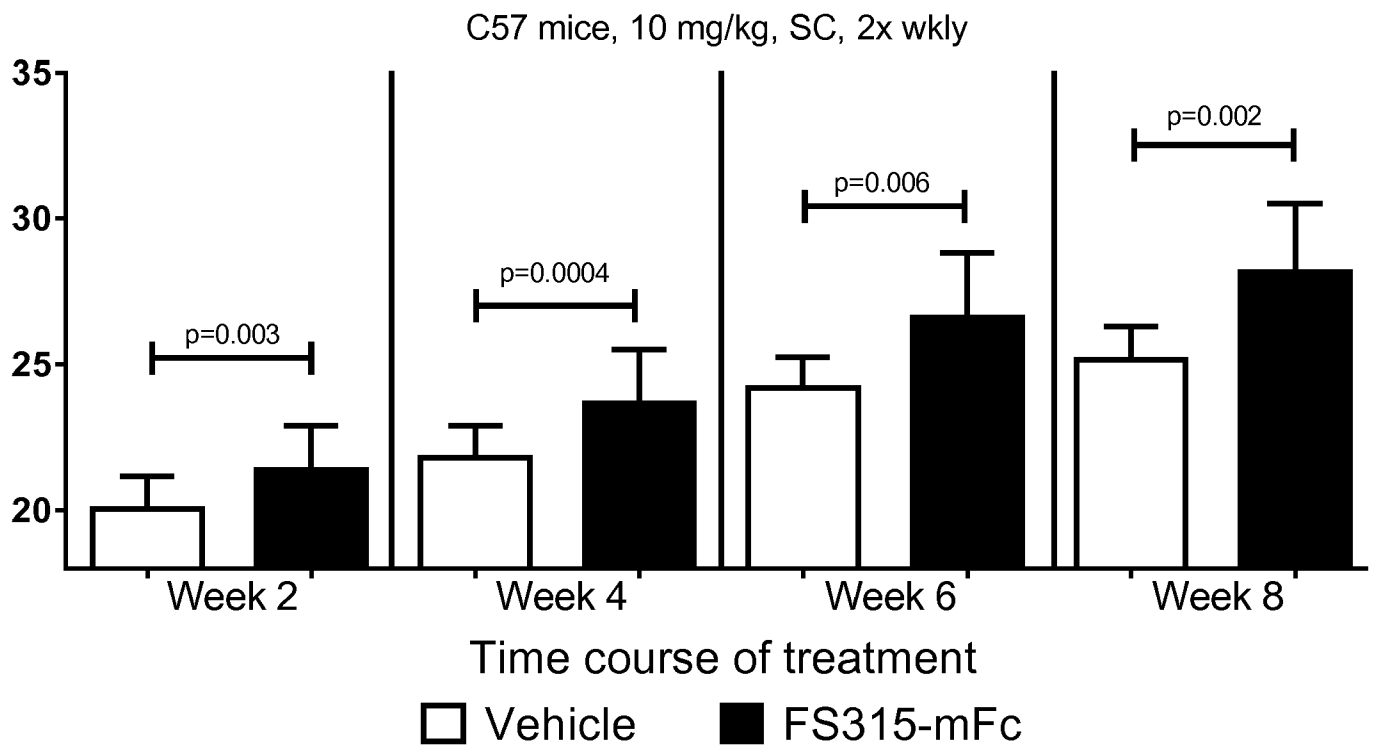


FIG. 16

Body weights of C57 mice treated twice weekly for 8 weeks with 10 mg/kg FS315-mFc via subcutaneous injection. P values were obtained using unpaired t-test.

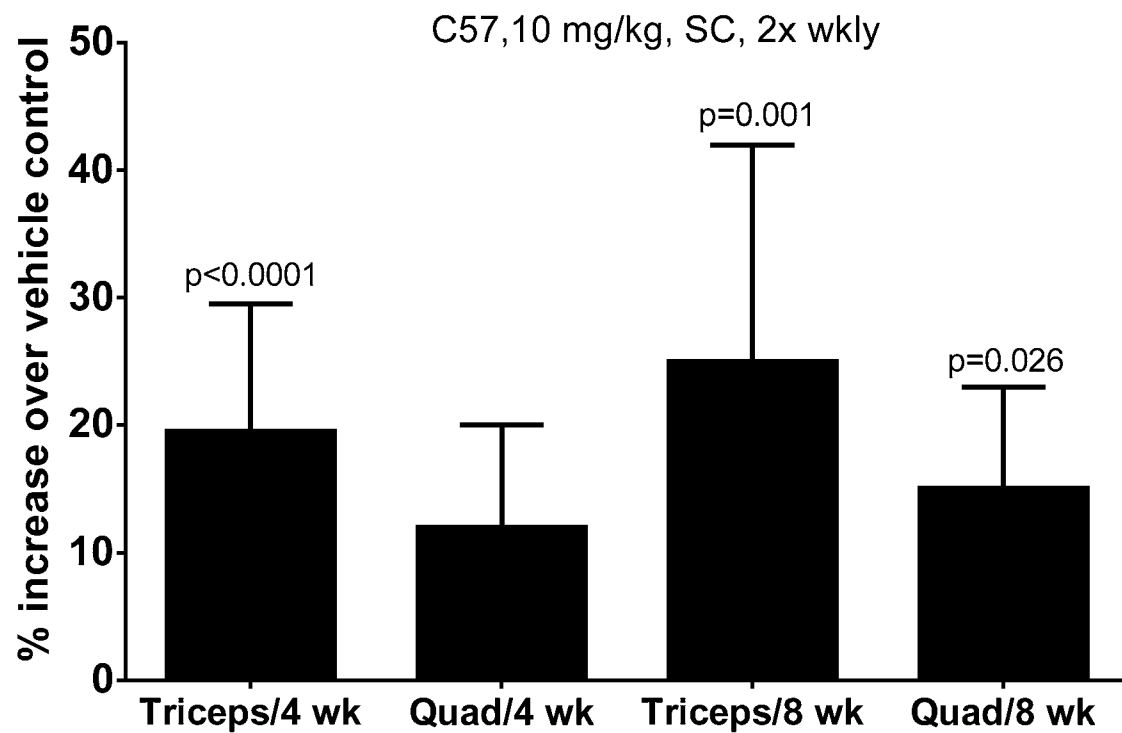


FIG.17

Percent change in weight for triceps and quadriceps muscles in FS315-mFc treated C57 mice at week 4 and 8. P values were obtained from unpaired t-test.

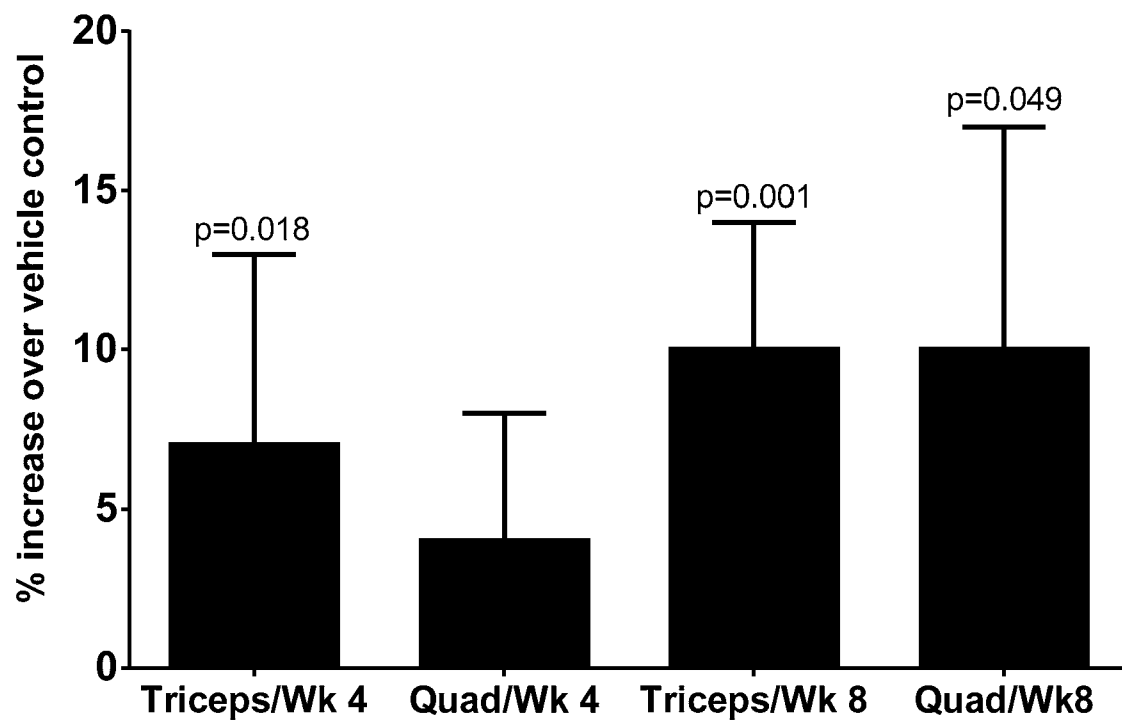
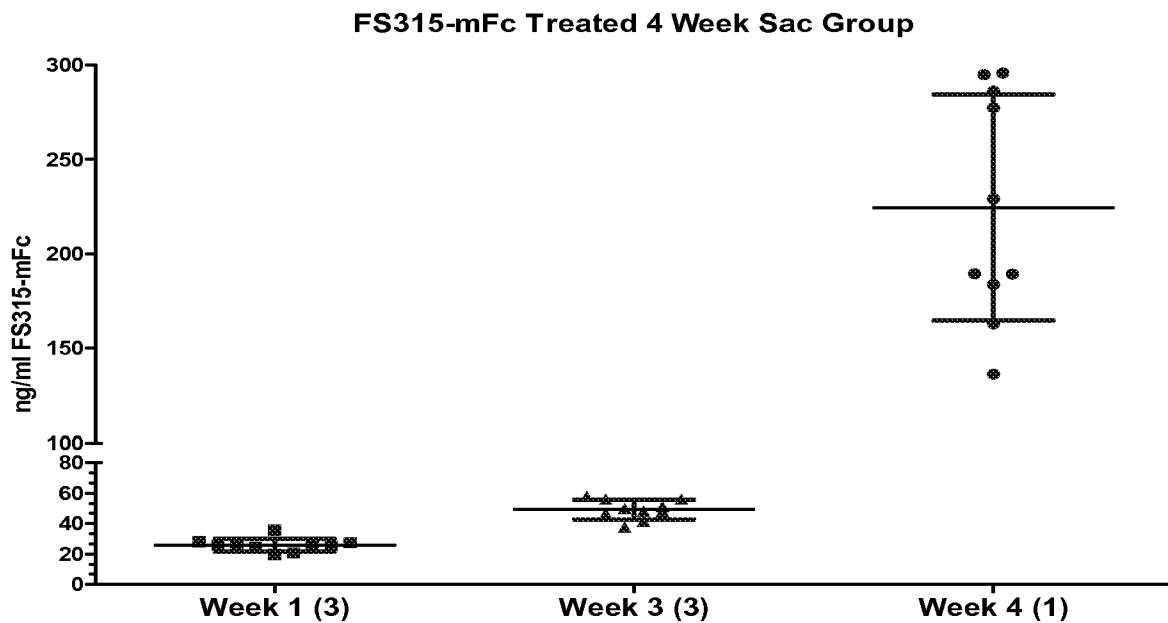


FIG. 18

Percent increase over vehicle control of triceps and quadriceps myofiber diameters in FS315-mFc treated C57 mice at week 4 and 8. P values were obtained from unpaired t-test.

A)



B)

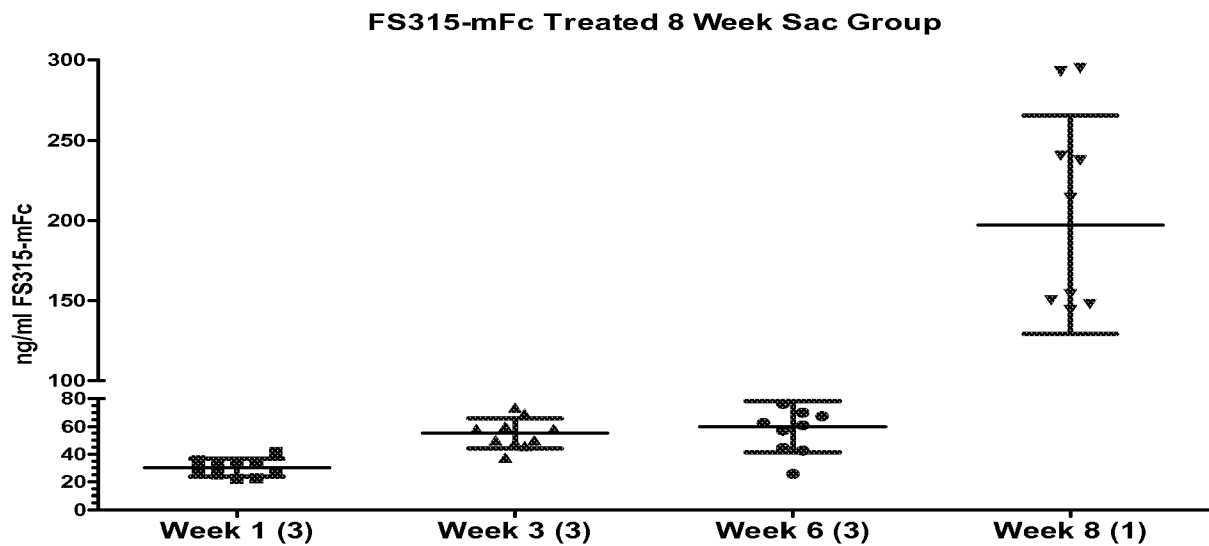


FIG.19

Levels of FS315-mFc in serum after twice weekly subcutaneous injection of 10 mg/kg into C57 mice. On the X-axis, “Week” refers to week of treatment course. The number of days post-injection of FS315-mFc, corresponding to when the serum was collected, is included in parentheses. The vehicle treated mice had no detectable follistatin in the serum.

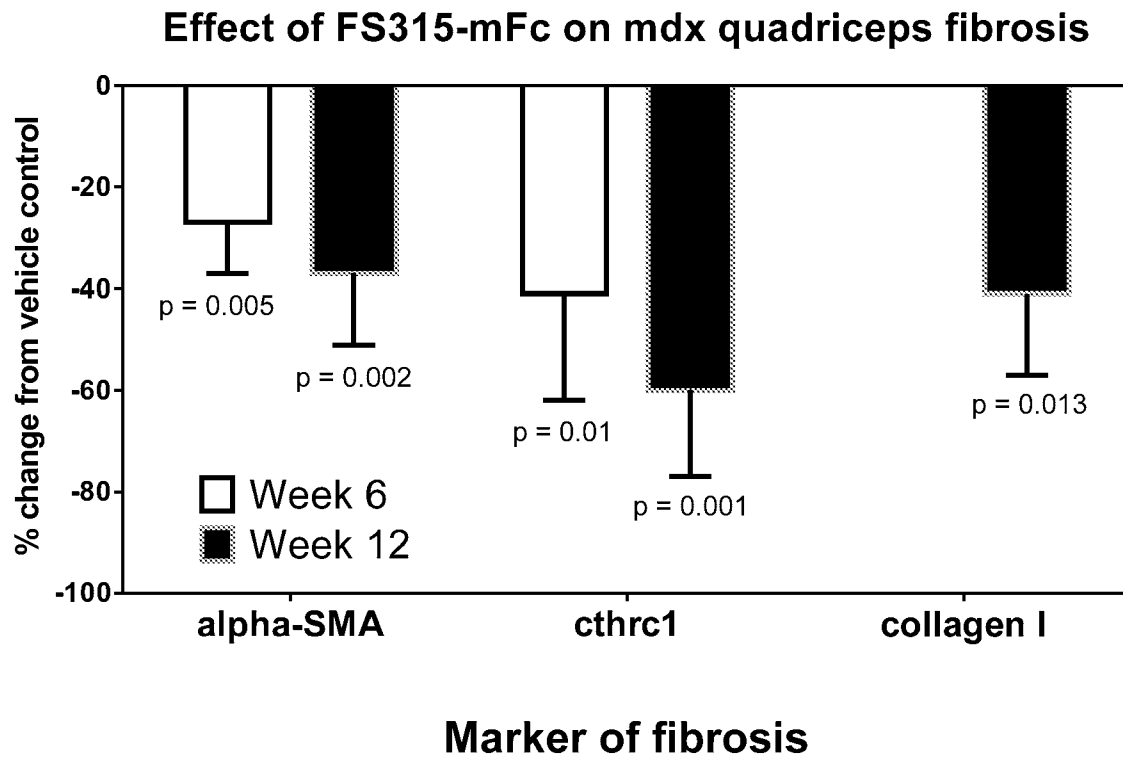


FIG. 20

mRNA levels of key markers of fibrosis after 6 and 12 weeks of FS315-mFc treatment of mdx mice. RT-PCR was used to quantitate mRNA levels of each protein in quadriceps of treated mice (n = 15 animals per time point). P-values were obtained from a one-way ANOVA followed by Tukey's posthoc pairwise comparison test or its nonparametric analogue.

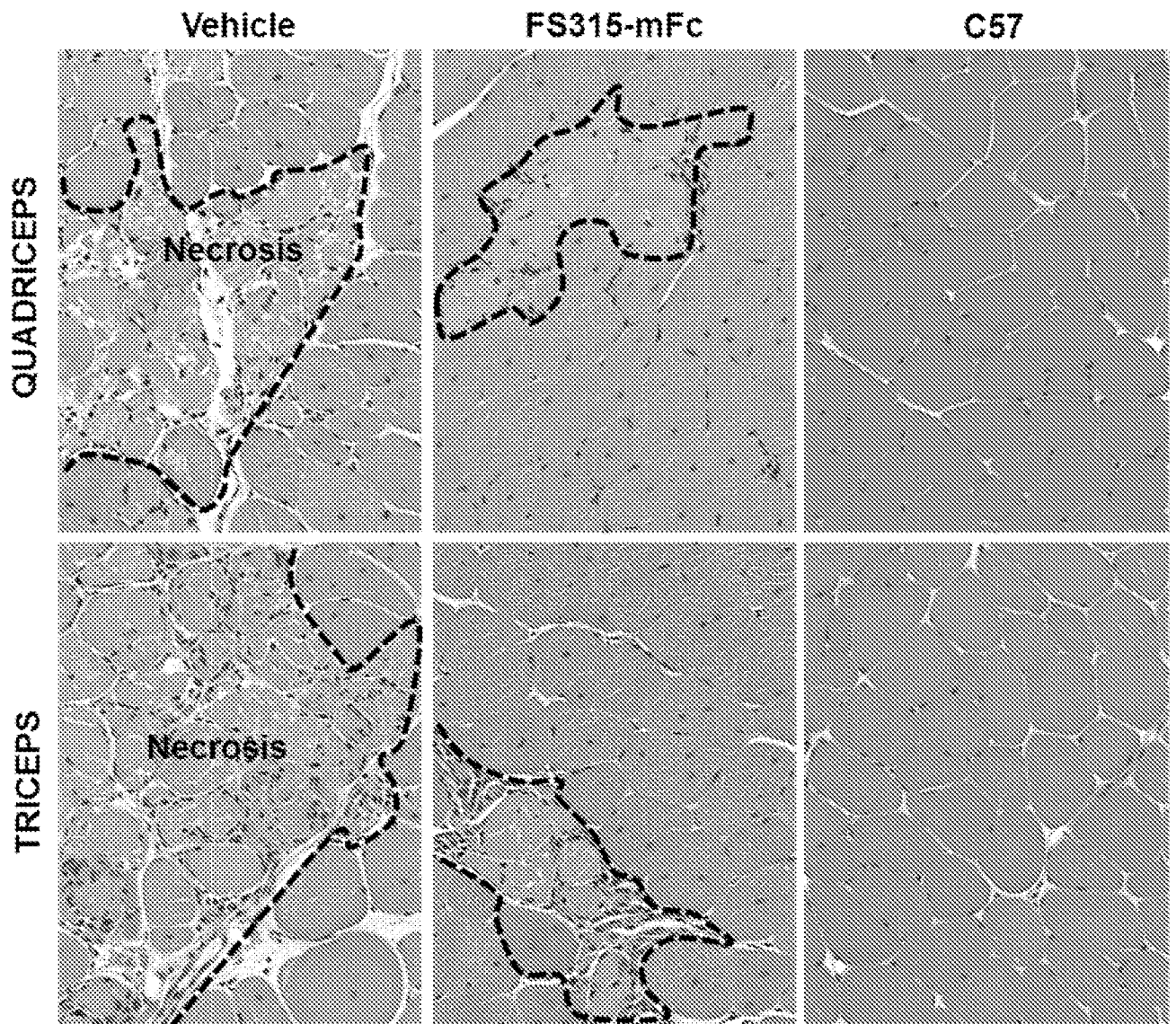


FIG. 21

H&E stained sections of quadriceps and triceps muscle after 6 weeks of twice weekly treatment with 10 mg/kg FS315-mFc. The outlined areas indicate necrotic tissue. The C57 panel represents healthy, unaffected muscle.

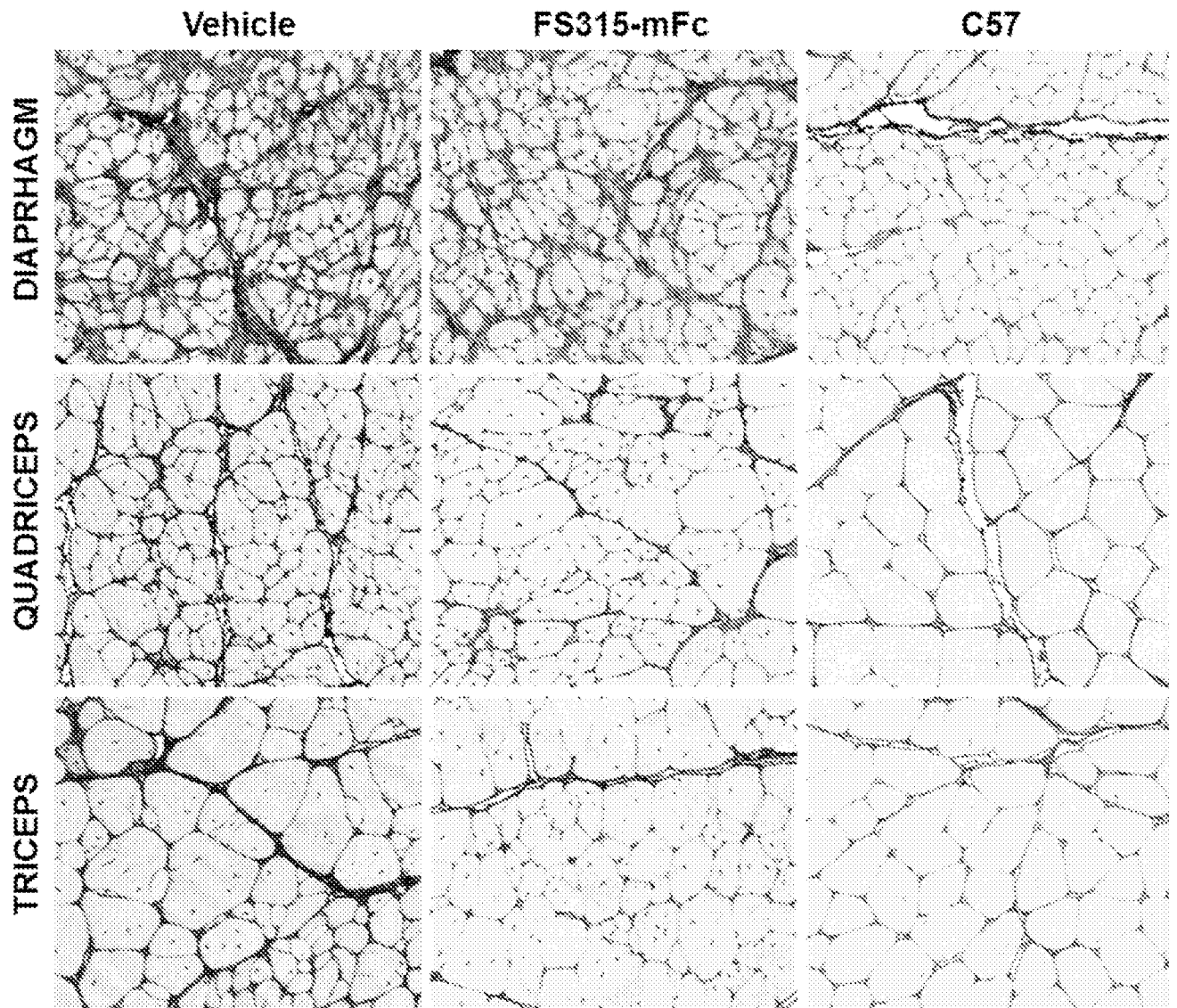


FIG. 22

Collagen I stained sections of diaphragm, quadriceps and triceps muscle after 12 weeks of twice weekly treatment with 10 mg/kg FS315-mFc. The brown stained areas indicate collagen I deposition and fibrosis. The C57 panel represents healthy, unaffected muscle.

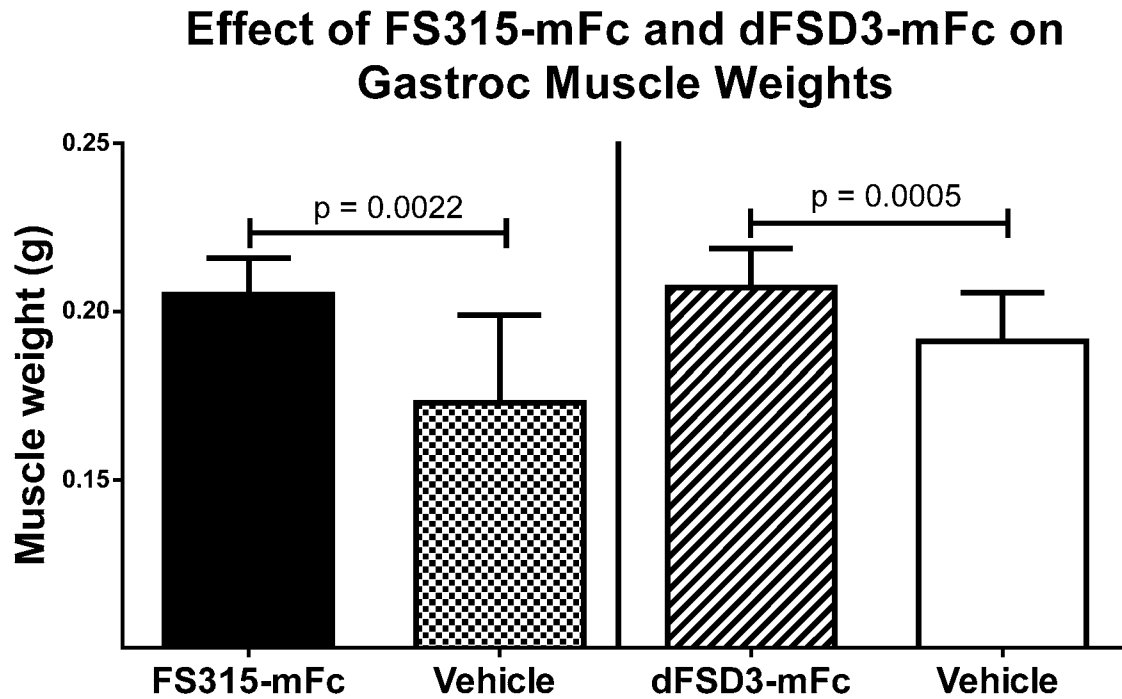


FIG. 23

The pharmacodynamic effect of FS315-mFc and dFSD3-mFc on injected muscle weights after twice weekly injections of 20 μ g directly into the gastrocnemius muscle for 4 weeks. P values were obtained from paired t-test (follistatin treated compared to vehicle control).

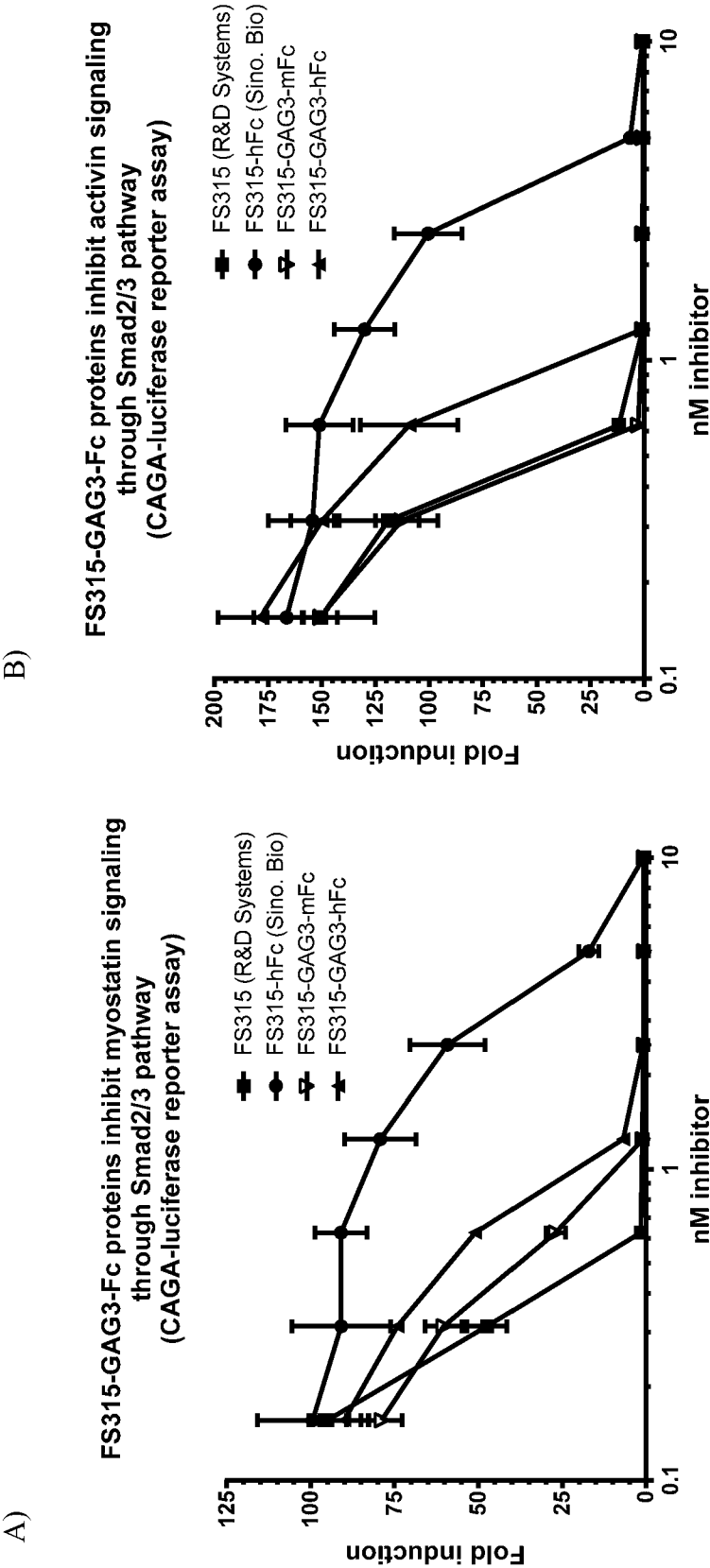


FIG. 24

FS315-GAG3-mFc and -hFc inhibit A) myostatin and B) activin A signaling through Smad2/3 pathway (CAGA-luciferase reporter assay) to a greater extent compared to the commercially available FS315-(9 linker)-hFc protein (Sino Biological).

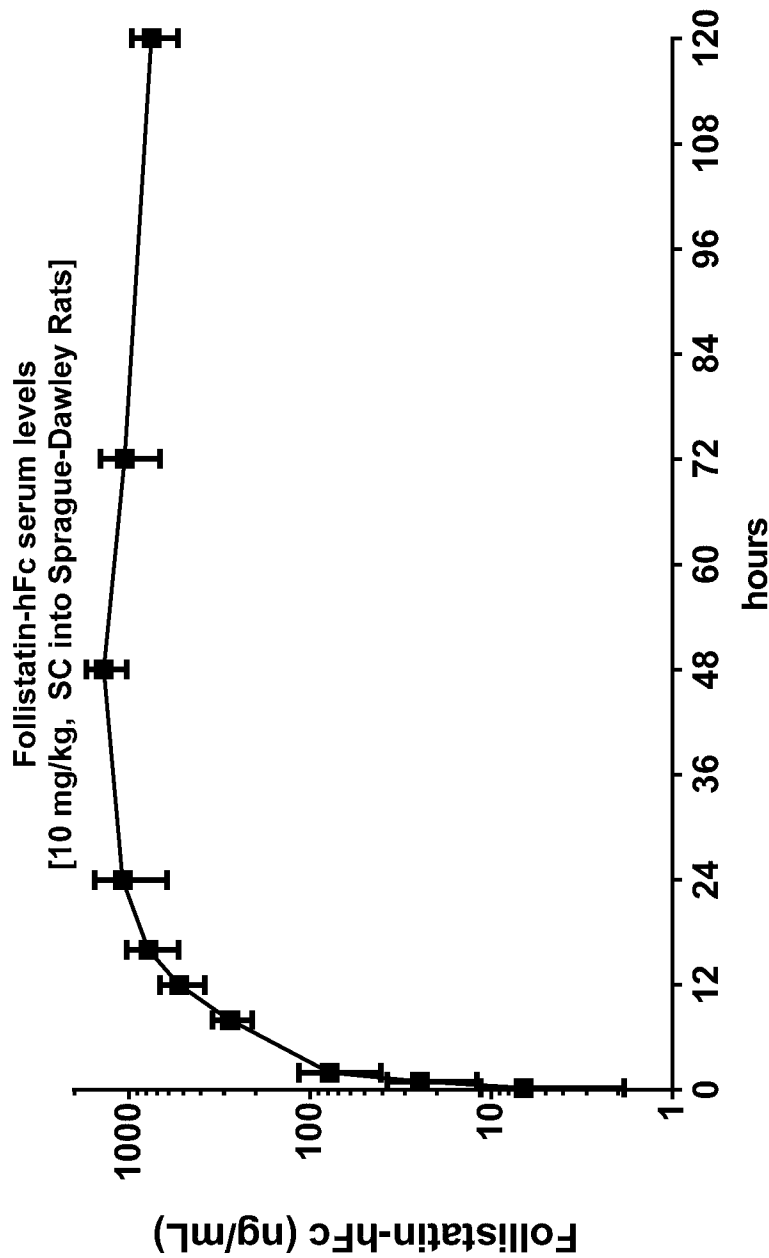


FIG. 25

FS315-hFc PK profile in rat serum after SC injection of 10 mg/kg. Estimated serum half-life ~ 3.5 days.