(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
03 October 2019 (03.10.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/191204 A1

(51) International Patent Classification:
A61K 38/17 (2006.01) *A61P 21/00* (2006.01)
A61K 47/68 (2017.01)

(21) International Application Number:
PCT/US2019/024243

(22) International Filing Date:
27 March 2019 (27.03.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/649,235 28 March 2018 (28.03.2018) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*

WO 2019/191204 A1

(54) Title: FOLLISTATIN POLYPEPTIDES FOR THE TREATMENT OF MUSCLE CONTRACTURE

(57) Abstract: The disclosure provides methods of treating a subject having a disease or disorder associated with muscle contractures by administering a follistatin polypeptide, wherein the polypeptide includes truncated variants of follistatin, as well as fusion proteins comprising the same.

FOLLISTATIN POLYPEPTIDES FOR THE TREATMENT OF MUSCLE CONTRACTURE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority from U.S. Provisional Application No. 62/649,235, filed on March 28, 2018. The foregoing application is incorporated herein by reference in their entirety.

GOVERNMENT LICENSE RIGHTS

This invention was made with government support under K08 AR059750 awarded by the NIH. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Congenital contractures are abnormally short and stiff skeletal muscles presenting at birth that constitute a major and long-term source of disability in children. In most cases, congenital contractures can be attributed to reduced fetal movements, and causes can include neurological disease, connective tissue disease, muscle disease, space limitations within the uterus, intrauterine or fetal vascular compromise, or maternal disease. Kalampokas et al., ISRN Obst. and Gyn. 2012: 264918. Muscles developed under these conditions have short muscle and long tendon components to muscle-tendon units (MTUs), resulting in impairment of movement. Flinchum D. J Bone Joint Surg Am 1953;35-A(1):111-114; Ippolito et al. J Child Orthop 2009;3(3):171-178; and Sharrard WJ. J Bone Joint Surg Br 1967; 49(4):731-747. Approximately 1% of all live births show some form of contracture, which can range from single-joint immobility (such as clubfoot) to severe contractures of several limb joints (such as arthrogryposis multiplex congenita (AMC). Kalampokas at 264918; and Rink BD. Obst. & Gyn. Survey 2011;66(6):369-377.

Treatment of contractures is generally long-term, with the potential to achieve some level of ambulation in many patients. Therapies are focused on the specific symptoms experienced by each individual and may include physical therapy, removable splints, exercise, and/or surgery. Fassier et al., J. Children's Orthopaedics 2009;3(5):383-390; and Bevan et al., J. Pediatric Orthopedics 2007;27(5):594-600. These therapeutic strategies are generally focused on restoring flexibility but have not been designed to improve strength. Thus, there is a need for agents that restore flexibility and improve strength in a localized manner.

SUMMARY OF THE INVENTION

In some embodiments, the disclosure provides for a method of treating a subject having a disorder associated with muscle contractures, comprising administering to the subject an effective amount of a protein, wherein the protein comprises a follistatin polypeptide, or a biologically active fragment thereof. In some embodiments, the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 1, or a biologically active fragment thereof. In some embodiments, the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 2, or a biologically active fragment thereof.

In some embodiments, the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3, or a biologically active fragment thereof. In some embodiments, the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 4, or a biologically active fragment thereof. In some embodiments, the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, or a biologically active fragment thereof. In some embodiments, the follistatin polypeptide is conjugated to a heterologous moiety. In some embodiments, the heterologous moiety is a constant domain of an immunoglobulin. In some embodiments, the heterologous moiety is albumin.

In some embodiments, the disclosure provides for a method of treating a subject having a disorder associated with muscle contractures, comprising administering to the subject an effective amount of a protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence comprises a follistatin polypeptide, wherein the follistatin polypeptide consists of an amino acid sequence that is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15 or 16, and wherein the second amino acid sequence comprises a constant domain of an immunoglobulin.

In some embodiments, the disclosure provides for a method of treating a subject having a disorder associated with muscle contractures, comprising administering to the

subject an effective amount of a protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence comprises a follistatin polypeptide, wherein the follistatin polypeptide consists of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-4, 7-16 and 26-43, and wherein the second amino acid sequence comprises a constant domain of an immunoglobulin. In some embodiments, the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO: 38-43. In some embodiments, the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO: 26-28 and 32-34. In some embodiments, the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO: 29-31 and 35-37. In some embodiments, the first amino acid sequence comprises an amino acid sequence that begins at a residue corresponding to any one of amino acids 30-95 of SEQ ID NO: 3 and ends at a position corresponding to any one of amino acids 316-344 of SEQ ID NO: 3. In some embodiments, the first amino acid sequence comprises an amino acid sequence that begins at a residue corresponding to any one of amino acids 30-95 of SEQ ID NO: 1 and ends at a position corresponding to any one of amino acids 164-167 or 238-244 of SEQ ID NO: 1. In some embodiments, the first amino acid sequence consists of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15 or 16.

In some embodiments, the first amino acid sequence consists of the amino acid sequence of SEQ ID NO: 15 or 16. In some embodiments, a linker polypeptide is positioned between the first amino acid sequence and second amino acid sequence, wherein the linker directly connects the C-terminal portion of the first amino acid sequence to the N-terminal portion of the second amino acid sequence, and wherein the linker is 1-10 amino acids in length. In some embodiments, the linker polypeptide comprises the sequence TGGG (SEQ ID NO: 49). In some embodiments, the linker polypeptide consists of the sequence TGGG (SEQ ID

NO: 49). In some embodiments, the second amino acid sequence comprises a constant domain of an IgG immunoglobulin. In some embodiments, the second amino acid sequence comprises a constant domain of an IgG immunoglobulin that has reduced or no substantial ADCC and/or CDC activity relative to human IgG1. In some embodiments, the second amino acid sequence comprises a constant domain of an IgG immunoglobulin selected from the group: IgG1, IgG2 and IgG4. In some embodiments, the second amino acid sequence comprises an Fc portion of an immunoglobulin. In some embodiments, the second amino acid sequence comprises an IgG1 constant domain. In some embodiments, the second amino acid sequence comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 17. In some embodiments, the second amino acid sequence comprises an IgG2 constant domain. In some embodiments, the IgG2 constant domain comprises an Fc portion of an IgG immunoglobulin that has reduced ADCC activity relative to human IgG2 . In some embodiments, the IgG2 constant domain comprises an Fc portion of an IgG immunoglobulin that has reduced CDC activity relative to human IgG2. In some embodiments, the second amino acid sequence comprises an Fc portion of an IgG immunoglobulin selected from the group: IgG1, IgG2, IgG4 and an IgG2/4 hybrid. In some embodiments, the second amino acid sequence comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 18. In some embodiments, the follistatin polypeptide ends at a position corresponding to an amino acid selected from the group consisting of amino acid 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, and 305 of SEQ ID NO: 4. In some embodiments, the follistatin polypeptide does not include residues corresponding to the amino acids selected from the group consisting of amino acids 289-315, 290-315, 291-315, 292-315, 293-315, 294-315, 295-315, 296-315, 297-315, 298-315, 299-315, 300-315, 301-315, 302-315, 303-315, 304-315, and 305-315 of SEQ ID NO: 4. In some embodiments, the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO: 43, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is optionally absent. In some embodiments, the protein comprises the amino acid sequence of SEQ ID NO: 43, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is optionally absent. In some embodiments, the protein comprises an amino acid sequence that is at least 80%,

81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO: 42, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is optionally absent. In some embodiments, the protein comprises the amino acid sequence of SEQ ID NO: 42, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is optionally absent. In some embodiments, the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is absent. In some embodiments, the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is included. In some embodiments, the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is absent. In some embodiments, the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is included. In some embodiments, the protein comprises the amino acid sequence of SEQ ID NO: 42. In some embodiments, the protein comprises the amino acid sequence of SEQ ID NO: 43. In some embodiments, the follistatin polypeptide comprises an unmasked heparin binding domain. In some embodiments, the heparin binding domain comprises the endogenous follistatin heparin binding sequence of SEQ ID NO: 5. In some embodiments, the heparin binding domain comprises a heterologous heparin binding sequence. In some embodiments, the follistatin polypeptide binds to one or more ligands selected from the group consisting of: myostatin, GDF-11, activin A and activin B with a KD less than 1 nM, 100 pM, 50 pM or 10 pM. In some embodiments, the follistatin polypeptide binds to myostatin with a KD less than 1 nM, 100 pM, 50 pM or 10 pM. In some embodiments, the follistatin polypeptide binds to activin A with a KD less than 1 nM, 100 pM, 50 pM or 10 pM. In some embodiments, the follistatin polypeptide binds to activin B with a KD less than 1 nM, 100 pM, 50 pM or 10 pM. In some embodiments, the subject has muscle contractures associated with a neurological disease of the brain, spine, or peripheral nerve. In some embodiments, the subject has muscle contractures associated with a connective tissue defect. In some embodiments, the connective tissue defect is diastrophic dysplasia. In some embodiments, the subject has muscle contractures associated with muscle abnormalities. In some embodiments, the muscle abnormalities are selected from the group consisting of muscular dystrophy and mitochondrial abnormalities. In some embodiments, the subject has muscle contractions associated with a congenital contracture. In some embodiments, the congenital contracture in the subject was caused by uterine space limitations from when the subject was in utero. In some embodiments, the space limitations within the uterus were associated with a condition selected from the group consisting of oligohydramnios, fibroids, uterine malformations, and multiple pregnancy. In some embodiments, the congenital contractures in the subject are a

result of intrauterine or fetal vascular compromise from when the subject was in utero. In some embodiments, the intrauterine or fetal vascular compromise result in impaired development of nerves or anterior horn cell death. In some embodiments, the congenital contracture is a result of maternal disease. In some embodiments, the maternal disease is selected from the group consisting of diabetes mellitus, multiple sclerosis, myasthenia gravis, infection, drug usage, or trauma. In some embodiments, the subject has muscle contractions associated with dysgenesis of the nervous system as observed in chromosomal abnormalities. In some embodiments, the chromosomal abnormalities are selected from the group consisting of trisomy 18 or trisomy 21. In some embodiments, the subject has muscle contractions associated with dysplasias of the brainstem nuclei or spinal cord. In some embodiments, the dysplasias of the brainstem nuclei or spinal cord are selected from the group consisting of Mobius syndrome, Pierre-Robin syndrome, prune belly syndrome, and Zellweger syndrome. In some embodiments, the subject has muscle contractions associated with Arthrogryposis multiplex congenita (AMC). In some embodiments, the AMC is selected from the group consisting of amyoplasia, distal arthrogryposis, a systemic connective tissue disorder, multiple pterygium syndromes, and fetal crowding. In some embodiments, the subject has muscle contractions associated with an isolated congenital contracture. In some embodiments, the subject has one or more of the following characteristics: decreased flexibility of the joints, muscle weakness, shortening of a muscle; and/or shortening of a joint. In some embodiments, the method comprises administering an effective amount of the protein by an intramuscular route of administration to a targeted muscle of the subject. In some embodiments, an effective amount of the protein is administered intramuscularly. In some embodiments, an effective amount of the protein is administered intravenously. In some embodiments, the subject is a mammal. In some embodiments, the subject is a human. In some embodiments, the subject is an adult or juvenile. In some embodiments, the subject is an infant or a fetus. In some embodiments, the protein is administered prior to, during, or following surgical correction of the muscle contracture. In some embodiments, the protein is administered in combination with stretch therapy. In some embodiments, the protein is administered in combination with electrostimulation therapy. In some embodiments, the administration of the protein in combination with stretch therapy provides at least 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater shortening of the tendon as compared to stretch therapy in combination with electrostimulation therapy. In some embodiments, the

administration of the protein in combination with stretch therapy provides at least 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater shortening of the tendon as compared to stretch therapy in combination with vehicle. In some embodiments, the administration of the protein in combination with stretch therapy provides 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% decrease in tendon length. In some embodiments, the protein inhibits activin A or GDF-11 in a cell-based reporter gene assay with a lower IC50 than the IC50 of a follistatin polypeptide comprising SEQ ID NO: 2. In some embodiments, the protein binds heparin with a greater affinity than a follistatin polypeptide comprising SEQ ID NO: 2. In some embodiments, the protein does not have a systemic effect when administered intramuscularly. In some embodiments, the neurological disease of the brain, spine, or peripheral nerve is selected from the group consisting of multiple sclerosis, poliomyelitis, Parkinson's disease, dementia, Cerebral Palsy, dysplasia of the brainstem nuclei, and dysplasia of the spinal cord. In some embodiments, the subject has muscle contractures associated with being bedbound, chronic illness, serious injury, or post-surgical or disuse atrophy. In some embodiments, the post-surgical or disuse atrophy is selected from the group consisting of hip fracture, total hip arthroplasty, total knee arthroplasty, and rotator cuff surgery. In some embodiments, the serious injury is selected from the group consisting of head injury and stroke. In some embodiments, the subject has muscle contractures associated with an inflammatory disorder. In some embodiments, the inflammatory disorder is rheumatoid arthritis.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Patent Office upon request and payment of the necessary fee.

Figure 1 shows the full, unprocessed amino acid sequence of human follistatin 315 (SEQ ID NO: 3). The leader sequence is italicized in bold font, the follistatin N-terminal region (FSN) is indicated by single underlining, and the three follistatin domains (FSDs) are indicated by double underlining. In particular, follistatin domain I (FSDI) is indicated in red font, follistatin domain II (FSDII) is indicated in blue font, and the follistatin domain III (FSDIII) is indicated in green font.

Figure 2 shows the effect of 4 weeks treatment, by subcutaneous injection, with either FST(288)-Fc, FST(315)-Fc, or ActRIIB-Fc on lean tissue mass in mice. Vehicle was Tris-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. TBS by unpaired t-test. #, $P < 0.05$ vs. FST groups by unpaired t-test. FST(288)-Fc, FST(315)-Fc, and ActRIIB-Fc treatment resulted in significant increases in lean tissue mass compared to vehicle control mice. The increase in lean tissue mass of ActRIIB-Fc treated mice was significantly greater than the increases in lean tissue mass observed in either FST(288)-Fc or FST(315)-Fc treated mice.

Figure 3 shows the effect of 4 weeks treatment, by subcutaneous injection twice per week, with either FST(288)-Fc, FST(315)-Fc, or ActRIIB-Fc on grip strength in mice. Vehicle was Tris-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. TBS by unpaired t-test. #, $P < 0.05$ vs. FST groups by unpaired t-test. ActRIIB-Fc treatment increased grip strength in mice. No increased grip strength was observed in FST(288)-Fc or FST(315)-Fc treated mice.

Figure 4 shows the effect of 4 weeks treatment, by subcutaneous injection twice per week, with either FST(288)-IgG1, FST(315)-IgG1, or ActRIIB-Fc on pectoralis (Pecs), tibialis anterior (TA), gastrocnemius (Gastroc), and femoris muscle mass in mice. Vehicle was Tris-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. TBS by unpaired t-test. #, $P < 0.05$ vs. FST groups by unpaired t-test. ActRIIB-Fc treatment significantly increased pectoralis, tibialis anterior, gastrocnemius, and femoris muscle mass in mice, but little to no increase in muscle mass was observed in FST(288)-IgG1 or FST(315)-IgG1 treated mice.

Figure 5 shows the effect of 4 weeks treatment, by subcutaneous injection, with either FST(288)-IgG1 or FST(315)-IgG1 on serum levels of follicle-stimulating hormone (FSH). Vehicle was Tris-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. TBS by unpaired t-test. FST(315)-IgG1 treatment resulted in a significant decrease in serum FSH levels in comparison to vehicle control mice. In contrast, FST(288)-IgG1 treatment had no effect on serum FSH levels

Figure 6 shows the effect of 4 weeks treatment, by subcutaneous injection twice weekly, with either FST(288)-IgG1, FST(315)-IgG1, or ActRIIB-mFc on lean tissue mass in mice. Vehicle was Tris-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. TBS by unpaired t-test. ActRIIB-mFc treatment resulted in significant increases in lean tissue mass compared to vehicle control mice. No increases in lean tissue mass were observed in either FST(288)-IgG1 or FST(315)-IgG1 treated mice.

Figure 7 shows the effect of 4 weeks treatment, by intramuscular injection into the right gastrocnemius twice weekly, with either FST(288)-IgG1, FST(315)-IgG1, or ActRIIB-mFc on gastrocnemius muscle mass in mice. Vehicle was Tris-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. TBS by unpaired t-test. #, $P < 0.05$ right, injected gastrocnemius muscle vs. left, non-injected, gastrocnemius muscle by unpaired t-test. FST(288)-IgG1, FST(315)-IgG1, and ActRIIB-mFc treatment significantly increased muscle mass in the right, injected gastrocnemius muscle. ActRIIB-mFc treatment also significantly increased muscle mass in the left, non-injected gastrocnemius muscle. In contrast, there was not observed increase in the left, non-injected gastrocnemius muscle in FST(288)-IgG1 or FST(315)-IgG1 treated mice.

Figure 8 shows the effect of 3 weeks treatment, by intramuscular injection into the right gastrocnemius twice weekly, with varying doses of FST(288)-IgG1, on gastrocnemius muscle mass in mice, expressed as a ratio over the uninjected, left gastrocnemius. Vehicle was phosphate-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. PBS by unpaired t-test. Increasing doses of FST(288)-IgG1 caused an increasing hypertrophy of the injected gastrocnemius muscle relative to the uninjected muscle.

Figure 9 shows the effect of 4 weeks treatment, by intramuscular injection into the left gastrocnemius twice weekly, with FST(291)-IgG1. Vehicle was phosphate-buffered saline. Data are means ± SEM. *, $P < 0.05$ vs. PBS by unpaired t-test. Intramuscular administration of FST(291)-IgG2 caused marked increase in muscle mass in the injected gastrocnemius muscle relative to the uninjected muscle and relative to controls.

Figure 10 shows a diagram of the overall experimental design related to the treatment of immobilization-induced contractures. This diagram illustrates the breakdown of experimental groups and timepoints for the experiments in Example 8. Colored boxes correspond to treatment group values in Figures 12 and 13.

Figure 11 shows an illustration of contracture formation following immobilization in the mouse. Figure 11A is a diagram comparing the positioning of splint components to immobilize the ankle plantarflexed. Figures 11B and 11C depict casting of the ankle in the plantarflexed position at 1 week of life (Figure 11B), which results in a stable ankle deformity at 3 weeks of life (Figure 11C). Figure 11D shows that upon dissection of the tibialis anterior (TA) muscle, lengthening of the tendon in the immobilized limb is apparent. Figure 11E shows that epoxy-embedded, toluidine blue stained tissue did not reveal histological abnormalities in the muscle or tendon tissue of mice following immobilization.

Figure 12 illustrates the impact of stretch and electrostimulation on muscle-tendon unit (MTU) abnormalities induced by immobilization at 90 degrees. Figure 12A is a diagram of the studies comparing limbs that have not undergone immobilization, limbs which underwent immobilization which received no treatment, limbs which underwent immobilization which received 3-stage stretch therapy, and limbs which underwent immobilization which received a combination therapy of 3-stage stretch therapy and electrostimulation. A summary infographic of the average MTU length within each growth is shown at the bottom of the diagram. Figure 12B provides graphical depictions of tendon length (as % of the MTU; top), sarcomere number (middle), and sarcomere number/tendon length (bottom) measurements for the unsplinted (gray) and immobilized (colored) limbs in each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistics for tendon length and sarcomere number were calculated using the Mann-Whitney test, and statistics for sarcomere number/tendon length were calculated using ANOVA. Dark gray bars represent the limbs that have not undergone immobilization or treatment in the study animals. Dark blue bars represent limbs that had undergone immobilization, but no treatment. Dark green bars represent limbs that had undergone immobilization, followed by treatment with stretch in three stages. Light green bars represent limbs that had undergone immobilization and a combination therapy including stretch in three stages and electrostimulation.

Figure 13 illustrates the impact of stretch and FST288 treatment on MTU abnormalities induced by immobilization at 90 degrees. Figure 13A is a diagram of the studies comparing limbs that have not undergone immobilization, limbs that have not undergone immobilization which were treated with FST-288 injection, limbs which underwent immobilization which received 5-stage stretch treatment and vehicle; and limbs which underwent immobilization which received a combination therapy of 5-stage stretch treatment and FST-288 injection. A summary infographic of the average MTU length within each group is shown at the bottom of the diagram. Figure 13B provides graphical depictions of tendon length (as % of the MTU; top), sarcomere number (middle), and sarcomere number/tendon length (bottom) measurements for the unsplinted (gray) and immobilized (colored) limbs in each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistics for tendon length and sarcomere number were calculated using the Mann-Whitney test, and statistics for sarcomere number/tendon length were calculated using ANOVA. Dark gray bars represent the limbs that have not undergone immobilization or treatment in the study animals. Light gray bars represent unsplinted limbs that have received only FST(288)-IgG2. White bars

represent unsplinted limbs that have received only vehicle. Orange bars represent limbs that have muscle contractures due to ankle immobilization, followed by treatment with stretch therapy in 5 stages. Peach bars represent limbs that have muscle contractures due to ankle immobilization, followed by treatment with FST(288)-IgG2 and stretch therapy in 5 stages.

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DETAILED DESCRIPTION

1. Overview

In certain aspects, the present disclosure relates to methods of using follistatin polypeptides to treat muscle contractures. As used herein, the term "follistatin" refers to a family of follistatin (FST) proteins and follistatin-related proteins, derived from any species. Follistatin is an autocrine glycoprotein that is expressed in nearly all tissues of higher animals. It was initially isolated from follicular fluid and was identified as a protein fraction that inhibited follicle-stimulating hormone (FSH) secretion from the anterior pituitary, and therefore was designated as FSH-suppressing protein (FSP). Subsequently, its primary function has been determined to be the binding and neutralization of members of the TGF-β superfamily including, for example, activin, a paracrine hormone that enhances secretion of FSH in the anterior pituitary.

The term "follistatin polypeptide" is used to refer to polypeptides comprising any naturally occurring polypeptide of the follistatin family as well as any variants thereof (including mutants, fragments, fusions, and peptidomimetic forms) that retain a useful activity, including, for example, ligand binding (e.g., myostatin, GDF-11, activin A, activin B) or heparin binding. For example, follistatin polypeptides include polypeptides comprising an amino acid sequence derived from the sequence of any known follistatin having a sequence at least about 80% identical to the sequence of a follistatin polypeptide, and preferably at least 85%, 90%, 95%, 97%, 99% or greater identity. The term "follistatin polypeptide" may refer to fusion proteins that comprise any of the polypeptides mentioned above along with a heterologous (non-follistatin) portion. An amino acid sequence is understood to be heterologous to follistatin if it is not uniquely found in the long (315 amino acid) form of human follistatin, represented by SEQ ID NO: 3. Many examples of heterologous portions are provided herein, and such heterologous portions may be immediately adjacent, by amino acid sequence, to the follistatin polypeptide portion of a

fusion protein, or separated by intervening amino acid sequence, such as a linker or other sequence.

Follistatin is a single-chain polypeptide with a range of molecular weights from 31 to 49 kDa based on alternative mRNA splicing and variable glycosylation of the protein. The alternatively spliced mRNAs encode two proteins of 315 amino acids (i.e., FST315) and 288 amino acids (i.e., FST288); follistatin 315 can be further proteolytically degraded to follistatin 303 (FST303). Analysis of the amino acid sequence has revealed that the native human follistatin polypeptide comprises five domains (from the N-terminal side): a signal sequence peptide (amino acids 1-29 of SEQ ID NO:1), an N-terminal domain (FSN) (amino acids 30-94 of SEQ ID NO:1), follistatin domain I (FSDI) (amino acids 95-164 of SEQ ID NO:1), follistatin domain II (FSDII) (amino acids (168-239 of SEQ ID NO:1), and follistatin domain III (FSDIII) (amino acids 245-316 of SEQ ID NO:1). See PNAS, U.S.A., 1988, Vol. 85, No 12, pp 4218-4222.

The human follistatin-288 (FST288) precursor has the following amino acid sequence, with the signal peptide indicated in bold, the N-terminal domain (FSN) indicated by single underlining, and the follistatin domains I-III (FSI, FSII, FSIII) indicated by double underlining.

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTEL
SKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDC
GPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARC
KEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQC
TGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEA
ACSSGVLLEVKHSGSCN (SEQ ID NO:1)

The processed (mature) human follistatin variant FST(288) has the following amino acid sequence with the N-terminal domain indicated by single underlining, and the follistatin domains I-III indicated by double underlining. Moreover, it will be appreciated that any of the initial amino acids G or N, prior to the first cysteine may be removed by processing or intentionally eliminated without any consequence, and polypeptides comprising such slightly smaller polypeptides are further included.

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWM
IFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKG

PVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTC
VVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLGR
SIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKS
DEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSCN (SEQ ID
NO:2)

The human follistatin-315 (FST315) precursor has the following amino acid sequence, with the signal peptide indicated in bold, the N-terminal domain (FSN) indicated by single underlining, and the follistatin domains I-III (FSI, FSII, FSIII) indicated by double underlining (NCBI Accession Number AAH04107.1; 344 amino acids).

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTEL
SKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDC
GPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARC
KEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQC
TGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEA
ACSSGVLLEVKHSGSCNSISEDTEEEEEDEDQDYSFPISSILEW (SEQ
ID NO:3)

The processed (mature) human FST(315) has the following amino acid sequence with the N-terminal domain indicated by single underlining, and the follistatin domains I-III indicated by double underlining. Moreover, it will be appreciated that any of the initial amino acids G or N, prior to the first cysteine may be removed by processing or intentionally eliminated without any consequence, and polypeptides comprising such slightly smaller polypeptides are further included.

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWM
IFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKG
PVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTC
VVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLGR
SIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKS
DEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSCNSISEDTEEEEED
EDQDYSFPISSILEW (SEQ ID NO:4)

Follistatin proteins herein may be referred to as FST. If followed by a number, such as FST(288), this indicates that the protein is the 288 form of follistatin. If presented as

FST(288)-Fc, this indicates a C-terminal Fc fusion to the FST(288), which may or may not include an intervening linker. The Fc in this instance may be any immunoglobulin Fc portion as that term is defined herein. If presented as FST(288)-IgG2, this indicates a C-terminal Fc fusion to the FST(288) of the Fc portion of human IgG2.

Activins are dimeric polypeptide growth factors and belong to the TGF- β superfamily. There are three activins (A, B, and AB) that are homo/heterodimers of two closely related β subunits ($\beta_A\beta_A$, $\beta_B\beta_B$, and $\beta_A\beta_B$). Additional activins C and E have been identified, although the function of these proteins is poorly understood. In the TGF- β superfamily, activins are unique and multifunctional factors that can stimulate hormone production in ovarian and placental cells, support neuronal cell survival, influence cell-cycle progress positively or negatively depending on cell type, and induce mesodermal differentiation at least in amphibian embryos (DePaolo et al., 1991, Proc SocEp Biol Med. 198:500-512; Dyson et al., 1997, Curr Biol. 7:81-84; Woodruff, 1998, Biochem Pharmacol. 55:953-963). Moreover, erythroid differentiation factor (EDF) isolated from the stimulated human monocytic leukemic cells was found to be identical to activin A (Murata et al., 1988, PNAS, 85:2434). It was suggested that activin A acts as a natural regulator of erythropoiesis in the bone marrow. In several tissues, activin signaling is antagonized by its related heterodimer, inhibin. For example, during the release of follicle-stimulating hormone (FSH) from the pituitary, activin promotes FSH secretion and synthesis, while inhibin prevents FSH secretion and synthesis. Activin has also been implicated as a negative regulator of muscle mass and function, and activin antagonists can promote muscle growth or counteract muscle loss in vivo. Link and Nishi, Exp Cell Res. 1997 Jun 15;233(2):350-62; He et al., Anat Embryol (Berl). 2005 Jun;209(5):401-7; Souza et al. Mol Endocrinol. 2008 Dec;22(12):2689-702; Am J Physiol Endocrinol Metab. 2009 Jul;297(1):E157-64; Gilson et al. Zhou et al. Cell. 2010 Aug 20;142(4):531-43.

Growth and Differentiation Factor-8 (GDF8) is also known as myostatin. GDF8 is a negative regulator of skeletal muscle mass. GDF8 is highly expressed in the developing and adult skeletal muscle. The GDF8 null mutation in transgenic mice is characterized by a marked hypertrophy and hyperplasia of the skeletal muscle (McPherron et al., Nature, 1997, 387:83-90). Similar increases in skeletal muscle mass are evident in naturally occurring mutations of GDF8 in cattle (Ashmore et al., 1974, Growth, 38:501-507; Swatland and Kieffer, J. Anim. Sci., 1994, 38:752-757; McPherron and Lee, Proc. Natl. Acad. Sci. USA, 1997, 94:12457-12461; and Kambadur et al., Genome Res., 1997, 7:910-915) and, strikingly,

in humans (Schuelke et al., N Engl J Med 2004;350:2682-8). Studies have also shown that muscle wasting associated with HIV-infection in humans is accompanied by increases in GDF8 protein expression (Gonzalez-Cadavid et al., PNAS, 1998, 95:14938-43). In addition, GDF8 can modulate the production of muscle-specific enzymes (e.g., creatine kinase) and modulate myoblast cell proliferation (WO 00/43781). The GDF8 propeptide can noncovalently bind to the mature GDF8 domain dimer, inactivating its biological activity (Miyazono et al. (1988) J. Biol. Chem., 263: 6407-6415; Wakefield et al. (1988) J. Biol. Chem., 263; 7646-7654; and Brown et al. (1990) Growth Factors, 3: 35-43). Other proteins which bind to GDF8 or structurally related proteins and inhibit their biological activity include follistatin, and potentially, follistatin-related proteins (Gamer et al. (1999) Dev. Biol., 208: 222-232).

The terms used in this specification generally have their ordinary meanings in the art, within the context of this invention and in the specific context where each term is used. Certain terms are discussed below or elsewhere in the specification, to provide additional guidance to the practitioner in describing the compositions and methods of the invention and how to make and use them. The scope or meaning of any use of a term will be apparent from the specific context in which the term is used.

"Homologous," in all its grammatical forms and spelling variations, refers to the relationship between two proteins that possess a "common evolutionary origin," including proteins from superfamilies in the same species of organism, as well as homologous proteins from different species of organism. Such proteins (and their encoding nucleic acids) have sequence homology, as reflected by their sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions.

The term "sequence similarity," in all its grammatical forms, refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin. However, in common usage and in the instant application, the term "homologous," when modified with an adverb such as "highly," may refer to sequence similarity and may or may not relate to a common evolutionary origin.

"Percent (%) sequence identity" or "percent (%) identical" with respect to a reference polypeptide (or nucleotide) sequence is defined as the percentage of amino acid residues (or nucleic acids) in a candidate sequence that are identical to the amino acid residues (or nucleic acids) in the reference polypeptide (nucleotide) 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, BLAST-2, ALIGN, EMBOSS Needle, or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid (nucleic acid) sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

"Agonize", in all its grammatical forms, refers to the process of activating a protein and/or gene (e.g., by activating or amplifying that protein's gene expression or by inducing an inactive protein to enter an active state) or increasing a protein's and/or gene's activity.

"Antagonize", in all its grammatical forms, refers to the process of inhibiting a protein and/or gene (e.g., by inhibiting or decreasing that protein's gene expression or by inducing an active protein to enter an inactive state) or decreasing a protein's and/or gene's activity.

The terms "about" and "approximately" as used in connection with a numerical value throughout the specification and the claims denotes an interval of accuracy, familiar and acceptable to a person skilled in the art.

Numeric ranges disclosed herein are inclusive of the numbers defining the ranges.

The terms "a" and "an" include plural referents unless the context in which the term is used clearly dictates otherwise. The terms "a" (or "an"), as well as the terms "one or more," and "at least one" can be used interchangeably herein. Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two or more specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to

encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

Throughout this specification, the word "comprise" or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or groups of integers but not the exclusion of any other integer or group of integers. As used herein, the term "comprises" also encompasses the use of the narrower terms "consisting" and "consisting essentially of."

The term "consisting essentially of" is limited to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the invention(s) disclosed herein.

The terms "polypeptide", "oligopeptide", "peptide" and "protein" are used interchangeably herein to refer to chains of amino acids of any length. The chain may be linear or branched, it may comprise modified amino acids, and/or may be interrupted by non-amino acids. The terms also encompass an amino acid chain that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. It is understood that the polypeptides can occur as single chains or associated chains.

The term "biologically active", in all its grammatical forms, and when used in the context of a follistatin polypeptide or variant or fragment thereof, refers to a polypeptide with the ability to bind a ligand from at least one of the (1) activin or (2) bone morphogenic protein (BMP) class of ligands. In some embodiments, a biologically active polypeptide or fragment thereof inhibits the activity of a ligand from at least one of the (1) activin or (2) bone morphogenic protein (BMP) class of ligands. In some embodiments, a biologically active follistatin polypeptide or variant or fragment thereof inhibits activin A or GDF-11 in a cell-based reporter gene assay with a lower IC50 than the IC50 of a follistatin polypeptide comprising the amino acid sequence of SEQ ID NO: 2. In some embodiments, a biologically active follistatin polypeptide or variant or fragment thereof inhibits activin A or GDF-11 in a cell-based reporter gene assay with an equal IC50 as compared to the IC50 of a follistatin polypeptide comprising the amino acid sequence of SEQ ID NO: 2. In some embodiments, a biologically active follistatin polypeptide or variant or fragment thereof binds to one or more

ligands selected from the group consisting of: myostatin, GDF-11, activin A and activin B with a KD less than 1 nM, 100 pM, 50 pM or 10 pM. In some embodiments, a biologically active follistatin polypeptide or variant or fragment thereof binds heparin with a greater affinity as compared to a follistatin polypeptide comprising the amino acid sequence of SEQ ID NO: 4. In some embodiments, a biologically active follistatin polypeptide or variant or fragment thereof binds heparin with an equal binding affinity to a follistatin polypeptide comprising the amino acid sequence of SEQ ID NO: 4.

2. Follistatin Polypeptides

In certain aspects, the disclosure relates to follistatin polypeptides (e.g., FST-Fc polypeptides). In some embodiments, the follistatin proteins are truncated forms exemplified by polypeptides comprising SEQ ID NO: 2, 7, 8, 9, 10, 11, 12, 13, 14, 15 or 16, and variants thereof. In some embodiments, any of the follistatin polypeptides, fragments, functional variants, and modified forms disclosed herein may have similar, the same or improved biological activities as compared to a wild-type follistatin polypeptide (*e.g.*, a polypeptide having the amino acid sequence of SEQ ID NO: 2 or 4). For example, in some embodiments, a follistatin variant of the disclosure may bind to and inhibit function of a follistatin ligand (e.g., activin A, activin AB, activin B, and GDF8). In some embodiments, a follistatin polypeptide modulates growth of tissues, particularly muscle. Examples of follistatin polypeptides include polypeptides comprising, consisting essentially of or consisting of the amino acid sequences by any of SEQ ID NOs: 1-16 and 26-43 or biologically active fragments thereof, as well as polypeptides comprising, consisting essentially of or consisting of amino acid sequences that are at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence of any of SEQ ID NOs: 1-16 and 26-43, or biologically active fragments thereof. Variations on these polypeptides may be prepared according to the following guidance. Unless stated otherwise, the numbering of amino acids in the follistatin polypeptides is based on the sequence of SEQ ID NO: 1, regardless of whether the native leader sequence is used. As described above, follistatin is characterized by three cysteine-rich regions (i.e., FS domains I-III) that are believed to mediate follistatin-ligand binding. Furthermore, researchers have demonstrated that polypeptide constructs comprising only one of the three FS-binding domains (e.g., FSDI) retains strong affinity towards certain follistatin-ligands (e.g., myostatin) and is biologically active *in vivo*. See Nakatani *et al.*, The FASEB Journal, Vol. 22477-487 (2008). Therefore, variant follistatin polypeptides of the

disclosure may comprise one or more active portions of a follistatin protein. For example, constructs of the disclosure may begin at a residue corresponding to amino acids 30-95 of SEQ ID NO: 3 and end at a position corresponding to amino acids 316-344 of SEQ ID NO: 3. Other examples include constructs that begin at a position from 30-95 of SEQ ID NO: 1 and end at a position corresponding to amino acids 164-167 or 238-244 of SEQ ID NO: 1. Others may include any of SEQ ID Nos. 7-16. Further examples include constructs that end at a position corresponding to an amino acid selected from the group consisting of the amino acid corresponding to amino acid 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, and 305 of SEQ ID NO: 4. In some embodiments, follistatin polypeptides and constructs of the disclosure may comprise follistatin polypeptides which do not include residues corresponding to the amino acids selected from the group consisting of amino acids 289-315, 290-315, 291-315, 292-315, 293-315, 294-315, 295-315, 296-315, 297-315, 298-315, 299-315, 300-315, 301-315, 302-315, 303-315, 304-315, and 305-315 of SEQ ID NO: 4.

Follistatin polypeptides of the disclosure may include any naturally occurring domain of a follistatin protein as well as variants thereof (e.g., mutants, fragments, and peptidomimetic forms) that retain a useful activity. For example, it is well-known that FST(315) and FST(288) have high affinity for both activin (activin A and activin B) and myostatin (and the closely related GDF11) and that the follistatin domains (e.g., FSN and FSD I-III) are thought to be involved in the binding of such TGF-β ligands. However, it believed that each of these three domains may have a different affinity for these TGF-β ligands. For example, a recent study has demonstrated that polypeptide constructs comprising only the N-terminal domain (FSN) and two FSDI domains in tandem retained high affinity for myostatin, demonstrated little or no affinity for activin and promoted systemic muscle growth when introduced into a mouse by gene expression (Nakatani *et al.*, The FASEB Journal, Vol. 22477-487 (2008)).

Additionally, the FSDI domain contains the heparin binding domain of human follistatin, which has the amino acid sequence of KKCRMNKKNKPR (SEQ ID NO: 5). This heparin binding domain can be represented as BBXBXXBBXBXB (SEQ ID NO:6) wherein "B" means a basic amino acid, particularly lysine (K) or arginine (R). Accordingly, the present disclosure encompasses, in part, variant follistatin proteins that demonstrate selective binding and/or inhibition of a given TGF-β ligand relative to the naturally occurring FST protein (e.g., maintaining high-affinity for myostatin while having a significantly reduced affinity for activin).

In certain aspects, the disclosure includes polypeptides comprising the FSN domain, as set forth below, and, for example, one or more heterologous polypeptide, and moreover, it will be appreciated that any of the initial amino acids G or N, prior to the first cysteine may be deleted, as in the example shown below (SEQ ID NO:8).

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP NCIPCKET (SEQ ID NO:7)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC IPCKET (SEQ ID NO:8)

In certain aspects, the disclosure includes polypeptides comprising the FSDI domain which contains the minimal core activities of myostatin (and/or GDF11) binding along with heparin binding as set forth below, and, for example, one or more heterologous polypeptide.

CENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCK EQPELEVQYQGRC (SEQ ID NO:9)

An FSDI sequence may be advantageously maintained in structural context by expression as a polypeptide further comprising the FSN domain. Accordingly, the disclosure includes polypeptides comprising the FSN-FSDI sequence, as set forth below (SEQ ID NO:10), and, for example, one or more heterologous polypeptide, and moreover, it will be appreciated that any of the initial amino acids G or N, prior to the first cysteine may be removed by processing or intentionally eliminated without any consequence, and polypeptides comprising such slightly smaller polypeptides are further included.

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL LKARCKEQPELEVQYQGRC (SEQ ID NO:10)

As demonstrated by Nakani et al., an FSN-FSDI-FSDI construct is sufficient to confer systemic muscle growth when genetically expressed in a mouse, and accordingly the disclosure includes polypeptides comprising the amino acid sequences below and, for example, one or more heterologous polypeptide.

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL LKARCKEQPELEVQYQGRCKKTCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKG PVCGLDGKTYRNECALLKARCKEQPELEVQYQGRC (SEQ ID NO:11)

The FSDI sequence confers myostatin and GDF11 binding. It has been demonstrated that activins, particularly activin A but also activin B, are also negative regulators of muscle, and therefore a follistatin polypeptide that inhibits both the myostatin/GDF11 group and the activin A/activin B group may provide a more potent muscle effect. Moreover, in view of the findings herein demonstrating the low systemic availability of certain follistatin polypeptides, particularly those comprising a heparin binding domain, and more particularly in a homodimeric form, such as an Fc fusion, safety concerns associated with the known effects of activin inhibition on the reproductive axis and other tissues are alleviated. Given that FSDII confers activin A and B binding, the disclosure provides polypeptides comprising FSDI and FSDII (SEQ ID NO: 12), as well as FSN-FSDI-FSDII constructs (SEQ ID NOS: 13) and, for example, one or more heterologous polypeptide.

CENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCK
EQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCG
NDGVTYSSACHLRKATCLLGRSIGLAYEGKC (SEQ ID NO:12)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKC (SEQ ID NO:13)

As described in the Examples, a follistatin polypeptide of 291 amino acids (representing a truncation of the naturally occurring FST-315) has advantageous properties. Accordingly, unprocessed (SEQ ID NO: 14) and mature FST(291) (SEQ ID NO: 15) polypeptides are included in the disclosure and may be combined with heterologous proteins. Moreover, it will be appreciated that any of the initial amino acids G or N, prior to the first cysteine may be removed by processing or intentionally eliminated without any consequence, and polypeptides comprising such slightly smaller polypeptides are further included, such as the example shown below (SEQ ID NO:16).

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTEL
SKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDC
GPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARC
KEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQC

TGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEA
ACSSGVLLEVKHSGSCNSIS (SEQ ID NO:14)

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCL
WDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNSIS (SEQ ID NO:15)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWD
FKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSC
NSIS (SEQ ID NO:16)

In certain embodiments, the present invention relates to antagonizing a ligand of follistatin (also referred to as a follistatin ligand) with a subject follistatin polypeptide (e.g., an FST-IgG fusion polypeptide). Thus, compositions and methods of the present disclosure are useful for treating disorders associated with abnormal activity of one or more ligands of follistatin. Exemplary ligands of follistatin include some TGF-β family members, such as activin A, activin B, myostatin (GDF8) and GDF11.

The follistatin variations described herein may be combined in various ways with each other or with heterologous amino acid sequences. For example, variant follistatin proteins of the disclosure include polypeptides that comprise one or more FS domains selected from FSDI (amino acids 95-164 of SEQ ID NO: 1 (i.e., SEQ ID NO: 2), FSDII (amino acids 168-239 of SEQ ID NO: 1), or FSDIII (amino acids 245-316 of SEQ ID NO: 1) as well as proteins that comprise one or more FS domains selected from a sequence at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to FSDI (amino acids 95-164 of SEQ ID NO: 1 (i.e., SEQ ID NO: 2)), FSDII (amino acids 168-239 of SEQ ID NO: 1), or

FSDIII (amino acids 245-316 of SEQ ID NO: 1). These FS domains may be combined in any order within a variant follistatin polypeptide of the disclosure provided that such recombinant proteins maintain the desired activity including, for example, follistatin ligand-binding activity (e.g., myostatin) and biological activity (e.g., inducing muscle mass and/or strength). Examples of such follistatin variant polypeptides include, for example, polypeptides having domain structures such as FSDI-FSDII-FSDIII, FSDI-FSDIII, FSDI-FSDI-FSDIII, FSDI-FSDII, FSDI-FSDI, FSN-FSDI-FSDII-FSDIII, FSN-FSDI-FSDII, FSN-FSDI-FSDI, FSN-FSDI-FSDIII, FSN-FSDI-FSDI-FSDIII, and polypeptides obtained by fusing other heterologous polypeptides to the N-termini or the C-termini of these polypeptides. These domains may be directly linked or liked via a linker polypeptide. Optionally, polypeptide linkers may be any sequence and may comprise 1-50, preferably 1-10, and more preferably 1-5 amino acids. In certain aspects, preferred linkers contain no cysteine amino acids.

As referenced herein, "follistatin variants" includes follistatin polypeptides that are fragments and/or mutants/modified polypeptides as compared to a reference wildtype follistatin protein (*e.g.*, a follistatin protein having the amino acid sequence of any of SEQ ID NOs: 1-4). In some embodiments, follistatin variants of the disclosure have reduced or abolished binding affinity for one or more follistatin ligands as compared to a wildtype follistatin polypeptide (*e.g.*, a polypeptide having the amino acid sequence of SEQ ID NO: 4). In certain aspects, the disclosure provides follistatin variants that have reduced or abolished binding affinity for activin as compared to a wildtype follistatin polypeptide (*e.g.*, a polypeptide having the amino acid sequence of SEQ ID NO: 4). In certain aspects, the disclosure provides follistatin variants that have reduced or abolished binding affinity for activin but retain high affinity for myostatin as compared to a wildtype follistatin polypeptide (*e.g.*, a polypeptide having the amino acid sequence of SEQ ID NO: 4). In certain aspects, the disclosure provides follistatin variants that have reduced or abolished binding affinity for GDF11 as compared to a wildtype follistatin polypeptide (*e.g.*, a polypeptide having the amino acid sequence of SEQ ID NO: 4).

In some embodiments, follistatin fragments or variants of the disclosure have increased binding affinity for heparin. In some embodiments, follistatin fragments or variants of the disclosure have a binding affinity for heparin which is equivalent to the binding affinity of a follistatin polypeptide comprising SEQ ID NO: 2. In some embodiments, follistatin fragments or variants have a binding affinity for heparin that is at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the binding affinity for heparin of a follistatin

polypeptide comprising SEQ ID NO: 2. In some embodiments, follistatin fragments or variants of the disclosure have a binding affinity for heparin which is greater than the binding affinity of a follistatin polypeptide comprising SEQ ID NO: 2. In some embodiments, follistatin fragments or variants of the disclosure have a binding affinity for heparin which is greater than the binding affinity of a follistatin polypeptide comprising SEQ ID NO: 4. In some embodiments, follistatin fragments or variants of the disclosure have an unmasked heparin binding domain. In some embodiments, follistatin fragments or variants of the disclosure comprise a heparin binding domain which comprises the endogenous follistatin heparin binding sequence of SEQ ID NO: 5. In some embodiments, follistatin fragments or variants of the disclosure comprise a heterologous heparin binding sequence.

In certain aspects, the disclosure provides follistatin fragments or variants that do not comprise a sequence corresponding to the FSDII domain or functionally active FSDII domain. For example, follistatin polypeptides of the disclosure may include a variant obtained through partial or complete deletion of the FSDII domain. In certain aspects, such follistatin variants include the deletion of one or more cysteine residues within the FSDII region or substitution with non-cysteine amino acids.

The follistatin proteins of the disclosure may comprise a signal sequence. The signal sequence can be a native signal sequence of a follistatin protein (e.g., amino acids 1-29 of SEQ ID NO:1) or a signal sequence from another protein, such as tissue plasminogen activator (TPA) signal sequence or a honey bee melatin (HBM) signal sequence. In some embodiments, the signal sequence is removed during processing of the follistatin protein.

Further N-linked glycosylation sites (N-X-S/T) may be added to a follistatin polypeptide, and may increase the serum half-life of an FST-Fc fusion protein. N-X-S/T sequences may be generally introduced at positions outside the ligand-binding pocket. N-X-S/T sequences may be introduced into the linker between the follistatin sequence and the Fc or other fusion component. Such a site may be introduced with minimal effort by introducing an N in the correct position with respect to a pre-existing S or T, or by introducing an S or T at a position corresponding to a pre-existing N. Any S that is predicted to be glycosylated may be altered to a T without creating an immunogenic site, because of the protection afforded by the glycosylation. Likewise, any T that is predicted to be glycosylated may be altered to an S. Accordingly, a follistatin variant may include one or more additional, non-endogenous N-linked glycosylation consensus sequences.

In certain embodiments, the present disclosure contemplates making functional variants by modifying the structure of a follistatin polypeptide for such purposes as enhancing therapeutic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Modified follistatin polypeptides can also be produced, for instance, by amino acid substitution, deletion, or addition. For instance, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (e.g., conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Whether a change in the amino acid sequence of a follistatin polypeptide results in a functional homolog can be readily determined by assessing the ability of the variant follistatin polypeptide to produce a response in cells in a fashion similar to the wild-type follistatin polypeptide, or to bind to one or more ligands, such as activin or myostatin in a fashion similar to wild-type follistatin.

In certain embodiments, the present invention contemplates specific mutations of the follistatin polypeptides so as to alter the glycosylation of the polypeptide. Such mutations may be selected so as to introduce or eliminate one or more glycosylation sites, such as O-linked or N-linked glycosylation sites. Asparagine-linked glycosylation recognition sites generally comprise a tripeptide sequence, asparagine-X-threonine (where "X" is any amino acid) which is specifically recognized by appropriate cellular glycosylation enzymes. The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the wild-type follistatin polypeptide (for O-linked glycosylation sites). A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Another means of increasing the number of carbohydrate moieties on a follistatin polypeptide is by chemical or enzymatic coupling of glycosides to the follistatin polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine; (b) free carboxyl groups; (c) free sulfhydryl groups such as those of cysteine; (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in

Aplin and Wriston (1981) CRC Crit. Rev. Biochem., pp. 259-306, incorporated by reference herein. Removal of one or more carbohydrate moieties present on an ActRIIB polypeptide may be accomplished chemically and/or enzymatically. Chemical deglycosylation may involve, for example, exposure of the follistatin polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the amino acid sequence intact. Chemical deglycosylation is further described by Hakimuddin et al. (1987) Arch. Biochem. Biophys. 259:52 and by Edge et al. (1981) Anal. Biochem. 118:131. Enzymatic cleavage of carbohydrate moieties on follistatin polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. (1987) Meth. Enzymol. 138:350. The sequence of a follistatin polypeptide may be adjusted, as appropriate, depending on the type of expression system used, as mammalian, yeast, insect and plant cells may all introduce differing glycosylation patterns that can be affected by the amino acid sequence of the peptide. In some embodiments, follistatin proteins for use in humans will be expressed in a cell line (*e.g.*, a mammalian cell line) that provides proper glycosylation, such as HEK293 or CHO cell lines, although other expression cell lines are expected to be useful as well.

This disclosure further contemplates a method of generating variants, particularly sets of combinatorial variants of an follistatin polypeptide, including, optionally, truncation variants; pools of combinatorial mutants are especially useful for identifying functional variant sequences. The purpose of screening such combinatorial libraries may be to generate, for example, follistatin polypeptide variants that have altered properties, such as altered pharmacokinetics, or altered ligand binding as compared to a wildtype follistatin polypeptide (*e.g.*, a polypeptide having the amino acid sequence of SEQ ID NO: 2 or 4). A variety of screening assays are provided below, and such assays may be used to evaluate variants. For example, a follistatin polypeptide variant may be screened for its ability to bind to a follistatin ligand, and/or to prevent binding of a follistatin ligand to a follistatin polypeptide.

The activity of a follistatin polypeptide or its variants may also be tested in a cell-based or *in vivo* assay. For example, the effect of a follistatin polypeptide variant on the expression of genes involved in muscle production may be assessed. This may, as needed, be performed in the presence of one or more recombinant follistatin ligand proteins (e.g., activin A), and cells may be transfected so as to produce a follistatin polypeptide and/or variants thereof, and optionally, a follistatin ligand. Likewise, a follistatin polypeptide may be

administered to a mouse or other animal, and one or more muscle properties, such as muscle mass or strength may be assessed. In some embodiments, any of the follistatin polypeptides disclosed herein may be administered to an animal model of muscle contractures, and the effects of the follistatin polypeptide on the animal model may be assessed (*see, e.g.*, Example 8). Such assays are either described in the application or are well known and routine in the art. A responsive reporter gene may be used in such cell lines to monitor effects on downstream signaling.

Combinatorially-derived variants can be generated which have a selective potency relative to a naturally occurring follistatin polypeptide. Such variant proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding a wild-type follistatin polypeptide. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other processes which result in destruction of, or otherwise inactivation of a native follistatin polypeptide. Such variants, and the genes which encode them, can be utilized to alter follistatin polypeptide levels by modulating the half-life of the follistatin polypeptides. For instance, a short half-life can give rise to more transient biological effects and, when part of an inducible expression system, can allow tighter control of recombinant follistatin polypeptide levels within the cell.

In certain embodiments, the follistatin polypeptides of the disclosure may further comprise post-translational modifications in addition to any that are naturally present in the follistatin polypeptides. Such modifications include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. As a result, the modified follistatin polypeptides may contain non-amino acid elements, such as polyethylene glycols, lipids, poly- or mono-saccharide, and phosphates. Effects of such non-amino acid elements on the functionality of a follistatin polypeptide may be tested as described herein for other follistatin polypeptide variants. When a follistatin polypeptide is produced in cells by cleaving a nascent form of the follistatin polypeptide, post-translational processing may also be important for correct folding and/or function of the protein. Different cells (such as CHO, HeLa, MDCK, 293, WI38, NIH-3T3 or HEK293) have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the follistatin polypeptides.

In certain aspects, functional variants or modified forms of the follistatin polypeptides include fusion proteins having at least a portion of a follistatin polypeptide and one or more fusion domains. Well known examples of such fusion domains include, but are not limited to, polyhistidine, Glu-Glu, glutathione S transferase (GST), thioredoxin, protein A, protein G, an immunoglobulin heavy chain constant region (e.g., an Fc), maltose binding protein (MBP), or human serum albumin. A fusion domain may be selected so as to confer a desired property. For example, some fusion domains are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt-conjugated resins are used. Many of such matrices are available in "kit" form, such as the Pharmacia GST purification system and the QIAexpress™ system (Qiagen) useful with (HIS_6) fusion partners. As another example, a fusion domain may be selected so as to facilitate detection of the follistatin polypeptides. Examples of such detection domains include the various fluorescent proteins (e.g., GFP) as well as "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated proteins can then be isolated from the fusion domain by subsequent chromatographic separation. In certain preferred embodiments, a follistatin polypeptide is fused with a domain that stabilizes the follistatin polypeptide *in vivo* (a "stabilizer" domain). By "stabilizing" is meant anything that increases serum half-life, regardless of whether this is because of decreased destruction, decreased clearance by the kidney, or other pharmacokinetic effect. Fusions with the Fc portion of an immunoglobulin are known to confer desirable pharmacokinetic properties on a wide range of proteins. Likewise, fusions to human serum albumin can confer desirable properties. Other types of fusion domains that may be selected include multimerizing (e.g., dimerizing, tetramerizing) domains and functional domains (that confer an additional biological function, such as further stimulation of muscle growth).

As specific examples, the present disclosure provides fusion proteins comprising follistatin polypeptides fused to a polypeptide comprising a heterologous moiety/domain. In some embodiments, the heterologous moiety is serum albumin. In some embodiments, the

heterologous moiety is a constant domain of an immunoglobulin, such as a CH1, CH2 or CH3 domain of an immunoglobulin or an Fc. Fc domains derived from human IgG1 and IgG2 are provided below (SEQ ID NO: 17 and SEQ ID NO: 18, respectively). As described herein, an IgG2, IgG4 or IgG2/4 Fc domain is particularly advantageous for fusion with follistatin polypeptides that retain heparin binding activity because these Fc species have reduced CDC and/or ADCC activity which may be harmful to the cells to which these heparin binding polypeptides may adhere. Other mutations are known that decrease either CDC or ADCC activity, and collectively, any of these variants are included in the disclosure and may be used as advantageous components of a follistatin fusion protein. In some embodiments, any of the follistatin polypeptides disclosed herein is conjugated to an Fc domain comprising an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 17, or fragments thereof. In some embodiments, any of the follistatin polypeptides disclosed herein is conjugated to an Fc domain comprising an amino acid sequence that is at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 18, or fragments thereof. Optionally, the Fc domain of SEQ ID NO: 17 (or variant or fragment thereof) has one or more mutations at residues such as Asp-265, Lys-322, and Asn-434 (numbered in accordance with the corresponding full-length IgG1). In certain cases, the mutant Fc domain having one or more of these mutations (e.g., Asp-265 mutation) has reduced ability of binding to the Fcγ receptor relative to a wildtype Fc domain. In other cases, the mutant Fc domain having one or more of these mutations (e.g., Asn-434 mutation) has increased ability of binding to the MHC class I-related Fc-receptor (FcRN) relative to a wildtype Fc domain.

Examples of human IgG1 and IgG2 amino acid sequences that may be employed are shown below:

IgG1

THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
(SEQ ID NO:17)

IgG2

VECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDG
VEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISK
TKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK (SEQ
ID NO:18)

It is understood that different elements of the fusion proteins may be arranged in any manner that is consistent with the desired functionality. For example, a follistatin polypeptide may be placed C-terminal to a heterologous moiety/domain, or, alternatively, a heterologous moiety/domain may be placed C-terminal to a follistatin polypeptide. The follistatin polypeptide domain and the heterologous domain need not be adjacent in a fusion protein, and additional domains or amino acid sequences may be included C- or N-terminal to either domain or between the domains. In some embodiments, the follistatin polypeptide is conjugated directly to the heterologous moiety/domain. In other embodiments, the follistatin polypeptide is conjugated to the heterologous moiety/domain by means of a linker. In some embodiments, the linker is a glycine, threonine and/or serine rich linker. Other near neutral amino acids, such as, but not limited to, Asn, Pro and Ala, may also be used in the linker sequence. In some embodiments, the linker comprises various permutations of amino acid sequences containing Gly and Thr. In some embodiments, the linker comprises various permutations of amino acid sequences containing Gly and Ser. In some embodiments, the linker has a length of at least 3, 4, 5, 7, 10, 12, 15, 20, 21, 25, 30, 35, 40, 45 or 50 amino acids. In some embodiments, the linker comprises GlyGlyGly (GGG) (SEQ ID NO: 48), or repetitions thereof. In some embodiments, the linker comprises the amino acid sequence of ThrGlyGlyGly (TGGG) (SEQ ID NO: 49) or repetitions thereof. In some embodiments, the linker is 1-5, 1-10 or 1-15 amino acids in length. In some embodiments, the linker consists of ThrGlyGlyGly (TGGG) (SEQ ID NO: 49). In some embodiments, the linker is greater than 10 amino acids in length. 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 an amino acid sequence that is at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to GAPGGGGGAAAAAGGGGGGAP (SEQ ID NO: 50) or fragments thereof. In some embodiments, the linker comprises an amino acid sequence that is at least 80%, 85%, 90%,

95%, 96%, 97%, 98%, 99% or 100% identical to GAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAP (SEQ ID NO: 51), or fragments thereof. In some embodiments, the linker comprises a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identical to GAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGGGAPGGGGGAAAAAGGGGG GAP (SEQ ID NO: 52), or fragments thereof. In some embodiments, the linker does not consist of or comprise the amino acid sequence of ALEVLFQGP (SEQ ID NO: 53).

As used herein, the term "immunoglobulin Fc domain" or simply "Fc" is understood to mean the carboxyl-terminal portion of an immunoglobulin chain constant region, preferably an immunoglobulin heavy chain constant region, or a portion thereof. For example, an immunoglobulin Fc region may comprise 1) a CH1 domain, a CH2 domain, and a CH3 domain, 2) a CH1 domain and a CH2 domain, 3) a CH1 domain and a CH3 domain, 4) a CH2 domain and a CH3 domain, or 5) a combination of two or more domains and an immunoglobulin hinge region. In a preferred embodiment the immunoglobulin Fc region comprises at least an immunoglobulin hinge region a CH2 domain and a CH3 domain, and preferably lacks the CH1 domain. It is also understood that a follistatin polypeptide may comprise only a domain of an immunoglobulin, such as a CH1 domain, a CH2 domain or a CH3 domain. Many of these domains confer desirable pharmacokinetic properties as well as dimerization or higher order multimerization.

In one embodiment, the class of immunoglobulin from which the heavy chain constant region is derived is IgG (Igγ) (γ subclasses 1, 2, 3, or 4). Other classes of immunoglobulin, IgA (Igα), IgD (Igδ), IgE (Igε) and IgM (Igμ), may be used. The choice of appropriate immunoglobulin heavy chain constant region is discussed in detail in U.S. Pat. Nos. 5,541,087 and 5,726,044. The choice of particular immunoglobulin heavy chain constant region sequences from certain immunoglobulin classes and subclasses to achieve a particular result is considered to be within the level of skill in the art. In certain embodiments, the constant domain of an IgG immunoglobulin has reduced or no substantial ADCC and/or CDC activity relative to native human IgG1. The portion of the DNA construct encoding the immunoglobulin Fc region preferably comprises at least a portion of a hinge domain, and preferably at least a portion of a CH_3 domain of Fc gamma or the homologous domains in any of IgA, IgD, IgE, or IgM.

Furthermore, it is contemplated that substitution or deletion of amino acids within the immunoglobulin heavy chain constant regions may be useful in the practice of the methods and compositions disclosed herein. One example would be to introduce amino acid substitutions in the upper CH2 region to create an Fc variant with reduced affinity for Fc receptors (Cole *et al.* (1997) J. Immunol. 159:3613). Additionally, in many instances, the C-terminal lysine, or K, will be removed and thus any of the polypeptides described herein may omit the C-terminal K that is found in an Fc domain, such as those shown in SEQ ID NO: 17 or SEQ ID NO: 18.

In certain embodiments, the final (carboxy-terminal) lysine, or K, of the follistatin polypeptide is absent. For example, the protein may comprise an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NOs: 42 or 43, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 or 43, respectively, is optionally absent.

In certain embodiments, the follistatin polypeptides of the present disclosure contain one or more modifications that are capable of stabilizing the follistatin polypeptides. For example, such modifications enhance the *in vitro* half-life of the follistatin polypeptides, enhance circulatory half-life of the follistatin polypeptides or reducing proteolytic degradation of the follistatin polypeptides. Such stabilizing modifications include, but are not limited to, fusion proteins (including, for example, fusion proteins comprising a follistatin polypeptide and a stabilizer domain), modifications of a glycosylation site (including, for example, addition of a glycosylation site to a follistatin polypeptide), and modifications of carbohydrate moiety (including, for example, removal of carbohydrate moieties from a follistatin polypeptide). In the case of fusion proteins, a follistatin polypeptide is fused to a stabilizer domain such as an IgG molecule (e.g., an Fc domain). As used herein, the term "stabilizer domain" not only refers to a fusion domain (e.g., Fc) as in the case of fusion proteins, but also includes nonproteinaceous modifications such as a carbohydrate moiety, or nonproteinaceous polymer, such as polyethylene glycol.

In certain embodiments, the present invention makes available isolated and/or purified forms of the follistatin polypeptides, which are isolated from, or otherwise substantially free of, other proteins.

In certain embodiments, follistatin polypeptides (unmodified or modified) of the disclosure can be produced by a variety of art-known techniques. For example, such follistatin polypeptides can be synthesized using standard protein chemistry techniques such as those described in Bodansky, M. Principles of Peptide Synthesis, Springer Verlag, Berlin (1993) and Grant G. A. (ed.), Synthetic Peptides: A User's Guide, W. H. Freeman and Company, New York (1992). In addition, automated peptide synthesizers are commercially available (e.g., Advanced ChemTech Model 396; Milligen/Biosearch 9600). Alternatively, the follistatin polypeptides, fragments or variants thereof may be recombinantly produced using various expression systems (e.g., E. coli, Chinese Hamster Ovary cells, COS cells, baculovirus) as is well known in the art (also see below). In a further embodiment, the modified or unmodified follistatin polypeptides may be produced by digestion of naturally occurring or recombinantly produced full-length follistatin polypeptides by using, for example, a protease, e.g., trypsin, thermolysin, chymotrypsin, pepsin, or paired basic amino acid converting enzyme (PACE). Computer analysis (using a commercially available software, e.g., MacVector, Omega, PCGene, Molecular Simulation, Inc.) can be used to identify proteolytic cleavage sites. Alternatively, such follistatin polypeptides may be produced from naturally occurring or recombinantly produced full-length follistatin polypeptides such as standard techniques known in the art, such as by chemical cleavage (e.g., cyanogen bromide, hydroxylamine).

3. Nucleic Acids Encoding Follistatin Polypeptides

In certain aspects, the invention provides isolated and/or recombinant nucleic acids encoding any of the follistatin polypeptides disclosed herein. The subject nucleic acids may be single-stranded or double stranded. Such nucleic acids may be DNA or RNA molecules. These nucleic acids are may be used, for example, in methods for making follistatin polypeptides.

For example, the following sequence encodes a naturally occurring human follistatin precursor polypeptide (SEQ ID NO: 19) (NCBI Accession Number BC004107.2, 1032 bp):

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atggtccgcgcgaggcaccagccgggtgggctttgcctcctgctgctgctgctctg
ccagttcatggaggaccgcagtgcccaggctgggaactgctggctccgtcaagcga
agaacggccgctgccaggtcctgtacaagaccgaactgagcaaggaggagtgctgc
agcaccggccggctgagcacctcgtggaccgaggaggacgtgaatgacaacacact
cttcaagtggatgattttcaacgggggcgcccccaactgcatcccctgtaaagaaa
cgtgtgagaacgtggactgtggacctgggaaaaaatgccgaatgaacaagaagaac
aaaccccgctgcgtctgcgccccggattgttccaacatcacctggaagggtccagt
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ctgcgggctggatgggaaaacctaccgcaatgaatgtgcactcctaaaggcaagat
gtaaagagcagccagaactggaagtccagtaccaaggcagatgtaaaaagacttgt
cgggatgttttctgtccaggcagctccacatgtgtggtggaccagaccaataatgc
ctactgtgtgacctgtaatcggatttgcccagagcctgcttcctctgagcaatatc
tctgtgggaatgatggagtcacctactccagtgcctgccacctgagaaaggctacc
tgcctgctgggcagatctattggattagcctatgagggaaagtgtatcaaagcaaa
gtcctgtgaagatatccagtgcactggtgggaaaaaatgtttatgggatttcaagg
ttgggagaggccggtgttccctctgtgatgagctgtgccctgacagtaagtcggat
gagcctgtctgtgccagtgacaatgccacttatgccagcgagtgtgccatgaagga
agctgcctgctcctcaggtgtgctactggaagtaaagcactccggatcttgcaact
ccatttcggaagacaccgaggaagaggaggaagatgaagaccaggactacagcttt
cctatatcttctattctagagtgg

The following sequence encodes the mature FST(315) polypeptide (SEQ ID NO: 20).

gggaactgctggctccgtcaagcgaagaacggccgctgccaggtcctgtacaagacc
gaactgagcaaggaggagtgctgcagcaccggccggctgagcacctcgtggaccgag
gaggacgtgaatgacaacacactcttcaagtggatgattttcaacgggggcgccccc
aactgcatcccctgtaaagaaacgtgtgagaacgtggactgtggacctgggaaaaaa
tgccgaatgaacaagaagaacaaaccccgctgcgtctgcgccccggattgttccaac
atcacctggaagggtccagtctgcgggctggatgggaaaacctaccgcaatgaatgt
gcactcctaaaggcaagatgtaaagagcagccagaactggaagtccagtaccaaggc
agatgtaaaaagacttgtcgggatgttttctgtccaggcagctccacatgtgtggtg
gaccagaccaataatgcctactgtgtgacctgtaatcggatttgcccagagcctgct
tcctctgagcaatatctctgtgggaatgatggagtcacctactccagtgcctgccac
ctgagaaaggctacctgcctgctgggcagatctattggattagcctatgagggaaag
tgtatcaaagcaaagtcctgtgaagatatccagtgcactggtgggaaaaaatgttta
tgggatttcaaggttgggagaggccggtgttccctctgtgatgagctgtgccctgac
agtaagtcggatgagcctgtctgtgccagtgacaatgccacttatgccagcgagtgt
gccatgaaggaagctgcctgctcctcaggtgtgctactggaagtaaagcactccgga
tcttgcaactccatttcggaagacaccgaggaagaggaggaagatgaagaccaggac
tacagctttcctatatcttctattctagagtgg

The following sequence encodes the FST(288) polypeptide (SEQ ID NO: 21).

gggaactgctggctccgtcaagcgaagaacggccgctgccaggtcctgtacaagacc
gaactgagcaaggaggagtgctgcagcaccggccggctgagcacctcgtggaccgag
gaggacgtgaatgacaacacactcttcaagtggatgattttcaacgggggcgccccc
aactgcatcccctgtaaagaaacgtgtgagaacgtggactgtggacctgggaaaaaa
tgccgaatgaacaagaagaacaaaccccgctgcgtctgcgccccggattgttccaac
atcacctggaagggtccagtctgcgggctggatgggaaaacctaccgcaatgaatgt
gcactcctaaaggcaagatgtaaagagcagccagaactggaagtccagtaccaaggc
agatgtaaaaagacttgtcgggatgttttctgtccaggcagctccacatgtgtggtg
gaccagaccaataatgcctactgtgtgacctgtaatcggatttgcccagagcctgct
tcctctgagcaatatctctgtgggaatgatggagtcacctactccagtgcctgccac
ctgagaaaggctacctgcctgctgggcagatctattggattagcctatgagggaaag
tgtatcaaagcaaagtcctgtgaagatatccagtgcactggtgggaaaaaatgttta
tgggatttcaaggttgggagaggccggtgttccctctgtgatgagctgtgccctgac
agtaagtcggatgagcctgtctgtgccagtgacaatgccacttatgccagcgagtgt
gccatgaaggaagctgcctgctcctcaggtgtgctactggaagtaaagcactccgga
tcttgcaac

The following sequence encodes the mature FST(291) polypeptide (SEQ ID NO: 22).

```
gggaactgctggctccgtcaagcgaagaacggccgctgccaggtcctgtacaagacc
gaactgagcaaggaggagtgctgcagcaccggccggctgagcacctcgtggaccgag
gaggacgtgaatgacaacacactcttcaagtggatgattttcaacgggggcgccccc
aactgcatcccctgtaaagaaacgtgtgagaacgtggactgtggacctgggaaaaaa
tgccgaatgaacaagaagaacaaaccccgctgcgtctgcgccccggattgttccaac
atcacctggaagggtccagtctgcgggctggatgggaaaacctaccgcaatgaatgt
gcactcctaaaggcaagatgtaaagagcagccagaactggaagtccagtaccaaggc
agatgtaaaaagacttgtcgggatgttttctgtccaggcagctccacatgtgtggtg
gaccagaccaataatgcctactgtgtgacctgtaatcggatttgcccagagcctgct
tcctctgagcaatatctctgtgggaatgatggagtcacctactccagtgcctgccac
ctgagaaaggctacctgcctgctgggcagatctattggattagcctatgagggaaag
tgtatcaaagcaaagtcctgtgaagatatccagtgcactggtgggaaaaaatgttta
tgggatttcaaggttgggagaggccggtgttccctctgtgatgagctgtgccctgac
agtaagtcggatgagcctgtctgtgccagtgacaatgccacttatgccagcgagtgt
gccatgaaggaagctgcctgctcctcaggtgtgctactggaagtaaagcactccgga
tcttgcaactccatttcgtgg
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In certain aspects, the subject nucleic acids encoding follistatin polypeptides are further understood to include nucleic acids that are variants of SEQ ID NOs: 19-22. Variant nucleotide sequences include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants; and will, therefore, include coding sequences that differ from the nucleotide sequence of the coding sequence designated in SEQ ID NOs: 19-22.

In certain embodiments, the disclosure provides isolated or recombinant nucleic acid sequences that are at least 80%, 85%, 90%, 95%, 96% 97%, 98%, 99%, or 100% identical to SEQ ID NOs: 19-22, and particularly those portions thereof that are derived from follistatin (nucleotides corresponding to amino acids 95-164 of SEQ ID NO:1). One of ordinary skill in the art will appreciate that nucleic acid sequences complementary to SEQ ID NOs: 19-22, and variants of SEQ ID NO: 19-22 are also within the scope of this disclosure. In further embodiments, the nucleic acid sequences of the disclosure can be isolated, recombinant, and/or fused with a heterologous nucleotide sequence, or in a DNA library.

In other embodiments, nucleic acids of the invention also include nucleotide sequences that hybridize under highly stringent conditions to the nucleotide sequence designated in SEQ ID NOs: 19-22, complement sequence of SEQ ID NOs: 19-22, or fragments thereof (e.g., nucleotides 19-22).

One of ordinary skill in the art will understand readily that appropriate stringency conditions that promote DNA hybridization can be varied. One of ordinary skill in the art

will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids that differ from the nucleic acids as set forth in SEQ ID NOs: 19-22 due to degeneracy in the genetic code are also within the scope of the disclosure. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations that do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this disclosure.

In certain embodiments, the recombinant nucleic acids of the disclosure may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate to the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the disclosure. The promoters may be either naturally occurring promoters, or hybrid promoters that

combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

In certain aspects, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding a follistatin polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the follistatin polypeptide. Accordingly, the term regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology*: *Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding a follistatin polypeptide. Such useful expression control sequences, include, for example, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, RSV promoters, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

A recombinant nucleic acid of the disclosure can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant follistatin polypeptide include plasmids and other vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-

derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

Some mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and in transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant polypeptides by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

In certain embodiments, a vector will be designed for production of the subject follistatin polypeptides in CHO cells, such as a Pcmv-Script vector (Stratagene, La Jolla, Calif.), pcDNA4 vectors (Invitrogen, Carlsbad, Calif.) and pCI-neo vectors (Promega, Madison, Wisc.). As will be apparent, the subject gene constructs can be used to cause expression of the subject follistatin polypeptides in cells propagated in culture, e.g., to produce proteins, including fusion proteins or variant proteins, for purification.

This disclosure also pertains to a host cell transfected with a recombinant gene including a coding sequence (e.g., SEQ ID NOs: 19-22) for one or more of the subject follistatin polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, a follistatin polypeptide of the disclosure may be expressed in bacterial cells such as

E. coli, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present disclosure further pertains to methods of producing the subject follistatin polypeptides. For example, a host cell transfected with an expression vector encoding a follistatin polypeptide can be cultured under appropriate conditions to allow expression of the follistatin polypeptide to occur. The follistatin polypeptide may be secreted and isolated from a mixture of cells and medium containing the follistatin polypeptide. Alternatively, the follistatin polypeptide may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The subject follistatin polypeptides can be isolated from cell culture medium, host cells, or both, using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the follistatin polypeptides. In a preferred embodiment, the follistatin polypeptide is a fusion protein containing a domain that facilitates its purification.

In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant follistatin polypeptide, can allow purification of the expressed fusion protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified follistatin polypeptide (e.g., see Hochuli et al., (1987) *J. Chromatography* 411:177; and Janknecht et al., *PNAS USA* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be

annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

4. Exemplary Therapeutic Uses

In certain embodiments, any of the follistatin polypeptides disclosed herein, including for example FST(288)-IgG1, FST(288)-IgG2, FST(291)-IgG1, FST(291)-IgG2 (*e.g.*, a polypeptide comprising the amino acid sequence of SEQ ID NO: 42 or 43), FST(315)-IgG1, FST(315)-IgG2, can be used for treating or preventing any of the diseases or conditions disclosed herein, including diseases or disorders that are associated with muscle contractures (e.g., Arthrogryposis multiplex congenita). These diseases, disorders or conditions are generally referred to herein as "disorders associated with muscle contractures." In certain embodiments, the present disclosure provides methods of treating or preventing an individual in need thereof by administering to the individual a therapeutically effective amount of a follistatin polypeptide as described herein. These methods are particularly aimed at therapeutic and prophylactic treatments of animals, and more particularly, humans.

As used herein, a therapeutic that "prevents" a disorder or condition refers to a compound that, in a statistical sample, reduces the occurrence of the disorder or condition in the treated sample relative to an untreated control sample, or delays the onset or reduces the severity of one or more symptoms of the disorder or condition relative to the untreated control sample.

The terms "treatment", "treating", "alleviation" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect, and may also be used to refer to improving, alleviating, and/or decreasing the severity of one or more symptoms of a condition being treated. The effect may be prophylactic in terms of completely or partially delaying the onset or recurrence of a disease, condition, or symptoms thereof, and/or may be therapeutic in terms of a partial or complete cure for a disease or condition and/or adverse effect attributable to the disease or condition. "Treatment" as used herein covers any treatment of a disease or condition of a mammal, particularly a human, and includes: (a) preventing the disease or condition from occurring in a subject which may be predisposed to the disease or condition but has not yet been diagnosed as having it; (b) inhibiting the disease or condition (e.g., arresting its development); or (c) relieving the

disease or condition (e.g., causing regression of the disease or condition, providing improvement in one or more symptoms).

The terms "patient", "subject", or "individual" are used interchangeably herein and refer to either a human or a non-human animal. These terms include mammals, such as humans, non-human primates, laboratory animals, livestock animals (including bovines, porcines, camels, etc.), companion animals (e.g., canines, felines, other domesticated animals, etc.) and rodents (e.g., mice and rats). In particular embodiments, the patient, subject or individual is a human. In some embodiments, the subject is a fetus. In some embodiments, the subject is an infant. In some embodiments, the subject is a juvenile. In some embodiments, the subject is an adult.

Muscle contractures are generally associated with a limited range of motion in the joint and muscles which appear functionally short. Muscle contractures may be caused by congenital conditions in utero or by non-congenital conditions which arise in infants, juveniles, or adults. Congenital muscle contractures are caused by numerous conditions which either prevent or restrict movement during the terminal stages of muscle differentiation in utero (e.g. fetal vascular compromise). Non-congenital muscle contractures arise from multiple conditions which either prevent or restrict movement for extensive periods of time (e.g. stroke). Some common characteristics found in muscle contractures include, but are not limited to decreased flexibility of the joints, muscle weakness, shortening of a muscle, and/or shortening of a joint. Thus, disorders associated with muscle contractures include congenital or non-congenital conditions which result in tightening or shortening of muscles, tendons, ligaments or other tissue, leading to restriction of normal movement. In some embodiments, any of the methods of the disclosure treats or prevents of the symptoms associated with muscle contractures, including any of the symptoms described herein.

The disclosure provides methods of treating or preventing muscle contractures associated with various congenital or non-congenital conditions, by administering to a subject an effect amount of a protein, wherein the protein comprises a follistatin polypeptide, or a biologically active fragment thereof, including a follistatin polypeptide of the foregoing, which may be collectively referred to herein as "therapeutic agents." In some embodiments, the disclosure provides methods of treating or preventing a muscle contracture that is associated with a neurological disease of the brain, spine, or peripheral nerve. Also provided are methods and compositions for treating muscle contractures associated with connective

tissue defects, muscle abnormalities, dysplasia of the brainstem nuclei, dysplasia of the spinal cord, dysgenesis of the nervous system as observed in chromosomal abnormalities, or Arthrogryposis multiplex congenital. In addition, the disclosure provides methods and compositions for treating or preventing congenital contractures.

In some embodiments, compositions (e.g., FST-Fc polypeptides) of the invention are useful for treating or preventing muscle contractures associated with a neurological disease of the brain, spine, or peripheral nerve. Examples of disorders of this kind include, but are not limited to multiple sclerosis, poliomyelitis, Parkinson's disease, dementia, Cerebral Palsy, dysplasia of the brainstem nuclei, or dysplasia of the spinal cord. Further examples include, but are not limited to Mobius syndrome, Pierre-Robin syndrome, prune belly syndrome, and Zellweger syndrome.

Other examples of conditions include muscle contractures associated with an inflammatory disorder (e.g. rheumatoid arthritis), a connective tissue defect (e.g. diastrophic dysplasia), and muscle abnormalities (e.g. muscular dystrophy or mitochondrial abnormalities).

In certain embodiments, compositions (e.g., FST-Fc polypeptides) of the invention are used as part of a treatment for muscle contractures associated with muscular dystrophy. The term "muscular dystrophy" refers to a group of degenerative muscle diseases characterized by gradual weakening and deterioration of skeletal muscles and sometimes the heart and respiratory muscles. Muscular dystrophies are genetic disorders characterized by progressive muscle wasting and weakness that begin with microscopic changes in the muscle. As muscles degenerate over time, the person's muscle strength declines. Examples of muscular dystrophies that can be treated with a regimen including the subject follistatin polypeptides include: Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Emery-Dreifuss Muscular Dystrophy (EDMD), Limb-Girdle Muscular Dystrophy (LGMD), Facioscapulohumeral Muscular Dystrophy (FSH or FSHD) (also known as Landouzy-Dejerine), Myotonic Dystrophy (MMD) (also known as Steinert's Disease), Oculopharyngeal Muscular Dystrophy (OPMD), Distal Muscular Dystrophy (DD), Congenital Muscular Dystrophy (CMD).

Duchenne Muscular Dystrophy (DMD) was first described by the French neurologist Guillaume Benjamin Amand Duchenne in the 1860s. Becker Muscular Dystrophy (BMD) is named after the German doctor Peter Emil Becker, who first described this variant of DMD

in the 1950s. DMD is one of the most frequent inherited diseases in males, affecting one in 3,500 boys. DMD occurs when the dystrophin gene, located on the short arm of the X chromosome, is broken. Since males only carry one copy of the X chromosome, they only have one copy of the dystrophin gene. Without the dystrophin protein, muscle is easily damaged during cycles of contraction and relaxation. While early in the disease muscle compensates by regeneration, later on muscle progenitor cells cannot keep up with the ongoing damage and healthy muscle is replaced by non-functional fibro-fatty tissue.

BMD results from different mutations in the dystrophin gene. BMD patients have some dystrophin, but it is either insufficient in quantity or poor in quality. Having some dystrophin protects the muscles of those with BMD from degenerating as badly or as quickly as those of people with DMD.

Recent research demonstrates that blocking or eliminating function of GDF8 (a follistatin ligand) *in vivo* can effectively treat at least certain symptoms in DMD and BMD patients. Thus, the subject follistatin polypeptides may act as GDF8 inhibitors (antagonists), and constitute an alternative means of blocking the functions of GDF8 *in vivo* in DMD and BMD patients.

Similarly, the subject follistatin polypeptides provide an effective means to increase muscle mass in other disease conditions that are in need of muscle growth. For example, effective therapies for muscle contractures promote longitudinal muscle growth in the localized muscle.

In still other embodiments, any of the follistatin polypeptides disclosed herein may be useful in the treatment or prevention of congenital contractures. As used herein, the term "congenital contractures" refers to the development of abnormally short or stiff skeletal muscle fibers at birth. The formation of congenital muscle contractures has a common pathophysiological association (decreased limb movement during development) and a characteristic pathological phenotype (short muscle components and long tendon components) of the muscle tendon unit (MTU). Approximately 1% of all live births show some sort of contracture, which can range from single-joint immobility (such as clubfoot) to severe contractures of several limb joints (such as arthrogryposis multiplex congenita (AMC)). Rink BD. Obst. & Gyn. Survey 2011;66(6):369-377. Congenital contractures or conditions include, but are not limited to congenital contractures as a result of uterine space limitations from when the subject was in utero. Examples of disorders leading to space

limitations within the uterus include, but are not limited to, oligohydramnios, fibroids, uterine malformations, and multiple pregnancy. Further congenital contractures or conditions include, but are not limited to congenital contractures as a result of intrauterine or fetal vascular compromise from when the subject was in utero (e.g., impaired development of nerves or anterior horn cell death), maternal disease (e.g., diabetes mellitus, multiple sclerosis, myasthenia gravis, infection, drug usage, or trauma), Arthrogryposis multiplex congenital (e.g., amyoplasia, distal arthrogryposis, a systemic connective tissue disorder, multiple pterygium syndromes, or fetal crowding), and isolated congenital contracture.

In still other embodiments, compositions (e.g., FST-Fc polypeptides) of the invention are used as part of a treatment for muscle contractures associated with dysgenesis of the nervous system as observed in chromosomal abnormalities, such as trisomy 18 or trisomy 21.

Muscle contractures of patients with a post-surgical or disuse atrophy of one or muscles may be treated with the follistatin polypeptides disclosed herein including atrophy after: Hip Fracture; Total Hip Arthroplasty (THA); Total Knee Arthroplasty (TKA) or Rotator Cuff surgery. Further disuse atrophy contractures or conditions include, but are not limited to, muscle contractures associated with being bedbound, chronic illness, serious injury, head injury, or stroke.

Any of the follistatin polypeptides of the disclosure can be administered to the subject alone, or in combination with one or more agents or therapeutic modalities, e.g., therapeutic agents, which are useful for treating muscle contractures associated with a neurological disease of the brain, spine, or peripheral nerve. In certain embodiments, if the disease is caused by or associated with aberrant inflammation or aberrant immune system function, the method comprises administering any of the follistatin polypeptides disclosed herein in combination with a second agent or therapeutic modality, wherein the second agent or therapeutic modality is chosen from one or more of: glatiramer acetate, interferon-β, mitoxantrone, natalizumab, inhibitors of MMPs including inhibitor of MMP-9 and MMP-2, short-acting β2-agonists, long-acting β2-agonists, anticholinergics, corticosteroids, systemic corticosteroids, mast cell stabilizers, leukotriene modifiers, methylxanthines, β2-agonists, albuterol, levalbuterol, pirbuterol, artformoterol, formoterol, salmeterol, anticholinergics including ipratropium and tiotropium; corticosteroids including beclomethasone, budesonide, fluticasone, mometasone, triamcinolone, methyprednisolone, prednisolone; prednisone; leukotriene modifiers including montelukast, zafirlukast, and zileuton; mast cell stabilizers

including cromolyn and nedocromil; methylxanthines including theophylline; combination drugs including ipratropium and albuterol, fluticasone and salmeterol, budesonide and formoterol; antihistamines including hydroxyzine, diphenhydramine, loratadine, cetirizine, and hydrocortisone; immune system modulating drugs including tacrolimus and pimecrolimus; cyclosporine; azathioprine; mycophenolatemofetil; and combinations thereof.

Any of the follistatin polypeptides of the disclosure can be administered to the subject alone, or in combination with one or more therapeutic modalities, which are useful for treating muscle contractures. In certain embodiments, the therapy comprises physical therapy using passive range of motion and splinting before contractures occur or after contractures have occurred. In certain embodiments, the therapy comprises daily standing and/or walking. In certain embodiments, the therapy comprises passive stretching of muscles and joints. In certain embodiments, the therapy comprises positioning the limbs to promote extension and oppose flexion. In certain embodiments, the therapy comprises splinting the affected region. Examples of splinting include ankle-foot orthotics, long leg knee-ankle-foot orthoses, nighttime resting splints, daytime splints, and serial night casting. In certain embodiments, the therapy comprises surgical correction. Examples of surgeries include tendo achilles lengthening, posterior tibialis lengthening, and other surgeries which improve the range of motion or positioning of an affected region. In certain embodiments, the follistatin polypeptides of the disclosure can be administered prior to, during, or following surgical correction of the muscle contracture. In certain embodiments, the therapy comprises massage of the affected region.

In certain embodiments, the therapy comprises stretch therapy. In certain embodiments, administration of the follistatin polypeptide in combination with stretch therapy provides at least 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater shortening of the tendon as compared to stretch therapy in combination with vehicle. In certain embodiments, the administration of the protein in combination with stretch therapy provides 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% decrease in tendon length. In certain embodiments, the therapy comprises electrostimulation therapy. In certain embodiments, administration of the follistatin polypeptide in combination with stretch therapy provides at least 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater

shortening of the tendon as compared to stretch therapy in combination with electrostimulation therapy.

Any of the follistatin polypeptides of the disclosure and any of the co-therapeutic agents or co-therapies of the disclosure can be administered in the same formulation or separately. In the case of separate administration, the follistatin polypeptide can be administered before, after, or concurrently with the co-therapeutic or co-therapy. One agent may precede or follow administration of the other agent by intervals ranging from minutes to weeks. In embodiments where two or more different kinds of therapeutic agents are applied separately to a subject, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that these different kinds of agents would still be able to exert an advantageously combined effect on the target tissues or cells.

In certain embodiments, compositions (e.g., FST-Fc polypeptides) of the invention are administered parenterally. Examples of methods of parental administration include administering an effective amount of the protein by an intramuscular route of administration to a targeted muscle of the subject. As exemplified herein, the polypeptides of the disclosure can be locally injected by intramuscular injection to increase muscle mass and strength of the localized muscle. In other embodiments, compositions of the invention are administered intravenously, orally, or subcutaneously. In certain embodiments, compositions of the invention are administered systemically or locally. In other embodiments, compositions of the invention are not administered systemically. In still other embodiments, compositions of the invention do not have a systemic effect when administered intramuscularly.

5. Pharmaceutical Compositions

In certain embodiments, compounds (e.g., any of the follistatin polypeptides disclosed herein) of the present invention are formulated with a pharmaceutically acceptable carrier. For example, a follistatin polypeptide can be administered alone or as a component of a pharmaceutical formulation (i.e., a therapeutic composition). The subject compounds may be formulated for administration in any convenient way for use in human or veterinary medicine.

In certain embodiments, the therapeutic method of the invention includes administering the composition topically, systemically, or locally as an implant or device. When administered, the therapeutic composition for use in this invention is preferably in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be

encapsulated or injected in a viscous form for delivery to a target tissue site (e.g., bone, cartilage, muscle, fat or neurons), for example, a site having tissue damage. Topical administration may be suitable for wound healing and tissue repair. Therapeutically useful agents other than the follistatin polypeptides, which may also optionally be included in the composition as described above, may alternatively or additionally, be administered simultaneously or sequentially with the subject compounds (e.g., follistatin polypeptides) in the methods of the invention.

In certain embodiments, compositions of the present invention may include a matrix capable of delivering one or more therapeutic compounds (e.g., follistatin polypeptides) to a target tissue site, providing a structure for the developing tissue and optimally capable of being resorbed into the body. For example, the matrix may provide slow release of the follistatin polypeptides. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the subject compositions will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid and polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are non-biodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above-mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

In certain embodiments, methods of the invention can be administered for orally, e.g., in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of an

agent as an active ingredient. An agent may also be administered as a bolus, electuary or paste.

In solid dosage forms for oral administration (capsules, tablets, pills, dragees, powders, granules, and the like), one or more therapeutic compounds of the present invention may be mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose, and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds; (7) wetting agents, such as cetyl alcohol and glycerol monostearate; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and (10) coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming, and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol, and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Certain compositions disclosed herein may be administered topically, either to skin or to mucosal membranes. The topical formulations may further include one or more of the wide variety of agents known to be effective as skin or stratum corneum penetration enhancers. Examples of these are 2-pyrrolidone, N-methyl-2-pyrrolidone, dimethylacetamide, dimethylformamide, propylene glycol, methyl or isopropyl alcohol, dimethyl sulfoxide, and azone. Additional agents may further be included to make the formulation cosmetically acceptable. Examples of these are fats, waxes, oils, dyes, fragrances, preservatives, stabilizers, and surface active agents. Keratolytic agents such as those known in the art may also be included. Examples are salicylic acid and sulfur.

Dosage forms for the topical or transdermal administration include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches, and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants that may be required. The ointments, pastes, creams and gels may contain, in addition to a subject compound of the invention (e.g., a follistatin polypeptide), excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to a subject compound, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

In certain embodiments, pharmaceutical compositions suitable for parenteral administration may comprise one or more follistatin polypeptides in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and

injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

In some embodiments, any of the polypeptides disclosed herein may be administered parenterally. Exemplary methods of parental administration include administering an effective amount of the protein by an intramuscular route of administration to a targeted muscle of the subject. As exemplified herein, the polypeptides of the disclosure can be locally injected by intramuscular injection to increase muscle mass and strength of the localized muscle.

The compositions of the invention may also contain adjuvants, such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents that delay absorption, such as aluminum monostearate and gelatin.

It is understood that the dosage regimen will be determined by the attending physician, considering various factors that modify the action of the subject compounds of the invention (e.g., follistatin polypeptides). The various factors will depend upon the disease to be treated.

In certain embodiments, the present invention also provides gene therapy for the *in vivo* production of follistatin polypeptides or other compounds disclosed herein. Such therapy would achieve its therapeutic effect by introduction of the follistatin polynucleotide sequences into cells or tissues having the disorders as listed above. Delivery of follistatin polynucleotide sequences can be achieved using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system. Preferred for therapeutic delivery of follistatin polynucleotide sequences is the use of targeted liposomes.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited

to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. Retroviral vectors can be made target-specific by attaching, for example, a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody. Those of skill in the art will recognize that specific polynucleotide sequences can be inserted into the retroviral genome or attached to a viral envelope to allow target specific delivery of the retroviral vector containing the follistatin polynucleotide. In one preferred embodiment, the vector is targeted to bone, cartilage, muscle or neuron cells/tissues.

Alternatively, tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes gag, pol and env, by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Another targeted delivery system for follistatin polynucleotides is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome. Liposomes are artificial membrane vesicles which are useful as delivery vehicles in vitro and in vivo. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (see e.g., Fraley, et al., Trends Biochem. Sci., 6:77, 1981). Methods for efficient gene transfer using a liposome vehicle, are known in the art, see e.g., Mannino, et al., Biotechniques, 6:682, 1988. The composition of the liposome is usually a combination of phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and

distearoylphosphatidylcholine. The targeting of liposomes is also possible based on, for example, organ-specificity, cell-specificity, and organelle-specificity and is known in the art.

EXEMPLIFICATION

The invention now being generally described will be more readily understood by reference to the following examples, which are included merely for purposes of illustrating certain embodiments of the present invention. These examples are not intended to limit the invention.

Example 1: Generation of Follistatin-Fc Proteins

Follistatin (FST) is known to have complex pharmacokinetic behavior. The short form FST(288) is reported to be more effective at blocking ligands and binds to cell surfaces in part due to its unmasked heparin binding domain. FST(315) is thought to be less effective but less attracted to cell surfaces due to the acid rich C-terminal amino acid sequence, which neutralizes the heparin binding domain. In the literature, follistatin is generally reported as having systemic effects. Experiments were designed to determine whether a follistatin construct could be produced that would tend to have effects in the tissue of administration (such as an injected muscle), and whether dimerization of follistatin would provide enhanced tissue retention. The Fc domains of immunoglobulins are known to form dimers. To explore the effects of follistatin-Fc fusion proteins on muscle and other tissues, and to evaluate the effects of Fc-mediated dimerization on the pharmacokinetic properties of follistatin polypeptides, fusion proteins containing FST(288) or FST(315) fused to an Fc portion of an IgG1 were generated. A TGGG linker sequence was selected to join each follistatin polypeptide to the Fc portion.

For each FST-IgG1 construct, the following three leader sequences were considered:

(1) Follistatin leader: MVRARHQPGGLCLLLLLLCQFMEDRSAQA (SEQ ID NO: 23)

(2) Tissue plasminogen activator (TPA): MDAMKRGLCCVLLLCGAVFVSP (SEQ ID NO: 24)

(3) Honey bee melittin (HBML): MKFLVNVALVFMVVYISYIYA (SEQ ID NO: 25)

The selected FST-Fc proteins incorporate the follistatin leader. The FST(288)-IgG1 fusion has the unprocessed and mature amino acid sequences shown below.

Unprocessed FST(288)-IgG1 (SEQ ID NO:26)

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCS
TGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKP
RCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDV
FCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLG
RSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCA
SDNATYASECAMKEAACSSGVLLEVKHSGSCNTGGGTHTCPPCPAPELLGGPSVFLF
PPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREE
MTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK
SRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Mature FST(288)-IgG1 (SEQ ID NO:27)

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCL
WDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPE
VKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
APIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQ
PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK

The initial "GN" sequence may be removed, yielding the following polypeptide. (SEQ ID NO: 28)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWD
FKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSC
NTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAP
IEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK

The FST(315)-IgG1 fusion has the unprocessed and mature amino acid sequences shown below.

Unprocessed FST(315)-IgG1 (SEQ ID NO: 29)

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCS
TGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKP
RCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDV
FCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLG
RSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCA
SDNATYASECAMKEAACSSGVLLEVKHSGSCNSISEDTEEEEEDEDQDYSFPISSIL
EWTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS
PGK

Mature FST(315)-IgG1 (SEQ ID NO: 30)

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCL
WDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNSISEDTEEEEEDEDQDYSFPISSILEWTGGGTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
VSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMT
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The initial "GN" sequence may be removed, yielding the following polypeptide. (SEQ ID NO: 31)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWD
FKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSC
NSISEDTEEEEEDEDQDYSFPISSILEWTGGGTHTCPPCPAPELLGGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVS
VLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQ
QGNVFSCSVMHEALHNHYTQKSLSLSPGK

Proteins were expressed in HEK-293 cells or CHO cells and purified from conditioned media by filtration and protein A chromatography. In some instances anion exchange and hydrophobic interaction chromatography and/or gel filtration was also used.

Protein activity was assessed by binding to activin A or GDF11. In each case, the proteins bind with a K_D of less than 10 pM.

Example 2: The Effect of Systemic Administration of Follistatin-Fc Proteins on Muscle Mass and Strength in Mice

The ability of follistatin-Fc proteins to increase muscle mass and strength in wild-type mice was determined after systemic administration. An ActRIIB-Fc fusion protein that is well-known to stimulate substantial whole-body increases in lean muscle mass was used as a positive control.

C57BL/6 mice were dosed (10 mg/kg; subcutaneously (s.c.)) twice/week for four weeks with the FST(288)-IgG1 protein, the human FST(315)-IgG1 protein, or the human ActRIIB-Fc protein. Mice were subjected to whole-body nuclear magnetic resonance (NMR) scanning to determine the percent change of whole body lean tissue mass. ActRIIB-Fc treated mice exhibited a significant (approximately 35%) increase in lean tissue when compared to the vehicle-control group. Mice treated with either the FST(288)-IgG1 or FST(315)-IgG1 protein exhibited little increase in lean tissue mass compared to the control cohort. See Figure 2. At the end of the study, pectoralis, tibialis anterior (TA), gastrocnemius, and femoris muscles were dissected and weighed. As shown in Figure 4, ActRIIB-Fc treatment significantly increased muscle mass in each of these muscle groups. In contrast, little to no increase in muscle mass was observed in either the FST(288)-IgG1 or FST(315)-IgG1 treatment groups. See Figure 2.

During the course of this study, mice were also examined for changes in muscle strength. The force a mouse exerts when pulling a force transducer is measured to determine forelimb grip strength. Mice treated with the ActRIIB-Fc protein exhibited increased muscle strength. In contrast, there was no increase in grip strength observed in either the FST(288)-IgG1 or FST(315)-IgG1 treatment groups. See Figure 3.

Together, the results confirm that systemic administration of ActRIIB-Fc profoundly increases both muscle mass and strength in mice when compared to vehicle-control animals. In contrast, there was little to no increase in muscle mass or strength observed in mice treated with either the follistatin-Fc fusion protein FST(288)-IgG1 or FST(315)-IgG1. Therefore, it appears that follistatin-Fc fusions proteins have little or no effect on muscle mass or strength *in vivo* when administered systemically.

Example 3: The Effect of Systemic Administration of Follistatin-Fc Proteins on FSH levels.

Follistatin is primarily characterized for its ability to bind and inhibit members of the TGF-beta superfamily of signaling proteins. In particular, follistatin is known to be a potent

inhibitor of activin activity. Activin is a potent inducer of follicle-stimulating hormone (FSH) production. FSH is synthesized and secreted by gonadotrophs of the anterior pituitary gland and regulates growth and development during pubertal maturation and various reproductive processes in the body. To assess systemic effects of follistatin-Fc polypeptides, effects on FSH levels were evaluated.

Treatment (10 mg/kg; subcutaneously (s.c.) twice/week) with FST(288)-IgG1 resulted in circulating levels of the drug at 3.836 (± 5.22) μg/mL. Similar treatment with FST(315)-IgG1 resulted in substantially higher serum levels of the drug at 19.31 (± 1.85) μg/mL. As indicated in Figure 5, FST(288)-IgG1 did not have any significant effects on serum levels of FSH, suggesting that this FST(288)-IgG1 treatment regime does not significantly affect systemic activin activity. In contrast, FST(315)-IgG1 treatment resulted in a decrease in circulating levels of FSH, indicating that systemic administration of FST(315)-IgG1 has an effect on systemic activin signaling. Overall, these data indicate that use of a follistatin polypeptide with an unmasked heparin binding domain, fused to an Fc domain that mediates dimerization, such as FST(288)-IgG1 results in a protein that has little or no systemic activity, while an FST(315)-IgG1, with a masked heparin binding domain, may be used to achieve systemic effects.

Example 4: The Effect of Local Administration of Follistatin-Fc Proteins on Muscle Mass and Strength in Mice

While there were no significant effects after systemic administration, a similar experimental approach was used to determine if follistatin can be used to locally increase muscle mass and strength in wild-type mice after intramuscular (i.m.) administration.

C57BL/6 mice were dosed (50 micrograms; i.m. into the right gastrocnemius muscle) twice/week for four weeks with the FST(288)-Fc protein, the FST(315)-Fc protein, or the human ActRIIB-Fc protein. At various time points after initial treatment, mice were subjected to whole-body nuclear magnetic resonance (NMR) scanning to determine the percent change of whole body lean tissue mass. As shown in Figure 6, ActRIIB-Fc treated mice exhibited a significant increase in lean tissue when compared to the vehicle-control group. In contrast, neither mice treated with the FST(288)-Fc nor FST(315)-Fc protein exhibited a significant increase in lean tissue mass compared to the control cohort. At the end of the study, both the right, injected gastrocnemius muscle and the left, contralateral gastrocnemius muscle were dissected and weighed. As shown in Figure 7, ActRIIB-Fc treatment significantly increased muscle mass in both the right and left gastrocnemius

muscles in comparison to vehicle-treated mice. Therefore, ActRIIB-Fc has systemic effects on increasing muscle mass even when restricted to local administration in a single muscle. In contrast, both FST(288)-Fc and FST(315)-Fc resulted in significant increases in muscle mass of the right gastrocnemius muscle but had no effect on the mass of the contralateral muscle. Therefore, contrary to the effects observed after systemic administration, it appears that follistatin protein is a potent stimulator of muscle mass when directly administered into a muscle. Furthermore, follistatin appears to have a distinct advantage over other agents like ActRIIB-Fc in that its effects on muscle mass are localized to the site of administration, indicating that follistatin can be used for targeted therapy of a selected muscle, or muscle groups, without affecting the normal growth/activity of surrounding, non-targeted muscles.

The serum levels of follistatin-Fc fusion protein were closely monitored after i.m. administration. Treatment with FST(288)-IgG1 resulted in a circulating levels of the drug at 0.156 (± 0.245) μg/mL. Similar treatment with FST(315)-IgG1 resulted in slightly higher serum levels of the drug at 3.58 (± 1.73) μg/mL, but these levels were substantially lower than those observed after systemic administration of FST(315)-IgG1. As both FST(288)-IgG1 and FST(315)-IgG1 circulate in patient serum at lower levels after i.m. injection than is observed after systemic administration of FST(288)-IgG1 (i.e., 3.836 (± 5.22) μg/mL), neither FST(288)-IgG1 nor FST(315)-IgG1 would be expected to have significant effects on serum levels of FSH as FST(288)-IgG1 had no such effect after s.c. administration. See Figure 5. Accordingly, these data indicate that both FST(288)-IgG1 and FST(315)-IgG1 would be particularly well-suited for promoting targeted muscle growth in patients that are reproductively active or have a desire to minimize effects on the reproductive system.

A similar experiment was conducted to establish a dose-response curve of the effects of FST(288)-IgG1 on muscle mass and quality. C57BL/6 mice were dosed with varying amounts (1 to 100 micrograms); i.m. into the right gastrocnemius muscle twice/week for four weeks. As shown in Figure 8, the selective increase in the muscle mass of the injected muscle versus the contralateral muscle was greater with greater doses of FST(288)-IgG1. Muscle cross sections revealed the enhanced muscle mass to be the result of muscle fiber hypertrophy, rather than hypoplasia.

Example 5: Fc Optimization of Locally-Acting Follistatin-Fc Fusion Proteins

As described in the preceding Examples, follistatin-Fc fusion proteins such as FST(288)-IgG1 and FST(315)-IgG1 have poor systemic effects on muscle and other tissues,

and particularly FST(288) forms of the protein are active at the site of injection. FST(288) binds to cells by virtue of the heparin binding domain and this binding can be eliminated by exogenous heparin. As a consequence, immunoglobulin domains known to mediate CDC and ADCC effects on targeted cells may cause damage to cells treated with the heparin-binding follistatin constructs. Such damage could manifest as an immune reaction in the targeted tissue or in decreased growth of the targeted tissue. Therefore versions of follistatin polypeptides were generated employing the Fc portion of human IgG2, which is an example of an IgG constant domain that is known to have diminished capability to stimulate CDC and ADCC activity. This experiment was conducted to ascertain whether follistatin-Fc fusion proteins using alternative Fc domains would retain activity.

FST(288) or FST(315) were fused to an Fc portion of an IgG2 to generate fusion proteins. A TGGG linker sequence was selected to join each follistatin polypeptide to the Fc portion.

For each FST-IgG2 construct, the follistatin leader was employed.

The FST(288)-IgG2 fusion has the unprocessed and mature amino acid sequences shown below.

Unprocessed FST(288)-IgG2 (SEQ ID NO: 32)

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCS
TGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKP
RCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDV
FCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLG
RSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCA
SDNATYASECAMKEAACSSGVLLEVKHSGSCNTGGGVECPPCPAPPVAGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRV
VSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMT
KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSR
WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Which is encoded by the following nucleic acid sequence (SEQ ID NO: 44)

atggtccgcgcgaggcaccagccgggtgggctttgcctcctgctgctgctctgccagttcatggaggaccgcagtgcccaggct
gggaactgctggctccgtcaagcgaagaacggccgctgccaggtcctgtacaagaccgaactgagcaaggaggagtgctgcagca
ccggccggctgagcacctcgtggaccgaggaggacgtgaatgacaacacactcttcaagtggatgattttcaacgggggcgccccc
aactgcatcccctgtaaagaaacgtgtgagaacgtggactgtggacctgggaaaaaatgccgaatgaacaagaagaacaaaccccg
ctgcgtctgcgccccggattgttccaacatcacctggaagggtccagtctgcgggctggatgggaaaacctaccgcaatgaatgtgca
ctcctaaaggcaagatgtaaagagcagccagaactggaagtccagtaccaaggcagatgtaaaaagacttgtcgggatgttttctgtc

caggcagctccacatgtgtggtggaccagaccaataatgcctactgtgtgacctgtaatcggatttgcccagagcctgcttcctctgagc
aatatctctgtgggaatgatggagtcacctactccagtgcctgccacctgagaaaggctacctgcctgctgggcagatctattggattag
cctatgagggaaagtgtatcaaagcaaagtcctgtgaagatatccagtgcactggtgggaaaaaatgtttatgggatttcaaggttggg
agaggccggtgttccctctgtgatgagctgtgccctgacagtaagtcggatgagcctgtctgtgccagtgacaatgccacttatgccag
cgagtgtgccatgaaggaagctgcctgctcctcaggtgtgctactggaagtaaagcactccggatcttgcaacaccggtggtggagtc
gagtgcccaccgtgcccagcaccacctgtggcaggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctccc
ggacccctgaggtcacgtgcgtggtggtggacgtgagccacgaagaccccgaggtccagttcaactggtacgtggacggcgtgga
ggtgcataatgccaagacaaagccacgggaggagcagttcaacagcacgttccgtgtggtcagcgtcctcaccgtcgtgcaccagg
actggctgaacggcaaggagtacaagtgcaaggtctccaacaaaggcctcccagccccatcgagaaaaccatctccaaaaccaaa
gggcagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaaccaggtcagcctgacctgcctg
gtcaaaggcttctaccccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacacctccca
tgctggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctc
cgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaatgagaattc

Mature FST(288)-IgG2 (SEQ ID NO: 33)

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCL
WDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNTGGGVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
FNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAP
IEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK

The initial "GN" sequence may be removed, yielding the following polypeptide. (SEQ ID NO: 34)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWD
FKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSC
NTGGGVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFN
WYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIE
KTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
YKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

The FST(315)-IgG2 fusion has the unprocessed and mature amino acid sequences shown below.

Unprocessed FST(315)-IgG2 (SEQ ID NO: 35)

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCS
TGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKP
RCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDV
FCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLG
RSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCA
SDNATYASECAMKEAACSSGVLLEVKHSGSCNSISEDTEEEEEDEDQDYSFPISSIL
EWTGGGVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQF
NWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPI
EKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPEN
NYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG
K

Which is encoded by the following nucleic acid sequence (SEQ ID NO: 45)

atggtccgcgcgaggcaccagccgggtgggctttgcctcctgctgctgctctgccagttcatggaggaccgcagtgc
ccaggctgggaactgctggctccgtcaagcgaagaacggccgctgccaggtcctgtacaagaccgaactgagcaaggaggagtgc
tgcagcaccggccggctgagcacctcgtggaccgaggaggacgtgaatgacaacacactcttcaagtggatgattttcaacgggggt
gcccccaactgcatcccctgtaaagaaacgtgtgagaacgtggactgtggacctgggaaaaaatgccgaatgaacaagaagaacaa
accccgctgcgtctgcgccccggattgttccaacatcacctggaagggtccagtctgcgggctggatgggaaaacctaccgcaatga
atgtgcactcctaaaggcaagatgtaaagagcagccagaactggaagtccagtaccaaggcagatgtaaaaagacttgtcgggatgtt
ttctgtccaggcagctccacatgtgtggtggaccagaccaataatgcctactgtgtgacctgtaatcggatttgcccagagcctgcttcct
ctgagcaatatctctgtgggaatgatggagtcacctactccagtgcctgccacctgagaaaggctacctgcctgctgggcagatctatt
ggattagcctatgagggaaagtgtatcaaagcaaagtcctgtgaagatatccagtgcactggtgggaaaaaatgtttatgggatttcaag
gttgggagaggccggtgttccctctgtgatgagctgtgccctgacagtaagtcggatgagcctgtctgtgccagtgacaatgccacttat
gccagcgagtgtgccatgaaggaagctgcctgctcctcaggtgtgctactggaagtaaagcactccggatcttgcaactccatttcgga
agacaccgaggaagaggaggaagatgaagaccaggactacagctttcctatatcttctattctagagtggaccggtggtggagtcga
gtgcccaccgtgcccagcaccacctgtggcaggaccgtcagtcttcctcttcccccaaaacccaaggacaccctcatgatctcccgg
acccctgaggtcacgtgcgtggtggtggacgtgagccacgaagaccccgaggtccagttcaactggtacgtggacggcgtggaggt
gcataatgccaagacaaagccacgggaggagcagttcaacagcacgttccgtgtggtcagcgtcctcaccgtcgtgcaccaggact
ggctgaacggcaaggagtacaagtgcaaggtctccaacaaaggcctcccagcccccatcgagaaaaccatctccaaaaccaaagg
gcagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaaccaggtcagcctgacctgcctggtc
aaaggcttctaccccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaagaccacacctcccatgc
tggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgt
gatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaatgagaattc

Mature FST(315)-IgG2 (SEQ ID NO: 36)

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCL
WDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNSISEDTEEEEEDEDQDYSFPISSILEWTGGGVECPPCPAPPVAGPSVFLFPPKP
KDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVS
VLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQ
QGNVFSCSVMHEALHNHYTQKSLSLSPGK

The initial "GN" sequence may be removed, yielding the following polypeptide. (SEQ ID NO: 37)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWD
FKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSC
NSISEDTEEEEEDEDQDYSFPISSILEWTGGGVECPPCPAPPVAGPSVFLFPPKPKD
TLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVL
TVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQV
SLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQG
NVFSCSVMHEALHNHYTQKSLSLSPGK

Proteins were expressed in HEK-293 cells or CHO cells and purified from conditioned media by filtration and protein A chromatography. In some instances anion exchange and hydrophobic interaction chromatography and/or gel filtration was also used.

Protein activity was assessed by binding to activin A or GDF11. In each case, the proteins bind with a K_D of less than 10 pM. These data indicate that Follistatin-IgG2 fusion proteins can be generated and expressed and retain picomolar ligand binding activity.

Example 6: Optimized Locally-Acting Follistatin-Fc Fusion Proteins

To assess whether an optimal follistatin-Fc fusion protein could be generated, a variety of truncations between the C-terminus of FST(288) and FST(315) were generated. One of these truncations, ending at amino acid 291 and termed FST(291) showed superior expression properties compared to other forms and retained the desired heparin binding activity, despite containing a small portion of the masking domain of FST(315). This form was fused to the Fc portion of human IgG1 and IgG2 to generate FST(291)-IgG1 and FST(291)-IgG2.

A TGGG linker sequence was selected to join each follistatin polypeptide to the Fc portion.

For each FST-IgG1 construct, the follistatin leader was employed.

The FST(291)-IgG1 fusion has the unprocessed and mature amino acid sequences shown below.

Unprocessed FST(291)-IgG1 (SEQ ID NO: 38)

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCS
TGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKP
RCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDV
FCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLG
RSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCA
SDNATYASECAMKEAACSSGVLLEVKHSGSCNSISTGGGTHTCPPCPAPELLGGPSV
FLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT
VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Mature FST(291)-IgG1 (SEQ ID NO: 39)

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCL
WDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNSISTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHE
DPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKS
LSLSPGK

The initial “GN” sequence may be removed, yielding the following polypeptide. (SEQ ID NO: 40)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWD
FKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSC
NSISTGGGTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL
PAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK

The FST(291)-IgG2 fusion has the unprocessed and mature amino acid sequences shown below.

Unprocessed FST(291)-IgG2 (SEQ ID NO: 41)

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCS
TGRLSTSWTEEDVNDNTLFKWMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKP
RCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCKEQPELEVQYQGRCKKTCRDV
FCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLG
RSIGLAYEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCA
SDNATYASECAMKEAACSSGVLLEVKHSGSCNSISTGGGVECPPCPAPPVAGPSVFL
FPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNST
FRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQVYTLPPSRE
EMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVD
KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Mature FST(291)-IgG2 (SEQ ID NO: 42)

GNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAP
NCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNEC
ALLKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPA
SSEQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCL
WDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSG
SCNSISTGGGVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP
EVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGL
PAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG
QPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK

The initial "GN" sequence may be removed, yielding the following polypeptide. (SEQ ID NO: 43)

CWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDNTLFKWMIFNGGAPNC
IPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECAL
LKARCKEQPELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASS
EQYLCGNDGVTYSSACHLRKATCLLGRSIGLAYEGKCIKAKSCEDIQCTGGKKCLWD
FKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGVLLEVKHSGSC
NSISTGGGVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
QFNWYVDGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPA
PIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLS
PGK

Proteins were expressed in HEK-293 cells or CHO cells and purified from conditioned media by filtration and protein A chromatography. In some instances anion exchange and hydrophobic interaction chromatography and/or gel filtration was also used.

Protein activity was assessed by binding to activin A or GDF11. In each case, the proteins bind with a K_D of less than 10 pM.

Additional truncation experiments were conducted to identify Follistatin-IgG2 constructs, in the context of the TGGG linker, exhibiting an optimal ligand and heparin binding activity, so as to generate a polypeptide with high potency, a strong tendency towards retention in the treated tissue and low tendency to produce inflammatory or immune reaction in the treated tissue. For this purpose a series of constructs were generated, termed FST(278)-IgG2, FST(284)-IgG2, FST(291)-IgG2 and FST(303)-IgG2 and compared to each other and to FST(288)-IgG2 and FST-(315)-IgG2. Heparin binding was assessed by measuring protein recovery from cells in the presence or absence of heparin, quantitated by ELISA and expressed as a ratio of protein recovered in the presence of heparin to the protein recovered in the absence of heparin. As shown in the table below, FST(278)-IgG2, FST(284)-IgG2, FST(288)-IgG2 and FST(291)-IgG2 all show similar ratios of 3.00-4.00, while FST(303)-IgG2 and FST(315)-IgG2 show ratios of 1.50 and 0.97, respectively. This indicates that as more amino acids are included between position 291 and 303, the heparin binding activity is sharply reduced.

Heparin Binding of FST-IgG2 Truncations

FST-IgG2 Construct	Ratio (protein recovered with heparin/protein recovered without heparin)
FST(278)-IgG2	4.18
FST(284)-IgG2	3.54
FST(288)-IgG2	3.34
FST(291)-IgG2	3.00
FST(303)-IgG2	1.50
FST(315)-IgG2	0.97

Cell-based reporter gene assays (A-204 Reporter Gene Assay, described in WO/2006/012627) to assess inhibition of activin and GDF11 were conducted. As shown in

the table below, constructs extending beyond position 288 provided enhanced ligand inhibition.

Ligand Inhibition of FST-IgG2 Truncations

FST-IgG2 Construct	IC50 (ng/ml) Activin A	IC50 (ng/ml) GDF-11
FST(278)-IgG2	521	91
FST(284)-IgG2	369	123
FST(288)-IgG2	30	41
FST(291)-IgG2	20	26
FST(303)-IgG2	2	18
FST(315)-IgG2	10	15

Taking together the heparin binding and ligand inhibition data, it is apparent that FST-IgG2 constructs, in the context of the TGGG linker used here, or similar sized linkers (e.g., linkers sized 1-10 amino acids, optionally 3-8 amino acids), that end at position 291-302 will have enhanced ligand inhibition relative to FST(288)-IgG2 and enhanced heparin binding relative to FST(315)-IgG2, and that FST(291)-IgG2 represents an optimal protein for local administration and effect.

Example 7: The Effect of Local Administration of FST(291)-IgG2 Protein on Muscle Mass and Strength in Mice

The ability of the optimized FST(291)-IgG2 protein to locally increase muscle mass and strength in wild-type mice after intramuscular (i.m.) administration was assessed.

C57BL/6 mice were dosed (100 micrograms in 50 microliters; i.m. into the left gastrocnemius muscle) twice/week for four weeks with vehicle (PBS), FST(291)-IgG2 or a control Fc from IgG1. At the end of the study, both the left, injected gastrocnemius muscle and the right, contralateral gastrocnemius muscle were dissected and weighed. As shown in Figure 9, FST(291)-IgG2 treatment significantly increased muscle mass in the injected left gastrocnemius muscles, to a remarkable degree, in comparison to vehicle-treated mice, with

no effect observed on the contralateral muscle. Additionally, pectoral and femoris muscles were weighed and showed no change as a consequence of vehicle or FST(291)-IgG2 administration. Therefore, FST(291)-IgG2 has restricted effect on the injected muscle group with little or no systemic effect. Similar experiments have been conducted by injecting different muscle groups, including the triceps and the tibialis anterior. In each case, selective hypertrophy of the injected muscle was observed.

Additional experiments were conducted to directly compare the effects of FST(288)-IgG1 and FST(291)-IgG2 on muscle growth. While both constructs promoted significant increased muscle mass in the injected muscle (gastrocnemius), the FST(291)-IgG2 caused approximately a 42% increase in the injected muscle versus the contralateral muscle, while FST(288)-IgG1 caused approximately a 22% increase in injected muscle versus the contralateral muscle.

Accordingly, these data indicate that FST(291)-IgG2 is an optimal compound for promoting targeted muscle growth in patients in need thereof.

Example 8: The Effect of Local Administration of FST(288)-IgG2 Protein on Tendon Length and Muscle in a Mouse Model of Congenital Muscle Contractures

The effect of FST(288)-IgG2 on tendon length and muscle mass was assessed in a mouse model of congenital muscle contractures based on limb immobilization early in life in the mouse. Congenital muscle contractures are abnormally short and stiff skeletal muscles presenting at birth that constitute a major and long-term source of disability in children. They are typically characterized by muscles having short muscle and long tendon components to muscle-tendon units (MTUs), resulting in impairment of movement.

In order to establish a congenital muscle contracture mouse model, postnatal limb immobilization in genetically normal CD-1 mice was performed (Figure 10). The impact of ankle immobilization in mice from 1-3 weeks of life was evaluated. It was found that this immobilization was sufficient to produce muscle shortening and tendon lengthening similar to that seen in human muscle contracture cases (Figure 11), and a variety of casting strategies were developed to allow the assessment of therapies. Using this ankle immobilization model, the ability of stretch, electrostimulation, and intramuscular injection of a locally distributing follistatin analog FST(288)-IgG2 were assessed.

Effect of Stretch Therapy on Contractures Induced at 90 Degrees

Based on the successful proof-of-concept studies conducted using ankle immobilization at 90 degrees, stretch therapy was evaluated by incrementally plantarflexing the foot via increasing splinting angle between weeks of life 3-5. This strategy produces stepwise stretch of the tibialis anterior muscle to a final ankle position of full plantarflexion at 180 degrees. Stretch in 3 stages (120, 150, and 180 degrees) or 5 stages (108, 126, 144, 162, and 180 degrees) were evaluated and elicited essentially identical results (Figures 12 and 13). In terms of tendon length (calculated as tendon length/total muscle tendon unit length), there was a nonsignificant trend toward decreased tendon lengthening (TA tendons were 10+1% longer in immobilized limbs in comparison to non-immobilized limbs; p=ns, Figures 12 and 13). Measurement of sarcomere numbers and sarcomere number/tendon length calculations were consistent with a modest benefit to longitudinal muscle growth as a result of stretch therapy. Sarcomere number decreased 17+4% (p=0.026) in TA's from immobilized limbs in comparison to non-immobilized limbs following stretch therapy Figures 12 and 13). Sarcomere numbers were not significantly different from the 30+2% decrease seen in immobilized TA's without treatment. When viewed as the ratio of sarcomere number/tendon length, TA's from immobilized limbs showed decrease of 38+4% (p=0.0022, Figures 12 and 13) in comparison to non-immobilized limbs. These values are consistent with a modest benefit in comparison to the 48+3% decrease observed in untreated animals with contracture (p= 0.0079).

Effect of Electrostimulation and Stretch on Contractures Induced at 90 Degrees

Electrostimulation was added to the stretch therapy protocol to determine whether increased isometric contractile activity would promote longitudinal muscle growth. This "stretch plus electrostimulation" condition was tested in parallel to the efficacy of stretch therapy alone, using a splinting strategy involving 3 stages of progressive plantarflexion (120, 150, and 180 degrees). Evaluation of TA muscle and tendon lengths after 2 weeks of stretch + electrostimulation revealed that there was no additional benefit to tendon length (increased by 13+1% with stretch+electrostimulation in comparison to 13+2% with no treatment and 10+1% with stretch alone; p=ns; Figure 12). Similarly, decreases in sarcomere number (23+2%), and sarcomere number/tendon length (decreased by 42+2%) were not indicative of longitudinal muscle growth beyond that observed with stretch therapy alone (Figure 12).

Effect of FST(288)-IgG2 and Stretch on Contractures Induced at 90 Degrees

Follistatin is capable of producing axial myofiber growth, but its capacity to promote longitudinal muscle growth is unclear. Longitudinal muscle growth was evaluated using FST(288)-IgG2, a composition that was not expected to have therapeutic effects outside the injected leg. The impact of follistatin on longitudinal muscle growth was evaluated in mice with and without contracture between 3-5 weeks of life. In mice without ankle immobilization and lacking contractures, FST(288)-IgG2 exposure did not alter longitudinal muscle growth, as indicated by similar TA tendon/total lengths similar within1+1%, p=ns), sarcomere numbers (similar within 3+4%, p=ns), and sarcomere number/ tendon length (similar within 7+5%, p=ns) on injected in comparison to non-injected sides (Figure 13). To determine whether a physical stimulus for longitudinal muscle-tendon unit growth (such as stretch therapy) would promote longitudinal muscle growth in the context of FST(288)-IgG2 treatment, a group of animals with contractures formed by 90 degree ankle immobilization received five FST(288)-IgG2 injections and 5 stage stretch therapy between weeks 3-5. A cohort of animals receiving stretch therapy in 5 stages (at 108, 126, 144, 162, and 180 degrees) and Tris-buffered saline (vehicle) injections was used as a control, and these mice showed similar degrees of tendon lengthening in comparison to the earlier stretch therapy studies (TA tendon/total MTU lengths greater by 17+1% (p=0.005), sarcomere numbers were lower by 30+3% (p=0.0022), sarcomere number/tendon length were lower by 49+3% (p=0.0022); Figure 13). Surprisingly, treatment of animals with contracture with FST(288)-IgG2 in addition to 5 stage stretch therapy produced a significant improvement by producing less of an increase in tendon/total MTU length (increased by 10+1% in comparison to non-immobilized TA, p=ns) while producing less of a decrease in sarcomere number (decreased by 18+1% in comparison to non-immobilized TA, p=0.0331). Furthermore, sarcomere number/tendon length was also dramatically improved (decreased by 30+2% in comparison to non-immobilized TA, p=0.0073; Figure 13). Significant improvements in these parameters were also observed when comparing 5 way stretch therapy+ FST(288)-IgG2 to animals who received either no treatment or 3 stage stretch therapy+ electrostimulation.

These data demonstrate using twice per week intramuscular injection of FST(288)-IgG2 combined with stretch therapy results in the promotion of longitudinal muscle growth and less tendon lengthening over a two week treatment period.

Example 8 Materials and Methods

Live Animal Studies

CD-1 albino mice (Charles River Laboratories) without genetic abnormalities were used in the experiments described in Example 8. During the period of contracture generation and treatment, health of mice was monitored daily through visual inspection and measurement of body weight. Evidence of deterioration by physical examination or weight loss exceeding 20% were criteria for veterinary consultation or removal from the study.

Ankle Splinting to Produce Progressive Stretch

To assess the impact of progressive stretch on muscle-tendon unit growth in the tibialis anterior (TA) muscle, mice with contractures produced at 90 degrees had their ankles re-splinted in progressively plantarflexed positions over a 2 week period (from 3-5 weeks of age; Figure 10). Splinting to produce stretch was evaluated using 3 stages (at 120, 150 and 180 degrees) or 5 stages (at 108, 126, 144, 162, and 180 degrees). Some experimental groups also included additional therapeutic approaches beyond stretch therapy, as described below. Animals were euthanized at 5 weeks of life for tissue measurement and evaluation.

Assessment of Combined Stretch Therapy Plus Electrostimulation Therapy

To assess the impact of electrostimulation of muscle to improve longitudinal growth beyond what is seen with stretch treatment alone, awake mice with immobilization-induced muscle shortening produced at 90 degrees underwent leg electrostimulation for 30 min/day with concurrent progressive stretch in three stages over a 2-week period (from 3-5 weeks of age; Figure 10). Contraction of leg muscles was evoked by transcutaneous stimulation to the fibular and tibial nerves with a loop electrode (cathode) encircling the leg below the fibular head and a patch electrode (anode) against the shaved lower back. The procedure was developed in 13-15 day old awake mice and maximum current intensity was defined as the level that produced strong TA and gastrocnemius isometric contractions without activating thigh or tail muscles and causing vocalization and startle movements. At maximum, the contracting muscles of the splinted limb were palpably hard, and the leg, ankle and foot stiffened visibly from the opposing tensions developed by co contraction of the dorsi- and plantar-flexor muscles. Electrode (carbonized rubber) conductance was facilitated using Spectra 360 electrode gel (Parker Laboratories, Inc., Fairfield, NJ). The pattern and

waveform of stimulation used were based on those employed in humans to build muscle strength (Doucet BM, Lam A, Griffin L. Neuromuscular electrical stimulation for skeletal muscle function. Yale J Biol Med 2012;85(2):201-215) and were well tolerated 30 min/day by the awake mice constrained under soft nylon netting. Electrode impedance averaged 34±4 kOhms. The stimuli were generated by a Grass S88 stimulator producing square pulses of 11-28 V (16.1±2.1 V) at 50 Hz that were converted to biphasic pulses (400 μsec duration) via a SIU 5 isolator. The stimulation, delivered in a duty cycle of 1 second on, 3 seconds off trains, was repeated continuously for 30 minutes. Maximum contractions were elicited by stimulating currents averaging 13.2±1.7 mA (range 8.9-21.8 mA). A similar pattern of transcutaneous stimulation of the fibular nerve in adult rats employed currents of 9-18 mA and after 4 weeks generated higher force output and increased vascularity of the anterior compartment leg muscles.

Assessment of Stretch Therapy Plus FST(288)-IgG2 Injection

To assess the additional impact of FST-288 treatment on longitudinal muscle growth, mice with immobilization-induced muscle shortening produced at 90 degrees underwent intramuscular injections with FST-288 concurrently with progressive stretch over a 2-week period (from 3-5 weeks of age). Stretch was performed in five stages to allow injections and splint changes to be performed at the same time. Mice received semiweekly, intramuscular 20-30 μl injections of drug or vehicle into the lateral compartment (fibularis muscles). Additional control groups in this study included 1) mice without immobilization-induced muscle shortening or stretch treatment, but with intramuscular injection of FST-288 at the same location and 2) mice with immobilization-induced muscle shortening and five-stage stretch treatment, but with injection of vehicle (Tris-buffered saline) instead of FST-288.

Assessment of Bone, Muscle, and Tendon Growth

Animals were euthanized for anatomical evaluation and tissue collection at 5 weeks of life for the treatment experiments. Animals were euthanized using isoflurane followed by cervical dislocation using approved animal care and use protocols. The hindlimbs were removed by cutting above the knee and fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde, pH 7.2 and stored at 4°C. Both hindlimbs limbs were dissected and tibial lengths were measured on the treated and control sides to compare longitudinal bone growth.

Tibialis Anterior (TA) Muscle and Tendon Measurements

For measurement of the TA muscle-tendon unit, the distal tendon insertion at the first metatarsal bone was cut to free the muscle distally. The proximal insertions of the TA muscle fibers were then freed by blunt dissection from the tibia to preserve intact fiber lengths. TA muscle fiber and tendon lengths were measured with the aid of dissecting microscope magnification and a ruler graduated in 0.5 mm units, and measurements were made to the nearest 0.25 mm. The TA muscle belly length was measured from the proximal-most insertion on the tibia near the knee joint to the most distal insertion of muscle fibers on the tendon. The TA tendon was measured from the point of distal muscle fiber insertion to the tip of the cut distal insertion.

Determination of Sarcomere Number

Experiments evaluating the effects of stretch treatment, electrostimulation, and FST-288 injection also used the number of sarcomeres in series to quantify longitudinal muscle growth. Following measurement of muscle belly and tendon lengths, the TA muscles were further dissected aided by dissecting microscope magnification. The total muscle fiber length of single fibers was the distance between the attachment on distal tendon to the proximal origin on the periosteum. A 1-2 mm mid- fiber segment was then cut from the fibers whose length had been measured. The segment was mounted in Fluoromount aqueous medium (Millipore Sigma, St. Louis, MO) on a glass slide and coverslipped. The cross striations of individual fibers were brought into focus and photographed with a Spot RT3 digital camera (SPOT Imaging, division Diagnostic Instruments, Inc. Sterling Heights, MI) attached to a Nikon E600 light microscope using the 40x objective and the substage condenser set to enhance sarcomere cross striations. The images were opened in MetaMorph Imaging software 7.8 (Diagnostic Instruments, Inc., Sterling Heights, MI). The distance morphometry program was employed to measure the distance covered by 10 sarcomeres in series in 30 different fibers per muscle. The total number of sarcomeres per fiber was calculated by dividing the measured muscle fiber length by the average length of a single sarcomere for the same fibers.

Statistical testing

Data were first evaluated by the Kolmogorov-Smirnov test for normality, and Barlett's test for equal variances. Student's t-test or one-way ANOVA with post hoc Tukey-

Kramer testing was utilized for those passing. For those failing, and Box Cox transformation did not achieve normality (sarcomere number, tendon length and tendon%/MTU), nonparametric testing (Mann-Whitney or Kruskal-Wallis with post hoc Dunn's multiple comparison test) was utilized. Statistics were conducted with GraphPad Prism 7, and significance was accepted at $p<0.05$. See figure legends for specific statistical analysis of each data set.

When comparing within a group, the splinted side was compared with the contralateral nonsplinted limb. The contralateral control MTU may be influenced by the splinted side, but contralateral controls were not different from nontreated controls conducted in parallel. The values for each mouse were normalized by the ratio of the left (L) splinted to (R) right nonsplinted side. Group data compared in this manner included: L/R sarcomere number, L/R tendon length, sarcomere number/tendon length, L/R percent tendon length/MTU, and L/R sarcomere number/tendon length.

Animal Condition Assessments

Splinted mice were generally very tolerant of the ankle immobilization procedure. The tape casts were designed to immobilize the ankle joint, but permit growth of the leg and foot bones. The ratio percent of immobilized to non-immobilized tibia across all groups averaged 99.6±0.9%. Body weight was assessed during all experiments as an indicator of animal distress, with a threshold of 20% weight loss as a criterion for veterinary consultation. No study animals exhibited this degree of weight loss, and so this study exclusion criterion was never implemented.

Ankle Immobilization at 90 Degrees

The impact of ankle immobilization at 90 degrees was assessed as it 1) corresponds to a straightforward and reproducibly estimated angle that is also abnormal and 2) allows significant displacement in either the plantarflexed or dorsiflexed positions if stretch therapy is pursued (Figure 10). Mice with contracture due to immobilization at 90 degrees showed significant tendon lengthening when evaluated at 5 weeks of life without further immobilization or other interventions (TA tendon/total length increased 13+2% in comparison to non-immobilized TA, $p=0.012$; Figures 12 and 13). Decreased longitudinal muscle growth was demonstrated by a

decrease in TA sarcomere number (30+2% decrease in immobilized limbs in comparison to non-immobilized limbs, p=0.026) and the ratio of sarcomere number/tendon length (decreased by 48+3% when comparing the TA's of immobilized vs. non-immobilized limbs, p=0.0079) in animals with immobilization and no treatment in comparison to their non-immobilized limbs (Figures 12 and 13).

INCORPORATION BY REFERENCE

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

While specific embodiments of the subject matter have been discussed, the above specification is illustrative and not restrictive. Many variations will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

WE CLAIM:

1. A method of treating a subject having a disorder associated with muscle contractures, comprising administering to the subject an effective amount of a protein, wherein the protein comprises a follistatin polypeptide, or a biologically active fragment thereof.

2. The method of claim 1, wherein the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 1, or a biologically active fragment thereof.

3. The method of claim 1, wherein the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 2, or a biologically active fragment thereof.

4. The method of claim 1, wherein the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 3, or a biologically active fragment thereof.

5. The method of claim 1, wherein the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 4, or a biologically active fragment thereof.

6. The method of claim 1, wherein the follistatin polypeptide is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15, or a biologically active fragment thereof.

7. The method of claims 1-6, wherein the follistatin polypeptide is conjugated to a heterologous moiety.

8. The method of claim 7, wherein the heterologous moiety is a constant domain of an immunoglobulin.

9. The method of claim 7, wherein the heterologous moiety is albumin.

10. A method of treating a subject having a disorder associated with muscle contractures, comprising administering to the subject an effective amount of a protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence comprises a follistatin polypeptide, wherein the follistatin polypeptide consists of an amino acid sequence that is at least 80%, 85%, 90%, 92%, 93%, 94%, 95%, 96%, 97%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15 or 16, and wherein the second amino acid sequence comprises a constant domain of an immunoglobulin.

11. A method of treating a subject having a disorder associated with muscle contractures, comprising administering to the subject an effective amount of a protein comprising a first amino acid sequence and a second amino acid sequence, wherein the first amino acid sequence comprises a follistatin polypeptide, wherein the follistatin polypeptide consists of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to an amino acid sequence selected from the group consisting of SEQ ID NOs: 1-4, 7-16 and 26-43, and wherein the second amino acid sequence comprises a constant domain of an immunoglobulin.

12. The method of claim 11, wherein the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO: 38-43.

13. The method of claim 11, wherein the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO: 26-28 and 32-34.

14. The method of claim 11, wherein the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to a sequence selected from the group consisting of SEQ ID NO: 29-31 and 35-37.

15. The method of any one of claims 10-14, wherein the first amino acid sequence comprises an amino acid sequence that begins at a residue corresponding to any one of amino acids 30-95 of SEQ ID NO: 3 and ends at a position corresponding to any one of amino acids 316-344 of SEQ ID NO: 3.

16. The method of any one of claims 10-14, wherein the first amino acid sequence comprises an amino acid sequence that begins at a residue corresponding to any one of amino acids 30-95 of SEQ ID NO: 1 and ends at a position corresponding to any one of amino acids 164-167 or 238-244 of SEQ ID NO: 1.

17. The method of claim 10, wherein the first amino acid sequence consists of an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO: 15 or 16.

18. The method of claim 10, wherein the first amino acid sequence consists of the amino acid sequence of SEQ ID NO: 15 or 16.

19. The method of any one of claims 10-18, wherein a linker polypeptide is positioned between the first amino acid sequence and second amino acid sequence, wherein the linker directly connects the C-terminal portion of the first amino acid sequence to the N-terminal portion of the second amino acid sequence, and wherein the linker is 1-10 amino acids in length.

20. The method of claim 18, wherein a linker polypeptide is positioned between the first amino acid sequence and second amino acid sequence, wherein the linker directly connects the C-terminal portion of the first amino acid sequence to the N-terminal portion of the second amino acid sequence, and wherein the linker is 1-10 amino acids in length.

21. The method of claim 19, wherein the linker polypeptide comprises the sequence TGGG (SEQ ID NO: 49).

22. The method of claim 20, wherein the linker polypeptide consists of the sequence TGGG (SEQ ID NO: 49).

23. The method of any one of claims 10-22, wherein the second amino acid sequence comprises a constant domain of an IgG immunoglobulin.

24. The method of any one of claims 10-22, wherein the second amino acid sequence comprises a constant domain of an IgG immunoglobulin that has reduced or no substantial ADCC and/or CDC activity relative to human IgG1.

25. The method of any one of claims 10-22, wherein the second amino acid sequence comprises a constant domain of an IgG immunoglobulin selected from the group: IgG1, IgG2 and IgG4.

26. The method of any one of claims 10-22, wherein the second amino acid sequence comprises an Fc portion of an immunoglobulin.

27. The method of any one of claims 10-22, wherein the second amino acid sequence comprises an IgG1 constant domain.

28. The method of any one of claims 10-22, wherein the second amino acid sequence comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 17.

29. The method of any one of claims 10-22, wherein the second amino acid sequence comprises an IgG2 constant domain.

30. The method of claim 29, wherein the IgG2 constant domain comprises an Fc portion of an IgG immunoglobulin that has reduced ADCC activity relative to human IgG2.

31. The method of claim 29, wherein the IgG2 constant domain comprises an Fc portion of an IgG immunoglobulin that has reduced CDC activity relative to human IgG2.

32. The method of any one of claims 10-22, wherein the second amino acid sequence comprises an Fc portion of an IgG immunoglobulin selected from the group: IgG1, IgG2, IgG4 and an IgG2/4 hybrid.

33. The method of any one of claims 10-22, wherein the second amino acid sequence comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 18.

34. The method of any one of claims 1-33, wherein the follistatin polypeptide ends at a position corresponding to an amino acid selected from the group consisting of amino acid 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, and 305 of SEQ ID NO: 4.

35. The method of any one of claims 1-33, wherein the follistatin polypeptide does not include residues corresponding to the amino acids selected from the group consisting of amino acids 289-315, 290-315, 291-315, 292-315, 293-315, 294-315, 295-315, 296-315, 297-315, 298-315, 299-315, 300-315, 301-315, 302-315, 303-315, 304-315, and 305-315 of SEQ ID NO: 4.

36. The method of any one of claims 1-35, wherein the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID NO: 43, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is optionally absent.

37. The method of any one of claims 1-35, wherein the protein comprises the amino acid sequence of SEQ ID NO: 43, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is optionally absent.

38. The method of any one of claims 1-35, wherein the protein comprises an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to the sequence of SEQ ID

NO: 42, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is optionally absent.

39. The method of any one of claims 1-35, wherein the protein comprises the amino acid sequence of SEQ ID NO: 42, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is optionally absent.

40. The method of any one of claims 36-39, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is absent.

41. The method of any one of claims 36-39, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 43 is included.

42. The method of claim 38 or 39, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is absent.

43. The method of claim 38 or 39, wherein the final (carboxy-terminal) lysine (K) of SEQ ID NO: 42 is included.

44. The method of claim 39, wherein the protein comprises the amino acid sequence of SEQ ID NO: 42.

45. The method of claim 37, wherein the protein comprises the amino acid sequence of SEQ ID NO: 43.

46. The method of any one of claims 1-44, wherein the follistatin polypeptide comprises an unmasked heparin binding domain.

47. The method of claim 46, wherein the heparin binding domain comprises the endogenous follistatin heparin binding sequence of SEQ ID NO: 5.

48. The method of claim 46, wherein the heparin binding domain comprises a heterologous heparin binding sequence.

49. The method of any one of claims 1-48, wherein the follistatin polypeptide binds to one or more ligands selected from the group consisting of: myostatin, GDF-11, activin A and activin B with a KD less than 1 nM, 100 pM, 50 pM or 10 pM.

50. The method of any one of claims 1-48, wherein the follistatin polypeptide binds to myostatin with a KD less than 1 nM, 100 pM, 50 pM or 10 pM.

51. The method of any one of claims 1-50, wherein the follistatin polypeptide binds to activin A with a KD less than 1 nM, 100 pM, 50 pM or 10 pM.

52. The method of any one of claims 1-51, wherein the follistatin polypeptide binds to activin B with a KD less than 1 nM, 100 pM, 50 pM or 10 pM.

53. The method of any one of claims 1-52, wherein the subject has muscle contractures associated with a neurological disease of the brain, spine, or peripheral nerve.

54. The method of any one of claims 1-52, wherein the subject has muscle contractures associated with a connective tissue defect.

55. The method of claim 54, wherein the connective tissue defect is diastrophic dysplasia.

56. The method of any one of claims 1-52, wherein the subject has muscle contractures associated with muscle abnormalities.

57. The method of claim 56, wherein the muscle abnormalities are selected from the group consisting of muscular dystrophy and mitochondrial abnormalities.

58. The method of any one of claims 1-52, wherein the subject has muscle contractions associated with a congenital contracture.

59. The method of claim 58, wherein the congenital contracture in the subject was caused by uterine space limitations from when the subject was in utero.

60. The method of claim 59, wherein the space limitations within the uterus were associated with a condition selected from the group consisting of oligohydramnios, fibroids, uterine malformations, and multiple pregnancy.

61. The method of claim 58, wherein the congenital contractures in the subject are a result of intrauterine or fetal vascular compromise from when the subject was in utero.

62. The method of claim 61, wherein the intrauterine or fetal vascular compromise result in impaired development of nerves or anterior horn cell death.

63. The method of claim 58, wherein the congenital contracture is a result of maternal disease.

64. The method of claim 63, wherein the maternal disease is selected from the group consisting of diabetes mellitus, multiple sclerosis, myasthenia gravis, infection, drug usage, or trauma.

65. The method of any one of claims 1-52, wherein the subject has muscle contractions associated with dysgenesis of the nervous system as observed in chromosomal abnormalities.

66. The method of claim 65, wherein the chromosomal abnormalities are selected from the group consisting of trisomy 18 or trisomy 21.

67. The method of any one of claims 1-52, wherein the subject has muscle contractions associated with dysplasias of the brainstem nuclei or spinal cord.

68. The method of claim 67, wherein the dysplasias of the brainstem nuclei or spinal cord are selected from the group consisting of Mobius syndrome, Pierre-Robin syndrome, prune belly syndrome, and Zellweger syndrome.

69. The method of any one of claims 1-52, wherein the subject has muscle contractions associated with Arthrogryposis multiplex congenita (AMC).

70. The method of claim 69, wherein the AMC is selected from the group consisting of amyoplasia, distal arthrogryposis, a systemic connective tissue disorder, multiple pterygium syndromes, and fetal crowding.

71. The method of any one of claims 1-52, wherein the subject has muscle contractions associated with an isolated congenital contracture.

72. The method of any one of claims 1-52, wherein the subject has one or more of the following characteristics:

a. decreased flexibility of the joints
b. muscle weakness
c. shortening of a muscle; and/or
d. shortening of a joint.

73. The method of any one of claims 1-72, wherein the method comprises administering an effective amount of the protein by an intramuscular route of administration to a targeted muscle of the subject.

74. The method of any one of claims 1-72, wherein an effective amount of the protein is administered intramuscularly.

75. The method of any one of claims 1-72, wherein an effective amount of the protein is administered intravenously.

76. The method of any one of claims 1-75, wherein the subject is a mammal.

77. The method of any one of claims 1-75, wherein the subject is a human.

78. The method of any one of claims 1-77, wherein the subject is an adult or juvenile.

79. The method of any one of claims 1-78, wherein the subject is an infant or a fetus.

80. The method of any one of claims 1-79, wherein the protein is administered prior to, during, or following surgical correction of the muscle contracture.

81. The method of any one of claims 1-80, wherein the protein is administered in combination with stretch therapy.

82. The method of any one of claims 1-81, wherein the protein is administered in combination with electrostimulation therapy.

83. The method of any one of claims 1-82, wherein the administration of the protein in combination with stretch therapy provides at least 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater shortening of the tendon as compared to stretch therapy in combination with electrostimulation therapy.

84. The method of any one of claims 1-83, wherein the administration of the protein in combination with stretch therapy provides at least 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% greater shortening of the tendon as compared to stretch therapy in combination with vehicle.

85. The method of any one of claims 1-84, wherein the administration of the protein in combination with stretch therapy provides 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% decrease in tendon length.

86. The method of any one of claims 1-85, wherein the protein inhibits activin A or GDF-11 in a cell-based reporter gene assay with a lower IC50 than the IC50 of a follistatin polypeptide comprising SEQ ID NO: 2.

87. The method of any one of claims 1-86, wherein the protein binds heparin with a greater affinity than a follistatin polypeptide comprising SEQ ID NO: 2.

88. The method of any one of claims 1-87, wherein the protein does not have a systemic effect when administered intramuscularly.

89. The method of claim 53, wherein the neurological disease of the brain, spine, or peripheral nerve is selected from the group consisting of multiple sclerosis, poliomyelitis, Parkinson's disease, dementia, Cerebral Palsy, dysplasia of the brainstem nuclei, and dysplasia of the spinal cord.

90. The method of any one of claims 1-89, wherein the subject has muscle contractures associated with being bedbound, chronic illness, serious injury, or post-surgical or disuse atrophy.

91. The method of claim 90, wherein the post-surgical or disuse atrophy is selected from the group consisting of hip fracture, total hip arthroplasty, total knee arthroplasty, and rotator cuff surgery.

92. The method of claim 90, wherein the serious injury is selected from the group consisting of head injury and stroke.

93. The method of any one of claims 1-92, wherein the subject has muscle contractures associated with an inflammatory disorder.

94. The method of claim 93, wherein the inflammatory disorder is rheumatoid arthritis.

MVRARHQPGGLCLLLLLLCQFMEDRSAQAGNCWLRQAKNGRCQVLYKTELSKEECCSTGRLSTSWTEEDVNDWTL
FK
WMIFNGGAPNCIPCKETCENVDCGPGKKCRMNKKNKPRCVCAPDCSNITWKGPVCGLDGKTYRNECALLKARCKE
QP
FSI
ELEVQYQGRCKKTCRDVFCPGSSTCVVDQTNNAYCVTCNRICPEPASSEQYLCGNDGVTYSSACHLRKATCLLGR
SI
FSII
GLAVEGKCIKAKSCEDIQCTGGKKCLWDFKVGRGRCSLCDELCPDSKSDEPVCASDNATYASECAMKEAACSSGV
LL
FSIII
EVKHSGSCNSISEDTEEEEEDEDQDYSFPISSILEW

Figure 1

Figure 2

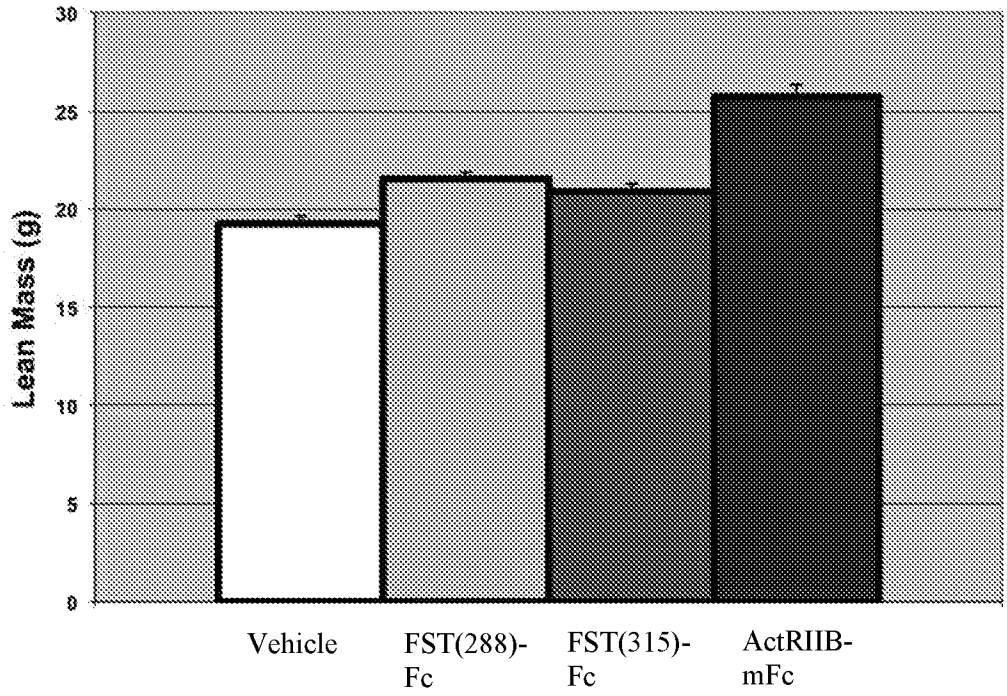

Lean Mass (g)
30
25
20
15
10
5
0
Vehicle
FST(288)-Fc
FST(315)-Fc
ActRIIB-mFc

Figure 3

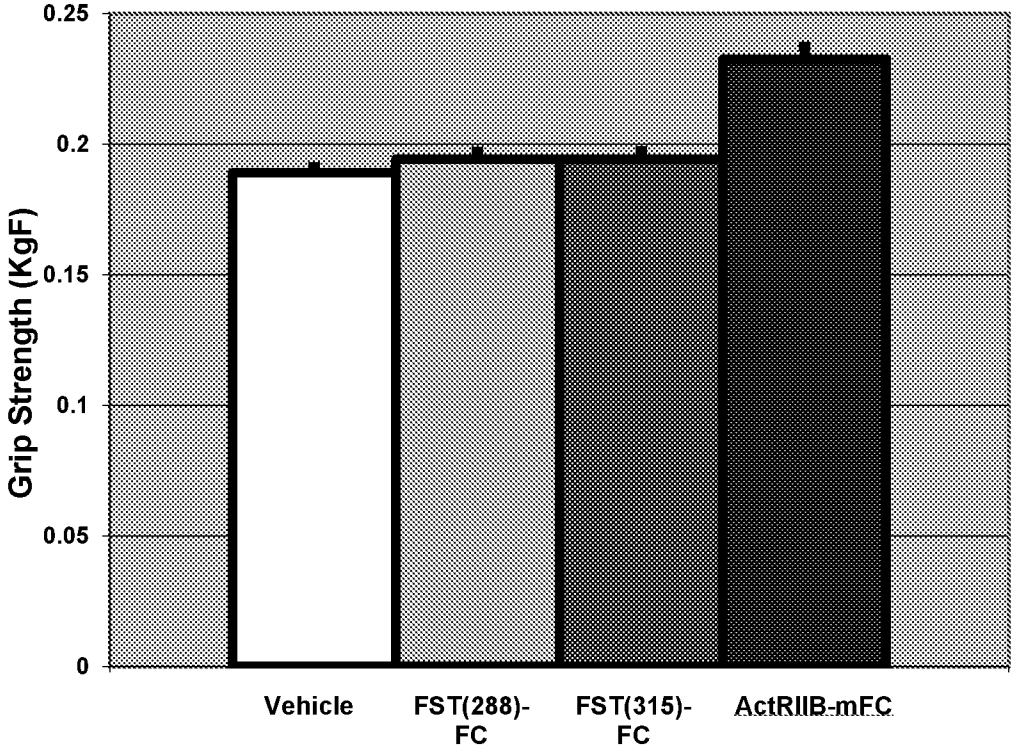

0.25
0.2
0.15
0.1
0.05
0
Grip Strength (KgF)
Vehicle
FST(288)-FC
FST(315)-FC
ActRIIB-mFC

Figure 4

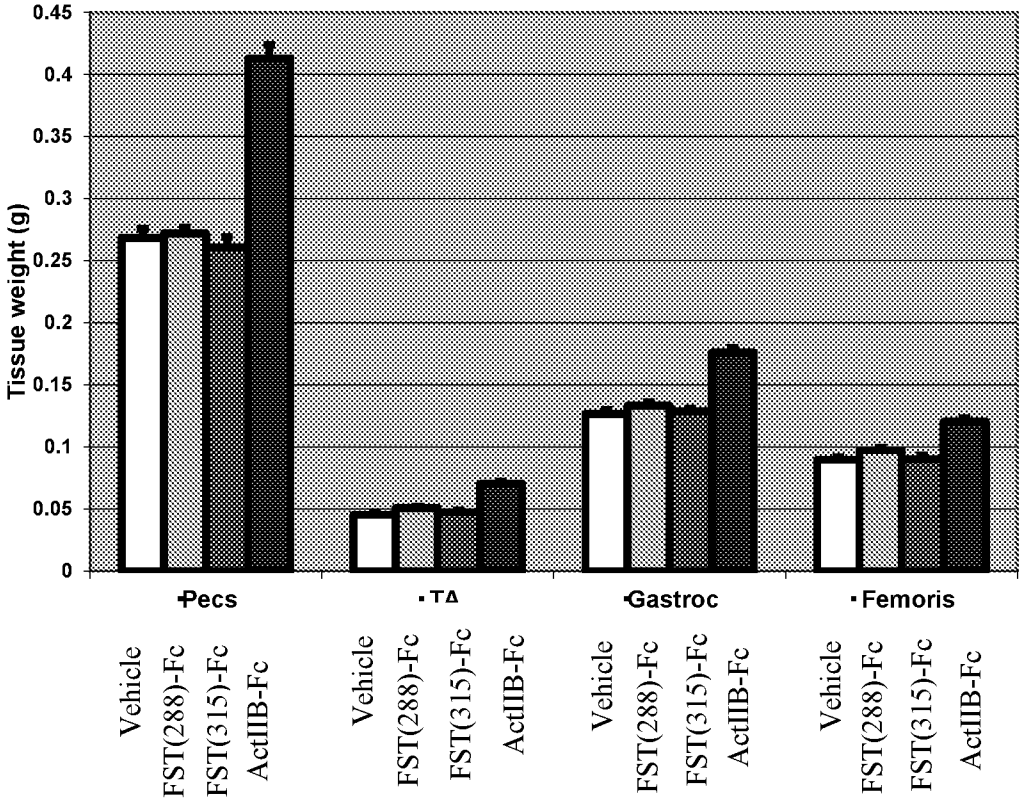

Tissue weight (g)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Pecs
TA
Gastroc
Femoris
Vehicle
FST(288)-Fc
FST(315)-Fc
ActIIB-Fc

Figure 5

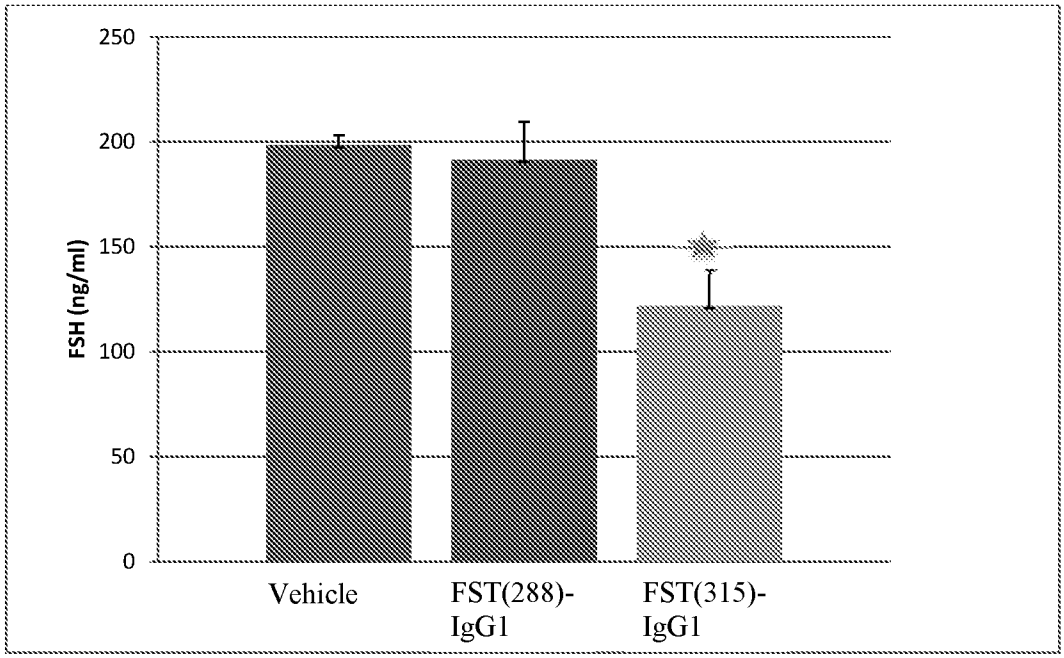


Figure 6

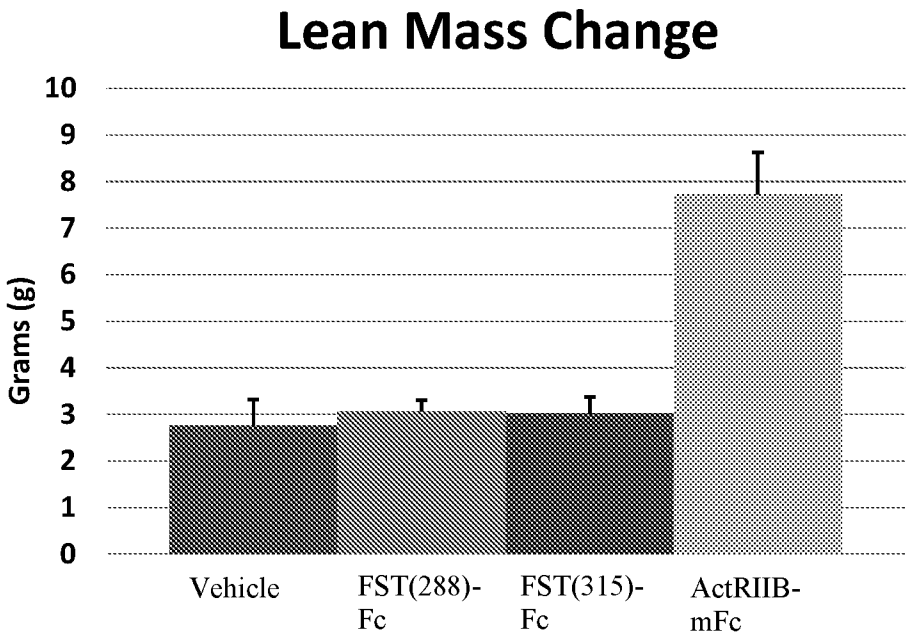

Lean Mass Change
Grams (g)
10
9
8
7
6
5
4
3
2
1
0
Vehicle
FST(288)-Fc
FST(315)-Fc
ActRIIB-mFc

Figure 7

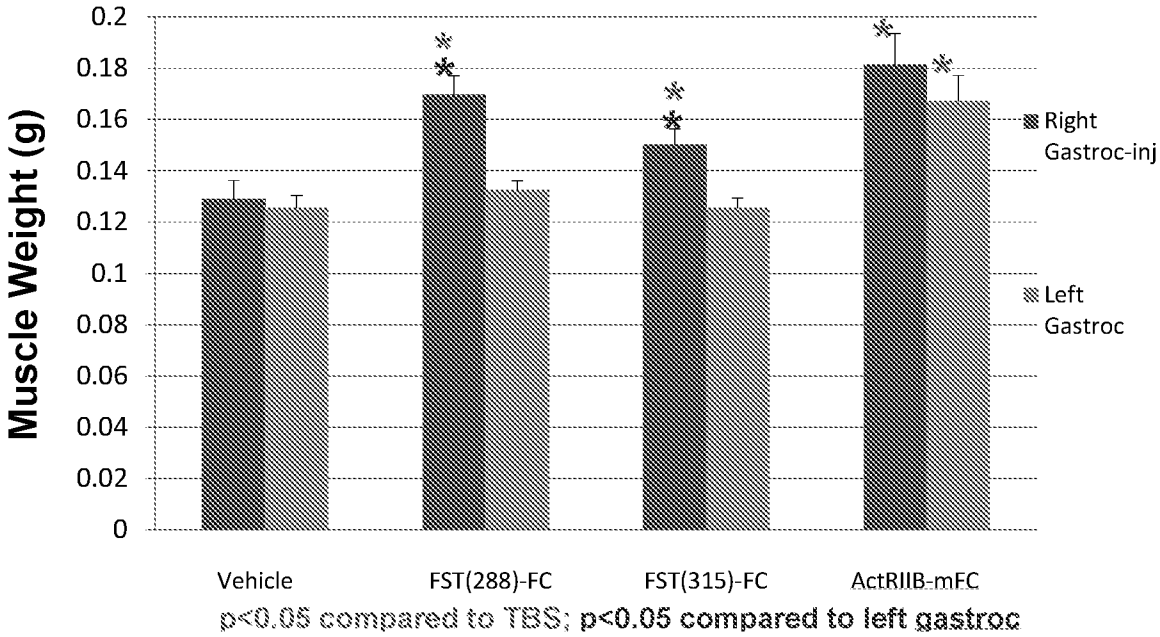
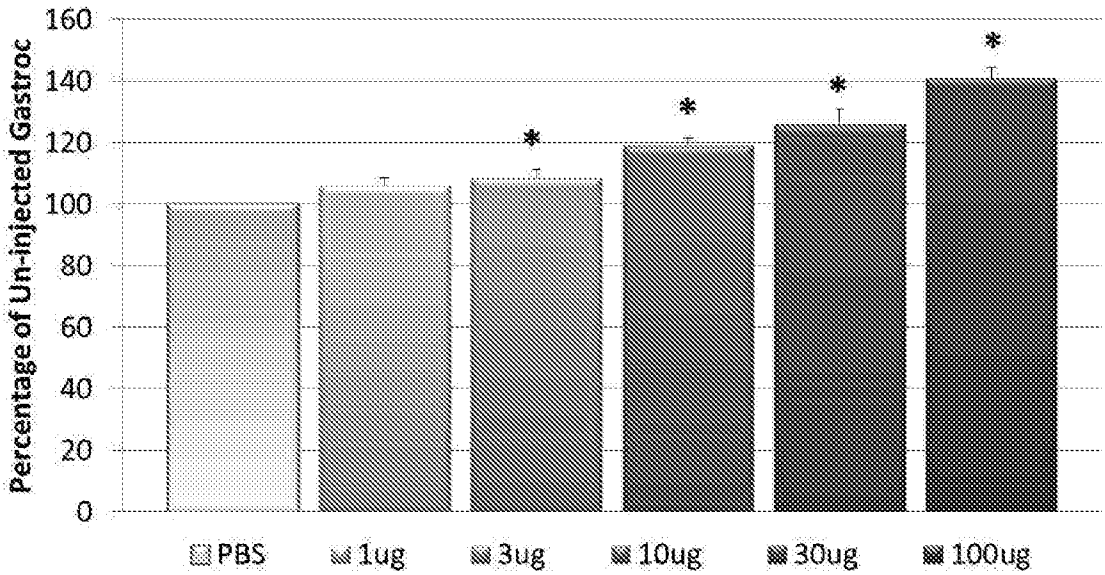

Muscle Weight (g)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Right Gastroc-inj
Left Gastroc
Vehicle
FST(288)-FC
FST(315)-FC
ActRIIB-mFC
p<0.05 compared to TBS; p<0.05 compared to left gastroc

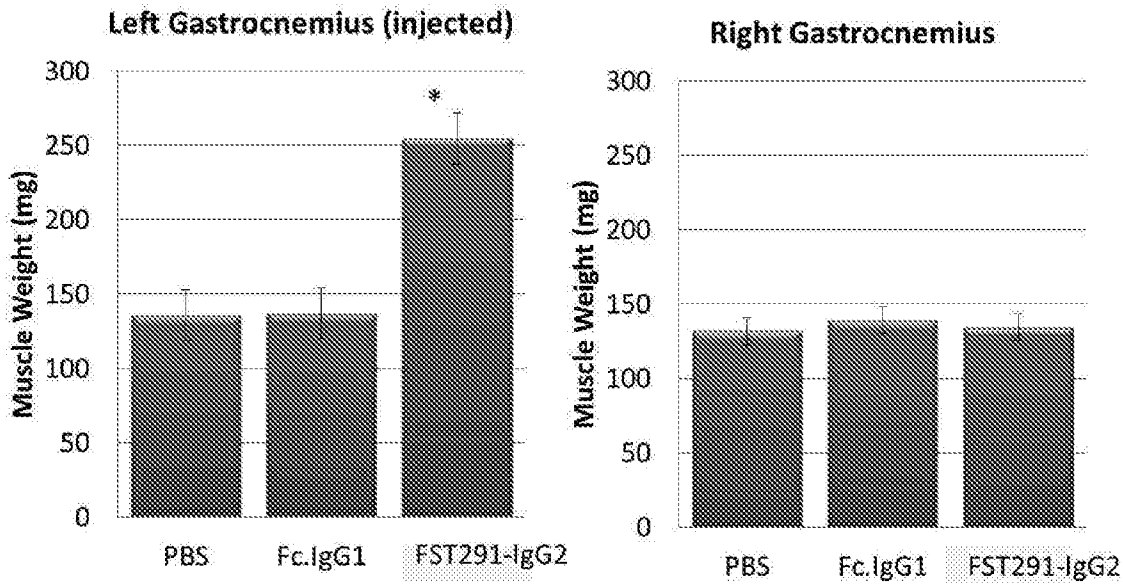
Figure 8



- Gastrocnemius muscle injected directly with FST(288)-IgG1

*p<0.05 vs PBS

Figure 9



*=p<0.05 vs PBS

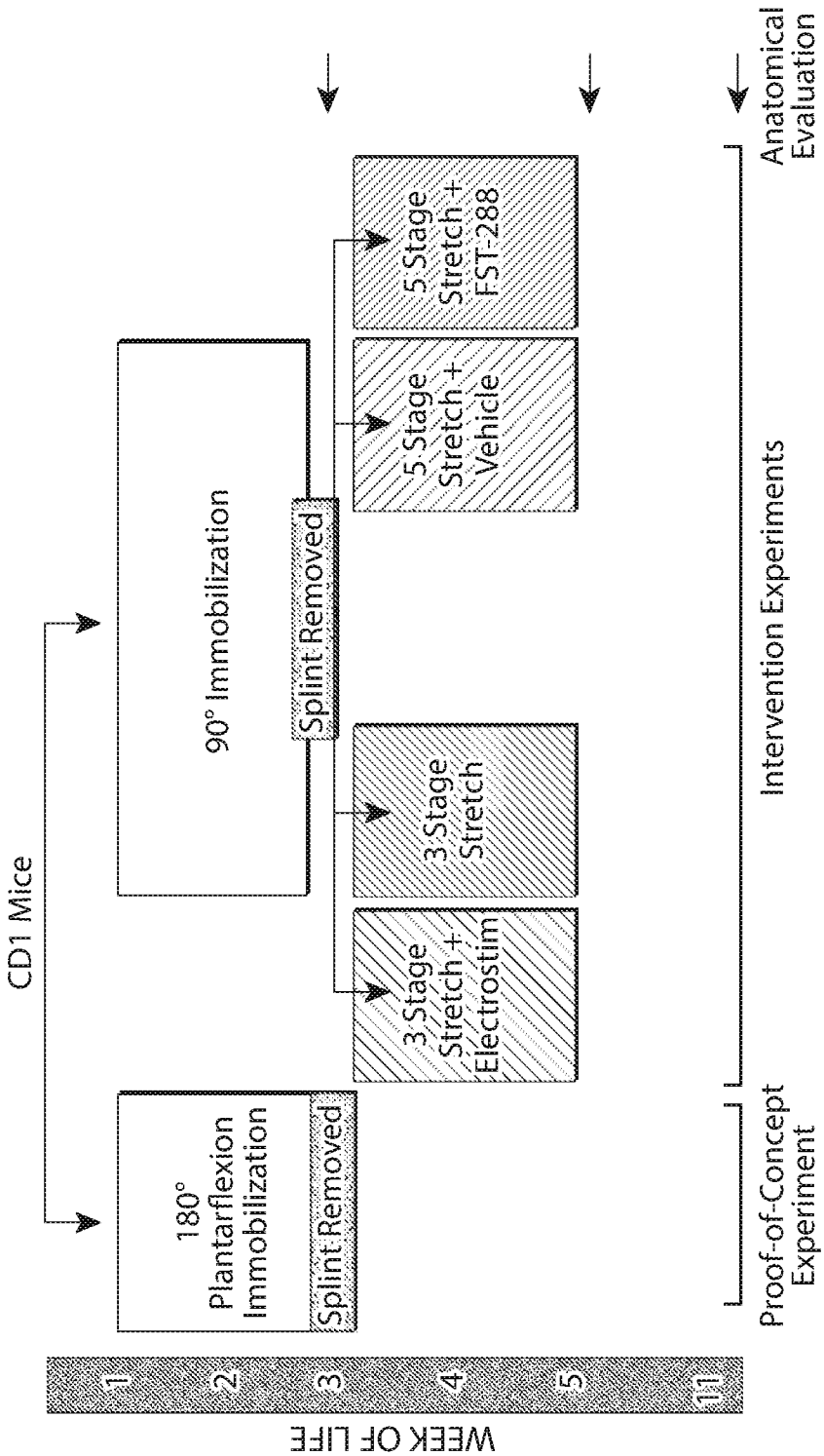

CD1 Mice
180° Plantarflexion Immobilization
Splint Removed
90° Immobilization
Splint Removed
3 Stage Stretch + Electrostim
3 Stage Stretch
5 Stage Stretch + Vehicle
5 Stage Stretch + FST-288
Anatomical Evaluation
Intervention Experiments
Proof-of-Concept Experiment
1
2
3
4
5
11
WEEK OF LIFE

Figure 10

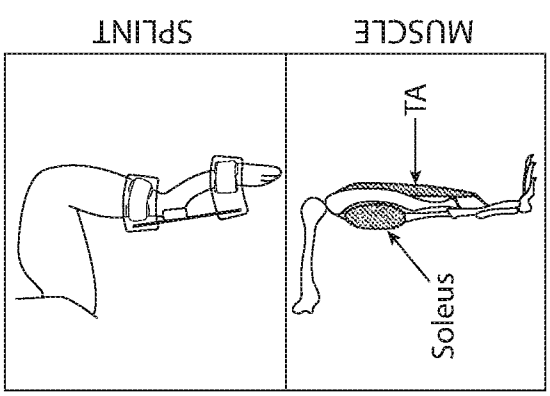


Figure 11A

Figure 11B

Figure 11C

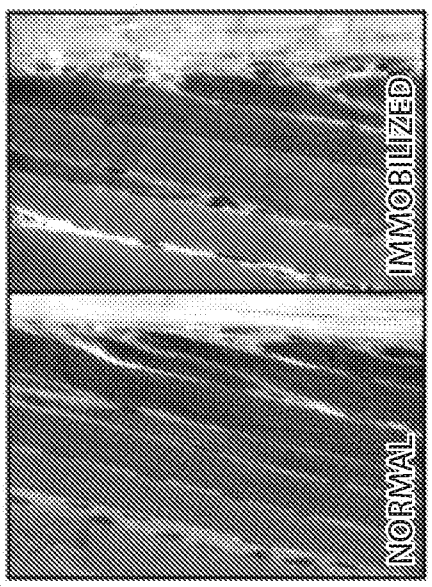


Figure 11E

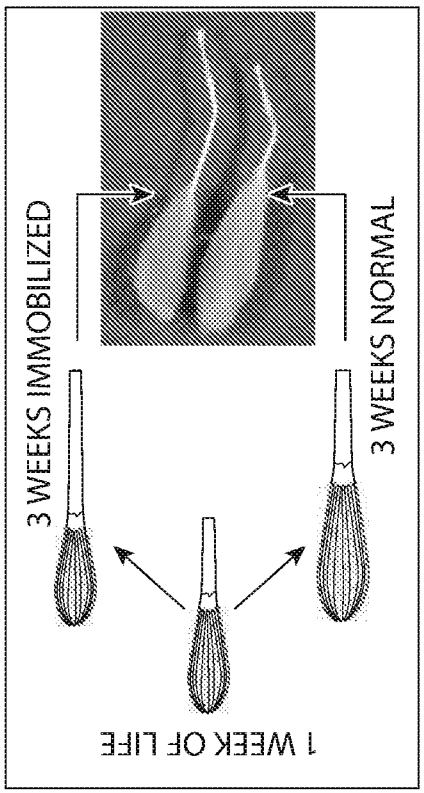


Figure 11D

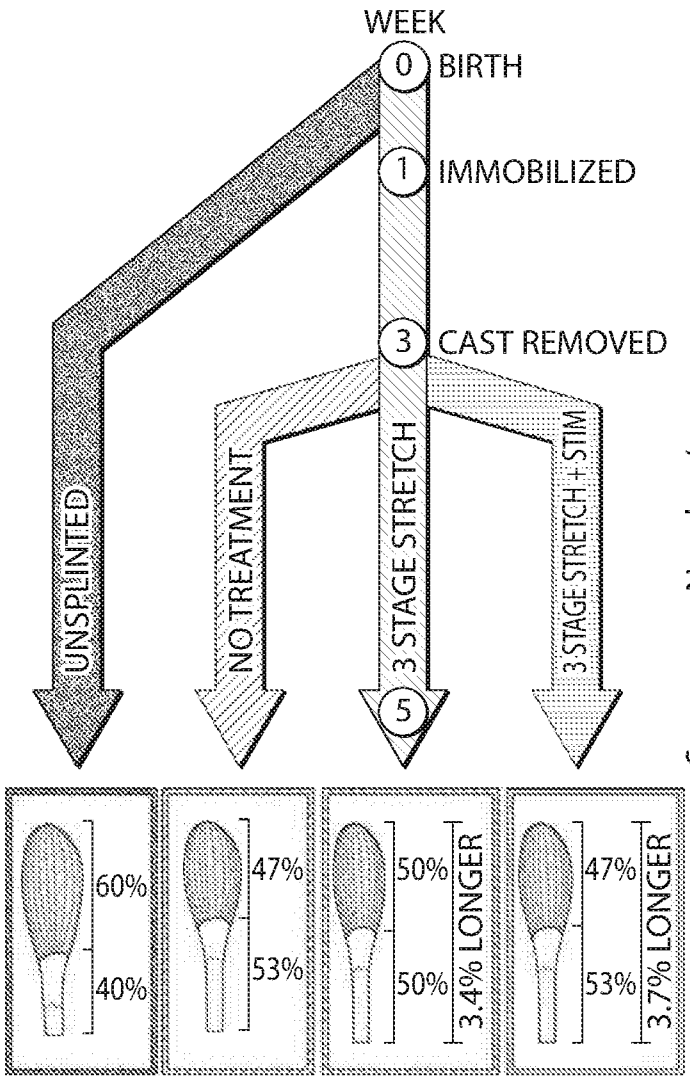


Figure 12A

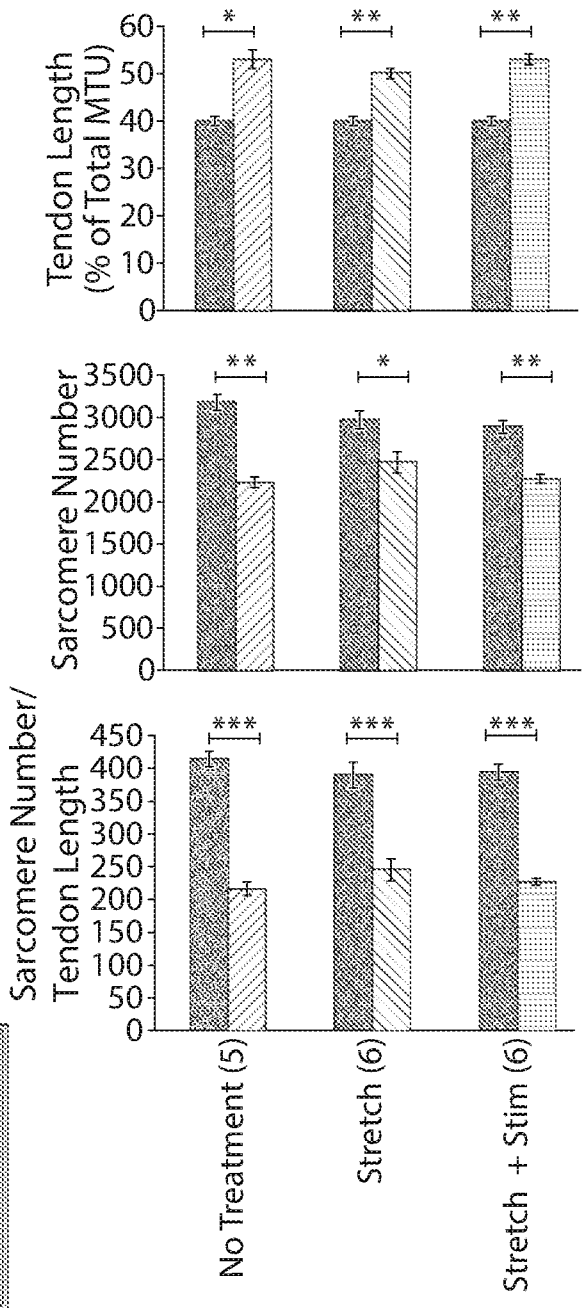


Figure 12B

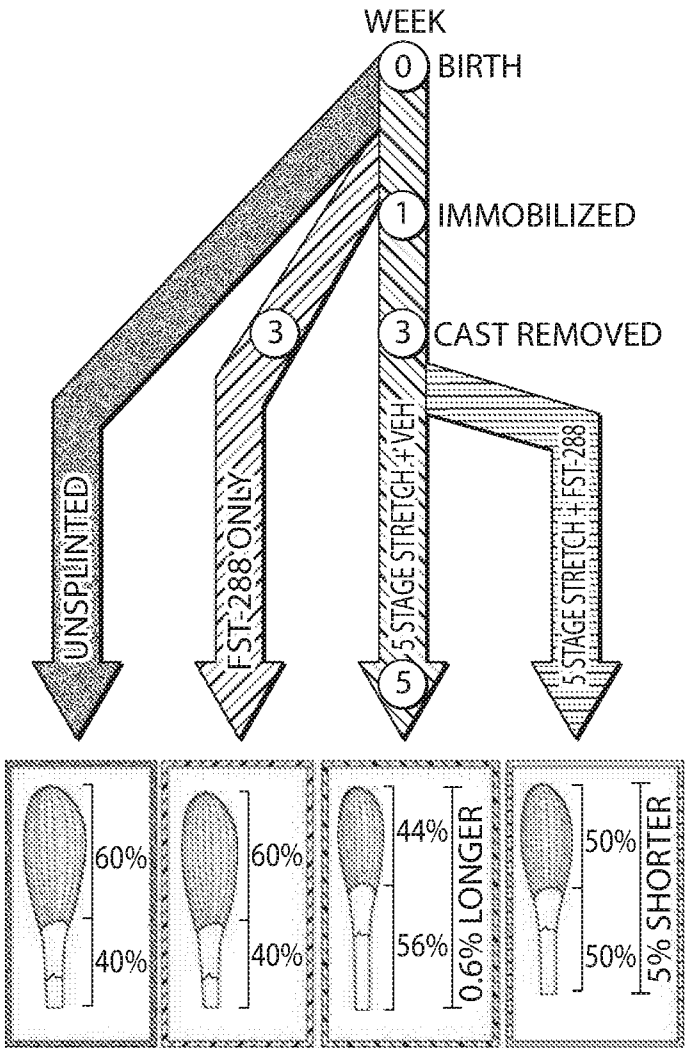


Figure 13A

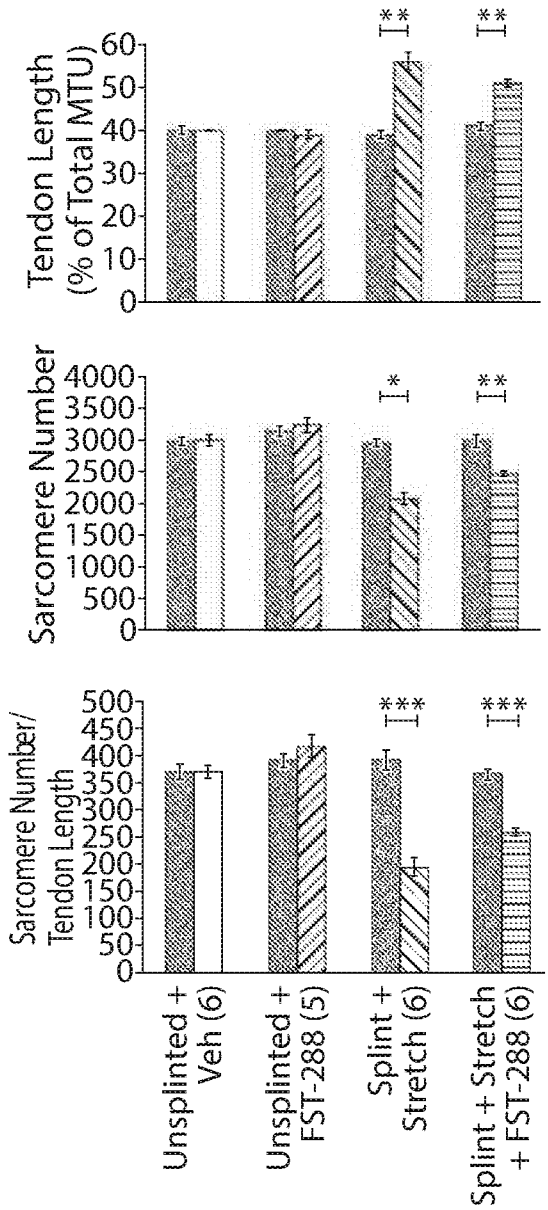


Figure 13B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/024243

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/024243

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/17 (2006.01) A61K 47/68 (2017.01) A61P 21/00 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: PATENW, MEDLINE, CAPLUS, EMBASE, Protein databases of GenomeQuest, Esp@cenet, Pubmed, and internal databases provided by IP Australia

Keywords: Follistatin, muscle, joint, tendon, contracture, shorten, tight, flexible, atrophy, muscular dystrophy, Parkinson, Rheumatoid Arthritis, inflammation, trisomy, congenital, in-utero, multiple pregnancy, oligohydramnios, dementia, arthrogryposis, as similar terms. SEQ ID NO: 1-4, 7-16, 26-43, and the Applicant's and Inventor's names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 July 2019	11 July 2019

Name and mailing address of the ISA/AU

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INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/US2019/024243**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/187977 A1 (ACCELERON PHARMA, INC.) 10 December 2015 See SEQ ID NO: 1-4, 7-16 and 26-43, [0008], [0088-0098], Examples and Claims	1-53, 56-58, 61-62, 72-78, 86-88, 90-91 and 93
X	WO 2017/152090 A2 (SHIRE HUMAN GENETIC THERAPIES, INC.) 08 September 2017 See SEQ ID NO: 1-5, and 75-100, [0093-97], Examples and Claims	1-17, 19, 23-36, 38, 46-53, 56-58, 72-79, 86-88 and 93
X	WO 2014/116981 A1 (SHIRE HUMAN GENETIC THERAPIES, INC.) 31 July 2014 See SEQ ID NO: 1-2, 8, 9, 16 and 17, [0112-114], Examples and Claims	1-8, 10-17, 19, 23-36, 38, 46-47, 49-53, 56-57, 72-78, 86-88 and 93
X	WO 2006/085988 A1 (VOYAGER PHARMACEUTICAL CORPORATION) 17 August 2006 See pp. 5 and Claims	1-6, 46-47, 49-53, 72, 76-78, 86-87 and 89
X	WO 2005/032578 A1 (MONASH UNIVERSITY) 14 April 2005 See pp. 34-35, Examples and Claims	1-7, 46-47, 49-53, 72-78, 86-87, 89-90, and 92-94
X	Rose, F. et al. 2009 "Delivery of recombinant follistatin lessens disease severity in a mouse model of spinal muscular atrophy", Human Molecular Genetics, Vol. 18, No. 6, pp. 997-1005 See Abstract and Materials and Methods	1-6, 46-47, 49-53, 56, 58, 61-62, 72, 76, 78-79, and 86
X	Yaden, B. et al. 2014 "Follistatin: A Novel Therapeutic for the Improvement of Muscle Regeneration", J Pharmacol Exp Ther, Vol. 349, pp. 355-371 See pp. 356 and Results	1-94
A	Cash, J. et al. 2012 "Characterization of Follistatin-Type Domains and Their Contribution to Myostatin and Activin A Antagonism" Mol Endocrinol, Vol. 26, No. 7, pp.1167-1178 See Whole Document	1-94
P,X	WO 2018/209242 A1 (SHIRE HUMAN GENETIC THERAPIES, INC.) 15 November 2018 See SEQ ID NO: 1-5, 75-100, and 117-120, [0108-112], Examples and Claims	1-17, 19, 23-36, 38, 46-53, 56-58, 72-79, 86-88 and 93

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2019/024243**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2015/187977 A1	10 December 2015	WO 2015187977 A1	10 Dec 2015
		AU 2015269333 A1	15 Dec 2016
		BR 112016028520 A2	24 Oct 2017
		CA 2950754 A1	10 Dec 2015
		CN 106795224 A	31 May 2017
		EA 201692529 A1	31 May 2017
		EP 3152237 A1	12 Apr 2017
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		KR 20170005891 A	16 Jan 2017
		MX 2016015868 A	05 Apr 2017
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		US 10010498 B2	03 Jul 2018
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		US 2018325808 A1	15 Nov 2018
		US 2018340013 A1	29 Nov 2018
WO 2017/152090 A2	08 September 2017	WO 2017152090 A2	08 Sep 2017
		AU 2017228475 A1	13 Sep 2018
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		CA 3016576 A1	08 Sep 2017
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		EA 201891990 A1	30 Apr 2019
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		MX 2018010470 A	06 Dec 2018
		SG 11201807400T A	27 Sep 2018
WO 2014/116981 A1	31 July 2014	WO 2014116981 A1	31 Jul 2014
		AU 2014209178 A1	21 May 2015
		AU 2014209178 B2	08 Nov 2018
		AU 2019200797 A1	21 Feb 2019
		CA 2898121 A1	31 Jul 2014
		CN 104902915 A	09 Sep 2015
		CN 104902915 B	06 Jul 2018
		EA 201590719 A1	30 Dec 2015

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2019/024243**

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
		EP 2948161 A1	02 Dec 2015
		EP 2948161 B1	12 Dec 2018
		HK 1213804 A1	15 Jul 2016
		HK 1217637 A1	20 Jan 2017
		JP 2016511751 A	21 Apr 2016
		JP 6475639 B2	27 Feb 2019
		JP 2019059730 A	18 Apr 2019
		KR 20150111347 A	05 Oct 2015
		MX 2015009631 A	30 Nov 2015
		US 2016083440 A1	24 Mar 2016
		US 9957309 B2	01 May 2018
		US 2018305421 A1	25 Oct 2018

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2019/024243**

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2006/085988 A1	17 August 2006	WO 2006085988 A1	17 Aug 2006
		AU 2002361744 A1	09 Jul 2003
		AU 2005327203 A1	17 Aug 2006
		BR 0215168 A	31 May 2005
		CA 2470576 A1	03 Jul 2003
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		CN 1612745 A	04 May 2005
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		EP 1503779 A2	09 Feb 2005
		EP 1863510 A1	12 Dec 2007
		JP 2005523885 A	11 Aug 2005
		JP 2008530088 A	07 Aug 2008
		MX PA04005885 A	17 May 2005
		NZ 533463 A	23 Feb 2007
		US 2003144203 A1	31 Jul 2003
		US 2005192225 A1	01 Sep 2005
		US 2006148697 A1	06 Jul 2006
		US 2006234918 A1	19 Oct 2006
		US 2007015713 A1	18 Jan 2007
		US 2007173454 A1	26 Jul 2007
		US 2007238647 A1	11 Oct 2007
		US 2009197796 A1	06 Aug 2009
		US 2010267620 A1	21 Oct 2010
		US 2011286998 A1	24 Nov 2011
		WO 03053219 A2	03 Jul 2003
		WO 2007011434 A2	25 Jan 2007
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		ZA 200603151 B	25 Feb 2009
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		CA 2541237 A1	14 Apr 2005
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2019/024243**

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
		EP 1677816 B1	12 Mar 2014
		JP 2007507429 A	29 Mar 2007
		US 2007248609 A1	25 Oct 2007
		US 8920806 B2	30 Dec 2014
		US 2015359846 A1	17 Dec 2015
WO 2018/209242 A1	15 November 2018	WO 2018209242 A1	15 Nov 2018
		US 2018362604 A1	20 Dec 2018

End of Annex

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