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(54) Title: A THERAPEUTIC METHOD OF INCREASING MUSCLE MASS IN A SUBJECT

(57) Abstract: The present disclosure provides a method of increasing muscle mass in a subject, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, and wherein the increased level of the protein is normalised before the onset of the increase in muscle mass.

A THERAPEUTIC METHOD OF INCREASING MUSCLE MASS IN A SUBJECT

FIELD

The present disclosure relates generally to a method of increasing muscle mass in a subject and to agents useful for same. The present disclosure also relates to a method of long term increase of muscle mass in a subject. The method of the present disclosure is useful, *inter alia*, in both agricultural applications and the treatment of individuals who would benefit from increased muscle mass, such as premature babies, cancer patients, the elderly and the chronically unwell.

BACKGROUND

Bibliographic details of the publications referred to in this specification are collected alphabetically at the end of the description.

Muscle tissue is a soft tissue which represents one of the four fundamental tissue types present in mammals. In vertebrates, there exist 3 classes of muscle tissue, as follows:

- Skeletal muscle or "voluntary muscle" is anchored by tendons (or by aponeuroses) to bone and is used to effect skeletal movement such as locomotion and maintaining posture. Though this postural control is generally maintained as an unconscious reflex, the muscles responsible react to conscious control like non-postural muscles. An average adult male comprises 42% skeletal muscle and an average adult female comprises 35% skeletal muscle (as a percentage of body mass).
- Smooth muscle or "involuntary muscle" is found within the walls of the organs and structures such as esophagus, stomach, intestines, bronchi, uterus, urethra, bladder, blood vessels and the arrector pili in the skin. Unlike skeletal muscle, smooth muscle is not under conscious control.
- Cardiac muscle (myocardium) is also a type of "involuntary muscle" but is more akin in structure to skeletal muscle. It is found only in the heart.

Cardiac and skeletal muscles are "striated" in that they contain sarcomeres that are packed into highly regular arrangements of bundles. The myofibrils of smooth muscle cells are not arranged in sarcomeres and so are not striated. While the sarcomeres in skeletal muscle are arranged in regular, parallel bundles, cardiac muscle sarcomeres connect at branching, irregular angles (called intercalated discs). Striated muscle contracts and relaxes in short, intense bursts, whereas smooth muscle sustains longer or even near-permanent contractions.

Skeletal (voluntary) muscle is further divided into two broad types: slow twitch and fast twitch:

- Type I, slow twitch, or "red" muscle, is dense with capillaries and is rich in mitochondria and myoglobulin, giving the muscle tissue its characteristic red colour. It can carry more oxygen and sustain aerobic activity using fats or carbohydrates as fuel. Slow twitch fibers contract for long periods of time but with little force.
- Type II, fast twitch muscle, has three major subtypes (IIa, IIx and IIb) that vary in both contractile speed and force generated. Fast twitch fibers contract quickly and powerfully but fatigue very rapidly, sustaining only short, anaerobic bursts of activity before muscle contraction becomes painful. They contribute most to muscle strength and have greater potential for increase in mass. Type IIb is anaerobic, glycolytic, "white" muscle that is least dense in mitochondria and myoglobin. In small animals (e.g., rodents) this is the major fast muscle type, explaining the pale colour of their flesh.

The density of mammalian skeletal muscle tissue is about 1.06 kg/liter. This can be contrasted with the density of adipose tissue (fat), which is 0.9196 kg/liter, making muscle tissue approximately 15% denser than fat tissue. In addition to its contractile role, skeletal muscle is vital for metabolic regulation, and secretes factors that influence other cells' functions.

The loss of muscle mass with chronic illness, inactivity, and senescence impacts these roles, leading to debilitating frailty and effects on other organs that exacerbate illness and increase the risk of medical complications. Diminishing muscle mass is a key predictor of poor prognosis and reduced survival prospects across diseases and age. The role of muscle in determining health is evident from animal studies, where altering muscle attributes can affect illness susceptibility and lifespan. Thus, interventions that preserve or restore functional muscle present opportunities to reduce the incidence of illness and death associated with the effects of frailty.

The interaction of muscle fibres and motor nerves via the neuromuscular junction is vital for muscle health. Disrupting interaction between muscles and nerves causes muscle wasting via proteolysis, and perturbed protein synthesis. This signature of "neurogenic" muscle atrophy is observed with nerve injury (e.g., denervation, neuropathy), degeneration (e.g., motor neuron disease, spinal muscular atrophy), disruption of neuromuscular junction architecture (e.g., myasthenia gravis, Duchenne muscular dystrophy), and prolonged inactivity (e.g., ICU myopathy). Critically,

neuromuscular junction deterioration is a defining feature of aging-related muscle wasting (sarcopenia), which is a cause of death in up to 25% of the elderly.

In terms of the intracellular signalling mechanisms which impact on skeletal muscle functionality, the TGFβ members myostatin and activin engage type-II/type-I activin co-receptors (ActRII/ActRI) to stimulate Smad2/3, and regulate genes that inhibit muscle growth, and promote muscle wasting. Active Smad2/3 proteins also inhibit Akt-mTOR-p70s6k signalling, to depress the main driver of protein synthesis in muscle. As myostatin and activin are elevated in some wasting states (e.g., cancer, cachexia, inflammation, aging), and inhibition in healthy muscles is anabolic, industry has sought to develop interventions and block myostatin and activin as therapeutics for frailty. So far, the most potent would-be therapeutics have been soluble activin receptors that work as "ligand traps" (i.e., Acceleron's ACE-031 and ACE-083 & Amgen's sActRIIb.Fc, now Atara Biotherapeutics' STM-434), although Novartis recently receive dFDA "breakthrough" status for an antibody antagonist of ActRIIB (BYM338). These interventions increase muscle mass in healthy animals and humans, and some models of wasting, by reducing Smad2/3 activity. However, ActRII ligand traps and antagonists impinge on the actions of other TGFβ family ligands that use ActRII, including BMP 7, 9, 10 and 14. Accordingly, interventions targeting ActRII are actually unsuitable for widespread use.

Given the prevalence of neurogenic muscle atrophy as a factor in frailty, interventions that can preserve, augment or restore muscle function have wide application for reducing the impact of frailty upon health and the aging process.

It will be clear to the skilled person from the foregoing, that there is a need in the art to develop means of effecting both the increase and maintenance of muscle mass in a subject.

SUMMARY

The present disclosure is based on the inventors' identification of a method of increasing muscle mass in a subject.

In producing the present disclosure, the inventors' studied the effects of administering an agent that modulates the level of a protein of the TGFβ signaling network on muscle mass increase. The inventors were able to show that administration of follistatin for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein administration of follistatin increased the level of a follistatin protein in the subject compared to a control level, and wherein normalisation of the level of follistatin protein in the subject occurred prior to the onset of muscle mass

increase. The inventors also demonstrated that such short term administration achieved a long term increase in muscle mass. This has not been achievable to date and represents a highly sought after outcome. Specifically, it avoids ongoing exposure of a subject to an agent, thereby minimising potential unwanted side effects, such as unwanted modulation of the inflammatory response in the subject. Still further, where an agent is administered to livestock in order to increase muscle mass for food production, the presence of artificially increased protein levels (e.g., follistatin levels) in the meat at the point of sale would be undesirable. Still further, it provides a viable alternative to steroid based methods for increasing muscle mass, which methods are prone to highly undesirable side effects and which require ongoing administration of the steroid to maintain muscle mass.

The findings by the inventors' provide the basis for methods of increasing muscle mass in a subject by administering follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein compared to a control level, and wherein the increased level of the follistatin protein is normalized before the onset of the increase in muscle mass.

It will be appreciated by the skilled person that administration of the follistatin or functional fragment thereof is for a period of time and in an amount sufficient to induce a long term increase in muscle mass (i.e., after cessation of administration), wherein the increase in muscle mass does not occur until after the level of follistatin in the subject has normalised.

The present disclosure provides a method of increasing muscle mass in a subject, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, and wherein the increased level of the protein is normalised before the onset of the increase in muscle mass.

In one example, the follistatin or functional fragment thereof is administered in the form selected from the group consisting of a protein or a vector comprising a nucleic acid encoding follistatin or functional fragment thereof, which is expressed *in vivo*. In one example, the protein is an antibody or antigen binding fragment thereof. In one example, the vector is a viral vector or a non-viral vector. For example, the follistatin or functional fragment thereof is administered in the form of a protein or a vector comprising a nucleic acid encoding follistatin or functional fragment thereof, which is expressed *in vivo*.

In one example, the follistatin or functional fragment thereof is administered in the form of a protein. For example, the follistatin or functional fragment thereof is a follistatin protein. In one example, the follistatin is recombinant derived or human. For example, the follistatin is recombinant follistatin. In one example, the follistatin is human follistatin. In one example, the follistatin is mammalian derived. For example, the follistatin is derived from a non-human mammal, such as a ruminant or non-ruminant mammal. For example, the non-human mammal is a sheep, a bovine, a porcine, a goat or an equine. In one example, the follistatin is derived from an aquatic or avian species, e.g., fish or poultry.

In one example, the follistatin or functional fragment thereof is administered in the form of a protein, wherein the protein is an antibody or antigen binding fragment thereof. In one example, the antibody or antigen binding fragment thereof is recombinant, chimeric, CDR grafted, humanized, synhumanized, primatized, deimmunized or human. For example, the antibody or antigen binding fragment thereof that binds to or specifically binds to the follistatin protein. This does not mean that the antibody or antigen binding protein does not bind to other proteins, only that the antibody or antigen binding protein is specific to a follistatin protein and does not bind proteins in general. This term also does not exclude e.g., a bispecific antibody or protein comprising binding regions thereof, which can specifically bind to a protein with one (or more) binding regions and can specifically bind to another protein with another binding region.

In one example, the follistatin or functional fragment thereof is administered in the form of a vector comprising a nucleic acid encoding follistatin or functional fragment thereof, which is expressed *in vivo*. For example, the follistatin or functional fragment thereof is administered in the form of a viral vector or a non-viral vector. For example, the vector carries a nucleic acid molecule encoding the follistatin or a functional fragment thereof. In one example, the follistatin or functional fragment thereof is administered in the form of a vector (e.g., a viral or non-viral vector) that carries a nucleic acid molecule encoding follistatin or a functional fragment thereof, which is expressed *in vivo*.

In one example, administration of the follistatin or functional fragment thereof increases the level of follistatin protein in the subject. For example, administration of the follistatin or functional fragment thereof transiently increases the level of follistatin protein in the subject. The increase in the level of follistatin protein in the subject is relative or compared to a control level. It will be appreciated that the increase in the level of follistatin protein in the subject occurs prior to normalisation of the level of the

follistatin protein and the onset of the increase in muscle mass. For example, the level of follistatin protein in the subject is increased (e.g., transiently increased) at a level of at least about 1.5 fold greater, or about 2 fold greater, or about 3 fold greater, or about 4 fold greater, or about 5 fold greater than the control level. In one example, the level of follistatin protein in the subject is increased to a level of at least 1.5 fold greater or 2 fold greater or 3 fold greater or 4 fold greater or 5 fold greater than the control level, prior to normalisation of the level of the protein in the subject and the onset of the increase in muscle mass.

In one example, the follistatin or functional fragment thereof is administered acutely to effect an increase in muscle mass, wherein the acute administration increases (i.e., transient increase) the level of follistatin protein in the subject compared to a control level.

It will be appreciated that the acute administration of the follistatin or functional fragment thereof is for a period of time and in an amount sufficient to induce a long term increase in muscle mass, wherein the increased level of the follistatin protein is normalised prior to (i.e., before) the onset of the increase in muscle mass.

In one example, the acute administration of the follistatin or functional fragment thereof is for a period of about 1 to about 5 minutes, or about 5 minutes to about 1 hour. For example, the acute administration of the follistatin or functional fragment thereof is for a period of about 5 minutes, or about 10 minutes, or about 15 minutes, or about 20 minutes, or about 30 minutes, or about 40 minutes, or about 50 minutes, or about 60 minutes. In one example, the acute administration of the follistatin or functional fragment thereof is for a period of about 1 hour to about 24 hours (or 1 day). For example, the acute administration of the follistatin or functional fragment thereof is for a period of about 1 hour, or about 1.5 hours, or about 2 hours, or about 2.5 hours, or about 3 hours, or about 3.5 hours, or about 4 hours, or about 4.5 hours, or about 5 hours, or about 6 hours, or about 7 hours, or about 8 hours, or 9 hours, or about 10 hours, or about 11 hours, or about 12 hours, or about 13 hours, or about 14 hours, or about 15 hours, or about 16 hours, or about 17 hours, or about 18 hours, or about 19 hours, or about 20 hours, or about 21 hours, or about 22 hours, or about 23 hours, or about 24 hours.

In one example, the follistatin or functional fragment thereof is administered for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of follistatin protein in the subject compared to a control level.

In one example, administration of the follistatin or functional fragment thereof is for a period of about 24 hours (i.e., 1 day) to about 5 days. For example, the follistatin or functional fragment thereof is administered for a period of about 1 day, or about 2 days, or about 3 days, or about 4 days, or about 5 days. In one example, the follistatin or functional fragment thereof is administered for a period of 2 days or about 48 hours. For example, the follistatin or functional fragment thereof is administered for a period of about 24 hours to about 72 hours. In one example, the follistatin or functional fragment thereof is administered for a period of about 24 hours, or about 28 hours, or about 32 hours, or about 36 hours, or about 40 hours, or about 44 hours, or about 48 hours, or about 52 hours, or about 56 hours, or about 60 hours, or about 64 hours, or about 68 hours, or about 72 hours.

In one example, the follistatin or functional fragment thereof is administered chronically to effect an increase in muscle mass in the subject, wherein the chronic administration increases the level of follistatin protein in the subject compared to a control level.

It will be appreciated that the chronic administration of the follistatin or functional fragment thereof is for a period of time and in an amount sufficient to induce a long term increase in muscle mass, wherein the increased level of the follistatin protein is normalised prior to (i.e., before) the onset of the increase in muscle mass.

In one example, the chronic administration of the follistatin or functional fragment thereof is for a period of about 5 days to about 28 days. For example, the chronic administration of the follistatin or functional fragment thereof is for a period of about 5 days, or about 6 days, or about 7 days, or about 8 days, or about 9 days, or about 10 days, or about 11 days, or about 12 days, or about 13 days, or about 14 days, or about 15 days, or about 16 days, or about 17 days, or about 18 days, or about 19 days, or about 20 days, or about 21 days, or about 22 days, or about 23 days, or about 24 days, or about 25 days, or about 26 days, or about 27 days, or about 28 days.

The present disclosure provides a method of increasing muscle mass in a subject, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of follistatin protein in the subject compared to a control level, and wherein the increased level of the protein is normalised before the onset of the increase in muscle mass. It will be appreciated that the normalised level (i.e., the level of follistatin protein in the subject after withdrawal of administration) is a steady state of follistatin protein in the subject.

In one example, the increased level of the follistatin protein is normalised to a steady state.

In one example, the steady state is a level that is similar or substantially equivalent to the level of the follistatin protein in the subject before administration of 5 the follistatin or functional fragment thereof.

In one example, the steady state is a level that is less than the level of the follistatin protein in the subject before administration of the follistatin or functional fragment thereof.

In another example, the steady state is a level that is higher than the level of the 10 follistatin protein in the subject before administration of the follistatin or functional fragment thereof but lower than the maximum increased level obtained during or following administration of the follistatin or functional fragment thereof.

In another example, the level of follistatin protein is normalised to a background or reference level. For example, the steady state is a background or reference level.

15 The present disclosure provides that the increased level of follistatin protein in the subject is normalised prior (i.e., before) to the onset of an increase in muscle mass.

In one example, the increased level of follistatin protein is normalised about 1 day to about 7 days after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass. For example, the 20 increased level of the follistatin protein is normalised about 1 day, or about 2 days, or about 3 days, or about 4 days, or about 5 days, or about 6 days, or about 7 days (i.e., 1 week) after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

In one example, the increased level of the follistatin protein is normalised about 25 1 week to about 6 weeks after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass. For example, the increased level of the follistatin protein is normalised about 1 week, or about 2 weeks, or about 3 weeks, or about 4 weeks, or about 5 weeks, or about 6 weeks after administration of the follistatin or functional fragment thereof to the subject, and 30 prior to the onset of the increase in muscle mass.

In one example, the increased level of the follistatin protein is normalised at least 20 days after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass. In one example, the increased level of the follistatin protein is normalised about 20 days, or about 21 days, 35 or about 22 days, or about 23 days, or about 24 days, or about 25 days, or about 26 days, or about 27 days, or about 28 days, or about 29 days or about 30 days after

administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

In one example, the increased level of the follistatin protein is normalised about 1 day to about 7 days after a first administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

In one example, the increased level of the follistatin protein is normalised about 1 week to about 6 weeks after a first administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

In another example, the increased level of the follistatin protein is normalised about 1 day to about 7 days after cessation of administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

In another example, the increased level of the follistatin protein is normalised about 1 week to about 6 weeks after cessation of administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

In a further example, the increased level of the follistatin protein is normalised about 1 day to about 7 days after an initial increase in the level of the follistatin protein in the subject, and prior to the onset of the increase in muscle mass.

In a further example, the increased level of the follistatin protein is normalised about 1 week to about 6 weeks after an initial increase in the level of the follistatin protein in the subject, and prior to the onset of the increase in muscle mass.

It will be appreciated by the skilled person that normalisation of the level of the follistatin protein can be achieve in a number of different ways depending on whether the follistatin or functional fragment thereof is administered as a protein or a vector carrying a nucleic acid molecule encoding follistatin which is expressed *in vivo*. For example, if the follistatin or functional fragment thereof is administered in the form of a protein, normalisation may achieved by withdrawal of treatment (i.e., stopping administration) and metabolism of the protein. In another example, if the follistatin or functional fragment thereof is administered in the form of a vector comprising, for example, an inducible promoter or control sequence, expression of the nucleic acid encoding follistatin or functional fragment thereof may be switched off.

In one example, increasing the level of follistatin protein in the subject modulates (i.e., increases or decreases) the level of a further protein of the TGFβ signalling network. For example, the further protein of the TGFβ signalling network is selected from the group consisting of GDF8 (Myostatin), GDF11 (BMP11), BMP2,

BMP4, BMP6, BMP7, BMP15 (GDF9B), Activin A, Activin B, SMAD1, SMAD3, SMAD5.

In one example, the method comprises increasing the systemic level of the follistatin protein in the subject.

5 In one example, the method comprises increasing the local level of the follistatin protein in the subject. For example, the level of follistatin protein is increased in the muscle requiring the increase in muscle mass.

In one example, the disclosure provides a method of increasing skeletal muscle mass. For example, the muscle is Type I skeletal muscle. In another example, the 10 muscle is Type II skeletal muscle. In another example, the muscle is smooth muscle. In a further example, the muscle is cardiac muscle.

In a further example, the present disclosure provides a method of increasing overall muscle mass in a subject.

In still a further example, the disclosure provides a method of increasing the 15 mass of at least one isolated muscle in a subject.

In one example, the follistatin or functional fragment thereof that increases the level of the protein is administered to the subject before or after the increase in muscle mass is required. For example, the follistatin or functional fragment thereof is administered prophylactically or therapeutically. In one example, the follistatin or 20 functional fragment thereof is administered before the increase of muscle mass is required (i.e., prophylactically). In another example, the follistatin or functional fragment thereof is administered after the increase of muscle mass is required (i.e., therapeutically).

In one example of any method described herein, the subject is in need of an 25 increase in muscle mass. For example, the subject is at risk of needing an increase in muscle mass. An exemplary subject at risk of needing an increase in muscle mass suffers from a wasting disorder associated with a condition. In one example, the subject is suffering from or at risk of developing a wasting disorder associated with a condition.

30 In one example, the wasting disorder selected from the group consisting of unintended body weight loss, cachexia, sarcopenia (e.g., muscle wasting associated with ageing), muscle wasting or other form of insufficient weight or body mass.

Symptoms of a wasting disorder associated with a condition will be apparent to the skilled person and include, for example:

35
- Loss of muscle mass;
- Loss of muscle strength;

- Uncontrolled twitching;
- Muscle cramping or spasms
- Joint pain;
- Loss of balance;
- Abnormal gait; and/or
- Stooped posture.

In one example, the wasting disorder is associated with a condition, wherein the condition is selected from the group consisting of age, cancer, metabolic acidosis, infectious disease, diabetes, human immunodeficiency virus (HIV), autoimmune immune deficiency syndrome (AIDS), autoimmune disorders, liver disease (e.g., cirrhosis of the liver), chronic inflammatory disorders, anorexia, heart disease, kidney disease, lung disease, osteoporosis, skeletal muscle disease, motor neuron disease, multiple sclerosis, muscle atrophy, malnutrition, low birth weight, inactivity or disuse, drug toxicity, burns, parasitic infection, trauma, surgery, nerve and vessel damage and neurodegenerative diseases including, but not limited to, myasthenia gravis, Guillain-Barré syndrome, Lou Gehrig's disease, muscular dystrophy and spinal-cord injuries. In another example, the wasting disorder is associated with age, for example, premature birth or aging.

In one example, the wasting disorder is cachexia.

In one example, the cachexia is associated with a condition selected from the group consisting of age, cancer, chronic illness, chronic kidney disease, AIDS, chronic heart failure, diabetes, cirrhosis of the liver and age. In one example, the cachexia is associated with premature birth. In another example, the cachexia is associated with ageing and/or frailty.

In a further example, the cachexia is associated with cancer. Numerous types of cancer are associated with cachexia, including solid tumors, carcinoma, neuroma, melanoma, leukemia, lymphoma, sarcoma, fibroma, thyroid cancer, bladder cancer, lung cancer, blastoma, bone cancer, bone tumor, brain stem glioma, brain tumor, breast cancer, bronchial tumor, cervical cancer, colon cancer, colorectal cancer, neuroepithelial tumor, endometrial cancer, endometrial uterine cancer, fallopian tube cancer, kidney cancer, oral cancer, myeloma, neoplasm, neurinoma, neuroblastoma, ovarian cancer, pancreatic cancer, prostate cancer, rectal cancer or renal cell carcinoma.

In one example, the present disclosure provides a method of increasing muscle mass in a subject to treat or prevent a wasting disorder which is associated with a condition, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase

in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, and wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

In one example, the present disclosure provides a method of increasing muscle mass in a subject to treat or prevent cachexia, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

In one example of any method described herein, the subject is a human or livestock. For example, the livestock is selected from the group consisting of a bovine (i.e., cattle), sheep, porcine (i.e., pigs), deer, goats, aquatic species (e.g., fish) and avian species (e.g., poultry). In one example of any method described herein, the subject is a bovine. In another example of any method described herein, the subject is a sheep. In a further example of any method described herein, the subject is a porcine. In one example of any method described herein, the subject is a deer. In another example of any method described herein, the subject is a goat. In a further example of any method described herein, the subject is an aquatic species, e.g., a fish. In one example of any method described herein, the subject is an avian species, e.g., poultry.

In one example, the present disclosure provides a method of increasing muscle mass in a livestock, the method comprising administering follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the livestock compared to a control level, wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

In one example, the present disclosure provides a method of increasing muscle mass in a subject to treat or prevent a wasting disorder which is associated with a condition, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

In one example, the present disclosure provides use of follistatin or a functional fragment thereof in the manufacture of a medicament for increasing muscle mass in a subject, the method comprising administering the follistatin or functional fragment

thereof to the subject for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

5 In another example, the present disclosure provides use of follistatin or a functional fragment thereof in the manufacture of a medicament for increasing muscle mass in a subject to treat or prevent a wasting disorder which is associated with a condition, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase 10 in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

In one example, the follistatin or functional fragment thereof is administered in the form of a composition. For example, the composition comprises follistatin or a 15 functional fragment thereof. In one example, the composition comprising follistatin or functional fragment thereof further comprises a pharmaceutical carrier and/or excipient.

In one example, the present disclosure provides a composition comprising follistatin or functional fragment thereof for use in increasing muscle mass in a subject to treat or prevent a wasting disorder which is associated with a condition, wherein the 20 method comprises administering to the subject the follistatin or functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, and wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

25 The present disclosure also provides a kit comprising follistatin or functional fragment thereof packaged with instructions for use in increasing muscle mass in a subject.

The present disclosure further provides a kit comprising follistatin or functional fragment thereof packaged with instructions for use in increasing muscle mass in a 30 subject in need thereof.

Exemplary effects of follistatin or functional fragments thereof to increase the level of a follistatin protein are described herein and are to be taken to apply *mutatis mutandis* to the examples of the disclosure set out in the previous four paragraphs.

35 **KEY TO SEQUENCE LISTING**

SEQ ID NO: 1 is an amino acid sequence of human follistatin.

SEQ ID NO: 2 is an amino acid sequence of FST317.
SEQ ID NO: 3 is an amino acid sequence of FST288.
SEQ ID NO: 4 is an amino acid sequence of bovine follistatin.
SEQ ID NO: 5 is an amino acid sequence of equine follistatin.
5 SEQ ID NO: 6 is an amino acid sequence of sheep follistatin.
SEQ ID NO: 7 is an amino acid sequence of porcine follistatin.
SEQ ID NO: 8 is an amino acid sequence of chicken follistatin.
SEQ ID NO: 9 is an amino acid sequence of goat follistatin.

10 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 illustrates that transient exposure to FST317 results in permanent increases in skeletal muscle mass. A) Excised tibialis muscles from treated mice maintained on a dox free diet (0/56), a dox diet for 28 days (28/0), or a dox diet for 28 days followed by 28 days of dox free diet (28/28). B) Mass of tibialis anterior muscles 15 as treated above normalised by bodyweight. N=9-12 ±SEM P-value *<0.05. C) Relative muscle mass of rAAV:TetON and rAAV:indFST317 to contralateral rAAV:TetON and rAAV:MCS treated muscles.

Figure 2 illustrates that transient exposure to FST317 results in permanent increases in skeletal muscle fiber size. A) Cross section of tibialis anterior muscles 20 treated with rAAV:TetON + rAAV:indFST317. B) Cross-sectional area of treated tibialis anterior muscles maintained on a dox free diet (0/56), a dox diet for 28 days (28/0), or a dox diet for 28 days followed by 28 days of dox free diet (28/28). C) Distribution of cross-sectional area of treated tibialis anterior muscles. The quartile distribution for each timepoint is inset.

25 **Figure 3** illustrates that sub-maximal transient exposure to FST317 results in permanent increases in skeletal muscle mass. A) Mass of tibialis anterior muscles normalised by bodyweight maintained on a dox free diet (0/30), a dox diet for 2 days (2/0), a dox diet for 2 days followed by 28 days of dox free diet (2/28), or a dox diet for 2 days followed by 84 days of dox free diet (2/84). B) Relative muscle mass of 30 rAAV:TetON and rAAV:indFST317 to contralateral rAAV:TetON and rAAV:MCS treated muscles. C) Peak tetanic force generated from treated tibialis anterior muscle from mice on the 2/84 day dox diet. D) Mass of extensor digitalis longitudus (EDL) muscle normalised to body weight treated with either with rAAV:TetON and rAAV:indFST317 or rAAV:TetON and rAAV:MCS in the contralateral muscle.

35 **Figure 4** illustrates that FST317 expression is absent upon doxycycline withdrawal. A) FST317 protein expression was determined by immunoblot analysis of

protein lysates extracted from rAAV:TetON and rAAV:indFST317 treated tibialis anterior muscles. B) FST317 relative gene expression as determined by RNA-Seq analysis. N=3-4 FDR-value *<0.05 n.d.= no difference. C) SMAD3 phosphorylation, TBC1D1 expression and ASB2 expression as determined by immunoblot analysis.

5 **Figure 5** illustrates that transient exposure to FST317 improves power to weight ratio compared to continual FST317 treatment. A) Maximum force output of TA muscles treated continuously with follistatin. B) Specific force output of TA muscle treated continuously with follistatin. C) Maximum force output of TA muscles treated transiently with follistatin. D) Specific force output of TA muscle treated continuously 10 with follistatin. N= 5 (A and B) N = 8 (C and D) Paired t-test * P< 0.05.

Figure 6 illustrates that transient exposure to FST317 alters gene expression in the tibialis anterior muscle in a unique and permanent way. A) Comparison of genes differentially expressed in muscle after transient follistatin treatment compared to genes differentially expressed with continual follistatin treatment. Genes right of the vertical 15 P-value cut-off line are significant with continual follistatin treatment. Genes above the horizontal P-value cut-off line are significant with transient follistatin treatment. Genes unique to transient treatment are in the left upper quadrant, genes unique to continual follistatin treatment are in the lower right quadrant, genes common to both treatments are in the upper right quadrant. B) Venn diagram of the differentially expressed genes 20 in (A). N = 3 P-value <0.05, FDR 0.01.

DETAILED DESCRIPTION

General

Throughout this specification, unless specifically stated otherwise or the context 25 requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or groups of compositions of matter.

Those skilled in the art will appreciate that the present disclosure is susceptible 30 to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications. The disclosure also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

35 The present disclosure is not to be limited in scope by the specific examples described herein, which are intended for the purpose of exemplification only.

Functionally-equivalent products, compositions and methods are clearly within the scope of the present disclosure.

Any example of the present disclosure herein shall be taken to apply mutatis mutandis to any other example of the disclosure unless specifically stated otherwise.

5 Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (for example, in cell culture, molecular genetics, immunology, immunohistochemistry, protein chemistry, and biochemistry).

Unless otherwise indicated, the techniques utilized in the present disclosure are 10 standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, 15 Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring 20 Harbor Laboratory, (1988), and J.E. Coligan et al. (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

Any discussion of a protein or antibody herein will be understood to include any variants of the protein or antibody produced during manufacturing and/or storage. For example, during manufacturing or storage an antibody can be deamidated (e.g., at an 25 asparagine or a glutamine residue) and/or have altered glycosylation and/or have a glutamine residue converted to pyroglutamate and/or have a N-terminal or C-terminal residue removed or “clipped” and/or have part or all of a signal sequence incompletely processed and, as a consequence, remain at the terminus of the antibody. It is understood that a composition comprising a particular amino acid sequence may be a 30 heterogeneous mixture of the stated or encoded sequence and/or variants of that stated or encoded sequence.

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

35 Throughout this specification the word “comprise”, and variations such as “comprises” and “comprising”, will be understood to imply the inclusion of a stated

element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

As used herein, the term “derived from” shall be taken to indicate that a specified integer or group of integers has originated from the species specified, but has 5 not necessarily been obtained directly from the specified source.

Further, as used herein the singular forms of “a”, “and” and “the” include plural referents unless the context clearly dictates otherwise.

As used herein, the term "about" refers to a range of values + 10% of a specified value. For example, the phrase "about 24 hours" includes ± 10% of 24 hours, or from 10 21.6 to 26.4 hours.

Selected Definitions

As used herein, the term “muscle” should be understood as a reference to the soft tissue which is made up of cells containing protein filaments of actin and myosin 15 that slide past one another, producing a contraction that changes both the length and the shape of the cell. Without limiting the present disclosure to any one theory or mode of action, there exist three types of muscle - Skeletal (striated), cardiac and smooth. Cardiac and smooth muscles contract involuntarily, whereas the skeletal muscles contract upon command. Skeletal muscles in turn can be divided into fast and slow 20 twitch fibers. The present disclosure is directed to increasing the muscle mass of skeletal muscle, smooth muscle and/or cardiac muscle.

As used herein, the term muscle “mass” should be understood as a reference to the size, weight and/or volume of the muscle. In this regard “increasing” muscle mass should be understood as occurring through changes in muscle cell growth by addition 25 of new protein filaments, an increase in the protein synthesis rate, an increase in cellular mechanisms associated with protein synthesis, and/or additional mass provided by undifferentiated satellite cells alongside existing muscle cells. In the context of the present disclosure, an increase in muscle mass may be determined by an increased in the functional characteristic of the muscle. For example, increased muscle function 30 (e.g., increase in muscle contractile force, twitch force, tetanic force and/or force:mass ratio).

As detailed hereinbefore, the method of the present disclosure is predicated on the unexpected determination that administering follistatin or a functional fragment thereof effects a long term increase in muscle mass, wherein the administration 35 increases the level of a follistatin protein in the subject compared to a control level and

wherein the increased level is normalised before the onset of the increase in muscle mass.

As used herein, the term "increased" means that the level of follistatin protein in the subject is increased, compared to a control level, for a defined period of time and
5 are then reduced or decreased before the onset of the increase in muscle mass.

As used herein, the term "normalisation" means returning the increased level of the follistatin protein to a steady state level. The steady state may be a level that is similar or substantially equivalent to the level of the follistatin protein in the subject before administration of the follistatin or functional fragment thereof; or a level that is
10 less than the level of the follistatin protein in the subject before administration of the follistatin or functional fragment thereof; or a level that is higher than the level of the follistatin protein in the subject before administration of the follistatin or functional fragment thereof but lower than the maximum increased level obtained during or following administration of the follistatin or functional fragment thereof; or a
15 background, control or reference level. Methods of determining the increased level of the follistatin protein and the steady state of follistatin protein in the subject will be apparent to the skilled person and/or are herein described. It will be apparent to the skilled person that the levels of follistatin protein are normalised (i.e., reach a steady state) before any observable increase in muscle mass has occurred. In this regard, it
20 should be understood that the intracellular signaling mechanisms and changes to metabolic processes which lead to the muscle mass having been increased may already have commenced. However, substantial and observable increase in muscle mass has not commenced. Accordingly, reference to the "onset" of an increase in muscle mass should be understood as a reference to the onset of an increase in muscle mass which is
25 noticeable either visually or by external size measurement of skeletal muscle.

In terms of the "normalisation" of the level of follistatin protein following administration of the follistatin or functional fragment thereof, it should be understood that this may be achieved by any suitable method that would be well known to those of skill in the art. For example, one may design the administration protocol such that the
30 local or systemic level of the follistatin protein is gradually metabolised and efficacy decreased such that normalised levels are effectively achieved. Alternatively, one may administer an antagonist of the follistatin protein, such as an antibody or soluble competitive receptor. To the extent that the follistatin or functional fragment thereof is provided via a gene construct, such as a vector, one may use an inducible promoter, or
35 a control sequence, such that expression of the nucleic acid encoding follistatin or functional fragment thereof can be induced and switched off when desired.

Reference to the "level" of the protein should be understood as a reference to the level of functionality of the protein (i.e., the functional level). This will most often be assessed by reference to the absolute level in the subject. However, in some circumstances the absolute levels may change only marginally but the functionality is
5 altered. For example, in one example the follistatin or functional fragment thereof which is administered may exhibit much higher and/or longer lived activity than naturally occurring follistatin. In another example, where follistatin protein levels are normalised, this may be achieved by antagonising existing follistatin proteins (such as via the use of an antibody) rather than waiting for the decrease in absolute levels which
10 will occur eventually.

As used herein, the term "acute" in reference to administration of the follistatin or functional fragment thereof generally refers to either a single or continuous administration, typically over several minutes, hours or possibly a day, or repetitive such treatments of relatively short duration over a confined time period of hours or
15 days.

As used herein, the term "chronic" in reference to administration of the follistatin or functional fragment thereof refers to a prolonged duration of administration that typically requires at least a day to a week of repetitive or continuous treatment and such treatment could continue over several weeks.

20 As used herein, the term "wasting disorder" refers to a disorder which involves, results at least in part from, or includes loss of weight, muscle atrophy, fatigue, weakness in someone who is not actively trying to lose weight. Wasting disorders are commonly characterized by inadvertent and/or uncontrolled (in the absence of medical intervention) loss of muscle and/or fat. The term encompasses cachexia or other forms
25 of wasting, e.g., denervation-induced wasting.

The term "wasting disorder associated with a condition" will be understood to mean a wasting that is observed in a subject suffering from a condition, i.e., the wasting may result from changes (e.g., metabolic changes) caused by the condition.

As used herein, the term "cachexia" will be understood to refer to metabolic
30 condition associated with an underlying (or another) condition, wherein cachexia is characterized by loss of body weight and loss of muscle with or without loss of fat mass. Cachexia is generally associated with increased protein catabolism due to underlying disease(s). Contributory factors to the onset of cachexia are anorexia and metabolic alterations (e.g., increased inflammatory status, increased muscle proteolysis
35 and impaired carbohydrate, protein and lipid metabolism). A prominent clinical feature of cachexia is weight loss in adults (optionally, corrected for fluid retention) or

growth failure in children (excluding endocrine disorders). Anorexia, inflammation, insulin resistance and increased muscle protein breakdown are frequently associated with cachexia. Cachexia is distinct from starvation, primary depression, malabsorption and hyperthyroidism and is associated with increased morbidity. Cachexia can be associated with or result from (directly or indirectly) various underlying disorders including cancer, metabolic acidosis (from decreased protein synthesis and increased protein catabolism), certain infectious diseases (e.g. bacterial infections, including tuberculosis, AIDS), some autoimmune disorders, addiction to drugs such as amphetamines or cocaine, chronic alcoholism and/or cirrhosis of the liver, chronic inflammatory disorders, anorexia, neurological conditions and/or neurodegenerative disease. In one example, cachexia is cancer cachexia (cachexia associated with cancer). In other examples, muscle wasting and/or unintended body weight loss associated with neurological conditions, immobility or impaired mobility due to various diseases such as neurodegenerative disease, multiple sclerosis, spinal cord injury, are included in the term. Cachexia can be diagnosed based on one or more of the following:

- Weight loss of at least 5% over a period of six months (in the absence of starvation);
- A BMI <20 together with weight loss; or
- Appendicular skeletal muscle index consistent with sarcopenia (males <7.26kg/m2; females <5.45kg/m2) together with weight loss.

As used herein, the term “unintended body weight loss” refers to a condition where the subject is incapable of maintaining a healthy body weight or loses a considerable amount of body weight, without actually attempting to reduce body weight. For example a body mass index of less than 18.5 (or any another BMI range defined by a medical specialist) is considered underweight.

For the purposes of the present disclosure, the term “body mass index” is calculated by the following formula: mass (kg)/(height (m)^2).

The term “total body mass” will be understood to mean a subject’s weight.

As used herein, the term “preventing”, “prevent” or “prevention” includes providing prophylaxis with respect to occurrence or recurrence of a wasting disorder associated with a condition in a subject, delaying the onset of a wasting disorder and reducing the incidence, likelihood, extent or risk of a wasting disorder associated with a condition. A subject may be predisposed to or at risk of developing the wasting disorder but has not yet developed the wasting disorder.

As used herein, the term "treating", "treat" or "treatment" includes providing treatment with respect to occurrence or recurrence of a wasting disorder associated with

a condition in a subject, reducing the severity, extent or duration of a wasting disorder associated with a condition. The term shall also be taken to mean reducing or eliminating at least one symptom of a specified condition or disease.

As used herein, a subject "at risk" of developing a disease or condition or relapse thereof or relapsing may or may not have detectable disease or symptoms of disease, and may or may not have displayed detectable disease or symptoms of disease prior to the treatment according to the present disclosure. "At risk" denotes that a subject has one or more risk factors, which are measurable parameters that correlate with development of the disease or condition, as known in the art and/or described herein.

An "effective amount" refers to at least an amount effective, at dosages and for periods of time necessary, to achieve the desired result, (i.e., treatment or prevention of a wasting disorder associated with a condition). An effective amount can be provided in one or more administrations. The effective amount may vary according to the disease or condition to be treated and also according to the weight, age, racial background, sex, health and/or physical condition and other factors relevant to the subject being treated. Typically, the effective amount will fall within a relatively broad range (e.g., a "dosage" range) that can be determined through routine trial and experimentation by a medical practitioner. The effective amount can be administered in a single dose or in a dose repeated once or several times over a treatment period.

As used herein, the term "condition" refers to a disruption of or interference with normal function, and is not to be limited to any specific condition, and will include diseases or disorders.

The term "recombinant" shall be understood to mean the product of artificial genetic recombination. Accordingly, in the context of a recombinant protein comprising an antibody variable region, this term does not encompass an antibody naturally-occurring within a subject's body that is the product of natural recombination that occurs during B cell maturation. However, if such an antibody is isolated, it is to be considered an isolated protein comprising an antibody variable region. Similarly, if nucleic acid encoding the protein is isolated and expressed using recombinant means, the resulting protein is a recombinant protein comprising an antibody variable region. A recombinant protein also encompasses a protein expressed by artificial recombinant means when it is within a cell, tissue or subject, e.g., in which it is expressed.

The term "protein" shall be taken to include a single polypeptide chain, i.e., a series of contiguous amino acids linked by peptide bonds or a series of polypeptide chains covalently or non-covalently linked to one another (i.e., a polypeptide complex).

For example, the series of polypeptide chains can be covalently linked using a suitable chemical or a disulfide bond. Examples of non-covalent bonds include hydrogen bonds, ionic bonds, Van der Waals forces, and hydrophobic interactions.

The term "polypeptide" or "polypeptide chain" will be understood from the 5 foregoing paragraph to mean a series of contiguous amino acids linked by peptide bonds.

The skilled artisan will be aware that an "antibody" is generally considered to be a protein that comprises a variable region made up of a plurality of polypeptide chains, e.g., a polypeptide comprising a light chain variable region (VL) and a polypeptide 10 comprising a heavy chain variable region (VH). An antibody also generally comprises constant domains, some of which can be arranged into a constant region, which includes a constant fragment or fragment crystallizable (Fc), in the case of a heavy chain. A VH and a VL interact to form a Fv comprising an antigen binding region that is capable of specifically binding to one or a few closely related antigens. Generally, a 15 light chain from mammals is either a κ light chain or a λ light chain and a heavy chain from mammals is α, δ, ε, γ, or μ. Antibodies can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass. The term "antibody" also encompasses humanized antibodies, primatized antibodies, human antibodies, synhumanized antibodies and chimeric antibodies.

20 As used herein, "variable region" refers to the portions of the light and/or heavy chains of an antibody as defined herein that is capable of specifically binding to an antigen and includes amino acid sequences of complementarity determining regions (CDRs); i.e., CDRl, CDR2, and CDR3, and framework regions (FRs). Exemplary variable regions comprise three or four FRs (e.g., FR1, FR2, FR3 and optionally FR4) 25 together with three CDRs. In the case of a protein derived from an IgNAR, the protein may lack a CDR2. VH refers to the variable region of the heavy chain. VL refers to the variable region of the light chain.

As used herein, the term "binds" in reference to the interaction of a protein or an antigen binding site thereof with an antigen means that the interaction is dependent 30 upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the antigen. For example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody binds to epitope "A", the presence of a molecule containing epitope "A" (or free, unlabeled "A"), in a reaction containing labeled "A" and the protein, will reduce the amount of labeled "A" bound to 35 the antibody.

As used herein, the term “specifically binds” or “binds specifically” shall be taken to mean that a protein reacts or associates more frequently, more rapidly, with greater duration and/or with greater affinity with a particular antigen or cell expressing same than it does with alternative antigens or cells. For example, a protein binds to follistatin with materially greater affinity (e.g., 5 fold or 10 fold or 20 fold or 40 fold or 60 fold or 80 fold to 100 fold or 150 fold or 200 fold) than it does to other members of the TGF-beta signalling network or to antigens commonly recognized by polyreactive natural antibodies (i.e., by naturally occurring antibodies known to bind a variety of antigens naturally found in humans). Generally, but not necessarily, reference to binding means specific binding, and each term shall be understood to provide explicit support for the other term.

The term "subject" as used herein includes humans, primates, non-human mammals (e.g., ruminants and non-ruminants), livestock animals (e.g., an equine (i.e., horse), a bovine (i.e., cattle), a sheep, a porcine (i.e., pig), aquatic species (i.e., fish), donkeys), laboratory test animals (e.g., mice, rats, guinea pigs), companion or domestic animals (e.g., a canine (i.e., dog), a feline (i.e., cat) and captive wild animals (e.g., a kangaroo, a deer, a fox). In one example, the subject is a human or a livestock. In another example, the subject is a human.

Methods of Increasing Muscle Mass

The present disclosure is predicated, in part, on the determination that muscle can be induced to undergo a long term increase in mass via the administration of follistatin or functional fragment thereof to increase the level of follistatin protein in a subject, wherein the level of follistatin protein is normalised prior to the onset of the increase in muscle mass. Accordingly, this finding has facilitated the development of methods of treating a subject, for example therapeutically or prophylactically, to stably increase muscle mass. This has particular relevance in terms of the treatment of premature babies, the elderly, the chronically unwell and cancer patients. Still further, in the context of the livestock industry, the present method provides a means of effectively increasing muscle mass without the concomitant problem of residual agents in the end stage food product, unlike the problems commonly associated with ongoing hormone administration to increase the rate of animal growth.

Accordingly, the present disclosure is directed to a method of increasing muscle mass in a subject, the method comprising administering follistatin or functional fragment thereof for a period of time and in an amount sufficient to increase the level of follistatin protein in the subject compared to a control level, wherein the increased

level of the follistatin protein is normalised before the onset of the increase in muscle mass.

Without limiting the present disclosure to any one theory or mode of action, muscles can be induced to grow larger by a number of factors, including hormone signaling, developmental factors, strength training and disease. Biological factors such as age and hormone levels can affect muscle hypertrophy.

At birth, muscles are still actively engaged in growth and remodelling. In premature infants or low-birth weight babies, muscle development may be delayed or halted. The skilled person would understand that the methods of the present disclosure can also be used to treat failure to thrive, prematurity or low birth weight babies. Without limiting the present disclosure to any one theory or mode of action, an increase in muscle mass may be induced in premature babies by increasing the number of cells that fuse in to a maturing muscle fiber; establishing or enhancing the connection between the muscle fiber and the motor nerve; developing myofibrils; developing supporting vasculature; or enhancing protein synthesis rate in the developing muscle fiber, which will continue in the mature state.

During puberty in males, hypertrophy occurs at an accelerated rate as the levels of growth-stimulating hormones produced by the body increase. Natural hypertrophy normally stops at full growth in the late teens.

Contrary to this, inactivity and starvation leads to atrophy of skeletal muscle, a decrease in muscle mass that may be accompanied by a smaller number and size of the muscle cells as well as lower protein content. Muscle atrophy may also result from the natural aging process or from disease. In humans, prolonged periods of immobilization, as in the cases of bed rest or astronauts flying in space, are known to result in muscle weakening and atrophy. Atrophy is of particular interest to the manned spaceflight community because the weightlessness experienced in spaceflight results in a loss of as much as 30% of mass in some muscles.

During aging, there is a gradual decrease in the ability to maintain skeletal muscle function and mass, known as sarcopenia. The exact cause of sarcopenia is unknown, but it may be due to a combination of the gradual failure in the "satellite cells" that help to regenerate skeletal muscle fibers, and a decrease in sensitivity to or the availability of, critical secreted growth factors that are necessary to maintain muscle mass and satellite cell survival. Sarcopenia is a normal aspect of aging, and is not actually a disease state, yet can be linked to many injuries in the elderly population as well as decreasing quality of life. There are also many diseases and conditions that cause muscle atrophy. Examples include cancer and AIDS, which induce a body

wasting disorder called cachexia. Other syndromes or conditions that can induce skeletal muscle atrophy are congestive heart disease and some diseases of the liver. Although muscle mass can currently be artificially increased using hormone treatments such as anabolic steroids, there are significant undesirable side effects and dangers associated with this, not least the development of male sexual characteristics, such as facial hair growth in women who take testosterone. The method of the present disclosure, however, provides a means of increasing the muscle mass without the use of a sex hormone. In this regard, the present disclosure overcomes these problems via the administration of an agent that transiently increases the level of follistatin or binding protein thereof.

Targeting the TGFβ signaling network

Methods of the present disclosure comprise administering follistatin or a functional fragment thereof, wherein the administration increases the level of a follistatin protein (i.e., a protein of the TGFβ signalling network) in the subject compared to a control level, wherein the level of the follistatin protein is normalized before the onset of an increase in muscle mass.

Reference to the "TGFβ signaling network" or TGFβ signaling pathway" refers to the transforming growth factor beta signaling pathway that is involved in many cellular process including cell growth, differentiation, apoptosis, cellular homeostasis and other cellular functions. The TGF beta superfamily includes follistatin, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), anti-müllerian hormone (AMH), Activin, Nodal and TGFβ's. Activation of the pathway results in activation and phosphorylation of receptor regulated SMADs. In the context of the present disclosure, the skilled person would understand that use of the term TGFβ signaling network encompasses the downstream pathway members (i.e., SMADs).

Members of the TGFβ superfamily will be apparent to the skilled person and include, for example, follistatin, GDF11 (BMP11), BMP2, BMP4, BMP6, BMP7, BMP15 (GDF9B), SMAD1, SMAD5, GDF8 (Myostatin), SMAD3, Activin A and Activin B.

Follistatin and Functional Fragments Thereof

The present disclosure provides methods of increasing muscle mass in a subject comprising administering follistatin or a functional fragment thereof. The follistatin or functional fragment thereof for use in the present disclosure may be in the form of a

protein, such as a recombinant or human protein, an antibody or antigen binding protein, or a vector (e.g., a viral or non-viral vector).

"Follistatin" is a glycoprotein that primarily functions to bind and neutralize members of the TGFβ superfamily. For the purposes of nomenclature only and not
5 limitation exemplary sequences of human follistatin are set out in NCBI Reference Sequence AAH04107 and in SEQ ID NO: 1. It should also be understood that the term "follistatin" includes any isoform (including FST317, FST288 and FST300) which may arise from alterative splicing of follistatin mRNA or mutant or polymorphic forms of follistatin. The term "FST317" refers to 317 amino acid mature follistatin and is the
10 most abundant and the sole form found in plasma. For the purposes of nomenclature only and not limitation exemplary sequences of human FST317 are set out in NCBI Reference AAA35851 and in SEQ ID NO: 2. The term "FST288" refers to the 288 amino acid length follistatin. For the purposes of nomenclature only and not limitation exemplary sequences of FST288 are set out in NCBI Reference ALC04452 and in SEQ
15 ID NO: 3. Additional sequence of follistatin can be determined using sequences provided herein and/or in publically available databases and/or determined using standard techniques (e.g., as described in Ausubel et al., (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present) or Sambrook et al., Molecular Cloning: A Laboratory Manual,
20 Cold Spring Harbor Laboratory Press (1989)). It should still further be understood to extend to any protein encoded by the follistatin gene, any subunit polypeptide, such as precursor forms which may be generated, and any follistatin protein, whether existing as a monomer, multimer or fusion protein. Furthermore, it should be understood that reference to follistatin extends to other non-human forms of the protein encoded by the
25 follistatin gene of that species. For example, other non-human sources of follistatin include bovines, sheep, poultry, porcine, fish, goats and equines. For the purposes of nomenclature only and not limitation exemplary sequences of bovine follistatin are set out in NCBI Reference Sequence ID: NP_786995.2 and SEQ ID NO: 4, equine follistatin are set out in NCBI Reference Sequence ID: NP_001075280.1 and SEQ ID
30 NO: 5, sheep follistatin are set out in GenBank Accession ID: AHH83718.1 and SEQ ID NO: 6, porcine follistatin are set out in NCBI Reference Sequence ID: NP_001003662.1 and SEQ ID NO: 7, chicken follistatin are set out in NCBI Reference Sequence ID: NP_990531.1 and SEQ ID NO: 8 and goat follistatin are set out in GenBank Accession ID: ADJ53357.1 and SEQ ID NO: 9.

35 Reference to a "functional fragment" of follistatin should be understood as a reference to a fragment of follistatin which exhibits follistatin functionality.

Forms of follistatin and functional fragments thereof suitable for use in the present disclosure will be apparent to the skilled person and include, for example:

(i) Wild-type follistatin (FS), comprising an N-terminal domain (ND) followed by three follistatin domains (FSD1, FSD2 and FSD3) with a heparin-binding
5 sequence located in FSD1 (amino acid sequence positions 72-86), and all known isoforms thereof, including FS317 and FS288.

(ii) Wild-type follistatin-like 3 protein (FSTL3), which is also known as follistatin-related gene product (FLRG) and follistatin-related protein (FSRP), comprising an N-terminal domain (N3D) followed by two follistatin-like 3 domains
10 (FS3D1 and FS3D2), and all known isoforms thereof.

(iii) Follistatin analogue having the structure ND-FSD1-FSD2 (i.e. wild-type minus FSD3).

(iv) Analogues of (i) and (iii) above with FSD1 substituted by FSD1', where FSD1' represents FSD1 with heparin-binding site removed.

15 (v) Analogues of (i) and (iii) above with FSD1 substituted by FSD1*, where FSD1* represents FSD1 with sequence prior to and including the heparin-binding sequence removed.

(vi) Hybrid forms of (i) and (iii) above where at least one of the domains is substituted by a corresponding FSTL3 domain N3D, FS3D1 and FS3D2.

20 (vii) Hybrid forms of (ii) above where at least one of the domains is substituted by a corresponding FS domain ND, FSD1, FSD1', FSD1* and FSD2.

(viii) Any of the above proteins modified by one or more deletions, insertions and/or mutations in ND, N3D, FSD1, FSD1', FSD1*, FS3D1, FSD2, FS3D2, and FSD3.

25 (ix) Genetically modified or codon optimized forms of follistatin.

In one example, increasing the level of follistatin protein in the subject modulates (i.e., increases or decreases) the level of a further protein of the TGFβ signalling network. For example, the further protein of the TGFβ signalling network is selected from the group consisting of GDF8 (Myostatin), GDF11 (BMP11), BMP2,
30 BMP4, BMP6, BMP7, BMP15 (GDF9B), Activin A, Activin B, SMAD1, SMAD3, SMAD5.

Protein based Agents

In one example of the any method described herein, the follistatin or functional
35 fragment thereof that increases the level of follistatin protein is a protein based agent.

As discussed herein, the follistatin proteins of the present disclosure can take various forms. An exemplary follistatin protein of the present disclosure binds or specifically binds to a follistatin protein. For example, the follistatin protein is a recombinant protein, such as a recombinant human protein. Exemplary proteins are
5 discussed herein.

Proteins Comprising Antibody Variable Regions

In one example of the any method described herein, the follistatin or functional fragment thereof is a protein comprising an antibody variable region that binds to or
10 specifically binds to a follistatin protein and increases the level of the follistatin protein.

In one example of any method described herein, the follistatin or functional fragment thereof is an antibody mimetic. For example, the follistatin or functional fragment thereof is a protein comprising an antigen binding domain of an immunoglobulin, e.g., an IgNAR, a camelid antibody or a T cell receptor.

15 In one example of any method described herein, the follistatin or functional fragment thereof is a domain antibody (e.g., comprising only a heavy chain variable region or only a light chain variable region that binds to follistatin or binding protein thereof) or a heavy chain only antibody (e.g., a camelid antibody or an IgNAR) or variable region thereof.

20 In one example of any method described herein, the follistatin or functional fragment thereof is a protein comprising a Fv. For example, the follistatin or functional fragment thereof is selected from the group consisting of:

(i) a single chain Fv fragment (scFv);
(ii) a dimeric scFv (di-scFv); or
25 (iv) a diabody;
(v) a triabody;
(vi) a tetrabody;
(vii) a Fab;
(viii) a F(ab')2;
30 (ix) a Fv; or
(x) one of (i) to (ix) linked to a constant region of an antibody, Fc or a heavy chain constant domain (CH) 2 and/or CH3.

In another example of any method described herein, the follistatin or functional fragment thereof is an antibody. Exemplary antibodies are full-length and/or naked
35 antibodies.

In one example of any method described herein, the follistatin or functional fragment thereof is a protein that is recombinant, chimeric, CDR grafted, humanized, synhumanized, primatized, deimmunized, human, sheep, bovine, porcine, goat, equine, or an aquatic or avian species.

5 As will be apparent to the skilled artisan, a sequence encoded by a nucleic acid includes all variants of that sequence that may be produced during expression.

In another example, an antibody or protein comprising a variable region thereof is produced using a standard method, e.g., as is known in the art.

10 *Gene Therapy*

In one example of the present disclosure, the follistatin or functional fragment thereof takes the form of genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule encoding the follistatin protein. The vector may, for example, be a viral vector or a non-viral vector.

15 Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as *gene therapy*, are disclosed in *Gene Transfer into Mammalian Somatic Cells in vivo*, N. Yang, Crit. Rev. Biotech. 12(4):335-356 (1992), which is hereby incorporated by reference.

Gene transfer methods for gene therapy fall into three broad categories: physical 20 (e.g., electroporation, direct gene transfer and particle bombardment), chemical (lipid-based carriers, or other non-viral vectors) and biological (virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. Additionally, vectors or the "naked" DNA of the gene 25 may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

Gene therapy methodologies can also be described by delivery site. Fundamental ways to deliver genes include *ex vivo* gene transfer, *in vivo* gene transfer, and *in vitro* gene transfer.

30 Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, to ferry the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, may be used to cross the cell membrane and provide the DNA into the interior of the cell. Another chemical method may include receptor-based endocytosis, which involves 35 binding a specific ligand to a cell surface receptor and enveloping and transporting it across the cell membrane.

Many gene therapy methodologies employ viral vectors such as retrovirus vectors to insert genes into cells. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors may be selected from the group including, but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV 40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors are the most widely utilized gene transfer vectors and are preferred. Adenoviral vectors may be delivered bound to an antibody that is in turn bound to collagen coated stents.

Mechanical methods of DNA delivery may be employed and include, but are not limited to, fusogenic lipid vesicles such as liposomes or other vesicles for membrane fusion, lipid particles of DNA incorporating cationic lipid such as lipofectin, polylysine-mediated transfer of DNA, direct injection of DNA, such as microinjection of DNA into germ or somatic cells, pneumatically delivered DNA-coated particles, such as the gold particles used in a "gene gun", inorganic chemical approaches such as calcium phosphate transfection and plasmid DNA incorporated into polymer coated stents. Ligand-mediated gene therapy may also be employed involving complexing the DNA with specific ligands to form ligand-DNA conjugates, to direct the DNA to a specific cell or tissue.

The DNA of the plasmid is preferably designed not to integrate into the genome of the cells. Non-integration of the transfected DNA would allow the transfection and expression of gene product proteins in terminally differentiated, non-proliferative tissues for a prolonged period of time without fear of mutational insertions, deletions, or alterations in the cellular or mitochondrial genome. Long-term, but not necessarily permanent, transfer of genes into specific cells may provide a useful option for prophylactic use. The DNA could be reinjected periodically to maintain the gene product level without mutations occurring in the genomes of the recipient cells. Non-integration of exogenous DNAs may allow for the presence of several different exogenous DNA constructs within one cell with all of the constructs expressing various gene products.

It will be appreciated that expression of the genes once delivered can be effected and controlled in a number of different ways, such as:

- Using optogenetics to control gene expression, wherein the vector comprises a control sequence encoding light-responsive proteins, which can be switched on/off using defined wavelengths;
- Using vectors comprising control sequences that are induced by non-immunosuppressive derivatives of rapamycin (rapalogs) which allow simultaneous control of expression and exocytosis of secreted therapeutic polypeptides; and
- Using vectors comprising heat shock proteins which are overexpressed in response to heat and/or stress signals (e.g., ultrasound), thereby switching on and off expression of the genes.

Methods of Administration

The present disclosure provides methods of increasing muscle mass in a subject comprising administering to the subject follistatin or functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level follistatin protein in the subject compared to a control level, wherein the increased level is normalised before the onset of the increase in muscle mass.

The period of administration of the follistatin or functional fragment thereof is sufficient to increase the level of the follistatin protein in the subject compared to a control level. For example, the period of administration of the follistatin or functional fragment thereof is sufficient to increase (e.g., transiently increase) the level of follistatin protein at least 1.5 fold greater or 2 fold greater or 3 fold greater or 4 fold greater or 5 fold greater than the control level. In one example, the follistatin or functional fragment thereof is administered for a period of between about 5 minutes to about 45 days. In one example, the follistatin or functional fragment thereof is administered for a period of about 5 minutes to about 1 hour. For example, the follistatin or functional fragment thereof is administered for a period of about 5 minutes, or about 10 minutes, or about 20 minutes, or about 30 minutes, or about 40 minutes, or about 50 minutes or about 1 hour. In another example, the follistatin or functional fragment thereof is administered for a period of about 1 hour to about 12 hours. For example, the follistatin or functional fragment thereof is administered for a period of about 1 hour, or about 1.5 hours, or about 2 hours, or about 2.5 hours, or about 3 hours, or about 3.5 hours, or about 4 hours, or about 4.5 hours, or about 5 hours, or about 5.5 hours, or about 6 hours, or about 7 hours, or about 8 hours, or about 9 hours, or about 10 hours, or about 11 hours, or about 12 hours. In a further example,

the follistatin or functional fragment thereof is administered for a period of about 12 hours to about 24 hours. For example, the follistatin or functional fragment thereof is administered for a period of about 12 hours, or about 13 hours, or about 14 hours, or about 15 hours, or about 16 hours, or about 17 hours, or about 18 hours, or about 19 hours, or about 20 hours, or about 21 hours, or about 22 hours, or about 23 hours, or about 24 hours. In another example, the follistatin or functional fragment thereof is administered for a period of about 1 day to about 7 days. For example, the follistatin or functional fragment thereof is administered for a period of about1 day, or about 2 days, or about 3 days, or about 4 days, or about 5 days, or about 6 days, or about 7 days. In one example, the follistatin or functional fragment thereof is administered for a period of. about 7 days minimally, but may be administered for 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, 24 days, 25 days, 26 days, 27 days, 28 days, 29 days, 30 days, 31 days, 32 days, 33 days, 34 days, 35 days, 36 days, 37 days, 38 days, 39 days, 40 days, 41 days, 42 days, 43 days, 44 days and 45 days.

The present disclosure provides a method of increasing muscle mass in a subject, comprising administering to the subject follistatin or functional fragment thereof that increases the level of a follistatin protein in the subject compared to a control level, wherein the increased level of follistatin protein is normalised before the onset of an increase in muscle mass.

In one example, the increased level of follistatin protein is normalised to a level of the follistatin protein in the subject before administration of the follistatin or functional fragment thereof, and prior to the onset of an increase in muscle mass. For example, the level of the follistatin protein is normalized to a baseline level.

In one example, the increased level of the follistatin protein is normalised about 1 week to about 6 weeks after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of an increase in muscle mass. For example, the increased level of the follistatin protein is normalised at least 20 days after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of an increase in muscle mass.

In some examples of the present disclosure, the increased level of the follistatin protein is normalised following a period after the initial increase or decrease in the level of the follistatin protein. Reference to the “initial” increase should be understood as a reference to the time point when the level is first increased in the subject. Without limiting the present disclosure, and in terms of reference to “increasing”, it should be understood that the levels are not necessarily maintained at a constant increased level

from the point in time of first increase to the time at which the level is normalised. For example, the follistatin or functional fragment thereof may be administered as a series of doses, which maintain a constant level until such time as normalisation is effected. Alternatively, there may be a single dose administration which is metabolised over time 5 and, after the initial increase in level, gradually decreases over a period of time until normalisation is achieved. Alternatively, administration of repeated doses of the follistatin or functional fragment thereof where levels are initially increased then decrease somewhat between dosage administrations may also be used. Accordingly, the increase in level may be a reference to a constant and continuous increased level 10 (such as can be achieved via the use of an inducible gene expression system which is switched on) or it may be single or multiple doses of the follistatin or functional fragment thereof to the subject, which may or may not maintain a consistent level in the patient.

It should also be understood that the increase in the level the follistatin protein 15 may be a local increase or a systemic increase. Where it is desirable to effect an increase in muscle mass in a discrete muscle, the follistatin or functional fragment thereof may be administered locally to the muscle in issue. However, if it is sought to achieve an overall increase in muscle mass for the subject then a systemic increase in the level of follistatin protein is more appropriate. It is well within the skill of the 20 person in the art to determine how best to proceed.

Accordingly, in one example there is provided a method of increasing overall muscle mass in a subject the method comprising administering follistatin or functional fragment thereof to increase the systemic level of follistatin protein in the subject wherein the increased level of follistatin protein is normalised before the onset of an 25 increase in muscle mass is observed.

In another example there is provided a method of increasing the mass of an isolated muscle in a subject the method comprising administering follistatin or functional fragment thereof, wherein the local level of follistatin protein in the muscle is increased compared to a control level and wherein the increased level is normalised 30 before the onset of an increase in muscle mass.

In terms of increasing the level follistatin protein it is preferable to know the background level of follistatin in the subject, relative to which the level of protein is increased and thereafter to which it is normalised. Methods of determining the level of follistatin protein in the subject will be apparent to the skilled person or are described 35 herein. In one example, the level of follistatin protein is normalised to a level in the

subject before administration of the agent. In another example, the level of follistatin protein is normalised to a background or reference level.

The "normal", "reference", "background" or "steady state" level may be determined using an appropriate biological sample which has been isolated or derived from the subject prior to treatment in accordance with the method of the present disclosure. However, it would be appreciated that it is likely to be most convenient to assess the levels relative to a standard level which reflects individual or collective results obtained from healthy individuals. The standard results which provide the normal level may be calculated by any suitable means which would be well known to the person of skill in the art. For example, a population of normal biological samples can be assessed in terms of the level of expression of the nucleic acid or protein thereby providing a standard value or range of values against which any future levels are assessed. It should also be understood that the normal level may be determined from the subjects of a specific cohort and for use with respect to test samples derived from that cohort. Accordingly, there may be determined a number of standard values or ranges which correspond to cohorts which differ in respect of characteristics such as age, gender, ethnicity or health status. Said "normal level" may be a discrete level or a range of levels.

Treating or Preventing a Wasting Disorder by Increasing Muscle Mass

As detailed hereinbefore, the applications for the method of the present disclosure are significant. Methods of the present disclosure (i.e., methods of increasing muscle mass) may be useful in the treatment or prevention of a wasting disorder associated with a condition. For example, in addition to treating conditions associated with a wasting disorder (such as cachexia), such as cancer and Alzheimer's disease, there is also enabled the more generalised treatment of:

- patients in whom an increase in muscle mass will assist in minimising the onset and/or progression of frailty;
- premature babies;
- low birth weight and/or failure to thrive babies;
- the chronically ill;
- AIDS patients;
- skeletal muscle atrophy, such as that induced by congestive heart disease or liver disease;
- physical injury or congenital physical defects;

- as a prophylactic treatment to individuals (even healthy individuals) at risk of cachexia or skeletal muscle atrophy such as newly diagnosed cancer patients, middle aged individuals wanting to slow the aging process or astronauts.

Beyond traditional prophylactics and therapeutics, the method of the present disclosure also has significant application to the livestock industry where increasing muscle mass of animals (whether for food or some other reason) is highly sought after.

In another aspect, the present disclosure is directed to a method of increasing muscle mass to treat or prevent a wasting disorder which is associated with a condition, said method comprising administering follistatin or functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein administration increases the level of follistatin protein in the subject compared to a control level, and wherein the increased level of follistatin protein is normalised before the onset of an increase in muscle mass.

In one example, the wasting disorder is selected from the group consisting of unintended body weight loss, cachexia, sarcopenia, muscle wasting, or other form of insufficient weight or body mass.

In one example said cachexia, sarcopenia, muscle wasting or other form of insufficient weight or body mass is associated with a condition selected from the group consisting of age, cancer, metabolic acidosis, infectious disease, diabetes, human immunodeficiency virus (HIV), autoimmune immune deficiency syndrome (AIDS), autoimmune disorders, liver disease (e.g., cirrhosis of the liver), chronic inflammatory disorders, anorexia, heart disease, kidney disease, lung disease, osteoporosis, skeletal muscle disease, motor neuron disease, multiple sclerosis, muscle atrophy, malnutrition, low birth weight, inactivity or disuse, drug toxicity, burns, parasitic infection, trauma, surgery, nerve and vessel damage and neurodegenerative diseases including myasthenia gravis, Guillain-Barré syndrome, Lou Gehrig's disease, muscular dystrophy and spinal-cord injuries.

Screening Assays

Methods of determining an increase in muscle mass in a subject will be apparent to the skilled person and include, for example, methods described herein.

Body Composition

There are numerous methods for assessing an increase in muscle mass. For example, an increase in muscle mass may be determined by assessing an increase in total body mass or an increase in lean body mass (i.e., the mass attributed to non-fat

tissue). Methods for assessing changes in lean body mass by methods that quantitatively estimate body composition are known in the art and include, for example, measuring weight, stature, abdominal circumference, and skinfold measurements. Additional methods include, for example, bioelectrical impedance, 5 dual-energy X-ray absorptiometry, body density, and total body water estimates.

An increase in muscle mass may also be determined by assessing an increase in muscle size, for example, by estimating external measurements (e.g., limb girth) or volumetric analysis (e.g., dual energy x-ray absorptiometry, ultrasound-based imaging, or magnetic resonance imaging).

10

In Vivo Assays

There are numerous *in vivo* assays for assessing muscle mass. For example, the present inventors have made use of a model in which the tibialis anterior muscles of mice are injected with a test compound or control and then the increase in muscle mass 15 is determined over time in the presence or absence of a test compound.

An increase muscle mass may be determined by assessing the cross-sectional area and fibre proportion following a treatment period. Methods for assessing cross-sectional area and fibre proportion are known in the art, e.g., as described in Davey *et al.* (Integrated expression analysis of muscle hypertrophy identifies Asb2 as a negative 20 regulator of muscle mass. JCI Insight. 2016;1(5)). For example, an increase in the mean size of muscle fibers is quantified by performing histological analysis of a muscle biopsy. An increase in the cross-sectional area and/or fibre proportion following the treatment period with the test compound compared to the control is indicative of an increase in muscle mass.

25 Assessing the peak tetanic force or contractile properties of the muscles can also be used to determine an increase in muscle mass. Methods for assessing peak tetanic force are known in the art, e.g., as described in Winbanks *et al.* (Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. J Cell Biol. 2012). Briefly, a series of electrical stimuli are delivered to the 30 muscles motor nerve via percutaneous electrodes, and the tension generated during contraction is recorded via a force transducer (e.g., using 305C-LR; Aurora Scientific). An increase in the peak tetanic force following the treatment period with the test compound compared to the control is indicative of an increase in muscle mass.

Assessing an increase in the force producing capacity of a muscle, or an increase 35 in resistance to fatigue when challenged can be used to determine an increase in muscle mass. Methods of assessing an increase in the force producing capacity are known in

the art and include, for example, measurement of grip strength and arm or leg dynamometer tests.

Assessing an increase in the fractional protein synthesis rate or the fractional protein breakdown can be used to determine an increase in muscle mass. Methods of assessing protein synthesis/breakdown are known in the art and include, for example, measuring protein incorporation in a muscle biopsy, blood or urine sample following administration of a protein or amino acid or isotopically labelled water.

Expression Assays

A follistatin or functional fragment thereof that increases muscle mass is identified by contacting a cell with the follistatin or functional fragment thereof and determining the level of follistatin protein. Suitable methods for determining gene expression at the nucleic acid level are known in the art and include, for example, quantitative polymerase chain reaction (qPCR) or microarray assays. Suitable methods for determining expression at the protein level are also known in the art and include, for example, enzyme-linked immunosorbent assay (ELISA), fluorescence linked immunosorbent assay (FLISA), immunofluorescence or Western blotting. It will be apparent to the skilled person that such methods are also suitable for measuring the level of follistatin protein before, during and after administration of the follistatin or functional fragment thereof. For example, such methods are suitable for determining increased levels, control levels, as well as normalised levels of follistatin protein levels in the subject.

Assessing the activity of cellular mechanisms associated with protein synthesis or protein breakdown can be used to determine an increase in muscle mass. For example, changes in the abundance of protein or activated protein state can be determined by methods known in the art, such as protein blotting or quantitative proteomics, or an increase in the transcription of genes encoding for the proteins associated with protein synthesis or protein breakdown.

Pharmaceutical Compositions

The present disclosure provides a method of increasing muscle mass in a subject comprising administration of follistatin or functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein administration increases the level of follistatin protein in the subject compared to a control level, and wherein the level of follistatin protein is normalised prior to the onset of the increase in muscle mass.

The compositions of the present disclosure can be administered in a variety of unit dosage forms depending upon the method of administration. Dosages for typical compositions are well known to those of skill in the art. Such dosages are typically advisory in nature and are adjusted depending on the particular therapeutic context, patient or organ tolerance, etc. The amount of follistatin or functional fragment thereof adequate to accomplish this is defined as a "therapeutically effective dose." The dosage schedule and amounts effective for this use, i.e., the "dosing regimen," will depend upon a variety of factors, including the formulation and concentration of the agent. In calculating the dosage regimen, the mode of administration also is taken into consideration. The dosage regimen must also take into consideration the pharmacokinetics, i.e., the pharmaceutical composition's rate of absorption, bioavailability, metabolism, clearance, and the like. (See, e.g., the latest Remington's; Egleton and Davis 1997 *Peptides* 18:1431-1439; Langer 1990 *Science* 249:1527-1533).

The pharmaceutical composition which comprises the follistatin or functional fragment thereof may be administered by any convenient means and is contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal. A broad range of doses may be applicable. Considering a patient, for example, from about 0.1 mg to about 1 mg of modulatory agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum response. For example, several divided doses may be administered daily, or weekly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation.

The composition may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). It may be administered as a nasal or oral spray or in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the agent is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

Routes of administration include, but are not limited to, respiratorally, intratracheally, nasopharyngeally, intravenously, intraperitoneally, subcutaneously,

intracranially, intradermally, intramuscularly, intraoccularly, intrathecally, intracereberally, intranasally, infusion, orally, rectally, *via* IV drip patch and implant.

In accordance with these methods, the agent defined in accordance with the present disclosure may be coadministered with one or more other compounds or 5 molecules. By "coadministered" is meant simultaneous administration in the same formulation or in two different formulations via the same or different routes or sequential administration by the same or different routes. For example, the subject composition may be administered together with another agent in order to enhance its effects. Alternatively, it may be administered together with another therapeutic agent 10 which is treating the disease condition that has resulted in the weight loss. For example, if the patient being treated suffers from cancer, then it is to be expected that the patient will simultaneously be treated with chemotherapy or radiation therapy, for example. In the context of AIDS, the patient is likely to be undergoing retroviral therapy. By "sequential" administration is meant a time difference of from seconds, 15 minutes, hours or days between the administration of the two types of molecules. These molecules may be administered in any order.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion or may be in 20 the form of a cream or other form suitable for topical application. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), 25 suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal antagonists, for example, parabens, chlorobutanol, phenol, sorbic acid, 30 thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of antagonists delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compound in 35 the required amount in the appropriate solvent with various the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally,

dispersions are prepared by incorporating the various sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are
5 vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

When the active ingredients are suitably protected they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or
10 it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. Such compositions and preparations should contain at
15 least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present disclosure are prepared
20 so that an oral dosage unit form contains between about 0.1 μg and 2000 mg of active compound.

The tablets, troches, pills, capsules and the like may also contain the components as listed hereafter: a binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating antagonist such as corn starch,
25 potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening antagonist such as sucrose, lactose or saccharin may be added or a flavouring antagonist such as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to
30 otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and
35 substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Kits and Other Compositions of Matter

Another example of the present disclosure provides kits containing follistatin or functional fragment thereof useful for increasing muscle mass in a subject according to
5 any method described herein.

In one example, the kit comprises (a) a container comprising follistatin or functional fragment thereof optionally in a pharmaceutically acceptable carrier or diluent; and (b) a package insert with instructions for increasing muscle mass in a subject by administering the follistatin or functional fragment thereof for a period of
10 time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of follistatin protein compared to a control level, and wherein the level of follistatin protein is normalised prior to the onset of the increase in muscle mass.

In accordance with this example of the disclosure, the package insert is on or
15 associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition that is effective for increasing muscle mass and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic
20 injection needle). At least one active agent in the composition is the follistatin or functional fragment thereof. The label or package insert indicates that the composition is used for treating a subject eligible for treatment, e.g., one in need of increasing muscle mass, with specific guidance regarding dosing amounts and intervals of the agent and any other medicament being provided. The kit may further comprise an
25 additional container comprising a pharmaceutically acceptable diluent buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution, and/or dextrose solution. The kit may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

30 The present disclosure is further described by reference to the following non-limiting examples.

EXAMPLES

Example 1: Transient exposure to FST317 results in permanent increases in
35 skeletal muscle mass

Recombinant adeno-associated virus-based vectors (rAAV vectors) were constructed to transduce mouse skeletal muscle with follistatin-expressing constructs as previously described in Winbanks *et al.*, (Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. J Cell Biol. 2012), Chen *et al.*, (Elevated expression of activins promotes muscle wasting and cachexia. FASEB J. 2014;28(4):1711-23) and Davey *et al.*, (Integrated expression analysis of muscle hypertrophy identifies Asb2 as a negative regulator of muscle mass. JCI Insight. 2016;1(5)).

The tibialis anterior muscles of C57Bl/6 mice were injected with a rAAV carrying a tetracycline inducible gene expression construct (rAAV:TetOn) and a TetOn-responsive follistatin 317 construct (rAAV:indFST317), or an empty vector control construct (rAAV:MCS and rAAV:TetOn) in the muscle of the contralateral leg.

Mice were administered doxycycline (at a dose of approximately 1.75mg/mouse/day) as supplemental chow to induce follistatin expression. Mice were maintained on either a doxycycline free diet for 56 days (0/56), a doxycycline diet for 28 days (28/0), or a doxycycline diet for 28 days followed by a doxycycline free diet for 28 days (28/28).

Figure 1 shows the effect of follistatin on the mass of tibialis anterior muscles following the treatment period. Transient treatment with follistatin increased skeletal muscle mass compared to untreated groups. The increase in skeletal muscle mass in follistatin treated groups was also observed following removal of doxycycline treatment. This data demonstrate that transient treatment with follistatin (an inhibitor of activin) induces a permanent increase in skeletal muscle mass.

Example 2: Transient exposure to FST317 results in permanent increases in skeletal muscle fibre size

The cross-sectional area of tibialis anterior muscles treated as described above were examined to determine the effect of follistatin treatment on muscle fibre diameter. Muscle fibre diameter was determined as previously described in Davey *et al.*, (Integrated expression analysis of muscle hypertrophy identifies Asb2 as a negative regulator of muscle mass. *JCI Insight*. 2016; 1(5)). Briefly, muscles were placed in cryomolds containing optimum cutting temperature (Grale Scientific, Victoria, Australia), frozen in liquid nitrogen-cooled isopentane and then stored at -80°C. Haematoxylin and eosin staining was performed as per standard protocols with 10-mm-thick sections and imaged at x400 magnification on an Olympus FSX-100 microscope. Image analysis was performed using the ImageJ software package. For each replicate a

minimum of 300 muscle fibers were measured for calculation of minimum feret diameter.

Histological examination of the cross-sectional area demonstrated that muscle fibre diameter was increased as a consequence of follistatin treatment. This effect was maintained following withdrawal of follistatin treatment.

Follistatin treatment increased the fibre diameter by at least 50% compared with control-treated muscles and this effect was maintained following withdrawal of follistatin treatment. The distribution of changes in fibre diameter was largely conserved across the distribution of muscle fibers as shown in Figure 2.

Example 3: Sub-maximal transient exposure to FST317 results in permanent increases in skeletal muscle mass

Mice were injected with rAAV vectors as described above and maintained on either a doxycycline free diet for 30 days (0/30), a doxycycline diet for 2 days (2/0), a doxycycline diet for 2 days followed by a doxycycline free diet for 28 days (2/28), or a doxycycline diet for 2 days followed by a doxycycline free diet for 84 days (2/84).

Muscles were analysed as previously described in Example 1. Additionally peak tetanic force generated from treated tibialis anterior muscle was analysed as previously described in Winbanks *et al.,* 2012. Briefly, the contractile properties of the mice tibialis anterior muscles were assessed *in situ* by delivering a series of electrical stimuli to the tibial motor nerve via percutaneous electrodes, and recording tension generated during contraction via a force transducer (305C-LR; Aurora Scientific) attached to the distal tendon with surgical silk suture. The mice were placed under tribromoethanol (Sigma-Aldrich) anesthesia before testing, and were humanely killed via cervical dislocation immediately at the conclusion of evaluation, while still deeply anesthetized. At the conclusion of the protocol, muscles were rapidly excised, dissected free of tendon and connective tissue, and weighed. Total muscle cross-sectional area (CSA) was calculated by dividing muscle mass by the product of fiber length and 1.06 mg/mm3, the density of mammalian skeletal muscle. Specific force was determined by normalizing maximum isometric tetanic force to CSA.

As shown in Figure 3 muscles examined after as little as 48 hours of doxycycline feeding exhibited evidence of increased mass and increased contractile properties as evidenced by increased peak tetanic force.

Example 4: FST317 expression is absent upon doxycycline withdrawal

Mice were injected with rAAV vectors as described above and maintained on either a doxycycline free diet for 30 days (0/30), a doxycycline diet for 2 days (2/0), a doxycycline diet for 2 days followed by a doxycycline free diet for 28 days (2/28), or a doxycycline diet for 2 days followed by a doxycycline free diet for 84 days (2/84).

5 The effect of follistatin treatment on protein and mRNA levels was examined in tibialis anterior muscles following the treatment period.

Protein was extracted from treated tibialis anterior muscles and follistatin protein expression determined by immunoblot analysis as previously described in Davey *et al.*, 2016. Briefly, skeletal muscles were lysed in RIPA buffer (25mM Tris-

10 HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), reduced with 2.5% β-mercaptoethanol, supplemented with protease and phosphatase inhibitor cocktail. Protein concentrations were determined using the bicinchoninic acid (BCA®) assay as per manufacturer's instructions (Thermo Fisher Scientific, Victoria, Australia). Proteins were separated and transferred to PVDF membranes (Millipore, Australia)

15 before blocking for 1 hour at room temperature (RT) in 5 % non-fat milk that was diluted in phosphate buffered saline containing 0.1 % Tween-20. Membranes were incubated overnight with primary antibodies (anti-GAPDH (#SC32233) from Santa Cruz Biotechnology, and anti-FST (#AF669) from R&D Systems) followed by incubation for 1 hour at RT with HRP-conjugated conjugated secondary antibodies and

20 detection using ECL detection reagent (GE Healthcare life sciences, Australia).

RNA was extracted from treated tibialis anterior muscles and the relative gene expression of follistatin compared to the contralateral control muscles was determined by RNA-Seq analysis. Briefly, RNA was extracted using standard methodology with TriZol reagent (ThermoFisher Scientific). The extracted RNA was used to generate

25 cDNA libraries from purified mRNA samples according to the manufacturer's instructions (TruSeq Standard RNA sample preparation kit, Illumina CA, USA). Transcriptome libraries were sequenced on an Illumina HiSeq2000 instrument (Illumina CA, USA), with the resulting 50 bp single-end reads mapped to the mouse genome (mm10 assembly) using TopHat. Gene transcript levels were determined via

30 Cuffdiff in the form of FPKM (RPKM) values by correcting for multi reads and using geometric normalization.

As shown in Figure 4A, follistatin protein expression was apparent after 2 days of doxycycline feeding and strongly detectable after 28 days of doxycycline administration. In contrast, follistatin was not observed in mice administered

35 rAAV:TetOn and rAAV:indFST without doxycycline treatment or in mice analysed following withdrawal of doxycycline treatment. Similarly, follistatin mRNA

expression was observed following both acute and chronic follistatin treatment but was undetectable following withdrawal of treatment (Figure 4B).

To determine whether the differences in FST-mediated effects were a consequence of differential FST expression or effects upon SMAD signaling, FST abundance and SMAD3 phosphorylation, TBC1D1 expression and ASB2 expression in response to rAAV:FST administration was examined. Briefly, skeletal muscles were lysed in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS), reduced with 2.5% β-mercaptoethanol, supplemented with protease and phosphatase inhibitor cocktail. Protein concentrations were determined using the bicinchoninic acid (BCA®) assay as per manufacturer's instructions (Thermo Fisher Scientific, Victoria, Australia). Proteins were separated and transferred to PVDF membranes (Millipore, Australia) before blocking for 1 hour at room temperature (RT) in 5 % non-fat milk that was diluted in phosphate buffered saline containing 0.1 % Tween-20. Membranes were incubated overnight with primary antibodies (anti-TBC1D1 (#4629) from Cell Signaling Technologies; anti-Asb2 (#SAB2701121) from Sigma-Aldrich; anti-pSMAD3$^{Ser423/425}$ (ab52903) and anti-SMAD3 (#9513)) followed by incubation for 1 hour at RT with HRP-conjugated conjugated secondary antibodies and detection using ECL detection reagent (GE Healthcare life sciences, Australia).

Follistatin treated muscles exhibited reduced SMAD3 phosphorylation however, this effect was reversed following withdrawal of follistatin treatment. A reduction in expression of the follistatin-responsive genes TBC1D but not ASB2 was also observed following follistatin treatment and shown to be restored following follistatin withdrawal.

These data demonstrate that follistatin expression is absent following withdrawal of treatment despite the permanent increase in muscle mass observed.

Example 5: Transient exposure to FST317 improves power to weight ratio compared to continual FST317 treatment

For transient expression, the tibialis anterior muscles of C57Bl/6 mice were injected with rAAV vectors as described above (i.e., a tetracycline inducible gene expression construct (rAAV:TetOn) and a TetOn-responsive follistatin 317 construct (rAAV:indFST317), or an empty vector control construct (rAAV:MCS and rAAV:TetOn) in the muscle of the contralateral leg) and maintained on a doxycycline diet for 2 days followed by a doxycycline free diet for 28 days (2/28). For continuous expression, the tibialis anterior muscles of C57Bl/6 mice were injected with rAAV:MCS or rAAV:FST317 in the contralateral muscle.

Contractile properties of the muscles were analysed as described above in Example 3. As shown in Figure 5 continuous follistatin treatment causes significant increases in the power output of striated muscle (Fig. 5A) but the power to weight ratio is reduced with continual follistatin treatment compared to untreated baseline muscles (Fig. 5B). Transient follistatin treatment of the tibialis anterior muscle also showed an increase in maximum force produced due to contraction (Fig. 5C). However, in contrast to the reduction in weight ratios with continuous follistatin treatment, transient treatment had no such reduction, and had normal power ratios that were similar to untreated baseline muscles (Fig. 5D).

These data demonstrate that transient follistatin treatment produces functionally improved skeletal muscles compared to muscles continuously treated with follistatin.

Example 6: Transient exposure to FST317 alters gene expression in the tibialis anterior muscle in a unique and permanent way

The tibialis anterior (TA) muscle of C57Bl/6 mice were injected with rAAV:TetON and rAAV:indFST317 or rAAV:TetON and rAAV:MCS in the contralateral muscle as described above and maintained on a doxycycline diet for 2 days followed by a doxycycline free diet for 28 days (2/28).

At the end of the treatment period, mice were humanely culled and the TA muscles excised and frozen in liquid nitrogen. RNA was extracted using standard methodology with TriZol reagent (ThermoFisher Scientific). Extracted RNA was used to generate cDNA libraries from purified mRNA samples according to the manufacturer's instructions (TruSeq Standard RNA sample preparation kit, Illumina CA, USA). Transcriptome libraries were sequenced on an Illumina HiSeq2000 instrument (Illumina CA, USA), with the resulting 50 bp single-end reads mapped to the mouse genome (mm10 assembly) using TopHat. Gene transcript levels were determined via Cuffdiff in the form of FPKM (RPKM) values by correcting for multi reads and using geometric normalization. Statistical analysis was performed in the Perseus software program. Comparisons between gene expression profiles were made with TA muscles with permanently increased follistatin levels described in Davey *et al*, (2016). Significant changes in gene expression were determined using t-tests corrected for multiple comparisons by permutation Bonferroni-Hochberg false discovery rate.

As shown in Figure 6, using RNA-seq gene expression profiling 125 genes were shown to be differentially expressed after transient follistatin treatment compared to untreated muscles. Changes in gene expression were compared to the gene expression profile of skeletal muscle that is continually treated with follistatin (Fig. 6A). As shown

in Figure 6B, the expression profiles diverge between treatment groups and differentially regulated genes could be sorted into three categories: (1) genes that were unique to continuous treatment with follistatin (557 genes); (2) genes that were unique to transient follistatin treatment (58 genes); and (3) genes that were differentially
5 expressed in both treatments (67 genes).

These data show that transient treatment with follistatin results in a gene expression profile that is distinct and unique from continuous follistatin treatment.

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CLAIMS

1. A method of increasing muscle mass in a subject, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, and wherein the increased level of the protein is normalised before the onset of the increase in muscle mass.

2. The method of claim 1, wherein the level of the follistatin protein in the subject is increased to a level of at least 1.5 fold greater or 2 fold greater or 3 fold greater or 4 fold greater or 5 fold greater than the control level, prior to normalisation of the level of the follistatin protein and the onset of the increase in muscle mass.

3. The method of claim 1 or 2, wherein the increased level of follistatin protein is normalised about 1 week to about 6 weeks after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

4. The method of any one of claims 1 to 3, wherein the increased level of the follistatin protein is normalised at least 20 days after administration of the follistatin or functional fragment thereof to the subject, and prior to the onset of the increase in muscle mass.

5. The method of any one of claims 1 to 4, wherein the follistatin or functional fragment thereof is administered in the form of a protein or vector comprising a nucleic acid encoding follistatin or functional fragment thereof, which is expressed *in vivo*.

6. The method of any one of claims 1 to 5, wherein the method comprises increasing the systemic level of the follistatin protein in the subject.

7. The method of any one of claims 1 to 5, wherein the method comprises increasing the local level of the follistatin protein in the subject.

8. The method of any one of claims 1 to 5, wherein the method comprises increasing the absolute level of the follistatin protein in the subject.

9. The method of any one of claims 1 to 8, wherein the muscle is skeletal muscle, smooth muscle or cardiac muscle.

5 10. The method of any one of claims 1 to 9, wherein the subject is suffering from or is at risk of developing a wasting disorder which is associated with a condition.

11. A method of increasing muscle mass in a subject to treat or prevent a wasting disorder which is associated with a condition, the method comprising administering to 10 the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, and wherein the increased level of the the follistatin protein is normalised before the onset of the increase in muscle mass.

15

12. The method of claim 10 or 11, wherein the wasting disorder is selected from the group consisting of unintended body weight loss, cachexia, sarcopenia, muscle wasting, or other form of insufficient weight or body mass.

20 13. The method of any one of claims 10 to 12, wherein the condition is selected from the group consisting of age, cancer, metabolic acidosis, infectious disease, diabetes, human immunodeficiency virus (HIV), autoimmune immune deficiency syndrome (AIDS), autoimmune disorders, liver disease (e.g., cirrhosis of the liver), chronic inflammatory disorders, anorexia, heart disease, kidney disease, lung disease, 25 osteoporosis, skeletal muscle disease, motor neuron disease, multiple sclerosis, muscle atrophy, malnutrition, low birth weight, inactivity or disuse, drug toxicity, burns, parasitic infection, trauma, surgery, nerve and vessel damage and neurodegenerative diseases including myasthenia gravis, Guillain-Barré syndrome, Lou Gehrig's disease, muscular dystrophy and spinal-cord injuries.

30

14. A method of increasing muscle mass in a subject to treat or prevent cachexia, the method comprising administering to the subject follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the 35 subject compared to a control level, and wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

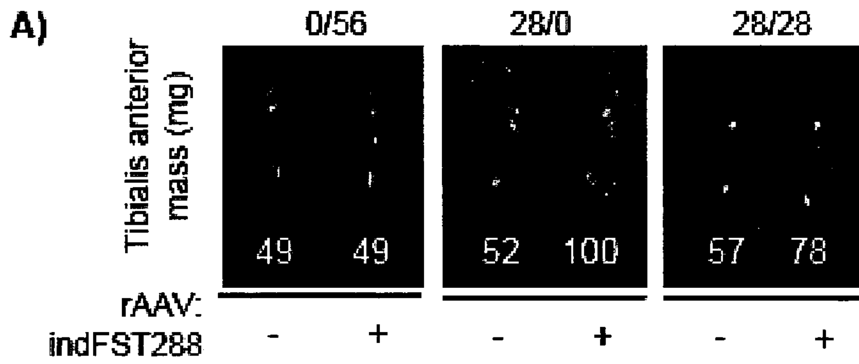
15. A method of increasing muscle mass in a livestock, the method comprising administering follistatin or a functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the livestock compared to a control level, and wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

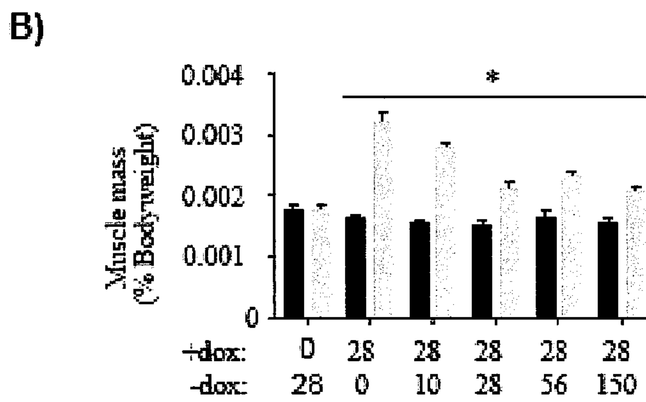
16. Use of follistatin or a functional fragment thereof in the manufacture of a medicament for increasing muscle mass in a subject, the method comprising administering to the subject the follistatin or functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of follistatin protein in the subject compared to a control level and wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

17. Use of follistatin or a functional fragment thereof in the manufacture of a medicament for increasing muscle mass in a subject to treat or prevent a wasting disorder which is associated with a condition, the method comprising administering to the subject the follistatin or functional fragment thereof for a period of time and in an amount sufficient to effect an increase in muscle mass, wherein the administration increases the level of a follistatin protein in the subject compared to a control level, and wherein the increased level of the follistatin protein is normalised before the onset of the increase in muscle mass.

18. The method of any one of claims 1 to 14 or the use of any one of claims 16 or 17, wherein the subject is selected from the group consisting of a human and livestock.

19. The method or use of claim 15 or 18, wherein the livestock is selected from the group consisting of a bovine (i.e., cattle), a sheep, a porcine (i.e., pig), an equine (i.e., horse), a deer, a goat, an aquatic species (e.g., fish) and an avian species (e.g., poultry).





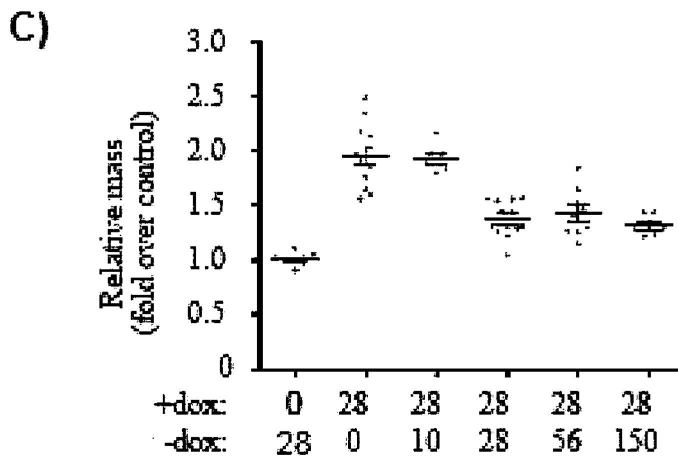


FIGURE 1

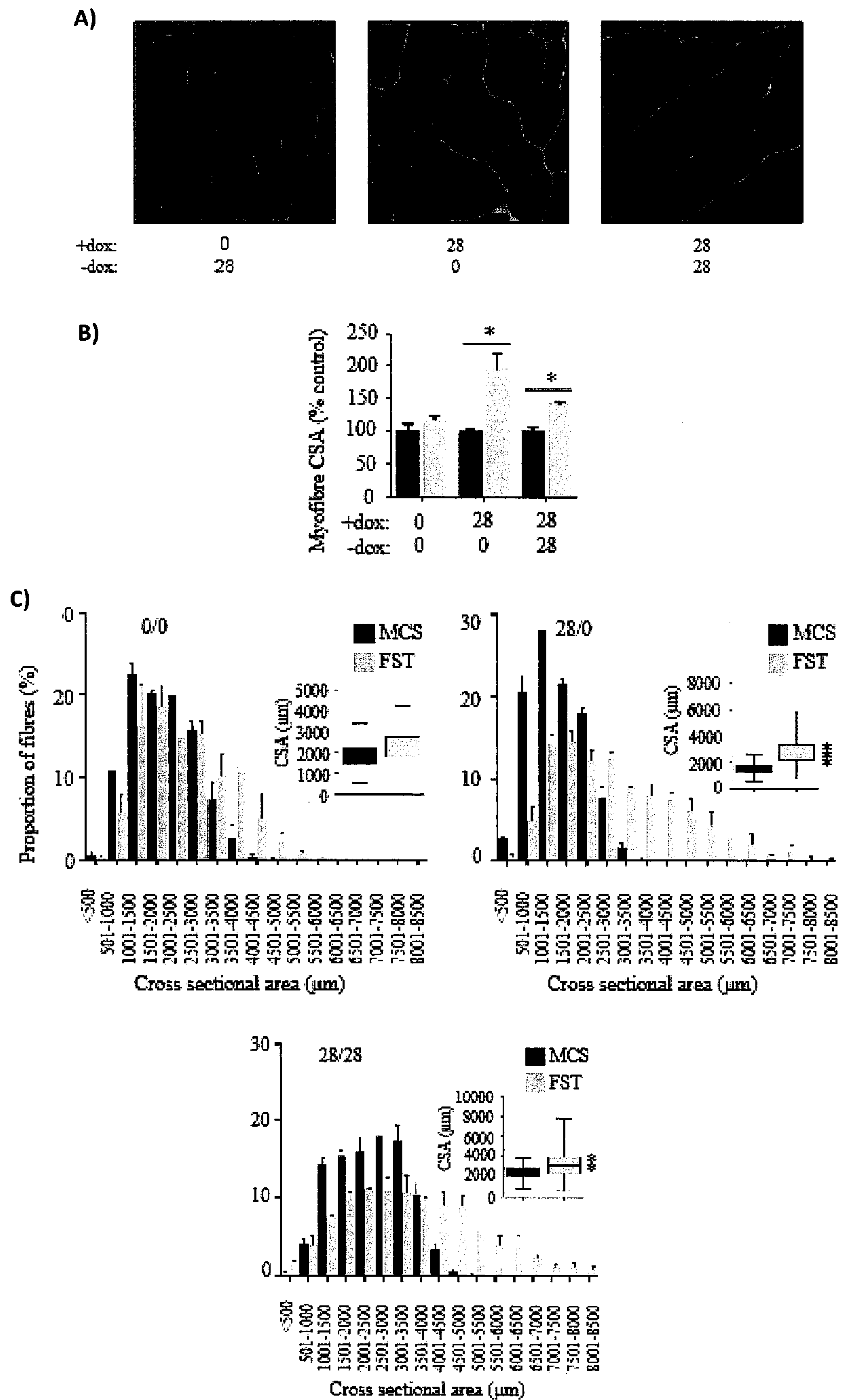


FIGURE 2

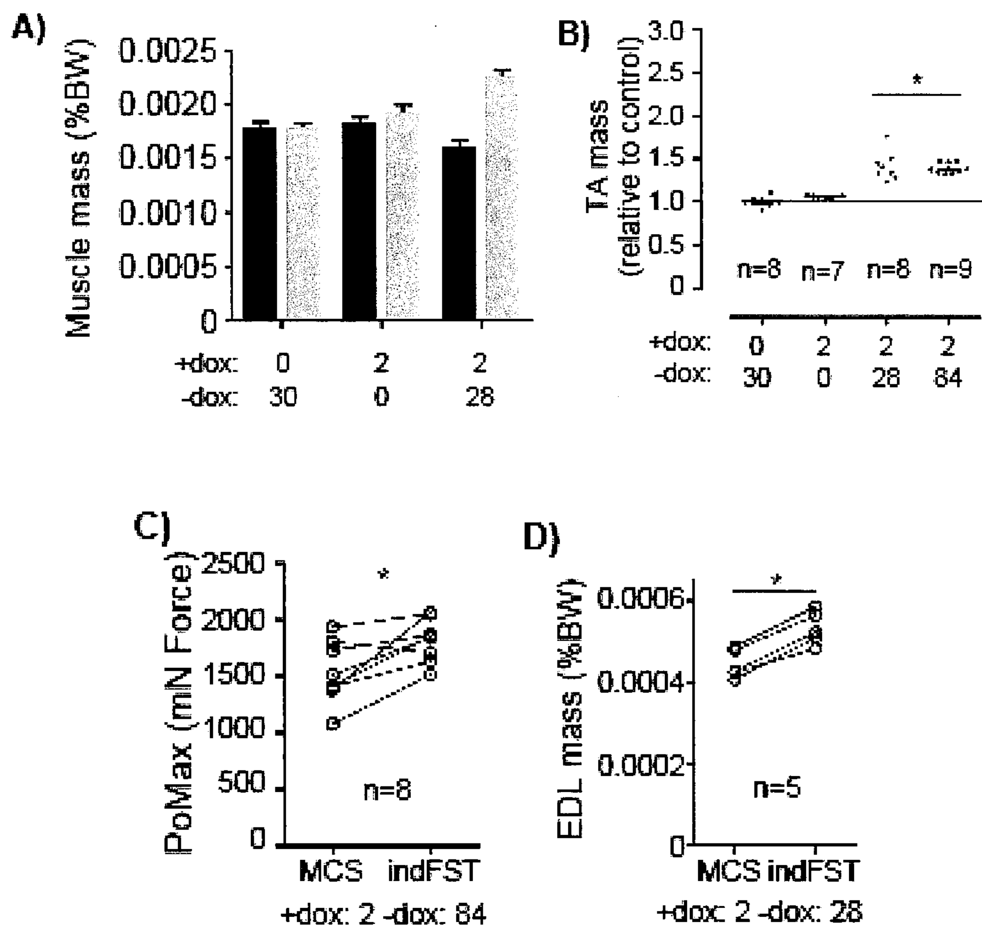
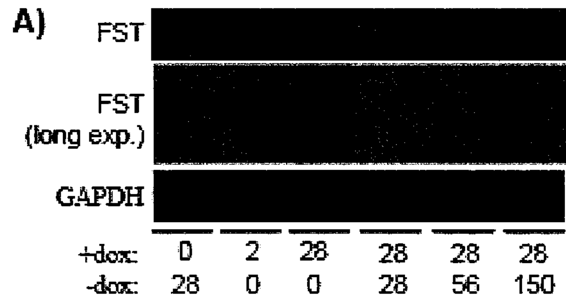
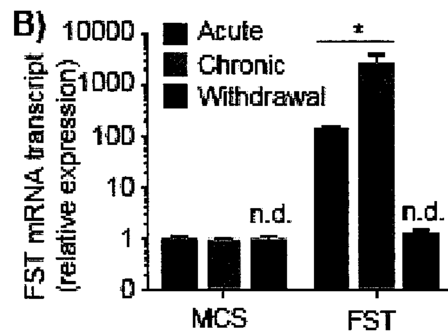


FIGURE 3





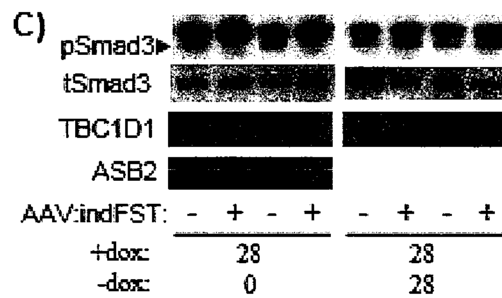


FIGURE 4

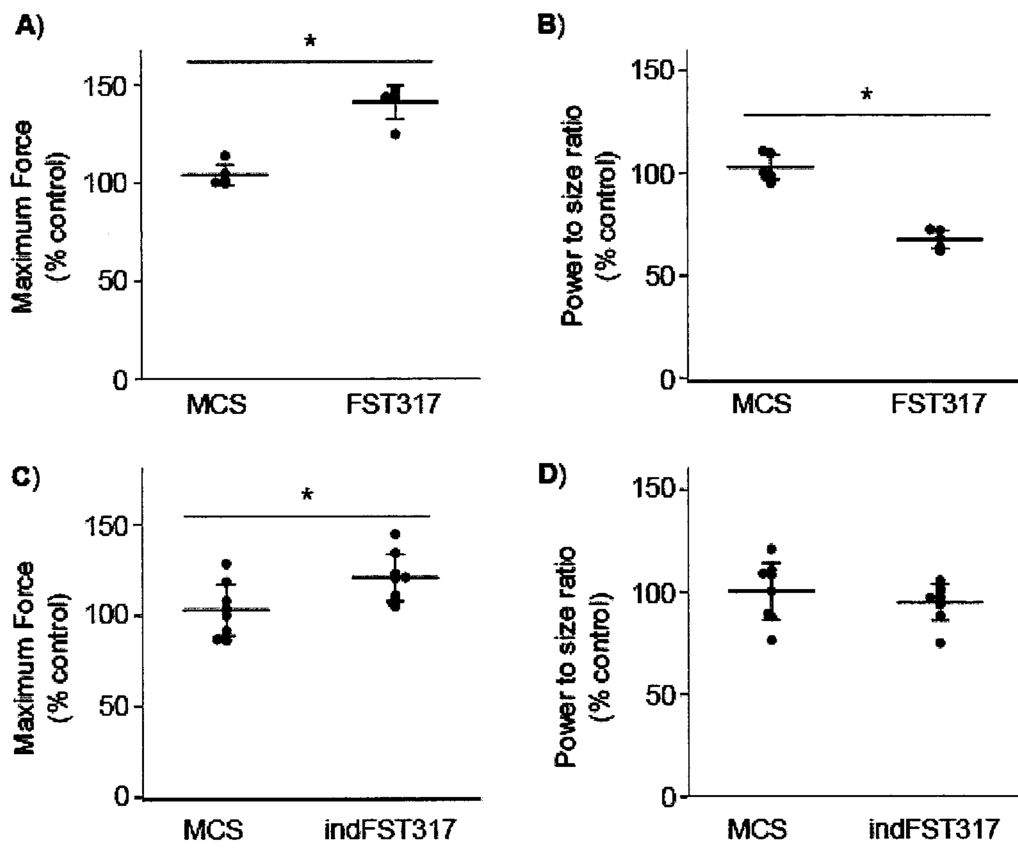
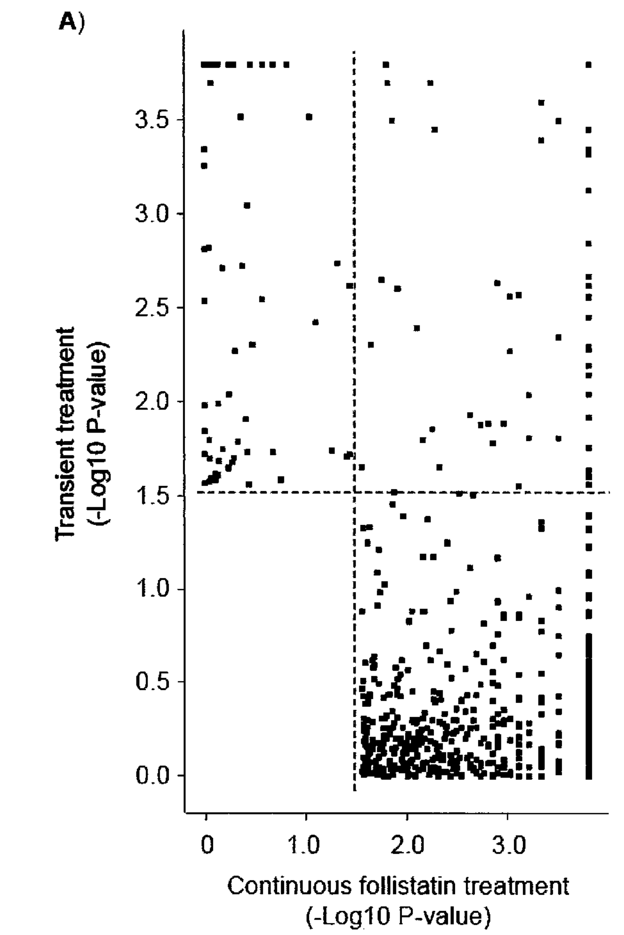


FIGURE 5

A)



B)

Continuous follistatin treatment: 557 exclusive genes
Transient follistatin treatment: 58 exclusive genes
Common genes: 67 genes
P-value <0.05

FIGURE 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2018/050973

A. CLASSIFICATION OF SUBJECT MATTER

A61K 38/17 (2006.01) A61K 48/00 (2006.01) A61P 21/06 (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)

PATENW (Epoque) CAPLUS, BIOSIS, EMBASE, MEDLINE CABA FSTA AGRICOLA: Keywords: Follistatin, activin binding protein, fsh suppressing protein, FS(T)_288, FS(T)_300, FS(T)_315, FS(T)_317, FS(T)_344, muscle increase, muscle wasting, atrophy, hypertrophy, regeneration, cachexia, sarcopenia, senescence, livestock, anabolic, author; Gregorevic, P. Davey, J. and like terms. Applicant(s)/Inventor(s) name searched in internal databases provided by IP Australia

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
- "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
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- "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
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Date of the actual completion of the international search	Date of mailing of the international search report
23 October 2018	23 October 2018

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INTERNATIONAL SEARCH REPORT	International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/AU2018/050973**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0275036 A1 (GREEN III et al.) 29 November 2007 Abstract, paragraphs 5, 10, 170-172	1-19
X	WO 2009/137880 A1 (AGRICULTURE VICTORIA SERVICES PTY LTD et al.) 19 November 2009 page 1, lines 1-13; page 6, lines 1-6 &21-33; page 8, line 22- page 9 line 2; Claims 2, 13, 17-19	1-19
X	WO 2003/053219 A2 (VOYAGER PHARMACEUTICAL CORPORATION) 03 July 2003 Paragraphs, 13, 32, 50, 51	1-19
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X	WO 2008/030367 A2 (THE GENERAL HOSPITAL CORPORATION et al.) 13 March 2008 Page 2, lines 7-15; page 50, lines 15-27	1-19
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X	Yaden, B.C. et al. 'Follistatin: A Novel Therapeutic for the Improvement of Muscle Regeneration ' The Journal of Pharmacology and Experimental Therapeutics, 2014, vol. 349 pages 355-371 Page 357 right hand column, paragraph under results section; page 358, right hand column- page 359 left hand column, first paragraph, figure 2	1-5, 7-19
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/AU2018/050973**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Abstract; page 27, right hand column, first paragraph under the results section; page 33, left hand column, first paragraph	1-19
T	WO 2018/176065 A1 (OREGON STATE UNIVERSITY) 27 September 2018 Pages 1 and 2	1-19

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:

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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:

   A sequence was filed with this application but was not used for the purpose of establishing this International Search Report and Opinion

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2018/050973**

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.

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