BINDING AGENTS WHICH INHIBIT MYOSTATIN

This application hereby claims benefit of United States provisional application serial number 60/435,923, filed December 20, 2002, the entire disclosure of which is relied upon and incorporated by reference.

FIELD OF THE INVENTION

The invention relates to growth factors and in particular to the growth factor myostatin and agents which bind myostatin and inhibit its activity.

BACKGROUND

Myostatin, also known as growth/differentiation factor 8 (GDF-8), is a transforming growth factor-ß (TGF-ß) family member known to be involved in regulation of skeletal muscle mass. Most members of the TGF-ß-GDF family are expressed non-specifically in many tissue types and exert a variety of pleitrophic actions. However, myostatin is largely expressed in the cells of developing and adult skeletal muscle tissue and plays an essential role in negatively controlling skeletal muscle growth (McPherron et al. *Nature* (London) **387**, 83-90 (1997)). Recent studies, however, indicate that low levels of myostatin expression can be measured in cardiac, adipose and pre-adipose tissues.

The myostatin protein has been highly conserved evolutionarily (McPherron et al. *PNAS USA* **94**:12457-12461 (1997)). The biologically active C-terminal region of myostatin has 100 percent sequence identity between human, mouse, rat, cow, chicken, and turkey sequences. The function of myostatin also appears to be conserved across species as well. This is evident from the phenotypes of animals having a mutation in the myostatin gene. Two breeds of cattle, the Belgian Blue (Hanset R., *Muscle Hypertrophy of Genetic Origin and its Use to Improve Beef Production*, eds, King, J.W.G. & Menissier, F. (Nijhoff, The Hague, The Netherlands) pp. 437-449) and the Piedmontese (Masoero, G. & Poujardieu, B, *Muscle Hypertrophy of Genetic Origin and its Use to Improve Beef Production.*, eds, King, J.W.G. & Menissier, F. (Nijhoff, The Hague,The Netherlands) pp. 450-459) are characterized by a "double muscling" phenotype and increase in muscle mass. These breeds were shown to contain mutations in the coding region of the myostatin gene (McPherron et al.(1997) *supra*). In addition, mice containing a targeted deletion of the gene encoding myostatin (Mstn) demonstrate a dramatic increase in muscle mass without a corresponding increase in fat. Individual muscles of Mstn $^{-/-}$ mice weigh approximately 100 to 200 percent more than those of control animals as a result of muscle fiber hypertrophy and hyperplasia (Zimmers et al. *Science* **296**, 1486 (2002)).

Administration of myostatin to certain strains of mice has been shown to create a condition similar to muscle wasting disorders found associated with cancer, AIDS, and muscular dystrophy, for example. Myostatin administered as myostatin-producing CHO cells to athymic nude mice resulted in a wasting effect with a high degree of weight loss, a decrease of as much as 50% of skeletal muscle mass in addition to fat wasting, and severe hypoglycemia (Zimmers et al. *supra*).

Loss of myostatin appears to result in the retention of muscle mass and reduction in fat accumulation with aging. It has been shown that age-related increases in adipose tissue mass and decrease in muscle mass were proportional to myostatin levels, as determined by a comparison of fat and muscle mass in Mstn $^{+/+}$ when compared with Mstn $^{-/-}$ adult knockout mice (McFerron et al. *J. Clin. Invest* **109**, 595 (2002)). Mstn $^{-/-}$ mice showed decreased fat accumulation with age compared with Mstn $^{+/+}$ mice.

In addition myostatin may play a role in maintaining blood glucose levels and may influence the development of diabetes in certain cases. It is known that, for example, skeletal muscle resistance to insulin-stimulated glucose uptake is the earliest known manifestation of non-insulin-dependent (type 2) diabetes mellitus (Corregan et al. *Endocrinology* **128**:1682 (1991)). It has now been shown that the lack of myostatin partially attenuates the obese and diabetes phenotypes of two mouse models, the agouti lethal yellow (A^y) (Yen et al. *FASEB J.* **8**:479 (1994)), and obese ($Lep^{ob/ob}$). Fat accumulation and total body weight of the $A^{y/a}$, Mstn $^{-/-}$ double mutant mouse was dramatically reduced compared with the $A^{y/a}$ Mstn $^{+/+}$ mouse (McFerron et al., (2002) *supra*). In addition, blood glucose levels in the $A^{y/a}$, Mstn $^{-/-}$ mice was dramatically lower than in $A^{y/a}$ Mstn $^{+/+}$ mice following exogenous glucose load, indicating that the lack of myostatin improved glucose metabolism. Similarly $Lep^{ob/ob}$ Mstn $^{-/-}$ mice showed decreased fat accumulation when compared with the $Lep^{ob/ob}$ Mstn $^{+/+}$ phenotype.

Therefore, there is considerable evidence from the phenotypes of over-expressing and knockout animals that myostatin may play a role in contributing to a number of metabolic disorders including disorders resulting in muscle wasting, diabetes, obesity and hyperglycemia.

SUMMARY OF THE INVENTION

The present invention is directed to binding agents which bind myostatin and inhibit its activity. The binding agents comprise at least one peptide capable of binding myostatin. The myostatin-binding peptides are preferably between about 5 and about 50 amino acids in length, more preferably between about 10 and 30 amino acids in length, and most preferably between about 10 and 25 amino acids in length. In one embodiment the myostatin-binding peptide comprises the amino acid sequence WMCPP (SEQ ID NO: 633). In another embodiment the

myostatin binding peptides comprise the amino acid sequence $\underline{C}a_1a_2\underline{W}a_3\underline{WMCPP}$ (SEQ ID NO: 352), wherein a_1, a_2 and a_3 are selected from a neutral hydrophobic, neutral polar, or basic amino acid. In another embodiment the myostatin binding peptide comprises the sequence $\underline{C}b_1b_2\underline{W}b_3\underline{WMCPP}$ (SEQ ID NO: 353), wherein b_1 is selected from any one of the amino acids T, I, or R; b_2 is selected from any one of R, S, Q; b_3 is selected from any one of P, R and Q, and wherein the peptide is beween 10 and 50 amino acids in length, and physiologically acceptable salts thereof. In another embodiment, the myostatin binding peptide comprises the formula:

$c_1c_2c_3c_4c_5c_6\underline{C}c_7c_8\underline{W}c_9\underline{WMCPP}c_{10}c_{11}c_{12}c_{13}$ (SEQ ID NO: 354), wherein:

c_1 is absent or any amino acid;
c_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_4 is absent or any amino acid;
c_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
c_7 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_8 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_9 is a neutral hydrophobic, neutral polar or basic amino acid; and
c_{10} to c_{13} is any amino acid; and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof.

A related embodiment the myostatin binding peptide comprises the formula:

$d_1d_2d_3d_4d_5d_6\underline{C}d_7d_8\underline{W}d_9\underline{WMCPP}$ $d_{10}d_{11}d_{12}d_{13}$ (SEQ ID NO: 355), wherein

d_1 is absent or any amino acid;
d_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_4 is absent or any amino acid;
d_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
d_7 is selected from any one of the amino acids T, I, or R;
d_8 is selected from any one of R, S, Q;
d_9 is selected from any one of P, R and Q, and
d_{10} to d_{13} is selected from any amino acid,

and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof.

Additional embodiments of binding agents comprise at least one of the following peptides:

(1) a peptide capable of binding myostatin, wherein the peptide comprises the sequence $\underline{WY}e_1e_2\underline{Y}e_3\underline{G}$, (SEQ ID NO: 356)

wherein e_1 is P, S or Y,
e_2 is C or Q, and

e_3 is G or H, wherein the peptide is between 7 and 50 amino acids in length, and physiologically acceptable salts thereof;

(2) a peptide capable of binding myostatin, wherein the peptide comprises the sequence f_1EMLf_2SLf_3f_4LL, (SEQ ID NO: 455),

wherein f_1 is M or I,
f_2 is any amino acid,
f_3 is L or F,
f_4 is E, Q or D;
and wherein the peptide is between 7 and 50 amino acids in length, and physiologically acceptable salts thereof;

(3) a peptide capable of binding myostatin wherein the peptide comprises the sequence Lg_1g_2LLg_3g_4L, (SEQ ID NO: 456), wherein

g_1 is Q, D or E,
g_2 is S, Q, D or E,
g_3 is any amino acid,
g_4 is L, W, F, or Y, and wherein the peptide is between 8 and 50 amino acids in length, and physiologically acceptable salts thereof;

(4) a peptide capable of binding myostatin, wherein the peptide comprises the sequence $h_1h_2h_3h_4h_5h_6h_7h_8h_9$ (SEQ ID NO: 457), wherein

h_1 is R or D,
h_2 is any amino acid,
h_3 is A, T S or Q,
h_4 is L or M,
h_5 is L or S,
h_6 is any amino acid,
h_7 is F or E,
h_8 is W, F or C,
h_9 is L, F, M or K, and wherein the peptide is between 9 and 50 amino acids in length, and physiologically acceptable salts thereof.

In one embodiment, the binding agents of the present invention further comprise at least one vehicle such as a polymer or an Fc domain, and may further comprise at least one linker sequence. In this embodiment, the binding agents of the present invention are constructed so that at least one myostatin-binding peptide is attached to at least one vehicle. The peptide or peptides are attached directly or indirectly through a linker sequence, to the vehicle at the N-terminal, C-terminal or an amino acid sidechain of the peptide. In this embodiment, the binding agents of the present invention have the following generalized structure:

$(X^1)_a$-F^1-$(X^2)_b$, or multimers thereof;

wherein F^1 is a vehicle; and X^1 and X^2 are each independently selected from

$-(L^1)_c-P^1$;

$-(L^1)_c-P^1-(L^2)_d-P^2$;

$-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$;

and $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$;

wherein P^1, P^2, P^3, and P^4 are peptides capable of binding myostatin; and

L^1, L^2, L^3, and L^4 are each linkers; and a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1, and physiologically acceptable salts thereof.

In various embodiments of binding agents having this generalized structure, the peptides P^1, P^2, P^3, and P^4 can be independently selected from one or more of any of the peptides comprising the sequences provided above. P^1, P^2, P^3, and P^4 are independently selected from one or more peptides comprising any of the following sequences: SEQ ID NO: 633, SEQ ID NO: 352, SEQ ID NO: 353, SEQ ID NO: 354, SEQ ID NO: 355, SEQ ID NO: 356, SEQ ID NO: 455, SEQ ID NO: 456, or SEQ ID NO: 457.

In a further embodiment, the binding agents comprise peptides fused to an Fc domain, either directly or indirectly, thereby providing peptibodies. The peptibodies of the present invention display a high binding affinity for myostatin and can inhibit the activity of myostatin as demonstrated both *in vitro* using cell based assays and in animals.

The present invention also provides nucleic acid molecules comprising polynucleotides encoding the peptides, peptibodies, and peptide and peptibody variants and derivatives of the present invention.

The present invention provides pharmaceutically acceptable compositions comprising one or more binding agents of the present invention.

The binding agents of the present invention inhibit myostatin activity *in vitro* and *in vivo*. The binding agents of the present invention increase lean muscle mass in a treated animal and decreases fat mass as a percentage of body weight of the animal. The myostatin binding agents of the present invention increase muscular strength in treated animal models.

The present invention provides methods of inhibiting myostatin activity in animals including humans by administering an effective dosage of one or more binding agents to the subject. The present invention provides methods of increasing lean muscle mass in animals including humans by administering an effective dosage of one or more binding agents. The present invention further provides methods of treating myostatin- related disorders by administering an therapeutically effective dosage of one or more myostatin binding agents in a pharmaceutically acceptable composition to a subject. The present invention provides methods of treating muscle wasting disorders including muscular dystrophy, muscle wasting due to cancer, AIDS, rheumatoid arthritis, renal failure, uremia, chronic heart failure, age-related sarcopenia, prolonged bed-rest, spinal chord injury, stroke, bone fracture. The present invention also provides

methods of treating metabolic disorders including obesity, diabetes, hyperglycemia, and bone loss.

The present invention also provides a method of increasing muscle mass in food animals by administering an effective dosage of one or more myostatin binding agents to the animal.

The present invention provides assays utilizing one or more myostatin binding agents to identify and quantitate myostatin in a sample. The assays may be diagnotic assays for measuring or monitoring myostatin levels in individuals with a myostatin related disorder or disease.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows myostatin activity as measured by expressed luciferase activity (y-axis) vrs. concentration (x-axis) for the TN8-19 peptide QGHCTRWPWMCPPY (Seq ID No: 32) and the TN8-19 peptibody (pb) to determine the IC_{50} for each using the C2C12 pMARE luciferase assay described in the Examples below. The peptibody has a lower IC_{50} value compared with the peptide.

Figure 2 is a graph showing the increase in total body weight for CD1 nu/nu mice treated with increasing dosages of the 1x mTN8-19-21 peptibody over a fourteen day period compared with mice treated with a huFc control, as described in Example 8.

Figure 3A shows the increase in the mass of the gastrocnemius muscle mass at necropsy of the mice treated in Figure 2 (Example 8). Figure 3B shows the increase in lean mass as determined by NMR on day 0 compared with day 13 of the experiment described in Example 8.

Figure 4 shows the increase in lean body mass as for CD1 nu/nu mice treated with biweekly injections of increasing dosages of 1x mTN8-19-32 peptibody as determined by NMR on day 0 and day 13 of the experiment described in Example 8.

Figure 5A shows the increase in body weight for CD1 nu/nu mice treated with biweekly injections of 1x mTN8-19-7 compared with 2x mTN8-19-7 and the control animal for 35 days as described in Example 8. Figure 5B shows the increase in lean carcass weight at necropsy for the 1x and 2x versions at 1 mg/kg and 3 mg/kg compared with the animals receiving the vehicle (huFc) (controls).

Figure 6A shows the increase in lean muscle mass vrs. body weight for aged *mdx* mice treated with either affinity matured 1x mTN8-19-33 peptibody or huFc vehicle at 10 mg/kg subcutaneously every other day for three months. Figure 6B shows the change in fat mass compared to body weight as determined by NMR for the same mice after 3 months of treatment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides binding agents capable of binding myostatin and inhibiting its activity. The myostatin binding agents can be used in assays, to identify, quantitate, or monitor the level of myostatin in an animal. The myostatin binding agents of the present invention reduce myostatin activity. The myostatin binding agents of the present invention increase lean muscle mass in animals, decrease fat mass as a percentage of body weight, and increase muscle strength. The myostatin binding agents of the present invention can be used to treat a variety of metabolic disorders in which myostatin plays a role, including muscle wasting disorders such as muscular dystrophies, muscle wasting due to cancer, AIDS, rheumatoid arthritis, renal failure, uremia, chronic heart failure, prolonged bed-rest, spinal chord injury, stroke, and age-related sarcopenia as well as other metabolic disorders including diabetes, obesity, hyperglycemia, and bone loss, by administering a therapeutic dosage of one or more binding agents in a pharmaceutically acceptable composition to a subject.

Myostatin

Myostatin, a growth factor also known as GDF-8, is known to be a negative regulator of skeletal muscle tissue. Myostatin is synthesized as an inactive preproprotein which is activated by proteolyic cleavage (Zimmers et al., *supra* (2002)). The precurser protein is cleaved to produce an NH_2-terminal inactive prodomain and an approximately 109 amino acid COOH-terminal protein in the form of a homodimer of about 25 kDa, which is the mature, active form (Zimmers et al, *supra* (2002)). It is now believed that the mature dimer circulates in the blood as an inactive latent complex bound to the propeptide (Zimmers et al, supra (2002).

As used herein the term "full-length myostatin" refers to the full-length human preproprotein sequence described in McPherron et al. *supra* (1997), as well as related full-length polypeptides including allelic variants and interspecies homologs which are also described in McPherron et al. (1997). As used herein, the term "prodomain" or "propeptide" refers to the inactive NH_2-terminal protein which is cleaved off to release the active COOH-terminal protein. As used herein the term "myostatin" or "mature myostatin" refers to the mature, biologically active COOH-terminal polypeptide, in monomer, dimer, multimeric form or other form. "Myostatin" or "mature myostatin" also refers to fragments of the biologically active mature myostatin, as well as related polypeptides including allelic variants, splice variants, and fusion peptides and polypeptides. The mature myostatin COOH-terminal protein has been reported to have 100% sequence identity among many species including human, mouse, chicken, porcine, turkey, and rat (Lee et al., *PNAS* **98**, 9306 (2001)). Myostatin may or may not include additional

terminal residues such as targeting sequences, or methionine and lysine residues and /or tag or fusion protein sequences, depending on how it is prepared.

As used herein the term "capable of binding to myostatin" or "having a binding affinity for myostatin" refers to a binding agent or peptide which binds to myostatin as demonstrated by as the phage ELISA assay, the BIAcore® or KinExA™ assays described in the Examples below.

As used herein, the term "capable of modifying myostatin activity" refers to the action of an agent as either an agonist or an antagonist with respect to at least one biological activity of myostatin. As used herein, "agonist" or "mimetic"activity refers an agent having biological activity comparable to a protein that interacts with the protein of interest, as described, for example, in International application WO 01/83525, filed May 2, 2001, which is incorporated herein by reference.

As used herein, the term "inhibiting myostatin activity" or "having antagonist activity"refers to the ability of a peptide or binding agent to reduce or block myostatin activity or signaling as demonstrated or *in vitro* assays such as, for example, the pMARE C2C12 cell-based myostatin activity assay or by *in vivo* animal testing as described below.

Structure of Myostatin Binding Agents

In one embodiment, the binding agents of the present invention comprise at least one myostatin binding peptide covalently attached to at least one vehicle such as a polymer or an Fc domain. The attachment of the myostatin-binding peptides to at least one vehicle is intended to increase the effectiveness of the binding agent as a therapeutic by increasing the biological activity of the agent and/or decreasing degradation *in vivo*, increasing half-life *in vivo*, reducing toxicity or immunogenicity *in vivo*. The binding agents of the present invention may further comprise a linker sequence connecting the peptide and the vehicle. The peptide or peptides are attached directly or indirectly through a linker sequence to the vehicle at the N-terminal, C-terminal or an amino acid sidechain of the peptide. In this embodiment, the binding agents of the present invention have the following structure:

$(X^1)_a$-F^1-$(X^2)_b$, or multimers thereof;

wherein F^1 is a vehicle; and X^1 and X^2 are each independently selected from

$-(L^1)_c-P^1$;

$-(L^1)_c-P^1-(L^2)_d-P^2$;

$-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$;

and $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$;

wherein P^1, P^2, P^3, and P^4 are peptides capable of binding myostatin; and

L^1, L^2, L^3, and L^4 are each linkers; and a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

Any peptide containing a cysteinyl residue may be cross-linked with another Cys-containing peptide, either or both of which may be linked to a vehicle. Any peptide having more than one Cys residue may form an intrapeptide disulfide bond, as well.

In one embodiment, the vehicle is an Fc domain, defined below. This embodiment is referred to as a "peptibody". As used herein, the term "peptibody" refers to a molecule comprising an antibody Fc domain attached to at least one peptide. The production of peptibodies is generally described in PCT publication WO 00/24782, published May 4, 2000, which is herein incorporated by reference. Exemplary peptibodies are provided as 1x and 2x configurations with one copy and two copies of the peptide (attached in tandem) respectively, as described in the Examples below.

Peptides

As used herein the term "peptide" refers to molecules of about 5 to about 90 amino acids linked by peptide bonds. The peptides of the present invention are preferably between about 5 to about 50 amino acids in length, more preferably between about 10 and 30 amino acids in length, and most preferably between about 10 and 25 amino acids in length, and are capable of binding to the myostatin protein.

The peptides of the present invention may comprise part of a sequence of naturally occuring proteins, may be randomized sequences derived from naturally occuring proteins, or may be entirely randomized sequences. The peptides of the present invention may be generated by any methods known in the art including chemical synthesis, digestion of proteins, or recombinant technology. Phage display and RNA-peptide screening, and other affinity screening techniques are particularly useful for generating peptides capable of binding myostatin.

Phage display technology is described, for example, in Scott *et al. Science* 249: 386 (1990); Devlin *et al.*, *Science* 249: 404 (1990); U.S. Patent No. 5,223,409, issued June 29, 1993; U.S. Patent No. 5,733,731, issued March 31, 1998; U.S. Patent No. 5,498,530, issued March 12, 1996; U.S. Patent No. 5,432,018, issued July 11, 1995; U.S. Patent No. 5,338,665, issued August 16, 1994; U.S. Patent No. 5,922,545, issued July 13, 1999; WO 96/40987, published December 19, 1996; and WO 98/15833, published April 16, 1998, each of which is incorporated herein by reference. Using phage libraries, random peptide sequences are displayed by fusion with coat proteins of filamentous phage. Typically, the displayed peptides are affinity-eluted either specifically or non-specifically against the target molecule. The retained phages may be enriched

by successive rounds of affinity purification and repropagation. The best binding peptides are selected for further analysis, for example, by using phage ELISA, described below, and then sequenced. Optionally, mutagenesis libraries may be created and screened to further optimize the sequence of the best binders (Lowman, *Ann Rev Biophys Biomol Struct* **26**:401-24 (1997)).

Other methods of generating the myostatin binding peptides include additional affinity selection techniques known in the art. A peptide library can be fused in the carboxyl terminus of the lac repressor and expressed in *E.coli*. Another *E. coli*-based method allows display on the cell's outer membrane by fusion with a peptidoglycan-associated lipoprotein (PAL). Hereinafter, these and related methods are collectively referred to as "*E. coli* display." In another method, translation of random RNA is halted prior to ribosome release, resulting in a library of polypeptides with their associated RNA still attached. Hereinafter, this and related methods are collectively referred to as "ribosome display." Other methods employ chemical linkage of peptides to RNA. See, for example, Roberts and Szostak, *Proc Natl Acad Sci USA*, 94: 12297-303 (1997). Hereinafter, this and related methods are collectively referred to as "RNA-peptide screening." Yeast two-hybrid screening methods also may be used to identify peptides of the invention that bind to myostatin. In addition, chemically derived peptide libraries have been developed in which peptides are immobilized on stable, non-biological materials, such as polyethylene rods or solvent-permeable resins. Another chemically derived peptide library uses photolithography to scan peptides immobilized on glass slides. Hereinafter, these and related methods are collectively referred to as "chemical-peptide screening." Chemical-peptide screening may be advantageous in that it allows use of D-amino acids and other analogues, as well as non-peptide elements. Both biological and chemical methods are reviewed in Wells and Lowman, *Curr Opin Biotechnol* 3: 355-62 (1992).

Additionally, selected peptides capable of binding myostatin can be further improved through the use of "rational design". In this approach, stepwise changes are made to a peptide sequence and the effect of the substitution on the binding affinity or specificity of the peptide or some other property of the peptide is observed in an appropriate assay. One example of this technique is substituting a single residue at a time with alanine, referred to as an "alanine walk" or an "alanine scan". When two residues are replaced, it is referred to as a "double alanine walk". The resultant peptide containing amino acid substitutions are tested for enhanced activity or some additional advantageous property.

In addition, analysis of the structure of a protein-protein interaction may also be used to suggest peptides that mimic the interaction of a larger protein. In such an analysis, the crystal structure of a protein may suggest the identity and relative orientation of critical residues of the protein, from which a peptide may be designed. See, for example, Takasaki et al., *Nature Biotech*

15:1266 (1977). These methods may also be used to investigate the interaction between a targeted protein and peptides selected by phage display or other affinity selection processes, thereby suggesting further modifications of peptides to increase binding affinity and the ability of the peptide to inhibit the activity of the protein.

In one embodiment, the peptides of the present invention are generated as families of related peptides. Exemplary petides are represented by SEQ ID NO: 1 through 132. These exemplary peptides were derived through an selection process in which the best binders generated by phage display technology were further analyzed by phage ELISA to obtain candidate peptides by an affinity selection technique such as phage display technology as described herein. However, the peptides of the present invention may be produced by any number of known methods including chemical synthesis as described below.

The peptides of the present invention can be further improved by the process of "affinity maturation". This procedure is directed to increasing the affinity or the activity of the peptides and peptibodies of the present invention using phage display or other selection technologies. Based on a consensus sequence, directed secondary phage display libraries, for example, can be generated in which the "core" amino acids (determined from the consensus sequence) are held constant or are biased in frequency of occurrence. Alternatively, an individual peptide sequence can be used to generate a biased, directed phage display library. Panning of such libraries under more stringent conditions can yield peptides with enhanced binding to myostatin, selective binding to myostatin, or with some additional desired property. However, peptides having the affinity matured sequences may then be produced by any number of known methods including chemical synthesis or recombinantly. These peptides are used to generate binding agents such as peptibodies of various configurations which exhibit greater inhibitory activity in cell-based assays and *in vivo* assays.

Example 6 below describes affinity maturation of the "first round" peptides described above to produce affinity matured peptides. Exemplary affinity matured peptibodies are presented in Tables IV and V. The resultant 1x and 2x peptibodies made from these peptides were then further characterized for binding affinity, ability to neutralize myostatin activity, specificity to myostatin as opposed to other TNFß family members, and for additional *in vitro* and *in vivo* activity, as described below. Affinity-matured peptides and peptibodies are referred to by the prefix "m" before their family name to distinguish them from first round peptides of the same family.

Exemplary first round peptides chosen for further affinity maturation according to the present invention included the following peptides: TN8-19 QGHCTRWPWMCPPY (SEQ ID NO: 33), and the linear peptides Linear-2 MEMLDSLFELLKDMVPISKA (SEQ ID NO: 104),

Linear-15 HHGWNYLRKGSAPQWFEAWV (SEQ ID NO: 117), Linear-17, RATLLKDFWQLVEGYGDN (SEQ ID NO: 119), Linear-20 YREMSMLEGLLDVLERLQHY (SEQ ID NO: 122), Linear-21 HNSSQMLLSELIMLVGSMMQ (SEQ ID NO: 123), Linear-24 EFFHWLHNHRSEVNHWLDMN (SEQ ID NO: 126). The affinity matured families of each of these is presented below in Tables IV and V.

The peptides of the present invention also encompass variants and derivatives of the selected peptides which are capable of binding myostatin. As used herein the term "variant" refers to peptides having one or more amino acids inserted, deleted, or substituted into the original amino acid sequence, and which are still capable of binding to myostatin. Insertional and substitutional variants may contain natural amino acids as well as non-naturally occuring amino acids. As used herein the term "variant" includes fragments of the peptides which still retain the ability to bind to myostatin. As used herein, the term "derivative" refers to peptides which have been modified chemically in some manner distinct from insertion, deletion, and substitution variants. Variants and derivatives of the peptides and peptibodies of the present invention are described more fully below.

Vehicles

As used herein the term "vehicle" refers to a molecule that may be attached to one or more peptides of the present invention. Preferably, vehicles confer at least one desired property on the binding agents of the present invention. Peptides alone are likely to be removed *in vivo* either by renal filtration, by cellular clearance mechanisms in the reticuloendothelial system, or by proteolytic degradation. Attachment to a vehicle improves the therapeutic value of a binding agent by reducing degradation of the binding agent and/or increasing half-life, reducing toxicity, reducing immunogenicity, and/or increasing the biological activity of the binding agent.

Exemplary vehicles include Fc domains; linear polymers such as polyethylene glycol (PEG), polylysine, dextran; a branched chain polymer (see for example U.S. Patent No. 4,289,872 to Denkenwalter *et al.*, issued September 15, 1981; U. S. Patent No. 5,229,490 to Tam, issued July 20, 1993; WO 93/21259 by Frechet *et al.*, published 28 October 1993); a lipid; a cholesterol group (such as a steroid); a carbohydrate or oligosaccharide; or any natural or synthetic protein, polypeptide or peptide that binds to a salvage receptor.

In one embodiment, the myostatin binding agents of the present invention have at least one peptide attached to at least one vehicle (F^1, F^2) through the N-terminus, C-terminus or a side chain of one of the amino acid residues of the peptide(s). Multiple vehicles may also be used; such as an Fc domain at each terminus or an Fc domain at a terminus and a PEG group at the other terminus or a side chain.

An Fc domain is one preferred vehicle. As used herein, the term "Fc domain" encompasses native Fc and Fc variant molecules and sequences as defined below. As used herein the term "native Fc" refers to a non-antigen binding fragment of an antibody or the amino acid sequence of that fragment which is produced by recombinant DNA techniques or by enzymatic or chemical cleavage of intact antibodies. A preferred Fc is a fully human Fc and may originate from any of the immunoglobulins, such as IgG1 and IgG2. However, Fc molecules that are partially human, or originate from non-human species are also included herein. Native Fc molecules are made up of monomeric polypeptides that may be linked into dimeric or multimeric forms by covalent (*i.e.*, disulfide bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (*e.g.*, IgG, IgA, IgE) or subclass (*e.g.*, IgG1, IgG2, IgG3, IgA1, IgGA2). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG (see Ellison *et al.* (1982), *Nucl Acids Res* **10**: 4071-9). The term "native Fc" as used herein is used to refer to the monomeric, dimeric, and multimeric forms.

As used herein, the term "Fc variant" refers to a modified form of a native Fc sequence provided that binding to the salvage receptor is maintained, as described, for example, in WO 97/34631 and WO 96/32478, both of which are incorporated herein by reference. Fc variants may be constructed for example, by substituting or deleting residues, inserting residues or truncating portions containing the site. The inserted or substituted residues may also be altered amino acids, such as peptidomimetics or D-amino acids. Fc variants may be desirable for a number of reasons, several of which are described below. Exemplary Fc variants include molecules and sequences in which:

1. Sites involved in disulfide bond formation are removed. Such removal may avoid reaction with other cysteine-containing proteins present in the host cell used to produce the molecules of the invention. For this purpose, the cysteine-containing segment at the N-terminus may be truncated or cysteine residues may be deleted or substituted with other amino acids (*e.g.*, alanyl, seryl). Even when cysteine residues are removed, the single chain Fc domains can still form a dimeric Fc domain that is held together non-covalently.

2. A native Fc is modified to make it more compatible with a selected host cell. For example, one may remove the PA sequence near the N-terminus of a typical native Fc, which may be recognized by a digestive enzyme in *E. coli* such as proline iminopeptidase. One may also add an N-terminal methionyl residue, especially when the molecule is expressed recombinantly in a bacterial cell such as *E. coli*.

3. A portion of the N-terminus of a native Fc is removed to prevent N-terminal heterogeneity when expressed in a selected host cell. For this purpose, one may delete any of the first 20 amino acid residues at the N-terminus, particularly those at positions 1, 2, 3, 4 and 5.

4. One or more glycosylation sites are removed. Residues that are typically glycosylated (*e.g.*, asparagine) may confer cytolytic response. Such residues may be deleted or substituted with unglycosylated residues (*e.g.*, alanine).

5. Sites involved in interaction with complement, such as the C1q binding site, are removed. For example, one may delete or substitute the EKK sequence of human IgG1. Complement recruitment may not be advantageous for the molecules of this invention and so may be avoided with such an Fc variant.

6. Sites are removed that affect binding to Fc receptors other than a salvage receptor. A native Fc may have sites for interaction with certain white blood cells that are not required for the fusion molecules of the present invention and so may be removed.

7. The ADCC site is removed. ADCC sites are known in the art. See, for example, *Molec Immunol* **29** (5):633-9 (1992) with regard to ADCC sites in IgG1. These sites, as well, are not required for the fusion molecules of the present invention and so may be removed.

8. When the native Fc is derived from a non-human antibody, the native Fc may be humanized. Typically, to humanize a native Fc, one will substitute selected residues in the non-human native Fc with residues that are normally found in human native Fc. Techniques for antibody humanization are well known in the art.

The term "Fc domain" includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by other means. As used herein the term "multimer" as applied to Fc domains or molecules comprising Fc domains refers to molecules having two or more polypeptide chains associated covalently, noncovalently, or by both covalent and non-covalent interactions. IgG molecules typically form dimers; IgM, pentamers; IgD, dimers; and IgA, monomers, dimers, trimers, or tetramers. Multimers may be formed by exploiting the sequence and resulting activity of the native Ig source of the Fc or by derivatizing such a native Fc. The term "dimer" as applied to Fc domains or molecules comprising Fc domains refers to molecules having two polypeptide chains associated covalently or non-covalently.

Additionally, an alternative vehicle according to the present invention is a non-Fc domain protein, polypeptide, peptide, antibody, antibody fragment, or small molecule (*e.g.*, a peptidomimetic compound) capable of binding to a salvage receptor. For example, one could use as a vehicle a polypeptide as described in U.S. Patent No. 5,739,277, issued April 14, 1998 to Presta *et al.* Peptides could also be selected by phage display for binding to the FcRn salvage receptor. Such salvage receptor-binding compounds are also included within the meaning of

"vehicle"and are within the scope of this invention. Such vehicles should be selected for increased half-life (*e.g.*, by avoiding sequences recognized by proteases) and decreased immunogenicity (*e.g.*, by favoring non-immunogenic sequences, as discovered in antibody humanization).

In addition, polymer vehicles may also be used to construct the binding agents of the present invention. Various means for attaching chemical moieties useful as vehicles are currently available, see, *e.g.*, Patent Cooperation Treaty ("PCT") International Publication No. WO 96/11953, entitled "N-Terminally Chemically Modified Protein Compositions and Methods," herein incorporated by reference in its entirety. This PCT publication discloses, among other things, the selective attachment of water soluble polymers to the N-terminus of proteins.

A preferred polymer vehicle is polyethylene glycol (PEG). The PEG group may be of any convenient molecular weight and may be linear or branched. The average molecular weight of the PEG will preferably range from about 2 kDa to about 100 kDa, more preferably from about 5 kDa to about 50 kDa, most preferably from about 5 kDa to about 10 kDa. The PEG groups will generally be attached to the compounds of the invention via acylation or reductive alkylation through a reactive group on the PEG moiety (*e.g.*, an aldehyde, amino, thiol, or ester group) to a reactive group on the inventive compound (e.g., an aldehyde, amino, or ester group). A useful strategy for the PEGylation of synthetic peptides consists of combining, through forming a conjugate linkage in solution, a peptide and a PEG moiety, each bearing a special functionality that is mutually reactive toward the other. The peptides can be easily prepared with conventional solid phase synthesis as known in the art. The peptides are "preactivated" with an appropriate functional group at a specific site. The precursors are purified and fully characterized prior to reacting with the PEG moiety. Ligation of the peptide with PEG usually takes place in aqueous phase and can be easily monitored by reverse phase analytical HPLC. The PEGylated peptides can be easily purified by preparative HPLC and characterized by analytical HPLC, amino acid analysis and laser desorption mass spectrometry.

Polysaccharide polymers are another type of water soluble polymer which may be used for protein modification. Dextrans are polysaccharide polymers comprised of individual subunits of glucose predominantly linked by a1-6 linkages. The dextran itself is available in many molecular weight ranges, and is readily available in molecular weights from about 1 kDa to about 70 kDa. Dextran is a suitable water-soluble polymer for use in the present invention as a vehicle by itself or in combination with another vehicle (e.g., Fc). See, for example, WO 96/11953 and WO 96/05309. The use of dextran conjugated to therapeutic or diagnostic immunoglobulins has been reported; see, for example, European Patent Publication No. 0 315 456, which is hereby

incorporated by reference. Dextran of about 1 kDa to about 20 kDa is preferred when dextran is used as a vehicle in accordance with the present invention.

Linkers

The binding agents of the present invention may optionally further comprise a "linker" group. Linkers serve primarily as a spacer between a peptide and a vehicles or between two peptides of the binding agents of the present invention. In one embodiment, the linker is made up of amino acids linked together by peptide bonds, preferably from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids. One or more of these amino acids may be glycosylated, as is understood by those in the art. In one embodiment, the 1 to 20 amino acids are selected from glycine, alanine, proline, asparagine, glutamine, and lysine. Preferably, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. Thus, exemplary linkers are polyglycines (particularly $(Gly)_5$, $(Gly)_8$), poly(Gly-Ala), and polyalanines. As used herein, the designation "g" refers to a glycine homopeptide linkers. As shown in Table II, "gn" refers to a 5x gly linker at the N terminus, while "gc" refers to 5x gly linker at the C terminus. Combinations of Gly and Ala are also preferred. One exemplary linker sequence useful for constructing the binding agents of the present invention is the following: gsgsatggsgstassgsgsatg (Seq ID No: 305). This linker sequence is referred to as the "k" or 1k sequence. The designations "kc", as found in Table II, refers to the k linker at the C–terminus, while the designation "kn", refers to the k linker at the N-terminus.

The linkers of the present invention may also be non-peptide linkers. For example, alkyl linkers such as $-NH-(CH_2)s-C(O)-$, wherein s = 2-20 can be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc. An exemplary non-peptide linker is a PEG linker, and has a molecular weight of 100 to 5000 kDa, preferably 100 to 500 kDa. The peptide linkers may be altered to form derivatives in the same manner as above.

Exemplary Binding Agents

The binding agents of the present invention comprise at least one peptide capable of binding myostatin. In one embodiment, the myostatin binding peptide is between about 5 and about 50 amino acids in length, in another, between about 10 and 30 amino acids in length, and in another, between about 10 and 25 amino acids in length. In one embodiment the myostatin binding peptide comprises the amino acid sequence WMCPP (SEQ ID NO: 633). In other embodiment, the myostatin binding peptide comprises the amino acid sequence

$\underline{C}a_1a_2\underline{W}a_3\underline{WMCPP}$ (SEQ ID NO: 352), wherein a_1, a_2 and a_3 are selected from a neutral hydrophobic, neutral polar, or basic amino acid. In another embodiment the myostatin binding peptide comprises the amino acid sequence $\underline{C}b_1b_2\underline{W}b_3\underline{WMCPP}$ (SEQ ID NO: 353), wherein b_1 is selected from any one of the amino acids T, I, or R; b_2 is selected from any one of R, S, Q; b_3 is selected from any one of P, R and Q, and wherein the peptide is beween 10 and 50 amino acids in length, and physiologically acceptable salts thereof.

In another embodiment, the myostatin binding peptide comprises the formula:

$c_1c_2c_3c_4c_5c_6\underline{C}c_7c_8\underline{W}c_9\underline{WMCPP}c_{10}c_{11}c_{12}c_{13}$ (SEQ ID NO: 354), wherein:

c_1 is absent or any amino acid;
c_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_4 is absent or any amino acid;
c_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
c_7 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_8 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_9 is a neutral hydrophobic, neutral polar or basic amino acid; and
c_{10} to c_{13} is any amino acid; and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof.

A related embodiment the myostatin binding peptide comprises the formula:

$d_1d_2d_3d_4d_5d_6\underline{C}d_7d_8\underline{W}d_9\underline{WMCPP}$ $d_{10}d_{11}d_{12}d_{13}$ (SEQ ID NO: 355), wherein

d_1 is absent or any amino acid;
d_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_4 is absent or any amino acid;
d_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
d_7 is selected from any one of the amino acids T, I, or R;
d_8 is selected from any one of R, S, Q;
d_9 is selected from any one of P, R and Q, and
d_{10} to d_{13} is selected from any amino acid,

and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof.

Additional embodiments of binding agents comprise at least one of the following peptides:

(1) a peptide capable of binding myostatin, wherein the peptide comprises the sequence $\underline{WY}e_1e_2\underline{Y}e_3\underline{G}$, (SEQ ID NO: 356)

wherein e_1 is P, S or Y,
e_2 is C or Q, and

e_3 is G or H, wherein the peptide is between 7 and 50 amino acids in length, and physiologically acceptable salts thereof.

(2) a peptide capable of binding myostatin, wherein the peptide comprises the sequence f_1EMLf_2SLf_3f_4LL, (SEQ ID NO: 455),

wherein f_1 is M or I,
f_2 is any amino acid,
f_3 is L or F,
f_4 is E, Q or D;
and wherein the peptide is between 7 and 50 amino acids in length, and physiologically acceptable salts thereof.

(3) a peptide capable of binding myostatin wherein the peptide comprises the sequence Lg_1g_2LLg_3g_4L, (SEQ ID NO: 456), wherein

g_1 is Q, D or E,
g_2 is S, Q, D or E,
g_3 is any amino acid,
g_4 is L, W, F, or Y, and wherein the peptide is between 8 and 50 amino acids in length, and physiologically acceptable salts thereof.

(4) a peptide capable of binding myostatin, wherein the peptide comprises the sequence $h_1h_2h_3h_4h_5h_6h_7h_8h_9$ (SEQ ID NO: 457), wherein

h_1 is R or D,
h_2 is any amino acid,
h_3 is A, T S or Q,
h_4 is L or M,
h_5 is L or S,
h_6 is any amino acid,
h_7 is F or E,
h_8 is W, F or C,
h_9 is L, F, M or K, and wherein the peptide is between 9 and 50 amino acids in length, and physiologically acceptable salts thereof.

In one embodiment, the binding agents of the present invention further comprise at least one vehicle such as a polymer or an Fc domain, and may further comprise at least one linker sequence. In this embodiment, the binding agents of the present invention are constructed so that at least one myostatin-binding peptide is covalently attached to at least one vehicle. The peptide or peptides are attached directly or indirectly through a linker sequence, to the vehicle at the N-terminal, C-terminal or an amino acid sidechain of the peptide. In this embodiment, the binding agents of the present invention have the following generalized structure:

$(X^1)_a$-F^1-$(X^2)_b$, or multimers thereof;

wherein F^1 is a vehicle; and X^1 and X^2 are each independently selected from

$-(L^1)_c-P^1$;

$-(L^1)_c-P^1-(L^2)_d-P^2$;

$-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$;

and $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$;

wherein P^1, P^2, P^3, and P^4 are peptides capable of binding myostatin; and

L^1, L^2, L^3, and L^4 are each linkers; and a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1.

In one embodiment of the binding agents having this generalized structure, the peptides P^1, P^2, P^3, and P^4 can be selected from one or more of any of the peptides comprising the sequences provided above. Peptides P^1, P^2, P^3, and P^4 can be selected from one or more peptides comprising any of the following sequences: SEQ ID NO: 633, SEQ ID NO: 352, SEQ ID NO: 353, SEQ ID NO: 354, SEQ ID NO: 355, SEQ ID NO: 356, SEQ ID NO: 455, SEQ ID NO: 456, or SEQ ID NO: 457.

In a further embodiment, the vehicles of binding agents having the general formula above are Fc domains. The peptides are therefore fused to an Fc domain, either directly or indirectly, thereby providing peptibodies. The peptibodies of the present invention display a high binding affinity for myostatin and can inhibit the activity of myostatin as demonstrated by *in vitro* assays and *in vivo* testing in animals provided herein.

The present invention also provides nucleic acid molecules comprising polynucleotides encoding the peptides, peptibodies, and peptide and peptibody variants and derivatives of the present invention. Exemplary nucleotides sequences are given below.

Variants and Derivatives of Peptides and Peptibodies

The binding agents of the present invention also encompass variants and derivatives of the peptides and peptibodies described herein. Since both the peptides and peptibodies of the present invention can be described in terms of their amino acid sequence, the terms "variants" and "derivatives" can be said to apply to a peptide alone, or a peptide as a component of a peptibody. As used herein, the term "peptide variants" refers to peptides or peptibodies having one or more amino acid residues inserted, deleted or substituted into the original amino acid sequence and which retain the ability to bind to myostatin and modify its activity. As used herein, fragments of the peptides or peptibodies are included within the definition of "variants".

It is understood that any given peptide or peptibody may contain one or two or all three types of variants. Insertional and substitutional variants may contain natural amino acids, as well as non-naturally occuring amino acids or both.

Peptide and peptibody variants also include mature peptides and peptibodies wherein leader or signal sequences are removed, and the resulting proteins having additional amino terminal residues, which amino acids may be natural or non-natural. Peptibodies with an

additional methionyl residue at amino acid position -1 (Met^{-1}-peptibody) are contemplated, as are peptibodies with additional methionine and lysine residues at positions -2 and -1 (Met^{-2}-Lys^{-1}-). Variants having additional Met, Met-Lys, Lys residues (or one or more basic residues, in general) are particularly useful for enhanced recombinant protein production in bacterial host cells.

Peptide or peptibody variants of the present invention also includes peptides having additional amino acid residues that arise from use of specific expression systems. For example, use of commercially available vectors that express a desired polypeptide as part of glutathione-S-transferase (GST) fusion product provides the desired polypeptide having an additional glycine residue at amino acid position-1 after cleavage of the GST component from the desired polypeptide. Variants which result from expression in other vector systems are also contemplated, including those wherein histidine tags are incorporated into the amino acid sequence, generally at the carboxy and/or amino terminus of the sequence.

In one example, insertional variants are provided wherein one or more amino acid residues, either naturally occurring or non-naturally occuring amino acids, are added to a peptide amino acid sequence. Insertions may be located at either or both termini of the protein, or may be positioned within internal regions of the peptibody amino acid sequence. Insertional variants with additional residues at either or both termini can include, for example, fusion proteins and proteins including amino acid tags or labels. Insertional variants include peptides in which one or more amino acid residues are added to the peptide amino acid sequence or fragment thereof.

Insertional variants also include fusion proteins wherein the amino and/or carboxy termini of the peptide or peptibody is fused to another polypeptide, a fragment thereof or amino acids which are not generally recognized to be part of any specific protein sequence. Examples of such fusion proteins are immunogenic polypeptides, proteins with long circulating half lives, such as immunoglobulin constant regions, marker proteins, proteins or polypeptides that facilitate purification of the desired peptide or peptibody, and polypeptide sequences that promote formation of multimeric proteins (such as leucine zipper motifs that are useful in dimer formation/stability).

This type of insertional variant generally has all or a substantial portion of the native molecule, linked at the N- or C-terminus, to all or a portion of a second polypeptide. For example, fusion proteins typically employ leader sequences from other species to permit the recombinant expression of a protein in a heterologous host. Another useful fusion protein includes the addition of an immunologically active domain, such as an antibody epitope, to facilitate purification of the fusion protein. Inclusion of a cleavage site at or near the fusion junction will facilitate removal of the extraneous polypeptide after purification. Other useful

fusions include linking of functional domains, such as active sites from enzymes, glycosylation domains, cellular targeting signals or transmembrane regions.

There are various commercially available fusion protein expression systems that may be used in the present invention. Particularly useful systems include but are not limited to the glutathione-S-transferase (GST) system (Pharmacia), the maltose binding protein system (NEB, Beverley, MA), the FLAG system (IBI, New Haven, CT), and the 6xHis system (Qiagen, Chatsworth, CA). These systems are capable of producing recombinant peptides and/or peptibodies bearing only a small number of additional amino acids, which are unlikely to significantly affect the activity of the peptide or peptibody. For example, both the FLAG system and the 6xHis system add only short sequences, both of which are known to be poorly antigenic and which do not adversely affect folding of a polypeptide to its native conformation. Another N-terminal fusion that is contemplated to be useful is the fusion of a Met-Lys dipeptide at the N-terminal region of the protein or peptides. Such a fusion may produce beneficial increases in protein expression or activity.

Other fusion systems produce polypeptide hybrids where it is desirable to excise the fusion partner from the desired peptide or peptibody. In one embodiment, the fusion partner is linked to the recombinant peptibody by a peptide sequence containing a specific recognition sequence for a protease. Examples of suitable sequences are those recognized by the Tobacco Etch Virus protease (Life Technologies, Gaithersburg, MD) or Factor Xa (New England Biolabs, Beverley, MA).

The invention also provides fusion polypeptides which comprise all or part of a peptide or peptibody of the present invention, in combination with truncated tissue factor (tTF). tTF is a vascular targeting agent consisting of a truncated form of a human coagulation-inducing protein that acts as a tumor blood vessel clotting agent, as described U.S. Patent Nos.: 5,877,289; 6,004,555; 6,132,729; 6,132,730; 6,156,321; and European Patent No. EP 0988056. The fusion of tTF to the anti-myostatin peptibody or peptide, or fragments thereof facilitates the delivery of anti-myostatin antagonists to target cells, for example, skeletal muscle cells, cardiac muscle cells, fibroblasts, pre-adipocytes, and possibly adipocytes.

In another aspect, the invention provides deletion variants wherein one or more amino acid residues in a peptide or peptibody are removed. Deletions can be effected at one or both termini of the peptibody, or from removal of one or more residues within the peptibody amino acid sequence. Deletion variants necessarily include all fragments of a peptide or peptibody.

In still another aspect, the invention provides substitution variants of peptides and peptibodies of the invention. Substitution variants include those peptides and peptibodies wherein one or more amino acid residues are removed and replaced with one or more alternative amino

acids, which amino acids may be naturally occurring or non-naturally occurring. Substitutional variants generate peptides or peptibodies that are "similar" to the original peptide or peptibody, in that the two molecules have a certain percentage of amino acids that are identical. Substitution variants include substitutions of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, and 20 amino acids within a peptide or peptibody, wherein the number of substitutions may be up to ten percent of the amino acids of the peptide or peptibody. In one aspect, the substitutions are conservative in nature, however, the invention embraces substitutions that are also non-conservative and also includes unconventional amino acids.

Identity and similarity of related peptides and peptibodies can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York (1988); Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York (1993); Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey (1994); Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press (1987); Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York (1991); and Carillo *et al.*, *SIAM J. Applied Math.*, 48:1073 (1988).

Preferred methods to determine the relatedness or percent identity of two peptides or polypeptides, or a polypeptide and a peptide, are designed to give the largest match between the sequences tested. Methods to determine identity are described in publicly available computer programs. Preferred computer program methods to determine identity between two sequences include, but are not limited to, the GCG program package, including GAP (Devereux *et al.*, *Nucl. Acid. Res.*, 12:387 (1984); Genetics Computer Group, University of Wisconsin, Madison, WI, BLASTP, BLASTN, and FASTA (Altschul *et al.*, *J. Mol. Biol.*, 215:403-410 (1990)). The BLASTX program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (*BLAST Manual,* Altschul *et al.* NCB/NLM/NIH Bethesda, MD 20894; Altschul *et al., supra* (1990)). The well-known Smith Waterman algorithm may also be used to determine identity.

Certain alignment schemes for aligning two amino acid sequences may result in the matching of only a short region of the two sequences, and this small aligned region may have very high sequence identity even though there is no significant relationship between the two full-length sequences. Accordingly, in certain embodiments, the selected alignment method will result in an alignment that spans at least ten percent of the full length of the target polypeptide being compared, *i.e.*, at least 40 contiguous amino acids where sequences of at least 400 amino acids are being compared, 30 contiguous amino acids where sequences of at least 300 to about 400 amino

acids are being compared, at least 20 contiguous amino acids where sequences of 200 to about 300 amino acids are being compared, and at least 10 contiguous amino acids where sequences of about 100 to 200 amino acids are being compared. For example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, WI), two polypeptides for which the percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). In certain embodiments, a gap opening penalty (which is typically calculated as 3X the average diagonal; the "average diagonal" is the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM 250 or BLOSUM 62 are used in conjunction with the algorithm. In certain embodiments, a standard comparison matrix (see Dayhoff *et al.*, *Atlas of Protein Sequence and Structure*, 5(3)(1978) for the PAM 250 comparison matrix; Henikoff *et al.*, *Proc. Natl. Acad. Sci* USA, 89:10915-10919 (1992) for the BLOSUM 62 comparison matrix) is also used by the algorithm.

In certain embodiments, for example, the parameters for a polypeptide sequence comparison can be made with the following: Algorithm: Needleman et al., J. Mol. Biol., 48:443-453 (1970); Comparison matrix: BLOSUM 62 from Henikoff et al., supra (1992); Gap Penalty: 12; Gap Length Penalty: 4; Threshold of Similarity: 0, along with no penalty for end gaps.

In certain embodiments, the parameters for polynucleotide molecule sequence (as opposed to an amino acid sequence) comparisons can be made with the following: Algorithm: Needleman et al., supra (1970); Comparison matrix: matches = +10, mismatch = 0; Gap Penalty: 50: Gap Length Penalty: 3

Other exemplary algorithms, gap opening penalties, gap extension penalties, comparison matrices, thresholds of similarity, etc. may be used, including those set forth in the Program Manual, Wisconsin Package, Version 9, September, 1997. The particular choices to be made will be apparent to those of skill in the art and will depend on the specific comparison to be made, such as DNA-to-DNA, protein-to-protein, protein-to-DNA; and additionally, whether the comparison is between given pairs of sequences (in which case GAP or BestFit are generally preferred) or between one sequence and a large database of sequences (in which case FASTA or BLASTA are preferred).

Stereoisomers (*e.g.*, D-amino acids) of the twenty conventional (naturally occuring) amino acids, non-naturally occuring amino acids such as α-, α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components

for peptides of the present invention. Examples of non-naturally occuring amino acids include, for example: aminoadipic acid, beta-alanine, beta-aminopropionic acid, aminobutyric acid, piperidinic acid, aminocaprioic acid, aminoheptanoic acid, aminoisobutyric acid, aminopimelic acid, diaminobutyric acid, desmosine, diaminopimelic acid, diaminopropionic acid, N-ethylglycine, N-ethylaspargine, hyroxylysine, all0-hydroxylysine, hydroxyproline, isodesmosine, allo-isoleucine, N-methylglycine, sarcosine, N-methylisoleucine, N-methylvaline, norvaline, norleucine, orithine, 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine, ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, σ-N-methylarginine, and other similar amino acids and amino acids (*e.g.*, 4-hydroxyproline).

Naturally occurring residues may be divided into (overlapping) classes based on common side chain properties:

1) neutral hydrophobic: Met, Ala, Val, Leu, Ile, Pro, Trp, Met, Phe;
2) neutral polar: Cys, Ser, Thr, Asn, Gln, Tyr, Gly;
3) acidic: Asp, Glu;
4) basic: His, Lys, Arg;
5) residues that influence chain orientation: Gly, Pro; and
6) aromatic: Trp, Tyr, Phe.

Substitutions of amino acids may be conservative, which produces peptides having functional and chemical characteristics similar to those of the original peptide. Conservative amino acid substitutions involve exchanging a member of one of the above classes for another member of the same class. Conservative changes may encompass unconventional amino acid residues, which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. These include peptidomimetics and other reversed or inverted forms of amino acid moieties.

Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class. These changes can result in substantial modification in the functional and/or chemical characteristics of the peptides. In making such changes, according to certain embodiments, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. They are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine

(-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is understood in the art. Kyte *et al.*, *J. Mol. Biol.*, 157:105-131 (1982). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, in certain embodiments, the substitution of amino acids whose hydropathic indices are within ±2 is included. In certain embodiments, those which are within ±1 are included, and in certain embodiments, those within ±0.5 are included.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biologically functional peptibody or peptide thereby created is intended for use in immunological embodiments, as in the present case. In certain embodiments, the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, *i.e.*, with a biological property of the protein.

The following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5) and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, in certain embodiments, the substitution of amino acids whose hydrophilicity values are within ±2 is included, in certain embodiments, those which are within ±1 are included, and in certain embodiments, those within ±0.5 are included. One may also identify epitopes from primary amino acid sequences on the basis of hydrophilicity. These regions are also referred to as "epitopic core regions."

Exemplary amino acid substitutions are set forth in Table 1 below.

Amino Acid Substitutions

Original Residues	Exemplary Substitutions	Preferred Substitutions
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, Glu, Asp	Gln
Asp	Glu, Gln, Asp	Glu
Cys	Ser, Ala	Ser

Gln	Asn, Glu, Asp	Asn
Glu	Asp, Gln, Asn	Asp
Gly	Pro, Ala	Ala
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleucine	Leu
Leu	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, 1,4 Diamino-butyric Acid, Gln, Asn	Arg
Met	Leu, Phe, Ile	Leu
Phe	Leu, Val, Ile, Ala, Tyr	Leu
Pro	Ala	Gly
Ser	Thr, Ala, Cys	Thr
Thr	Ser	Ser
Trp	Tyr, Phe	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Met, Leu, Phe, Ala, Norleucine	Leu

One skilled in the art will be able to produce variants of the peptides and peptibodies of the present invention by random substitution, for example, and testing the resulting peptide or peptibody for binding activity using the assays described herein.

Additionally, one skilled in the art can review structure-function studies or three-dimensional structural analysis in order to identify residues in similar polypeptides that are important for activity or structure. In view of such a comparison, one can predict the importance of amino acid residues in a protein that correspond to amino acid residues which are important for activity or structure in similar proteins. One skilled in the art may opt for chemically similar amino acid substitutions for such predicted important amino acid residues. The variants can then be screened using activity assays as described herein.

A number of scientific publications have been devoted to the prediction of secondary structure. See Moult J., *Curr. Op. in Biotech.*, 7(4):422-427 (1996), Chou *et al., Biochemistry*, 13(2):222-245 (1974); Chou *et al., Biochemistry*, 113(2):211-222 (1974); Chou *et al., Adv. Enzymol. Relat. Areas Mol. Biol.*, 47:45-148 (1978); Chou *et al., Ann. Rev. Biochem.*, 47:251-276 and Chou *et al., Biophys. J.*, 26:367-384 (1979). Moreover, computer programs are currently available to assist with predicting secondary structure. One method of predicting secondary structure is based upon homology modeling. For example, two polypeptides or proteins which

have a sequence identity of greater than 30%, or similarity greater than 40% often have similar structural topologies. The recent growth of the protein structural database (PDB) has provided enhanced predictability of secondary structure, including the potential number of folds within a protein's structure. *See* Holm *et al.*, *Nucl. Acid. Res.*, 27(1):244-247 (1999). It has been suggested (Brenner *et al., Curr. Op. Struct. Biol.*, 7(3):369-376 (1997)) that there are a limited number of folds in a given protein and that once a critical number of structures have been resolved, structural prediction will become dramatically more accurate.

Additional methods of predicting secondary structure include "threading" (Jones, D., *Curr. Opin. Struct. Biol.*, 7(3):377-87 (1997); Sippl *et al.*, *Structure*, 4(1):15-19 (1996)), "profile analysis" (Bowie *et al., Science*, 253:164-170 (1991); Gribskov *et al.*, *Meth. Enzym.*, 183:146-159 (1990); Gribskov *et al.*, *Proc. Nat. Acad. Sci.*, 84(13):4355-4358 (1987)), and "evolutionary linkage" (See Holm, *supra* (1999), and Brenner, *supra* (1997)).

In certain embodiments, peptide or peptibody variants include glycosylation variants wherein one or more glycosylation sites such as a N-linked glycosylation site, has been added to the peptibody. An N-linked glycosylation site is characterized by the sequence: Asn-X-Ser or Asn-X-Thr, wherein the amino acid residue designated as X may be any amino acid residue except proline. The substitution or addition of amino acid residues to create this sequence provides a potential new site for the addition of an N-linked carbohydrate chain. Alternatively, substitutions which eliminate this sequence will remove an existing N-linked carbohydrate chain. Also provided is a rearrangement of N-linked carbohydrate chains wherein one or more N-linked glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked sites are created.

The invention also provides "derivatives" of the peptides or peptibodies of the present invention. As used herein the term "derivative" refers to modifications other than, or in addition to, insertions, deletions, or substitutions of amino acid residues which retain the ability to bind to myostatin.

Preferably, the modifications made to the peptides of the present invention to produce derivatives are covalent in nature, and include for example, chemical bonding with polymers, lipids, other organic, and inorganic moieties. Derivatives of the invention may be prepared to increase circulating half-life of a peptibody, or may be designed to improve targeting capacity for the peptibody to desired cells, tissues, or organs.

The invention further embraces derivative binding agents covalently modified to include one or more water soluble polymer attachments, such as polyethylene glycol, polyoxyethylene

glycol, or polypropylene glycol, as described U.S. Patent Nos.: 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192; and 4,179,337. Still other useful polymers known in the art include monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone)-polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol) and polyvinyl alcohol, as well as mixtures of these polymers. Particularly preferred are peptibodies covalently modified with polyethylene glycol (PEG) subunits. Water-soluble polymers may be bonded at specific positions, for example at the amino terminus of the peptibodies, or randomly attached to one or more side chains of the polypeptide. The use of PEG for improving the therapeutic capacity for binding agents, *e.g.* peptibodies, and for humanized antibodies in particular, is described in US Patent No. 6, 133, 426 to Gonzales *et al.*, issued October 17, 2000.

The invention also contemplates derivatizing the peptide and/or vehicle portion of the myostatin binding agents. Such derivatives may improve the solubility, absorption, biological half-life, and the like of the compounds. The moieties may alternatively eliminate or attenuate any undesirable side-effect of the compounds and the like. Exemplary derivatives include compounds in which:

1. The derivative or some portion thereof is cyclic. For example, the peptide portion may be modified to contain two or more Cys residues (*e.g.*, in the linker), which could cyclize by disulfide bond formation.

2. The derivative is cross-linked or is rendered capable of cross-linking between molecules. For example, the peptide portion may be modified to contain one Cys residue and thereby be able to form an intermolecular disulfide bond with a like molecule. The derivative may also be cross-linked through its C-terminus.

3. One or more peptidyl [-C(O)NR-] linkages (bonds) is replaced by a non-peptidyl linkage. Exemplary non-peptidyl linkages are -CH_2-carbamate [-CH_2-OC(O)NR-], phosphonate, -CH_2-sulfonamide [-CH_2-$S(O)_2$NR-], urea [-NHC(O)NH-], -CH_2-secondary amine, and alkylated peptide [-$C(O)NR_6$- wherein R_6 is lower alkyl].

4. The N-terminus is derivatized. Typically, the N-terminus may be acylated or modified to a substituted amine. Exemplary N-terminal derivative groups include -NRR_1 (other than -NH_2), -$NRC(O)R_1$, -$NRC(O)OR_1$, -$NRS(O)_2R_1$, -$NHC(O)NHR_1$, succinimide, or benzyloxycarbonyl-NH- (CBZ-NH-), wherein R and R1 are each independently hydrogen or lower alkyl and wherein the phenyl ring may be substituted with 1 to 3 substituents selected from the group consisting of C_1-C_4 alkyl, C_1-C_4 alkoxy, chloro, and bromo.

5. The free C-terminus is derivatized. Typically, the C-terminus is esterified or amidated. For example, one may use methods described in the art to add (NH-CH_2-CH_2-NH_2)$_2$ to

compounds of this invention at the C-terminus. Likewise, one may use methods described in the art to add -NH_2, (or "capping" with an -NH_2 group) to compounds of this invention at the C-terminus. Exemplary C-terminal derivative groups include, for example, -$C(O)R_2$ wherein R_2 is lower alkoxy or -NR_3R_4 wherein R_3 and R_4 are independently hydrogen or C_1-C_8 alkyl (preferably C_1-C_4 alkyl).

6. A disulfide bond is replaced with another, preferably more stable, cross-linking moiety (*e.g.*, an alkylene). See, *e.g.*, Bhatnagar *et al.*, *J Med Chem* **39**: 3814-9 (1996), Alberts *et al.*, *Thirteenth Am Pep Symp*, 357-9 (1993).

7. One or more individual amino acid residues is modified. Various derivatizing agents are known to react specifically with selected side chains or terminal residues, as described in detail below.

Lysinyl residues and amino terminal residues may be reacted with succinic or other carboxylic acid anhydrides, which reverse the charge of the lysinyl residues. Other suitable reagents for derivatizing alpha-amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues may be modified by reaction with any one or combination of several conventional reagents, including phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginyl residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

Specific modification of tyrosyl residues has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side chain groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides (R'-N=C=N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutaminyl and asparaginyl residues may be deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Cysteinyl residues can be replaced by amino acid residues or other moieties either to eliminate disulfide bonding or, conversely, to stabilize cross-linking. See, *e.g.*, Bhatnagar et al., (*supra*).

Derivatization with bifunctional agents is useful for cross-linking the peptides or their functional derivatives to a water-insoluble support matrix or to other macromolecular vehicles. Commonly used cross-linking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Carbohydrate (oligosaccharide) groups may conveniently be attached to sites that are known to be glycosylation sites in proteins. Generally, O-linked oligosaccharides are attached to serine (Ser) or threonine (Thr) residues while N-linked oligosaccharides are attached to asparagine (Asn) residues when they are part of the sequence Asn-X-Ser/Thr, where X can be any amino acid except proline. X is preferably one of the 19 naturally occurring amino acids other than proline. The structures of N-linked and O-linked oligosaccharides and the sugar residues found in each type are different. One type of sugar that is commonly found on both is N-acetylneuraminic acid (referred to as sialic acid). Sialic acid is usually the terminal residue of both N-linked and O-linked oligosaccharides and, by virtue of its negative charge, may confer acidic properties to the glycosylated compound. Such site(s) may be incorporated in the linker of the compounds of this invention and are preferably glycosylated by a cell during recombinant production of the polypeptide compounds (*e.g.*, in mammalian cells such as CHO, BHK, COS). However, such sites may further be glycosylated by synthetic or semi-synthetic procedures known in the art.

Other possible modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, oxidation of the sulfur atom in Cys, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains [see, for example, Creighton, Proteins: Structure and Molecule Properties (W. H. Freeman & Co., San Francisco), pp. 79-86 (1983)].

Compounds of the present invention may be changed at the DNA level, as well. The DNA sequence of any portion of the compound may be changed to codons more compatible with the

chosen host cell. For *E. coli*, which is the preferred host cell, optimized codons are known in the art. Codons may be substituted to eliminate restriction sites or to include silent restriction sites, which may aid in processing of the DNA in the selected host cell. The vehicle, linker and peptide DNA sequences may be modified to include any of the foregoing sequence changes.

Additional derivatives include non-peptide analogs that provide a stabilized structure or lessened biodegradation, are also contemplated. Peptide mimetic analogs can be prepared based on a selected inhibitory peptide by replacement of one or more residues by nonpeptide moieties. Preferably, the nonpeptide moieties permit the peptide to retain its natural confirmation, or stabilize a preferred, *e.g.*, bioactive, confirmation which retains the ability to recognize and bind myostatin. In one aspect, the resulting analog/mimetic exhibits increased binding affinity for myostatin. One example of methods for preparation of nonpeptide mimetic analogs from peptides is described in Nachman *et al.*, *Regul Pept* **57**:359-370 (1995). If desired, the peptides of the invention can be modified, for instance, by glycosylation, amidation, carboxylation, or phosphorylation, or by the creation of acid addition salts, amides, esters, in particular C-terminal esters, and N-acyl derivatives of the peptides of the invention. The peptibodies also can be modified to create peptide derivatives by forming covalent or noncovalent complexes with other moieties. Covalently-bound complexes can be prepared by linking the chemical moieties to functional groups on the side chains of amino acids comprising the peptibodies, or at the N- or C-terminus.

In particular, it is anticipated that the peptides can be conjugated to a reporter group, including, but not limited to a radiolabel, a fluorescent label, an enzyme (*e.g.*, that catalyzes a colorimetric or fluorometric reaction), a substrate, a solid matrix, or a carrier (*e.g.*, biotin or avidin). The invention accordingly provides a molecule comprising a peptibody molecule, wherein the molecule preferably further comprises a reporter group selected from the group consisting of a radiolabel, a fluorescent label, an enzyme, a substrate, a solid matrix, and a carrier. Such labels are well known to those of skill in the art, *e.g.*, biotin labels are particularly contemplated. The use of such labels is well known to those of skill in the art and is described in, *e.g.*, U.S. Patent Nos.3,817,837; 3,850,752; 3,996,345; and 4,277,437. Other labels that will be useful include but are not limited to radioactive labels, fluorescent labels and chemiluminescent labels. U.S. Patents concerning use of such labels include, for example, U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; and 3,996,345. Any of the peptibodies of the present invention may comprise one, two, or more of any of these labels.

Methods of Making Peptides and Peptibodies

The peptides of the present invention can be generated using a wide variety of techniques known in the art. For example, such peptides can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automatic synthesizers are commercially available and can be used in accordance with known protocols. See, for example, Stewart and Young (*supra*); Tam *et al.*, *J Am Chem Soc*, **105**:6442, (1983); Merrifield, *Science* **232:**341-347 (1986); Barany and Merrifield, The Peptides, Gross and Meienhofer, eds, Academic Press, New York, 1-284; Barany *et al.*, *Int J Pep Protein Res*, **30**:705-739 (1987); and U.S. Patent No. 5,424,398, each incorporated herein by reference.

Solid phase peptide synthesis methods use a copoly(styrene-divinylbenzene) containing 0.1-1.0 mM amines/g polymer. These methods for peptide synthesis use butyloxycarbonyl (t-BOC) or 9-fluorenylmethyloxy-carbonyl(FMOC) protection of alpha-amino groups. Both methods involve stepwise syntheses whereby a single amino acid is added at each step starting from the C-terminus of the peptide (See, Coligan *et al.*, *Curr Prot Immunol*, Wiley Interscience, 1991, Unit 9). On completion of chemical synthesis, the synthetic peptide can be deprotected to remove the t-BOC or FMOC amino acid blocking groups and cleaved from the polymer by treatment with acid at reduced temperature (*e.g.*, liquid HF-10% anisole for about 0.25 to about 1 hours at 0°C). After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution that is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptides or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

Phage display techniques can be particularly effective in identifying the peptides of the present invention as described above. Briefly, a phage library is prepared (using *e.g.* ml 13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues. The inserts may represent, for example, a completely degenerate or biased array. Phage-bearing inserts that bind to the desired antigen are selected and this process repeated through several cycles of reselection of phage that bind to the desired antigen. DNA sequencing is conducted to identify the sequences of the expressed peptides. The minimal linear portion of the sequence that binds to the desired antigen can be determined in this way. The procedure can be repeated using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. These techniques may identify peptides of the invention with still greater binding affinity for myostatin than agents already identified herein.

Regardless of the manner in which the peptides are prepared, a nucleic acid molecule encoding each such peptide can be generated using standard recombinant DNA procedures. The nucleotide sequence of such molecules can be manipulated as appropriate without changing the amino acid sequence they encode to account for the degeneracy of the nucleic acid code as well as to account for codon preference in particular host cells.

The present invention also provides nucleic acid molecules comprising polynucleotide sequences encoding the peptides and peptibodies of the present invention. These nucleic acid molecules include vectors and constructs containing polynucleotides encoding the peptides and peptibodies of the present invention, as well as peptide and peptibody variants and derivatives. Exemplary nucleic acid molecules are provided in the Examples below.

Recombinant DNA techniques also provide a convenient method for preparing full length peptibodies and other large polypeptide binding agents of the present invention, or fragments thereof.. A polynucleotide encoding the peptibody or fragment may be inserted into an expression vector, which can in turn be inserted into a host cell for production of the binding agents of the present invention. Preparation of exemplary peptibodies of the present invention are described in Example 2 below.

A variety of expression vector/host systems may be utilized to express the peptides and peptibodies of the invention. These systems include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.*, baculovirus); plant cell systems transfected with virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with bacterial expression vectors (*e.g.*, Ti or pBR322 plasmid); or animal cell systems. One preferred host cell line is *E.coli* strain 2596 (ATCC # 202174), used for expression of peptibodies as described below in Example 2. Mammalian cells that are useful in recombinant protein productions include but are not limited to VERO cells, HeLa cells, Chinese hamster ovary (CHO) cell lines, COS cells (such as COS-7), W138, BHK, HepG2, 3T3, RIN, MDCK, A549, PC12, K562 and 293 cells.

The term "expression vector" refers to a plasmid, phage, virus or vector, for expressing a polypeptide from a polynucleotide sequence. An expression vector can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or sequence that encodes the binding agent which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular

secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an amino terminal methionyl residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final peptide product.

For example, the peptides and peptibodies may be recombinantly expressed in yeast using a commercially available expression system, *e.g.*, the Pichia Expression System (Invitrogen, San Diego, CA), following the manufacturer's instructions. This system also relies on the pre-pro-alpha sequence to direct secretion, but transcription of the insert is driven by the alcohol oxidase (AOX1) promoter upon induction by methanol. The secreted peptide is purified from the yeast growth medium using the methods used to purify the peptide from bacterial and mammalian cell supernatants.

Alternatively, the cDNA encoding the peptide and peptibodies may be cloned into the baculovirus expression vector pVL1393 (PharMingen, San Diego, CA). This vector can be used according to the manufacturer's directions (PharMingen) to infect Spodoptera frugiperda cells in sF9 protein-free media and to produce recombinant protein. The recombinant protein can be purified and concentrated from the media using a heparin-Sepharose column (Pharmacia).

Alternatively, the peptide or peptibody may be expressed in an insect system. Insect systems for protein expression are well known to those of skill in the art. In one such system, Autographa californica nuclear polyhedrosis virus (AcNPV) can be used as a vector to express foreign genes in Spodoptera frugiperda cells or in Trichoplusia larvae. The peptide coding sequence can be cloned into a nonessential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of the peptide will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein coat. The recombinant viruses can be used to infect S. frugiperda cells or Trichoplusia larvae in which the peptide is expressed (Smith *et al.*, *J Virol* **46:** 584 (1983); Engelhard *et al.*, *Proc Nat Acad Sci (USA)* **91**: 3224-7 (1994)).

In another example, the DNA sequence encoding the peptide can be amplified by PCR and cloned into an appropriate vector for example, pGEX-3X (Pharmacia). The pGEX vector is designed to produce a fusion protein comprising glutathione-S-transferase (GST), encoded by the vector, and a protein encoded by a DNA fragment inserted into the vector's cloning site. The primers for PCR can be generated to include for example, an appropriate cleavage site. Where the fusion moiety is used solely to facilitate expression or is otherwise not desirable as an attachment to the peptide of interest, the recombinant fusion protein may then be cleaved from the GST portion of the fusion protein. The pGEX-3X/specific binding agent peptide construct is transformed into *E. coli* XL-1 Blue cells (Stratagene, La Jolla CA), and individual transformants

isolated and grown. Plasmid DNA from individual transformants can be purified and partially sequenced using an automated sequencer to confirm the presence of the desired specific binding agent encoding nucleic acid insert in the proper orientation.

The fusion protein, which may be produced as an insoluble inclusion body in the bacteria, can be purified as follows. Host cells are collected by centrifugation; washed in 0.15 M NaCl, 10 mM Tris, pH 8, 1 mM EDTA; and treated with 0.1 mg/ml lysozyme (Sigma, St. Louis, MO) for 15 minutes at room temperature. The lysate can be cleared by sonication, and cell debris can be pelleted by centrifugation for 10 minutes at 12,000 X g. The fusion protein-containing pellet can be resuspended in 50 mM Tris, pH 8, and 10 mM EDTA, layered over 50% glycerol, and centrifuged for 30 min. at 6000 X g. The pellet can be resuspended in standard phosphate buffered saline solution (PBS) free of Mg++ and Ca++. The fusion protein can be further purified by fractionating the resuspended pellet in a denaturing SDS-PAGE (Sambrook *et al.*, *supra*). The gel can be soaked in 0.4 M KCl to visualize the protein, which can be excised and electroeluted in gel-running buffer lacking SDS. If the GST/fusion protein is produced in bacteria as a soluble protein, it can be purified using the GST Purification Module (Pharmacia).

The fusion protein may be subjected to digestion to cleave the GST from the peptide of the invention. The digestion reaction (20-40 mg fusion protein, 20-30 units human thrombin (4000 U/mg, Sigma) in 0.5 ml PBS can be incubated 16-48 hrs at room temperature and loaded on a denaturing SDS-PAGE gel to fractionate the reaction products. The gel can be soaked in 0.4 M KCl to visualize the protein bands. The identity of the protein band corresponding to the expected molecular weight of the peptide can be confirmed by amino acid sequence analysis using an automated sequencer (Applied Biosystems Model 473A, Foster City, CA). Alternatively, the identity can be confirmed by performing HPLC and/or mass spectometry of the peptides.

Alternatively, a DNA sequence encoding the peptide can be cloned into a plasmid containing a desired promoter and, optionally, a leader sequence (Better *et al.*, *Science* **240**:1041-43 (1988)). The sequence of this construct can be confirmed by automated sequencing. The plasmid can then be transformed into *E. coli* strain MC1061 using standard procedures employing CaCl2 incubation and heat shock treatment of the bacteria (Sambrook *et al.*, *supra*). The transformed bacteria can be grown in LB medium supplemented with carbenicillin, and production of the expressed protein can be induced by growth in a suitable medium. If present, the leader sequence can effect secretion of the peptide and be cleaved during secretion.

Mammalian host systems for the expression of recombinant peptides and peptibodies are well known to those of skill in the art. Host cell strains can be chosen for a particular ability to process the expressed protein or produce certain post-translation modifications that will be useful in providing protein activity. Such modifications of the protein include, but are not limited to,

acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Different host cells such as CHO, HeLa, MDCK, 293, WI38, and the like have specific cellular machinery and characteristic mechanisms for such post-translational activities and can be chosen to ensure the correct modification and processing of the introduced, foreign protein.

It is preferable that transformed cells be used for long-term, high-yield protein production. Once such cells are transformed with vectors that contain selectable markers as well as the desired expression cassette, the cells can be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The selectable marker is designed to allow growth and recovery of cells that successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell line employed.

A number of selection systems can be used to recover the cells that have been transformed for recombinant protein production. Such selection systems include, but are not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase genes, in tk-, hgprt- or aprt- cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for dhfr which confers resistance to methotrexate; gpt which confers resistance to mycophenolic acid; neo which confers resistance to the aminoglycoside G418 and confers resistance to chlorsulfuron; and hygro which confers resistance to hygromycin. Additional selectable genes that may be useful include trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine. Markers that give a visual indication for identification of transformants include anthocyanins, ß-glucuronidase and its substrate, GUS, and luciferase and its substrate, luciferin.

Purification and Refolding of Binding Agents

In some cases, the binding agents such as the peptides and/or peptibodies of this invention may need to be "refolded" and oxidized into a proper tertiary structure and disulfide linkages generated in order to be biologically active. Refolding can be accomplished using a number of procedures well known in the art. Such methods include, for example, exposing the solubilized polypeptide agent to a pH usually above 7 in the presence of a chaotropic agent. The selection of chaotrope is similar to the choices used for inclusion body solubilization, however a chaotrope is typically used at a lower concentration. Exemplary chaotropic agents are guanidine and urea. In most cases, the refolding/oxidation solution will also contain a reducing agent plus its oxidized form in a specific ratio to generate a particular redox potential which allows for disulfide shuffling to occur for the formation of cysteine bridges. Some commonly used redox couples include cysteine/cystamine, glutathione/dithiobisGSH, cupric chloride, dithiothreitol DTT/dithiane DTT,

and 2-mercaptoethanol (bME)/dithio-bME. In many instances, a co-solvent may be used to increase the efficiency of the refolding. Commonly used cosolvents include glycerol, polyethylene glycol of various molecular weights, and arginine.

It may be desirable to purify the peptides and peptibodies of the present invention. Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the crude fractionation of the proteinaceous and non-proteinaceous fractions. Having separated the peptide and/or peptibody from other proteins, the peptide or polypeptide of interest can be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity). Analytical methods particularly suited to the preparation of peptibodies and peptides or the present invention are ion-exchange chromatography, exclusion chromatography; polyacrylamide gel electrophoresis; isoelectric focusing. A particularly efficient method of purifying peptides is fast protein liquid chromatography or even HPLC.

Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of a peptibody or peptide of the present invention. The term "purified peptibody or peptide" as used herein, is intended to refer to a composition, isolatable from other components, wherein the peptibody or peptide is purified to any degree relative to its naturally-obtainable state. A purified peptide or peptibody therefore also refers to a peptibody or peptide that is free from the environment in which it may naturally occur.

Generally, "purified" will refer to a peptide or peptibody composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a peptide or peptibody composition in which the peptibody or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95% or more of the proteins in the composition.

Various methods for quantifying the degree of purification of the peptide or peptibody will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific binding activity of an active fraction, or assessing the amount of peptide or peptibody within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a peptide or peptibody fraction is to calculate the binding activity of the fraction, to compare it to the binding activity of the initial extract, and to thus calculate the degree of purification, herein assessed by a "-fold purification number." The actual units used to represent the amount of binding activity will, of course, be dependent upon the particular assay technique chosen to follow the purification and whether or not the peptibody or peptide exhibits a detectable binding activity.

Various techniques suitable for use in purification will be well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies (immunoprecipitation) and the like or by heat denaturation, followed by centrifugation; chromatography steps such as affinity chromatography (*e.g.*, Protein-A-Sepharose), ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of such and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified binding agent.

There is no general requirement that the binding agents of the present invention always be provided in their most purified state. Indeed, it is contemplated that less substantially purified binding agent products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low-pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of the peptide or peptibody, or in maintaining binding activity of the peptide or peptibody.

It is known that the migration of a peptide or polypeptide can vary, sometimes significantly, with different conditions of SDS/PAGE (Capaldi *et al.*, *Biochem Biophys Res Comm*, **76**: 425 (1977)). It will therefore be appreciated that under differing electrophoresis conditions, the apparent molecular weights of purified or partially purified binding agent expression products may vary.

Activity of Myostatin Binding Agents

After the construction of the binding agents of the present invention, they are tested for their ability to bind myostatin and inhibit or block myostatin activity. Any number of assays or animal tests may be used to determine the ability of the agent to inhibit or block myostatin activity. Several assays used for characterizing the peptides and peptibodies of the present invention are described in the Examples below. One assay is the C2C12 pMARE-luc assay which makes use of a myostatin-responsive cell line (C2C12 myoblasts) transfected with a luciferase reporter vector containing myostatin/activin response elements (MARE). Exemplary peptibodies are assayed by pre-incubating a series of peptibody dilutions with myostatin, and then exposing the cells to the incubation mixture. The resulting luciferase activity is determined, and a titration curve is generated from the series of peptibody dilutions. The IC_{50} (the concentration of

peptibody to achieve 50% inhibition of myostatin activity as measured by luciferase activity) was then determined. A second assay described below is a BIAcore® assay to determine the kinetic parameters k_a (association rate constant), k_d (dissociation rate constant), and K_D (dissociation equilibrium constant) for the myostatin binding agents. Lower dissociation equilibrium constants (K_D, expressed in nM) indicated a greater affinity of the peptibody for myostatin. Additional assays include blocking assays, to determine whether a binding agent such as a peptibody is neutralizing (prevents binding of myostatin to its receptor), or non-neutralizing (does not prevent binding of myostatin to its receptor); selectivity assays, which determine if the binding agents of the present invention bind selectively to myostatin and not to other TGFß family members; and KinEx A™ assays or solution-based equilibrium assays, which also determine K_D and are considered to be more sensitive in some circumstances. These assays are described in Example 3.

Figure 1 shows the IC_{50} of a peptide compared with the IC_{50} of the peptibody form of the peptide. This demonstrates that the peptibody is significantly more effective at inhibiting myostatin activity than the peptide alone. In addition, affinity-matured peptibodies generally exhibit improved IC_{50} and K_D values compared with the parent peptides and peptibodies. The IC_{50} values for a number of exemplary affinity matured peptibodies are shown in Table VII, Example 7 below. Additionally, in some instances, making a 2x version of a peptibody, where two peptides are attached in tandem, increase the activity of the peptibody both *in vitro* and *in vivo*.

In vivo activities are demonstrated in the Examples below. The activities of the binding agents include anabolic acitivity increasing lean muscle mass in animal models, as well as decreasing the fat mass with respect to total body weight in treated animal models, and increasing muscular strength in animal models.

Uses of the Myostatin Binding Agents

The myostatin binding agents of the present invention bind to myostatin and block or inhibit myostatin signaling within targeted cells. The present invention provides methods and reagents for reducing the amount or activity of myostatin in an animal by administering an effective dosage of one or more myostatin binding agents to the animal. In one aspect, the present invention provides methods and reagents for treating myostatin-related disorders in an animal comprising administering an effective dosage of one or more binding agents to the animal. These myostatin-related disorders include but are not limited to various forms of muscle wasting, as well as metabolic disorders such as diabetes and related disorders, and bone degenerative diseases such as osteoporosis.

As shown in the Example 8 below, exemplary peptibodies of the present invention dramatically increases lean muscle mass in the CD1 nu/nu mouse model. This *in vivo* activity correlates to the *in vitro* binding and inhibitory activity described below for the same peptibodies.

Muscle wasting disorders include dystrophies such as Duchenne's muscular dystrophy, progressive muscular dystrophy, Becker's type muscular dystrophy, Dejerine-Landouzy muscular dystrophy, Erb's muscular dystrophy, and infantile neuroaxonal muscular dystrophy. For example, blocking myostatin through use of antibodies *in vivo* improved the dystrophic phenotype of the *mdx* mouse model of Duchenne muscular dystrophy (Bogdanovich et al, *Nature* **420**, 28 (2002)). The peptibodies of the present invention increase lean muscle mass as a percentage of body weight and decreases fat mass as percentage of body weight when administered to an aged *mdx* mouse model.

Additional muscle wasting disorders arise from chronic disease such as amyotrophic lateral sclerosis, congestive obstructive pulmonary disease, cancer, AIDS, renal failure, and rheumatoid arthritis. For example, cachexia or muscle wasting and loss of body weight was induced in athymic nude mice by a systemically administered myostatin (Zimmers et al., *supra*). In another example, serum and intramuscular concentrations of myostatin-immunoreactive protein was found to be increased in men exhibiting AIDS-related muscle wasting and was inversely related to fat-free mass (Gonzalez-Cadavid et al., *PNAS USA* **95**: 14938-14943 (1998)). Additional conditions resulting in muscle wasting may arise from inactivity due to disability such as confinement in a wheelchair, prolonged bedrest due to stroke, illness, spinal chord injury, bone fracture or trauma, and muscular atrophy in a microgravity environment (space flight). For example, plasma myostatin immunoreactive protein was found to increase after prolonged bedrest (Zachwieja et al. *J Gravit Physiol.* **6**(2):11(1999). It was also found that the muscles of rats exposed to a microgravity environment during a space shuttle flight expressed an increased amount of myostatin compared with the muscles of rats which were not exposed (Lalani et al., *J.Endocrin* **167** (3):417-28 (2000)).

In addition, age-related increases in fat to muscle ratios, and age-related muscular atrophy appear to be related to myostatin. For example, the average serum myostatin-immunoreactive protein increased with age in groups of young (19-35 yr old), middle-aged (36-75 yr old), and elderly (76-92 yr old) men and women, while the average muscle mass and fat-free mass declined with age in these groups (Yarasheski et al. *J Nutr Aging* **6**(5):343-8 (2002)). It has also been shown that myostatin gene knockout in mice increased myogenesis and decreased adipogenesis (Lin et al., *Biochem Biophys Res Commun* **291**(3):701-6 (2002), resulting in adults with increased muscle mass and decreased fat accumulation and leptin secretion. Exemplary peptibodies improve the lean muscle mass to fat ratio in aged *mdx* mice as shown below.

In addition, myostatin has now been found to be expressed at low levels in heart muscle and expression is upregulated after cardiomyocytes after infarct (Sharma et al., *J Cell Physiol.* **180** (1):1-9 (1999)). Therefore, reducing myostatin levels in the heart muscle may improve recovery of heart muscle after infarct.

Myostatin also appears to influence metabolic disorders including type 2 diabetes, noninsulin-dependent diabetes mellitus, hyperglycemia, and obesity. For example, lack of myostatin has been shown to improve the obese and diabetic phenotypes of two mouse models (Yen et al. *supra*). It has been demonstrated in the Examples below that decreasing myostatin activity by administering the inhibitors of the present invention will decreases the fat to muscle ratio in an animal, including aged animal models. Therefore, decreasing fat composition by administering the inhibitors of the present invention will improve diabetes, obesity, and hyperglycemic conditions in animals.

In addition, increasing muscle mass by reducing myostatin levels may improve bone strength and reduce osteoporosis and other degenerative bone diseases. It has been found, for example, that myostatin-deficient mice showed increased mineral content and density of the mouse humerus and increased mineral content of both trabecular and cortical bone at the regions where the muscles attach, as well as increased muscle mass (Hamrick et al. *Calcif Tissue Int* **71**(1):63-8 (2002)). .

The present invention also provides methods and reagents for increasing muscle mass in food animals by administering an effective dosage of the myostatin binding agent to the animal. Since the mature C-terminal myostatin polypeptide is identical in all species tested, myostatin binding agents would be expected to be effective for increasing muscle mass and reducing fat in any agriculturally important species including cattle, chicken, turkeys, and pigs.

The binding agents of the present invention may be used alone or in combination with other therapeutic agents to enhance their therapeutic effects or decrease potential side effects. The binding agents of the present invention possess one or more desirable but unexpected combination of properties to improve the therapeutic value of the agents. These properties include increased activity, increased solubility, reduced degradation, increased half-life, reduced toxicity, and reduced immunogenicity. Thus the binding agents of the present invention are useful for extended treatment regimes. In addition, the properties of hydrophilicity and hydrophobicity of the compounds of the invention are well balanced, thereby enhancing their utility for both *in vitro* and especially *in vivo* uses. Specifically, compounds of the invention have an appropriate degree of solubility in aqueous media that permits absorption and bioavailability in the body, while also having a degree of solubility in lipids that permits the compounds to traverse the cell membrane to a putative site of action, such as a particular muscle mass.

The binding agents of the present invention are useful for treating a "subject" or any animal, including humans, when administered in an effective dosages in a suitable composition.

In addition, the mystatin binding agents of the present invention are useful for detecting and quantitating myostatin in a number of assays. These assays are described in more detail below.

In general, the binding agents of the present invention are useful as capture agents to bind and immobilize myostatin in a variety of assays, similar to those described, for example, in Asai, ed., Methods in Cell Biology, **37**, Antibodies in Cell Biology, Academic Press, Inc., New York (1993). The binding agent may be labeled in some manner or may react with a third molecule such as an anti-binding agent antibody which is labeled to enable myostatin to be detected and quantitated. For example, a binding agent or a third molecule can be modified with a detectable moiety, such as biotin, which can then be bound by a fourth molecule, such as enzyme-labeled streptavidin, or other proteins. (Akerstrom, *J Immunol* **135**:2589 (1985); Chaubert, *Mod Pathol* **10**:585 (1997)).

Throughout any particular assay, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures.

Non-competitive binding assays:

Binding assays can be of the non-competitive type in which the amount of captured myostatin is directly measured. For example, in one preferred "sandwich" assay, the binding agent can be bound directly to a solid substrate where it is immobilized. These immobilized agents then bind to myostatin present in the test sample. The immobilized myostatin is then bound with a labeling agent, such as a labeled antibody against myostatin, which can be detected . In another preferred "sandwich" assay, a second agent specific for the binding agent can be added which contains a detectable moiety, such as biotin, to which a third labeled molecule can specifically bind, such as streptavidin. (See, Harlow and Lane, Antibodies, A Laboratory Manual, Ch 14, Cold Spring Harbor Laboratory, NY (1988), which is incorporated herein by reference).

Competitive Binding Assays:

Binding assays can be of the competitive type. The amount of myostatin present in the sample is measured indirectly by measuring the amount of myostastin displaced, or competed away, from a binding agent by the myostatin present in the sample. In one preferred competitive

binding assay, a known amount of myostastin, usually labeled, is added to the sample and the sample is then contacted with the binding agent. The amount of labeled myostastin bound to the binding agent is inversely proportional to the concentration of myostastin present in the sample. (following the protocols found in, for example Harlow and Lane, Antibodies, A Laboratory Manual, Ch 14, pp. 579-583, *supra*).

In another preferred competitive binding assay, the binding agent is immobilized on a solid substrate. The amount of myostastin bound to the binding agent may be determined either by measuring the amount of myostatin present in a myostatin/binding agent complex, or alternatively by measuring the amount of remaining uncomplexed myostatin.

Other Binding Assays

The present invention also provides Western blot methods to detect or quantify the presence of myostatin in a sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight and transferring the proteins to a suitable solid support, such as nitrocellulose filter, a nylon filter, or derivatized nylon filter. The sample is incubated with the binding agents or fragments thereof that bind myostatin and the resulting complex is detected. These binding agents may be directly labeled or alternatively may be subsequently detected using labeled antibodies that specifically bind to the binding agent.

Diagnostic Assays

The binding agents or fragments thereof of the present invention may be useful for the diagnosis of conditions or diseases characterized by increased amounts of myostatin. Diagnostic assays for high levels of myostatin include methods utilizing a binding agent and a label to detect myostatin in human body fluids, extracts of cells or specific tissue extracts. For example, serum levels of myostatin may be measured in an individual over time to determine the onset of muscle wasting associated with aging or inactivity, as described, for example, in Yarasheski et al., *supra*. Increased myostatin levels were shown to correlate with average decreased muscle mass and fat-free mass in groups of men and women of increasing ages (Yarasheski et al., *supra*). The binding agents of the present invention may be useful for monitoring increases or decreases in the levels of myostatin with a given individual over time, for example. The binding agents can be used in such assays with or without modification. In a preferred diagnostic assay, the binding agents will be labeled by attaching, e.g., a label or a reporter molecule. A wide variety of labels and reporter molecules are known, some of which have been already described herein. In particular, the present invention is useful for diagnosis of human disease.

A variety of protocols for measuring myostatin proteins using binding agents of myostatin are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescence activated cell sorting (FACS).

For diagnostic applications, in certain embodiments the binding agents of the present invention typically will be labeled with a detectable moiety. The detectable moiety can be any one that is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; or an enzyme, such as alkaline phosphatase, βgalactosidase, or horseradish peroxidase (Bayer et al., *Meth Enz*, **184**: 138 (1990)).

Pharmaceutical Compositions

Pharmaceutical compositions of myostatin binding agents such as peptibodies described herein are within the scope of the present invention. Such compositions comprise a therapeutically or prophylactically effective amount of a myostatin binding agent, fragment, variant, or derivative thereof as described herein, in admixture with a pharmaceutically acceptable agent. In a preferred embodiment, pharmaceutical compositions comprise antagonist binding agents that inhibit myostatin partially or completely in admixture with a pharmaceutically acceptable agent. Typically, the myostastin binding agents will be sufficiently purified for administration to an animal.

The pharmaceutical composition may contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolarity, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulfite or sodium hydrogen-sulfite); buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, other organic acids); bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin); fillers; monosaccharides; disaccharides and other carbohydrates (such as glucose, mannose, or dextrins); proteins (such as serum albumin, gelatin or immunoglobulins); coloring; flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such

as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate 80, triton, tromethamine, lecithin, cholesterol, tyloxapal); stability enhancing agents (sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides (preferably sodium or potassium chloride, mannitol sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. (Remington's Pharmaceutical Sciences, 18th Edition, A.R. Gennaro, ed., Mack Publishing Company, 1990).

The optimal pharmaceutical composition will be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format, and desired dosage. See for example, Remington's Pharmaceutical Sciences, supra. Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the binding agent.

The primary vehicle or carrier in a pharmaceutical composition may be either aqueous or non-aqueous in nature. For example, a suitable vehicle or carrier may be water for injection, physiological saline solution or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. Other exemplary pharmaceutical compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefore. In one embodiment of the present invention, binding agent compositions may be prepared for storage by mixing the selected composition having the desired degree of purity with optional formulation agents (Remington's Pharmaceutical Sciences, supra) in the form of a lyophilized cake or an aqueous solution. Further, the binding agent product may be formulated as a lyophilizate using appropriate excipients such as sucrose.

The pharmaceutical compositions can be selected for parenteral delivery. Alternatively, the compositions may be selected for inhalation or for enteral delivery such as orally, aurally, opthalmically, rectally, or vaginally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

The formulation components are present in concentrations that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at slightly lower pH, typically within a pH range of from about 5 to about 8.

When parenteral administration is contemplated, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising the desired binding agent in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which a binding agent is

formulated as a sterile, isotonic solution, properly preserved. Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (polylactic acid, polyglycolic acid), beads, or liposomes, that provides for the controlled or sustained release of the product which may then be delivered via a depot injection. Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

In another aspect, pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils, such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate, triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents to increase the solubility of the ompounds and allow for the preparation of highly concentrated solutions.In another embodiment, a pharmaceutical composition may be formulated for inhalation. For example, a binding agent may be formulated as a dry powder for inhalation. Polypeptide or nucleic acid molecule inhalation solutions may also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions may be nebulized. Pulmonary administration is further described in PCT Application No. PCT/US94/001875, which describes pulmonary delivery of chemically modified proteins.

It is also contemplated that certain formulations may be administered orally. In one embodiment of the present invention, binding agent molecules that are administered in this fashion can be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. For example, a capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the binding agent molecule. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

Pharmaceutical compositions for oral administration can also be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral

administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combining active compounds with solid excipient and processing the resultant mixture of granules (optionally, after grinding) to obtain tablets or dragee cores. Suitable auxiliaries can be added, if desired. Suitable excipients include carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, and sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; gums, including arabic and tragacanth; and proteins, such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, and alginic acid or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations that can be used orally also include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with fillers or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Another pharmaceutical composition may involve an effective quantity of binding agent in a mixture with non-toxic excipients that are suitable for the manufacture of tablets. By dissolving the tablets in sterile water, or other appropriate vehicle, solutions can be prepared in unit dose form. Suitable excipients include, but are not limited to, inert diluents, such as calcium carbonate, sodium carbonate or bicarbonate, lactose, or calcium phosphate; or binding agents, such as starch, gelatin, or acacia; or lubricating agents such as magnesium stearate, stearic acid, or talc.

Additional pharmaceutical compositions will be evident to those skilled in the art, including formulations involving binding agent molecules in sustained- or controlled-delivery formulations. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art. See for example, PCT/US93/00829 that

describes controlled release of porous polymeric microparticles for the delivery of pharmaceutical compositions. Additional examples of sustained-release preparations include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices may include polyesters, hydrogels, polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., Biopolymers, **22**:547-556 (1983), poly (2-hydroxyethyl-methacrylate) (Langer et al., *J. Biomed. Mater. Res.*, **15**:167-277, (1981); Langer et al., *Chem. Tech.*, **12**:98-105(1982)), ethylene vinyl acetate (Langer et al., *supra*) or poly-D(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release compositions also include liposomes, which can be prepared by any of several methods known in the art. See e.g., Eppstein et al.,*PNAS* (USA), **82:**3688 (1985); EP 36,676; EP 88,046; EP 143,949.

The pharmaceutical composition to be used for *in vivo* administration typically must be sterile. This may be accomplished by filtration through sterile filtration membranes. Where the composition is lyophilized, sterilization using this method may be conducted either prior to or following lyophilization and reconstitution. The composition for parenteral administration may be stored in lyophilized form or in solution. In addition, parenteral compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Once the pharmaceutical composition has been formulated, it may be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or a dehydrated or lyophilized powder. Such formulations may be stored either in a ready-to-use form or in a form (e.g., lyophilized) requiring reconstitution prior to administration.

In a specific embodiment, the present invention is directed to kits for producing a single-dose administration unit. The kits may each contain both a first container having a dried protein and a second container having an aqueous formulation. Also included within the scope of this invention are kits containing single and multi-chambered pre-filled syringes (e.g., liquid syringes and lyosyringes).

An effective amount of a pharmaceutical composition to be employed therapeutically will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will thus vary depending, in part, upon the molecule delivered, the indication for which the binding agent molecule is being used, the route of administration, and the size (body weight, body surface or organ size) and condition (the age and general health) of the patient. Accordingly, the clinician may titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. A typical dosage may range from about 0.1mg/kg to up to about 100 mg/kg or more, depending on the factors mentioned

above. In other embodiments, the dosage may range from 0.1 mg/kg up to about 100 mg/kg; or 1 mg/kg up to about 100 mg/kg; or 5 mg/kg up to about 100 mg/kg.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models such as mice, rats, rabbits, dogs, pigs, or monkeys. An animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

The exact dosage will be determined in light of factors related to the subject requiring treatment. Dosage and administration are adjusted to provide sufficient levels of the active compound or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, the general health of the subject, the age, weight, and gender of the subject, time and frequency of administration, drug combination(s), reaction sensitivities, and response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or biweekly depending on the half-life and clearance rate of the particular formulation.

The frequency of dosing will depend upon the pharmacokinetic parameters of the binding agent molecule in the formulation used. Typically, a composition is administered until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as multiple doses (at the same or different concentrations/dosages) over time, or as a continuous infusion. Further refinement of the appropriate dosage is routinely made. Appropriate dosages may be ascertained through use of appropriate dose-response data.

The route of administration of the pharmaceutical composition is in accord with known methods, e.g. orally, through injection by intravenous, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, intra-ocular, intraarterial, intraportal, intralesional routes, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, urethral, vaginal, or rectal means, by sustained release systems or by implantation devices. Where desired, the compositions may be administered by bolus injection or continuously by infusion, or by implantation device.

Alternatively or additionally, the composition may be administered locally via implantation of a membrane, sponge, or another appropriate material on to which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device may be implanted into any suitable tissue or organ, and delivery of the desired molecule may be via diffusion, timed-release bolus, or continuous administration.

In some cases, it may be desirable to use pharmaceutical compositions in an *ex vivo* manner. In such instances, cells, tissues, or organs that have been removed from the patient are exposed to the pharmaceutical compositions after which the cells, tissues and/or organs are subsequently implanted back into the patient.

In other cases, a binding agent of the present invention such as a peptibody can be delivered by implanting certain cells that have been genetically engineered, using methods such as those described herein, to express and secrete the polypeptide. Such cells may be animal or human cells, and may be autologous, heterologous, or xenogeneic. Optionally, the cells may be immortalized. In order to decrease the chance of an immunological response, the cells may be encapsulated to avoid infiltration of surrounding tissues. The encapsulation materials are typically biocompatible, semi-permeable polymeric enclosures or membranes that allow the release of the protein product(s) but prevent the destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues.

Pharmaceutical compositions containing the binding agents of the present invention are administered to a subject to treat any myostatin-related disorders. These include muscle-wasting disorders including but not limited to muscular dystrophy, muscle wasting in cancer, AIDS, muscle atrophy, rheumatoid arthritis, renal failure/uremia, chronic heart failure, prolonged bed-rest, spinal chord injury, stroke, and aging related sarcopenia. In addition these compositions are administed to treat obesity, diabetes, hyperglycemia, and increase bone density,

The invention having been described, the following examples are offered by way of illustration, and not limitation.

Example 1

Identification of myostatin -binding peptides

Three filamentous phage libraries, TN8-IX ($5X10^9$ independent transformants), TN12-I ($1.4X10^9$ independent transformants), and linear ($2.3X10^9$ independent transformants) (Dyax Corp.) were used to select for myostatin binding phage. Each library was incubated on myostatin-coated surfaces and subjected to different panning conditions: non-specific elution, and specific elution using recombinant human activin receptor IIB/Fc chimera (R&D Systems, Inc., Minneapolis, Minnesota), or myostastin propeptide elution as described below. For all three libraries, the phages were eluted in a non-specific manner for the first round of selection, while the receptor and promyostatin was used in the second and third rounds of selection. The selection procedures were carried out as described below.

Preparation of myostatin

Myostatin protein was produced recombinantly in the *E.coli* K-12 strain 2596 (ATCC # 202174) as follows. Polynucleotides encoding the human promyostatin molecule were cloned into the pAMG21 expression vector (ATCC No. 98113), which was derived from expression vector pCFM1656 (ATCC No. 69576) and the expression vector system described in United States Patent No. 4,710,473, by following the procedure described in published International Patent Application WO 00/24782. The polynucleotides encoding promyostatin were obtained from a mammalian expression vector. The coding region was amplified using a standard PCR method and the following PCR primers to introduce the restriction site for *Nde*I and *Bam*HI.

5' primer: 5'-GAGAGAGAGCATATGAATGAGAACAGTGAGCAAAAAG-3' (Seq ID No: 292)

3'primer: 5'-AGAGAGGGATCCATTATGAGCACCCACAGCGGTC-3' (Seq ID No: 293)

The PCR product and vector were digested with both enzymes, mixed and ligated. The product of the ligation was transformed into *E. coli* strain #2596. Single colonies were checked microscopically for recombinant protein expression in the form of inclusion bodies. The plasmid was isolated and sequenced through the coding region of the recombinant gene to verify genetic fidelity.

Bacterial paste was generated from a 10L fermentation using a batch method at 37°C. The culture was induced with HSL at a cell density of 9.6 OD_{600} and harvested six hours later at a density of 104 OD_{600}. The paste was stored at -80°C. *E.coli* paste expressing promyostatin was lysed in a microfluidizer at 16,000psi, centrifuged to isolate the insoluble inclusion body fraction. Inclusion bodies were resuspended in guanidine hydrochloride containing dithiothreitol and solubilized at room temperature. This was then diluted 30 fold in an aqueous buffer. The refolded promyostatin was then concentrated and buffer exchanged into 20mM Tris pH 8.0, and applied to an anion exchange column. The anion exchange column was eluted with an increasing sodium chloride gradient. The fractions containing promyostatin were pooled. The promyostatin produced in *E.coli* is missing the first 23 amino acids and begins with a methionine before the residue 24 asparagine. To produce mature myostatin, the pooled promyostatin was enzymatically cleaved between the propeptide and mature myostatin C terminal. The resulting mixture was then applied to a C4-rpHPLC column using a increasing gradient of acetonitrile containing 0.1% trifluoroacetic acid. Fractions containing mature myostatin were pooled and dried in a speed-vac.

The recombinant mature myostatin produced from *E. coli* was tested in the myoblast C2C12 based assay described below and found to be fully active when compared with

recombinant murine myostatin commercially produced in a mammalian cell system (R&D Systems, Inc., Minneapolis, Minnesota). The *E.coli*-produced mature myostatin was used in the phage-display and screening assays described below.

Preparation of Myostatin-Coated Tubes

Myostatin was immobilized on 5 ml Immuno ™ Tubes (NUNC) at a concentration of 8 ug of myostatin protein in 1 ml of 0.1M sodium carbonate buffer (pH 9.6). The myostatin-coated Immuno™ Tube was incubated with orbital shaking for 1 hour at room temperature. Myostatin-coated Immuno ™ Tube was then blocked by adding 5 ml of 2% milk-PBS and incubating at room temperature for 1 hour with rotation. The resulting myostatin-coated Immuno ™ Tube was then washed three times with PBS before being subjected to the selection procedures. Additional Immuno ™ Tubes were also prepared for negative selections (no myostatin). For each panning condition, five to ten Immuno™ Tubes were subjected to the above procedure except that the Immuno ™ Tubes were coated with 1ml of 2% BSA-PBS instead of myostatin protein.

Negative Selection

For each panning condition, about 100 random library equivalents for TN8-IX and TN12-I libraries ($5X10^{11}$ pfu for TN8-IX, and $1.4X10^{11}$ pfu for TN12-I) and about 10 random library equivalents for the linear library ($2.3X10^{10}$ pfu) were aliquoted from the library stock and diluted to 1 ml with PBST (PBS with 0.05% Tween-20). The 1 ml of diluted library stock was added to an Immuno™ Tube prepared for the negative selection, and incubated for 10 minutes at room temperature with orbital shaking. The phage supernatant was drawn out and added to the second Immuno™ Tube for another negative selection step. In this way, five to ten negative selection steps were performed.

Selection for Myostatin Binding

After the last negative selection step above, the phage supernatant was added to the prepared myostatin coated Immuno™ Tubes. The Immuno ™ Tube was incubated with orbital shaking for one hour at room temperature, allowing specific phage to bind to myostatin. After the supernatant was discarded, the Immuno ™ Tube was washed about 15 times with 2% milk-PBS, 10 times with PBST and twice with PBS for the three rounds of selection with all three libraries (TN8-IX, TN12-I, and Linear libraries) except that for the second round of selections with TN8-IX and TN12-I libraries, the Immuno ™ Tube was washed about 14 times with 2% milk-PBS, twice with 2% BSA-PBS, 10 times with PBST and once with PBS.

Non-specific elution

After the last washing step, the bound phages were eluted from the Immuno ™ Tube by adding 1 ml of 100 mM triethylamine solution (Sigma, St. Louis, Missouri) with 10-minute

incubation with orbital shaking. The pH of the phage containing solution was then neutralized with 0.5 ml of 1 M Tris-HCl (pH 7.5).

Receptor (Human Activin Receptor) elution of bound phage

For round 2 and 3, after the last washing step, the bound phages were eluted from the Immuno ™ Tube by adding 1 ml of 1 μM of receptor protein (recombinant human activin receptor IIB/Fc chimera, R&D Systems, Inc., Minneapolis, Minnesota) with a 1-hour incubation for each condition.

Propeptide elution of bound phage

For round 2 and 3, after the last washing step, the bound phages were eluted from the Immuno ™ Tube by adding 1 ml of 1 μM propeptide protein (made as described above) with a 1-hour incubation for each condition.

Phage Amplification

Fresh *E.coli.* (XL-1 Blue MRF') culture was grown to OD_{600} = 0.5 in LB media containing 12.5 ug/ml tetracycline. For each panning condition, 20 ml of this culture was chilled on ice and centrifuged. The bacteria pellet was resuspended in 1 ml of the min A salts solution.

Each mixture from different elution methods was added to a concentrated bacteria sample and incubated at 37°C for 15 minutes. 2 ml of NZCYM media (2x NZCYM, 50 ug/ml Ampicillin) was added to each mixture and incubated at 37°C for 15 minutes. The resulting 4 ml solution was plated on a large NZCYM agar plate containing 50 ug/ml ampicillin and incubated overnight at 37°C.

Each of the bacteria/phage mixture that was grown overnight on a large NZCYM agar plate was scraped off in 35 ml of LB media, and the agar plate was further rinsed with additional 35 ml of LB media. The resulting bacteria/phage mixture in LB media was centrifuged to pellet the bacteria away. 50 ul of the phage supernatant was transferred to a fresh tube, and 12.5 ml of PEG solution (20% PEG8000, 3.5M ammonium acetate) was added and incubated on ice for 2 hours to precipitate phages. The precipitated phages were centrifuged down and resuspended in 6 ml of the phage resuspension buffer (250 mM NaCl, 100 mM Tris pH8, 1 mM EDTA). This phage solution was further purified by centrifuging away the remaining bacteria and precipitating the phage for the second time by adding 1.5 ml of the PEG solution. After a centrifugation step, the phage pellet was resuspended in 400 ul of PBS. This solution was subjected to a final centrifugation to rid of remaining bacteria debris. The resulting phage preparation was titered by a standard plaque formation assay (Molecular Cloning, Maniatis et al., 3rd Edition).

Additional rounds of selection and amplification

In the second round, the amplified phage (10^{11} pfu) from the first round was used as the input phage to perform the selection and amplification steps. The amplified phage (10^{11} pfu) from the second round in turn was used as the input phage to perform third round of selection and amplification. After the elution steps of the third round, a small fraction of the eluted phage was plated out as in the plaque formation assay above. Individual plaques were picked and placed into 96 well microtiter plates containing 100 ul of TE buffer in each well. These master plates were incubated at 4°C overnight to allow phages to elute into the TE buffer.

Clonal Analysis

Phage ELISA

The phage clones were subjected to phage ELISA and then sequenced. The sequences were ranked as discussed below.

Phage ELISA was performed as follows. An *E. Coli* XL-1 Blue MRF'culture was grown until OD_{600} reached 0.5. 30 ul of this culture was aliquoted into each well of a 96 well microtiter plate. 10 ul of eluted phage was added to each well and allowed to infect bacteria for 15 min at room temperature. About 120 ul of LB media containing 12.5 ug/ml of tetracycline and 50 ug/ml of ampicillin were added to each well. The microtiter plate was then incubated with shaking overnight at 37 °C. Myostatin protein (2 ug/ml in 0.1M sodium carbonate buffer, pH 9.6) was allowed to coat onto a 96 well Maxisorp™ plates (NUNC) overnight at 4°C. As a control, a separate Maxisorp™ plate was coated with 2% BSA prepared in PBS.

On the following day, liquid in the protein coated Maxisorp™ plates was discarded, washed three times with PBS and each well was blocked with 300 ul of 2% milk solution at room temperature for 1 hour. The milk solution was discarded, and the wells were washed three times with the PBS solution. After the last washing step, about 50 ul of PBST-4% milk was added to each well of the protein-coated Maxisorp™ plates. About 50 ul of overnight cultures from each well in the 96 well microtiter plate was transferred to the corresponding wells of the myostatin coated plates as well as the control 2% BSA coated plates. The 100 ul mixture in the two kinds of plates were incubated for 1 hour at room temperature. The liquid was discarded from the Maxisorp™ plates, and the wells were washed about three times with PBST followed by two times with PBS. The HRP-conjugated anti-M13 antibody (Amersham Pharmacia Biotech) was diluted to about 1:7,500, and 100 ul of the diluted solution was added to each well of the Maxisorp™ plates for 1 hour incubation at room temperature. The liquid was again discarded and the wells were washed about three times with PBST followed by two time with PBS. 100 ul of LumiGlo™ Chemiluminescent substrate (KPL) was added to each well of the Maxisorp™ plates and incubated for about 5 minutes for reaction to occur. The chemiluminescent unit of the Maxisorp ™ plates was read on a plate reader (Lab System).

Sequencing of the phage clones

For each phage clone, the sequencing template was prepared by a PCR method. The following oligonucleotide pair was used to amplify a 500 nucleotide fragment: primer #1: 5'-CGGCGCAACTATCGGTATCAAGCTG-3' (Seq ID No: 294) and primer #2: 5'-CATGTACCGTAACACTGAGTTTCGTC-3'(Seq ID No: 295). The following mixture was prepared for each clone.

Reagents	Volume (μL) / tube
distilled H_2O	26.25
50% glycerol	10
10X PCR Buffer (w/o $MgCl_2$)	5
25 mM $MgCl_2$	4
10 mM dNTP mix	1
100 μM primer 1	0.25
100 μM primer 2	0.25
Taq polymerase	0.25
Phage in TE (section 4)	3
Final reaction volume	50

A thermocycler (GeneAmp PCR System 9700, Applied Biosystem) was used to run the following program: [94°C for 5min; 94°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec.] x30 cycles; 72°C for 7 min; cool to 4°C. The PCR product from each reaction was cleaned up using the QIAquick Multiwell PCR Purification kit (Qiagen), following the manufacturer's protocol. The PCR cleaned up product was checked by running 10 ul of each PCR reaction mixed with 1 ul of dye (10X BBXS agarose gel loading dye) on a 1% agarose gel. The remaining product was then sequenced using the ABI 377 Sequencer (Perkin Elmer) following the manufacturer recommended protocol.

Sequence Ranking and Analysis

The peptide sequences that were translated from the nucleotide sequences were correlated to ELISA data. The clones that showed high chemiluminescent units in the myostatin-coated wells and low chemiluminescent units in the 2% BSA-coated wells were identified. The sequences that occurred multiple times were identified. Candidate sequences chosen based on these criteria were subjected to further analysis as peptibodies. Approximately 1200 individual clones were analyzed. Of these approximately 132 peptides were chosen for generating the peptibodies of the present invention. These are shown in Table I below. The peptides having

SEQ ID NO: 1 to 129 were used to generate peptibodies of the same name. The peptides having SEQ ID NO: 130 to 141 shown in Table 1 comprise two or more peptides from SEQ ID NO: 1 to 132 attached by a linker sequence. SEQ ID NO: 130 to 141 were also used to generate peptibodies of the same name.

Consensus sequences were determined for the TN-8 derived group of peptides. These are as follows:

KDXCXXWHWMCKPX (Seq ID No: 142)

WXXCXXXGFWCXNX (Seq ID No: 143)

IXGCXWWDXXCYXX (Seq ID No: 144)

XXWCVSPXWFCXXX (Seq ID No: 145)

XXXCPWFAXXCVDW (Seq ID No: 146)

For all of the above consensus sequences, the underlined "core sequences" from each consensus sequence are the amino acid which always occur at that position. "X" refers to any naturally occurring or modified amino acid. The two cysteines contained with the core sequences were fixed amino acids in the TN8-IX library.

TABLE I

PEPTIBODY NAME	SEQ.ID No	PEPTIDE SEQUENCE
Myostatin-TN8-Con1	1	KDKCKMWHWMCKPP
Myostatin-TN8-Con2	2	KDLCAMWHWMCKPP
Myostatin-TN8-Con3	3	KDLCKMWKWMCKPP
Myostatin-TN8-Con4	4	KDLCKMWHWMCKPK
Myostatin-TN8-Con5	5	WYPCYEFHFWCYDL
Myostatin-TN8-Con6	6	WYPCYEGHFWCYDL
Myostatin-TN8-Con7	7	IFGCKWWDVQCYQF
Myostatin-TN8-Con8	8	IFGCKWWDVDCYQF
Myostatin-TN8-Con9	9	ADWCVSPNWFCMVM
Myostatin-TN8-Con10	10	HKFCPWWALFCWDF
Myostatin-TN8-1	11	KDLCKMWHWMCKPP
Myostatin-TN8-2	12	IDKCAIWGWMCPPL
Myostatin-TN8-3	13	WYPCGEFGMWCLNV
Myostatin-TN8-4	14	WFTCLWNCDNE
Myostatin-TN8-5	15	HTPCPWFAPLCVEW
Myostatin-TN8-6	16	KEWCWRWKWMCKPE
Myostatin-TN8-7	17	FETCPSWAYFCLDI
Myostatin-TN8-8	18	AYKCEANDWGCWWL
Myostatin-TN8-9	19	NSWCEDQWHRCWWL
Myostatin-TN8-10	20	WSACYAGHFWCYDL
Myostatin-TN8-11	21	ANWCVSPNWFCMVM
Myostatin-TN8-12	22	WTECYQQEFWCWNL
Myostatin-TN8-13	23	ENTCERWKWMCPPK
Myostatin-TN8-14	24	WLPCHQEGFWCMNF
Myostatin-TN8-15	25	STMCSQWHWMCNPF

Myostatin-TN8-16	26	IFGCHWWDVDCYQF
Myostatin-TN8-17	27	IYGCKWWDIQCYDI
Myostatin-TN8-18	28	PDWCIDPDWWCKFW
Myostatin-TN8-19	29	QGHCTRWPWMCPPY
Myostatin-TN8-20	30	WQECYREGFWCLQT
Myostatin-TN8-21	31	WFDCYGPGFKCWSP
Myostatin-TN8-22	32	GVRCPKGHLWCLYP
Myostatin-TN8-23	33	HWACGYWPWSCKWV
Myostatin-TN8-24	34	GPACHSPWWWCVFG
Myostatin-TN8-25	35	TTWCISPMWFCSQQ
Myostatin-TN8-26	36	HKFCPPWAIFCWDF
Myostatin-TN8-27	37	PDWCVSPRWYCNMW
Myostatin-TN8-28	38	VWKCHWFGMDCEPT
Myostatin-TN8-29	39	KKHCQIWTWMCAPK
Myostatin-TN8-30	40	WFQCGSTLFWCYNL
Myostatin-TN8-31	41	WSPCYDHYFYCYTI
Myostatin-TN8-32	42	SWMCGFFKEVCMWV
Myostatin-TN8-33	43	EMLCMIHPVFCNPH
Myostatin-TN8-34	44	LKTCNLWPWMCPPL
Myostatin-TN8-35	45	VVGCKWYEAWCYNK
Myostatin-TN8-36	46	PIHCTQWAWMCPPT
Myostatin-TN8-37	47	DSNCPWYFLSCVIF
Myostatin-TN8-38	48	HIWCNLAMMKCVEM
Myostatin-TN8-39	49	NLQCIYFLGKCIYF
Myostatin-TN8-40	50	AWRCMWFSDVCTPG
Myostatin-TN8-41	51	WFRCFLDADWCTSV
Myostatin-TN8-42	52	EKICQMWSWMCAPP
Myostatin-TN8-43	53	WFYCHLNKSECTEP
Myostatin-TN8-44	54	FWRCAIGIDKCKRV
Myostatin-TN8-45	55	NLGCKWYEVWCFTY
Myostatin-TN8-46	56	IDLCNMWDGMCYPP
Myostatin-TN8-47	57	EMPCNIWGWMCPPV
Myostatin-TN12-1	58	WFRCVLTGIVDWSECFGL
Myostatin-TN12-2	59	GFSCTFGLDEFYVDCSPF
Myostatin-TN12-3	60	LPWCHDQVNADWGFCMLW
Myostatin-TN12-4	61	YPTCSEKFWIYGQTCVLW
Myostatin-TN12-5	62	LGPCPIHHGPWPQYCVYW
Myostatin-TN12-6	63	PFPCETHQISWLGHCLSF
Myostatin-TN12-7	64	HWGCEDLMWSWHPLCRRP
Myostatin-TN12-8	65	LPLCDADMMPTIGFCVAY
Myostatin-TN12-9	66	SHWCETTFWMNYAKCVHA
Myostatin-TN12-10	67	LPKCTHVPFDQGGFCLWY
Myostatin-TN12-11	68	FSSCWSPVSRQDMFCVFY
Myostatin-TN12-13	69	SHKCEYSGWLQPLCYRP
Myostatin-TN12-14	70	PWWCQDNYVQHMLHCDSP
Myostatin-TN12-15	71	WFRCMLMNSFDAFQCVSY
Myostatin-TN12-16	72	PDACRDQPWYMFMGCMLG
Myostatin-TN12-17	73	FLACFVEFELCFDS
Myostatin-TN12-18	74	SAYCIITESDPYVLCVPL

Myostatin-TN12-19	75	PSICESYSTMWLPMCQHN
Myostatin-TN12-20	76	WLDCHDDSWAWTKMCRSH
Myostatin-TN12-21	77	YLNCVMMNTSPFVECVFN
Myostatin-TN12-22	78	YPWCDGFMIQQGITCMFY
Myostatin-TN12-23	79	FDYCTWLNGFKDWKCWSR
Myostatin-TN12-24	80	LPLCNLKEISHVQACVLF
Myostatin-TN12-25	81	SPECAFARWLGIEQCQRD
Myostatin-TN12-26	82	YPQCFNLHLLEWTECDWF
Myostatin-TN12-27	83	RWRCEIYDSEFLPKCWFF
Myostatin-TN12-28	84	LVGCDNVWHRCKLF
Myostatin-TN12-29	85	AGWCHVWGEMFGMGCSAL
Myostatin-TN12-30	86	HHECEWMARWMSLDCVGL
Myostatin-TN12-31	87	FPMCGIAGMKDFDFCVWY
Myostatin-TN12-32	88	RDDCTFWPEWLWKLCERP
Myostatin-TN12-33	89	YNFCSYLFGVSKEACQLP
Myostatin-TN12-34	90	AHWCEQGPWRYGNICMAY
Myostatin-TN12-35	91	NLVCGKISAWGDEACARA
Myostatin-TN12-36	92	HNVCTIMGPSMKWFCWND
Myostatin-TN12-37	93	NDLCAMWGWRNTIWCQNS
Myostatin-TN12-38	94	PPFCQNDNDMLQSLCKLL
Myostatin-TN12-39	95	WYDCNVPNELLSGLCRLF
Myostatin-TN12-40	96	YGDCDQNHWMWPFTCLSL
Myostatin-TN12-41	97	GWMCHFDLHDWGATCQPD
Myostatin-TN12-42	98	YFHCMFGGHEFEVHCESF
Myostatin-TN12-43	99	AYWCWHGQCVRF
Myostatin-Linear-1	100	SEHWTFTDWDGNEWWVRPF
Myostatin-Linear-2	101	MEMLDSLFELLKDMVPISKA
Myostatin-Linear-3	102	SPPEEALMEWLGWQYGKFT
Myostatin-Linear-4	103	SPENLLNDLYILMTKQEWYG
Myostatin-Linear-5	104	FHWEEGIPFHVVTPYSYDRM
Myostatin-Linear-6	105	KRLLEQFMNDLAELVSGHS
Myostatin-Linear-7	106	DTRDALFQEFYEFVRSRLVI
Myostatin-Linear-8	107	RMSAAPRPLTYRDIMDQYWH
Myostatin-Linear-9	108	NDKAHFFEMFMFDVHNFVES
Myostatin-Linear-10	109	QTQAQKIDGLWELLQSIRNQ
Myostatin-Linear-11	110	MLSEFEEFLGNLVHRQEA
Myostatin-Linear-12	111	YTPKMGSEWTSFWHNRIHYL
Myostatin-Linear-13	112	LNDTLLRELKMVLNSLSDMK
Myostatin-Linear-14	113	FDVERDLMRWLEGFMQSAAT
Myostatin-Linear-15	114	HHGWNYLRKGSAPQWFEAWV
Myostatin-Linear-16	115	VESLHQLQMWLDQKLASGPH
Myostatin-Linear-17	116	RATLLKDFWQLVEGYGDN
Myostatin-Linear-18	117	EELLREFYRFVSAFDY
Myostatin-Linear-19	118	GLLDEFSHFIAEQFYQMPGG
Myostatin-Linear-20	119	YREMSMLEGLLDVLERLQHY
Myostatin-Linear-21	120	HNSSQMLLSELIMLVGSMMQ
Myostatin-Linear-22	121	WREHFLNSDYIRDKLIAIDG
Myostatin-Linear-23	122	QFPFYVFDDLPAQLEYWIA

Myostatin-Linear-24	123	EFFHWLHNHRSEVNHWLDMN
Myostatin-Linear-25	124	EALFQNFFRDVLTLSEREY
Myostatin-Linear-26	125	QYWEQQWMTYFRENGLHVQY
Myostatin-Linear-27	126	NQRMMLEDLWRIMTPMFGRS
Myostatin-Linear-29	127	FLDELKAELSRHYALDDLDE
Myostatin-Linear-30	128	GKLIEGLLNELMQLETFMPD
Myostatin-Linear-31	129	ILLLDEYKKDWKSWF
Myostatin-2xTN8-19 kc	130	QGHCTRWPWMCPPYGSGSATGGS GSTASSGSGSATGQGHCTRWPWM CPPY
Myostatin-2xTN8-con6	131	WYPCYEGHFWCYDLGSGSTASSG SGSATGWYPCYEGHFWCYDL
Myostatin-2xTN8-5 kc	132	HTPCPWFAPLCVEWGSGSATGGSG STASSGSGSATGHTPCPWFAPLCV EW
Myostatin-2xTN8-18 kc	133	PDWCIDPDWWCKFWGSGSATGGS GSTASSGSGSATGPDWCIDPDWW CKFW
Myostatin-2xTN8-11 kc	134	ANWCVSPNWFCMVMGSGSATGG SGSTASSGSGSATGANWCVSPNWF CMVM
Myostatin-2xTN8-25 kc	135	PDWCIDPDWWCKFWGSGSATGGS GSTASSGSGSATGPDWCIDPDWW CKFW
Myostatin-2xTN8-23 kc	136	HWACGYWPWSCKWVGSGSATGG SGSTASSGSGSATGHWACGYWPW SCKWV
Myostatin-TN8-29-19 kc	137	KKHCQIWTWMCAPKGSGSATGGS GSTASSGSGSATGQGHCTRWPWM CPPY
Myostatin-TN8-19-29 kc	138	QGHCTRWPWMCPPYGSGSATGGS GSTASSGSGSATGKKHCQIWTWM CAPK
Myostatin-TN8-29-19 kn	139	KKHCQIWTWMCAPKGSGSATGGS GSTASSGSGSATGQGHCTRWPWM CPPY
Myostatin-TN8-29-19-8g	140	KKHCQIWTWMCAPKGGGGGGGG QGHCTRWPWMCPPY
Myostatin-TN8-19-29-6gc	141	QGHCTRWPWMCPPYGGGGGGKK HCQIWTWMCAPK

Example 2

Generating peptibodies

Construction of DNA encoding peptide-Fc fusion proteins

Peptides capable of binding myostatin were used alone or in combination with each other to construct fusion proteins in which a peptide was fused to the Fc domain of human IgG1. The

amino acid sequence of the Fc portion of each peptibody is as follows (from amino terminus to carboxyl terminus):

DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV
SHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPS
RDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL
DSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK (Seq ID No: 296)

The peptide was fused in the N configuration (peptide was attached to the N-terminus of the Fc region), the C configuration (peptide was attached to the C-terminus of the Fc region), or the N,C configuration (peptide attached both at the N and C terminus of the Fc region). Separate vectors were used to express N-terminal fusions and C-terminal fusions. Each peptibody was constructed by annealing pairs of oligonucleotides ("oligos") to the selected phage nucleic acid to generate a double stranded nucleotide sequence encoding the peptide. These polynucleotide molecules were constructed as *Apa*L to *Xho*I fragments. The fragments were ligated into either the pAMG21-Fc N-terminal vector for the N-terminal orientation, or the pAMG21-Fc-C-terminal vector for the C-terminal orientation which had been previously digested with *Apa*LI and *Xho*I . The resulting ligation mixtures were transformed by electroporation into *E. coli* strain 2596 or 4167 cells (a hsdR- variant of strain 2596 cells) using standard procedures. Clones were screened for the ability to produce the recombinant protein product and to possess the gene fusion having a correct nucleotide sequence. A single such clone was selected for each of the modified peptides.

Many of constructs were created using an alternative vector designated pAMG21-2xBs-N(*Zeo*R) Fc. This vector is simlar to the above-described vector except that the vector digestion was performed with *Bsm*BI. Some constructs fused peptide sequences at both ends of the Fc. In those cases the vector was a composite of pAMG21-2xBs-N(*Zeo*R) Fc and pAMG21-2xBs-C-Fc.

Construction of pAMG21

Expression plasmid pAMG21 (ATCC No. 98113) is derived from expression vector pCFM1656 (ATCC No. 69576) and the expression vector system described in United States Patent No. 4,710,473, by following the procedure described in published International Patent Application WO 00/24782, all of which are incorporated herein by reference.

Fc N-terminal Vector

The Fc N-terminal vector was constructed using the pAMG21 Fc_Gly5_ Tpo vector as a template. A 5' PCR primer (below) was designed to remove the Tpo peptide sequence in pAMG Tpo Gly5 and replace it with a polylinker containing *Apa*LI and *Xho*I sites. Using this vector as a template, PCR was performed with Expand Long Polymerase, using the following 5' primer and a universal 3' primer:

5'primer: 5'-ACAAACAAACATATGGGTGCACAGAAAGCGGCCGCAAAAAAA CTCGAGGGTGGAGGCGGTGGGGACA-3' (Seq ID No: 297)

3' primer: 5'-GGTCATTACTGGACCGGATC-3' (Seq ID No: 298)

The resulting PCR product was gel purified and digested with restriction enzymes *Nde*I and *Bsr*GI. Both the plasmid and the polynucleotide encoding the peptide of interest together with its linker were gel purified using Qiagen (Chatsworth, CA) gel purification spin columns. The plasmid and insert were then ligated using standard ligation procedures, and the resulting ligation mixture was transformed into *E. coli* cells (strain 2596). Single clones were selected and DNA sequencing was performed. A correct clone was identified and this was used as a vector source for the modified peptides described herein.

Construction of Fc C-terminal Vector

The Fc C-terminal vector was constructed using pAMG21 Fc_Gly5_ Tpo vector as a template. A 3' PCR primer was designed to remove the Tpo peptide sequence and to replace it with a polylinker containing *Apa*LI and *Xho*I sites. PCR was performed with Expand Long Polymerase using a universal 5' primer and the 3' primer.

5' Primer: 5'-CGTACAGGTTTACGCAAGAAAATGG-3' (Seq ID No: 299)

3' Primer: 5'-TTTGTTGGATCCATTACTCGAGTTTTTTTGCGGCCGCT TTCTGTGCACCACCACCTCCACCTTTAC-3' (Seq ID No: 300)

The resulting PCR product was gel purified and digested with restriction enzymes *Bsr*GI and *Bam*HI. Both the plasmid and the polynucleotide encoding each peptides of interest with its linker were gel purified via Qiagen gel purification spin columns. The plasmid and insert were then ligated using standard ligation procedures, and the resulting ligation mixture was transformed into E. coli (strain 2596) cells. Strain 2596 (ATCC # 202174) is a strain of *E. coli* K-12 modified to contain the lux promoter and two lambda temperature sensitive repressors, the cI857s7 and the lac I^Q repressor. Single clones were selected and DNA sequencing was performed. A correct clone was identified and used as a source of each peptibody described herein.

Expression in *E. coli*.

Cultures of each of the pAMG21-Fc fusion constructs in *E. coli* strain 2596 were grown at 37°C in Terrific Broth medium (See Tartof and Hobbs, "Improved media for growing plasmid and cosmid clones", Bethesda Research Labs Focus, Volume 9, page 12, 1987, cited in aforementioned Sambrook et al. reference). Induction of gene product expression from the luxPR promoter was achieved following the addition of the synthetic autoinducer, N-(3-oxohexanoyl)-DL-homoserine lactone, to the culture medium to a final concentration of 20 nanograms per milliliter (ng/ml). Cultures were incubated at 37°C for an additional six hours. The bacterial cultures were then examined by microscopy for the presence of inclusion bodies and collected by

centrifugation. Refractile inclusion bodies were observed in induced cultures, indicating that the Fc-fusions were most likely produced in the insoluble fraction in *E. coli*. Cell pellets were lysed directly by resuspension in Laemmli sample buffer containing 10% β-mercaptoethanol and then analyzed by SDS-PAGE. In most cases, an intense coomassie-stained band of the appropriate molecular weight was observed on an SDS-PAGE gel.

Folding and purifying peptibodies

Cells were broken in water (1/10 volume per volume) by high pressure homogenization (3 passes at 15,000 PSI) and inclusion bodies were harvested by centrifugation (4000 RPM in J-6B for 30 minutes). Inclusion bodies were solubilized in 6 M guanidine, 50 mM Tris, 8 mM DTT, pH 8.0 for 1 hour at a 1/10 ratio at ambient temperature. The solubilized mixture was diluted 25 times into 4 M urea, 20% glycerol, 50 mM Tris, 160 mM arginine, 3 mM cysteine, 1 mM cystamine, pH 8.5. The mixture was incubated overnight in the cold. The mixture was then dialyzed against 10 mM Tris pH 8.5, 50 mM NaCl, 1.5 M urea. After an overnight dialysis the pH of the dialysate was adjusted to pH 5 with acetic acid. The precipitate was removed by centrifugation and the supernatant was loaded onto a SP-Sepharose Fast Flow column equilibrated in 10 mM NaAc, 50 mM NaCl, pH 5 , 4°C). After loading the column was washed to baseline with 10 mM NaAc, 50 mM NaCl, pH 5.2. The column was developed with a 20 column volume gradient from 50mM -500 mM NaCl in the acetate buffer. Alternatively, after the wash to baseline, the column was washed with 5 column volumes of 10 mM sodium phosphate pH 7.0 and the column developed with a 15 column volume gradient from 0-400 mM NaCl in phosphate buffer. Column fractions were analyzed by SDS-PAGE. Fractions containing dimeric peptibody were pooled. Fractions were also analyzed by gel filtration to determine if any aggregate was present.

A number of peptibodies were prepared from the peptides of Table I. The peptides were attached to the human IgG1 Fc molecule to form the peptibodies in Table II. Regarding the peptibodies in Table II, the C configuration indicates that the peptide named was attached at the C-termini of the Fc. The N configuration indicates that the peptide named was attached at the N-termini of the Fc. The N,C configuration indicates that one peptide was attached at the N-termini and one at the C-termini of each Fc molecule. The 2x designation indicates that the two peptides named were attached in tandem to each other and also attached at the N or the C termini, or both the N,C of the Fc, separated by the linker indicated. Two peptides attached in tandem separated by a linker, are indicated, for example, as Myostatin-TN8-29-19-8g, which indicates that TN8-29 peptide is attached via a $(gly)_8$ linker to TN8-19 peptide. The peptide(s) were attached to the Fc via a $(gly)_5$ linker sequence unless otherwise specified. In some instances the peptide(s) were attached via a k linker. The linker designated k or 1k refers to the gsgsatggsgstassgsgsatg (Seq ID

No: 301) linker sequence, with kc referring to the linker attached to the C–terminus of the Fc, and kn referring to the linker attached to the N-terminus of the Fc. In Table II below, column 4 refers to the linker sequence connecting the Fc to the first peptide and the fifth column refers to the configuration N or C or both.

Since the Fc molecule dimerizes in solution, a peptibody constructed so as to have one peptide will actually be a dimer with two copies of the peptide and two Fc molecules, and the 2X version having two peptides in tandem will actually be a dimer with four copies of the peptide and two Fc molecules.

Since the peptibodies given in Table II are expressed in *E. coli*, the first amino acid residue is Met (M). Therefore, the peptibodies in the N configuration are Met-peptide-linker-Fc, or Met-peptide-linker-peptide-linker-Fc, for example. Peptibodies in the C configuration are arranged as Met-Fc-linker-peptide or Met-Fc-linker-peptide-linker-peptide, for example. Peptibodies in the C,N configuration are a combination of both, for example, Met-peptide-linker-Fc-linker-peptide.

Nucleotide sequences encoding exemplary peptibodies are provided below in Table II. The polynucleotide sequences encoding an exemplary peptibody of the present invention includes a nucleotide sequence encoding the Fc polypeptide sequence such as the following:

5'-GACAAAACTCACACATGTCCACCTTGCCCAGCACCTGAACTC
CTGGGGGGACCGTCAGTTTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCA
TGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACG
AAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATA
ATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTG
GTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTAC
AAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATC
TCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCA
TCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAA
GGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCG
GAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCT
TCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACG
TCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAA
GAGCCTCTCCCTGTCTCCGGGTAAA-3' (Seq ID No: 301)

In addition, the polynucleotides encoding the ggggg linker such as the following are included:

5'-GGTGGAGGTGGTGGT-3' (Seq ID No: 302)

The polynucleotide encoding the peptibody also includes the codon encoding the methionine ATG and a stop codon such as TAA.

Therefore, the structure of the first peptibody in Table II is TN8-Con1 with a C configuration and a $(gly)_5$ linker is as follows: M-Fc-GGGGG-KDKCKMWHWMCKPP (Seq ID No: 303). Exemplary polynucleotides encoding this peptibody would be:

5'- ATGGACAAAACTCACACATGTCCACCTTGCCCAGCACCTGAA
CTCCTGGGGGGACCGTCAGTTTTCCTCTTCCCCCCAAAACCCAAGGACACCC
TCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCC
ACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGC
ATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGT
GTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAG
TACAAGTGCAAGGTCTCCAACAAAGCCCTCCCAGCCCCCATCGAGAAAACC
ATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCC
CCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTC
AAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAG
CCGGAGAACAACTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCC
TTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGG
AACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGC
AGAAGAGCCTCTCCCTGTCTCCGGGTAAAGGTGGAGGTGGTGGTAAGACAA
ATGCAAAATGTGGCACTGGATGTGCAAACCGCCG-3' (Seq ID No: 304)

TABLE II

Peptibody Name	Peptide	Nucleotide Sequence (Seq ID No)		
Myostatin-TN8-con1	KDKCKMWHWMCKPP	AAAGACAAATGCAAAATGTGGCACTG GATGTGCAAACCGCCG (Seq. ID No: 147)	5 gly	C
Myostatin-TN8-con2	KDLCAMWHWMCKPP	AAAGACCTGTGCGCTATGTGGCACTG GATGTGCAAACCGCCG (Seq. ID No: 148)	5 gly	C
Myostatin-TN8-con3	KDLCKMWKWMCKPP	AAAGACCTGTGCAAAATGTGGAAATG GATGTGCAAACCGCCG (Seq ID No: 149)	5 gly	C
Myostatin-TN8-con4	KDLCKMWHWMCKPK	AAAGACCTGTGCAAAATGTGGCACTG GATGTGCAAACCGAAA (Seq ID No: 150)	5 gly	C
Myostatin-TN8-con5	WYPCYEFHFWCYDL	TGGTACCCGTGCTACGAATTCCACTTC TGGTGCTACGACCTG (Seq ID No: 151)	5 gly	C
Myostatin-TN8-con5	WYPCYEFHFWCYDL	TGGTACCCGTGCTACGAATTCCACTTC TGGTGCTACGACCTG (Seq ID No: 152)	5 gly	N
Myostatin-TN8-con6	WYPCYEGHFWCYDL	TGGTACCCGTGCTACGAAGGTCACTT CTGGTGCTACGACCTG (Seq ID No: 153)	5 gly	C
Myostatin-TN8-con6	WYPCYEGHFWCYDL	TGGTACCCGTGCTACGAAGGTCACTT CTGGTGCTACGACCTG (Seq ID No: 154)	5 gly	N
Myostatin-TN8-con7	IFGCKWWDVQCYQF	ATCTTCGGTTGCAAATGGTGGGACGT TCAGTGCTACCAGTTC (Seq ID No: 155)	5 gly	C
Myostatin-TN8-con8	IFGCKWWDVDCYQF	ATCTTCGGTTGCAAATGGTGGGACGT TGACTGCTACCAGTTC (Seq ID No: 156)	5 gly	C
Myostatin-TN8-con8	IFGCKWWDVDCYQF	ATCTTCGGTTGCAAATGGTGGGACGT TGACTGCTACCAGTTC (Seq ID No: 157)	5 gly	N
Myostatin-TN8-	ADWCVSPNWFCMVM	GCTGACTGGTGCGTTTCCCCGAACTG	5 gly	C

con9		GTTCTGCATGGTTATG (Seq ID No: 158)		
Myostatin-TN8-con10	HKFCPWWALFCWDF	CACAAATTCTGCCCGTGGTGGGCTCT GTTCTGCTGGGACTTC (Seq ID No: 159)	5 gly	C
Myostatin-TN8-1	KDLCKMWHWMCKPP	AAAGACCTGTGCAAAATGTGGCACTG GATGTGCAAACCGCCG (Seq ID No: 160	5 gly	C
Myostatin-TN8-2	IDKCAIWGWMCPPL	ATCGACAAATGCGCTATCTGGGGTTG GATGTGCCCGCCGCTG (Seq ID No: 161)	5 gly	C
Myostatin-TN8-3	WYPCGEFGMWCLNV	TGGTACCCGTGCGGTGAATTCGGTAT GTGGTGCCTGAACGTT (Seq ID No: 162)	5 gly	C
Myostatin-TN8-4	WFTCLWNCDNE	TGGTTCACCTGCCTGTGGAACTGCGA CAACGAA (Seq ID No: 163)	5 gly	C
Myostatin-TN8-5	HTPCPWFAPLCVEW	CACACCCCGTGCCCGTGGTTCGCTCC GCTGTGCGTTGAATGG (Seq ID No: 164)	5 gly	C
Myostatin-TN8-6	KEWCWRWKWMCKPE	AAAGAATGGTGCTGGCGTTGGAAATG GATGTGCAAACCGGAA (Seq ID No: 165)	5 gly	C
Myostatin-TN8-7	FETCPSWAYFCLDI	TTCGAAACCTGCCCGTCCTGGGCTTA CTTCTGCCTGGACATC (Seq ID No: 166)	5 gly	C
Myostatin-TN8-7	FETCPSWAYFCLDI	TTCGAAACCTGCCCGTCCTGGGCTTA CTTCTGCCTGGACATC (Seq ID No: 167)	5 gly	N
Myostatin-TN8-8	AYKCEANDWGCWWL	GCTTACAAATGCGAAGCTAACGACTG GGGTTGCTGGTGGCTG (Seq ID No: 168)	5 gly	C
Myostatin-TN8-9	NSWCEDQWHRCWWL	AACTCCTGGTGCGAAGACCAGTGGCA CCGTTGCTGGTGGCTG (Seq ID No: 169)	5 gly	C
Myostatin-TN8-10	WSACYAGHFWCYDL	TGGTCCGCTTGCTACGCTGGTCACTTC TGGTGCTACGACCTG (Seq ID No: 170)	5 gly	C
Myostatin-TN8-11	ANWCVSPNWFCMVM	GCTAACTGGTGCGTTTCCCCGAACTG GTTCTGCATGGTTATG (Seq ID No: 171)	5 gly	C
Myostatin-TN8-12	WTECYQQEFWCWNL	TGGACCGAATGCTACCAGCAGGAATT CTGGTGCTGGAACCTG (Seq ID No: 172)	5 gly	C
Myostatin-TN8-13	ENTCERWKWMCPPK	GAAAACACCTGCGAACGTTGGAAATG GATGTGCCCGCCGAAA (Seq ID No: 173)	5 gly	C
Myostatin-TN8-14	WLPCHQEGFWCMNF	TGGCTGCCGTGCCACCAGGAAGGTTT CTGGTGCATGAACTTC (Seq ID No: 174)	5 gly	C
Myostatin-TN8-15	STMCSQWHWMCNPF	TCCACCATGTGCTCCCAGTGGCACTG GATGTGCAACCCGTTC (Seq ID No: 175)	5 gly	C
Myostatin-TN8-16	IFGCHWWDVDCYQF	ATCTTCGGTTGCCACTGGTGGGACGT TGACTGCTACCAGTTC (Seq ID No: 176)	5 gly	C
Myostatin-TN8-17	IYGCKWWDIQCYDI	ATCTACGGTTGCAAATGGTGGGACAT CCAGTGCTACGACATC (Seq ID No: 177)	5 gly	C
Myostatin-TN8-18	PDWCIDPDWWCKFW	CCGGACTGGTGCATCGATCCGGACTG GTGGTGCAAATTCTGG (Seq ID No: 178)	5 gly	C
Myostatin-TN8-19	QGHCTRWPWMCPPY	CAGGGTCACTGCACCCGTTGGCCGTG GATGTGCCCGCCGTAC (Seq ID No: 179)	5 gly	C
Myostatin-TN8-20	WQECYREGFWCLQT	TGGCAGGAATGCTACCGTGAAGGTTT CTGGTGCCTGCAGACC (Seq ID No: 180)	5 gly	C

Myostatin-TN8-21	WFDCYGPGFKCWSP	TGGTTCGACTGCTACGGTCCGGGTTTC AAATGCTGGTCCCCG (Seq ID No: 181)	5 gly	C
Myostatin-TN8-22	GVRCPKGHLWCLYP	GGTGTTCGTTGCCCGAAAGGTCACCT GTGGTGCCTGTACCCG (Seq ID No: 182)	5 gly	C
Myostatin-TN8-23	HWACGYWPWSCKWV	CACTGGGCTTGCGGTTACTGGCCGTG GTCCTGCAAATGGGTT (Seq ID No: 183)	5 gly	C
Myostatin-TN8-24	GPACHSPWWWCVFG	GGTCCGGCTTGCCACTCCCCGTGGTG GTGGTGCGTTTTCGGT (Seq ID No: 184)	5 gly	C
Myostatin-TN8-25	TTWCISPMWFCSQQ	ACCACCTGGTGCATCTCCCCGATGTG GTTCTGCTCCCAGCAG (Seq ID No: 185)	5 gly	C
Myostatin-TN8-26	HKFCPPWAIFCWDF	CACAAATTCTGCCCGCCGTGGGCTAT CTTCTGCTGGGACTTC (Seq ID No: 186)	5 gly	N
Myostatin-TN8-27	PDWCVSPRWYCNMW	CCGGACTGGTGCGTTTCCCCGCGTTG GTACTGCAACATGTGG (Seq ID No: 187)	5 gly	N
Myostatin-TN8-28	VWKCHWFGMDCEPT	GTTTGGAAATGCCACTGGTTCGGTAT GGACTGCGAACCGACC (Seq ID No: 188)	5 gly	N
Myostatin-TN8-29	KKHCQIWTWMCAPK	AAAAAACACTGCCAGATCTGGACCTG GATGTGCGCTCCGAAA (Seq ID No: 189)	5 gly	N
Myostatin-TN8-30	WFQCGSTLFWCYNL	TGGTTCCAGTGCGGTTCCACCCTGTTC TGGTGCTACAACCTG (Seq ID No: 190)	5 gly	N
Myostatin-TN8-31	WSPCYDHYFYCYTI	TGGTCCCCGTGCTACGACCACTACTTC TACTGCTACACCATC (Seq ID No: 191)	5 gly	N
Myostatin-TN8-32	SWMCGFFKEVCMWV	TCCTGGATGTGCGGTTTCTTCAAAGA AGTTTGCATGTGGGTT (Seq ID No: 192)	5 gly	N
Myostatin-TN8-33	EMLCMIHPVFCNPH	GAAATGCTGTGCATGATCCACCCGGT TTTCTGCAACCCGCAC (Seq ID No: 193)	5 gly	N
Myostatin-TN8-34	LKTCNLWPWMCPPL	CTGAAAACCTGCAACCTGTGGCCGTG GATGTGCCCGCCGCTG (Seq ID No: 194)	5 gly	N
Myostatin-TN8-35	VVGCKWYEAWCYNK	GTTGTTGGTTGCAAATGGTACGAAGC TTGGTGCTACAACAAA (Seq ID No: 195)	5 gly	N
Myostatin-TN8-36	PIHCTQWAWMCPPT	CCGATCCACTGCACCCAGTGGGCTTG GATGTGCCCGCCGACC (Seq ID No: 196)	5 gly	N
Myostatin-TN8-37	DSNCPWYFLSCVIF	GACTCCAACTGCCCGTGGTACTTCCT GTCCTGCGTTATCTTC (Seq ID No: 197)	5 gly	N
Myostatin-TN8-38	HIWCNLAMMKCVEM	CACATCTGGTGCAACCTGGCTATGAT GAAATGCGTTGAAATG (Seq ID No: 198)	5 gly	N
Myostatin-TN8-39	NLQCIYFLGKCIYF	AACCTGCAGTGCATCTACTTCCTGGG TAAATGCATCTACTTC (Seq ID No: 199)	5 gly	N
Myostatin-TN8-40	AWRCMWFSDVCTPG	GCTTGGCGTTGCATGTGGTTCTCCGAC GTTTGCACCCCGGGT (Seq ID No: 200)	5 gly	N
Myostatin-TN8-41	WFRCFLDADWCTSV	TGGTTTCGTTGTTTTCTTGATGCTGAT TGGTGTACTTCTGTT (Seq ID No: 201)	5 gly	N
Myostatin-TN8-42	EKICQMWSWMCAPP	GAAAAAATTTGTCAAATGTGGTCTTG GATGTGTGCTCCACCA (Seq ID No: 202)	5 gly	N
Myostatin-TN8-43	WFYCHLNKSECTEP	TGGTTTTATTGTCATCTTAATAAATCT	5 gly	N

		GAATGTACTGAACCA (Seq ID No: 203)		
Myostatin-TN8-44	FWRCAIGIDKCKRV	TTTTGGCGTTGTGCTATTGGTATTGAT AAATGTAAACGTGTT (Seq ID No: 204)	5 gly	N
Myostatin-TN8-45	NLGCKWYEVWCFTY	AATCTTGGTTGTAAATGGTATGAAGT TTGGTGTTTTACTTAT (Seq ID No: 205)	5 gly	N
Myostatin-TN8-46	IDLCNMWDGMCYPP	ATTGATCTTTGTAATATGTGGGATGGT ATGTGTTATCCACCA (Seq ID No: 206)	5 gly	N
Myostatin-TN8-47	EMPCNIWGWMCPPV	GAAATGCCATGTAATATTTGGGGTTG GATGTGTCCACCAGTT (Seq ID No: 207)	5 gly	N
Myostatin-TN12-1	WFRCVLTGIVDWSECF GL	TGGTTCCGTTGCGTTCTGACCGGTATC GTTGACTGGTCCGAATGCTTCGGTCT G (Seq ID No: 208)	5 gly	N
Myostatin-TN12-2	GFSCTFGLDEFYVDCSP F	GGTTTCTCCTGCACCTTCGGTCTGGAC GAATTCTACGTTGACTGCTCCCCGTTC (Seq ID No: 209)	5 gly	N
Myostatin-TN12-3	LPWCHDQVNADWGFC MLW	CTGCCGTGGTGCCACGACCAGGTTAA CGCTGACTGGGGTTTCTGCATGCTGT GG (Seq ID No: 210)	5 gly	N
Myostatin-TN12-4	YPTCSEKFWIYGQTCV LW	TACCCGACCTGCTCCGAAAAATTCTG GATCTACGGTCAGACCTGCGTTCTGT GG (Seq ID No: 211)	5 gly	N
Myostatin-TN12-5	LGPCPIHHGPWPQYCV YW	CTGGGTCCGTGCCCGATCCACCACGG TCCGTGGCCGCAGTACTGCGTTTACT GG (Seq ID No: 212)	5 gly	N
Myostatin-TN12-6	PFPCETHQISWLGHCLS F	CCGTTCCCGTGCGAAACCCACCAGAT CTCCTGGCTGGGTCACTGCCTGTCCTT C (Seq ID No: 213)	5 gly	N
Myostatin-TN12-7	HWGCEDLMWSWHPLC RRP	CACTGGGGTTGCGAAGACCTGATGTG GTCCTGGCACCCGCTGTGCCGTCGTC CG (Seq ID No: 214)	5 gly	N
Myostatin-TN12-8	LPLCDADMMPTIGFCV AY	CTGCCGCTGTGCGACGCTGACATGAT GCCGACCATCGGTTTCTGCGTTGCTTA C (Seq ID No: 215)	5 gly	N
Myostatin-TN12-9	SHWCETTFWMNYAKC VHA	TCCCACTGGTGCGAAACCACCTTCTG GATGAACTACGCTAAATGCGTTCACG CT (Seq ID No: 216)	5 gly	N
Myostatin-TN12-10	LPKCTHVPFDQGGFCL WY	CTGCCGAAATGCACCCACGTTCCGTT CGACCAGGGTGGTTTCTGCCTGTGGT AC (Seq ID No: 217)	5 gly	N
Myostatin-TN12-11	FSSCWSPVSRQDMFCV FY	TTCTCCTCCTGCTGGTCCCCGGTTTCC CGTCAGGACATGTTCTGCGTTTTCTAC (Seq ID No: 218)	5 gly	N
Myostatin-TN12-13	SHKCEYSGWLQPLCYR P	TCCCACAAATGCGAATACTCCGGTTG GCTGCAGCCGCTGTGCTACCGTCCG (Seq ID No: 219)	5 gly	N
Myostatin-TN12-14	PWWCQDNYVQHMLH CDSP	CCGTGGTGGTGCCAGGACAACTACGT TCAGCACATGCTGCACTGCGACTCCC CG (Seq ID No: 220)	5 gly	N
Myostatin-TN12-15	WFRCMLMNSFDAFQC VSY	TGGTTCCGTTGCATGCTGATGAACTCC TTCGACGCTTTCCAGTGCGTTTCCTAC (Seq ID No: 221)	5 gly	N
Myostatin-TN12-16	PDACRDQPWYMFMGC MLG	CCGGACGCTTGCCGTGACCAGCCGTG GTACATGTTCATGGGTTGCATGCTGG GT (Seq ID No: 222)	5 gly	N
Myostatin-TN12-	FLACFVEFELCFDS	TTCCTGGCTTGCTTCGTTGAATTCGAA	5 gly	N

17		CTGTGCTTCGACTCC (Seq ID No: 223)		
Myostatin-TN12-18	SAYCIITESDPYVLCVPL	TCCGCTTACTGCATCATCACCGAATCCGACCCGTACGTTCTGTGCGTTCCGCTG (Seq ID No: 224)	5 gly	N
Myostatin-TN12-19	PSICESYSTMWLPMCQHN	CCGTCCATCTGCGAATCCTACTCCACCATGTGGCTGCCGATGTGCCAGCACAAC (Seq ID No: 225)	5 gly	N
Myostatin-TN12-20	WLDCHDDSWAWTKMCRSH	TGGCTGGACTGCCACGACGACTCCTGGGCTTGGACCAAAATGTGCCGTTCCCAC (Seq ID No: 226)	5 gly	N
Myostatin-TN12-21	YLNCVMMNTSPFVECVFN	TACCTGAACTGCGTTATGATGAACACCTCCCCGTTCGTTGAATGCGTTTTCAAC (Seq ID No: 227)	5 gly	N
Myostatin-TN12-22	YPWCDGFMIQQGITCMFY	TACCCGTGGTGCGACGGTTTCATGATCCAGCAGGGTATCACCTGCATGTTCTAC (Seq ID No: 228)	5 gly	N
Myostatin-TN12-23	FDYCTWLNGFKDWKCWSR	TTCGACTACTGCACCTGGCTGAACGGTTTCAAAGACTGGAAATGCTGGTCCCGT (Seq ID No: 229)	5 gly	N
Myostatin-TN12-24	LPLCNLKEISHVQACVLF	CTGCCGCTGTGCAACCTGAAAGAAATCTCCCACGTTCAGGCTTGCGTTCTGTTC (Seq ID No: 230)	5 gly	N
Myostatin-TN12-25	SPECAFARWLGIEQCQRD	TCCCCGGAATGCGCTTTCGCTCGTTGGCTGGGTATCGAACAGTGCCAGCGTGAC (Seq ID No: 231)	5 gly	N
Myostatin-TN12-26	YPQCFNLHLLEWTECDWF	TACCCGCAGTGCTTCAACCTGCACCTGCTGGAATGGACCGAATGCGACTGGTTC (Seq ID No: 232)	5 gly	N
Myostatin-TN12-27	RWRCEIYDSEFLPKCWFF	CGTTGGCGTTGCGAAATCTACGACTCCGAATTCCTGCCGAAATGCTGGTTCTTC (Seq ID No: 233)	5 gly	N
Myostatin-TN12-28	LVGCDNVWHRCKLF	CTGGTTGGTTGCGACAACGTTTGGCACCGTTGCAAACTGTTC (Seq ID No: 234)	5 gly	N
Myostatin-TN12-29	AGWCHVWGEMFGMGCSAL	GCTGGTTGGTGCCACGTTTGGGGTGAAATGTTCGGTATGGGTTGCTCCGCTCTG (Seq ID No: 235)	5 gly	N
Myostatin-TN12-30	HHECEWMARWMSLDCVGL	CACCACGAATGCGAATGGATGGCTCGTTGGATGTCCCTGGACTGCGTTGGTCTG (Seq ID No: 236)	5 gly	N
Myostatin-TN12-31	FPMCGIAGMKDFDFCVWY	TTCCCGATGTGCGGTATCGCTGGTATGAAAGACTTCGACTTCTGCGTTTGGTAC (Seq ID No: 237)	5 gly	N
Myostatin-TN12-32	RDDCTFWPEWLWKLCERP	CGTGATGATTGTACTTTTTGGCCAGAATGGCTTTGGAAACTTTGTGAACGTCCA (Seq ID No: 238)	5 gly	N
Myostatin-TN12-33	YNFCSYLFGVSKEACQLP	TATAATTTTTGTTCTTATCTTTTTGGTGTTTCTAAAGAAGCTTGTCAACTTCCA (Seq ID No: 239)	5 gly	N
Myostatin-TN12-34	AHWCEQGPWRYGNICMAY	GCTCATTGGTGTGAACAAGGTCCATGGCGTTATGGTAATATTTGTATGGCTTAT (Seq ID No: 240)	5 gly	NC
Myostatin-TN12-35	NLVCGKISAWGDEACARA	AATCTTGTTTGTGGTAAAATTTCTGCTTGGGGTGATGAAGCTTGTGCTCGTGCT (Seq ID No: 241)	5 gly	N
Myostatin-TN12-	HNVCTIMGPSMKWFC	CATAATGTTTGTACTATTATGGGTCCA	5 gly	N

36	WND	TCTATGAAATGGTTTTGTTGGAATGAT (Seq ID No: 242)		C
Myostatin-TN12-37	NDLCAMWGWRNTIWCQNS	AATGATCTTTGTGCTATGTGGGGTTGGCGTAATACTATTTGGTGTCAAAATTCT (Seq ID No: 243)	5 gly	N C
Myostatin-TN12-38	PPFCQNDNDMLQSLCKLL	CCACCATTTTGTCAAAATGATAATGATATGCTTCAATCTCTTTGTAAACTTCTT (Seq ID No: 244)	5 gly	N
Myostatin-TN12-39	WYDCNVPNELLSGLCRLF	TGGTATGATTGTAATGTTCCAAATGAACTTCTTTCTGGTCTTTGTCGTCTTTTT (Seq ID No: 245)	5 gly	N
Myostatin-TN12-40	YGDCDQNHWMWPFTCLSL	TATGGTGATTGTGATCAAAATCATTGGATGTGGCCATTTACTTGTCTTTCTCTT (Seq ID No: 246)	5 gly	N C
Myostatin-TN12-41	GWMCHFDLHDWGATCQPD	GGTTGGATGTGTCATTTTGATCTTCATGATTGGGGTGCTACTTGTCAACCAGAT (Seq ID No: 247)	5 gly	N
Myostatin-TN12-42	YFHCMFGGHEFEVHCESF	TATTTTCATTGTATGTTTGGTGGTCATGAATTTGAAGTTCATTGTGAATCTTTT (Seq ID No: 248)	5 gly	N C
Myostatin-TN12-43	AYWCWHGQCVRF	GCTTATTGGTGTTGGCATGGTCAATGTGTTCGTTTT (Seq ID No: 249)	5 gly	N
Myostatin-Linear-1	SEHWTFTDWDGNEWWVRPF	TCCGAACACTGGACCTTCACCGACTGGGACGGTAACGAATGGTGGGTTCGTCCGTTC (Seq ID No: 250)	5 gly	N
Myostatin-Linear-2	MEMLDSLFELLKDMVPISKA	ATGGAAATGCTGGACTCCCTGTTCGAACTGCTGAAAGACATGGTTCCGATCTCCAAAGCT (Seq ID No: 251)	5 gly	N
Myostatin-Linear-3	SPPEEALMEWLGWQYGKFT	TCCCCGCCGGAAGAAGCTCTGATGGAATGGCTGGGTTGGCAGTACGGTAAATTCACC (Seq ID No: 252)	5 gly	N
Myostatin-Linear-4	SPENLLNDLYILMTKQEWYG	TCCCCGGAAAACCTGCTGAACGACCTGTACATCCTGATGACCAAACAGGAATGGTACGGT (Seq ID No: 253)	5 gly	N
Myostatin-Linear-5	FHWEEGIPFHVVTPYSYDRM	TTCCACTGGGAAGAAGGTATCCCGTTCCACGTTGTTACCCCGTACTCCTACGACCGTATG (Seq ID No: 254)	5 gly	N
Myostatin-Linear-6	KRLLEQFMNDLAELVSGHS	AAACGTCTGCTGGAACAGTTCATGAACGACCTGGCTGAACTGGTTTCCGGTCACTCC (Seq ID No: 255)	5 gly	N
Myostatin-Linear-7	DTRDALFQEFYEFVRSRLVI	GACACCCGTGACGCTCTGTTCCAGGAATTCTACGAATTCGTTCGTTCCCGTCTGGTTATC (Seq ID No: 256)	5 gly	N
Myostatin-Linear-8	RMSAAPRPLTYRDIMDQYWH	CGTATGTCCGCTGCTCCGCGTCCGCTGACCTACCGTGACATCATGGACCAGTACTGGCAC (Seq ID No: 257)	5 gly	N
Myostatin-Linear-9	NDKAHFFEMFMFDVHNFVES	AACGACAAAGCTCACTTCTTCGAAATGTTCATGTTCGACGTTCACAACTTCGTTGAATCC (Seq Id No: 258)	5 gly	N
Myostatin-Linear-10	QTQAQKIDGLWELLQSIRNQ	CAGACCCAGGCTCAGAAAATCGACGGTCTGTGGGAACTGCTGCAGTCCATCCGTAACCAG (Seq ID No: 259)	5 gly	N
Myostatin-Linear-11	MLSEFEEFLGNLVHRQEA	ATGCTGTCCGAATTCGAAGAATTCCTGGGTAACCTGGTTCACCGTCAGGAAGCT (Seq ID No: 260)	5 gly	N
Myostatin-Linear-	YTPKMGSEWTSFWHN	TACACCCCGAAAATGGGTTCCGAATG	5 gly	N

12	RIHYL	GACCTCCTTCTGGCACAACCGTATCC ACTACCTG (Seq ID No: 261)		
Myostatin-Linear-13	LNDTLLRELKMVLNSL SDMK	CTGAACGACACCCTGCTGCGTGAACT GAAAATGGTTCTGAACTCCCTGTCCG ACATGAAA (Seq ID No: 262)	5 gly	N
Myostatin-Linear-14	FDVERDLMRWLEGFM QSAAT	TTCGACGTTGAACGTGACCTGATGCG TTGGCTGGAAGGTTTCATGCAGTCCG CTGCTACC (Seq ID No: 263)	5 gly	N
Myostatin-Linear-15	HHGWNYLRKGSAPQW FEAWV	CACCACGGTTGGAACTACCTGCGTAA AGGTTCCGCTCCGCAGTGGTTCGAAG CTTGGGTT (Seq ID No: 264)	5 gly	N
Myostatin-Linear-16	VESLHQLQMWLDQKL ASGPH	GTTGAATCCCTGCACCAGCTGCAGAT GTGGCTGGACCAGAAACTGGCTTCCG GTCCGCAC (Seq ID No: 265)	5 gly	N
Myostatin-Linear-17	RATLLKDFWQLVEGY GDN	CGTGCTACCCTGCTGAAAGACTTCTG GCAGCTGGTTGAAGGTTACGGTGACA AC (Seq ID No: 266)	5 gly	N
Myostatin-Linear-18	EELLREFYRFVSAFDY	GAAGAACTGCTGCGTGAATTCTACCG TTTCGTTTCCGCTTTCGACTAC (Seq ID No: 267)	5 gly	N
Myostatin-Linear-19	GLLDEFSHFIAEQFYQ MPGG	GGTCTGCTGGACGAATTCTCCCACTTC ATCGCTGAACAGTTCTACCAGATGCC GGGTGGT (Seq ID No: 268)	5 gly	N
Myostatin-Linear-20	YREMSMLEGLLDVLER LQHY	TACCGTGAAATGTCCATGCTGGAAGG TCTGCTGGACGTTCTGGAACGTCTGC AGCACTAC (Seq ID No: 269)	5 gly	N
Myostatin-Linear-21	HNSSQMLLSELIMLVG SMMQ	CACAACTCCTCCCAGATGCTGCTGTC CGAACTGATCATGCTGGTTGGTTCCA TGATGCAG (Seq ID No: 270)	5 gly	N
Myostatin-Linear-22	WREHFLNSDYIRDKLI AIDG	TGGCGTGAACACTTCCTGAACTCCGA CTACATCCGTGACAAACTGATCGCTA TCGACGGT (Seq ID No: 271)	5 gly	N
Myostatin-Linear-23	QFPFYVFDDLPAQLEY WIA	CAGTTCCCGTTCTACGTTTTCGACGAC CTGCCGGCTCAGCTGGAATACTGGAT CGCT (Seq ID No: 272)	5 gly	N
Myostatin-Linear-24	EFFHWLHNHRSEVNH WLDMN	GAATTCTTCCACTGGCTGCACAACCA CCGTTCCGAAGTTAACCACTGGCTGG ACATGAAC (Seq ID No: 273)	5 gly	N
Myostatin-Linear-25	EALFQNFFRDVLTLSER EY	GAAGCTCTTTTTCAAAATTTTTTTCGT GATGTTCTTACTCTTTCTGAACGTGAA TAT (Seq ID No: 274)	5 gly	N C
Myostatin-Linear –26	QYWEQQWMTYFRENG LHVQY	CAATATTGGGAACAACAATGGATGAC TTATTTTCGTGAAAATGGTCTTCATGT TCAATAT (Seq ID No: 275)	5 gly	N
Myostatin-Linear-27	NQRMMLEDLWRIMTP MFGRS	AATCAACGTATGATGCTTGAAGATCT TTGGCGTATTATGACTCCAATGTTTGG TCGTTCT (Seq ID No: 276)	5 gly	N C
Myostatin-Linear-29	FLDELKAELSRHYALD DLDE	TTTCTTGATGAACTTAAAGCTGAACTT TCTCGTCATTATGCTCTTGATGATCTT GATGAA (Seq ID No: 277)	5 gly	N
Myostatin-Linear-30	GKLIEGLLNELMQLETF MPD	GGTAAACTTATTGAAGGTCTTCTTAAT GAACTTATGCAACTTGAAACTTTTATG CCAGAT (Seq ID No: 278)	5 gly	N C
Myostatin-Linear-31	ILLLDEYKKDWKSWF	ATTCTTCTTCTTGATGAATATAAAAAA GATTGGAAATCTTGGTTT (Seq ID No: 279)	5 gly	N

Myostatin-2XTN8-19 kc	QGHCTRWPWMCPPYGSGSATGGSGSTASSGSGSATGQGHCTRWPWMCPPY	CAGGGCCACTGTACTCGCTGGCCGTGGATGTGCCCGCCGTACGGTTCTGGTTCCGCTACCGGTGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCTGCTACTGGTCAGGGTCACTGCACTCGTTGGCCATGGATGTGTCCACCGTAT (Seq ID No: 280)	1k	N
Myostatin-2XTN8-CON6	WYPCYEGHFWCYDLGSGSTASSGSGSATGWYPCYEGHFWCYDL	TGGTATCCGTGTTATGAGGGTCACTTCTGGTGCTACGATCTGGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTTGGTACCCGTGCTACGAAGGTCACTTTTGGTGTTATGATCTG (Seq ID No: 281)	5 gly	C
Myostatin-2XTN8-5 kc	HTPCPWFAPLCVEWGSGSATGGSGSTASSGSGSATGHTPCPWFAPLCVEW	CACACTCCGTGTCCGTGGTTTGCTCCGCTGTGCGTTGAATGGGGTTCTGGTTCCGCTACTGGTGGTTCCGGTTCCACTGCTTCTTCTGGTTCCGGTTCTGCAACTGGTCACACCCCGTGCCCGTGGTTTGCACCGCTGTGTGTAGAGTGG (Seq ID No: 282)	1k	C
Myostatin-2XTN8-18 kc	PDWCIDPDWWCKFWGSGSATGGSGSTASSGSGSATGPDWCIDPDWWCKFW	CCGGATTGGTGTATCGACCCGGACTGGTGGTGCAAATTCTGGGGTTCTGGTTCCGCTACCGGTGGTTCCGGTTCCACTGCTTCTTCTGGTTCCGGTTCTGCAACTGGTCCGGACTGGTGCATCGACCCGGATTGGTGGTGTAAATTTTGG (Seq ID No: 283)	1k	C
Myostatin-2XTN8-11 kc	ANWCVSPNWFCMVMGSGSATGGSGSTASSGSGSATGANWCVSPNWFCMVM	CCGGATTGGTGTATCGACCCGGACTGGTGGTGCAAATTCTGGGGTTCTGGTTCCGCTACCGGTGGTTCCGGTTCCACTGCTTCTTCTGGTTCCGGTTCTGCAACTGGTCCGGACTGGTGCATCGACCCGGATTGGTGGTGTAAATTTTGG (Seq ID No; 284)	1k	C
Myostatin-2XTN8-25 kc	PDWCIDPDWWCKFWGSGSATGGSGSTASSGSGSATGPDWCIDPDWWCKFW	ACCACTTGGTGCATCTCTCCGATGTGGTTCTGCTCTCAGCAGGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCTGCAACTGGTACTACTTGGTGTATCTCTCCAATGTGGTTTTGTTCTCAGCAA (Seq ID No: 285)	1k	C
Myostatin-2XTN8-23 kc	HWACGYWPWSCKWVGSGSATGGSGSTASSGSGSATGHWACGYWPWSCKWV	CACTGGGCATGTGGCTATTGGCCGTGGTCCTGCAAATGGGTTGGTTCTGGTTCCGCTACCGGTGGTTCCGGTTCCACTGCTTCTTCTGGTTCCGGTTCTGCAACTGGTCACTGGGCTTGCGGTTACTGGCCGTGGTCTTGTAAATGGGTT (Seq ID No: 286)	1k	C
Myostatin-TN8-29-19 kc	KKHCQIWTWMCAPKGSGSATGGSGSTASSGSGSATGQGHCTRWPWMCPPY	AAAAAACACTGTCAGATCTGGACTTGGATGTGCGCTCCGAAAGGTTCTGGTTCCGCTACCGGTGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTCAGGGTCACTGCACTCGTTGGCCATGGATGTGTCCGCCGTAT (Seq ID No: 287)	1k	C
Myostatin-TN8-19-29 kc	QGHCTRWPWMCPPYGSGSATGGSGSTASSGSGSATGKKHCQIWTWMC	CAGGGTCACTGCACCCGTTGGCCGTGGATGTGCCCGCCGTACGGTTCTGGTTCCGCTACCGGTGGTTCTGGTTCCACTG	1k	C

	APK	CTTCTTCTGGTTCCGGTTCTGCTACTG GTAAAAAACACTGCCAGATCTGGACT TGGATGTGCGCTCCGAAA (Seq ID No: 288)		
Myostatin-TN8-29-19 kn	KKHCQIWTWMCAPKG SGSATGGSGSTASSGSG SATGQGHCTRWPWMC PPY	AAAAAACACTGTCAGATCTGGACTTG GATGTGCGCTCCGAAAGGTTCTGGTT CCGCTACCGGTGGTTCTGGTTCCACTG CTTCTTCTGGTTCCGGTTCCGCTACTG GTCAGGGTCACTGCACTCGTTGGCCA TGGATGTGTCCGCCGTAT (Seq ID No: 289)	1k	N
Myostatin-TN8-29-19-8g	KKHCQIWTWMCAPKG GGGGGGGQGHCTRWP WMCPPY	AAAAAACACTGCCAGATCTGGACTTG GATGTGCGCTCCGAAAGGTGGTGGTG GTGGTGGCGGTGGCCAGGGTCACTGC ACCCGTTGGCCGTGGATGTGTCCGCC GTAT (Seq ID No: 290)	8 gly	C
Myostatin-TN8-19-29-6gc	QGHCTRWPWMCPPYG GGGGGKKHCQIWTWM CAPK	CAGGGTCACTGCACCCGTTGGCCGTG GATGTGCCCGCCGTACGGTGGTGGTG GTGGTGGCAAAAAACACTGCCAGATC TGGACTTGGATGTGCGCTCCGAAA (Seq ID No: 291)	6 gly	C

Example 3

In vitro Assays

C2C12 Cell Based Myostatin Activity Assay

This assay demonstrates the myostatin neutralizing capability of the inhibitor being tested by measuring the extent that binding of myostatin to its receptor is inhibited.

A myostatin-responsive reporter cell line was generated by transfection of C2C12 myoblast cells (ATCC No: CRL-1772) with a pMARE-luc construct. The pMARE-luc construct was made by cloning twelve repeats of the CAGA sequence, representing the myostatin/activin response elements (Dennler et al. EMBO 17: 3091-3100 (1998)) into a pLuc-MCS reporter vector (Stratagene cat # 219087) upstream of the TATA box. The myoblast C2C12 cells naturally express myostatin/activin receptors on its cell surface. When myostatin binds the cell receptors, the Smad pathway is activated, and phosphorylated Smad binds to the response element (Macias-Silva et al. Cell 87:1215 (1996)), resulting in the expression of the lucerase gene. Luciferase activity is then measured using a commercial luciferase reporter assay kit (cat # E4550, Promega, Madison, WI) according to manufacturer's protocol. A stable line of C2C12 cells that had been transfected with pMARE-luc (C2C12/pMARE clone #44) was used to measure myostatin activity according to the following procedure.

Equal numbers of the reporter cells (C2C12/pMARE clone #44) were plated into 96 well cultures. A first round screening using two dilutions of peptibodies was performed with the myostatin concentration fixed at 4 nM. Recombinant mature myostatin was pre-incubated for 2

hours at room temperature with peptibodies at 40 nM and 400 nM respectively. The reporter cell culture was treated with the myostatin with or without peptibodies for six hours. Myostatin activity was measured by determining the luciferase activity in the treated cultures. This assay was used to initially identify peptibody hits that inhibited the myostatin signaling activity in the reporter assay. Subsequently, a nine point titration curve was generated with the myostatin concentration fixed at 4 nM. The myostatin was preincubated with each of the following nine concentrations of peptibodies: 0.04 mM, 0.4 nM, 4 nM, 20 nM, 40 nM, 200 nM, 400 nM, 2 uM and 4 uM for two hours before adding the mixture to the reporter cell culture. The IC_{50} values were for a number of examplary peptibodies are provided in Tables III and for affinity matured peptibodies, in Table VIII.

BIAcore® assay

An affinity analysis of each candidate myostatin peptibody was performed on a BIAcore®3000 (Biacore, Inc., Piscataway, NJ), apparatus using sensor chip CM5, and 0.005 percent P20 surfactant (Biacore, Inc.) as running buffer. Recombinant mature myostatin protein was immobilized to a research grade CM5 sensor chip (Biacore, Inc.) via primary amine groups using the Amine Coupling Kit (Biacore, Inc.) according to the manufacturer's suggested protocol.

Direct binding assays were used to screen and rank the peptibodies in order of their ability to bind to immobilized myostatin. Binding assays were carried by injection of two concentrations (40 and 400 nM) of each candidate myostatin-binding peptibody to the immobilized myostatin surface at a flow rate of 50 µl/min for 3 minutes. After a dissociation time of 3 minutes, the surface was regenerated. Binding curves were compared qualitatively for binding signal intensity, as well as for dissociation rates. Peptibody binding kinetic parameters including k_a (association rate constant), k_d (dissociation rate constant) and K_D (dissociation equilibrium constant) were determined using the BIA evaluation 3.1 computer program (Biacore, Inc.). The lower the dissociation equilibrium constants (expressed in nM), the greater the affinity of the peptibody for myostatin. Examples of peptibody K_D values are shown in Table III and Table VI for affinity-matured peptibodies below.

Blocking assay on ActRIIB/Fc surface

Blocking assays were carried out using immobilized ActRIIB/Fc (R&D Systems, Minneapolis, MN) and myostatin in the presence and absence of peptibodies with the BIAcore® assay system. Assays were used to classify peptibodies as non-neutralizing (those which did not prevent myostatin binding to ActRIIB/Fc) or neutralizing (those that prevented myostatin binding

to ActRIIB/Fc). Baseline myostatin-ActRIIB/Fc binding was first determined in the absence of any peptibody.

For early screening studies, peptibodies were diluted to 4 nM, 40 nM, and 400 nM in sample buffer and incubated with 4 nM myostatin (also diluted in sample buffer). The peptibody: ligand mixtures were allowed to reach equilibrium at room temperature (at least 5 hours) and then were injected over the immobilized ActRIIB/Fc surface for 20 to 30 minutes at a flow rate of 10 uL/min. An increased binding response (over control binding with no peptibody) indicated that peptibody binding to myostatin was non-neutralizing. A decreased binding response (compared to the control) indicated that peptibody binding to myostatin blocked the binding of myostatin to ActRIIB/Fc. Selected peptibodies were further characterized using the blocking assay of a full concentration series in order to derive IC_{50} values (for neutralizing peptibodies) or EC_{50} (for non-neutralizing peptibodies). The peptibody samples were serially diluted from 200 nM to 0.05 mM in sample buffer and incubated with 4 mM myostatin at room temperature to reach equilibrium (minimum of five hours) before injected over the immobilized ActRIIB/Fc surface for 20 to 30 minutes at a flow rate of 10 uL/min. Following the sample injection, bound ligand was allowed to dissociate from the receptor for 3 minutes. Plotting the binding signal vrs. peptibody concentration, the IC_{50} values for each peptibody in the presence of 4 nM myostatin were calculated. It was found, for example, that the peptibodies TN8-19, L2 and L17 inhibit myostatin activity in cell-based assay, but binding of TN-8-19 does not block myostatin/ActRIIB/Fc interactions, indicating that TN-8-19 binds to a different epitope than that observed for the other two peptibodies.

Epitope binning for peptibodies

A purified peptibody was immobilized on a BIAcore chip to capture myostatin before injection of a second peptibody, and the amount of secondary peptibody bound to the captured myostatin was determined. Only peptibodies with distinct epitopes will bind to the captured myostatin, thus enabling the binning of peptibodies with similar or distinct epitope binding properties. For example, it was shown that peptibodies TN8-19 and L23 bind to different epitopes on myostatin.

Selectivity Assays

These assays were performed using BIAcore® technology, to determine the selectivity of binding of the peptibodies to other TGFß family members. ActRIIB/Fc, TGFßRII/Fc and BMPR-1A/Fc (all obtained from R & D Systems, Minneapolis, MN) were covalently coupled to research grade sensor chips according to manufacturer's suggested protocol. Because BIAcore assays

detects changes in the refractive index, the difference between the response detected with injection over the immobilized receptor surfaces compared with the response detected with injection over the control surface in the absence of any peptibody represents the actual binding of Activin A, TGFβ1, TGFβ3, and BMP4 to the receptors, respectively. With pre-incubation of peptibodies and TGFβ molecules, a change (increase or decrease) in binding response indicates peptibody binding to the TGFβ family of molecules. The peptibodies of the present invention all bind to myostatin but not to Activin A, TGFβ1, TGFβ3, or BMP4.

KinEx A™ Equilibrium Assays

Solution-based equilibrium-binding assays using KinExA™ technology (Sapidyne Instruments, Inc.) were used to determine the dissociation equilibrium (K_D) of myostatin binding to peptibody molecules. This solution-based assay is considered to be more sensitive than the BIAcore assay in some instances. Reacti-Gel™ 6X was pre-coated with about 50 ug/ml myostatin for over-night, and then blocked with BSA. 30pM and 100pM of peptibody samples were incubated with various concentrations (0.5 pM to 5 nM) of myostatin in sample buffer at room temperature for 8 hours before being run through the myostatin-coated beads. The amount of the bead-bound peptibody was quantified by fluorescent (Cy5) labeled goat anti-human-Fc antibody at 1 mg/ml in superblock. The binding signal is proportional to the concentration of free peptibody at equilibrium with a given myostatin concentration. K_D was obtained from the nonlinear regression of the competition curves using a dual-curve one-site homogeneous binding model provided in the KinEx A™ software (Sapidyne Instruments, Inc.).

Example 4

Comparison of Myostatin Inhibitors

The ability of three exemplary first-round peptibodies to bind to (K_D) and inhibit (IC_{50}) were compared with the K_D and IC_{50} values obtained for the soluble receptor fusion protein actRIIB/Fc (R &D Systems, Inc., Minneapolis, Minn.). The IC_{50} values were determined using the pMARE luc cell-based assay described in Example 3 and the K_D values were determined using the Biacore® assay described in Example 3.

TABLE III

Inhibitor	IC_{50} (nM)	K_D (nM)
ActRIIB/Fc	~83	~7
2xTN8-19-kc	~9	~2
TN8-19	~23	~2

TN8-29	~26	~60
TN12-34	~30	-----
Linear-20	~11	-----

The peptibodies have an IC_{50} that is improved over the receptor/Fc inhibitor and binding affinities which are comparable in two peptibodies with the receptor/Fc.

Example 5

Comparison of Ability of Peptide and Peptibody to Inhibit Myostatin

The following peptide sequence: QGHCTRWPWMCPPY (TN8-19) (SEQ ID NO: 33) was used to construct the corresponding peptibody TN8-19(pb) according to the procedure described in Example 2 above. Both the peptide alone and the peptibody were screened for myostatin inhibiting activity using the C2C12 based assay described in Example 3 above. It can be seen from Figure 1 the IC_{50} (effective concentration for fifty percent inhibition of myostatin) for the peptibody is significantly less than that of the peptide, and thus the ability of the peptide to inhibit myostatin activity has been substantially improved by placing it in the peptibody configuration.

Example 6

Generation of Affinity-Matured Peptides and Peptibodies

Several of the first round peptides used for peptibody generation were chosen for affinity maturation. The selected peptides included the following: the cysteine constrained TN8-19, QGHCTRWPWMCPPY (SEQ ID NO: 33), and the linear peptides Linear-2 MEMLDSLFELLKDMVPISKA (SEQ ID NO: 104); Linear-15 HHGWNYLRKGSAPQWFEAWV (SEQ. ID NO: 117); Linear-17 RATLLKDFWQLVEGYGDN (SEQ ID NO: 119); Linear-20 YREMSMLEGLLDVLERLQHY (SEQ ID NO: 122), Linear-21 HNSSQMLLSELIMLVGSMMQ (SEQ ID NO: 123), Linear-24 EFFHWLHNHRSEVNHWLDMN (SEQ ID NO: 126). Based on a consensus sequence, directed secondary phage display libraries were generated in which the "core"amino acids (determined from the consensus sequence) were either held constant or biased in frequency of occurrence. Alternatively, an individual peptide sequence could be used to generate a biased, directed phage display library. Panning of such libraries under more stringent conditions can yield peptides with enhanced binding to myostatin, selective binding to myostatin, or with some additional desired property.

Production of doped oligos for libraries

Oligonucleotides were synthesized in a DNA synthesizer which were 91% "doped" at the core sequences, that is, each solution was 91% of the represented base (A, G, C, or T), and 3% of each of the other 3 nucleotides. For the TN8-19 family, for example, a 91% doped oligo used for the construction of a secondary phage library was the following:

5'-CAC AGT GCA CAG GGT NNK NNK NNK caK ggK caK tgK acK cgK tgK

ccK tgK atK tgK ccK ccK taK NNK NNK NNK CAT TCT CTC GAG ATC A-3' (SEQ ID NO: 634)

wherein "N" indicates that each of the four nucleotides A, T, C, and G were equally represented, K indicates that G and T were equally represented, and the lower case letter represents a mixture of 91% of the indicated base and 3% of each of the other bases. The family of oligonucleotides prepared in this manner were PCR amplified as described above, ligated into a phagemid vectors, for example, a modified pCES1 plasmid (Dyax), or any available phagemid vector according to the protocol described above. The secondary phage libraries generated were all 91% doped and had between 1 and 6.5x 10^9 independent transformants. The libraries were panned as described above, but with the following conditions:

Round 1 Panning:
Input phage number: 10^{12} - 10^{13} cfu of phagemid
Selection method: Nunc Immuno Tube selection
Negative selection: 2 X with Nunc Immuno Tubes coated with 2% BSA at 10 min. each
Panning coating: Coat with 1 μg of Myostatin protein in 1 ml of 0.1M Sodium carbonate buffer (pH 9.6)
Binding time: 3 hours
Washing conditions: 6 X 2%-Milk-PBST; 6 X PBST; 2 X PBS
Elution condition: 100 mM TEA elution

Round 2 Panning:
Input phage number: 10^{11} cfu of phagemid
Selection method: Nunc Immuno Tube selection
Negative selection: 2 X with Nunc Immuno Tubes coated with 2% BSA at 30 min. each
Panning coating: Coat with 1 μg of Myostatin protein in 1 ml of 0.1M Sodium carbonate buffer (pH 9.6)
Binding time: 1 hour
Washing conditions: 15 X 2%-Milk-PBST, 1 X 2%-Milk-PBST for 1 hr., 10 X 2%-BSA-PBST, 1 X 2%-BSA-PBST for 1 hr., 10 X PBST and 3 X PBS
Elution condition: 100 mM TEA elution

Round 3 Panning:
Input phage number: 10^{10} cfu of phagemid
Selection method: Nunc Immuno Tube selection
Negative selection: 6 X with Nunc Immuno Tubes coated with 2% BSA at 10 min. each
Panning coating: Coat with 0.1 μg of Myostatin protein in 1 ml of 0.1M Sodium carbonate buffer (pH 9.6)
Binding time: 1 hour

Washing conditions: 15 X 2%-Milk-PBST, 1 X 2%-Milk-PBST for 1 hr., 10 X 2%-BSA-PBST, 1 X 2%-BSA-PBST for 1 hr., 10 X PBST and 3 X PBS
Elution condition: 100 mM TEA elution

Panning of the secondary libraries yielded peptides with enhanced binding to myostatin. Individual selected clones were subjected phage ELISA, as described above, and sequenced.

The following affinity matured TN8-19 family of peptides are shown in Table IV below.

Affinity- matured peptibody	TABLE IV SEQ ID NO:	Peptide sequence
mTN8-19-1	305	VALHGQCTRWPWMCPPQREG
mTN8-19-2	306	YPEQGLCTRWPWMCPPQTLA
mTN8-19-3	307	GLNQGHCTRWPWMCPPQDSN
mTN8-19-4	308	MITQGQCTRWPWMCPPQPSG
mTN8-19-5	309	AGAQEHCTRWPWMCAPNDWI
mTN8-19-6	310	GVNQGQCTRWRWMCPPNGWE
mTN8-19-7	311	LADHGQCIRWPWMCPPEGWE
mTN8-19-8	312	ILEQAQCTRWPWMCPPQRGG
mTN8-19-9	313	TQTHAQCTRWPWMCPPQWEG
mTN8-19-10	314	VVTQGHCTLWPWMCPPQRWR
mTN8-19-11	315	IYPHDQCTRWPWMCPPQPYP
mTN8-19-12	316	SYWQGQCTRWPWMCPPQWRG
mTN8-19-13	317	MWQQGHCTRWPWMCPPQGWG
mTN8-19-14	318	EFTQWHCTRWPWMCPPQRSQ
mTN8-19-15	319	LDDQWQCTRWPWMCPPQGFS
mTN8-19-16	320	YQTQGLCTRWPWMCPPQSQR
mTN8-19-17	321	ESNQGQCTRWPWMCPPQGGW
mTN8-19-18	322	WTDRGPCTRWPWMCPPQANG
mTN8-19-19	323	VGTQGQCTRWPWMCPPYETG
mTN8-19-20	324	PYEQGKCTRWPWMCPPYEVE
mTN8-19-21	325	SEYQGLCTRWPWMCPPQGWK
mTN8-19-22	326	TFSQGHCTRWPWMCPPQGWG
mTN8-19-23	327	PGAHDHCTRWPWMCPPQSRY
mTN8-19-24	328	VAEEWHCRRWPWMCPPQDWR
mTN8-19-25	329	VGTQGHCTRWPWMCPPQPAG
mTN8-19-26	330	EEDQAHCRSWPWMCPPQGWV
mTN8-19-27	331	ADTQGHCTRWPWMCPPQHWF
mTN8-19-28	332	SGPQGHCTRWPWMCAPQGWF
mTN8-19-29	333	TLVQGHCTRWPWMCPPQRWV
mTN8-19-30	334	GMAHGKCTRWAWMCPPQSWK
mTN8-19-31	335	ELYHGQCTRWPWMCPPQSWA
mTN8-19-32	336	VADHGHCTRWPWMCPPQGWG
mTN8-19-33	337	PESQGHCTRWPWMCPPQGWG
mTN8-19-34	338	IPAHGHCTRWPWMCPPQRWR
mTN8-19-35	339	FTVHGHCTRWPWMCPPYGWV
mTN8-19-36	340	PDFPGHCTRWRWMCPPQGWE
mTN8-19-37	341	QLWQGPCTQWPWMCPPKGRY
mTN8-19-38	342	HANDGHCTRWQWMCPPQWGG
mTN8-19-39	343	ETDHGLCTRWPWMCPPYGAR
mTN8-19-40	344	GTWQGLCTRWPWMCPPQGWQ
mTN8-19 con1	345	VATQGQCTRWPWMCPPQGWG
mTN8-19 con2	346	VATQGQCTRWPWMCPPQRWG

mTN8 con6-1	347	QREWYPCYGGHLWCYDLHKA
mTN8 con6-2	348	ISAWYSCYAGHFWCWDLKQK
mTN8 con6-3	349	WTGWYQCYGGHLWCYDLRRK
mTN8 con6-4	350	KTFWYPCYDGHFWCYNLKSS
mTN8 con6-5	351	ESRWYPCYEGHLWCFDLTET

The consensus sequence derived from the affinity- matured TN-8-19- 1 through Con2 (excluding the mTN8 con6 sequences) shown above is: $\underline{C}a_1a_2\underline{W}a_3\underline{WMCPP}$ (SEQ ID NO: 352). All of these peptide comprise the sequence WMCPP (SEQ ID NO: 633). The underlined amino acids represent the core amino acids present in all embodiments, and a_1, a_2 and a_3 are selected from a neutral hydrophobic, neutral polar, or basic amino acid. In one embodiment of this consensus sequence, $\underline{C}b_1b_2\underline{W}b_3\underline{WMCPP}$ (SEQ ID NO: 353), b_1 is selected from any one of the amino acids T, I, or R; b_2 is selected from any one of R, S, Q; and b_3 is selected from any one of P, R and Q. All of the peptides comprise the sequence WMCPP (SEQ ID NO: 633). A more detailed analysis of the affinity matured TN8 sequences comprising SEQ ID NO: 352 provides the following formula:

$c_1c_2c_3c_4c_5c_6\underline{C}c_7c_8\underline{W}c_9\underline{WMCPP}c_{10}c_{11}c_{12}c_{13}$ (SEQ ID NO: 354), wherein:

c_1 is absent or any amino acid;
c_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_4 is absent or any amino acid;
c_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
c_7 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_8 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_9 is a neutral hydrophobic, neutral polar or basic amino acid; and wherein
c_{10} to c_{13} is any amino acid.

In one embodiment of the above formulation, b_7 is selected from any one of the amino acids T, I, or R; b_8 is selected from any one of R, S, Q; and b_9 is selected from any one of P, R and Q. This provides the following sequence:

$d_1d_2d_3d_4d_5d_6\underline{C}d_7d_8\underline{W}d_9\underline{WMCPP}$ $d_{10}d_{11}d_{12}d_{13}$ (SEQ ID NO: 355).

d_1 is absent or any amino acid;
d_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_4 is absent or any amino acid;
d_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
d_7 is selected from any one of the amino acids T, I, or R;
d_8 is selected from any one of R, S, Q;
d_9 is selected from any one of P, R and Q

and d_{10} through d_{13} are selected from any amino acid.

The consensus sequence of the mTN8 con6 series is WYe_1e_2Ye_3G, (SEQ ID NO: 356) wherein e_1 is P, S or Y; e_2 is C or Q, and e_3 is G or H.

In addition to the TN-19 affinity matured family, additional affinity matured peptides were produced from the linear L-2, L-15, L-17, L-20, L-21, and L-24 first round peptides. These families are presented in Table V below.

TABLE V

Affinity matured peptibody	SEQ ID NO:	Peptide Sequence
L2	104	MEMLDSLFELLKDMVPISKA
mL2-Con1	357	RMEMLESLLELLKEIVPMSKAG
mL2-Con2	358	RMEMLESLLELLKEIVPMSKAR
mL2-1	359	RMEMLESLLELLKDIVPMSKPS
mL2-2	360	GMEMLESLFELLQEIVPMSKAP
mL2-3	361	RMEMLESLLELLKDIVPISNPP
mL2-4	362	RIEMLESLLELLQEIVPISKAE
mL2-5	363	RMEMLQSLLELLKDIVPMSNAR
mL2-6	364	RMEMLESLLELLKEIVPTSNGT
mL2-7	365	RMEMLESLFELLKEIVPMSKAG
mL2-8	366	RMEMLGSLLELLKEIVPMSKAR
mL2-9	367	QMELLDSLFELLKEIVPKSQPA
mL2-10	368	RMEMLDSLLELLKEIVPMSNAR
mL2-11	369	RMEMLESLLELLHEIVPMSQAG
mL2-12	370	QMEMLESLLQLLKEIVPMSKAS
mL2-13	371	RMEMLDSLLELLKDMVPMTTGA
mL2-14	372	RIEMLESLLELLKDMVPMANAS
mL2-15	373	RMEMLESLLQLLNEIVPMSRAR
mL2-16	374	RMEMLESLFDLLKELVPMSKGV
mL2-17	375	RIEMLESLLELLKDIVPIQKAR
mL2-18	376	RMELLESLFELLKDMVPMSDSS
mL2-19	377	RMEMLESLLEVLQEIVPRAKGA
mL2-20	378	RMEMLDSLLQLLNEIVPMSHAR
mL2-21	379	RMEMLESLLELLKDIVPMSNAG
mL2-22	380	RMEMLQSLFELLKGMVPISKAG
mL2-23	381	RMEMLESLLELLKEIVPNSTAA
mL2-24	382	RMEMLQSLLELLKEIVPISKAG
mL2-25	383	RIEMLDSLLELLNELVPMSKAR
L-15	117	HHGWNYLRKGSAPQWFEAWV
mL15-con1	384	QVESLQQLLMWLDQKLASGPQG
mL15-1	385	RMELLESLFELLKEMVPRSKAV
mL15-2	386	QAVSLQHLLMWLDQKLASGPQH

mL15-3	387	DEDSLQQLLMWLDQKLASGPQL
mL15-4	388	PVASLQQLLIWLDQKLAQGPHA
mL15-5	389	EVDELQQLLNWLDHKLASGPLQ
mL15-6	390	DVESLEQLLMWLDHQLASGPHG
mL15-7	391	QVDSLQQVLLWLEHKLALGPQV
mL15-8	392	GDESLQHLLMWLEQKLALGPHG
mL15-9	393	QIEMLESLLDLLRDMVPMSNAF
mL15-10	394	EVDSLQQLLMWLDQKLASGPQA
mL15-11	395	EDESLQQLLIYLDKMLSSGPQV
mL15-12	396	AMDQLHQLLIWLDHKLASGPQA
mL15-13	397	RIEMLESLLELLDEIALIPKAW
mL15-14	398	EVVSLQHLLMWLEHKLASGPDG
mL15-15	399	GGESLQQLLMWLDQQLASGPQR
mL15-16	400	GVESLQQLLIFLDHMLVSGPHD
mL15-17	401	NVESLEHLMMWLERLLASGPYA
mL15-18	402	QVDSLQQLLIWLDHQLASGPKR
mL15-19	403	EVESLQQLLMWLEHKLAQGPQG
mL15-20	404	EVDSLQQLLMWLDQKLASGPHA
mL15-21	405	EVDSLQQLLMWLDQQLASGPQK
mL15-22	406	GVEQLPQLLMWLEQKLASGPQR
mL15-23	407	GEDSLQQLLMWLDQQLAAGPQV
mL15-24	408	ADDSLQQLLMWLDRKLASGPHV
mL15-25	409	PVDSLQQLLIWLDQKLASGPQG
L-17	119	RATLLKDFWQLVEGYGDN
mL17-con1	410	DWRATLLKEFWQLVEGLGDNLV
mL17-con2	411	QSRATLLKEFWQLVEGLGDKQA
mL17-1	412	DGRATLLTEFWQLVQGLGQKEA
mL17-2	413	LARATLLKEFWQLVEGLGEKVV
mL17-3	414	GSRDTLLKEFWQLVVGLGDMQT
mL17-4	415	DARATLLKEFWQLVDAYGDRMV
mL17-5	416	NDRAQLLRDFWQLVDGLGVKSW
mL17-6	417	GVRETLLYELWYLLKGLGANQG
mL17-7	418	QARATLLKEFCQLVGCQGDKLS
mL17-8	419	QERATLLKEFWQLVAGLGQNMR
mL17-9	420	SGRATLLKEFWQLVQGLGEYRW
mL17-10	421	TMRATLLKEFWLFVDGQREMQW
mL17-11	422	GERATLLNDFWQLVDGQGDNTG
mL17-12	423	DERETLLKEFWQLVHGWGDNVA
mL17-13	424	GGRATLLKELWQLLEGQGANLV
mL17-14	425	TARATLLNELVQLVKGYGDKLV
mL17-15	426	GMRATLLQEFWQLVGGQGDNWM
mL17-16	427	STRATLLNDLWQLMKGWAEDRG
mL17-17	428	SERATLLKELWQLVGGWGDNFG
mL17-18	429	VGRATLLKEFWQLVEGLVGQSR
mL17-19	430	EIRATLLKEFWQLVDEWREQPN

mL17-20	431	QLRATLLKEFLQLVHGLGETDS
mL17-21	432	TQRATLLKEFWQLIEGLGGKHV
mL17-22	433	HYRATLLKEFWQLVDGLREQGV
mL17-23	434	QSRVTLLREFWQLVESYRPIVN
mL17-24	435	LSRATLLNEFWQFVDGQRDKRM
mL17-25	436	WDRATLLNDFWHLMEELSQKPG
mL17-26	437	QERATLLKEFWRMVEGLGKNRG
mL17-27	438	NERATLLREFWQLVGGYGVNQR
L-20	122	YREMSMLEGLLDVLERLQHY
mL20-1	439	HQRDMSMLWELLDVLDGLRQYS
mL20-2	440	TQRDMSMLDGLLEVLDQLRQQR
mL20-3	441	TSRDMSLLWELLEELDRLGHQR
mL20-4	442	MQHDMSMLYGLVELLESLGHQI
mL20-5	443	WNRDMRMLESLFEVLDGLRQQV
mL20-6	444	GYRDMSMLEGLLAVLDRLGPQL
mL20 con1	445	TQRDMSMLEGLLEVLDRLGQQR
mL20 con2	446	WYRDMSMLEGLLEVLDRLGQQR
L-21	123	HNSSQMLLSELIMLVGSMMQ
mL21-1	447	TQNSRQMLLSDFMMLVGSMIQG
mL21-2	448	MQTSRHILLSEFMMLVGSIMHG
mL21-3	449	HDNSRQMLLSDLLHLVGTMIQG
mL21-4	450	MENSRQNLLRELIMLVGNMSHQ
mL21-5	451	QDTSRHMLLREFMMLVGEMIQG
mL21 con1	452	DQNSRQMLLSDLMILVGSMIQG
L-24	126	EFFHWLHNHRSEVNHWLDMN
mL24-1	453	NVFFQWVQKHGRVVYQWLDINV
mL24-2	454	FDFLQWLQNHRSEVEHWLVMDV

The affinity matured peptides provided in Tables IV and V are then assembed into peptibodies as described above and assayed using the in vivo assays.

The affinity matured L2 peptides comprise a consensus sequence of f_1EMLf_2SLf_3f_4LL, (SEQ ID NO: 455), wherein f_1 is M or I; f_2 is any amino acid; f_3 is L or F; and f_4 is E, Q or D.

The affinity matured L15 peptide family comprise the sequence Lg_1g_2LLg_3g_4L, (SEQ ID NO: 456), wherein g_1 is Q, D or E, g_2 is S, Q, D or E, g_3 is any amino acid, and g_4 is L, W, F, or Y. The affinity matured L17 family comprises the sequence: $h_1h_2h_3h_4h_5h_6h_7h_8h_9$ (SEQ ID NO: 457) wherein h_1 is R or D; h_2 is any amino acid; h_3 is A, T S or Q; h_4 is L or M; h_5 is L or S; h_6 is any amino acid; h_7 is F or E; h_8 is W, F or C; and h_9 is L, F, M or K. Consensus sequences may also be determined for the mL20, mL21 and mL24 families of peptides shown above.

Peptibodies were constructed from these affinity matured peptides as described above, using a linker attached to the Fc domain of human IgG1, having SEQ ID NO: 296, at the N-

terminus (N configuration), at the C terminus (C configuration) of the Fc, or at both the N and C terminals (N,C configurations), as described in Example 2 above. The peptides named were attached to the C or N terminals via a 5 glycine (5G), 8 glycine or k linker sequence. In the 2X peptibody version the peptides were linked with linkers such as 5 gly, 8 gly or k. Affinity matured peptides and peptibodies are designated with a small "m" such as mTN8-19-22 for example. Peptibodies of the present invention further contain two splice sites where the peptides were spliced into the phagemid vectors. The position of these splice sites are AQ—peptide—LE. The peptibodies generally include these additional amino acids (although they are not included in the peptide sequences listed in the tables). In some peptibodies the LE amino acids were removed from the peptides sequences. These peptibodies are designated –LE.

Exemplary peptibodies, and exemplary polynucleotide sequences encoding them, are provided in Table VI below. This table includes examples of peptibody sequences (as opposed to peptide only), such as the 2x mTN8-19-7 (SEQ ID NO: 615) and the peptibody with the LE sequences deleted (SEQ ID NO: 617). By way of explanation, the linker sequences in the 2x versions refers to the linker between the tandem peptides. These peptibody sequences contain the Fc, linkers, AQ and LE sequences. The accompanying nucleotide sequence encodes the peptide sequence in addition to the AQ/LE linker sequences, if present, but does not encode the designated linker.

TABLE VI

Peptibody Name	Peptide	Nucleotide Sequence (SEQ ID No)	Linker	Term –inus
mL2–Con1	RMEMLESLLELL KEIVPMSKAG	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGAAATTGTTCC AATGTCTAAAGCTGGT (SEQ ID NO: 458)	5 gly	N
mL2–Con2	RMEMLESLLELL KEIVPMSKAR	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGAAATTGTTCC AATGTCTAAAGCTCGT (SEQ ID NO: 459)	5 gly	N
mL2–1	RMEMLESLLELL KDIVPMSKPS	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGATATTGTTCC AATGTCTAAACCATCT (SEQ ID NO: 460)	5 gly	N
mL2–2	GMEMLESLFELL QEIVPMSKAP	GGTATGGAAATGCTTGAATCTCTTT TTGAACTTCTTCAAGAAATTGTTCC AATGTCTAAAGCTCCA (SEQ ID NO: 461)	5 gly	N
mL2–3	RMEMLESLLELL KDIVPISNPP	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGATATTGTTCC AATTTCTAATCCACCA (SEQ ID NO: 462)	5 gly	N

mL2–4	RIEMLESLLELLQ EIVPISKAE	CGTATTGAAATGCTTGAATCTCTTC TTGAACTTCTTCAAGAAATTGTTCC AATTTCTAAAGCTGAA (SEQ ID NO: 463)	5 gly	N
mL2–5	RMEMLQSLLELL KDIVPMSNAR	CGTATGGAAATGCTTCAATCTCTTC TTGAACTTCTTAAAGATATTGTTCC AATGTCTAATGCTCGT (SEQ ID NO: 464)	5 gly	N
mL2–6	RMEMLESLLELL KEIVPTSNGT	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGAAATTGTTCC AACTTCTAATGGTACT (SEQ ID NO: 465)	5 gly	N
mL2–7	RMEMLESLFELL KEIVPMSKAG	CGTATGGAAATGCTTGAATCTCTTT TTGAACTTCTTAAAGAAATTGTTCC AATGTCTAAAGCTGGT (SEQ ID NO: 466)	5 gly	N
mL2–8	RMEMLGSLLELL KEIVPMSKAR	CGTATGGAAATGCTTGGTTCTCTTC TTGAACTTCTTAAAGAAATTGTTCC AATGTCTAAAGCTCGT(SEQ ID NO: 467)	5 gly	N
mL2–9	QMELLDSLFELL KEIVPKSQPA	CAAATGGAACTTCTTGATTCTCTTT TTGAACTTCTTAAAGAAATTGTTCC AAAATCTCAACCAGCT (SEQ ID NO: 468)	5 gly	N
mL2–10	RMEMLDSLLELL KEIVPMSNAR	CGTATGGAAATGCTTGATTCTCTTC TTGAACTTCTTAAAGAAATTGTTCC AATGTCTAATGCTCGT (SEQ ID NO: 469)	5 gly	N
mL2–11	RMEMLESLLELL HEIVPMSQAG	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTCATGAAATTGTTCC AATGTCTCAAGCTGGT (SEQ ID NO: 470)	5 gly	N
mL2–12	QMEMLESLLQLL KEIVPMSKAS	CAAATGGAAATGCTTGAATCTCTTC TTCAACTTCTTAAAGAAATTGTTCC AATGTCTAAAGCTTCT (SEQ ID NO: 471)	5 gly	N
mL2–13	RMEMLDSLLELL KDMVPMTTGA	CGTATGGAAATGCTTGATTCTCTTC TTGAACTTCTTAAAGATATGGTTCC AATGACTACTGGTGCT (SEQ ID NO: 472)	5 gly	N
mL2–14	RIEMLESLLELLK DMVPMANAS	CGTATTGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGATATGGTTCC AATGGCTAATGCTTCT (SEQ ID NO: 473)	5 gly	N
mL2–15	RMEMLESLLQLL NEIVPMSRAR	CGTATGGAAATGCTTGAATCTCTTC TTCAACTTCTTAATGAAATTGTTCC AATGTCTCGTGCTCGT (SEQ ID NO: 474)	5 gly	N
mL2–16	RMEMLESLFDLL KELVPMSKGV	CGTATGGAAATGCTTGAATCTCTTT TTGATCTTCTTAAAGAACTTGTTCC AATGTCTAAAGGTGTT (SEQ ID NO: 475)	5 gly	N
mL2–17	RIEMLESLLELLK DIVPIQKAR	CGTATTGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGATATTGTTCC AATTCAAAAAGCTCGT (SEQ ID NO: 476)	5 gly	N

mL2–18	RMELLESLFELLK DMVPMSDSS	CGTATGGAACTTCTTGAATCTCTTT TTGAACTTCTTAAAGATATGGTTCC AATGTCTGATTCTTCT (SEQ ID NO: 477)	5 gly	N
mL2–19	RMEMLESLLEVL QEIVPRAKGA	CGTATGGAAATGCTTGAATCTCTTC TTGAAGTTCTTCAAGAAATTGTTCC ACGTGCTAAAGGTGCT (SEQ ID NO: 478)	5 gly	N
mL2–20	RMEMLDSLLQLL NEIVPMSHAR	CGTATGGAAATGCTTGATTCTCTTC TTCAACTTCTTAATGAAATTGTTCC AATGTCTCATGCTCGT (SEQ ID NO: 479)	5 gly	N
mL2–21	RMEMLESLLELL KDIVPMSNAG	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGATATTGTTCC AATGTCTAATGCTGGT (SEQ ID NO: 480)	5 gly	N
mL2–22	RMEMLQSLFELL KGMVPISKAG	CGTATGGAAATGCTTCAATCTCTTT TTGAACTTCTTAAAGGTATGGTTCC AATTTCTAAAGCTGGT (SEQ ID NO: 481)	5 gly	N
mL2–23	RMEMLESLLELL KEIVPNSTAA	CGTATGGAAATGCTTGAATCTCTTC TTGAACTTCTTAAAGAAATTGTTCC AAATTCTACTGCTGCT (SEQ ID NO: 482)	5 gly	N
mL2–24	RMEMLQSLLELL KEIVPISKAG	CGTATGGAAATGCTTCAATCTCTTC TTGAACTTCTTAAAGAAATTGTTCC AATTTCTAAAGCTGGT (SEQ ID NO: 483)	5 gly	N
mL2–25	RIEMLDSLLELLN ELVPMSKAR	CGTATTGAAATGCTTGATTCTCTTC TTGAACTTCTTAATGAACTTGTTCC AATGTCTAAAGCTCGT (SEQ ID NO: 484)	5 gly	N
mL17–Con1	DWRATLLKEFW QLVEGLGDNLV	GATTGGCGTGCTACTCTTCTTAAAG AATTTTGGCAACTTGTTGAAGGTCT TGGTGATAATCTTGTT (SEQ ID NO: 485)	5 gly	N
mL17–1	DGRATLLTEFWQ LVQGLGQKEA	GATGGTCGTGCTACTCTTCTTACTG AATTTTGGCAACTTGTTCAAGGTCT TGGTCAAAAAGAAGCT (SEQ ID NO: 486)	5 gly	N
mL17–2	LARATLLKEFWQ LVEGLGEKVV	CTTGCTCGTGCTACTCTTCTTAAAG AATTTTGGCAACTTGTTGAAGGTCT TGGTGAAAAAGTTGTT (SEQ ID NO: 487)	5 gly	N
mL17–3	GSRDTLLKEFWQ LVVGLGDMQT	GGTTCTCGTGATACTCTTCTTAAAG AATTTTGGCAACTTGTTGTTGGTCT TGGTGATATGCAAACT (SEQ ID NO: 488)	5 gly	N
mL17–4	DARATLLKEFWQ LVDAYGDRMV	GATGCTCGTGCTACTCTTCTTAAAG AATTTTGGCAACTTGTTGATGCTTA TGGTGATCGTATGGTT (SEQ ID NO: 489)	5 gly	N
mL17–5	NDRAQLLRDFWQ LVDGLGVKSW	AATGATCGTGCTCAACTTCTTCGTG ATTTTTGGCAACTTGTTGATGGTCT TGGTGTTAAATCTTGG (SEQ ID NO: 490)	5 gly	N

mL17–6	GVRETLLYELWY LLKGLGANQG	GGTGTTCGTGAAACTCTTCTTTATG AACTTTGGTATCTTCTTAAAGGTCT TGGTGCTAATCAAGGT (SEQ ID NO: 491)	5 gly	N
mL17–7	QARATLLKEFCQ LVGCQGDKLS	CAAGCTCGTGCTACTCTTCTTAAAG AATTTTGTCAACTTGTTGGTTGTCA AGGTGATAAACTTTCT (SEQ ID NO: 492)	5 gly	N
mL17–8	QERATLLKEFWQ LVAGLGQNMR	CAAGAACGTGCTACTCTTCTTAAA GAATTTTGGCAACTTGTTGCTGGTC TTGGTCAAAATATGCGT (SEQ ID NO: 493)	5 gly	N
mL17–9	SGRATLLKEFWQ LVQGLGEYRW	TCTGGTCGTGCTACTCTTCTTAAAG AATTTTGGCAACTTGTTCAAGGTCT TGGTGAATATCGTTGG (SEQ ID NO: 494)	5 gly	N
mL17–10	TMRATLLKEFWL FVDGQREMQW	ACTATGCGTGCTACTCTTCTTAAAG AATTTTGGCTTTTTGTTGATGGTCA ACGTGAAATGCAATGG (SEQ ID NO: 495)	5 gly	N
mL17–11	GERATLLNDFWQ LVDGQGDNTG	GGTGAACGTGCTACTCTTCTTAATG ATTTTTGGCAACTTGTTGATGGTCA AGGTGATAATACTGGT (SEQ ID NO: 496)	5 gly	N
mL17–12	DERETLLKEFWQ LVHGWGDNVA	GATGAACGTGAAACTCTTCTTAAA GAATTTTGGCAACTTGTTCATGGTT GGGGTGATAATGTTGCT (SEQ ID NO: 497)	5 gly	N
mL17–13	GGRATLLKELWQ LLEGQGANLV	GGTGGTCGTGCTACTCTTCTTAAAG AACTTTGGCAACTTCTTGAAGGTCA AGGTGCTAATCTTGTT (SEQ ID NO: 498)	5 gly	N
mL17–14	TARATLLNELVQ LVKGYGDKLV	ACTGCTCGTGCTACTCTTCTTAATG AACTTGTTCAACTTGTTAAAGGTTA TGGTGATAAACTTGTT (SEQ ID NO: 499)	5 gly	N
mL17–15	GMRATLLQEFWQ LVGGQGDNWM	GGTATGCGTGCTACTCTTCTTCAAG AATTTTGGCAACTTGTTGGTGGTCA AGGTGATAATTGGATG (SEQ ID NO: 500)	5 gly	N
mL17–16	STRATLLNDLWQ LMKGWAEDRG	TCTACTCGTGCTACTCTTCTTAATG ATCTTTGGCAACTTATGAAAGGTTG GGCTGAAGATCGTGGT (SEQ ID NO: 501)	5 gly	N
mL17–17	SERATLLKELWQ LVGGWGDNFG	TCTGAACGTGCTACTCTTCTTAAAG AACTTTGGCAACTTGTTGGTGGTTG GGGTGATAATTTTGGT (SEQ ID NO: 502)	5 gly	N
mL17–18	VGRATLLKEFWQ LVEGLVGQSR	GTTGGTCGTGCTACTCTTCTTAAAG AATTTTGGCAACTTGTTGAAGGTCT TGTTGGTCAATCTCGT (SEQ ID NO: 503)	5 gly	N

2x mTN8–Con6–(N)–1K	M–GAQ–WYPCYEGHFWCYDL–GSGSATGGSGSTASSGSGSATG–WYPCYEGHFWCYDL–LE–5G–FC (SEQ ID NO: 504)	TGGTATCCGTGTTATGAGGGTCACTTCTGGTGCTACGATCTGGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTTGGTACCCGTGCTACGAAGGTCACTTTTGGTGTTATGATCTG (SEQ ID NO: 505)	1K	N
2x mTN8–Con6–(C)–1K	FC–5G–AQ–WYPCYEGHFWCYDL–GSGSATGGSGSTASSGSGSATG–WYPCYEGHFWCYDL–LE (SEQ ID NO: 506)	TGGTATCCGTGTTATGAGGGTCACTTCTGGTGCTACGATCTGGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTTGGTACCCGTGCTACGAAGGTCACTTTTGGTGTTATGATCTG (SEQ ID NO: 507)	1K	C
2x mTN8–Con7–(N)–1K	M–GAQ–IFGCKWWDVQCYQF–GSGSATGGSGSTASSGSGSATG–IFGCKWWDVQCYQF–LE–5G–FC (SEQ ID NO: 508)	ATCTTTGGCTGTAAATGGTGGGACGTTCAGTGCTACCAGTTCGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTATCTTCGGTTGCAAGTGGTGGGATGTACAGTGTTATCAGTTT (SEQ ID NO: 509)	1K	N
2x mTN8–Con7–(C)–1K	FC–5G–AQ–IFGCKWWDVQCYQF–GSGSATGGSGSTASSGSGSATG–IFGCKWWDVQCYQF–LE (SEQ ID NO: 510)	ATCTTTGGCTGTAAATGGTGGGACGTTCAGTGCTACCAGTTCGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTATCTTCGGTTGCAAGTGGTGGGATGTACAGTGTTATCAGTTT (SEQ ID NO: 511)	1K	C
2x mTN8–Con8–(N)–1K	M–GAQ–IFGCKWWDVDCYQF–GSGSATGGSGSTASSGSGSATG–IFGCKWWDVDCYQF–LE–5G–FC (SEQ ID NO: 512)	ATCTTTGGCTGTAAGTGGTGGGACGTTGACTGCTACCAGTTCGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTATCTTCGGTTGCAAATGGTGGGACGTTGATTGTTATCAGTTT (SEQ ID NO: 513)	1K	N
2x mTN8–Con8–(C)–1K	FC–5G–AQ–IFGCKWWDVDCYQF–GSGSATGGSGSTASSGSGSATG–IFGCKWWDVDCYQF–LE (SEQ ID NO: 514)	ATCTTTGGCTGTAAGTGGTGGGACGTTGACTGCTACCAGTTCGGTTCTGGTTCCACTGCTTCTTCTGGTTCCGGTTCCGCTACTGGTATCTTCGGTTGCAAATGGTGGGACGTTGATTGTTATCAGTTT (SEQ ID NO: 515)	1K	C
ML15–Con1	QVESLQQLLMWLDQKLASGPQG	CAGGTTGAATCCCTGCAGCAGCTGCTGATGTGGCTGGACCAGAAACTGGCTTCCGGTCCGCAGGGT (SEQ ID NO: 516)	5 gly	C
ML15–1	RMELLESLFELLKEMVPRSKAV	CGTATGGAACTGCTGGAATCCCTGTTCGAACTGCTGAAAGAAATGGTTCCGCGTTCCAAAGCTGTT (SEQ ID NO: 517)	5 gly	C

mL15–2	QAVSLQHLLMW LDQKLASGPQH	CAGGCTGTTTCCCTGCAGCACCTGC TGATGTGGCTGGACCAGAAACTGG CTTCCGGTCCGCAGCAC (SEQ ID NO: 518)	5 gly	C
mL15–3	DEDSLQQLLMWL DQKLASGPQL	GACGAAGACTCCCTGCAGCAGCTG CTGATGTGGCTGGACCAGAAACTG GCTTCCGGTCCGCAGCTG (SEQ ID NO: 519)	5 gly	C
mL15–4	PVASLQQLLIWL DQKLAQGPHA	CCGGTTGCTTCCCTGCAGCAGCTGC TGATCTGGCTGGACCAGAAACTGG CTCAGGGTCCGCACGCT (SEQ ID NO: 520)	5 gly	C
mL15–5	EVDELQQLLNWL DHKLASGPLQ	GAAGTTGACGAACTGCAGCAGCTG CTGAACTGGCTGGACCACAAACTG GCTTCCGGTCCGCTGCAG (SEQ ID NO: 521)	5 gly	C
mL15–6	DVESLEQLLMWL DHQLASGPHG	GACGTTGAATCCCTGGAACAGCTG CTGATGTGGCTGGACCACCAGCTG GCTTCCGGTCCGCACGGT (SEQ ID NO: 522)	5 gly	C
mL15–7	QVDSLQQVLLWL EHKLALGPQV	CAGGTTGACTCCCTGCAGCAGGTT CTGCTGTGGCTGGAACACAAACTG GCTCTGGGTCCGCAGGTT (SEQ ID NO: 523)	5 gly	C
mL15–8	GDESLQHLLMWL EQKLALGPHG	GGTGACGAATCCCTGCAGCACCTG CTGATGTGGCTGGAACAGAAACTG GCTCTGGGTCCGCACGGT (SEQ ID NO: 524)	5 gly	C
mL15–9	QIEMLESLLDLLR DMVPMSNAF	CAGATCGAAATGCTGGAATCCCTG CTGGACCTGCTGCGTGACATGGTTC CGATGTCCAACGCTTTC (SEQ ID NO: 525)	5 gly	C
mL15–10	EVDSLQQLLMWL DQKLASGPQA	GAAGTTGACTCCCTGCAGCAGCTG CTGATGTGGCTGGACCAGAAACTG GCTTCCGGTCCGCAGGCT (SEQ ID NO: 526)	5 gly	C
mL15–11	EDESLQQLLIYLD KMLSSGPQV	GAAGACGAATCCCTGCAGCAGCTG CTGATCTACCTGGACAAAATGCTG TCCTCCGGTCCGCAGGTT (SEQ ID NO: 527)	5 gly	C
mL15–12	AMDQLHQLLIWL DHKLASGPQA	GCTATGGACCAGCTGCACCAGCTG CTGATCTGGCTGGACCACAAACTG GCTTCCGGTCCGCAGGCT (SEQ ID NO: 528)	5 gly	C
mL15–13	RIEMLESLLELLD EIALIPKAW	CGTATCGAAATGCTGGAATCCCTG CTGGAACTGCTGGACGAAATCGCT CTGATCCCGAAAGCTTGG (SEQ ID NO: 529)	5 gly	C
mL15–14	EVVSLQHLLMWL EHKLASGPDG	GAAGTTGTTTCCCTGCAGCACCTGC TGATGTGGCTGGAACACAAACTGG CTTCCGGTCCGGACGGT (SEQ ID NO: 530)	5 gly	C
mL15–15	GGESLQQLLMWL DQQLASGPQR	GGTGGTGAATCCCTGCAGCAGCTG CTGATGTGGCTGGACCAGCAGCTG GCTTCCGGTCCGCAGCGT (SEQ ID NO: 531)	5 gly	C

mL15–16	GVESLQQLLIFLD HMLVSGPHD	GGTGTTGAATCCCTGCAGCAGCTG CTGATCTTCCTGGACCACATGCTGG TTTCCGGTCCGCACGAC (SEQ ID NO: 532)	5 gly	C
mL15–17	NVESLEHLMMW LERLLASGPYA	AACGTTGAATCCCTGGAACACCTG ATGATGTGGCTGGAACGTCTGCTG GCTTCCGGTCCGTACGCT (SEQ ID NO: 533)	5 gly	C
mL15–18	QVDSLQQLLIWL DHQLASGPKR	CAGGTTGACTCCCTGCAGCAGCTG CTGATCTGGCTGGACCACCAGCTG GCTTCCGGTCCGAAACGT (SEQ ID NO: 534)	5 gly	C
mL15–19	EVESLQQLLMWL EHKLAQGPQG	GAAGTTGAATCCCTGCAGCAGCTG CTGATGTGGCTGGAACACAAACTG GCTCAGGGTCCGCAGGGT (SEQ ID NO: 535)	5 gly	C
mL15–20	EVDSLQQLLMWL DQKLASGPHA	GAAGTTGACTCCCTGCAGCAGCTG CTGATGTGGCTGGACCAGAAACTG GCTTCCGGTCCGCACGCT (SEQ ID NO: 536)	5 gly	C
mL15–21	EVDSLQQLLMWL DQQLASGPQK	GAAGTTGACTCCCTGCAGCAGCTG CTGATGTGGCTGGACCAGCAGCTG GCTTCCGGTCCGCAGAAA (SEQ ID NO: 537)	5 gly	C
mL15–22	GVEQLPQLLMWL EQKLASGPQR	GGTGTTGAACAGCTGCCGCAGCTG CTGATGTGGCTGGAACAGAAACTG GCTTCCGGTCCGCAGCGT (SEQ ID NO: 538)	5 gly	C
mL15–23	GEDSLQQLLMWL DQQLAAGPQV	GGTGAAGACTCCCTGCAGCAGCTG CTGATGTGGCTGGACCAGCAGCTG GCTGCTGGTCCGCAGGTT (SEQ ID NO: 539)	5 gly	C
mL15–24	ADDSLQQLLMW LDRKLASGPHV	GCTGACGACTCCCTGCAGCAGCTG CTGATGTGGCTGGACCGTAAACTG GCTTCCGGTCCGCACGTT (SEQ ID NO: 540)	5 gly	C
mL15–25	PVDSLQQLLIWL DQKLASGPQG	CCGGTTGACTCCCTGCAGCAGCTG CTGATCTGGCTGGACCAGAAACTG GCTTCCGGTCCGCAGGGT (SEQ ID NO: 541)	5 gly	C
mL17–Con2	QSRATLLKEFWQ LVEGLGDKQA	CAGTCCCGTGCTACCCTGCTGAAA GAATTCTGGCAGCTGGTTGAAGGT CTGGGTGACAAACAGGCT (SEQ ID NO: 542)	5 gly	C
mL17–19	EIRATLLKEFWQL VDEWREQPN	GAAATCCGTGCTACCCTGCTGAAA GAATTCTGGCAGCTGGTTGACGAA TGGCGTGAACAGCCGAAC (SEQ ID NO: 543)	5 gly	C
mL17–20	QLRATLLKEFLQL VHGLGETDS	CAGCTGCGTGCTACCCTGCTGAAA GAATTCCTGCAGCTGGTTCACGGTC TGGGTGAAACCGACTCC (SEQ ID NO: 544)	5 gly	C
mL17–21	TQRATLLKEFWQ LIEGLGGKHV	ACCCAGCGTGCTACCCTGCTGAAA GAATTCTGGCAGCTGATCGAAGGT CTGGGTGGTAAACACGTT (SEQ ID NO: 545)	5 gly	C

mL17–22	HYRATLLKEFWQ LVDGLREQGV	CACTACCGTGCTACCCTGCTGAAA GAATTCTGGCAGCTGGTTGACGGT CTGCGTGAACAGGGTGTT (SEQ ID NO: 546)	5 gly	C
mL17–23	QSRVTLLREFWQ LVESYRPIVN	CAGTCCCGTGTTACCCTGCTGCGTG AATTCTGGCAGCTGGTTGAATCCTA CCGTCCGATCGTTAAC (SEQ ID NO: 547)	5 gly	C
mL17–24	LSRATLLNEFWQ FVDGQRDKRM	CTGTCCCGTGCTACCCTGCTGAACG AATTCTGGCAGTTCGTTGACGGTCA GCGTGACAAACGTATG (SEQ ID NO: 548)	5 gly	C
mL17–25	WDRATLLNDFW HLMEELSQKPG	TGGGACCGTGCTACCCTGCTGAAC GACTTCTGGCACCTGATGGAAGAA CTGTCCCAGAAACCGGGT (SEQ ID NO: 549)	5 gly	C
mL17–26	QERATLLKEFWR MVEGLGKNRG	CAGGAACGTGCTACCCTGCTGAAA GAATTCTGGCGTATGGTTGAAGGT CTGGGTAAAAACCGTGGT (SEQ ID NO: 550)	5 gly	C
mL17–27	NERATLLREFWQ LVGGYGVNQR	AACGAACGTGCTACCCTGCTGCGT GAATTCTGGCAGCTGGTTGGTGGTT ACGGTGTTAACCAGCGT (SEQ ID NO: 551)	5 gly	C
mTN8Con6–1	QREWYPCYGGHL WCYDLHKA	CAGCGTGAATGGTACCCGTGCTAC GGTGGTCACCTGTGGTGCTACGAC CTGCACAAAGCT (SEQ ID NO: 552)	5 gly	C
mTN8Con6–2	ISAWYSCYAGHF WCWDLKQK	ATCTCCGCTTGGTACTCCTGCTACG CTGGTCACTTCTGGTGCTGGGACCT GAAACAGAAA (SEQ ID NO: 553)	5 gly	C
mTN8Con6–3	WTGWYQCYGGH LWCYDLRRK	TGGACCGGTTGGTACCAGTGCTAC GGTGGTCACCTGTGGTGCTACGAC CTGCGTCGTAAA (SEQ ID NO: 554)	5 gly	C
mTN8Con6–4	KTFWYPCYDGHF WCYNLKSS	AAAACCTTCTGGTACCCGTGCTAC GACGGTCACTTCTGGTGCTACAAC CTGAAATCCTCC (SEQ ID NO: 545)	5 gly	C
mTN8Con6–5	ESRWYPCYEGHL WCFDLTET	GAATCCCGTTGGTACCCGTGCTAC GAAGGTCACCTGTGGTGCTTCGAC CTGACCGAAACC (SEQ ID NO: 546)	5 gly	C
mL24–1	NVFFQWVQKHG RVVYQWLDINV	AATGTTTTTTTTCAATGGGTTCAAA AACATGGTCGTGTTGTTTATCAATG GCTTGATATTAATGTT (SEQ ID NO: 557)	5 gly	C
mL24–2	FDFLQWLQNHRS EVEHWLVMDV	TTTGATTTTCTTCAATGGCTTCAAA ATCATCGTTCTGAAGTTGAACATTG GCTTGTTATGGATGTT (SEQ ID NO: 558)	5 gly	C
mL20–1	HQRDMSMLWEL LDVLDGLRQYS	CATCAACGTGATATGTCTATGCTTT GGGAACTTCTTGATGTTCTTGATGG TCTTCGTCAATATTCT (SEQ ID NO: 559)	5 gly	C
mL20–2	TQRDMSMLDGLL EVLDQLRQQR	ACTCAACGTGATATGTCTATGCTTG ATGGTCTTCTTGAAGTTCTTGATCA ACTTCGTCAACAACGT (SEQ ID	5 gly	C

		NO: 560)		
mL20–3	TSRDMSLLWELL EELDRLGHQR	ACCTCCCGTGACATGTCCCTGCTGT GGGAACTGCTGGAAGAACTGGACC GTCTGGGTCACCAGCGT (SEQ ID NO: 561)	5 gly	C
mL20–4	MQHDMSMLYGL VELLESLGHQI	ATGCAACATGATATGTCTATGCTTT ATGGTCTTGTTGAACTTCTTGAATC TCTTGGTCATCAAATT (SEQ ID NO: 562)	5 gly	C
mL20–5	WNRDMRMLESL FEVLDGLRQQV	TGGAATCGTGATATGCGTATGCTTG AATCTCTTTTTGAAGTTCTTGATGG TCTTCGTCAACAAGTT (SEQ ID NO: 563)	5 gly	C
mL20–6	GYRDMSMLEGLL AVLDRLGPQL	GGTTATCGTGATATGTCTATGCTTG AAGGTCTTCTTGCTGTTCTTGATCG TCTTGGTCCACAACTT (SEQ ID NO: 564)	5 gly	C
mL20 Con1	TQRDMSMLEGLL EVLDRLGQQR	ACTCAACGTGATATGTCTATGCTTG AAGGTCTTCTTGAAGTTCTTGATCG TCTTGGTCAACAACGT (SEQ ID NO: 565)	5 gly	C
mL20 Con2	WYRDMSMLEGL LEVLDRLGQQR	TGGTACCGTGACATGTCCATGCTG GAAGGTCTGCTGGAAGTTCTGGAC CGTCTGGGTCAGCAGCGT (SEQ ID NO: 566)	5 gly	C
mL21–1	TQNSRQMLLSDF MMLVGSMIQG	ACTCAAAATTCTCGTCAAATGCTTC TTTCTGATTTTATGATGCTTGTTGG TTCTATGATTCAAGGT (SEQ ID NO: 567)	5 gly	C
mL21–2	MQTSRHILLSEFM MLVGSIMHG	ATGCAAACTTCTCGTCATATTCTTC TTTCTGAATTTATGATGCTTGTTGG TTCTATTATGCATGGT (SEQ ID NO: 568)	5 gly	C
mL21–3	HDNSRQMLLSDL LHLVGTMIQG	CACGACAACTCCCGTCAGATGCTG CTGTCCGACCTGCTGCACCTGGTTG GTACCATGATCCAGGGT (SEQ ID NO: 569)	5 gly	C
mL21–4	MENSRQNLLRELI MLVGNMSHQ	ATGGAAAACTCCCGTCAGAACCTG CTGCGTGAACTGATCATGCTGGTTG GTAACATGTCCCACCAG (SEQ ID NO: 570)	5 gly	C
mL21–5	QDTSRHMLLREF MMLVGEMIQG	CAGGACACCTCCCGTCACATGCTG CTGCGTGAATTCATGATGCTGGTTG GTGAAATGATCCAGGGT (SEQ ID NO: 571)	5 gly	C
mL21 Con1	DQNSRQMLLSDL MILVGSMIQG	GACCAGAACTCCCGTCAGATGCTG CTGTCCGACCTGATGATCCTGGTTG GTTCCATGATCCAGGGT (SEQ ID NO: 572)	5 gly	C
mTN8–19–1	VALHGQCTRWP WMCPPQREG	GTTGCTCTTCATGGTCAATGTACTC GTTGGCCATGGATGTGTCCACCAC AACGTGAAGGT (SEQ ID NO: 573)	5 gly	C

mTN8–19–2	YPEQGLCTRWPW MCPPQTLA	TATCCAGAACAAGGTCTTTGTACTC GTTGGCCATGGATGTGTCCACCAC AAACTCTTGCT (SEQ ID N: 574)	5 gly	C
mTN8–19–3	GLNQGHCTRWP WMCPPQDSN	GGTCTGAACCAGGGTCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGACTCCAAC (SEQ ID NO: 575)	5 gly	C
mTN8–19–4	MITQGQCTRWPW MCPPQPSG	ATGATTACTCAAGGTCAATGTACTC GTTGGCCATGGATGTGTCCACCAC AACCATCTGGT (SEQ ID NO: 576)	5 gly	C
mTN8–19–5	AGAQEHCTRWP WMCAPNDWI	GCTGGTGCTCAGGAACACTGCACC CGTTGGCCGTGGATGTGCGCTCCG AACGACTGGATC (SEQ ID NO: 577)	5 gly	C
mTN8–19–6	GVNQGQCTRWR WMCPPNGWE	GGTGTTAACCAGGGTCAGTGCACC CGTTGGCGTTGGATGTGCCCGCCG AACGGTTGGGAA (SEQ ID NO: 578)	5 gly	C
mTN8–19–7	LADHGQCIRWPW MCPPEGWE	CTGGCTGACCACGGTCAGTGCATC CGTTGGCCGTGGATGTGCCCGCCG GAAGGTTGGGAA (SEQ ID NO: 579)	5 gly	C
mTN8–19–8	ILEQAQCTRWPW MCPPQRGG	ATCCTGGAACAGGCTCAGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGCGTGGTGGT (SEQ ID NO: 580)	5 gly	C
mTN8–19–9	TQTHAQCTRWP WMCPPQWEG	ACTCAAACTCATGCTCAATGTACTC GTTGGCCATGGATGTGTCCACCAC AATGGGAAGGT (SEQ ID NO: 581)	5 gly	C
mTN8–19–10	VVTQGHCTLWP WMCPPQRWR	GTTGTTACTCAAGGTCATTGTACTC TTTGGCCATGGATGTGTCCACCACA ACGTTGGCGT (SEQ ID NO: 582)	5 gly	C
mTN8–19–11	IYPHDQCTRWPW MCPPQPYP	ATTTATCCACATGATCAATGTACTC GTTGGCCATGGATGTGTCCACCAC AACCATATCCA (SEQ ID NO: 583)	5 gly	C
mTN8–19–12	SYWQGQCTRWP WMCPPQWRG	TCTTATTGGCAAGGTCAATGTACTC GTTGGCCATGGATGTGTCCACCAC AATGGCGTGGT (SEQ ID NO: 584)	5 gly	C
mTN8–19–13	MWQQGHCTRWP WMCPPQGWG	ATGTGGCAACAAGGTCATTGTACT CGTTGGCCATGGATGTGTCCACCA CAAGGTTGGGGT (SEQ ID NO: 585)	5 gly	C
mTN8–19–14	EFTQWHCTRWP WMCPPQRSQ	GAATTCACCCAGTGGCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGCGTTCCCAG (SEQ ID NO: 586)	5 gly	C
mTN8–19–15	LDDQWQCTRWP WMCPPQGFS	CTGGACGACCAGTGGCAGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGGTTTCTCC (SEQ ID NO: 587)	5 gly	C
mTN8–19–16	YQTQGLCTRWP WMCPPQSQR	TATCAAACTCAAGGTCTTTGTACTC GTTGGCCATGGATGTGTCCACCAC AATCTCAACGT (SEQ ID NO: 588)	5 gly	C
mTN8–19–17	ESNQGQCTRWP WMCPPQGGW	GAATCTAATCAAGGTCAATGTACT CGTTGGCCATGGATGTGTCCACCA CAAGGTGGTTGG (SEQ ID NO: 589)	5 gly	C

mTN8–19–18	WTDRGPCTRWP WMCPPQANG	TGGACCGACCGTGGTCCGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGCTAACGGT (SEQ ID NO: 590)	5 gly	C
mTN8–19–19	VGTQGQCTRWP WMCPPYETG	GTTGGTACCCAGGGTCAGTGCACC CGTTGGCCGTGGATGTGCCCGCCG TACGAAACCGGT (SEQ ID NO: 591)	5 gly	C
mTN8–19–20	PYEQGKCTRWP WMCPPYEVE	CCGTACGAACAGGGTAAATGCACC CGTTGGCCGTGGATGTGCCCGCCG TACGAAGTTGAA (SEQ ID NO: 592)	5 gly	C
mTN8–19–21	SEYQGLCTRWPW MCPPQGWK	TCCGAATACCAGGGTCTGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGGTTGGAAA (SEQ ID NO: 593)	5 gly	C
mTN8–19–22	TFSQGHCTRWPW MCPPQGWG	ACCTTCTCCCAGGGTCACTGCACCC GTTGGCCGTGGATGTGCCCGCCGC AGGGTTGGGGT (SEQ ID NO: 594)	5 gly	C
mTN8–19–23	PGAHDHCTRWP WMCPPQSRY	CCGGGTGCTCACGACCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGTCCCGTTAC (SEQ ID NO: 595)	5 gly	C
mTN8–19–24	VAEEWHCRRWP WMCPPQDWR	GTTGCTGAAGAATGGCACTGCCGT CGTTGGCCGTGGATGTGCCCGCCG CAGGACTGGCGT (SEQ ID NO: 596)	5 gly	C
mTN8–19–25	VGTQGHCTRWP WMCPPQPAG	GTTGGTACCCAGGGTCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGCCGGCTGGT (SEQ ID NO: 597)	5 gly	C
mTN8–19–26	EEDQAHCRSWP WMCPPQGWV	GAAGAAGACCAGGCTCACTGCCGT TCCTGGCCGTGGATGTGCCCGCCG CAGGGTTGGGTT (SEQ ID NO: 598)	5 gly	C
mTN8–19–27	ADTQGHCTRWP WMCPPQHWF	GCTGACACCCAGGGTCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGCACTGGTTC (SEQ ID NO: 599)	5 gly	C
mTN8–19–28	SGPQGHCTRWPW MCAPQGWF	TCCGGTCCGCAGGGTCACTGCACC CGTTGGCCGTGGATGTGCGCTCCG CAGGGTTGGTTC (SEQ ID NO: 600)	5 gly	C
mTN8–19–29	TLVQGHCTRWP WMCPPQRWV	ACCCTGGTTCAGGGTCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGCGTTGGGTT (SEQ ID NO: 601)	5 gly	C
mTN8–19–30	GMAHGKCTRWA WMCPPQSWK	GGTATGGCTCACGGTAAATGCACC CGTTGGGCTTGGATGTGCCCGCCG CAGTCCTGGAAA (SEQ ID NO: 602)	5 gly	C
mTN8–19–31	ELYHGQCTRWP WMCPPQSWA	GAACTGTACCACGGTCAGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGTCCTGGGCT (SEQ ID NO: 603)	5 gly	C
mTN8–19–32	VADHGHCTRWP WMCPPQGWG	GTTGCTGACCACGGTCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGGTTGGGGT (SEQ ID NO: 604	5 gly	C
mTN8–19–33	PESQGHCTRWPW MCPPQGWG	CCGGAATCCCAGGGTCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGGTTGGGGT (SEQ ID NO: 605)	5 gly	C

mTN8–19–34	IPAHGHCTRWPW MCPPQRWR	ATCCCGGCTCACGGTCACTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGCGTTGGCGT (SEQ ID NO: 606)	5 gly	C
mTN8–19–35	FTVHGHCTRWP WMCPPYGWV	TTCACCGTTCACGGTCACTGCACCC GTTGGCCGTGGATGTGCCCGCCGT ACGGTTGGGTT (SEQ ID NO: 607)	5 gly	C
mTN8–19–36	PDFPGHCTRWRW MCPPQGWE	CCAGATTTTCCAGGTCATTGTACTC GTTGGCGTTGGATGTGTCCACCAC AAGGTTGGGAA (SEQ ID NO: 608)	5 gly	C
mTN8–19–37	QLWQGPCTQWP WMCPPKGRY	CAGCTGTGGCAGGGTCCGTGCACC CAGTGGCCGTGGATGTGCCCGCCG AAAGGTCGTTAC (SEQ ID NO: 609)	5 gly	C
mTN8–19–38	HANDGHCTRWQ WMCPPQWGG	CACGCTAACGACGGTCACTGCACC CGTTGGCAGTGGATGTGCCCGCCG CAGTGGGGTGGT (SEQ ID NO: 610)	5 gly	C
mTN8–19–39	ETDHGLCTRWPW MCPPYGAR	GAAACCGACCACGGTCTGTGCACC CGTTGGCCGTGGATGTGCCCGCCG TACGGTGCTCGT (SEQ ID NO: 611)	5 gly	C
mTN8–19–40	GTWQGLCTRWP WMCPPQGWQ	GGTACCTGGCAGGGTCTGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGGTTGGCAG (SEQ ID NO: 612)	5 gly	C
mTN8–19 Con1	VATQGQCTRWP WMCPPQGWG	GTTGCTACCCAGGGTCAGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGGGTTGGGGT (SEQ ID NO: 613)	5 gly	C
mTN8–19 Con2	VATQGQCTRWP WMCPPQRWG	GTTGCTACCCAGGGTCAGTGCACC CGTTGGCCGTGGATGTGCCCGCCG CAGCGTTGGGGT (SEQ ID NO: 614)	5 gly	C
2X mTN8–19–7	FC–5G–AQ–LADHGQCIRWPW MCPPEGWELEGS GSATGGSGSTASS GSGSATGLADHG QCIRWPWMCPPE GWE–LE (SEQ ID NO: 615)	CTTGCTGATCATGGTCAATGTATTC GTTGGCCATGGATGTGTCCACCAG AAGGTTGGGAACTCGAGGGTTCCG GTTCCGCTACCGGCGGCTCTGGCTC CACTGCTTCTTCCGGTTCCGGTTCT GCTACTGGTCTGGCTGACCACGGT CAGTGCATCCGTTGGCCGTGGATG TGCCCGCCGGAAGGTTGGGAACTG GAA (SEQ ID NO: 616)	1K	C
2X mTN8–19–7 ST—GG del2x LE	FC–5G–AQ–LADHGQCIRWPW MCPPEGWEGSGS ATGGSGGGASSG SGSATGLADHGQ CIRWPWMCPPEG WE (SEQ ID NO: 617)	CTTGCTGATCATGGTCAATGTATTC GTTGGCCATGGATGTGTCCACCAG AAGGTTGGGAAGGTTCCGGTTCCG CTACCGGCGGCTCTGGCGGTGGCG CTTCTTCCGGTTCCGGTTCTGCTAC TGGTCTGGCTGACCACGGTCAGTG CATCCGTTGGCCGTGGATGTGTCCA CCAGAAGGTTGGGAA (SEQ ID NO: 618)	1K	C

2X mTN8–19–21	FC–5G–AQ–SEYQGLCTRWPWMCPPQGWKLEGSGSATGGSGSTASSGSGSATGSEYQGLCTRWPWMCPPQGWK –LE (SEQ ID NO: 619)	TCTGAATATCAAGGTCTTTGTACTCGTTGGCCATGGATGTGTCCACCACAAGGTTGGAAACTCGAGGGTTCCGGTTCCGCTACCGGCGGCTCTGGCTCCACTGCTTCTTCCGGTTCCGGTTCTGCTACTGGTTCTGAGTATCAAGGCCTCTGTACTCGCTGGCCATGGATGTGTCCACCACAAGGCTGGAAGCTGGAA (SEQ ID NO: 620)	1K	C
2X mTN8–19–21 ST—GG del2x LE	FC–5G–AQ–SEYQGLCTRWPWMCPPQGWKGSGSATGGSGGGASSGSGSATGSEYQGLCTRWPWMCPPQGWK (SEQ ID NO: 621)	TCTGAATATCAAGGTCTTTGTACTCGTTGGCCATGGATGTGTCCACCACAAGGTTGGAAAGGTTCCGGTTCCGCTACCGGCGGCTCTGGCGGTGGCGCTTCTTCCGGTTCCGGTTCTGCTACTGGTTCTGAGTATCAAGGCCTCTGTACTCGCTGGCCATGGATGTGTCCACCACAAGGTTGGAAA (SEQ ID NO: 622)	1K	C
2X mTN8–19–22	FC–5G–AQ–TFSQGHCTRWPWMCPPQGWGLEGSGSATGGSGSTASSGSGSATGTFSQGHCTRWPWMCPPQGWG –L E (SEQ ID NO: 623)	ACTTTTTCTCAAGGTCATTGTACTCGTTGGCCATGGATGTGTCCACCACAAGGTTGGGGTCTCGAGGGTTCCGGTTCCGCTACCGGCGGCTCTGGCTCCACTGCTTCTTCCGGTTCCGGTTCTGCTACTGGTACTTTTTCTCAAGGCCATTGTACTCGCTGGCCATGGATGTGTCCACCACAAGGCTGGGGCCTGGAA (SEQ ID NO: 624)	1K	C
2X mTN8–19–32	FC–5G–AQ–VADHGHCTRWPWMCPPQGWGLEGSGSATGGSGSTASSGSGSATGVADHGHCTRWPWMCPPQGWG–LE (SEQ ID NO: 625)	GTTGCTGATCATGGTCATTGTACTCGTTGGCCATGGATGTGTCCACCACAAGGTTGGGGTCTCGAGGGTTCCGGTTCCGCAACCGGCGGCTCTGGCTCCACTGCTTCTTCCGGTTCCGGTTCTGCTACTGGTGTTGCTGACCACGGTCACTGCACCCGTTGGCCGTGGATGTGCCCGCCGCAGGGTTGGGGTCTGGAA (SEQ ID NO: 626)	1K	C
2X mTN8–19–32 ST—GG del2x LE	FC–5G–AQ–VADHGHCTRWPWMCPPQGWGGSGSATGGSGGGASSGSGSATGVADHGHCTRWPWVCPPQGWG (SEQ ID NO: 627)	GTTGCTGATCATGGTCATTGTACTCGTTGGCCATGGATGTGTCCACCACAAGGTTGGGGTGGTTCCGGTTCCGCTACCGGCGGCTCTGGCGGTGGTGCTTCTTCCGGTTCCGGTTCTGCTACTGGTGTTGCTGACCACGGTCACTGCACCCGTTGGCCGTGGGTGTGTCCACCACAAGGTTGGGGT (SEQ ID NO: 628)	1K	C

2X mTN8–19–33	FC–5G–AQ–PESQGHCTRWPWMCPPQGWGLEGSGSATGGSGSTASSGSGSATGPESQGHCTRWPWMCPPQGWGLE (SEQ ID NO: 629)	CCAGAATCTCAAGGTCATTGTACTCGTTGGCCATGGATGTGTCCACCACAAGGTTGGGGTCTCGAGGGTTCCGGTTCCGCTACCGGCGGCTCTGGCTCCACTGCTTCTTCCGGTTCCGGTTCTGCTACTGGTCCGGAATCCCAGGGTCACTGCACCCGTTGGCCGTGGATGTGCCCGCCGCAGGGTTGGGGTCTGGAA (SEQ ID NO: 630)	1K	C
2X mTN8–19–33 ST—GG del2x LE	FC–5G–AQ–PESQGHCTRWPWMCPPQGWGGSGSATGGSGGGASSGSGSATGPESQGHCTRWPWMCPPQGWG (SEQ ID NO: 631)	CCAGAATCTCAAGGTCATTGTACTCGTTGGCCATGGATGTGTCCACCACAAGGTTGGGGTGGTTCCGGTTCCGCTACCGGCGGCTCTGGCGGTGGTGCTTCTTCCGGTTCCGGTTCTGCTACTGGTCCGGAATCCCAGGGTCACTGCACCCGTTGGCCGTGGATGTGTCCACCACAAGGTTGGGGT (SEQ ID NO: 632)	1K	C

Example 7

In vitro screening of affinity matured peptibodies

The following exemplary peptibodies were screened according to the protocols set forth above to obtain the following K_D and IC_{50} values. Table VII shows the range of K_D values for selected affinity matured peptibodies compared with the parent peptibodies, as determined by KinExA™ solution based assays or BIAcore® assays. These values demonstrate increased binding affinity of the affinity matured peptibodies for myostatin compared with the parent peptibodies. Table VIII shows IC_{50} values for a number of affinity matured peptibodies. A range of values is given in this table.

TABLE VII

peptibodies	K_D
TN8-19 (parent)	> 1 nM
2xmTN8-19 (parent)	> 1 nM
1x mTN8-19-7	10 pM
2x mTN8-19-7	12 pM
1x mTN8-19-21	6 pM
2x mTN8-19-21	6 pM
1x mTN8-19-32	9 pM
1x mTN8-19-33	21 pM
2x mTN8-19-33	3 pM
1x mTN8-19-22	4 pM
1x mTN8-19-con1	20 pM

TABLE VIII

Affinity Matured Peptibody	IC_{50} (nM)
mTN8-19 Con1	1.0 – 4.4
mTN8-19-2	7.508-34.39
mTN8-19-4	16.74
mTN8-19-5	7.743 – 3.495
mTN8-19-6	17.26
mTN8-19-7	1.778
mTN8-19-9	22.96-18.77
mTN8-19-10	5.252 - 7.4
mTN8-19-11	28.66
mTN8-19-12	980.4
mTN8-19-13	20.04
mTN8-19-14	4.065 – 6.556
mTN8-19-16	4.654
mTN8-19-21	2.767-3.602
mTN8-19-22	1.927-3.258
mTN8-19-23	6.584
mTN8-19-24	1.673-2.927
mTN8-19-27	4.837-4.925
mTN8-19-28	4.387
mTN8-19-29	6.358
mTN8-19-32	1.842-3.348
mTN8-19-33	2.146-2.745
mTN8-19-34	5.028 – 5.069
mTN8Con6-3	86.81
mTN8Con6-5	2385
mTN8-19-7(-LE)	1.75-2.677
mTN8-19-21(-LE)	2.49
mTN8-19-33(-LE)	1.808
2xmTN8-19-7	0.8572 -2.649
2xmTN8-19-9	1.316-1.228
2xmTN8-19-14	1.18-1.322
2xmTN8-19-16	0.9903 -1.451
2xmTN8-19-21	0.828 -1.434
2xmTN8-19-22	0.9937-1.22
2xmTN8-19-27	1.601-3.931
2xmTN8-19-7(-LE)	1.077-1.219
2xmTN8-19-21(-LE)	0.8827-1.254
2xmTN8-19-33(-LE)	1.12-1.033
mL2-7	90.24
mL2-9	105.5
mL15-7	32.75
mL15-9	354.2
mL20-2	122.6
mL20-3	157.9
mL20-4	160

Example 8

In vivo Anabolic Activity of Exemplary Peptibodies

The CD1 nu/nu mouse model (Charles River Laboratories, Massachusettes) was used to determine the *in vivo* efficacy of the peptibodies of the present invention which included the human Fc region (huFc). This model responded to the inhibitors of the present invention with a rapid anabolic response which was associated with increased dry muscle mass and an increase in myofibrillar proteins but was not associated with accumulation in body water content.

In one example, the efficacy of 1x peptibody mTN8-19-21 *in vivo* was demonstrated by the following experiment. A group of 10 8 week old CD1 nu/nu mice were treated twice weekly or once weekly with dosages of 1mg/kg, 3 mg/kg and 10 mg/kg (subcutaneous injection). The control group of 10 8 week old CD1 nu/nu mice received a twice weekly (subcutaneous) injection of huFc (vehicle) at 10 mg/kg. The animals were weighed every other day and lean body mass determined by NMR on day 0 and day 13. The animals are then sacrified at day 14 and the size of the gastrocnemius muscle determined. The results are shown in Figures 2 and 3. Figure 2 shows the increase in total body weight of the mice over 14 days for the various dosages of peptibody compared with the control. As can be seen from Figure 2 all of the dosages have show an increase in body weight compared with the control, with all of the dosages showing statistically significant increases over the control by day 14. Figure 3 shows the change in lean body mass on day 0 and day 13 as determined by NMR, as well as the change in weight of the gastrocnemius muscle dissected from the animals at day 14.

In another example, the 1x mTN8-19-32 peptibody was administered to CD1 nu/nu mice in a biweekly injection of 1 mg/kg, 3 mg/kg, 10 mg/kg, and 30 mg/kg compared with the huFc control (vehicle). The peptibody- treated animals show an increase in total body weight (not shown) as well as lean body mass on day 13 compared with day 0 as determined by NMR measurement. The increase in lean body mass is shown in Figure 4.

In another example, a 1x affinity-matured peptibody was compared with a 2x affinity-matured peptibody for *in vivo* anabolic efficacy. CD1 nu/nu male mice (10 animals per group) were treated with twice weekly injections of 1 mg/kg and 3 mg/kg of 1x mTN8-19-7 and 2x mTN8-19-7 for 35 days, while the control group (10 animals) received twice weekly injections of huFc (3 mg/kg). As shown in Figure 5, treatment with the 2x peptibody resulted in a greater body weight gain and leans carcass weight at necropsy compared with the 1x peptibody or control.

Example 9

Increase in muscular strength

Normal age-matched male 4 month old male C57B/6 mice were treated for 30 days with 2 injections per week subcutaneous injections 5 mg/kg per week of 2x mTN8-19-33, 2x mTN8-19-7, and huFc vehicle control group (10 animals/group). The animals were allowed to recover without any further injections. Gripping strength was measured on day 18 of the recovery period. Griping strength was measured using a Columbia Instruments meter, model 1027 dsm (Columbus, Ohio). Peptibody treatment resulted in significant increase in gripping strength, with 2x mTN8-19-33 pretreated animals showing a 14 % increase in gripping strength compared with the control-treated mice, while 2x mTN8-19-7 showed a 15% increase in gripping strength compared with the control treated mice.

Example 10

Pharmacokinetics

In vivo phamacokinetics experiments were performed using representative peptibodies without the LE sequences. 10 mg/kg and 5mg/kg dosages were administered to CD1 nu/nu mice and the following parameters determined: Cmax (ug/mL), area under the curve (AUC) (ug-hr/mL), and half-life (hr). It was found that the 2x versions of the affinity matured peptibodies have a significantly longer half-life than the 1x versions. For example 1x affinity matured mTN8-19-22 has a half-life in the animals of about 50.2 hours, whereas 2x mTN8-19-22 has a half- life of about 85.2 hours. Affinity matured 1x mTN8-7 has a half-life of about 65 hours, whereas 2x mTN8-19-7 has a half-life of about 106 hours.

Example 11

Treatment of *mdx* Mice

The peptibodies of the present invention have been shown to increase lean muscle mass in an animal and are useful for the treatment of a variety of disorders which involve muscle wasting. Muscular dystrophy is one of those disorders. The mouse model for Duchenne's muscular dystrophy is the Duchenne *mdx* mouse (Jackson Laboratories, Bar Harbor, Maine). Aged (10 month old) *mdx* mice were injected with either the peptibody 1x mTN8-19-33 (n=8/group) or with the vehicle huFc protein (N=6/group) for a three month period of time. The dosing schedule was every other day, 10 mg/kg, by subcutaneous injection. The peptibody treatment had a positive effect on increasing and maintaining body mass for the aged *mdx* mice. Significant increases in body weight were observed in the peptibody-treated group compared to the hu-Fc-treated control group, as shown in Figure 6A. In addition, NMR analysis revealed that the lean body mass to fat

mass ratio was also significantly increased in the aged *mdx* mice as a result of the peptibody treatment compared with the control group, and that the fat percentage of body weight decreased in the peptibody treated mice compared with the control group, as shown in Figure 6B.

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The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those
10 skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

CLAIMS

What is claimed is:

1. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the amino acid sequence WMCPP (SEQ ID NO: 633), and physiologically acceptable salts thereof.

2. The binding agent of claim 1, wherein the peptide is between 5 and 50 amino acids in length.

3. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the amino acid sequence $\underline{C}a_1a_2\underline{W}a_3\underline{WMCPP}$ (SEQ ID NO: 352), wherein a_1, a_2 and a_3 are selected from a neutral hydrophobic, neutral polar, or basic amino acid, and wherein the peptide is between 10 and 50 amino acids in length, and physiologically acceptable salts thereof.

4. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the sequence $\underline{C}b_1b_2\underline{W}b_3\underline{WMCPP}$ (SEQ ID NO: 353), wherein

b_1 is selected from any one of the amino acids T, I, or R;
b_2 is selected from any one of R, S, Q;
b_3 is selected from any one of P, R and Q,
and wherein the peptide is beween 10 and 50 amino acids in length, and physiologically acceptable salts thereof.

5. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the sequence $c_1c_2c_3c_4c_5c_6\underline{C}c_7c_8\underline{W}c_9\underline{WMCPP}c_{10}c_{11}c_{12}c_{13}$ (SEQ ID NO: 354), wherein:

c_1 is absent or any amino acid;
c_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_4 is absent or any amino acid;
c_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
c_7 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_8 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_9 is a neutral hydrophobic, neutral polar or basic amino acid; and
c_{10} to c_{13} is any amino acid; and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof.

6. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the sequence

$d_1d_2d_3d_4d_5d_6Cd_7d_8Wd_9WMCPP\ d_{10}d_{11}d_{12}d_{13}$ (SEQ ID NO: 355), wherein

d_1 is absent or any amino acid;
d_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_4 is absent or any amino acid;
d_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
d_7 is selected from any one of the amino acids T, I, or R;
d_8 is selected from any one of R, S, Q;
d_9 is selected from any one of P, R and Q, and
d_{10} to d_{13} is selected from any amino acid,

and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof.

7. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the sequence $WYe_1e_2Ye_3G$, (SEQ ID NO: 356)

wherein e_1 is P, S or Y,
e_2 is C or Q, and
e_3 is G or H, and wherein the peptide is between 7 and 50 amino acids in length, and physiologically acceptable salts thereof.

8. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the sequence $f_1EMLf_2SLf_3f_4LL$, (SEQ ID NO: 455),
wherein f_1 is M or I,

f_2 is any amino acid,
f_3 is L or F,
f_4 is E, Q or D;
and wherein the peptide is between 7 and 50 amino acids in length, and physiologically acceptable salts thereof.

9. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the sequence $Lg_1g_2LLg_3g_4L$, (SEQ ID NO: 456), wherein

g_1 is Q, D or E,
g_2 is S, Q, D or E,
g_3 is any amino acid,
g_4 is L, W, F, or Y, and wherein the peptide is between 8 and 50 amino acids in length, and physiologically acceptable salts thereof.

10. A binding agent comprising at least one peptide capable of binding myostatin, wherein the peptide comprises the sequence $h_1h_2h_3h_4h_5h_6h_7h_8h_9$ (SEQ ID NO: 457), wherein

h_1 is R or D,
h_2 is any amino acid,
h_3 is A, T S or Q,
h_4 is L or M,
h_5 is L or S,
h_6 is any amino acid,
h_7 is F or E,
h_8 is W, F or C,
h_9 is L, F, M or K, and wherein the peptide is between 9 and 50 amino acids in length, and physiologically acceptable salts thereof.

11. A binding agent wherein said agent has the structure:

$(X^1)_a$-F^1-$(X^2)_b$, or multimers thereof;

wherein F^1 is a vehicle; and X^1 and X^2 are each independently selected from

$-(L^1)_c-P^1$;

$-(L^1)_c-P^1-(L^2)_d-P^2$;

$-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$;

and $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$;

wherein P^1, P^2, P^3, and P^4 are peptides capable of binding myostatin, and each independently comprise the amino acid sequence WMCPP (SEQ ID NO: 633);

wherein L^1, L^2, L^3, and L^4 are each linkers;

and a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1, and physiologically acceptable salts thereof.

12. The binding agent of claim 11 wherein one or more of the myostatin binding peptides comprise the amino acid sequence $\underline{C}a_1a_2\underline{W}a_3\underline{WMCPP}$ (SEQ ID NO: 352), wherein a_1, a_2 and a_3 are selected from a neutral hydrophobic, neutral polar, or basic amino acid, and wherein the peptide is between 10 and 50 amino acids in length, and physiologically acceptable salts thereof.

13. The binding agent of claim 11, wherein one or more of the myostatin binding peptides each independently comprise the amino acid sequence $\underline{C}b_1b_2\underline{W}b_3\underline{WMCPP}$ (SEQ ID NO: 353), wherein

b_1 is selected from any one of the amino acids T, I, or R;
b_2 is selected from any one of R, S, Q;
b_3 is selected from any one of P, R and Q,

and wherein the peptide is beween 10 and 50 amino acids in length, and physiologically acceptable salts thereof.

14. The binding agent of claim 11, wherein one or more of the myostatin binding peptides each independently comprise the sequence $c_1c_2c_3c_4c_5c_6\underline{C}c_7c_8\underline{W}c_9\underline{WMCPP}c_{10}c_{11}c_{12}c_{13}$ (SEQ ID NO: 354), wherein:

c_1 is absent or any amino acid;
c_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_4 is absent or any amino acid;
c_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
c_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
c_7 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_8 is a neutral hydrophobic, neutral polar, or basic amino acid;
c_9 is a neutral hydrophobic, neutral polar or basic amino acid; and
c_{10} to c_{13} is any amino acid; and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof..

15. The binding agent of claim 11, wherein one or more of the myostatin binding peptides each independently comprise the sequence $d_1d_2d_3d_4d_5d_6\underline{C}d_7d_8\underline{W}d_9\underline{WMCPP}$ $d_{10}d_{11}d_{12}d_{13}$ (SEQ ID NO: 355), wherein

d_1 is absent or any amino acid;
d_2 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_3 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_4 is absent or any amino acid;
d_5 is absent or a neutral hydrophobic, neutral polar, or acidic amino acid;
d_6 is absent or a neutral hydrophobic, neutral polar, or basic amino acid;
d_7 is selected from any one of the amino acids T, I, or R;
d_8 is selected from any one of R, S, Q;
d_9 is selected from any one of P, R and Q, and
d_{10} to d_{13} is selected from any amino acid,
and wherein the peptide is between 20 and 50 amino acids in length, and physiologically acceptable salts thereof.

16. A binding agent wherein said agent has the structure:

$(X^1)_a$-F^1-$(X^2)_b$, or multimers thereof;

wherein F^1 is a vehicle; and X^1 and X^2 are each independently selected from

$-(L^1)_c-P^1$;

$-(L^1)_c-P^1-(L^2)_d-P^2$;

$-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$;

and $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$;

wherein P^1, P^2, P^3, and P^4 are peptides capable of binding myostatin, and wherein one or more of P^1, P^2, P^3, and P^4 each independently are selected from the group consisting of :

(a) a peptide comprising the amino acid sequence $\underline{WY}e_1e_2\underline{Y}e_3\underline{G}$, (SEQ ID NO: 356), wherein e_1 is P, S or Y, e_2 is C or Q, and e_3 is G or H;

(b) a peptide comprising the amino acid sequence $f_1\underline{EMLf_2SL}f_3f_4\underline{LL}$, (SEQ ID NO: 455), wherein f_1 is M or I, f_2 is any amino acid, f_3 is L or F, and f_4 is E, Q or D;

(c) a peptide comprising the amino acid sequence $\underline{L}g_1g_2\underline{LL}g_3g_4\underline{L}$, (SEQ ID NO: 456), wherein g_1 is Q, D or E, g_2 is S, Q, D or E, g_3 is any amino acid, and g_4 is L, W, F, or Y; and

(d) a peptide comprising the amino acid sequence $h_1h_2h_3h_4h_5h_6h_7h_8h_9$ (SEQ ID NO: 457), wherein h_1 is R or D, h_2 is any amino acid, h_3 is A, T S or Q, h_4 is L or M, h_5 is L or S, h_6 is any amino acid, h_7 is F or E, h_8 is W, F or C, and h_9 is L, F, M or K;

wherein the peptide is between 9 and 50 amino acids in length,

wherein L^1, L^2, L^3, and L^4 are each linkers;

and a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1, and physiologically acceptable salts thereof.

17. A binding agent wherein said agent has the structure:

$(X^1)_a-F^1-(X^2)_b$, or multimers thereof;

wherein F^1 is a vehicle; and X^1 and X^2 are each independently selected from

$-(L^1)_c-P^1$;

$-(L^1)_c-P^1-(L^2)_d-P^2$;

$-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3$;

and $-(L^1)_c-P^1-(L^2)_d-P^2-(L^3)_e-P^3-(L^4)_f-P^4$;

wherein P^1, P^2, P^3, and P^4 are peptides capable of binding myostatin, and are independently selected from SEQ ID NO: 305 through 351 and SEQ ID NO: 357 through 454;

wherein L^1, L^2, L^3, and L^4 are each linkers;

and a, b, c, d, e, and f are each independently 0 or 1, provided that at least one of a and b is 1, and physiologically acceptable salts thereof.

18. The binding agent of any one of claims 11 through 17 , wherein the vehicle is an Fc domain.

19. A polynucleotide sequence encoding the binding agent of claim 18.

20. An expression vector comprising the polynucleotide of claim 19.

21. A host cell comprising the expression vector of claim 20.

22. The host cell of claim 21, wherein the cell is a procaryotic cell.

23. The host cell of claim 21, wherein the cell is a eucaryotic cell.

24. A pharmaceutical composition comprising an effective amount of the binding agent of any one of claims 1 or 11 in admixture with a pharmaceutically acceptable carrier thereof.

25. The pharmaceutical composition of claim 24, wherein the vehicle is an Fc domain.

26. A method of inhibiting myostatin activity in a subject comprising administering an effective amount of the binding agent of any one of claims 1, 3 or 11 to the subject.

27. A method of increasing lean muscle mass in a subject comprising administering the composition of claim 24 to the subject.

28. The method of claim 27, wherein the subject is a food animal.

29. A method of increasing the ratio of lean muscle mass to fat in a subject comprising administering a therapeutically effective amount of the composition of claim 24 to the subject.

30. A method of treating a muscle-wasting disease in a subject comprising administering a therapeutically effective amount of the composition of claim 24 to the subject.

31. The method of claim 30, wherein the disease is selected from muscular dystrophy, amyotrophic lateral sclerosis, congestive obstructive pulmonary disease, chronic heart failure, cancer, AIDs, renal failure, uremia, rheumatoid arthritis, age-related sarcopenia, and muscle-wasting due to prolonged bedrest, spinal chord injury, stroke, bone fracture, and aging.

32. A method of treating a myostatin-related metabolic disorder in a subject comprising administering a therapeutically effective amount of the composition of claim 24 to the subject.

33. The method of claim 32 wherein the metabolic disorder is selected from diabetes, obesity, hyperglycemia, and bone loss.

34. A method of detecting myostatin in a sample comprising contacting the sample with a binding agent of claims 1 or 11, and detecting the bound complex.

35. A method of measuring myostatin in a sample comprising contacting the sample with a binding agent of claims 1 or 11, and measuring the bound complex.

36. A method of diagnosing a myostatin related disorder in a subject comprising contacting a sample taken from the subject with a binding agent of claims 1 or 11, and detecting the bound complex.