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(54) Title: LEFTY, LEFTY DERIVATIVES AND USES THEROF

(57) Abstract: The disclosure relates to Lefty derivatives and the uses of Lefty polypeptides as antagonists of the function of certain ligands such as Nodal, GDF-8 (Myostatin), and GDF-11. These derivatives may be fused to other functional heterologous proteins such as IgG, especially the Fc portion of IgG. Accrording to the disclosure, Lefty polypeptides are useful in the treatment of a variety of disorders, including, for example, neuronal diseases, muscle and bone conditions, and metabolic disorders.

LEFTY, LEFTY DERIVATIVES AND USES THEREOF

BACKGROUND OF THE INVENTION

Myostatin, or growth/differentiation factor 8 (GDF-8), belongs to the transforming growth factor-β (TGF-β) superfamily (McPherron et al., Nature 387:83-90 (1997)). The human myostatin gene has been cloned (Nestor et al. Proc. Natl. Acad. Sci. 95:14938-43 (1998)). Myostatin is present in human skeletal muscle in both type 1 and type 2 fibers. Myostatin negatively regulates the growth and development of skeletal muscle (Nestor et al., supra).

Myostatin knock-out mice are significantly larger than wild-type mice and have a large and widespread increase in skeletal muscle mass (McPherron et al., Nature 387:83-90 (1997). Two breeds of cattle, characterized by increased muscle mass, have mutations in the myostatin coding sequence (McPherron et al., Proc. Natl. Acad. Sci. 94:12457-61 (1997)). The serum and intramuscular concentrations of immunoreactive myostatin are increased in HIV-infected men with muscle wasting compared with healthy men, and correlate inversely with the fat-free mass index (Nestor et al., supra). Recently, a human child with an apparent loss-of-function mutation in myostatin was identified (Schuelke et al., N Engl J Med. 2004 Jun 24;350(26):2682-8). The infant had a marked increase in skeletal muscle mass. Taken together, these data provide genetic and physiological evidence that myostatin is a negative regulator of skeletal muscle growth in humans and contributes to muscle wasting in HIV-infected men.

In view of the above findings, a need exists for a manner of regulating myostatin activity, particularly in individuals who experience muscle wasting as a result of a condition or disease state such as, for example, aging, Autoimmune Deficiency Syndrome (AIDS), Multiple Sclerosis, and cancer. Additionally, GDF-11 is a protein that is closely related to myostatin and participates in neurological functions. Thus, regulators of myostatin are likely to find use in the regulation of GDF-11 and neurological processes as well.

The present invention provides methods and compositions which may be

utilized to help individuals with a variety of conditions associated with signaling mediated by TGF-β family members, including myostatin and GDF-11.

SUMMARY OF THE INVENTION

The disclosure provides new uses for Lefty polypeptides and provides a variety of Lefty derivative polypeptides. In part, the disclosure provides methods for using Lefty polypeptides as antagonists of myostatin and/or GDF-11. Accordingly, the disclosure provides methods for using Lefty polypeptides to treat a host of disorders related muscle or neurological function. In part, the disclosure provides regions of Lefty polypeptides that are functionally significant for the inhibition of Nodal, myostatin and/or GDF-11, and regions that may be readily modified without substantially affecting the inhibition of Nodal, myostatin and/or GDF-11. Therefore, the disclosure provides Lefty derivative polypeptides that retain Nodal, myostatin and/or GDF-11 antagonist function. Lefty derivative polypeptides may also exhibit desirable features such as improved solublitity or improved pharmacokinetics.

In certain aspects, the disclosure provides recombinant Lefty derivative polypeptides. Lefty derivative polypeptides are polypeptides that bear structural and functional relationship to naturally occurring Lefty polypeptides but have an amino acid sequence that is not identical to that of the mouse Lefty-1 and Lefty-2 polypeptides or the human Lefty-A or Lefty-B polypeptides. Lefty derivative polypeptides retain the ability to bind to one or more of Nodal, myostatin and/or GDF-11. In particular, the disclosure provides that Lefty proteins may be viewed as containing five regions, Regions 1 - 5. See Figures 1-5. The general cystine knot structure (maintained primarily by a series of cross-linked cysteine residues) and Regions 2 and 4 are expected to be primarily responsible for the binding of Lefty to Nodal, myostatin and GDF-11. Regions 1, 3 and 5, and particularly the C-terminal portion of Region 1 and Region 3 as a whole, are not expected to participate significantly in binding, and accordingly, these regions are attractive targets for modifying the amino acid sequences of Lefty proteins. Preferably, recombinant

Lefty derivative polypeptides are selected to inhibit signaling mediated by a protein such as an ActRII receptor, myostatin, Nodal, and/or GDF-11 in a biochemical binding assay or a cell-based assay.

In certain embodiments, a recombinant Lefty derivative polypeptide comprises an amino acid sequence as set forth in the formula: -A-X-B-, wherein A consists of an amino acid sequence at least 85%, 90%, 95%, 98% or 100% identical to the sequence of Region 2 of human Lefty A (SEQ ID NO:5) or Lefty B (SEQ ID NO:7); and wherein B consists of an amino acid sequence at least 85%, 90%, 95%, 98% or 100% identical to the sequence of Region 4 of human Lefty A or B, SEQ ID Nos: 6 and 8, respectively. Region 2 of Lefty A has the sequence CRQEMYIDLQGMKWAKNWVLEPPG FLAYECVGT (SEQ ID NO: 5). Region 2 of Lefty B has the sequence CRQEMYIDLQGMKWAENWVLEPPGFLAYECVGT (SEQ ID NO: 7). Region 4 of Lefty A has the sequence CIASETASLPMIVSIKEGGRTRPQVVSLPNMRVQKC (SEQ ID NO: 6) and Region 4 of Lefty B has the sequence CIASETDSLPMIVSIKEGGRTRPQVVSLPNMRVQKC (SEQ ID NO: 8).

A recombinant Lefty derivative polypeptide will preferably bind to one or more of Nodal, myostatin and GDF-11, preferably with a K_D of less than 10^{-6}, 10^{-7}, 10^{-8} or 10^{-9}. X may consist of zero, one or more than one one amino acid, except that X should be selected so as to maintain the functional activity of the Lefty derivative polypeptide. X may comprise an amino acid sequence that has low immunogenicity. X may include a site for post-translational modification, preferably glycosylation. X may be less than 500, 400, 300, 200, 100, 50 or less than 25 amino acids. X may include an additional domain, such as a dimerization domain, a domain that binds to Nodal, myostatin and/or GDF-11 or a domain that otherwise confers a desirable property such as improved solubility or improved pharmacokinetics.

A recombinant Lefty derivative polypeptide may or may not include amino acids that are N-terminal to the A portion of the -A-X-B- formula, and also may or

may not include amino acids that are C-terminal to the B portion of the -A-X-B- formula. Accordingly, a Lefty derivative polypeptide comprising a sequence of formula -A-X-B- should be understood to include, as optional embodiments, sequence that is N-terminal to A and/or sequence that is C-terminal to B. If specific reference to these portions is needed, such regions may be referred to as "W" and "Y". Thus, a derivative Lefty polypeptide may consist essentially of a sequence represented as W-A-X-B, A-X-B-Y or W-A-X-B-Y. The sequence of W and Y, if such sequence is included at all, is relatively unconstrained and will be selected so as to retain any of the desirable functional activities of the Lefty derivative polypeptide. W and Y may comprise an amino acid sequence that has low immunogenicity. W and Y may include a site for post-translational modification, preferably glycosylation. W and Y may be less than 500, 400, 300, 200, 100, 50 or less than 25 amino acids. W and Y may include an additional domain, such as a dimerization domain, a domain that binds to Nodal, myostatin and/or GDF-11 or a domain that otherwise confers a desirable property such as improved solubility or improved pharmacokinetics. In one embodiment, W includes a "long" Region 1 sequence corresponding to the sequence resulting after cleavage of the most N-terminal RXXR propeptide cleavage site of a Lefty protein. In one embodiment, W includes a "short" sequence corresponding to the sequence resulting after cleavage of the second RXXR propeptide cleavage site. In further embodiments, W may include some or all of the propeptide sequence, in which case W will generally be altered such that the first and/or second RXXR cleavage site is altered to reduce or eliminate cleavage. A preferred method for altering the RXXR cleavage site is to include a site for glycosylation which site either alters the sequence of the RXXR site or results in a glycosylation that occludes the cleavage site or otherwise inhibits cleavage. Any of portions W, X or Y may be at least 85% identical to a naturally occurring region 1, 3 or 5, respectively, of a naturally occurring Lefty polypeptide, particularly human Lefty A or B, and may also be at least 90%, 95%, 98%, 99% or 100% identical to such sequence.

The recombinant Lefty derivative polypeptide may comprise an amino acid sequence that is at least 85% identical to the cystine knot portion of a human Lefty

polypeptide. The recombinant Lefty derivative polypeptide may comprise an amino acid sequence that is at least 85% identical to a human Lefty polypeptide sequence selected from the group consisting of: amino acids 22-353 of SEQ ID NO:1 and amino acids 22-353 of SEQ ID NO:2, wherein one or both RXXR cleavage sequences are altered so as to prevent cleavage at the altered sequence.

As demonstrated herein, mature Lefty A polypeptides initiating at the first or second RXXR cleavage site, and N- or C- terminal Fc fusions thereof, have myostatin binding activity. Accordingly, a recombinant Lefty polypeptide may be a "long" form (e.g., 34 kDa form) or a "short" form (e.g., 28 kDa form) depending on the size of the sequence, corresponding to Region 1, between the propeptide cleavage site and the beginning of the cystine knot domain. A long form may be a Lefty derivative polypeptide in which the second RXXR cleavage site is altered to eliminate cleavage and permit consistent production of the longer form. A long form may also be produced by expression of a sequence that retains the second RXXR cleavage site in a cell line or culture conditions that are deficient for cleavage activity.

A Lefty derivative polypeptide may include one or more sequence alterations that introduce one or more sites for post-translational modification. Glycosylation is a preferred post-translational modification, and such modification will preferably be positioned in portions W, X or Y of the Lefty derivative polypeptide.

As noted above, a Lefty derivative polypeptide may include one or more additional domains, fused to the amino- or the carboxy- terminus or within any of Regions W, X or Y. As described herein, Lefty may be a potent antagonist in a dimeric or multimeric form, and therefore, the additional domain may be a dimerization or multimerization domain. It is expected that the propeptide functions naturally to mediate Lefty dimerization, and therefore, the the dimerization domain may comprise a Lefty propeptide sequence of either a short or long form. A dimerization domain may be an Fc domain. A dimerization domain may comprise an immunoglobulin Fab constant domain, and the Fab constant domain may be selected, for example, from an immunoglobulin heavy chain constant region and an

immunoglobulin light chain constant region. Other dimerization or multimerization domains may be chosen, such as a leucine zipper domain. A leucine zipper domain may comprise at least four leucine heptads, and may be, for example, a Fos or a Jun leucine zipper domain. Amino acid linkers may be interposed between any Lefty amino acid sequence and any additional domain(s). An additional domain may be a domain that binds to and, preferably, inhibits Nodal, myostatin and/or GDF-11. For example, Lefty is expected to block the Type II receptor binding site of BMP proteins, and therefore a second domain that block the Type I receptor (e.g., ALK4 or ALK7) binding site would be useful to improve the antagonistic properties of the Lefty molecule. A domain that competitively inhibits the binding of Nodal, myostatin and/or GDF-11 to ALK4 or ALK7 may be, for example, (a) an extracellular portion of ALK4; (b) an extracellular portion of ALK7; (c) an antigen-binding portion of an antibody that binds Nodal, myostatin and/or GDF-11; and (d) a randomized polypeptide that has been selected for binding to Nodal, myostatin and/or GDF-11.

In certain aspects, a recombinant Lefty derivative polypeptide comprises a heterogenous sequence that mediates secretion of the recombinant Lefty derivative polypeptide, such as a honey bee melatin leader sequence.

The disclosure further provides recombinant polynucleotides comprising a nucleotide sequence encoding a Lefty derivative polypeptide disclosed herein. The recombinant polynucleotide may include a promoter sequence operably linked to the nucleotide sequence encoding the Lefty derivative polypeptide. Such nucleic acids may be introduced into cells, such as mammalian cells (e.g., human or CHO cells). Such cells may be used in a method of making a recombinant Lefty derivative polypeptide, comprising: a) culturing a cell encoding the recombinant Lefty derivative polypeptide under conditions suitable for expression of the recombinant Lefty derivative polypeptide; and b) recovering the recombinant Lefty derivative polypeptide so expressed.

As noted above, it is now expected that Lefty will act as a potent antagonist in a dimeric or multimeric form. Therefore, one may prepare an isolated Lefty

polypeptide complex comprising: a first Lefty polypeptide and a second Lefty polypeptide, wherein the first and second Lefty polypeptides are associated to form a complex, and wherein the complex binds to a TGF-β family member selected from the group consisting of: myostatin, Nodal and GDF-11. The Lefty polypeptide complex may be a heterodimer (or multimer) or a homodimer (or multimers).

In certain aspects, the disclosure provides pharmaceutical preparations comprising any of the various Lefty derivatives or dimeric Lefty polypeptides.

In certain aspects, the disclosure provides new uses for Lefty polypeptides, including Lefty derivative polypeptides disclosed herein. In one embodiment, the disclosure provides methods for treating a subject having a disorder associated with muscle loss or insufficient muscle growth, comprising administering to the subject an effective amount of a composition comprising a Lefty polypeptide. In another embodiment, the disclosure provides methods for treating a disorder associated with neurodegeneration, comprising administering to the subject an effective amount of a composition comprising a Lefty polypeptide. In a further embodiment, a Lefty polypeptide may be used to promote weight loss and to treat disorders relating to body fat content or body weight, such as obesity and Type II diabetes. In certain embodiments, Lefty polypeptides may be used to bind to and/or inhibit the activity of Nodal, myostatin and/or GDF-11 in vitro or in vivo. The Lefty polypeptide may be a wildtype Lefty polypeptide or fragments thereof, a recombinant Lefty derivative polypeptide, as well as a dimerized Lefty polypeptide.

The disclosure further provides for the use of a Lefty polypeptide for making a medicament for the treatment of a disorder associated with abnormal amount, development or metabolic activity of muscle tissue or a disorder associated with neurodegeneration or a disorder relating to body fat content or body weight.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic of the conserved cystine knot structure of members of the TGF-β superfamily, based on the structure of BMP-7. Cysteines that form the core of the structure are indicated as C1 – C6. In the traditional

receptor ligand members of the family, Loops A and B mediate binding to Type II receptors, while the region between C3 and C4 contains an alpha helix and other amino acids that mediate dimerization and binding to Type I receptors. The region between C3 and C4 also contains a cysteine that is critical for the formation of a disulfide linkage between monomers. In the Dan family of ligand binding "traps", Loops A and B participate in binding to the target BMP ligand while the region between C3 and C4 is generally smaller than in the receptor ligands. As described herein, Lefty proteins may be mapped onto this structure and divided into five functional regions. Region 1 is the portion of the mature polypeptide that is N-terminal to C1 (the first cysteine of the cystine knot domain). Regions 2 and 4 correspond to Loops A and B and participate in ligand binding (ligands such as Nodal, myostatin and GDF-11). Region 3 of Lefty proteins lacks the alpha helix and the cysteine that participates in intermolecular crosslinking. Region 5 is the portion that is C-terminal to the final cysteine of the cystine knot domain.

Figure 2 shows an alignment of the cystine knot domains of several members of the TGF-β family. PDRC, Gremlin, Cerberus and Noggin are members of the ligand "trap" grouping – proteins that bind to and inhibit the activity of various receptor ligand members of the TGF-β family. BMP-2, GDF-8 and TGF-β are traditional receptor ligands that form disulfide-crosslinked dimers and bind and activate Type I and Type II receptor-mediated signaling. Lefty, shown at center, is a Lefty polypeptide. The regions corresponding to the Loops A and B, which mediate Type II receptor binding in BMP receptor ligands, are underlined at bottom. The region corresponding to the "Dimerization Loop" is overlined at top, and a box shows the Type I receptor binding helix of BMP-2. The cysteine that participates in monomer-monomer disulfide bond formation is shown in red. Notably, the Dimerization Loop is longer in BMP-2, GDF-8 and TGF-β than in Lefty or the trap family members. Conserved cysteines are shown in green. The SEQ ID Nos. for each of these sequences is as follows: PDRC (SEQ ID NO:17); Gremlin (SEQ ID NO:18); Cerberus (SEQ ID NO:19); Dan (SEQ ID NO:20); Noggin (SEQ ID NO:21); Human Lefty A (SEQ ID NO:22); BMP-2 (SEQ ID NO:23); GDF-8 (SEQ ID NO:24); TGF-β (SEQ ID NO:25).

Figure 3 shows an alignment of the Lefty proteins, human Lefty-A and -B and murine lefty-1 and lefty-2. Regions corresponding to the signal peptide, the RXXR cleavage sites (which mark the C-terminus of the propeptide), the conserved cysteines ("C") of the cystine knot and the locations of sequence variations that are associated with human disorders (R314K, S342K) are shown. SEQ ID Nos. 1-4.

Figure 4 shows the human Lefty A and Lefty B amino acid sequences (NCBI RefSeq ID NP_003231 and NP_066277, respectively; SEQ ID Nos. 1 and 2, respectively.) The signal sequence is underlined with a dotted line. RXXR cleavage sites are underlined. The cystine knot domain is double underlined.

Figure 5 shows the amino acid sequences of the 34 kDa mature forms of human Lefty A and Lefty B which result from cleavage at the first RXXR cleavage site (SEQ ID Nos. 9 and 10 respectively). Regions 1 through 5, as described in Figure 1, are indicated. Regions 1 and 5 have a broken underline. Regions 2 and 4 have a single underline. Region 3 has a double underline. Shaded regions correspond to regions that may be modified or deleted to generate Lefty variants that retain Nodal, myostatin and/or GDF-11 inhibiting activity.

Figure 6 is a schematic diagram showing various human Lefty A constructs that were prepared and tested for myostatin binding. The larger forms (34 kDa, or "Lefty 34") included an alteration to inactivate the second RXXR cleavage site. The natural signal sequence was replaced (along with two alanine residues C-terminal thereto) with a honey bee melatin leader sequence. The shorter forms (28 kDa, or "Lefty 28") contained the melatin leader sequence followed by an amino-terminally truncated Lefty sequence beginning with the Leucine that immediately follows the second RXXR cleavage sequence. N- and C- terminal Fc fusions were prepared as shown.

Figure 7 shows data from a Biacore™ binding assay. GDF-11 was immobilized on a Biacore chip and conditioned medium from cells expressing the varius Lefty constructs was passaged through the chip. The rising curve indicates protein binding, which decreases during the subsequent wash phase. Control samples show little or no binding. These data demonstrate that Lefty 34 and Lefty

28 bind directly to myostatin.

DETAILED DESCRIPTION OF THE INVENTION

1. Overview

The disclosure relates to the discovery that Lefty proteins bind to GDF-11, in addition to Nodal. Given the sequence conservation between myostatin and GDF-11, Lefty proteins are expected to bind and inhibit both myostatin and GDF-11. The disclosure also relates to the surprising insight that Lefty proteins will be potent as binders when dimerized or multimerized. Accordingly, the disclosure provides Lefty polypeptides and fusion proteins that form dimers or multimers. Furthermore, the disclosure provides regions of various Lefty polypeptides that may be modified to obtain Lefty derivatives that may retain the ability to inhibit Nodal, myostatin and/or GDF-11. Such derivatives may exhibit one or more desirable features, including, for example, improved protein expression, improved solubility, decreased tendency to adsorb non-specifically to surfaces (improved "handling"), improved serum half-life, improved tissue distribution and decreased immunogenicity.

Murine Lefty-1 and Lefty-2 genes are expressed asymmetrically prior to the appearance of anatomic left-right (LR) differences (Meno C, et al., 1996, Nature 381:151-155; Meno C, et al., 1997, Genes Cells 2:513-524; Meno C, et al., 1998, Cell, 94:287-297). Both are expressed on the left side of the embryo in the floor-plate and in the lateral-plate mesoderm; Lefty-2 is expressed more strongly in lateral-plate mesoderm than in floor-plate, whereas the reverse is true for Lefty-1. Two human Lefty proteins, Lefty-A and Lefty-B, were identified as homologous to the murine proteins (Kosaki et al., 1999, Am J Hum Genet, 64:712-721). In humans, two mutations in Lefty-A are associated with left-right axis malformations. Lefty-A is also referred to as Ebaf (endometrum bleeding-associated factor) (Kothapalli et al., 1997, J Clin Invest, 99:2342-2350). Human Lefty-A and Lefty-B are 96% identical in amino acid sequence. Mouse lefty-1 and lefty-2 are 90% identical in amino acid sequence. Human Lefty-A is 81% identical to each of the mouse lefty proteins, and human Lefty-B is 82% identical to each of the mouse lefty

proteins.

TGF-β family members are generally encoded as prepro-proteins that undergo secretion and cleavage to remove a signal sequence and further cleavage at a dibasic or RXXR site, which breaks the peptide bond between the mature carboxy-terminal portion and the amino-terminal propeptide region. Lefty proteins have two, rather than one, putative cleavage sites that release carboxy-terminal mature protein, and multiple cleavage forms of Lefty can be found in cell cultures. As shown herein, both long (34 kDa) and short (28 kDa) forms of mature Lefty retain certain key activities. Among members of the TGF-β family, propeptides have different functional attributes. In some instances, the propeptide portion is released upon cleavage and plays no further functional role. In other instances, the propeptide portion associates with the mature protein, generally either increasing bioavailability of the mature portion or inhibiting the activity of the mature portion, or both. No role for the Lefty propeptide has been proposed. However, in view of the present disclosure it is expected that, in physiological conditions, the propeptide region of Lefty polypeptides may associate with the mature portion and mediate the formation of dimers.

The mature portions of TGF-β family members generally contain a conserved cystine-knot structure. This structure is illustrated in Figure 1, as deduced from the solved structure of BMP-7. The cystine knot include two loops, designated Loop 1 and Loop 2, that participate in type II receptor binding. A third region contains amino acids that participate in Type I receptor binding and an α-helix and a conserved cysteine that are usually essential for formation of homo- or hetero-dimers and the covalent stabilization of such dimers (Thisse C, et al., 1999, Development, 126:229-240; Meno C, et al., 1996, Nature 381:151-155; Meno C, et al., 1997, Genes Cells 2:513-524). The binding of TGF-β ligand to the Type I and Type II receptors normally activates the canonical TGF-β signaling pathway, typically marked by activation of one or more SMAD proteins. Lefty proteins are atypical members of the TGF-β family. Unlike most other TGF-β family members, Lefty proteins lack the α-helix and a conserved cysteine that mediate dimerization. For these reasons, most published reports indicate that Lefty proteins are monomeric

(see, e.g., Sakuma et al., 2002). Thus, it is a surprising insight of the present disclosure that Lefty proteins bind to target molecules in a dimeric form and may naturally perform their physiological roles in a dimeric conformation.

Numerous members of the TGF-β family act as antagonists of the canonical TGF-β receptor-ligand signaling pathway. The Dan family of antagonists have a conserved cystine knot domain. These proteins dimerize and bind to a target ligand of the TGF-β family, inhibiting the signaling mediated by that ligand. Another antagonist, Noggin, functions similarly. These antagonists are referred to herein as "traps".

Lefty also acts as an antagonist. Genetic evidence suggests that Lefty functions as an antagonist of Nodal. In embryonic patterning, Lefty is thought to restrict the range and duration of Nodal activity (e.g., Meno C, et al., 1999, Mol Cell, 4:287-298). Lefty-induced inhibition of Nodal signaling was rescued by excess ActRIIA or ActRIIB, suggesting that Lefty antagonizes Nodal signaling through competive binding to the common receptor ActRIIA or ActRIIB (Sakuma et al., 2002, Genes Cells 7:401-412). This mode of antagonism is consistent with the presumed monomeric nature of Lefty, and presents a model for antagonism that is distinct from the mechanism proposed for the "trap" proteins. In one report, Lefty was reported to bind directly to Nodal (Chen and Shen, 2004, Curr. Biol. 14: 618-624).

The present disclosure reports the discovery that Lefty may directly bind to and antagonize numerous ligands of the TGF-β family, and may do so as a dimer. Accordingly, the disclosure provides Lefty polypeptides for inhibiting Nodal, myostatin (GDF-8) and/or GDF-11 function. The present disclosure further provides Lefty polypeptide derivatives, meaning variants and fragments of naturally occurring Lefty polypeptides that retain the ability to inhibit Nodal, myostatin and/or GDF-11. Examples of such derivatives include N-terminally truncated versions of Lefty, Lefty polypeptide containing alterations in any or all of Regions 1, 3 and 5 (see Figures 1-4), glycosylated (or other post-translationally modified) forms of Lefty and fusion proteins comprising any of the various Lefty polypeptides disclosed

herein. These so-called "Lefty derivatives" can be used to reduce the severity of a pathologic condition, which is characterized, at least in part, by an undesirable level of Nodal, myostatin and/or GDF-11 activity. For instance, the pharmaceutical preparations of the present disclosure can be administered in an amount effective to prevent, ameliorate or reduce the severity of a variety of disorders related to neurological or muscular function.

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

"About" and "approximately" shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typically, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values.

Alternatively, and particularly in biological systems, the terms "about" and "approximately" may mean values that are within an order of magnitude, preferably within 5-fold and more preferably within 2-fold of a given value. Numerical quantities given herein are approximate unless stated otherwise, meaning that the term "about" or "approximately" can be inferred when not expressly stated.

The methods of the disclosure may include steps of comparing sequences to each other, including wild-type sequence to one or more mutants / sequence variants Such comparisons typically comprise alignments of polymer sequences, e.g., using sequence alignment programs and/or algorithms that are well known in the art (for example, BLAST, FASTA and MEGALIGN, to name a few). The skilled artisan can readily appreciate that, in such alignments, where a mutation contains a residue insertion or deletion, the sequence alignment will introduce a "gap" (typically represented by a dash, or "A") in the polymer sequence not containing the inserted

or deleted residue.

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

The term "sequence similarity" refers to the degree of identity or correspondence between nucleic acid or amino acid sequences that may or may not share a common evolutionary origin.

A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other micleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook *et al. Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. For preliminary screening for homologous nucleic acids, low stringency hybridization conditions, corresponding to a T_m (melting temperature) of 55 °C, can be used, e.g., 5 × SSC, 0.1% SDS, 0.25% milk, and no formamide; or 30% formamide, 5 × SSC, 0.5% SDS).

Moderate stringency hybridization conditions correspond to a higher T_m, e.g., 40% formamide, with 5 × or 6 × SSC. High stringency hybridization conditions correspond to the highest T_m, e.g., 50% formamide, 5 × or 6 × SSC. SSC is 0.15 M NaCl, 0.015 M Na-citrate.

"High stringency conditions" are understood to encompass conditions of hybridization which allow hybridization of structurally related, but not structurally dissimilar, nucleic acids. The term "stringent" is a term of art which is understood by the skilled artisan to describe any of a number of alternative hybridization and

wash conditions which allow annealing of only highly complementary nucleic acids.

Exemplary high stringent hybridization conditions is equivalent to about 20-27 °C below the melting temperature (T_m) of the DNA duplex formed in about 1 M salt. Many equivalent procedures exist and several popular molecular cloning manuals describe suitable conditions for stringent hybridization and, furthermore, provide formulas for calculating the length of hybrids expected to be stable under these conditions (see e.g. *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6 or 13.3.6; or pages 9.47-9.57 of Sambrook, *et al.* (1989) *Molecular Cloning*, 2nd ed., Cold Spring Harbor Press).

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m, for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of micleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (see Sambrook *et al.*, *supra*, 9.51). For hybridization with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook *et al.*, *supra*, 11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides; preferably at least about 15 nucleotides; and more preferably the length is at least about 20 nucleotides.

Unless specified, the term "standard hybridization conditions" refers to a T_m of about 55 °C, and utilizes conditions as set forth above. In a preferred embodiment, the T_m is 60 °C; in a more preferred embodiment, the T_m is 65 °C. In a specific embodiment, "high stringency" refers to hybridization and/or washing conditions at 68 °C in 0.2 × SSC, at 42 °C in 50% formamide, 4 × SSC, or under conditions that afford levels of hybridization equivalent to those observed under

either of these two conditions.

Suitable hybridization conditions for oligonucleotides (e.g., for oligonucleotide probes or primers) are typically somewhat different than for full-length nucleic acids (e.g., full-length cDNA), because of the oligonucleotides' lower melting temperature. Because the melting temperature of oligonucleotides will depend on the length of the oligonucleotide sequences involved, suitable hybridization temperatures will vary depending upon the oligonucleotide molecules used. Exemplary temperatures may be 37 °C (for 14-base oligonucleotides), 48 °C (for 17-base oligonucleotides), 55 °C (for 20-base oligonucleotides) and 60 °C (for 23-base oligonucleotides). Exemplary suitable hybridization conditions for oligonucleotides include washing in 6 × SSC, 0.05% sodium pyrophosphate, or other conditions that afford equivalent levels of hybridization.

A protein or polypeptide, including an enzyme, may be a "native" or "wild-type," meaning that it occurs in nature; or it may be a "mutant," "variant," or "modified," meaning that it has been made, altered, derived, or is in some way different or changed from a native protein or from another mutant.

"Lefty protein" or "Lefty polypeptide" refers to mammalian Lefty proteins, such as the murine Lefty proteins or human Lefty proteins, and other proteins which share sequence homology and functional attributes of the mammalian Lefty proteins, including any Lefty derivatives. Exemplary amino acid sequences for Lefty proteins include:

Murine Lefty-1 protein (NCBI RefSeq ID NP_034224) (SEQ ID NO:3):

```
   1 mpflwlcwal walslvslre altgeqilgs llqqlqldqp pvldkadveg
mvipshvrtq
  61 yvallqhsha srsrgkrfsq nlrevagrfl vsetsthllv fgmeqrlppn
selvqavlrl
 121 fqepvprtal rrqkrlsphs ararvtiewl rfrddgsnrt alidsrlvsi
hesgwkafdv
 181 teavnfwqql srprqplllq vsvqrehlgp gtwsshklvr faaqgtpdgk
gqgepqlelh
 241 tldlkdygaq gncdpeapvt egtrccrqem yldlqgmkwa enwileppgf
ltyecvgscl
 301 qlpesltsrw pflgprqcva semtslpmiv svkeggrtrp qvvslpnmrv
qtcscasdga
 361 liprrlqp
```

Murine Lefty-2 protein (NCBI RefSeq ID NP_796073) (SEQ ID NO:4):

```
      1 mkslwlcwal wvlplagpga amteeqvlss llqqlqlsqa ptldsadvee
maipthvrsq
     61 yvallqgsha drsrgkrfsq nfrevagrfl msetsthllv fgmeqrlppn
selvqavlrl
    121 fqepvprtal rrferlsphs ararvtiewl rvredgsnrt alidsrlvsi
hesgwkafdv
    181 teavnfwqql srprqplllq vsvqrehlgp gtwsahklvr faaqgtpdgk
gqgepqlelh
    241 tldlkdygaq gncdpevpvt egtrccrqem yldlqgmkwa enwileppgf
ltyecvgscl
    301 qlpesltigw pflgprqcva semtslpmiv svkeggrtrp qvvslpnmrv
qtcscasdga
    361 liprgidl
```

Human Lefty-A protein (NCBI RefSeq ID NP_003231) (SEQ ID NO:1):

```
      1 mwplwlcwal wvlplagpga alteeqllgs llrqlqlsev pvldradmek
lvipahvraq
     61 yvvllrrshg drsrgkrfsq sfrevagrfl aseasthllv fgmeqrlppn
selvqavlrl
    121 fqepvpkaal hrhgrlsprs aqarvtvewl rvrddgsnrt slidsrlvsv
hesgwkafdv
    181 teavnfwqql srprqplllq vsvqrehlgp lasgahklvr fasqgapagl
gepqlelhtl
    241 dlrdygaqgd cdpeapmteg trccrqemyi dlqgmkwakn wvleppgfla
yecvgtcgqp
    301 pealafnwpf lgprqciase taslpmivsi keggrtrpqv vslpnmrvqk
cscasdgalv
    361 prrlqp
```

RXXR cleavage sites are underlined. The cysteine knot domain is double underlined.

Human Lefty-B protein (NCBI RefSeq ID NP_066277) (SEQ ID NO:2):

```
      1 mqplwlcwal wvlplaspga altgeqllgs llrqlqlkev ptldradmee
lvipthvraq
     61 yvallqrshg drsrgkrfsq sfrevagrfl aleasthllv fgmeqrlppn
selvqavlrl
    121 fqepvpkaal hrhgrlsprs ararvtvewl rvrddgsnrt slidsrlvsv
hesgwkafdv
    181 teavnfwqql srprqplllq vsvqrehlgp lasgahklvr fasqgapagl
gepqlelhtl
    241 dlgdygaqgd cdpeapmteg trccrqemyi dlqgmkwaen wvleppgfla
yecvgtcrqp
    301 pealafkwpf lgprqciase tdslpmivsi keggrtrpqv vslpnmrvqk
cscasdgalv
    361 prrlqp
```

RXXR cleavage sites are underlined. The cysteine knot domain is double

underlined.

Murine Lefty-1 cDNA (NCBI RefSeq ID NM_010094) (SEQ ID NO:13):

```
   1 aggacacctc agggacacac acatccaagg ctcctcttcc cggacagcac
catgccattc
  61 ctgtggctct gctgggcact ctgggcactg tcgctggtta gcctcaggga
agccctgacc
 121 ggagagcaga tcctgggcag cctgctgcaa cagctgcagc tcgatcaacc
gccagtcctg
 181 gacaaggctg atgtggaagg gatggtcatc ccctcgcacg tgaggactca
gtatgtggcc
 241 ctgctacaac acagccatgc cagccgctcc cgaggcaaga ggttcagcca
gaaccttcga
 301 gaggtggcag gcaggttcct ggtgtcagag acctccactc acctgctagt
gttcggaatg
 361 gagcagcggc tgccgcctaa cagcgagctg gtgcaggctg tgctgcggct
gttccaggag
 421 cctgtgccca gaacagctct ccggaggcaa aagaggctgt ccccacacag
tgcccgggct
 481 cgggtcacca ttgaatggct gcgcttccgc gacgacggct ccaaccgcac
tgcccttatc
 541 gattctaggc tcgtgtccat ccacgagagc ggctggaagg ccttcgacgt
gaccgaggcc
 601 gtgaacttct ggcagcagct gagccggccg aggcagccgc tgctgctcca
ggtgtcggtg
 661 cagagggagc atctggggcc gggaacctgg agctcacaca agttggttcg
tttcgcggcg
 721 caggggacgc cggatggcaa ggggcagggc gagccacagc tggagctgca
cacgctggac
 781 ctcaaggact atggagctca aggcaattgt gaccccgagg caccagtgac
tgaaggcacc
 841 cgatgctgtc gccaggagat gtacctggac ctgcagggga tgaagtgggc
cgagaactgg
 901 atcctagaac cgccagggtt cctgacatat gaatgtgtgg gcagctgcct
gcagctaccg
 961 gagtccctga ccagcaggtg gccatttctg gggcctcggc agtgtgtcgc
ctcagagatg
1021 acctccctgc ccatgattgt cagcgtgaag gagggaggca ggaccaggcc
tcaagtggtc
1081 agcctgccca acatgagggt gcagacctgt agctgcgcct cagatggggc
gctcataccc
1141 aggaggctgc agccataggc gcggggtgtg gcttccccaa ggatgtgcct
ttcatgcaaa
1201 tctgaagtgc tcattatact gggagagctg gggattctaa ctccctaatg
ggcaatccct
1261 gtgtgtgctc tttgcttcct ctgaagtagc ctcatcccta aatttttacc
ttcgaggaat
1321 gtgactcgct ggcccctgga ggcgctctga cccagtggtc tctgtccttc
atattgttca
1381 ctgcactgta tgcgaagcac ttacatgtat agatactgca aaccaaggac
agaatcccca
1441 attgccattg ttcccttaat ttgtcgctga atctgggctg agtcccagtc
ttgactctgg
1501 acctaagcca caagttgggc aaacatgtcc aacctaggca atactggctt
tgctagatgt
```

1561 gaataaaata tgctttgttt tgt

Murine Lefty-2 cDNA (NCBI RefSeq ID NM_177099) (SEQ ID NO:14):

1 gtcccaagaa cttttcaggg cacttttagg gacgcatata tccacgattc ctcctgggca
61 gcgccatgaa gtccctgtgg ctttgctggg cactctgggt actgcccctg gctggccctg
121 gggcagcgat gaccgaggaa caggtcctga gcagtctact gcagcagctg cagctcagcc
181 aggcccccac cctggacagc gcggatgtgg aggagatggc catccctacc cacgtgaggt
241 cccagtatgt ggccctgctg cagggaagtc acgctgaccg ctcccgaggc aagaggttca
301 gccagaattt tcgagaggtg gcaggcaggt tcctgatgtc agagacctcc actcacctgc
361 tagtgttcgg aatggagcag cggctgccgc ctaacagcga gctggtgcag gctgtgctgc
421 ggctgttcca ggagcctgtg cccagaacag ctctccggag gtttgagagg ctgtccccac
481 acagtgcccg ggctcgggtc accattgaat ggctgagagt ccgtgaggat ggctccaatc
541 gcactgccct catcgactct aggctcgtgt ccatccacga gagcggctgg aaggccttcg
601 acgtgaccga ggccgtgaac ttctggcagc agctgagccg gccgaggcag ccgctgctgc
661 tccaggtgtc ggtgcagagg gagcatctgg ggccggggac ctggagcgca cacaagttgg
721 tccgtttcgc ggcgcagggg acgccggacg gcaaggggca gggcgagcca cagctggagc
781 tgcacacgct ggacctcaag gactacggag ctcaaggcaa ttgtgacccc gaggtaccag
841 tgactgaagg cacccgatgc tgtcgccagg agatgtacct ggacctgcag gggatgaagt
901 gggccgagaa ctggatccta gaaccgccag ggttcctgac gtatgaatgt gtgggcagct
961 gcctgcagct accagagtcc ctgaccatcg ggtggccatt tctggggcct cggcagtgtg
1021 ttgcctcaga gatgacctcc ttgcccatga ttgtcagtgt gaaggaggga ggcaggacca
1081 ggcctcaagt ggtcagcctg cccaacatga gggtgcagac ctgtagctgc gcctcagatg
1141 gggcgctcat acccaggggg atagatctgt agtctccctg tccacagatg tattctcagt
1201 gagcttgtcc taacttagtg ctctcgtcag acctttgctc tacagtcttg gttttcttgt
1261 ccatcaccca gtttaagcac ttacatgggt aaatcatgtc actccagtag gacacactga
1321 ccccacttag ccaaggacat ggctatgcag tgaacaggtt cgcatctgag tctgttttct
1381 ggccagaact cagcttaatg tacaacaaaa ccctacggtg agaacagggg aatcaaaagc
1441 tcgtttactc ttacaccgtg attactggca tcaacgtacc atgtcaggga ctgcccacag

1501 caggctggga gggagacatc tcagaagcct gcggcagctc cttgtgaaaa accgttgttc
1561 ccatttctcc taaccttagc cctagacaag agctgtatag atttcatgtg tgtgactgct
1621 tttcagttgg ccttggtgtt catagttatt ctatattatt tgactttcct actcctttct
1681 ccttctgccc tggtgaattc tatgaaacta gatgttcctt gatgtaatga ttcttaaaca
1741 attaaaaagt tgaggcatgg gacacagcac agcacagtcc tgatggccca ggtgcatgct
1801 gtagatgtat tctgtgtgct cttatcttgg aaacaatgca ataactttgc aatgttagtt
1861 cagattaatg tttgacttgc aaagaaagtt tgaagaaatt attagaaagt gaaatagagc
1921 caacactggg atcccgaaaa gaaaaaagct attgaagtta tgaaataagt tttgcacaaa
1981 atttgagagt gtttcctgga taagcaagta tagaatacat aaaatcttat attagtaaaa
2041 ctaagccaaa acaccgggac tcttaggagg gtcactgcgt gcaatgtgca gaagcagaaa
2101 gctggcagaa ctgccgagtt aagggtgtac ctgagtcttt ctggccattg cctggcagct
2161 ttgcccatgt catttattgt cagagcttca cgggaaaatg caagtagccg acttcggagc
2221 tctgagctct ggagtataat aagtcaaaag gtaaagttta aataatgata agtttgcaat
2281 aattattatt ttggccagag gcctgggaat aggggaagct tgaaactctg ggggaacaat
2341 tataattctt gattctttgt gtgatgtggg tattgttttg aatttgattt ggcaacgatt
2401 atacaatgtc ttttttttcct atctgcattt ggagtatcaa taaaagactg gggcaagaga
2461 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
2521 aaaaaaaaaa aaaa

Human Lefty-A cDNA (NCBI RefSeq ID NM_003240) (SEQ ID NO:15):

1 acacccagct gcctgagacc ctccttcaac ctccctagag gacagcccca ctctgcctcc
61 tgctccccca gggcagcacc atgtggcccc tgtggctctg ctgggcactc tgggtgctgc
121 ccctggctgg ccccggggcg gccctgaccg aggagcagct cctgggcagc ctgctgcggc
181 agctgcagct cagcgaggtg cccgtactgg acagggccga catggagaag ctggtcatcc
241 ccgcccacgt gagggcccag tatgtagtcc tgctgcggcg cagccacggg gaccgctccc
301 gcggaaagag gttcagccag agcttccgag aggtggccgg caggttcctg gcgtcggagg
361 ccagcacaca cctgctggtg ttcggcatgg agcagcggct gccgcccaac agcgagctgg
421 tgcaggccgt gctgcggctc ttccaggagc cggtccccaa ggccgcgctg cacaggcacg

481 ggcggctgtc cccgcgcagc gcccaggccc gggtgaccgt cgagtggctg cgcgtccgcg
541 acgacggctc caaccgcacc tccctcatcg actccaggct ggtgtccgtc cacgagagcg
601 gctggaaggc cttcgacgtg accgaggccg tgaacttctg gcagcagctg agccggcccc
661 ggcagccgct gctgctacag gtgtcggtgc agagggagca tctgggcccg ctggcgtccg
721 gcgcccacaa gctggtccgc tttgcctcgc agggggcgcc agccgggctt ggggagcccc
781 agctggagct gcacaccctg gacctcaggg actatggagc tcagggcgac tgtgaccctg
841 aagcaccaat gaccgagggc acccgctgct gccgccagga gatgtacatt gacctgcagg
901 ggatgaagtg ggccaagaac tgggtgctgg agcccccggg cttcctggct tacgagtgtg
961 tgggcacctg ccagcagccc ccggaggccc tggccttcaa ttggccattt ctggggccgc
1021 gacagtgtat cgcctcggag actgcctcgc tgcccatgat cgtcagcatc aaggagggag
1081 gcaggaccag gccccaggtg gtcagcctgc ccaacatgag ggtgcagaag tgcagctgtg
1141 cctcggatgg ggcgctcgtg ccaaggaggc tccagccata ggcgcctggt gtatccattg
1201 agccctctaa ctgaacgtgt gcatagaggt ggtcttaatg taggtcttaa ctttatactt
1261 agcaagttac tccatcccaa tttagtgctc ctgtgtgacc ttcgccctgt gtccttccat
1321 ttcctgtctt tcccgtccat cacccatcct aagcacttac gtgagtaaat aatgcagctc
1381 agatgctgag ctctagtagg aaatgctggc atgctgatta caagatacag ctgagcaatg
1441 cacacatttt cagctgggag tttctgttct ctggcaaatt cttcactgag tctggaacaa
1501 taatacccta tgattagaac tggggaaaca gaactgaatt gctgtgttat atgaggaatt
1561 aaaaccttca aatctctatt tcccccaaat actgacccat tctggacttt tgtaaacata
1621 cctaggcccc tgttcccctg agagggtgct aagaggaagg atgaagggct tcaggctggg
1681 ggcagtggac agggaattgg gatacctgga ttctggttct gacagggcca caagctagga
1741 tctctaacaa acgcagaagg ctttggctcg tcatttcctc ttaaaaagga ggagctgggc
1801 ttcagctcta agaacttcat tgccctgggg atcagacagc ccctacctac ccctgcccac
1861 tcctctggag actgagcctt gcccgtgcat atttaggtca tttcccacac tgtcttagag
1921 aacttgtcac cagaaaccac atgtatttgc atgtttttg ttaatttagc taaagcaatt
1981 gaatgtagat actcagaaga aataaaaaat gatgtttcaa aaaaaaaaaa aaaaaaaaaa
2041 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
2101 aa

Human Lefty-B cDNA (NCBI RefSeq ID NM_020997) (SEQ ID NO:16):

```
        1 gcctgagacc ctcctgcagc cttctcaagg gacagcccca ctctgcctct
tgctcctcca
       61 gggcagcacc atgcagcccc tgtggctctg ctgggcactc tgggtgttgc
ccctggccag
      121 ccccggggcc gccctgaccg gggagcagct cctgggcagc ctgctgcggc
agctgcagct
      181 caaagaggtg cccaccctgg acagggccga catggaggag ctggtcatcc
ccacccacgt
      241 gagggcccag tacgtggccc tgctgcagcg cagccacggg gaccgctccc
gcggaaagag
      301 gttcagccag agcttccgag aggtggccgg caggttcctg gcgttggagg
ccagcacaca
      361 cctgctggtg ttcggcatgg agcagcggct gccgcccaac agcgagctgg
tgcaggccgt
      421 gctgcggctc ttccaggagc cggtccccaa ggccgcgctg cacaggcacg
ggcggctgtc
      481 cccgcgcagc gcccgggccc gggtgaccgt cgagtggctg cgcgtccgcg
acgacggctc
      541 caaccgcacc tccctcatcg actccaggct ggtgtccgtc cacgagagcg
gctggaaggc
      601 cttcgacgtg accgaggccg tgaacttctg gcagcagctg agccggcccc
ggcagccgct
      661 gctgctacag gtgtcggtgc agagggagca tctgggcccg ctggcgtccg
gcgcccacaa
      721 gctggtccgc tttgcctcgc agggggcgcc agccgggctt ggggagcccc
agctggagct
      781 gcacaccctg gaccttgggg actatggagc tcagggcgac tgtgaccctg
aagcaccaat
      841 gaccgagggc acccgctgct gccgccagga gatgtacatt gacctgcagg
ggatgaagtg
      901 ggccgagaac tgggtgctgg agcccccggg cttcctggct tatgagtgtg
tgggcacctg
      961 ccggcagccc ccggaggccc tggccttcaa gtggccgttt ctggggcctc
gacagtgcat
     1021 cgcctcggag actgactcgc tgcccatgat cgtcagcatc aaggagggag
gcaggaccag
     1081 gccccaggtg gtcagcctgc ccaacatgag ggtgcagaag tgcagctgtg
cctcggatgg
     1141 tgcgctcgtg ccaaggaggc tccagccata ggcgcctagt gtagccatcg
agggacttga
     1201 cttgtgtgtg tttctgaagt gttcgagggt accaggagag ctggcgatga
ctgaactgct
     1261 gatggacaaa tgctctgtgc tctctagtga gccctgaatt tgcttcctct
gacaagttac
     1321 ctcacctaat ttttgcttct caggaatgag aatctttggc cactggagag
cccttgctca
     1381 gttttctcta ttcttattat tcactgcact atattctaag cacttacatg
tggagatact
     1441 gtaacctgag ggcagaaagc ccaatgtgtc attgtttact tgtcctgtca
ctggatctgg
     1501 gctaaagtcc tccaccacca ctctggacct aagacctggg gttaagtgtg
ggttgtgcat
     1561 ccccaatcca gataataaag actttgtaaa acatgaataa aacacatttt
attctaaaaa
     1621 aaaaaaaaaa aaaaaaaaaa aaaaaaa
```

It is expected that Lefty proteins also exist in other species, including all mammals.

Unless specifically stated otherwise, "Lefty polypeptide" includes both wild-type (including naturally occurring alleles) and altered ("derivative") Lefty forms, including various truncated and variant versions of Lefty that retain the ability to inhibit the activity of a TGF-β family member such as Nodal, myostatin or GDF-11.

As used herein, the term "Lefty activity" refers to one or more of the activities which are exhibited by the Lefty proteins of the present disclosure. In particular, "Lefty activity" includes the ability to bind to one or more of Nodal, myostatin and GDF-11.

"Specifically binds" includes reference to the preferential association of a ligand, in whole or part, with a particular target molecule (i.e., "binding partner" or "binding moiety") relative to compositions lacking that target molecule. It is, of course, recognized that a certain degree of non-specific interaction may occur between the subject Lefty polypeptides and other non-target proteins. Typically specific binding results in a much stronger association between the Lefty polypeptide and the target protein (e.g., Nodal, myostatin and/or GDF-11) than between the Lefty polypeptide and other proteins.

2. Lefty derivatives

The disclosure provides novel derivatives of Lefty proteins. The term "Lefty derivatives" includes altered forms of known Lefty proteins, such as variants induced by mutagenesis, insertions or deletions, and fragments of Lefty proteins, which variants retain Nodal, myostatin and/or GDF-11 binding activity. Lefty derivatives also include proteins sharing structural and/or functional similarity to known Lefty proteins, including those proteins which are described further herein. Such proteins may have amino acid sequences sharing significant sequence identity (e.g., at least about 50%, 60%, 70%, 80%, 90%, 95%, 99% or more) with the human or mouse Lefty proteins, over the full-length, the mature portion, or at least within

the Regions 2 and 4 of the human or mouse Lefty. Lefty derivatives also include proteins that have amino acid sequences that are encoded by nucleic acid sequences that hybridize under stringent conditions with the coding sequences for human or mouse Lefty, particularly that portion of the coding sequence for the mature portion, especially Regions 2 and 4. The term "Lefty derivative" excludes any of SEQ ID Nos. 1-4.

As described in Figures 1-5, Lefty proteins may be divided into portions, including a signal sequence, a propeptide, and a mature portion. The mature portion can be viewed as having five regions. Region 1 is the portion of the mature polypeptide that is N-terminal to C1 (the first cysteine of the cystine knot domain). Regions 2 and 4 correspond to Loops A and B and participate in ligand binding (ligands such as Nodal, myostatin and GDF-11). Region 3 of Lefty proteins lacks the alpha helix and the cysteine that participates in intermolecular crosslinking. Region 5 is the portion that is C-terminal to the final cysteine of the cystine knot domain. The propeptide will be long or short, depending on whether the upstream (N-terminal) or downstream RXXR cleavage site is used. Conversely, the mature portion, if expressed with a propeptide, will be short or long, depending on which RXXR cleavage site is used.

It is expected that Regions 2 and 4 mediate binding to Nodal, myostatin and GDF-11, and accordingly, any of Regions 1, 3 and 5 may be altered, in so far as ligand binding is retained. It is expected that the basic cystine knot structure will generally be conserved in Lefty derivatives that retain ligand binding activity.

Lefty proteins from other species, especially those of mammals, can be readily obtained by standard molecular biology protocols, such as PCR, low stringency hybridization, or antibody-mediated screening of expression libraries using antibodies cross-reacting with identified Lefty homologs in target species.

In certain embodiments, isolated fragments of the Lefty polypeptides can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding a Lefty polypeptide (e.g., SEQ ID Nos: 13-16). In addition, fragments can be chemically synthesized using techniques known

in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments that can function to bind to Nodal, myostatin and/or GDF-11, or for a cell-based activity, such as stimulation of muscle growth.

In certain embodiments, a Lefty derivative has an amino acid sequence that is at least 75% identical to an amino acid sequence as set forth in any of SEQ ID Nos. 1-4, 9, 10, 11, 12 or 22. In certain cases, the Lefty derivative has an amino acid sequence at least 80%, 85%, 90%, 95%, 97%, 98%, 99% or 100% identical to an amino acid sequence as set forth in any of SEQ ID Nos. 1-4, 9, 10, 11, 12 or 22. Preferably such variants retain the ability to bind to to Nodal, myostatin and/or GDF-11.

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

In certain embodiments, the present invention contemplates making mutations in a proteolytic cleavage site of a Lefty sequence to make the site less susceptible to proteolytic cleavage. Computer analysis (using a commercially available software, e.g., MacVector, Omega, PCGene, Molecular Simulation, Inc.)

can be used to identify proteolytic cleavage sites. As will be recognized by one of skill in the art, most of the described mutations, variants or modifications may be made at the nucleic acid level or, in some cases, by post translational modification or chemical synthesis. Such techniques are well known in the art.

In certain embodiments, the present invention contemplates specific mutations of the Lefty sequences so as to alter the glycosylation of the polypeptide. Such mutations may be selected so as to introduce or eliminate one or more glycosylation sites, such as O-linked or N-linked glycosylation sites. Asparagine-linked glycosylation recognition sites generally comprise a tripeptide sequence, asparagine-X-threonine (where "X" is any amino acid) which are specifically recognized by appropriate cellular glycosylation enzymes. The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the sequence of the wild-type Lefty (for O-linked glycosylation sites). A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Another means of increasing the number of carbohydrate moieties on a Lefty polypeptide is by chemical or enzymatic coupling of glycosides to the Lefty polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine; (b) free carboxyl groups; (c) free sulfhydryl groups such as those of cysteine; (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline; (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan; or (f) the amide group of glutamine. Removal of one or more carbohydrate moieties present on a Lefty polypeptide may be accomplished chemically and/or enzymatically. Chemical deglycosylation may involve, for example, exposure of the Lefty polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the amino acid sequence intact. Chemical deglycosylation is further described by Hakimuddin et al. (1987) Arch. Biochem. Biophys. 259:52 and by Edge et al. (1981) Anal. Biochem. 118:131. Enzymatic

cleavage of carbohydrate moieties on Lefty polypeptide can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. (1987) Meth. Enzymol. 138:350. The nucleic acid and/or amino acid sequence of a propeptide may be adjusted, as appropriate, depending on the type of expression system used, as mammalian, yeast, insect and plant cells may all introduce differing glycosylation patterns that can be affected by the amino acid sequence of the peptide.

Examples of sequence modifications that can be made in Lefty-A are set forth below:

LTEEQLLGSLLRQLQLSEVPVLDRADMEKLVIPAHVRAQYVVLLRRS HGDRSRGKRFSQSFREVAGRFLASEASTHLLVFGMEQRLPPNSELVQAVLR LFQEPVPKAAL(N)HR(T/G)HGR(N)LSPRSAQARVTVEWLRVRDDGSNRTSLI DSRLVSVHESGWKAFDVTEAVNFWQQLSRPRQPLLLQVSVQREHLGPLASG AHKLVRFASQGAPAGLGEPQLELHTLDLRDYGAQGDCDPEAP(N)MTE(N)G TRCCRQEMYIDLQGMKWAKNWVLEPPGFLAYECVGTCQQPPEA(N)LA(T)F NW(S)P(T)FLGPRQCIASETASLPMIVSIKEGGRTRPQVVSLPNMRVQKCSCA SDGALVPRRLQP

The first and second cleavage sites are underlined. Possible alterations, which may be used together, individually, or in combinations, are shown in brackets following the amino acid that would be altered. In this version, the polypeptide will be cleaved at the first site to give rise to a 34 kDa Lefty-A polypeptide beginning with the bold font pheylalanine (F). The second RXXR cleavage site may be altered to eliminate cleavage (e.g., R -> G change) or to eliminate cleavage and introduce a glycosylation site (other changes shown). Other mutations are shown to introduce glycosylation sites at the C-terminal end of Region 1 and in Region 3. Similar mutations may be made in any of SEQ ID Nos. 2-4.

This disclosure contemplates methods of generating mutants, including sets of combinatorial mutants of Lefty polypeptides, as well as truncation mutants; pools of combinatorial mutants are especially useful for identifying functional variant sequences. The purpose of screening such combinatorial libraries may be to

generate, for example, Lefty derivatives which can act as antagonists of Nodal, myostatin and/or GDF-11. For example, a Lefty polypeptide variant may be screened for ability to bind to a Nodal, myostatin and/or GDF-11 polypeptide, or for the ability to prevent binding of a Nodal, myostatin and/or GDF-11 to a cell expressing a receptor, such as an ActRIIA or B. The activity of a Lefty polypeptide variant may also be tested in a cell-based or in vivo assay. For example, the effect of a Lefty polypeptide variant on the expression of genes involved in muscle cell growth or myostatin-sensitive promoter may be assessed. Likewise, a Lefty polypeptide may be administered to a mouse or other animal, and one or more bone properties, such as density or volume may be assessed. The healing rate for bone fractures may also be evaluated. The effect of a Lefty polypeptide on gene expression changes caused by any of myostatin, Nodal or GDF-11 may be assessed. For example, an A-204 reporter gene assay may be used to evaluate the effects of Lefty polypeptides on signaling by GDF-11 and myostatin or other TGF-beta family members. A cell line, (e.g., human rhabdomyosarcoma cell line) may be transfected with a reporter vector that places a reporter gene (e.g., luciferase) under direction of a TGF-beta signaling-sensitive regulatory element (e.g., pGL3(CAGA)12 described in Dennler et al, 1998, EMBO 17: 3091-3100.) The CAGA12 motif is present in TGF-Beta responsive genes (PAI-1 gene), and this type of vector is of general use for factors signaling through Smad2 and 3. Lefty polypeptides may be tested for effects on the reporter gene activity.

Combinatorially-derived variants can be generated which have a selective potency relative to a naturally occurring Lefty polypeptide. Likewise, mutagenesis can give rise to variants which have intracellular half-lives dramatically different than the corresponding wild-type propeptide. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation.

A combinatorial library may be produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential Lefty polypeptide sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential Lefty polypeptide nucleotide sequences are expressible as

individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display).

There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate vector for expression. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al., (1981) Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) Annu. Rev. Biochem. 53:323; Itakura et al., (1984) Science 198:1056; Ike et al., (1983) Nucleic Acid Res. 11:477). Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) Science 249:386-390; Roberts et al., (1992) PNAS USA 89:2429-2433; Devlin et al., (1990) Science 249: 404-406; Cwirla et al., (1990) PNAS USA 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, Lefty polypeptide variants can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) Biochemistry 33:1565-1572; Wang et al., (1994) J. Biol. Chem. 269:3095-3099; Balint et al., (1993) Gene 137:109-118; Grodberg et al., (1993) Eur. J. Biochem. 218:597-601; Nagashima et al., (1993) J. Biol. Chem. 268:2888-2892; Lowman et al., (1991) Biochemistry 30:10832-10838; and Cunningham et al., (1989) Science 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) Virology 193:653-660; Brown et al., (1992) Mol. Cell Biol. 12:2644-2652; McKnight et al., (1982) Science 232:316); by saturation mutagenesis (Meyers et al., (1986) Science 232:613); by PCR mutagenesis (Leung et al., (1989) Method Cell Mol Biol 1:11-19); or by random mutagenesis, including chemical mutagenesis, etc. (Miller et al., (1992) A Short Course in Bacterial Genetics, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) Strategies in Mol Biol 7:32-34). Linker scanning mutagenesis, particularly in a

combinatorial setting, is an attractive method for identifying truncated (bioactive) forms of Lefty polypeptides.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of Lefty polypeptides. The most widely used technique for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In certain embodiments, the Lefty polypeptides of the present invention include peptidomimetics. As used herein, the term "peptidomimetic" includes chemically modified peptides and peptide-like molecules that contain non-naturally occurring amino acids, peptoids, and the like. Peptidomimetics provide various advantages over a peptide, including enhanced stability when administered to a subject. Methods for identifying a peptidomimetic are well known in the art and include the screening of databases that contain libraries of potential peptidomimetics. For example, the Cambridge Structural Database contains a collection of greater than 300,000 compounds that have known crystal structures (Allen et al., Acta Crystallogr. Section B, 35:2331 (1979)). Where no crystal structure of a target molecule is available, a structure can be generated using, for example, the program CONCORD (Rusinko et al., J. Chem. Inf. Comput. Sci. 29:251 (1989)). Another database, the Available Chemicals Directory (Molecular Design Limited, Informations Systems; San Leandro Calif.), contains about 100,000 compounds that are commercially available and also can be searched to identify potential peptidomimetics of the Lefty polypeptides. For instance, non-hydrolyzable

peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al., (1986) J. Med. Chem. 29:295; and Ewenson et al., in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), b-turn dipeptide cores (Nagai et al., (1985) Tetrahedron Lett 26:647; and Sato et al., (1986) J Chem Soc Perkin Trans 1:1231), and b-aminoalcohols (Gordon et al., (1985) Biochem Biophys Res Commun 126:419; and Dann et al., (1986) Biochem Biophys Res Commun 134:71).

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

The various Lefty polypeptides may be prepared as fusion proteins. A fusion protein may include one or more additional polypeptide portion that enhance one or more of in vivo stability, in vivo half life, uptake/administration, tissue localization or distribution, formation of protein complexes, and/or purification. For example, a

fusion protein may include an immunoglobulin Fc domain and/or a purification subsequence selected from: an epitope tag, a FLAG tag, a polyhistidine sequence, and a GST fusion. A Lefty polypeptide may include one or more modified amino acid residues selected from: a glycosylated amino acid, a PEGylated amino acid, a farnesylated amino acid, an acetylated amino acid, a biotinylated amino acid, an amino acid conjugated to a lipid moiety, and an amino acid conjugated to an organic derivatizing agent.

A fusion protein or coupled protein system (e.g. non-fusion covalent linkage by crosslinking) may also include a second antagonist domain, which is a polypeptide affinity reagent that selectively binds to Nodal, myostatin and/or GDF-11 and competes with the binding of an ALK7 or ALK4 receptor. The affinity reagent may be an antibody agent. An antibody agent may be, for example, a recombinant antibody; a monoclonal antibody; a VH domain; a VL domain; an scFv; an Fab fragment; an Fab' fragment; an F(ab')2; an Fv; or a disulfide linked Fv, a fully human antibody or a humanized chimeric antibody, or an antigen binding fragment thereof. An affinity reagent is a peptide or scaffolded peptide that selectively binds to Nodal, myostatin and/or Lefty and competes with the binding of an ALK7 or ALK4 receptor. An affinity reagent may include a Nodal, myostatin and/or GDF-11 binding domain of ALK7 or ALK4. For example, an extracellular domain of ALK7 or ALK4 (preferably human ALK7 or ALK4) may be used. The affinity reagent may be a small organic molecule that selectively binds to Nodal, myostatin and/or GDF-11 and competes with the binding of an ALK7 or ALK4 receptor.

An example of a human ALK7 ligand binding domain is shown below:

LKCVCLLCDSSNFTCQTEGACWASVMLTNGKEQVIKSCVSLPELNA QVFCHSSNNVTKTECCFTDFCNNITLHLP (SEQ ID NO:26)

An example of a human ALK4 myostatin binding domain is shown below:

ALLCACTSCLQANYTCETDGACMVSIFNLDGMEHHVRTCIPKVELVP AGK PFYCLSSEDLRNTHCCYTDY (SEQ ID NO:27)

It is understood that different elements of the fusion proteins may be arranged in any manner that is consistent with the desired functionality. For example, a Lefty polypeptide may be placed C-terminal to a heterologous domain, or, alternatively, a heterologous domain may be placed C-terminal to a Lefty polypeptide. The propeptide domain and the heterologous domain need not be adjacent in a fusion protein, and additional domains or amino acid sequences may be included C- or N-terminal to either domain or between the domains.

In certain embodiments, the Lefty polypeptides of the present invention contain one or more modifications that are capable of stabilizing the Lefty polypeptides. For example, such modifications may enhance the in vitro half life of the propeptides, enhance circulatory half life of the propeptides or reducing proteolytic degradation of the propeptides. Such stabilizing modifications include, but are not limited to, fusion proteins (including, for example, fusion proteins comprising a Lefty polypeptide and a stabilizer domain), modifications of a glycosylation site (including, for example, addition of a glycosylation site to a Lefty polypeptide), and modifications of carbohydrate moiety (including, for example, removal of carbohydrate moieties from a Lefty polypeptide). In the case of fusion proteins, a Lefty polypeptide is fused to a stabilizer domain such as an IgG molecule (e.g., an Fc domain). As used herein, the term "stabilizer domain" not only refers to a fusion domain (e.g., Fc) as in the case of fusion proteins, but also includes nonproteinaceous modifications such as a carbohydrate moiety, or nonproteinaceous polymer, such as polyethylene glycol (PEG). PEG may be affixed to Lefty polypeptides in a variety of sizes, ranging from 1000 D to 50,000 D or more molecular weight polymers. PEG polymers may be affixed to propeptides in a selective, residue specific manner, particularly when directed against the N-terminal amine or an engineered cysteine. PEG polymers may also be affixed in a relatively uncontrolled reaction, in which primary amines and/or sulfhydryl groups may be reacted. The stoichiometry may range from 1:1 (PEG:peptide) to 2:1 and higher.

In certain embodiments, the Lefty polypeptide is fused with an immunoglobulin Fc domain. In a preferred embodiment, the Fc domain is an IgG1 Fc fragment. An IgG1 Fc fragment may include various alterations, including, for

example, mutations that reduce binding to Fcγ Receptor and mutations that decreased binding to MHC class I-related Fc-receptor (FcRN). Examples of mutations include mutations in the an Fc portion at positions 265 (Asp to Ala), 322 (Lys to Ala), and 434 (Asn to Ala).

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

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

3. Nucleic Acids Encoding Lefty Polypeptides

In certain aspects, the invention provides isolated and/or recombinant nucleic acids encoding any of the Lefty polypeptides, including derivatives, disclosed herein. The subject nucleic acids may be single-stranded or double stranded. Such nucleic acids may be DNA or RNA molecules. These nucleic acids are may be

used, for example, in methods for making Lefty polypeptides or as direct therapeutic agents (e.g., in a gene therapy approach).

In other embodiments, nucleic acids of the invention also include nucleotide sequences that hybridize under highly stringent conditions to the nucleotide sequence designated in SEQ ID Nos: 13-16, complement sequence of SEQ ID Nos: 13-16, or fragments thereof. As discussed above, one of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. One of ordinary skill in the art will understand readily that appropriate stringency conditions which promote DNA hybridization can be varied. For example, one could perform the hybridization at 6.0 x sodium chloride/sodium citrate (SSC) at about 45 °C, followed by a wash of 2.0 x SSC at 50 °C. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50 °C to a high stringency of about 0.2 x SSC at 50 °C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22 °C, to high stringency conditions at about 65 °C. Both temperature and salt may be varied, or temperature or salt concentration may be held constant while the other variable is changed. In one embodiment, the invention provides nucleic acids which hybridize under low stringency conditions of 6 x SSC at room temperature followed by a wash at 2 x SSC at room temperature.

Isolated nucleic acids which differ from the nucleic acids as set forth in SEQ ID Nos: 13-16 due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-5% of the nucleotides) of the nucleic acids encoding a particular protein may exist among

individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

In certain embodiments, the recombinant nucleic acids of the invention may be operably linked to one or more regulatory nucleotide sequences in an expression construct. Regulatory nucleotide sequences will generally be appropriate to the host cell used for expression. Numerous types of appropriate expression vectors and suitable regulatory sequences are known in the art for a variety of host cells. Typically, said one or more regulatory nucleotide sequences may include, but are not limited to, promoter sequences, leader or signal sequences, ribosomal binding sites, transcriptional start and termination sequences, translational start and termination sequences, and enhancer or activator sequences. Constitutive or inducible promoters as known in the art are contemplated by the invention. The promoters may be either naturally occurring promoters, or hybrid promoters that combine elements of more than one promoter. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome. In a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selectable marker genes are well known in the art and will vary with the host cell used.

In certain aspects of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding a Lefty polypeptide and operably linked to at least one regulatory sequence. Regulatory sequences are art-recognized and are selected to direct expression of the Lefty polypeptide. Accordingly, the term regulatory sequence includes promoters, enhancers, and other expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology*: *Methods in Enzymology*, Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding a Lefty polypeptide. Such useful expression control sequences, include, for example, the

CMV promoter, the early and late promoters of SV40, tet promoter, adenovirus or cytomegalovirus immediate early promoter, RSV promoters, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other protein encoded by the vector, such as antibiotic markers, should also be considered.

A recombinant nucleic acid of the invention can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells (yeast, avian, insect or mammalian), or both. Expression vehicles for production of a recombinant Lefty polypeptides include plasmids and other vectors. For instance, suitable vectors include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

Some mammalian expression vectors contain both prokaryotic sequences to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or

Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant SLC5A8 polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

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

This invention also pertains to a host cell transfected with a recombinant gene including a coding sequence for one or more of the subject Lefty polypeptides. The host cell may be any prokaryotic or eukaryotic cell. For example, a Lefty polypeptide of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (e.g., using a baculovirus expression system), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

Accordingly, the present invention further pertains to methods of producing the subject Lefty polypeptides. For example, a host cell transfected with an expression vector encoding a Lefty polypeptide can be cultured under appropriate

conditions to allow expression of the Lefty polypeptide to occur. The Lefty polypeptide may be secreted and isolated from a mixture of cells and medium containing the propeptide. Alternatively, the polypeptide may be retained cytoplasmically or in a membrane fraction and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for particular epitopes of the Lefty polypeptide. In a preferred embodiment, the Lefty polypeptide is a fusion protein containing a domain which facilitates its purification (purification domain). For example, a fusion gene coding for a purification leader sequence or C-terminal tail, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant Lefty polypeptide, can allow purification of the expressed fusion protein by affinity chromatography using a Ni^{2+} metal resin. The purification sequence can then be subsequently removed by treatment with enterokinase to provide the purified Lefty polypeptide (e.g., see Hochuli et al., (1987) *J. Chromatography* 411:177; and Janknecht et al., *PNAS USA* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

4. Examplary Therapeutic Uses

The subject Lefty polypeptides, such as the full-length and the N-terminally truncated Lefty derivatives, can be used in a number of therapeutic settings to treat a number of diseases resulting from or exacerbated by the presence of myostatin, Nodal or GDF-11.

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

Duchenne Muscular Dystrophy (DMD) was first described by the French neurologist Guillaume Benjamin Amand Duchenne in the 1860s. Becker Muscular Dystrophy (BMD) is named after the German doctor Peter Emil Becker, who first described this variant of DMD in the 1950s. DMD is one of the most frequent inherited diseases in males, affecting one in 3,500 boys. DMD occurs when the dystrophin gene, located on the short arm of the X chromosome, is broken. Since males only carry one copy of the X chromosome, they only have one copy of the dystrophin gene. Without the dystrophin protein, muscle is easily damaged during cycles of contraction and relaxation. While early in the disease muscle compensates by regeneration, later on muscle progenitor cells cannot keep up with the ongoing

damage and healthy muscle is replaced by non-functional fibro-fatty tissue.

In DMD, boys begin to show signs of muscle weakness as early as age 3. The disease gradually weakens the skeletal or voluntary muscles, those in the arms, legs and trunk. By the early teens or even earlier, the boy's heart and respiratory muscles may also be affected. BMD is a much milder version of DMD. Its onset is usually in the teens or early adulthood, and the course is slower and far less predictable than that of DMD. (Though DMD and BMD affect boys almost exclusively, in rare cases they can affect girls.

Until the 1980s, little was known about the cause of any kind of muscular dystrophy. In 1986, the dystrophin gene deficiency was identified as the cause of DMD. BMD results from different mutations in the same gene. BMD patients have some dystrophin, but it's either insufficient in quantity or poor in quality. Having some dystrophin protects the muscles of those with BMD from degenerating as badly or as quickly as those of people with DMD.

Recent results demonstrate that blocking or eliminating Myostatin function *in vivo* can effectively treat at least certain symptoms in DMD and BMD patients (Bogdanovich *et al.*, *supra*; Wagner *et al.*, *supra*). Thus, the subject Lefty derivatives, especially the N-terminally truncated versions thereof, constitute an alternative means of blocking the function of myostatin *in vivo* in DMD and BMD patients.

Similarly, the subject Lefty derivatives, especially the N-terminally truncated versions thereof, provide an effective means to increase muscle mass in other disease conditions that are in need of muscle growth. For example, Gonzalez-Cadavid *et al.* (*supra*) reported that that myostatin expression correlates inversely with fat-free mass in humans and that increased expression of the myostatin gene is associated with weight loss in men with AIDS wasting syndrome. By inhibiting the function of myostatin in AIDS patients, at least certain symptoms of AIDS may be alleviated, if not completely eliminated, thus significantly improving quality of life in AIDS patients.

Since loss of myostatin function is also associated with fat loss without

diminution of nutrient intake (Zimmers *et al.*, *supra*; McPherron and Lee, *supra*), the subject Lefty derivatives, especially the N-terminally truncated versions thereof, may further be used as a therapeutic agent for slowing or preventing the development of obesity and type II diabetes. It should also be noted that Lefty derivatives may affect obesity for reasons unrelated to myostatin.

The cancer anorexia-cachexia syndrome is among the most debilitating and life-threatening aspects of cancer. Progressive weight loss in cancer anorexia-cachexia syndrome is a common feature of many types of cancer and is responsible not only for a poor quality of life and poor response to chemotherapy, but also a shorter survival time than is found in patients with comparable tumors without weight loss. Associated with anorexia, fat and muscle tissue wasting, psychological distress, and a lower quality of life, cachexia arises from a complex interaction between the cancer and the host. It is one of the most common causes of death among cancer patients and is present in 80% at death. It is a complex example of metabolic chaos effecting protein, carbohydrate, and fat metabolism. Tumors produce both direct and indirect abnormalities, resulting in anorexia and weight loss. Currently, there is no treatment to control or reverse the process.

Cancer anorexia-cachexia syndrome affects cytokine production, release of lipid-mobilizing and proteolysis-inducing factors, and alterations in intermediary metabolism. Although anorexia is common, a decreased food intake alone is unable to account for the changes in body composition seen in cancer patients, and increasing nutrient intake is unable to reverse the wasting syndrome. Cachexia should be suspected in patients with cancer if an involuntary weight loss of greater than five percent of premorbid weight occurs within a six-month period.

Since systemic overexpression of myostatin in adult mice was found to induce profound muscle and fat loss analogous to that seen in human cachexia syndromes (Zimmers *et al.*, *supra*), the subject Lefty derivatives, especially the N-terminally truncated versions thereof as a pharmaceutical composition can be beneficially used as a Myostatin antagonist / blocker to prevent, treat, or alleviate the symptoms of the cachexia syndrome, where muscle growth is desired.

In certain embodiments, the subject Lefty polypeptides can be used to form pharmaceutical compositions that can be beneficially used to prevent, treat, or alleviate symptoms of a host of diseases involving neurodegeneration. The subject Lefty polypeptide as a pharmaceutical composition can be beneficially used to prevent, treat, or alleviate symptoms of diseases with neurodegeneration, including Alzheimer's Disease (AD), Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), Huntington's disease, etc.

Alzheimer's disease (AD) is a chronic, incurable, and unstoppable central nervous system (CNS) disorder that occurs gradually, resulting in memory loss, unusual behavior, personality changes, and a decline in thinking abilities. These losses are related to the death of specific types of brain cells and the breakdown of connections between them.

AD has been described as childhood development in reverse. In most people with AD, symptoms appear after the age 60. The earliest symptoms include loss of recent memory, faulty judgment, and changes in personality. Later in the disease, those with AD may forget how to do simple tasks like washing their hands. Eventually people with AD lose all reasoning abilities and become dependent on other people for their everyday care. Finally, the disease becomes so debilitating that patients are bedridden and typically develop coexisting illnesses. AD patients most commonly die from pneumonia, 8 to 20 years from disease onset.

Parkinson's disease (PD) is a chronic, incurable, and unstoppable CNS disorder that occurs gradually and results in uncontrolled body movements, rigidity, tremor, and gait difficulties. These motor system problems are related to the death of brain cells in an area of the brain that produces dopamine - a chemical that helps control muscle activity.

In most people with PD, symptoms appear after age 50. The initial symptoms of PD are a pronounced tremor affecting the extremities, notably in the hands or lips. Subsequent characteristic symptoms of PD are stiffness or slowness of movement, a shuffling walk, stooped posture, and impaired balance. There are wide ranging secondary symptoms such as memory loss, dementia, depression, emotional

changes, swallowing difficulties, abnormal speech, sexual dysfunction, and bladder and bowel problems. These symptoms will begin to interfere with routine activities, such as holding a fork or reading a newspaper. Finally, people with PD become so profoundly disabled that they are bedridden. People with PD usually die from pneumonia.

Amyotrophic lateral sclerosis (ALS; Lou Gehrig's disease; motor neuron disease) is a chronic, incurable, and unstoppable CNS disorder that attacks the motor neurons, components of the CNS that connect the brain to the skeletal muscles. In ALS, the motor neurons deteriorate and eventually die, and though a person's brain normally remains fully functioning and alert, the command to move never reaches the muscles.

Most people who have ALS are between 40 and 70 years old. The first motor neurons that weaken are those leading to the arms or legs. Those with ALS may have trouble walking, they may drop things, fall, slur their speech, and laugh or cry uncontrollably. Eventually the muscles in the limbs begin to atrophy from disuse. This muscle weakness will become debilitating and a person will need a wheel chair or become unable to function out of bed. Most ALS patients die from respiratory failure or from complications of ventilator assistance like pneumonia, 3-5 years from disease onset. Because ALS symptomology relates to a loss of muscle function, treatments that enhance muscle fiber growth or retention, such as anti-myostatin treatments, may be effective.

The causes of these neurological diseases has remained largely unknown. They are conventionally defined as distinct diseases, yet clearly show extraordinary similarities in basic processes and commonly demonstrate overlapping symptoms far greater than would be expected by chance alone. Current disease definitions fail to properly deal with the issue of overlap and a new classification of the neurodegenerative disorders has been called for.

Huntington's disease (HD) is another neurodegenerative disease resulting from genetically programmed degeneration of neurons in certain areas of the brain. This degeneration causes uncontrolled movements, loss of intellectual faculties, and

emotional disturbance. HD is a familial disease, passed from parent to child through a dominant mutation in the wild-type gene. Some early symptoms of HD are mood swings, depression, irritability or trouble driving, learning new things, remembering a fact, or making a decision. As the disease progresses, concentration on intellectual tasks becomes increasingly difficult and the patient may have difficulty feeding himself or herself and swallowing. The rate of disease progression and the age of onset vary from person to person.

Tay-Sachs disease and Sandhoff disease are glycolipid storage diseases caused by the lack of lysosomal β-hexosaminidase (Gravel *et al.*, in *The Metabolic Basis of Inherited Disease*, eds. Scriver et al., McGraw-Hill, New York, pp. 2839-2879, 1995). In both disorders, G_{M2} ganglioside and related glycolipidssubstrates for β-hexosaminidaseaccumulate in the nervous system and trigger acute neurodegeneration. In the most severe forms, the onset of symptoms begins in early infancy. A precipitous neurodegenerative course then ensues, with affected infants exhibiting motor dysfunction, seizure, visual loss, and deafness. Death usually occurs by 2-5 years of age. Neuronal loss through an apoptotic mechanism has been demonstrated (Huang *et al.*, *Hum. Mol. Genet.* **6**: 1879-1885, 1997).

It is well-known that apoptosis plays a role in AIDS pathogenesis in the immune system. However, HIV-1 also induces neurological disease. Shi *et al.* (*J. Clin. Invest.* **98**: 1979-1990, 1996) examined apoptosis induced by HIV-1 infection of the central nervous system (CNS) in an *in vitro* model and in brain tissue from AIDS patients, and found that HIV-1 infection of primary brain cultures induced apoptosis in neurons and astrocytes *in vitro*. Apoptosis of neurons and astrocytes was also detected in brain tissue from 10/11 AIDS patients, including 5/5 patients with HIV-1 dementia and 4/5 nondemented patients.

Neuronal loss is a also a salient feature of prion diseases, such as Creutzfeldt-Jakob disease in human, BSE in cattle (mad cow disease), Scrapie Disease in sheep and goats, and feline spongiform encephalopathy (FSE) in cats.

The subject Lefty polypeptides are also useful to prevent, treat, and alleviate symptoms of various PNS disorders, such as the ones described below. The PNS is

composed of the nerves that lead to or branch off from the CNS. The peripheral nerves handle a diverse array of functions in the body, including sensory, motor, and autonomic functions. When an individual has a peripheral neuropathy, nerves of the PNS have been damaged. Nerve damage can arise from a number of causes, such as disease, physical injury, poisoning, or malnutrition. These agents may affect either afferent or efferent nerves. Depending on the cause of damage, the nerve cell axon, its protective myelin sheath, or both may be injured or destroyed.

The term peripheral neuropathy encompasses a wide range of disorders in which the nerves outside of the brain and spinal cord—peripheral nerves—have been damaged. Peripheral neuropathy may also be referred to as peripheral neuritis, or if many nerves are involved, the terms polyneuropathy or polyneuritis may be used.

Peripheral neuropathy is a widespread disorder, and there are many underlying causes. Some of these causes are common, such as diabetes, and others are extremely rare, such as acrylamide poisoning and certain inherited disorders. The most common worldwide cause of peripheral neuropathy is leprosy. Leprosy is caused by the bacterium *Mycobacterium leprae*, which attacks the peripheral nerves of affected people. According to statistics gathered by the World Health Organization, an estimated 1.15 million people have leprosy worldwide.

Leprosy is extremely rare in the United States, where diabetes is the most commonly known cause of peripheral neuropathy. It has been estimated that more than 17 million people in the United States and Europe have diabetes-related polyneuropathy. Many neuropathies are idiopathic - no known cause can be found. The most common of the inherited peripheral neuropathies in the United States is Charcot-Marie-Tooth disease, which affects approximately 125,000 persons.

Another of the better known peripheral neuropathies is Guillain-Barré syndrome, which arises from complications associated with viral illnesses, such as cytomegalovirus, Epstein-Barr virus, and human immunodeficiency virus (HIV), or bacterial infection, including Campylobacter jejuni and Lyme disease. The worldwide incidence rate is approximately 1.7 cases per 100,000 people annually.

Other well-known causes of peripheral neuropathies include chronic alcoholism, infection of the varicella-zoster virus, botulism, and poliomyelitis. Peripheral neuropathy may develop as a primary symptom, or it may be due to another disease. For example, peripheral neuropathy is only one symptom of diseases such as amyloid neuropathy, certain cancers, or inherited neurologic disorders. Such diseases may affect the peripheral nervous system (PNS) and the central nervous system (CNS), as well as other body tissues.

Other PNS diseases treatable with the subject Lefty derivatives, especially the N-terminally truncated Lefty derivatives include: Brachial Plexus Neuropathies (Diseases of the cervical and first thoracic roots, nerve trunks, cords, and peripheral nerve components of the brachial plexus. Clinical manifestations include regional pain, paresthesia; muscle weakness, and decreased sensation in the upper extremity. These disorders may be associated with trauma, including birth injuries; thoracic outlet syndrome; neoplasms, neuritis, radiotherapy; and other conditions. See Adams *et al.*, Principles of Neurology, 6th ed, pp1351-2); Diabetic Neuropathies (Peripheral, autonomic, and cranial nerve disorders that are associated with disbetes mellitus. These conditions usually result from diabetic microvascular injury involving small blood vessels that supply nerves (vasa nervorum). Relatively common conditions which may be associated with diabetic neuropathy include third nerve palsy; mononeuropathy; mononeuropathy multiplex; diabetic amyotrophy; a painful polyneuropathy; autonomic neuropathy; and thoracoabdominal neuropathy. See Adams *et al.*, Principles of Neurology, 6th ed, p1325); Mononeuropathies (Disease or trauma involving a single peripheral nerve in isolation, or out of proportion to evidence of diffuse peripheral nerve dysfunction. Mononeuropathy multiplex refers to a condition characterized by multiple isolated nerve injuries. Mononeuropathies may result from a wide variety of causes, including ischemia; traumatic injury; compression; connective tissue diseases; cumulative trauma disorders; and other conditions); Neuralgia (Intense or aching pain that occurs along the course or distribution of a peripheral or cranial nerve); Peripheral Nervous System Neoplasms (Neoplasms which arise from peripheral nerve tissue. This includes neurofibromas; Schwannomas; granular cell tumors; and malignant peripheral nerve sheath tumors.

See DeVita Jr *et al.*, Cancer: Principles and Practice of Oncology, 5th ed, pp1750-1); Nerve Compression Syndromes (Mechanical compression of nerves or nerve roots from internal or external causes. These may result in a conduction block to nerve impulses, due to, for example, myelin sheath dysfunction, or axonal loss. The nerve and nerve sheath injuries may be caused by ischemia; inflammation; or a direct mechanical effect); Neuritis (A general term indicating inflammation of a peripheral or cranial nerve. Clinical manifestation may include pain; paresthesias; paresis; or hyperthesia); Polyneuropathies (Diseases of multiple peripheral nerves. The various forms are categorized by the type of nerve affected (e.g., sensory, motor, or autonomic), by the distribution of nerve injury (e.g., distal vs. proximal), by nerve component primarily affected (e.g., demyelinating vs. axonal), by etiology, or by pattern of inheritance).

Myostatin inhibitors may also promote bone growth and a combination of bone and muscle strengthening, making the Lefty polypeptides disclosed herein useful for treating disorders such as osteoporosis, frailty, low bone density and tumor induced bone loss.

While the above description provides likely mechanisms of action by which Lefty polypeptides, including Lefty derivatives, may achieve desirable therapeutic effects, such effects may also result from alternative mechanisms or several different mechanisms. It is quite likely that Lefty polypeptides will inhibit additional members of the TGF-beta family, and accordingly, such activity may contribute to a therapeutic effect.

5. Exemplary Formulations

The subject compositions may be used alone, or as part of a conjoint therapy with other compounds / pharmaceutical compositions. In certain embodiments, a pharmaceutical preparation comprising a Lefty polypeptide will be in compliance with guidelines established by the relevant regulatory agency (e.g., the Food and Drug Administration in the U.S.). Such a preparation will typically be substantially pyrogen-free.

The Lefty polypeptides for use in the subject methods may be conveniently formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the phosphopeptide therapeutics, its use in the pharmaceutical preparation of the disclosure is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (*Remington's Pharmaceutical Sciences*. Mack Publishing Co., Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations."

Pharmaceutical formulations of the present disclosure can also include veterinary compositions, e.g., pharmaceutical preparations of the Lefty therapeutics suitable for veterinary uses, e.g., for the treatment of live stock (cow, sheep, goat, pig, and horse, etc.) or domestic animals, e.g., cats and dogs.

Methods of disclosure may also be provided by rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a therapeutic at a particular target site.

The pharmaceutical compositions according to the present disclosure may be administered as either a single dose or in multiple doses. The pharmaceutical compositions of the present disclosure may be administered either as individual therapeutic agents or in combination with other therapeutic agents. The treatments of

the present disclosure may be combined with conventional therapies, which may be administered sequentially or simultaneously. The pharmaceutical compositions of the present disclosure may be administered by any means that enables the Lefty polypeptides to reach the targeted cells / tissues / organs. In some embodiments, routes of administration include those selected from the group consisting of oral, intravesically, intravenous, intraarterial, intraperitoneal, local administration into the blood supply of the organ in which the targeted cells reside or directly into the cells. Intravenous administration is the preferred mode of administration. It may be accomplished with the aid of an infusion pump.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal and intrastermal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, intravesically, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracisternally and topically, as by powders, ointments or drops, including buccally and sublingually.

Regardless of the route of administration selected, the compounds of the present disclosure, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present disclosure, are formulated into pharmaceutically acceptable dosage forms such as described below or by other

conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this disclosure may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present disclosure employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular phosphopeptide therapeutic employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the disclosure employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In general, a suitable daily dose of a compound of the disclosure will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, intracerebrovenitricular and subcutaneous doses of the compounds of this disclosure for a patient will range from about 0.0001 to about 100 mg per kilogram of body weight per day.

If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

The term "treatment" is intended to encompass also prophylaxis, therapy and

cure.

The patient receiving this treatment is any animal in need, including primates, in particular humans, and other non-human mammals such as equines, cattle, swine and sheep; and poultry and pets in general.

The compound of the disclosure can be administered as such or in admixtures with pharmaceutically acceptable carriers and can also be administered in conjunction with other antimicrobial agents such as penicillins, cephalosporins, aminoglycosides and glycopeptides. Conjunctive therapy, thus includes sequential, simultaneous and separate administration of the active compound in a way that the therapeutical effects of the first administered one is not entirely eliminated when the subsequent is administered.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present disclosure include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook *et al.*, (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel *et al.*, "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Md. (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson *et al.*, "Recombinant DNA", Scientific American Books, New York; Birren *et al.* (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites *et al.* (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262;

3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, Calif. (1990); Marshak *et al.*, "Strategies for Protein Purification and Characterization—A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXEMPLIFICATION

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

Example 1. Construction, expression, and purification of Lefty polypeptides

The various Lefty-A constructs described in Figure 6 were subcloned into pAID4 vector and plasmid cDNAs were transfected (10ug DNA) transiently into Cos cells by lipofectamine method. After six hours, growth media was added. Twenty four hours later, media was changed to serum free media and harvested after 48 hours. After centrifugation, supernatant was collected and kept at -20C.

The constructs produced and tested replace the endogenous Lefty-A signal sequence with a honey melatin sequence. The following Lefty-A sequence was fused to the melatin for expression of the 34 kDa (long) form:

LTEEQLLGSLLRQLQLSEVPVLDRADMEKLVIPAHVRAQYVVLLRRSHGDR
SRGKRFSQSFREVAGRFLASEASTHLLVFGMEQRLPPNSELVQAVLRLFQEP
VPKAALHGHGRLSPRSAQARVTVEWLRVRDDGSNRTSLIDSRLVSVHESGW
KAFDVTEAVNFWQQLSRPRQPLLLQVSVQREHLGPLASGAHKLVRFASQG
APAGLGEPQLELHTLDLRDYGAQGDCDPEAPMTEGTRCCRQEMYIDLQGM
KWAKNWVLEPPGFLAYECVGTCQQPPEALAFNWPFLGPRQCIASETASLPM
IVSIKEGGRTRPQVVSLPNMRVQKCSCASDGALVPRRLQP

The bold "F" indicates the expected first amino acid of the mature 34 kDa form. The underlined "G" shows the site of an R -> G alteration to eliminate the second RXXR cleavage site.

The following sequence was fused to the melatin leader for expression of the 28 kDa (short) form of Lefty-A:

LSPRSAQARVTVEWLRVRDDGSNRTSLIDSRLVSVHESGWKAFDVTEAVNF
WQQLSRPRQPLLLQVSVQREHLGPLASGAHKLVRFASQGAPAGLGEPQLEL
HTLDLRDYGAQGDCDPEAPMTEGTRCCRQEMYIDLQGMKWAKNWVLEPP
GFLAYECVGTCQQPPEALAFNWPFLGPRQCIASETASLPMIVSIKEGGRTRPQ
VVSLPNMRVQKCSCASDGALVPRRLQP

The various Fc fusions described in Figure 6 were generated based on the above "Lefty 34" and "Lefty 28" constructs.

Example 2. Lefty-A polypeptides bind to myostatin

BiaCore chip analysis was carried out with each of the Lefty constructs. GDF-11 is a close homolog of myostatin that regulates neurological processes. GDF-11 was immobilized on a BiaCore CM5 chip using standard amine coupling procedure. As shown in Figure 7, each of the Lefty constructs binds to myostatin.

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

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

CLAIMS:

1. A recombinant Lefty derivative polypeptide comprising an amino acid sequence as set forth in the formula: -A-X-B-,

wherein A consists essentially of an amino acid sequence at least 85% identical to the sequence of Region 2 of SEQ ID NO:1;

wherein B consists essentially of an amino acid sequence at least 85% identical to the sequence of Region 4 of SEQ ID NO:1;

wherein X consists of zero, one or more than one amino acid; and

wherein the recombinant, Lefty derivative polypeptide binds to one or more of Nodal, myostatin and GDF-11.

2. The recombinant Lefty derivative polypeptide of claim 1, wherein the recombinant Lefty derivative polypeptide comprises an amino acid sequence that is at least 85% identical to the cystine knot portion of a human Lefty polypeptide.

3. The recombinant Lefty derivative polypeptide of claim 1, wherein the recombinant Lefty derivative polypeptide comprises an amino acid sequence that is at least 85% identical to a human Lefty polypeptide sequence selected from the group consisting of: amino acids 22-353 of SEQ ID NO:1 and amino acids 22-353 of SEQ ID NO:2, wherein one or both RXXR cleavage sequences are altered so as to prevent cleavage at the altered sequence.

4. The recombinant Lefty derivative polypeptide of claim 1, wherein A consists of an amino acid sequence at least 95% identical to the sequence of Region 2 of SEQ ID NO:1 and wherein B consists of an amino acid sequence at least 95% identical to the sequence of Region 4 of SEQ ID NO:1.

5. The recombinant Lefty derivative polypeptide of claim 1, wherein A is selected from the group consisting of: CRQEMYIDLQGMKWAKNWVLEPPG FLAYECVGT (SEQ ID NO: 5) and CRQEMYIDLQGMKWAENWVLEPPGFLAYECVGT (SEQ ID NO: 7), and wherein B is selected from the group consisting of: CIASETASLPMIVSIKEGGRTRPQVVSLPNMRVQKC (SEQ ID NO: 6) and CIASETDSLPMIVSIKEGGRTRPQVVSLPNMRVQKC (SEQ ID NO: 8).

6. The recombinant Lefty derivative polypeptide of claim 1, wherein X comprises an amino acid sequence that has low immunogenicity.

7. The recombinant Lefty derivative polypeptide of claim 1, wherein X comprises a glycosylation site.

8. The recombinant Lefty derivative polypeptide of claim 1, wherein the length of X is between 0-50 amino acids.

9. The recombinant Lefty derivative polypeptide of claim 1, wherein X comprises a dimerization domain.

10. The recombinant Lefty derivative polypeptide of any of claims 1-8, wherein the polypeptide is fused to an additional domain.

11. The recombinant Lefty derivative polypeptide of claim 10, wherein the additional domain is a dimerization domain.

12. The recombinant Lefty derivative polypeptide of claim 10, wherein the additional domain is fused to the carboxyl or amino terminus of the Lefty polypeptide.

13. The recombinant Lefty derivative polypeptide of claim 9 or 11, wherein the dimerization domain comprises a Lefty propeptide sequence.

14. The recombinant Lefty derivative polypeptide of claim 9 or 11, wherein the dimerization domain comprises an immunoglobulin Fab constant domain.

15. The recombinant Lefty derivative polypeptide of claim 14, wherein said immunoglobulin Fab constant domain is selected from an immunoglobulin heavy chain constant region and an immunoglobulin light chain constant region.

16. The recombinant Lefty derivative polypeptide of claim 9 or 11, wherein the dimerization domain is a leucine zipper domain.

17. The recombinant Lefty derivative polypeptide of claim 16, wherein said leucine zipper domain comprises at least four leucine heptads.

18. The recombinant Lefty derivative polypeptide of claim 17, wherein said leucine zipper domain is selected from the group consisting of a Fos and a

Jun leucine zipper domain.

19. The recombinant Lefty derivative polypeptide of claim 11, further comprising a linker sequence interposed between and covalently joining the Lefty polypeptide and the dimerization domain.

20. The recombinant Lefty derivative polypeptide of claim 1, wherein X comprises a domain that binds Nodal, myostatin and/or GDF-11.

21. The recombinant Lefty derivative polypeptide of claim 1, wherein the additional domain is a domain that binds Nodal, myostatin and/or GDF-11.

22. The recombinant Lefty derivative polypeptide of claim 20 or 21, wherein the domain that binds Nodal, myostatin and/or GDF-11 inhibits the binding of Nodal, myostatin and/or GDF-11 to a Type I receptor.

23. The recombinant Lefty derivative polypeptide of claim 22 wherein the domain that binds Nodal, myostatin and/or GDF-11 competitively inhibits the binding of Nodal, myostatin and/or GDF-11 to a Type I receptor selected from the group consisting of ALK4 and ALK7.

24. The recombinant Lefty derivative polypeptide of claim 22, wherein the domain that binds Nodal, myostatin and/or GDF-11 is selected from the group consisting of:

(a) an extracellular portion of ALK4;

(b) an extracellular portion of ALK7;

(c) an antigen-binding portion of an antibody that binds Nodal, myostatin and/or GDF-11; and

(d) a randomized polypeptide that has been selected for binding to Nodal, myostatin and/or GDF-11.

25. The recombinant Lefty derivative polypeptide of any of claims 1-24, wherein said modified Lefty polypeptide inhibits signaling mediated by a protein selected from an ActRII receptor, myostatin, Nodal, and GDF-11 in a cell.

26. The recombinant Lefty derivative polypeptide of any of claims 1-25, wherein said modified Lefty polypeptide comprises a heterogenous sequence that mediates secretion of the recombinant Lefty derivative polypeptide.

27. The recombinant Lefty derivative polypeptide of claim 26, wherein said

heterogenous sequence that mediates secretion of the recombinant Lefty derivative polypeptide is a honey bee melatin leader sequence.

28. A recombinant polynucleotide comprising a nucleotide sequence encoding a Lefty derivative polypeptide of any of claims 1-27.

29. The recombinant polynucleotide of claim 28, further comprising a promoter sequence operably linked to the nucleotide sequence encoding the Lefty derivative polypeptide.

30. A cell transformed with a recombinant polynucleotide of claim 29.

31. The cell of claim 30, wherein the cell is a mammalian cell.

32. The cell of claim 31, wherein the cell is a human cell.

33. A method of making a recombinant Lefty derivative polypeptide, comprising:

a) culturing a cell of claim 30 under conditions suitable for expression of the recombinant Lefty derivative polypeptide; and

b) recovering the recombinant Lefty derivative polypeptide so expressed.

34. An isolated Lefty polypeptide complex comprising:

a) a first Lefty polypeptide; and

b) a second Lefty polypeptide,

wherein the first and second Lefty polypeptides are associated to form a complex, and wherein the complex binds to a TGF-β family member selected from the group consisting of: myostatin, Nodal and GDF-11.

35. The Lefty polypeptide complex of claim 34, wherein the polypeptide complex is a homodimer.

36. A pharmaceutical preparation comprising a modified Lefty polypeptide of any of claims 1-26 or 34-35.

37. A method for inhibiting the activity of GDF-11 and/or myostatin in vivo, the method comprising administering to the subject an effective amount of a Lefty polypeptide.

38. A method for treating a subject having a disorder associated with muscle loss or insufficient muscle growth, comprising administering to the subject an effective amount of a composition comprising a Lefty polypeptide.

39. The method of claim 38, wherein the subject has a condition selected from muscle atrophy, ALS, and a muscle wasting disorder.

40. The method of claim 39, wherein the muscle wasting disorder is selected from the group consisting of cachexia, anorexia, DMD syndrome, BMD syndrome, AIDS wasting syndrome, muscular dystrophies, neuromuscular diseases, motor neuron diseases, diseases of the neuromuscular junction, and inflammatory myopathies.

41. A method for treating a subject having a disorder associated with neurodegeneration, comprising administering to the subject an effective amount of a composition comprising a Lefty polypeptide.

42. The method of claim 41, wherein the disorder is selected from the group consisting of Alzheimer's Disease (AD), Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS), Huntington's disease (HD).

43. A method for inhibiting the activity of GDF-11 and/or myostatin in vitro, the method comprising administering to the cells an effective amount of a Lefty polypeptide.

44. A method for decreasing the body fat content or reducing the rate of increase in body fat content in a subject, comprising administering to a subject in need thereof an effective amount of a Lefty polypeptide.

45. A method for treating a disorder associated with undesirable body weight gain in a subject, comprising administering to a subject in need thereof an effective amount of a Lefty polypeptide.

46. The method of claim 45, wherein said disorder is selected from the group consisting of obesity, non-insulin dependent diabetes mellitus (NIDDM), cardiovascular disease, cancer, hypertension, osteoarthritis, stroke, respiratory problems, and gall bladder disease.

47. The method of any of claims 37-46 wherein the Lefty polypeptide is selected from a wildtype Lefty polypeptide or fragments thereof, a recombinant Lefty derivative polypeptide, and a dimerized Lefty polypeptide.

48. Use of a Lefty polypeptide for making a medicament for the treatment of a disorder associated with abnormal amount, development or metabolic activity of muscle tissue.

49. Use of a Lefty polypeptide for making a medicament for the treatment of a disorder associated with neurodegeneration.

50. Use of a Lefty polypeptide for making a medicament for the treatment of a disorder associated with undesirable body fat content.

51. The use of claim 50, wherein said disorder is selected from the group consisting of obesity and non-insulin dependent diabetes mellitus (NIDDM).

52. The use of any of claims 48-51 wherein the Lefty polypeptide is selected from a wildtype Lefty polypeptide or fragments thereof, a recombinant Lefty derivative polypeptide, and a dimerized Lefty polypeptide.

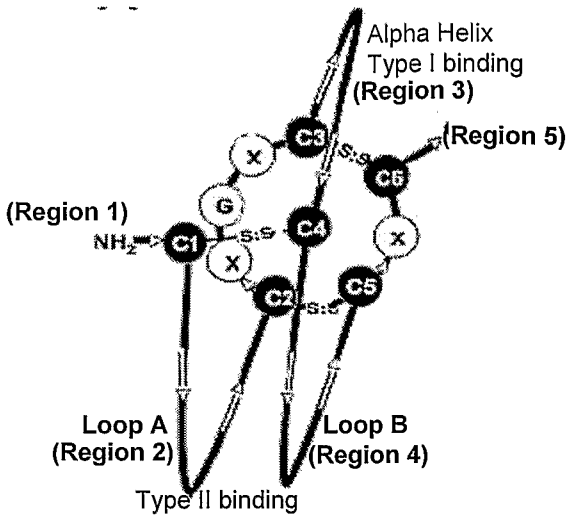
Alpha Helix
Type I binding
(Region 3)
(Region 5)
(Region 1)
Loop A
(Region 2)
Loop B
(Region 4)
Type II binding

Figure 1

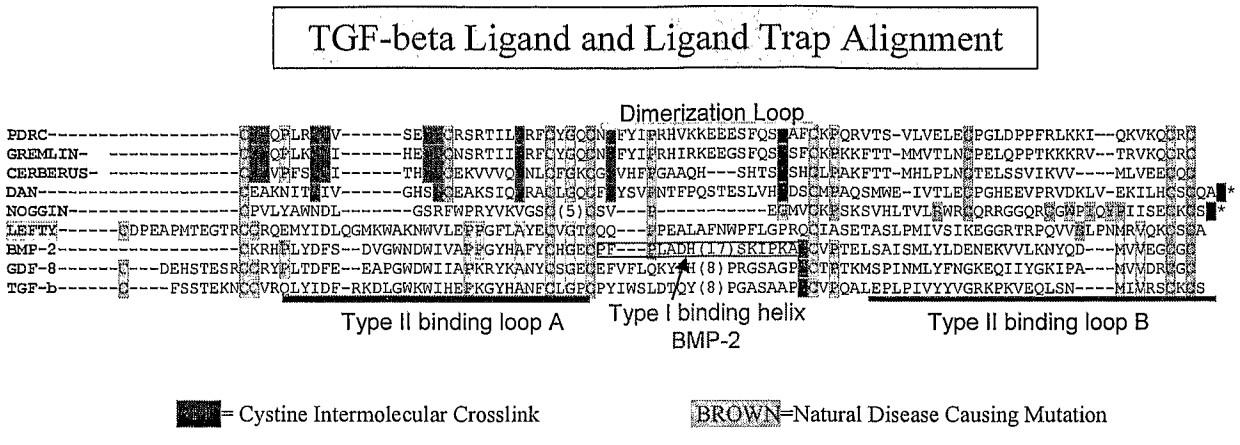
TGF-beta Ligand and Ligand Trap Alignment
Dimerization Loop
Type II binding loop A
Type I binding helix
BMP-2
Type II binding loop B
= Cystine Intermolecular Crosslink
BROWN=Natural Disease Causing Mutation
(X)= Number of Deleted Residues

Figure 2

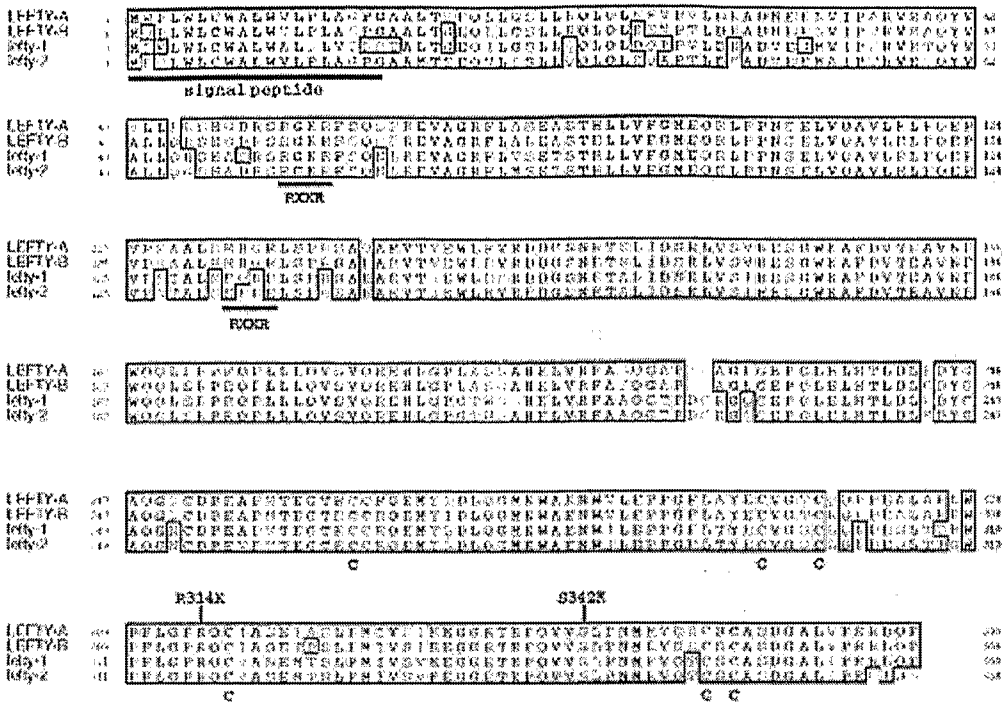

Figure 3

Human Lefty-A protein (NCBI RefSeq ID NP_003231):

```
  1 MWPLWLCWAL WVLPLAGPGA ALTEEQLLGS LLRQLQLSEV PVLDRADMEK LVIPAHVRAQ
 61 YVVLLRRSHG DRSRGKRFSQ SFREVAGRFL ASEASTHLLV FGMEQRLPPN SELVQAVLRL
121 FQEPVPKAAL HRHGRLSPRS AQARVTVEWL RVRDDGSNRT SLIDSRLVSV HESGWKAFDV
181 TEAVNFWQQL SRPRQPLLLQ VSVQREHLGP LASGAHKLVR FASQGAPAGL GEPQLELHTL
241 DLRDYGAQGD CDPEAPMTEG TRCCRQEMYI DLQGMKWAKN WVLEPPGFLA YECVGTCQQP
301 PEALAFNWPF LGPRQCIASE TASLPMIVSI KEGGRTRPQV VSLPNMRVQK CSCASDGALV
361 PRRLQP
```

Human Lefty-B protein (NCBI RefSeq ID NP_066277):

```
  1 MQPLWLCWAL WVLPLASPGA ALTGEQLLGS LLRQLQLKEV PTLDRADMEE LVIPTHVRAQ
 61 YVALLQRSHG DRSRGKRFSQ SFREVAGRFL ALEASTHLLV FGMEQRLPPN SELVQAVLRL
121 FQEPVPKAAL HRHGRLSPRS ARARVTVEWL RVRDDGSNRT SLIDSRLVSV HESGWKAFDV
181 TEAVNFWQQL SRPRQPLLLQ VSVQREHLGP LASGAHKLVR FASQGAPAGL GEPQLELHTL
241 DLGDYGAQGD CDPEAPMTEG TRCCRQEMYI DLQGMKWAEN WVLEPPGFLA YECVGTCRQP
301 PEALAFKWPF LGPRQCIASE TDSLPMIVSI KEGGRTRPQV VSLPNMRVQK CSCASDGALV
361 PRRLQP
```

Figure 4

Human Lefty-A protein (34 kDa mature form):

```
  1  FSQSFREVAG RFLASEASTH LLVFGMEQRL PPNSELVQAV LRLFQEPVPK AALHRHGRLS
                                     (REGION 1)

 61  PRSAQARVTV EWLRVRDDGS NRTSLIDSRL VSVHESGWKA FDVTEAVNFW QQLSRPRQPL
                                     (REGION 1, CONT.)

121  LLQVSVQREH LGPLASGAHK LVRFASQGAP AGLGEPQLEL HTLDLRDYGA QGDCDPEAPM
                                     (REGION 1, CONT.)

181  TEGTRCCRQE MYIDLQGMKW AKNWVLEPPG FLAYECVGTC QQPPEALAFN WPFLGPRQCI
               (REGION 2)                              (REGION 3)

241  ASETASLPMI VSIKEGGRTR PQVVSLPNMR VQKCSCASDG ALVPRRLQP
       (REGION 4)                          (REGION 5)
```

Human Lefty-B protein (34 kDa mature form):

```
  1  FSQSFREVAG RFLALEAST HLLVFGMEQR LPPNSELVQA VLRLFQEPVPK AALHRHGRLS
                                     (REGION 1)

 61  PRSARARVTV EWLRVRDDG SNRTSLIDSR LVSVHESGWK AFDVTEAVNFW QQLSRPRQPL
                                     (REGION 1, CONT.)

121  LLQVSVQREH LGPLASGAH KLVRFASQGA PAGLGEPQLE LHTLDLGDYGA QGDCDPEAPM
                                     (REGION 1, CONT.)

181  TEGTRCCRQE MYIDLQGMK WAENWVLEPP GFLAYECVGT CRQPPEALAFK WPFLGPRQCI
               (REGION 2)                              (REGION 3)

241  ASETDSLPMI VSIKEGGRT RPQVVSLPNM RVQKCSCASD GALVPRRLQP
       (REGION 4)                          (REGION 5)
```

Figure 5

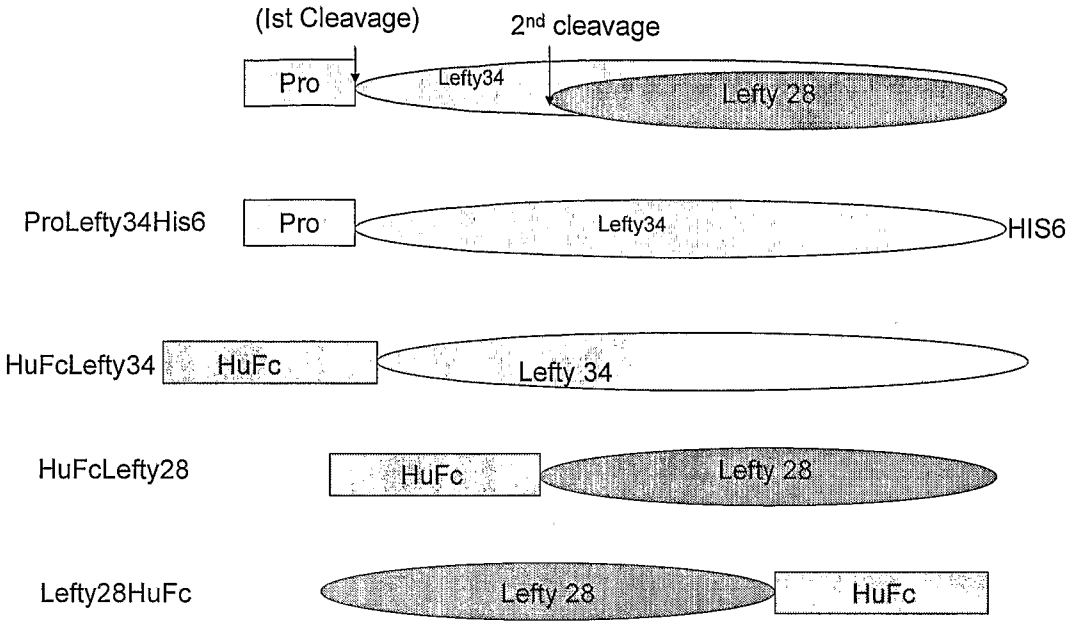
(Ist Cleavage)
2nd cleavage
Pro
Lefty34
Lefty 28
ProLefty34His6
Pro
Lefty34
HIS6
HuFcLefty34
HuFc
Lefty 34
HuFcLefty28
HuFc
Lefty 28
Lefty28HuFc
Lefty 28
HuFc

Figure 6

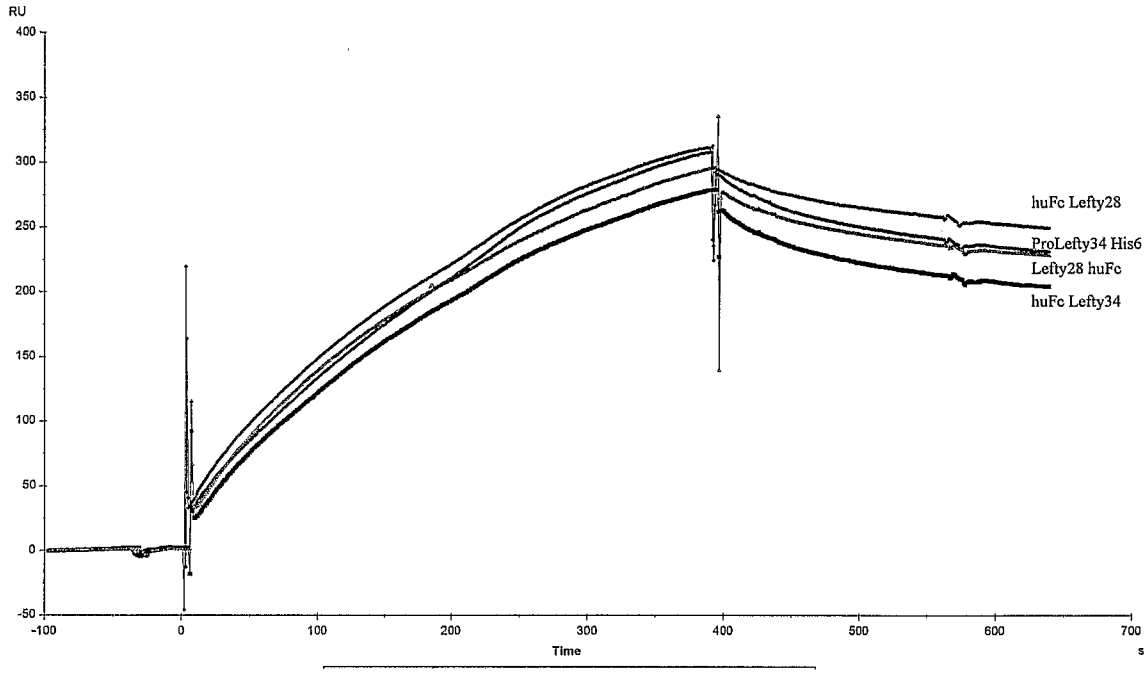
RU
400
350
300
250
200
150
100
50
0
-50
-100
0
100
200
300
400
500
600
700
Time
s
huFc Lefty28
ProLefty34 His6
Lefty28 huFc
huFc Lefty34

Figure 7