

## STABILIZED INSULIN-LIKE GROWTH FACTOR POLYPEPTIDES

### Background of the Invention

Insulin-like growth factors (IGFs) are part of a complex system that cells use to communicate with their physiologic environment. This complex system (often referred to as the insulin-like growth factor axis) consists of two cell-surface receptors (IGF-1R and IGF-2R), two ligands (IGF-1 and IGF-2), a family of six high-affinity IGF-binding proteins (IGFBP 1-6), and associated IGFBP degrading enzymes (proteases). This system is important not only for the regulation of normal physiology but also for a number of pathological states (Glass, Nat Cell Biol 5:87-90, 2003).

The IGF axis has been shown to play roles in the promotion of cell proliferation and the inhibition of cell death (apoptosis). IGF-1 is mainly secreted by the liver as a result of stimulation by human growth hormone (hGH). Almost every cell in the human body is affected by IGF-1, especially cells in muscles, cartilage, bones, liver, kidney, nerves, skin and lungs. In addition to the insulin-like effects, IGF-1 can also regulate cell growth. IGF-1 and IGF-2 are regulated by a family of gene products known as the IGF-binding proteins. These proteins help to modulate IGF action in complex ways that involve both inhibiting IGF action by preventing binding to the IGF receptors as well as promoting IGF action through aiding delivery to the receptors and increasing IGF half life in the blood stream. There are at least six characterized binding proteins (IGFBP1-6).

In its mature form, human IGF-1 (gpetlcgaelvdaqlfvcgdrfyfnkptgygssrrapqtgividccfrscdlrrlem ycaplpaksa; SEQ ID NO:1), also called somatomedin, is a small protein of 70 amino acids that has been shown to stimulate growth of a wide range of cells in culture. The mature protein is initially encoded by three known splice variant mRNAs. The open reading frame of each mRNA encodes a precursor protein containing the 70 amino acid IGF-1 and a particular E-peptide at the C-terminus, depending on the particular IGF-1 mRNA. These E-peptides have been termed the Ea (rsvraqrhtdmpktqkevhlnasrgsagnknym; SEQ ID NO:2), Eb (rsvraqrhtdmpktqkyqppstnknt ksqrkgwpgkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk; SEQ ID NO:3), and Ec (rsvraqrhtdmpktqkyqppstnkntksqrkgstfeerk; SEQ ID NO:4) peptides and range from 35 to 87 amino acids in

length and encompass a common sequence region at the N-terminus and a variable sequence region at the C-terminus. For example, the wild-type open reading frame for the IGF-1-Ea encodes a polypeptide of 105 amino acids (gpetlcgaelvdaqlqfvcgdrgrfyfinkptgygssrrapqtgvideccfrscdlrrlemypcaplkpaksarsvraqrhtdmptkqevhlknasrgsagnknyrm; SEQ ID NO:5). In physiological expression, the E-peptides are cleaved off of the precursor by endogenous proteases to yield the mature 70 amino acid IGF-1 known to be bioactive. In certain contexts, one to three of the N-terminal amino acids of IGF-1 are known to be cleaved under physiological conditions, yielding active IGF-1 having between 67-70 amino acids. IGF-2 gene expression and processing is characterized by similar attributes except that only one E-peptide (rdvstpptvlpdnfprypvgkffqydtwkqstqrlrrglpallrarrghvlakeleafreakhrplialptqdpahggappemasnrk; SEQ ID NO:6) for human IGF-2 has been identified for the 156 amino acid precursor (ayrpsetlcggelvdltqfvcgdrgrfyfsrpsrsvrrsrgiveeccfrscdlalletycatpakserdvstpptvlpdnfprypvgkffqydtwkqstqrlrrglpallrarrghvlakeleafreakhrplialptqdpahggappemasnrk; SEQ ID NO:7). Both IGF-1 and IGF-2 appear to be poor drug candidates, since these proteins are quickly degraded by endogenous proteases in the serum of patients. One strategy that has been contemplated is to stabilize IGF-1 as a drug by forming a complex with one of its binding proteins.

### Summary of the Invention

The invention is based on the discovery that a precursor IGF-1 or IGF-2 protein containing substantially its E-peptide is bioactive and stabilized in the presence of serum, resulting in an IGF-1 or IGF-2 polypeptide that is useful as a pharmaceutical. In the compositions of the invention, the normal cleavage of the E-peptide from IGF-1 is avoided, for example, by mutating or deleting either of the arginine at position 1 or the serine at position 2 of the E-peptides (corresponding to positions 71 and 72 in the wild-type precursor IGF-1). In IGF-2, the cleavage is avoided, for example, by mutating or deleting either the arginine at position 1 or the aspartic acid at position 2 of the E-peptide (corresponding to positions 68 and 69 in the wild-type precursor IGF-2). Other modifications of an IGF precursor protein can avoid or reduce this cleavage.

In addition, further modifications of the IGF-1 precursor amino acid sequence can confer additional pharmaceutical benefits. For example, the polypeptides of the invention can exhibit

increased affinity for the IGF-1 receptor or decreased binding ability to an inhibitory IGF-1 or IGF-2 binding protein.

For the sake of clarity and consistency, the numbering of amino acid residues in IGF-1 or IGF-2 precursor or mature proteins throughout this application and in the claims is based on the wild-type precursor protein sequence numbering without signal peptide.

Accordingly, the invention includes a polypeptide containing a human IGF-1 precursor protein, where the cleavage of the E-peptide from IGF-1 by a protease is reduced by modification of the precursor protein. The E-peptide can be the Ea, Eb, or Ec peptide. At the N-terminus of the precursor, amino acids G1, P2, or E3 of the precursor protein can be deleted or mutated, as can R36 (e.g., R36A) and R37 (e.g., R37A).

The precursor protein can further include the N-linked glycosylation consensus sequence NXS/T, for example by insertion of amino acids 93-102 of Ea between amino acids N95 and T96 of the Eb. In general, the precursor protein can include an oligosaccharide covalently linked to an amino acid side chain of the precursor protein, such as an arginine side chain of the precursor protein.

In addition, a residue of the precursor protein can be replaced by a non-natural amino acid (e.g., one that includes an acetylene or azido group). Such non-natural amino acids can facilitate linkage of a poly(ethylene glycol) moiety to a side-chain of the precursor protein, though typical protein pegylation strategies are well known in the art.

The precursor protein can further include one or more additional E-peptides linked to the C-terminus of the precursor protein. For example, a polypeptide can include, from N-terminus to C-terminus, (1) an IGF-1 precursor protein having a first Eb peptide, where G1, P1, and E1 are deleted, either R36 or R37 or both are mutated, R71 and S72 are deleted, and the last seven C-terminal amino acids of the first Eb peptide are deleted; (2) a second Eb peptide, where R71, S72, and the last seven C-terminal amino acids of the second Eb peptide are deleted; (3) a third

Eb peptide, where R71, S72, and the last seven C-terminal amino acids of the third Eb peptide are deleted; and (4) a fourth Eb peptide, where R71 and S72 are deleted.

5 An effective means of preventing cleavage of the E-peptide from the IGF-1 is the deletion or mutation of R71 or S72.

10 Similarly, the invention includes a human IGF-2 precursor protein where the cleavage of the E-peptide from IGF-2 by a protease is reduced by modification of the precursor protein. In particular, deletion or mutation of R68 or D69 can be an effective means of avoiding protease digestion of the IGF-2 precursor protein.

In addition, any E-peptide of IGF-1 can be combined with an IGF-2 and any E-peptide of IGF-2 can be combined with IGF-1 to provide the benefits described herein.

15 The invention further includes a method of treating a musculoskeletal disease, diabetes, neuronal cell death by administering a therapeutically effective amount of a polypeptide of the invention. Likewise, the invention includes the use of a polypeptide of the invention for the manufacture of a medicament for the treatment of a musculoskeletal disease, diabetes, neuronal cell death, or anemia.

20 In another embodiment, the invention includes a pegylated IGF-1 without an E-peptide but having introduced therein a non-natural amino acid as the site of pegylation. Any of the modified, pegylated IGF-1 containing a non-natural amino acid as disclosed herein, without an E-peptide, is also included in the invention.

25 The invention also includes veterinary methods and uses of administering an effective amount of the polypeptide of the invention to obtain a desired effect.

30 The veterinary uses include (i) enhancing the rate and/or extent of growth in an animal, (ii) enhancing the efficiency of their conversion of feed into body tissue, (iii) enhancing milk production in lactating animals, (iv) treating animal wasting symptoms associated with cachexia,

trauma, or other consumption diseases, and (v) treating lactating animals for improvement in neonatal health.

All cited references or documents are hereby incorporated by reference.

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#### Brief Description of the Drawings

**Figs. 1A-1C** are Western blots of polypeptides of the invention and wild-type IGF-1 precursor after zero or 16-hour incubation in the presence or absence of 10 % human serum at 37°C.

Expression vectors encoding various IGF-1 constructs were transfected into Cos7 cells, and the conditioned culture medium obtained. The "3mut" refers to a hIGF-1-E-peptide precursor having the following three sets of modifications: deletion of G1, P2, and E3; mutation of Arg 37 to Ala (R37A); and deletion of R71 and S72. Fig. 1A shows the Western blot results (using antibody to IGF-1) for the wild-type and 3mut precursor containing Ea. Fig. 1B shows the Western blot results (using antibody to hIGF-1) for the wild-type and 3mut precursor containing Eb. Fig. 1C shows the Western blot results (using antibody to hIGF-1) for the wild-type and 3mut precursor containing Ec.

**Figs. 2A-2D** are line graphs showing the biological activity of various IGF-1 polypeptides ("ligands"). Biological activity was measured by stimulation of C2C12 myoblasts with Cos7-expressed polypeptides. The stimulated C2C12 cells were then assayed for the relative amounts of total AKT and phosphorylated AKT. Long-R3-IGF-1 is a commercially available reagent (Sigma Product No. I-1271) that consists of the mature human IGF-1 amino acid sequence, with an E3R mutation and an additional 13 amino acid N-terminal extension peptide. Fig. 2A shows the activity of IGF-1-Ea3mut. Fig. 2B shows the activity of IGF-1-Eb3mut. Fig. 2C shows the activity of IGF-1-Eab3mut, which is a 3mut construct in which Ea amino acids 93 to 102 were inserted between amino acids 95 and 96 of Eb. Fig. 2D shows the activity of IGF-1-Ec3mut.

**Figs. 3A-3D and 4A-4D** are line graphs showing whether IGF-1 precursor polypeptides of the invention maintain selectivity to the appropriate receptor by assaying for receptor phosphorylation in response to ligand binding. Figs 3A and 3B test the receptor selectivity of IGF-1-Ea3mut against the IGF-1 receptor (Fig. 3A) and the insulin receptor (Fig. 3B). Figs. 3C

and 3D tests the receptor selectivity of IGF-1-Eb3mut against the IGF-1 receptor (Fig. 3C) and the insulin receptor (Fig. 3D). Figs. 4A and 4B test the receptor selectivity of IGF-1-Ec3mut against the IGF-1 receptor (Fig. 4A) and the insulin receptor (Fig. 4B). Figs. 4C and 4D tests the receptor selectivity of IGF-1-Eab3mut against the IGF-1 receptor (Fig. 4C) and the insulin receptor (Fig. 4D). "IGF1-R3" refers to the Long-R3-IGF-1 described above. The polypeptide listed as "IGF1Eab" refers to construct in which Ea amino acids 93 to 102 were inserted between amino acids 95 and 96 of Eb.

Fig. 5 is a Western blot showing relative AKT phosphorylation upon stimulation of C2C12 myotubes (as a result of 3 to 4 days of differentiation of C2C12 myocytes) by different ligands. The IGF-1Eb multimer refers to the construct schematically shown in Fig. 6A.

Figs. 6A and 6B are schematic representations of two of the polypeptides of the invention. Fig. 6A shows an IGF-1-Eb precursor polypeptide with four sets of modifications: deletion of G1, P2, and E3; mutation of R37 to A; deletion of R71 and S72; and deletion of the last seven C-terminal amino acids. In addition, the polypeptide is lengthened by the addition of two more Eb peptides (but without R71 and S72 and without the last seven C-terminal amino acids) and the addition of a final Eb peptide (buth without R71 and S72) at the C-terminus of the polypeptide. This construct is often referred to as the IGF-1-Eb multimer. Fig. 6B shows an IGF-1-Eab precursor polypeptide with four sets of modifications: deletion of G1, P2, and E3; mutation of R37 to A; deletion of R71 and S72; and insertion of Ea amino acids 93 to 102 between amino acids 95 and 96 of Eb.

Fig. 7A is a sequence alignment of the human IGF-1 (SEQ ID NO:1) with corresponding animal IGF-1. All animal species analyzed and their corresponding GenBank accession numbers for the sequence are given. G1, P2, E3 is conserved in all analyzed species except Sterlet (where S2 replaces P2). R36 and R37 are conserved in all analyzed species.

Fig. 7B is a graph showing the phylogeny of the analyzed amino acid sequences compared to human IGF-1 (SEQ ID NO:1). Below the tree is a scale indicating the number of "Amino Acid Substitutions" per 100 residues for protein sequences. The Kimura distance formula is used to

calculate distance values, derived from the number of non-gap mismatches and corrected for silent substitutions. The values computed are the mean number of differences per site and fall between zero and 1. Zero represents complete identity and 1 represents no identity. The phylogenetic tree scale uses these values multiplied by 100.

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**Fig. 8A** is a sequence alignment of the human Ea peptide (SEQ ID NO:2) with various animal Ea peptides. All animal species analyzed and their corresponding GenBank accession numbers for the sequence are given. R71 and S72 are conserved in all analyzed species.

10 **Fig. 8B** is a graph showing the phylogeny of the analyzed amino acid sequences compared to human IGF-1 Ea peptide (SEQ ID NO:2).

**Fig. 9A** is a sequence alignment of the human Eb peptide (SEQ ID NO:3) with various animal Eb peptides. All animal species analyzed and their corresponding GenBank accession numbers for the sequence are given. R71 and S72 are conserved in all analyzed species.

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**Fig. 9B** is a graph showing the phylogeny of the analysed amino acid sequences compared to human IGF-1 Eb peptide (SEQ ID NO:3).

20 **Fig. 10A** is a sequence alignment of the human Ec peptide (SEQ ID NO:4) with various animal Ec peptides. All animal species analyzed and their corresponding GenBank accession numbers for the sequence are given. R71 and S72 are conserved in all analyzed species.

25 **Fig. 10B** is a graph showing the phylogeny of the analyzed amino acid sequences compared to human IGF-1 Ec peptide (SEQ ID NO:4).

**Fig. 11A** is a sequence alignment of the human IGF-2 (SEQ ID NO:7) with corresponding animal IGF-2. All animal species analyzed and their corresponding GenBank accession numbers for the sequence are given. R68 is conserved in all analyzed species; D69 is conserved except for chimpanzee, where a histidine resides in that position.

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Fig. 11B is a graph showing the phylogeny of the analyzed amino acid sequences compared to human IGF-2 (SEQ ID NO:7).

Fig. 12A is a sequence alignment of the human IGF-2 E-peptide (SEQ ID NO:6) with various animal IGF-2 E-peptides. All animal species analyzed and their corresponding GenBank accession numbers for the sequence are given. R68 is conserved in all analyzed species; D69 is conserved except for Chimpanzee, where a histidine resides in that position.

Fig. 12B is a graph showing the phylogeny of the analyzed amino acid sequences compared to human IGF-2 E peptide (SEQ ID NO:6).

#### Detailed Description of the Invention

The invention relates to new IGF-1 and IGF-2 precursor polypeptides containing substantially an E-peptide that has been modified to prevent, reduce, or avoid the typical protease cleavage responsible for releasing the active IGF-1 or IGF-2 from its E-peptides. The utility of the polypeptides of the invention is based on the surprising discovery that such precursor polypeptides are biologically active, stable and beneficial as pharmaceuticals.

#### Screening for Active IGF Precursor Polypeptides

The usefulness of any polypeptide of the invention can be assessed using the following assays.

**Stability** A polypeptide of the invention should have sufficient stability in the presence of endogenous proteases, such as in human serum, to be an effective drug. To assess stability, an expression vector encoding the polypeptide can be transfected into Cos7 cells (ATCC) in a DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The culture medium containing secreted polypeptides can be applied to further analysis, or in the alternative, the expression vector can encode readily available tags, such as a hexa-histidine tag, in the polypeptide to facilitate efficient purification of the expressed polypeptides in the Cos7 cultures. However prepared, the polypeptide sample is incubated in normal human serum (Sigma) or in PBS for various times (e.g., 0, 1, 5, 10, and 16 hours), subjected to polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and the relevant

proteins visualized using a primary antibody against human IGF-1 or IGF-2 and a secondary antibody, e.g., conjugated to horseradish peroxidase. Any number of similar blotting and detection techniques, some using fluorescent dyes or even radionuclides, can be used. The intensity of the precursor band versus the intensity of the IGF-1 or IGF-2 band should indicate the degree to which the precursor polypeptide is cleaved under various conditions. A polypeptide of the invention that is exposed to human serum for 16 hours at 37°C can exhibit a ratio of uncleaved precursor to cleaved mature IGF of about 1:2 to 1:0.1, e.g., about 1:1 to 1:0.5, particularly a ratio of about 1:1 or a ratio of about 1:0.5. Typically, the precursor should exhibit a ratio of at least 1:1.

*AKT Phosphorylation* A polypeptide of the invention should maintain the ability to signal through the IGF-1 receptor. (Both IGF-1 and IGF-2 signal through the IGF-1 receptor.) To determine this signaling ability, one can assess whether a downstream intracellular target, AKT, is phosphorylated in response to ligand binding at the cell surface. For analysis of AKT phosphorylation, C2C12 myoblasts are starved in serum-free medium and then stimulated with different ligands. Cells are lysed and cleared by centrifugation. AKT phosphorylation and total AKT levels are analyzed by ELISA using PathScan phospho AKT (Ser473) sandwich ELISA kit and PathScan AKT sandwich ELISA kit (Cell Signaling), respectively.

*IGF-1 Receptor Specificity* A polypeptide of the invention preferably maintains the specificity for the IGF-1 receptor and should bind to the related insulin receptor with low affinity. To assess receptor specificity, polypeptide samples are added to serum-starved NIH3T3 cells overexpressing the IGF-1 receptor or the insulin receptor, and the level of IGF-1 receptor phosphorylation or insulin receptor phosphorylation is determined by lysing the cells and subjecting the lysates to ELISA using the DuoSet IC human phospho-IGF-1 receptor and insulin receptor ELISA kit (R&D Systems).

*In Vivo Testing in Mouse Models of Hypertrophy* To determine whether a polypeptide of the invention can act to increase skeletal muscle mass under a context that already leads to muscle hypertrophy, one can subject treated and untreated animals to exercise and determine whether animals receiving the polypeptide have developed larger muscles than untreated animals.

### Exercise Models

One model known in the art is based on the use of a voluntary running wheel with user-variable loads (see, e.g., Konhilas et al., *Am J Physiol Heart Circ Physiol* 289:H455-H465, 2005). The voluntary cage wheel eliminates physical and psychological insults that are common in forced exercised models, and are therefore more appropriate for evaluating candidate drugs that are used in relatively healthy individuals for whom increases in muscle mass is desirable.

Any suitable mouse strain can be used. For example, male C57Bl/6J mice can be randomly assigned to experimental (e.g., receiving IGF precursor polypeptide) and control groups. Animals are individually housed in a cage containing an exercise wheel; sedentary control animals are housed in identical cages without a wheel. The exercise wheels are described in Allen et al., *J Appl Physiol* 90:1900-1908, 2001. Briefly, the system consists of an 11.5 cm-diameter wheel with a 5.0 cm-wide running surface (model 6208, PetSMART, Phoenix, AZ) equipped with a digital magnetic counter (model BC 600, Sigma Sport, Olney, IL) that is activated by wheel rotation. In addition, each wheel is engineered with a resistance mechanism allowing adjustment of the load. This is accomplished by attaching stainless steel fishing line to the cage top and wrapping the wire around an immovable pulley that is secured to the cage wheel at the axis of rotation so as to not contribute to the wheel load. The wire is again secured to the cage top with a spring and screw. This design permits fine adjustments of the wheel load, which is evenly distributed throughout the rotation of the wheel. Daily exercise values for time and distance run are recorded for each exercised animal throughout the duration of the exercise period. All animals are given water and standard hard rodent chow ad libitum. Voluntary running (cage wheel exposure) can begin at an average age of about 12 weeks for all groups. Each group continues running under varying resistance, depending on experimental group, for 50 days until the animals are about 19 weeks of age. The load on the wheel is determined by hanging known weights on the wheel until the wheel was slightly displaced. All exercise groups begin with no load on the cage wheel for the first week. However, the "no-load" condition is actually 2 g, which is determined as the load necessary to maintain wheel inertia and frictional load. Considering a wheel acclimatization period of 1 week, wheel loads can be changed at one-week intervals, except for higher loads, which can be changed after 2 weeks. The range of loads

can be anywhere from 2 g to up to 12 g. Exercised and sedentary control animals are euthanized by cervical dislocation under inhaled anesthesia immediately after the end of the specific exercise period. Body mass is measured, and specific muscles are rapidly excised, washed, and frozen for histological or biochemical assays at a future date.

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Alternative exercise hypertrophy models are also available to the skilled artisan. See, e.g., the treadmill exercise model described in Lerman et al., J Appl Physiol 92:2245-2255, 2002.

#### Clenbuterol Injection Model

10 Clenbuterol is a  $\beta_2$ -adrenergic agonist with growth-promoting properties that cause a documented increase in muscle mass. The precise mechanism of clenbuterol action remains unclear, although a reduction in muscle protein degradation has been proposed. In the clinic, clenbuterol is used as an anti-asthma drug, but it appears to be mostly misused as a body-building agent to increase muscle mass in both humans and show animals.

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Five mice are given a daily injection of clenbuterol (3 mg/kg, subcutaneous (s.c.)) for 3, 7, or 14 days to induce muscle hypertrophy. Mice injected with PBS serves as negative control. The animals are monitored daily (visual inspection) for any adverse reactions (i.e. unkempt coat, lethargic) to the treatment. Clenbuterol treatment has the potential to make mice more fearful or  
20 aggressive, so mice should be especially monitored for fighting if housed in groups. Mice are mobile, and can eat and drink normally. Mice are monitored daily until they are euthanized on day 3, 7, or 14, and tissue collected for further analysis.

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*In Vivo Testing in Muscle Atrophy Models* In various skeletal muscle atrophy models, an IGF precursor polypeptide of the invention can be tested for the ability to maintain muscle mass under conditions that generally reduce muscle mass. With the example models described below, the skilled artisan can readily design and implement controlled experiments involving the administration and use of IGF precursor polypeptides to determine whether such polypeptides can increase muscle mass.

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For example, C57Bl6/2 male mice are purchased from The Jackson Laboratories. Mice are purchased so that they are about 9 weeks at the start of each experiment. Generally mice are housed in microisolator cages with normal rodent chow. At the start of each experiment mice are weighed. At the end of each experiment, generally mice are euthanized by CO<sub>2</sub> inhalation  
5 followed by cervical dislocation, and muscle tissues harvested for further processing. Mice are weighed to provide "end body weight." Skeletal muscles that can be harvested are tibialis anterior, extensor digitorum longus, soleus, and gastrocnemius muscles. Other tissues harvested occasionally are: heart, liver, spleen, kidneys, testes, and brain. All muscles and tissues are completely dissected and weighed on a balance capable of measuring to 0.0001g. Tissues are  
10 then snap-frozen in liquid nitrogen for later RNA and protein extraction, or snap-frozen embedded in OCT on a cork disc. Muscles frozen on a cork disc for later cryosectioning are immersed in isopentane cooled to a thick slush by liquid nitrogen. All samples are stored at -80°C.

#### 15 Dexamethasone Treatment

A pharmacological method of inducing muscle wasting in mice is daily intraperitoneal injection with dexamethasone at 20mg/kg. Dexamethasone is a synthetic member of the glucocorticoid class of hormones. It acts as an anti-inflammatory and immunosuppressant, with a potency of about 40 times that of hydrocortisone. Dexamethasone is used to treat many inflammatory and  
20 autoimmune conditions, e.g. rheumatoid arthritis. It is also given to cancer patients undergoing chemotherapy, to counteract certain side-effects of their antitumor treatment. Dexamethasone causes muscle atrophy both in mice and in human patients.

Mice are injected intraperitoneally (ip) with dexamethasone for 3, 7, or 14 days. On the terminal  
25 day subjects are euthanized using CO<sub>2</sub>, and the leg muscles harvested. The animals are monitored daily (visual inspection) for any adverse reactions (i.e. unkempt coat, lethargic) to the treatment. Mice are usually mobile, and can eat and drink normally. Mice injected with PBS are the negative control.

#### 30 Cast Immobilization

Physical disuse of various muscle groups results in atrophy of those muscles. Ankle joint fixation ("pinned heel" or casting) has proven to be a highly useful and reproducible way to induce physical immobilization of rat and mouse hindlimb musculature.

- 5 Mice are anesthetized with isoflurane for immobilization. The ankle and knee joints are fixed at 90 degrees with a light-weight casting material (VET-LITE) around the joints. The material is soaked in warm water and then wrapped around the limb, leaving the toes and hip joint free. The joints are maintained in at 90° positions until the casting material has dried. The contralateral leg serves as control. The mice are then allowed to recover from anesthesia and housed in normal  
10 micro isolator cages. Casting has not been observed to cause excessive stress, and animals freely move about the cage to feed and drink. The mice are however monitored daily for any adverse events affecting body weight, activity, and irritations.

- Once a cast is applied to a mouse, the animal is monitored daily to make sure that the cast  
15 remains in place, as chewing can occur. The animals can move, drink, and feed after recovery of anesthesia, and they do not require special bedding, caging or other assistance.

#### Denervation

- Generally, mice are anesthetized with isoflurane gas for denervation. Using aseptic surgical  
20 procedures (three washes of betadine with a final ethanol wash), the right sciatic nerve is isolated in the mid-thigh and a 2 to 5 mm piece cut out. The contralateral leg serves as control.

- More specifically, the skin incision is closed with a suture clip, and the animals injected with a single dose of buprenorphine before being allowed to recover from the anesthesia. Three, seven,  
25 or 14 days after surgery animals are euthanized by CO<sub>2</sub> inhalation followed by cervical dislocation, and muscles (gastrocnemius complex, tibialis anterior, extensor digitorum longus, soleus) are removed for histological and biochemical analyses.

- Given that the sciatic nerve is transected, the effected limb is rendered immobile to induce  
30 skeletal muscle atrophy of the muscles involved. The animal can otherwise move, drink, and feed after recovery of anesthesia and they do not require special bedding, caging or other

assistance. Nonetheless, animals are monitored immediately post-surgery and through recovery (1-2 hrs). In addition, the incision sites and general animal health are monitored for 3 days post-surgery. The suture clip is removed 7 to 10 days after surgery.

## 5 Genetic Models

Genetically manipulated transgenic mice can also be used as models of muscle atrophy. For example, the so-called Mini Mice (The Jackson Laboratory, Stock No. 003258) contains a knock out mutation in the IGF-1 gene that results in abnormally decreased postnatal growth, as well as low body weight and size. For additional information, see Powell-Braxton et al., Genes Dev  
10 7:2609-2617, 1993. In addition, the so-called Midi Mice (The Jackson Laboratory, Stock No. 003259) contains a different mutation in the IGF-1 gene that results in a hypomorph exhibiting low adult body weight and other cardiovascular phenotypes. For additional information, see Lembo et al., J Clin Invest 98:2648-2655, 1996.

## 15 Critical and Optional Mutations or Modifications in the IGF Precursors

*Critical Mutations* The invention is based in part on the observation that an IGF precursor polypeptide that contains substantially its E-peptide remains bioactive and stable in the presence of serum. To ensure that the E-peptide is not cleaved by endogenous proteases targeting the dibasic protease site, in general either of the two N-terminal dibasic amino acids of the E-peptide  
20 in the precursor is deleted, mutated, or otherwise masked. In the case of hIGF-1, these two amino acids are R71 and S72, while in the case of hIGF-2, these first two amino acids are R68 and D69.

A variety of modifications enables this prevention of cleavage:

- 25 (1) Deletion of one or both dibasic residues
- (2) Mutate one or both dibasic residues to a non-basic amino acid, such as alanine
- (3) Insert one or more non-basic amino acids between the dibasic residues
- (4) Place a glycosylation site near the dibasic residues sufficient to mask the protease site
- (5) Site-directed pegylation using replacement of either dibasic residue, or insertion near or  
30 between the dibasic residues, with a non-natural amino acid, as described below.

In addition, residues K68 and K65 appear to play a role in IGF-1/E-peptide cleavage; accordingly, mutations or deletions of these residues can be incorporated into any tactic directed to the dibasic amino acids as described above.

5    *Mutations at the N-terminus of Mature IGF* In certain embodiments of the invention, the IGF precursor polypeptides have deletions or mutations of the first few N-terminal amino acids. In the case of IGF-1, any of the first three N-terminal amino acids can be deleted or mutated, whereas in the case of IGF-2, any of the first six N-terminal amino acids can be deleted or mutated. It has been observed that certain N-terminal amino acids are naturally cleaved in vivo,  
10    and the introduction of these mutations or deletions minimizes the in vivo associations of the polypeptides of the invention with IGF binding proteins (IGFBPs). The interaction of IGF-1 and IGF-2 with the IGF-1 receptor is regulated by IGFBPs. All six IGFBPs have been shown to inhibit IGF action (particularly IGFBP5), but in some instances a stimulatory effect has been observed. At least 99% of the IGF in the circulation is normally bound to IGFBPs. The most  
15    abundant IGFBP in the circulation after the neonatal period is IGFBP3 which can bind both IGF-1 and IGF-2 with similar affinities. The naturally occurring truncated IGF-1 (bearing deletion of G1, P2, and E3) binds to IGFBP3 with several times lower affinity than natural IGF-1. In addition, G3 is important for IGFBP binding, and G6 plays a similar role in the IGF-2 peptide.

20    Accordingly, in the case of the hIGF-1 precursor, any of G1, P2, or E3 can be deleted or mutated either alone or in combination. When a mutation is desired, a mutation to alanine can be introduced. In another example, in the case of hIGF-2 precursor, any of P4, S5, and E6 can be deleted or mutated either alone or in combination. When a mutation is desired, a mutation to alanine can be introduced.

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*Mutations at Residues 36 and 37* IGF-1 can be cleaved by serine proteases present in human serum. Mutation of either R36 or R37 to A can prevent cleavage of IGF-1 at this predicted cleavage site between R36 and R37. In the case of hIGF-2, R38 can be mutated or deleted to prevent this deleterious cleavage.

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*Use of Glycosylation* The in vivo half-life of the polypeptides of the invention can be improved by the addition of N-linked glycosylation sites into either the IGF or the E-peptide portions of the precursor when expressed in mammalian or other eukaryotic cells capable of N-linked glycosylation. It has been shown in vitro that human IGF-1 Ea is glycosylated at N92 and N100, as these portions of Ea fits the consensus N-linked glycosylation sequence of N-X-S/T, where X can be any amino acid and the third amino acid of the triplet is either S or T. It is also know that the adjacent amino acid context of the consensus will affect how strongly the asparagine is glycosylated. Therefore, one strategy to introduce a glycosylation site into Eb or Ec is to insert Ea amino acids around the consensus sequence into roughly the same part of Eb or Ec. A particular implementation of this strategy is illustrated in the Examples below. In any event, any other consensus N-linked glycosylation site, including surrounding context amino acids, known to the skilled artisan can be inserted into a precursor polypeptide of the invention. In addition, O-linked glycosylation of a polypeptide of the invention can be accomplished by choosing the particular host used for production of the polypeptide. For example, use of certain yeast strains for IGF-1 expression results in the addition of oligosaccharides on a serines or threonines. See, e.g., US Patent No. 5,273,966.

*Addition of Poly(ethylene glycol)* Conjugation to poly(ethylene glycol) (PEG; pegylation) have proven to be beneficial in prolonging the half-life of therapeutic proteins drugs. It is expected that pegylation of the IGF precursor polypeptides of the invention may result in similar pharmaceutical advantages. Methods of pegylation of IGF-1 are well known in the art. See, for example, US Patent Application Publication 2006/0154865, which describes the beneficial properties of lysine-monopegylated IGF-1. Such lysine-monopegylation can be adapted for the precursor IGF polypeptides of the invention. In addition, pegylation can be achieved in any part of a polypeptide of the invention by the introduction of a nonnatural amino acid. Certain nonnatural amino acids can be introduced by the technology described in Deiters et al., J Am Chem Soc 125:11782-11783, 2003; Wang and Schultz, Science 301:964-967, 2003; Wang et al., Science 292:498-500, 2001; Zhang et al., Science 303:371-373, 2004 or in US Patent No. 7,083,970. Briefly, some of these expression systems involve site-directed mutagenesis to introduce a nonsense codon, such as an amber TAG, into the open reading frame encoding a polypeptide of the invention. Such expression vectors are then introduced into a host that can

utilize a tRNA specific for the introduced nonsense codon and charged with the nonnatural amino acid of choice. Particular nonnatural amino acids that are beneficial for purpose of conjugating moieties to the polypeptides of the invention include those with acetylene and azido side chains. The IGF precursor polypeptides containing these novel amino acids can then be  
5 pegylated at these chosen sites in the protein. In addition, such pegylated IGF molecules without the E-peptide are also useful as therapeutics.

*Multimers of E-Peptides* In certain pharmacological contexts, it is beneficial to increase the size of a peptide or protein drug to ensure that the drug remains on one side of the blood- brain  
10 barrier or the other. Since mature IGF molecules are relatively short peptides, even if the E-peptide remains attached, it can be beneficial to increase the size of the polypeptides of the invention. One means of doing so is to provide multimers of E-peptides at the C-terminus of the IGF precursor polypeptide, as illustrated in certain Examples described below.

*C-Terminal Deletion of E-Peptides* It is suspected that the free cysteine at position 81 of Eb may result in homodimerization or other effects that, when present in the polypeptides of the invention, might lead to lower activity drugs. Thus, deletion or mutation of C81 in Eb can optimize drug activity. In a particular example, deletion of the last seven amino acids of Eb (i.e., amino acids 81-87) is beneficial.  
20

*Other Mutations or Modifications* Additional mutations or modifications of IGF that can be incorporated into the IGF precursor polypeptides of the invention are described in US Patent No. 5,077,276; and US Patent Application Publication Nos. 2005/0287151, 2006/0211606, and 2006/0166328.  
25

The invention should be construed, in addition to human IGF-1 and IGF-2, to include all known and unknown non-human animal precursor IGF-1 or IGF-2 sequences containing substantially its E-peptide wherein the normal cleavage of the E-peptide is avoided or reduced according to modifications of the present invention.  
30

The preferred type of IGF to be used depends upon the species of the subject being treated.

It is preferred that the IGF is species-matched, for example, when a cow is being treated, the preferred type of IGF is bovine IGF.

Although all forms of IGF are likely to have an effect in different subjects due to the high  
5 sequence homologies, species matching will avoid potential adverse immunological complications stemming from the induction of an immune response to an IGF from a different species.

In one embodiment of the invention, modified non-human animal precursor IGF-1 sequences are  
10 provided.

Preferred are precursor IGF-1 sequences containing substantially its E-peptide wherein the normal cleavage of the E-peptide is avoided or reduced according to modifications of the present invention from a vertebrate animal.

For example, such sequences include but are not limited to sequences from a mouse, rat, cow, pig, horse, sheep, goat, bird, dog, cat, fish and the like, from any source whether natural, synthetic, or recombinant.

In another embodiment of the invention, modified non-human animal precursor IGF-2 sequences are provided.

Preferred are precursor IGF-2 sequences containing substantially its E-peptide wherein the normal cleavage of the E-peptide is avoided or reduced according to modifications of the present  
25 invention from a vertebrate animal.

For example, such sequences include but are not limited to sequences from a mouse, rat, cow, pig, horse, sheep, goat, bird, dog, cat, fish and the like from any source, whether natural, synthetic, or recombinant.

Therapeutic Use of IGF Precursor Polypeptides

*Indications* The invention also includes the use of an IGF precursor polypeptide of the invention in the manufacture of a medicament for the treatment or prevention of a musculoskeletal disease. In addition, the invention includes use of IGF precursor polypeptides to increase muscle or bone mass in an individual, whether or not such an individual is at risk for or has a musculoskeletal disease.

In particular, the musculoskeletal disease can be muscle atrophy. There are many causes of muscle atrophy, including as a result of treatment with a glucocorticoid such as cortisol, dexamethasone, betamethasone, prednisone, methylprednisolone, or prednisolone. The muscle atrophy can also be a result of denervation due to nerve trauma or a result of degenerative, metabolic, or inflammatory neuropathy (e.g., Guillian-Barré syndrome, peripheral neuropathy, or exposure to environmental toxins or drugs). In addition, the muscle atrophy can be a result of an adult motor neuron disease, infantile spinal muscular atrophy, juvenile spinal muscular atrophy, autoimmune motor neuropathy with multifocal conduction block, paralysis due to stroke or spinal cord injury, skeletal immobilization due to trauma, prolonged bed rest, voluntary inactivity, involuntary inactivity, metabolic stress or nutritional insufficiency, cancer, AIDS, fasting, rhabdomyolysis, a thyroid gland disorder, diabetes, benign congenital hypotonia, central core disease, nemaline myopathy, myotubular (centronuclear) myopathy, burn injury, chronic obstructive pulmonary disease, liver disease, sepsis, renal failure, congestive heart failure, or ageing.

The musculoskeletal disease can also be a muscular dystrophy syndrome, such as Duchenne, Becker, myotonic, fascioscapulohumeral, Emery-Deifuss, oculopharyngeal, scapulohumeral, limb girdle, a congenital muscular dystrophy, or hereditary distal myopathy. The musculoskeletal disease can also be osteoporosis, a bone fracture, short stature, or dwarfism.

IGF-1 is suggested as a treatment for insulin-insensitive diabetes, since IGF-1 can also bind heterodimers of IGF-1 receptor and insulin receptor. Accordingly, the polypeptides of the invention can be used to treat diabetes.

IGF-1 is neurotrophic and increases survival of neurons. It has been suggested that IGF-1 can be used to treat instances of motor-neuron death such as seen in amyotrophic lateral sclerosis (ALS), brain atrophy, ageing, and dementia. Accordingly, the polypeptides of the invention can be used to treat conditions associated with neuronal death, such as ALS, brain atrophy, or dementia.

IGF-1 increases both white and red blood cell populations and has an additive effect to administration of erythropoietin. Accordingly, the polypeptides of the invention can be used to treat anemia.

Since IGF-1 and IGF-2 are ubiquitous and essential regulators of cell division and vertebrate growth, they may be advantageously used in a variety of veterinary methods to exogenously enhance or maintain growth in an animal. Some examples include, but are not limited to:

- (i) enhancing rate and/or extent of growth in an animal, for example, enhancing muscle growth in swine, cattle, poultry and fish;
- (ii) enhancing the efficiency of their conversion of feed into body tissue (lean to fat ratio), for example, in swine, cattle, sheep, poultry and fish; and
- (iii) enhancing milk production in lactating animals, for example, dairy cattle, sheep, goats.

Other veterinary therapeutic applications include, but are not limited to:

- (iv) treating animal wasting symptoms associated with cachexia, trauma or other consumption diseases, for example, in companion animals such as dogs, cats, and horses; and
- (v) treating lactating animals for improvement in neonatal health, for example, lactating sows for improvement in neonatal performance.

*Methods of Administration* The polypeptides of the invention can be delivered in a variety of ways, including the use of gene delivery vehicles. Methods known in the art for the therapeutic delivery of agents such as proteins or nucleic acids can be used for the therapeutic delivery of a polypeptide of the invention, e.g., cellular transfection, gene therapy, direct administration with a delivery vehicle, or pharmaceutically acceptable carrier, indirect delivery by providing recombinant cells containing a nucleic acid encoding the polypeptide.

Various delivery systems are known and can be used to administer the polypeptide of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the protein, receptor-mediated endocytosis (see, e.g., Wu and Wu, J Biol Chem 262:4429-4432, 1987), construction of a nucleic acid as part of a retroviral, adeno-associated viral, adenoviral, poxviral (e.g., avipoxviral, particularly fowlpoxviral) or other vector, etc. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, pulmonary, intranasal, intraocular, epidural, and oral routes. The polypeptides can be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, e.g., by injection, by means of a catheter, or by means of an implant, the implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, fibers, or commercial skin substitutes.

In another embodiment, the active agent can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533, 1990). In yet another embodiment, the active agent can be delivered in a controlled release system. In one embodiment, a pump may be used. In another embodiment, polymeric materials can be used (see Howard et al., J Neurosurg 71:105, 1989). In another embodiment where the active agent of the invention is a nucleic acid encoding a polypeptide of the invention, the nucleic acid can be administered in vivo to promote expression

of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see, for example, US Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface  
5 receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see, e.g., Joliot et al., Proc. Natl. Acad. Sci. USA 88:1864-1868, 1991), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

10 *Cellular Transfection and Gene Therapy* The present invention encompasses the use of nucleic acids encoding polypeptides of the invention for transfection of cells in vitro and in vivo. These nucleic acids can be inserted into any of a number of well-known vectors for transfection of target cells and organisms. The nucleic acids are transfected into cells ex vivo and in vivo, through the interaction of the vector and the target cell. The compositions are administered (e.g.,  
15 by injection into a muscle) to a subject in an amount sufficient to elicit a therapeutic response.

In another aspect, the invention provides a method of treating a target site, i.e., a target cell or tissue, in a human or other animal including transfecting a cell with a nucleic acid encoding a polypeptide of the invention, wherein the nucleic acid includes an inducible promoter operably  
20 linked to the nucleic acid encoding the targeting fusion polypeptide. For gene therapy procedures in the treatment or prevention of human disease, see for example, Van Brunt Biotechnology 6:1149-1154, 1998.

*Combination Therapies* In numerous embodiments, the polypeptides of the present invention  
25 can be administered in combination with one or more additional compounds or therapies. For example, multiple polypeptides can be co-administered in conjunction with one or more therapeutic compounds. The combination therapy may encompass simultaneous or alternating administration. In addition, the combination may encompass acute or chronic administration. The polypeptides of the invention can be administered in combination with anabolic agents such  
30 as testosterone or specific androgen receptor modulators (SARMs). Additional anabolic agents include growth hormone (GH) or molecules that induce GH release. Ghrelin is particularly

useful in a combination therapy for cachexia, since Ghrelin can cause an increase in appetite. In a similar vein, the polypeptides of the invention can be combined with protein supplements to increase anabolism, or combined with physical therapy or exercise to increase body weight. Any molecule that inhibits myostatin is also a candidate for combination therapy.

5

*Pharmaceutical Compositions.* The present invention also provides pharmaceutical compositions comprising a IGF precursor protein of the invention and a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized

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pharmacopeia for use in animals or humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour,

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chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin.

25

In some embodiments, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water

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for injection or saline can be provided so that the ingredients can be mixed prior to administration.

The polypeptides of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

10 The amount of a polypeptide of the invention which will be effective in the treatment of a condition or disease can be determined by standard clinical techniques based on the present description. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the condition, and should be decided according to the judgment of the practitioner and each subject's circumstances. However, suitable dosage ranges for intravenous administration are generally about 20-5000 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 µg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems. In particular, a possible dosage regimen can be about 60 to 120 µg/kg body weight, subcutaneous injection, twice daily.

#### Veterinary Uses

In addition to the aforementioned methods of administration in humans, there may be additional considerations for veterinary administration.

The dosage may differ when administered to a healthy animal versus those animals suffering from a disease. An assessment of the appropriate dosage can easily be made by those skilled in the art using assays known in the art, for example, the myoblast proliferation assay (Example 79) or the mammary epithelial tissue assay (Example 80) as described below. General assays to measure IGF are also known in the art, such as those in Example 81.

Those skilled in the art will recognize that some species of animal exhibit seasonal fertility influenced by the length of the photoperiod. Any embodiment of a veterinary method or use may optionally include starting the treatment method at a specific time within the animal's reproductive cycle in order to achieve the desired effect. Those skilled in the art will know that reproductive status and cycle can easily be determined, and, if desired, synchronized by the use of an appropriate regimen.

When used for veterinary indications, in addition to methods previously mentioned for human use, the IGF-1 or IGF-2 peptide of the present invention can also be used as an oral drench, or a supplement to oral or solid feeds for animals.

The invention is further described but not limited by the following Examples.

### Examples

#### Example 1

A DNA expression vector encoding the hIGF-1-Ea precursor polypeptide containing the following modifications was constructed: deletion of G1, deletion of P2, and deletion of E3; mutation of R37 to A; and deletion of R71 and deletion of S72. These mutations are sometimes referred to as "3mut" throughout the present disclosure. This results in the following secreted protein sequence:

tlcgaelvdaqlqfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlknasr  
gsagnknyrm (SEQ ID NO:8)

Cos7 cells (available from ATCC) were maintained in DMEM containing 10% fetal bovine serum, 100U/ml penicillin, and 100 µg/ml streptomycin and plated at a density of  $1 \times 10^6$  cells per 10-cm plate. These cell cultures were transfected with 8 µg of expression plasmid using Fugene (Roche) according to manufacturer's instructions. Twenty-four hours post-transfection, cells were washed once and cultured in serum-free medium for 48 hours. Supernatants were collected and stored at -80°C.

In order to assess polypeptide stability in human serum, supernatants collected from the Cos7 cells transfected with wild-type (wt) hIGF-1Ea, and hIGF-1Ea3mut were incubated for 16 hours at 37°C either in the absence or presence of 10% human serum (Sigma). Samples were separated by 18% SDS-PAGE, and immunoblotting was performed using goat polyclonal antibody to human IGF-1. The results in Fig. 1A indicate that, while the wt.hIGF-1Ea was substantially degraded after incubation with serum for 16 hours, the hIGF-1Ea3mut was stabilized. Densitometry indicated that the ratio of uncleaved to cleaved IGF-1 was about 1:6.2, while the ratio for hIGF-1Ea3mut was about 1:0.68, showing that these mutations result in a stabilized polypeptide.

To confirm that the hIGF-1Ea3mut was able to signal through the IGF-1R, AKT phosphorylation of cells in contact with the polypeptide was measured. C2C12 were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Invitrogen) containing 10% fetal bovine serum (AMIMED), 100U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and 2 mM glutamine (Invitrogen). For analysis of AKT phosphorylation, the C2C12 cells were plated at a density of  $0.15 \times 10^6$  cells per well of a 6-well plate and were cultured in growth medium for 72 hours. Cells were starved for four hours in serum-free medium and then stimulated with different ligands at 37°C for 30 minutes. Cells were lysed with PhosphoSafe buffer (Cell Signaling) containing various protease inhibitors and cleared by centrifugation at 14,000 x g for 15 minutes at 4°C. AKT phosphorylation and total AKT levels were analyzed by ELISA using PathScan phospho AKT (Ser473) sandwich ELISA kit and PathScan AKT sandwich ELISA kit (Cell Signaling), respectively. The AKT phosphorylation results are summarized in Fig. 2A, which indicate that the hIGF-1Ea3mut was able to activate the IGF-1R cellular pathway to a similar extent as the long-R3-IGF-1 positive control reagent and the recombinant IGF-1. In addition, the data in Fig. 5 directly shows that hIGF-1Ea3mut led to AKT phosphorylation.

Next, to ensure that the receptor specificity of the hIGF-1Ea3mut remained with the IGF-1R, various ligands were added to cultures of NIH3T3 overexpressing either IGF-1R or insulin receptor (InsR). These cells were cultured under the same conditions as described above for Cos7 cells. For analysis of IGF-1R and InsR phosphorylation, NIH3T3-IGF1R and NIH3T3-

InsR cells were plated at a density of  $0.2 \times 10^6$  cells per well of a 6-well plate and were cultured in growth medium for 24 hours. Cells were starved for 18 hours in serum-free medium and then stimulated with different ligands at 37°C for 10 minutes. Cells were lysed as described above for the AKT experiment, and IGF-1R and InsR phosphorylation levels were analyzed by ELISA using DuoSet IC human phosphor-IGF1R and -InsR ELISA kit (R&D Systems). The results summarized in Figs. 3A and 3B indicate that this IGF-1 precursor polypeptide retains specificity for the IGF-1 receptor and should bind to the related insulin receptor with low affinity.

### Example 2

- 10 A DNA expression vector encoding the hIGF-1-Eb precursor polypeptide containing the following mutations was constructed: deletion of G1, deletion of P2, and deletion of E3; mutation of R37 to A; and deletion of R71 and deletion of S72 (i.e., the "3mut"). This results in the following secreted protein sequence:

15 tlcgaelvdaqlfvcgdrfyfnkptgygsssarapqtgividccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
ntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:9)

- The polypeptide was assayed in accordance with the procedures described in Example 1 above. Fig. 1B and use of densitometry indicated that the ratio of uncleaved to cleaved IGF-1 was about 1:9, while the ratio for hIGF-1Eb3mut was about 1:1, showing that these modifications result in a stabilized polypeptide. Fig. 2B indicates that the hIGF-1Eb3mut was able to activate the IGF-1R cellular pathway to a similar extent as the long-R3-IGF-1 positive control reagent and the recombinant IGF-1. In addition, the data in Fig. 5 directly shows that hIGF-1Eb3mut led to AKT phosphorylation. The results summarized in Figs. 3C and 3D indicate that this IGF-1 precursor polypeptide retains specificity for the IGF-1 receptor and should bind to the related insulin receptor with low affinity.

### Example 3

- 30 A DNA expression vector encoding the hIGF-1-Ec precursor polypeptide containing the following mutations was constructed: deletion of G1, deletion of P2, and deletion of E3;

mutation of R37 to A; and deletion of R71 and deletion of S72 (i.e., the "3mut"). This results in the following secreted protein sequence:

tlcgaelvdaqlqfvcgdrfyfnkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnkn  
5 tksqrrkgstfeerk (SEQ ID NO:10)

The polypeptide was assayed in accordance with the procedures described in Example 1 above. Fig. 1C and use of densitometry indicated that the ratio of uncleaved to cleaved IGF-1 was about 1:5, while the ratio for hIGF-1Ec3mut was about 1:0.96, showing that these modifications result in a stabilized polypeptide. Fig. 2D indicates that the hIGF-1Ec3mut was able to activate the IGF-1R cellular pathway to a similar extent as the long-R3-IGF-1 positive control reagent and the recombinant IGF-1. In addition, the data in Fig. 5 directly shows that hIGF-1Ec3mut led to AKT phosphorylation. The results summarized in Figs. 4A and 4B indicate that this IGF-1 precursor polypeptide retains specificity for the IGF-1 receptor and should bind to the related insulin receptor with low affinity.

#### Example 4

A DNA expression vector encoding the hIGF-1-Eab chimeric precursor polypeptide containing the following modifications to the hIGF-1-Eb peptide was constructed: deletion of G1, deletion of P2, and deletion of E3; mutation of R37 to A; deletion of R71 and deletion of S72 (i.e., the "3mut"); and insertion of Ea amino acids 93 to 102 between amino acids 95 and 96 of Eb. The insertion creates a putative N-linked glycosylation signal at N92. This results in the following secreted protein sequence:

25 tlcgaelvdaqlqfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnkn  
nasrgsagnkntksqrrkgwphgpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:11)

The polypeptide was assayed in accordance with some of the procedures described in Example 1 above. Fig. 2C indicates that the hIGF-1Eab3mut was able to activate the IGF-1R cellular pathway to a similar extent as the long-R3-IGF-1 positive control reagent and the recombinant

IGF-1. The results summarized in Figs. 4C and 4D indicate that this IGF-1 precursor polypeptide retains specificity for the IGF-1 receptor and does not activate the insulin receptor.

#### Example 5

- 5 A DNA expression vector encoding the hIGF-1-Eb multimer precursor polypeptide containing the following mutations was constructed: deletion of G1, deletion of P2, deletion of E3, deletion of R36, deletion of R37, deletion of R71, deletion of S72, deletion of the last seven C-terminal amino acids of Eb; and insertion to the C-terminus of this precursor of two additional Eb peptides both without the R71 and S72 and last seven C-terminal amino acids and a fourth and final Eb peptide without the R71 and S72. Fig. 6A shows a schematic drawing of this construct. This results in the following secreted protein sequence:

15 tlcgaelvdaqlfvcgrgyfinkptgygssapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnknt  
ksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaersvraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeq  
kegteaslqirgkkkeqrreigsnaersvraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqr  
eigsnaersvraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaecrgkkgk  
(SEQ ID NO:12)

- This polypeptide was subjected to an AKT phosphorylation assay as described in Example 1.  
20 Fig. 5 indicates that this hIGF-1-Eb multimer was able to signal through the IGF-1R pathway.

#### Example 6

- A hIGF-1-Eb precursor polypeptide of the invention as shown schematically in Fig. 6B can be expressed. This construct contains the following modifications: deletion of G1, deletion of P2,  
25 deletion of E3, deletion of R36, deletion of R37, deletion of R71, deletion of S72; and the insertion of Ea amino acids 93-102 between amino acids 95 and 96 of Eb, thereby creating an N-linked glycosylation site at position N92 and N100. This results in the following secreted protein sequence:

30 tlcgaelvdaqlfvcgrgyfinkptgygssapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnkna  
srgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaecrgkkgk (SEQ ID NO:13)

Example 7

A hIGF-2-E precursor polypeptide of the invention having the following modifications can be expressed: deletion of P4, deletion of S5, and deletion of E6; mutation of R38 to A; and deletion of R68 and deletion of D69. This results in the following secreted protein sequence:

ayrtlcggelvdltlqfvcgdrfyfsrpsrsvrsargiveeccfrscdlalletcatpaksevstptvlpdnfprypvgkffqydtwkq  
stqrlrrglpallrarrghvlakeleafreakrhrplialptqdpahggappemasnrk (SEQ ID NO:14)

Example 8

A hIGF-1-Ea precursor polypeptide of the invention having the following mutations can be expressed: deletion of G1 and deletion of P2; mutation of E3 to X where X is a nonnatural amino acid that is pegylated; mutation of R37 to A; and deletion of R71 and deletion of S72. This results in the following secreted protein sequence:

Xtlcgaelvdaqlqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplpaksavraqrhtdmpktqkevhlkna  
srgsagnknyrm (SEQ ID NO:15)

Examples 9-78 ( $\Delta$  = deletion)

9) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ R71

tlcgaelvdaqlqfvcgdrfyfnkptgygssrapqtgivedccfrscdlrrlemycaplpaksavraqrhtdmpktqkevhlkna  
rgsagnknyrm (SEQ ID NO:16)

10) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ S72

tlcgaelvdaqlqfvcgdrfyfnkptgygssrapqtgivedccfrscdlrrlemycaplpaksarvraqrhtdmpktqkevhlkna  
rgsagnknyrm (SEQ ID NO:17)

10) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ R71,  $\Delta$ S72

tlcgaelvdalqfvcgdrfyfnkptgygsssarapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlknasr  
gsagnknyrm (SEQ ID NO:18)

11) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71

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tlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlknas  
rgsagnknyrm (SEQ ID NO:19)

12) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ S72

10

tlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkevhlknas  
rgsagnknyrm (SEQ ID NO:20)

13) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3,  $\Delta$ R37;  $\Delta$ R71

15

tlcgaelvdalqfvcgdrfyfnkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlknasr  
gsagnknyrm (SEQ ID NO:21)

14) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3,  $\Delta$ R37;  $\Delta$ S72

20

tlcgaelvdalqfvcgdrfyfnkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkevhlknasr  
gsagnknyrm (SEQ ID NO:22)

15) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3;  $\Delta$ R37;  $\Delta$ R71,  $\Delta$ S72

25

tlcgaelvdalqfvcgdrfyfnkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlknasrg  
sagnknyrm (SEQ ID NO:23)

16) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ R71

30

tlcgaelvdalqfvcgdrfyfinkptgygsssarapqtgivdeccfrscdlrrlemycaplkpaksasvraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteasqirgkkkeqrreigsmaecrgkkgk (SEQ ID NO:24)

17) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ S72

5

tlcgaelvdalqfvcgdrfyfinkptgygsssarapqtgivdeccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteasqirgkkkeqrreigsmaecrgkkgk (SEQ ID NO:25)

18) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71

10

tlcgaelvdalqfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksasvraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteasqirgkkkeqrreigsmaecrgkkgk (SEQ ID NO:26)

19) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ S72

15

tlcgaelvdalqfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteasqirgkkkeqrreigsmaecrgkkgk (SEQ ID NO:27)

20) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

20

tlcgaelvdalqfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
ntksqrrkgwpkthpggeqkegteasqirgkkkeqrreigsmaecrgkkgk (SEQ ID NO:28)

21) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3,  $\Delta$ R37;  $\Delta$ R71

25

tlcgaelvdalqfvcgdrfyfinkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksasvraqrhtdmpktqkyqppstnk  
ntksqrrkgwpkthpggeqkegteasqirgkkkeqrreigsmaecrgkkgk (SEQ ID NO:29)

22) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3,  $\Delta$ R37;  $\Delta$ S72

30

tlcgaelvdalqfvcgdrfyfnkptgygssrapqgtgideccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstnk  
ntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:30)

23) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3,  $\Delta$ R37;  $\Delta$ R71,  $\Delta$ S72

5

tlcgaelvdalqfvcgdrfyfnkptgygssrapqgtgideccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
tksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:31)

24) hIGF-1-Ec:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ R71

10

tlcgaelvdalqfvcgdrfyfnkptgygssrapqgtgideccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:32)

25) hIGF-1-Ec:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ S72

15

tlcgaelvdalqfvcgdrfyfnkptgygssrapqgtgideccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:33)

26) hIGF-1-Ec:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ R71,  $\Delta$ S72

20

tlcgaelvdalqfvcgdrfyfnkptgygssrapqgtgideccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
ntksqrrkgstfeerk (SEQ ID NO:34)

27) hIGF-1-Ec:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E2; R37A;  $\Delta$ R71

25

tlcgaelvdalqfvcgdrfyfnkptgygssrapqgtgideccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:35)

28) hIGF-1-Ec:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ S72

30

tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgideccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:36)

29) hIGF-1-Ec: ΔG1, ΔP2, ΔE3; R37A; ΔR71, ΔS72

5

tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgideccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
ntksqrrkgstfeerk (SEQ ID NO:37)

30) hIGF-1-Ec: ΔG1, ΔP2, ΔE3, ΔR37, ΔR71

10

tlcgaelvdaqlfvcgdrfyfinkptgygssrapqtgideccfrscdlrrlemycaplkpaksasvraqrhtdmpktqkyqppstn  
ntksqrrkgstfeerk (SEQ ID NO:38)

31) hIGF-1-Ec: ΔG1, ΔP2, ΔE3, ΔR37, ΔS72

15

tlcgaelvdaqlfvcgdrfyfinkptgygssrapqtgideccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstn  
ntksqrrkgstfeerk (SEQ ID NO:39)

32) hIGF-1-Eab: ΔG1, ΔP2, ΔE3; R36A; ΔR71; insertion of Ea aa 93-102 between aa 95 and 96  
of Eb (i.e., "Eab")

20

tlcgaelvdaqlfvcgdrfyfinkptgygssrapqtgideccfrscdlrrlemycaplkpaksasvraqrhtdmpktqkyqppstn  
knasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:40)

33) hIGF-1-Eab: ΔG1, ΔP2, ΔE3; R37A; ΔR71

25

tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgideccfrscdlrrlemycaplkpaksasvraqrhtdmpktqkyqppstn  
knasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:41)

34) hIGF-1-Eab: ΔG1, ΔP2, ΔE3, ΔR37, ΔR71

30

tlcgaelvdalqfvcgdrfyfinkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksasvraqrhtdmpktqkyqppstnk  
nasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:42)

35) hIGF-1-Eab:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ S72

tlcgaelvdalqfvcgdrfyfinkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstnk  
knasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:43)

36) hIGF-1-Eab:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ S72

tlcgaelvdalqfvcgdrfyfinkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstnk  
knasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:44)

37) hIGF-1-Eab:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3,  $\Delta$ R37,  $\Delta$ S72

tlcgaelvdalqfvcgdrfyfinkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksarvraqrhtdmpktqkyqppstnk  
nasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:45)

38) hIGF-1-Eab:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R36A;  $\Delta$ R71,  $\Delta$ S72

tlcgaelvdalqfvcgdrfyfinkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
nasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:46)

39) hIGF-1-Eab:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3,  $\Delta$ R37,  $\Delta$ R71,  $\Delta$ S72

tlcgaelvdalqfvcgdrfyfinkptgygssrapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
asrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:47)

40) hIGF-1-Ea:  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

gtlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlkna  
srgsagnknyrm (SEQ ID NO:48)

41) hIGF-1-Eb:  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

5

gtlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:49)

42) hIGF-1-Eb multimer: ( $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A)-3xEb( $\Delta$ R71,  $\Delta$ S72,  $\Delta$ C-term 7 aa)-Eb( $\Delta$ R71,  
 $\Delta$ S72)

10

tlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
ntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnntksqrrkgwpkthpggeq  
kegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnntksqrrkgwpkthpggeqkegteaslqirgkkkeqrre  
gsrnaevraqrhtdmpktqkyqppstnntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ  
ID NO:50)

15

43) hIGF-1-Eb multimer: ( $\Delta$ P2,  $\Delta$ E3; R37A)-3xEb( $\Delta$ R71,  $\Delta$ S72,  $\Delta$ C-term 7 aa)-Eb( $\Delta$ R71,  
 $\Delta$ S72)

20

gtlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnntksqrrkgwpkthpgge  
qkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnntksqrrkgwpkthpggeqkegteaslqirgkkkeqr  
eigsrnaevraqrhtdmpktqkyqppstnntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk  
(SEQ ID NO:51)

25

44) hIGF-1-Ec:  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

gtlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:52)

30

45) hIGF-1-Ea:  $\Delta E3$ ; R37A;  $\Delta R71$ ,  $\Delta S72$

gptlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlkn  
asrgsagnknyrm (SEQ ID NO:53)

5

46) hIGF-1-Eb:  $\Delta E3$ ; R37A;  $\Delta R71$ ,  $\Delta S72$

gptlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppst  
nkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:54)

10

47) hIGF-1-Eb multimer: ( $\Delta G1$ ,  $\Delta P2$ ,  $\Delta E3$ ; R37A)-3xEb( $\Delta R71$ ,  $\Delta S72$ ,  $\Delta C$ -term 7 aa)-Eb( $\Delta R71$ ,  $\Delta S72$ )

tlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
ntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeq  
kegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrrei  
gsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ  
ID NO:55)

15

20 48) hIGF-1-Eb multimer: ( $\Delta E3$ ; R37A)-3xEb( $\Delta R71$ ,  $\Delta S72$ ,  $\Delta C$ -term 7 aa)-Eb( $\Delta R71$ ,  $\Delta S72$ )

gptlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppst  
nkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpg  
geqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeq  
rreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk  
(SEQ ID NO:56)

25

49) hIGF-1-Ec:  $\Delta E3$ ; R37A;  $\Delta R71$ ,  $\Delta S72$

gptlcgaelvdaqlfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppst  
nkntksqrrkgstfeerk (SEQ ID NO:57)

30

50) hIGF-1-Ea:  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

5 gptlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlkn  
asrgsagnknyrm (SEQ ID NO:58)

51) hIGF-1-Eb:  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

10 gptlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppst  
nkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:59)

52) hIGF-1-Ec:  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

15 gptlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppst  
nkntksqrrkgstfeerk (SEQ ID NO:60)

53) hIGF-1-Eab:  $\Delta$ E3; R37A;  $\Delta$  R71,  $\Delta$ S72

20 gptlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppst  
nknasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:61)

54) hIGF-1-Eb multimer: ( $\Delta$ E3; R37A)-3xEb( $\Delta$ R71,  $\Delta$ S72,  $\Delta$ C-term 7 aa)-Eb( $\Delta$ R71,  $\Delta$ S72)

25 gptlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppst  
nkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpg  
geqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeq  
reigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk  
(SEQ ID NO:62)

30 55) hIGF-1-Ea: E3A; R37A;  $\Delta$ R71,  $\Delta$ S72

gpatlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlk  
nasrgsagnknyrm (SEQ ID NO:63)

56) hIGF-1-Eb: E3A; R37A;  $\Delta$ R71,  $\Delta$ S72

5

gpatlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqpps  
tnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:64)

57) hIGF-1-Ec: E3A; R37A;  $\Delta$ R71,  $\Delta$ S72

10

gpatlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqpps  
tnkntksqrrkgstfeerk (SEQ ID NO:65)

58) hIGF-1-Eab: E3A; R37A;  $\Delta$ R71,  $\Delta$ S72

15

gpatlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqpps  
tnknasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:66)

59) hIGF-1-Eb multimer: (E3A; R37A)-3xEb( $\Delta$ R71,  $\Delta$ S72,  $\Delta$ C-term 7 aa)-Eb( $\Delta$ R71,  $\Delta$ S72)

20

gpatlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqpps  
tnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpg  
geqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeq  
rreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk

25

(SEQ ID NO:67)

60) hIGF-1-Ea:  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71,  $\Delta$ S72

30

gtlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhlkna  
srgsagnknyrm (SEQ ID NO:68)

61) hIGF-1-Eb: ΔP2, ΔE3; R37A; ΔR71, ΔS72

gtlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:69)

5

62) hIGF-1-Ec: ΔP2, ΔE3; R37A; ΔR71, ΔS72

gtlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:181)

10

63) hIGF-1-Eab: ΔP2, ΔE3; R37A; ΔR71, ΔS72

gtlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
knasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:70)

15

64) hIGF-1-Eb multimer: (ΔP2, ΔE3; R37A)-3xEb(ΔR71, ΔS72, ΔC-term 7 aa)-Eb(ΔR71, ΔS72)

gtlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpgge  
qkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqr  
eigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk  
(SEQ ID NO:71)

20

25 65) hIGF-1-Eb: ΔG1, ΔP2; E3X; R37A; ΔR71, ΔS72

Xtlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivdeccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:72)

30 66) hIGF-1-Ec: ΔG1, ΔP2; E3X; R37A; ΔR71, ΔS72

Xtlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:73)

67) hIGF-1-Eab:  $\Delta$ G1,  $\Delta$ P2; E3X; R37A;  $\Delta$ R71,  $\Delta$ S72

5

Xtlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
knasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:74)

68) hIGF-1-Eb multimer: ( $\Delta$ G1,  $\Delta$ P2; E3X; R37A)-3xEb( $\Delta$ R71,  $\Delta$ S72,  $\Delta$ C-term 7 aa)-Eb( $\Delta$ R71,  
10  $\Delta$ S72)

Xtlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpgge  
qkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrr  
15 eigsrnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk  
(SEQ ID NO:75)

69) hIGF-1-Ea:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71; S72X

20 tlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplpaksaXvraqrhtdmpktqkevhlkna  
srgsagnknyrm (SEQ ID NO:76)

70) hIGF-1-Eb:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71; S72X

25 tlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplpaksaXvraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaecrgkkgk (SEQ ID NO:77)

71) hIGF-1-Ec:  $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A;  $\Delta$ R71; S72X

30 tlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplpaksaXvraqrhtdmpktqkyqppstn  
kntksqrrkgstfeerk (SEQ ID NO:78)

72) hIGF-1-Eab:  $\Delta G1$ ,  $\Delta P2$ ,  $\Delta E3$ ; R37A;  $\Delta R71$ ; S72X

tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivedccfrscdlrrlemycaplkpaksXvraqrhtdmpktqkyqppstn  
5 knasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaecrgkkgk (SEQ ID NO:79)

73) hIGF-1-Eb multimer: ( $\Delta G1$ ,  $\Delta P2$ ,  $\Delta E3$ ; R37A)-Eb( $\Delta R71$ ; S72X;  $\Delta C$ -term 7 aa)-2xEb( $\Delta R71$ ,  $\Delta S72$ ,  $\Delta C$ -term 7 aa)-Eb( $\Delta R71$ ,  $\Delta S72$ )

10 tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivedccfrscdlrrlemycaplkpakXsavraqrhtdmpktqkyqppstn  
kntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpgge  
qkegteaslqirgkkkeqrreigsnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqr  
eigsnaevraqrhtdmpktqkyqppstnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaecrgkkgk  
(SEQ ID NO:80)

15 74) hIGF-1-Ea:  $\Delta G1$ ,  $\Delta P2$ ,  $\Delta E3$ ; R37A;  $\Delta R71$ ,  $\Delta S72$ ; N92X

tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkevhkXas  
rgsagnknyrm (SEQ ID NO:81)

20 75) hIGF-1-Eb:  $\Delta G1$ ,  $\Delta P2$ ,  $\Delta E3$ ; R37A;  $\Delta R71$ ,  $\Delta S72$ ; C142X

tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
ntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaeXrgkkgk (SEQ ID NO:82)

25 76) hIGF-1-Eab:  $\Delta G1$ ,  $\Delta P2$ ,  $\Delta E3$ ; R37A;  $\Delta R71$ ,  $\Delta S72$ ; C151X

tlcgaelvdaqlfvcgdrfyfinkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
nasrgsagnkntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsnaeXrgkkgk (SEQ ID NO:83)

78) hIGF-1-Eb multimer ( $\Delta$ G1,  $\Delta$ P2,  $\Delta$ E3; R37A)-3xEb( $\Delta$ R71,  $\Delta$ S72,  $\Delta$ C-term 7 aa)-Eb( $\Delta$ R71,  $\Delta$ S72; C71X)

tlcgaelvdalqfvcgdrfyfnkptgygssraapqtgivedccfrscdlrrlemycaplkpaksavraqrhtdmpktqkyqppstnk  
 5 ntksqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnknktsqrrkgwpkthpggeq  
 kegteaslqirgkkkeqrreigsrnaevraqrhtdmpktqkyqppstnknktsqrrkgwpkthpggeqkegteaslqirgkkkeqrrei  
 gsrnaevraqrhtdmpktqkyqppstnknktsqrrkgwpkthpggeqkegteaslqirgkkkeqrreigsrnaeXrgkkgk (SEQ  
 ID NO:84)

#### 10 Example 79: Myoblast Proliferation Assay

The myoblast proliferation assay provides a reliable in vitro indicator of IGF activity and is used as a model for factors affecting embryonic myoblasts and adult satellite cells. Factors active in this system behave similarly in primary cultures of myoblasts. The enhancement of myoblast proliferation in vitro by a peptide of this invention indicates its activity in causing increased  
 15 myoblast proliferation and, therefore, an increase in ultimate myofiber number in utero. In addition, similar enhancement of myoblast proliferation indicates that peptides of this invention can be used to enhance adult muscle hypertrophy, e.g. via stimulation of satellite muscle cell proliferation.

#### 20 Example 80: Mammary Epithelial Tissue Assay

In lactating animals, the amount of mammary epithelial tissue is a limiting factor in milk production, as these are the cells which produce and secrete milk. Employing in vitro systems, epithelial cells obtained from mammary glands of animals can be stimulated by the modified IGF-1 or IGF-2 of the present invention to proliferate and produce increased quantities of milk  
 25 constituents. It can further be demonstrated that mammary epithelial cells stimulated to proliferate in one such in vitro cell system can be reimplanted in cleared mammary fat pads and be stimulated to proliferate and/or produce milk in lactating female animals.

#### Example 81: Measurement of IGF-1 or IGF-2 in Blood or Other Body Fluids

30 The effective amount of the peptide administered parenterally per dose can be measured by a dose-response curve. For example, modified IGF peptides of the invention can be measured in

the blood or body fluids of the subject to be treated to determine the dosing. Alternatively, one can administer increasing amounts of the peptide to the subject and check the serum levels of the subject for modified IGF-1 and IGF-2. The amount of peptide to be employed can be calculated on a molar basis based on these serum levels of modified IGF-1 or IGF-2.

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One method for determining appropriate dosing of the peptide entails measuring an IGF peptide of the invention in a biological fluid such as a body or blood fluid. Measuring such levels can be done by any means, including RIA and ELISA. After measuring IGF levels, the fluid is contacted with the peptide using single or multiple doses. After this contacting step, the IGF

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levels are re-measured in the fluid. If the fluid IGF levels have fallen by an amount sufficient to produce the desired efficacy for which the molecule is to be administered, then the dose of the molecule can be adjusted to produce maximal efficacy. This method may be carried out in vitro or in vivo. Preferably, this method is carried out in vivo, i.e., after the fluid is extracted from a subject and the IGF levels measured, the peptide herein is administered to the mammal using

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single or multiple doses (that is, the contacting step is achieved by administration to an animal), and then the IGF levels are re-measured from fluid extracted from the animal.

Another method for determining dosing is to use antibodies to the peptide or another detection method for the peptide in the LIFA format.

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#### Example 82: In Vivo Pharmacokinetics of hIGF-1-Ec 3mut

Adult male mice (n = 3/group) received an intravenous (i.v.) bolus injection of rhIGF-1 at 1 mg/kg, and hIGF-1-Ec 3mut (described in Example 3) at 1.55 mg/kg. Serial blood specimens were collected at 5, 15, 30 and 60 minutes after administration of test material. Serum concentrations of rhIGF-1 and hIGF-1-Ec 3mut were determined by ELISA. This assay is specific for hIGF-1.

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Equimolar doses of rhIGF-1 and hIGF-1-Ec 3mut were administered i.v. in mice. The results show significantly higher levels of the hIGF-1-Ec 3mut protein as compared to rhIGF-1 at all examined time points, indicating that the hIGF-1-Ec 3mut is metabolically more stable than the 70 amino acid-long IGF-1.

30

| time (min) | IGF-1-Ec 3mut (nM) | IGF-1 (nM) |
|------------|--------------------|------------|
| 5          | 201.4              | 54.7       |
| 15         | 65.3               | 14.3       |
| 30         | 12                 | 2.4        |
| 60         | 0.76               | 0.2        |

5 What is claimed is

## Claims

1. A polypeptide comprising a human IGF-I precursor protein, wherein the cleavage of the E-peptide from IGF-I by a protease is reduced by modification of the precursor protein and wherein one or more of the following precursor protein residues is deleted or mutated: G1, P2, E3, R36 and R37.
2. The polypeptide of claim 1, wherein the precursor protein comprises the Ea peptide.
3. The polypeptide of claim 1, wherein the precursor protein comprises the Eb peptide.
4. The polypeptide of claim 3, wherein the last seven C-terminal amino acids of Eb are deleted.
5. The polypeptide of claim 1, wherein the precursor protein comprises the Ec peptide.
6. The polypeptide of any preceding claim, wherein G1 of the precursor protein is deleted or mutated.
7. The polypeptide of any preceding claim, wherein P2 of the precursor protein is deleted or mutated.
8. The polypeptide of any preceding claim, wherein E3 of the precursor protein is deleted or mutated.
9. The polypeptide of any preceding claim, wherein R36 of the precursor protein is deleted or mutated.
10. The polypeptide of any preceding claim, wherein R36 is mutated to alanine.
11. The polypeptide of any preceding claim, wherein R37 of the precursor protein is deleted or mutated.
12. The polypeptide of any preceding claim, wherein R37 is mutated to alanine.
13. The polypeptide of any preceding claim, further comprising the N-linked glycosylation consensus sequence NXS/T.
14. The polypeptide of claim 3, further comprising amino acids 93-102 of Ea inserted between amino acids N95 and T96 of the Eb.

15. The polypeptide of any preceding claim, further comprising an oligosaccharide covalently linked to an amino acid side chain of the precursor protein.
16. The polypeptide of claim 15, wherein the oligosaccharide is covalently linked to an arginine side chain of the precursor protein.
17. The polypeptide of any preceding claim, wherein a residue of the precursor protein is replaced by a non-natural amino acid.
18. The polypeptide of claim 17, wherein the non-natural amino acid comprises an acetylene or azido group.
19. The polypeptide of any preceding claim, further comprising a poly(ethylene glycol) moiety covalently attached to a side-chain of the precursor protein.
20. The polypeptide of any preceding claim, further comprising an additional E- peptide linked to the C-terminus of the precursor protein.
21. A polypeptide comprising, from N-terminus to C-terminus,
  - an IGF-I precursor protein comprising a first Eb peptide, wherein
    - G1, P2 , and E3 are deleted,
    - R36 and R37 are deleted,
    - R71 and S72 are deleted, and
    - the last seven C-terminal amino acids of the first Eb peptide are deleted;
  - a second Eb peptide; wherein R71, S72, and the last seven C terminal amino acids of the second Eb peptide are deleted;
  - a third Eb peptide, wherein R71, S72, and the last seven C-terminal amino acids of the third Eb peptide are deleted; and
  - a fourth Eb peptide, wherein R71 and S72 are deleted.
22. The polypeptide of any of claims 1 to 20, wherein R71 or S72 of the precursor protein is deleted or mutated.
23. A polypeptide comprising a human IGF-2 precursor protein, wherein the cleavage of the E-peptide from IGF-2 by a protease is reduced by modification of the precursor protein, and wherein one or more of the following precursor protein residues is deleted or mutated: P4, S5, E6 and R38.
24. The polypeptide of claim 23, wherein P4 of the precursor protein is deleted or mutated.
25. The polypeptide of claim 23 or claim 24, wherein S5 of the precursor protein is deleted or mutated.

26. The polypeptide of any one of claims 23 to 25, wherein E6 of the precursor protein is deleted or mutated.
27. The polypeptide of any one of claims 23 to 26, wherein R38 of the precursor protein is deleted or mutated.
28. The polypeptide of any one of claims 23 to 27, wherein R68 or D69 of the precursor protein is deleted or mutated.
29. The use of the polypeptide of any of claims 1 to 28 for the manufacture of a medicament for the treatment of a musculoskeletal disease, diabetes, neuronal cell death, or anemia.
30. A polypeptide according to any one of claims 1 to 28 for use in the treatment of a musculoskeletal disease, diabetes, neuronal cell death, or anemia.
31. A nucleic acid encoding the polypeptide of any one of claims 1-28.
32. A vector comprising a nucleic acid according to claim 31.
33. A cell transfected with a vector according to claim 32.