NOVEL WNT COMPOSITIONS AND THERAPEUTIC USES OF SUCH COMPOSITIONS

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is FATE_095_00WO_ST25.txt. The text file is 124 KB, was created on January 11, 2011, and is being submitted electronically via EFS-Web.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 61/431,701, filed January 11, 2012, which is incorporated by reference in its entirety.

BACKGROUND

Technical Field

The invention relates generally to novel Wnt compositions and therapeutic methods of using the same. The Wnt polypeptides of the invention and compositions thereof may be used therapeutically, for example for promoting muscle regeneration by promoting stem cell expansion and muscle hypertrophy.

Description of the Related Art

The Wnt family of genes encodes over twenty cysteine-rich, secreted Wnt glycoproteins that act by binding to Frizzled (Fzd) receptors on target cells. Frizzled receptors are a family of G-protein coupled receptor proteins. Binding of different members of the Wnt-family to certain members of the Fzd family can initiate signaling by one of several distinct pathways. In the "canonical pathway," activation of the signaling molecule, Disheveled, leads to the inactivation of glycogen synthase kinase-3 (GSK-3β), a cytoplasmic serine-threonine kinase. The GSK-3β target,

β-catenin, is thereby stabilized and translocates to the nucleus where it activates TCF (T-cell-factor)-dependant transcription of specific promoters (Wodarz, 1998, Dierick, 1999). "Non-canonical" Wnt pathway activation includes a subset of interactions between Wnt and Fzd that may activate Ca^{2+}pathway signaling and potentially PI3K signaling, Rho pathway signaling, and planar cell polarity (PCP) pathway signaling.

Wnts are secreted glycoproteins that function as paracrine or autocrine signals active in several primitive cell types. Although Wnt proteins are secreted from cells, they are found to be hydrophobic and are post-translationally modified by addition of a lipid moiety at a conserved cysteine residue and a conserved serine residue. These lipid modifications are widely accepted to be important for the biological activity and secretion of Wnt proteins. Lipidation and the low solubility of lipidated Wnts, however, are associated with low production yields when detergents are not used during formulation and thus, present a unique challenge for clinical scale production of Wnt. Thus, while Wnts have a tremendous potential for use as therapeutics in a variety of clinical settings, the therapeutic potential of Wnts has yet to be fully realized due to Wnt insolubility and corresponding insufficient production as a purified, biologically active therapeutic.

Accordingly, the art is in need of soluble, novel Wnt polypeptides that retain Wnt biological activity, methods for generating the novel Wnts on a clinical scale, and methods of using the novel Wnts to promote tissue formation, regeneration, maintenance and repair.

BRIEF SUMMARY

The invention provides modified Wnt polypeptides comprising one or more amino acids that reduce lipidation of the Wnt polypeptide. In a particular embodiment, the Wnt polypeptide comprises one or more amino acid deletions, insertions, or substitutions that reduce lipidation of the Wnt polypeptide.

In one embodiment, the polypeptide is a Wnt polypeptide that actives a non-canonical Wnt signaling pathway.

In a particular embodiment, a Wnt polypeptide that actives a non-canonical Wnt signaling pathway is selected from the group consisting of: Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11.

In some embodiments of the invention, the polypeptide is a Wnt7a or Wnt 5a polypeptide.

In particular embodiments, the invention provides a modified Wnt7a polypeptide having decreased lipidation relative to the lipidation of the Wnt7a polypeptide corresponding to any one of SEQ ID NOs: 2 and 6-11. In other embodiments, the invention provides a modified Wnt7a polypeptide comprising an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 73 of any one of SEQ ID NOs: 2 and 6-11. In some embodiments, the invention provides a Wnt7a polypeptide comprising an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 206 of any one of SEQ ID NOs: 2 and 6-11. In particular embodiments, the invention provides a Wnt7a polypeptide comprising amino acid deletions, insertions, or substitutions at the amino acid positions corresponding to positions 73 and 206 of any one of SEQ ID NOs: 2 and 6-11.

In some embodiments, the invention provides a Wnt7a polypeptide comprising an Alanine at the amino acid position corresponding to position 73 or 206 of any one of SEQ ID NOs: 2 and 6 – 11. In other embodiments, the invention provides a Wnt7a polypeptide comprising Alanine at the amino acid positions corresponding to positions 73 and 206 of any one of SEQ ID NOs: 2 and 6-11. The invention also provides a composition comprising any of the embodiments herein wherein the Wnt7a polypeptide is a human or mouse Wnt7a polypeptide.

In particular embodiments, the invention provides a modified Wnt5a polypeptide having decreased lipidation relative to the lipidation of the Wnt5a polypeptide corresponding to any one of SEQ ID NOs: 15 and 19-23. In other embodiments, the invention provides a modified Wnt5a polypeptide comprising an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 104 of any one of SEQ ID NOs: 15 and 19-23. In some embodiments, the invention provides a Wnt5a polypeptide comprising an amino acid deletion, insertion,

or substitution at the amino acid position corresponding to position 244 of any one of SEQ ID NOs: 15 and 19-23. In particular embodiments, the invention provides a Wnt5a polypeptide comprising amino acid deletions, insertions, or substitutions at the amino acid positions corresponding to positions 104 and 244 of any one of SEQ ID NOs: 15 and 19-23.

In some embodiments, the invention provides a Wnt5a polypeptide comprising an Alanine at the amino acid position corresponding to position 104 or 244 of any one of SEQ ID NOs: 15 and 19-11. In other embodiments, the invention provides a Wnt5a polypeptide comprising Alanine at the amino acid positions corresponding to positions 104 and 244 of any one of SEQ ID NOs: 15 and 19-23. The invention also provides a composition comprising any of the embodiments herein wherein the Wnt5a polypeptide is a human or mouse Wnt5a polypeptide.

In some embodiments, the invention provides a Wnt polypeptide comprising an amino acid sequence as set forth in any one of SEQ ID NOs: 3-5, 12-13, and 16-18.

In various embodiments, the present invention contemplates, in part, a fusion polypeptide comprising a Wnt polypeptide comprising an amino acid sequence as set forth in any one of SEQ ID NOs: 3-5, 12-13, and 16-18.

In one embodiment, the fusion polypeptide comprises a native signal peptide, a heterologous signal peptide, or a hybrid of a native and a heterologous signal peptide.

In a particular embodiment, the heterologous signal peptide is selected from the group consisting of: a CD33 signal peptide, an immunoglobulin signal peptide, a growth hormone signal peptide, an erythropoietin signal peptide, an albumin signal peptide, a secreted alkaline phosphatase signal peptide, and a viral signal peptide.

In a certain embodiment, the heterologous signal peptide is a CD33 signal peptide, an IgGκ signal peptide, or an IgGμ signal peptide.

In additional embodiments, the fusion polypeptide comprises a heterologous protease cleavage site.

In one embodiment, the heterologous protease cleavage site is selected from the group consisting of: a tobacco etch virus (TEV) protease cleavage site, a

heparin cleavage site, a thrombin cleavage site, an enterokinase cleavage site and a Factor Xa cleavage site.

In further embodiments, the fusion polypeptide comprises an epitope tag selected from the group consisting of: a HIS6 epitope, a MYC epitope, a FLAG epitope, a V5 epitope, a VSV-G epitope, and an HA epitope.

In particular embodiments, the fusion polypeptide comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 3-5 and 12-13, has increased production, secretion, or solubility compared to a corresponding native Wnt polypeptide as set forth in SEQ ID NOs: 2 and 6-11.

In certain embodiments, the fusion polypeptide comprises an amino acid sequence as set forth in any one of SEQ ID NOs: 16-18, has increased production, secretion, or solubility compared to a corresponding native Wnt polypeptide as set forth in SEQ ID NOs: 15 and 19-23.

The invention also provides polynucleotides encoding Wnt polypeptides having one or more amino acids that reduce lipidation of the Wnt polypeptide. Some embodiments of the invention provide a vector comprising a polynucleotide encoding a Wnt polypeptide having one or more amino acids that reduce lipidation of the Wnt polypeptide. The invention also provides a host cell comprising such vector, and Wnt polypeptides produced by the host cell.

The invention also provides compositions comprising the Wnt polypeptides, polynucleotides, and vectors of the invention. In some embodiments, the composition comprises a pharmaceutically-acceptable salt, carrier, or excipient, and in some embodiments, the composition is soluble in an aqueous solution. In particular embodiments of the invention, the composition is formulated for injection. In certain embodiments, the composition is formulated without a detergent. In related embodiments, detergent is substantially absent from the formulation of the composition. In another related embodiment, the formulated composition is substantially free of detergent. In more specific embodiments the composition is formulated for one or more of intravenous injection, intracardiac injection, subcutaneous injection, intraperitoneal injection, or direct injection into a muscle.

In some embodiments of the invention, the composition promotes tissue formation, regeneration, maintenance or repair. In particular embodiments, the tissue is muscle, and in more specific embodiments the muscle is skeletal, cardiac, or smooth muscle.

In other embodiments, the composition of the invention promotes stem cell expansion. In some embodiments, the stem cell is an adult stem cell, and in particular embodiments, the adult stem cell is a satellite stem cell.

In some embodiments, the composition of the invention promotes muscle hypertrophy or prevents atrophy.

The invention additionally provides a method for treating or preventing muscle loss comprising administering to a subject a composition having a Wnt polypeptide comprising one or more amino acids that reduce lipidation of the Wnt polypeptide. In some embodiments, the composition comprises a pharmaceutically-acceptable salt, carrier, or excipient, and in particular embodiments the composition is soluble in an aqueous solution. In other particular embodiments, the composition is formulated for injection, and in even more particular embodiments, the composition is formulated for one or more of intravenous injection, intracardiac injection, subcutaneous injection, intraperitoneal injection, or direct injection into muscle.

In certain embodiments, the composition is formulated without a detergent. In related embodiments, detergent is substantially absent from the formulation of the composition. In another related embodiment, the formulated composition is substantially free of detergent.

In some embodiments of the method of the invention, the subject has or is at risk of having a disease or condition affecting muscle. In particular embodiments, the disease is a degenerative disease, and in more particular embodiments the degenerative disease is muscular dystrophy. In even more particular embodiments, the muscular dystrophy is selected from Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Emery-Dreifuss muscular dystrophy, Landouzy-Dejerine muscular dystrophy, facioscapulohumeral muscular dystrophy (FSH), Limb-Girdle muscular dystrophies, von Graefe-Fuchs muscular dystrophy, oculopharyngeal

muscular dystrophy (OPMD), Myotonic dystrophy (Steinert's disease) and congenital muscular dystrophies.

In other embodiments of the method, the disease or condition affecting muscle is a wasting disease, muscular attenuation, muscle atrophy, ICU-induced weakness, prolonged disuse, surgery-induced weakness, or a muscle degenerative disease. In more particular embodiments, the condition is muscle atrophy associated with muscle disuse, immobilization, surgery-induced weakness, or injury.

In some embodiments, administering the composition promotes muscle atrophy. In particular embodiments, the muscle is skeletal muscle or cardiac muscle.

In other embodiments of the method of the invention, administering the composition promotes satellite cell expansion.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 shows a ClustalW alignment of all 19 human Wnt polypeptide sequences. Conserved potential post-translational lipidation sites are shaded in grey and aligned with Cysteine 73 and Serine 206 of Human Wnt 7a. Asparagine residues thought to be sites of glycosylation are underlined.

Figure 2 shows a ClustalW alignment of the conserved Wnt7a polypeptide sequences from various species.

Figure 3 shows Myoblast hypertrophy on stimulation with non-canonical Wnts. **Figure 3a** shows a representative image of *in vitro* myoblast hypertrophy stimulated by a non-canonical Wnt treatment (Wnt7a). **Figure 3b** shows data of *in vitro* myoblast fiber hypertrophy induced by certain Wnt treatments. 100 fibers were counted for each of 3 biological replicates for each treatment group and individual counts in microns and replicate medians are displayed.

Figure 4 shows a schematic representation of the various constructed Wnt7a variants. Wild type human Wnt7a sequence is shown in white, variants with specific point mutations leading to amino acid changes are seen in white with amino acid changes as indicated. Replacement of the naturally occurring Wnt7a secretion signal peptide with that of human IgG Kappa chain is indicated as grey shading. Amino

or carboxy-terminal fusions of immunoglobulin Fc regions were constructed with linker regions as indicated.

Figure 5 shows a SDS-PAGE, Western blot of Wnt7a expressed and secreted from HEK293 cells in mammalian tissue culture. The increased expression and subsequent secretion of Wnt polypeptides with exogenous secretion signal peptides from CD33 or IgG Kappa chain can be clearly seen over that of the native signal peptide.

Figure 6 shows High Performance Liquid Chromatography (HPLC) traces detecting the detergent CHAPS in solution. **Figure 6a** shows the standard curve for chaps in Phosphate Buffered Saline to calibrate the system. **Figure 6b** shows preparations of modified Wnt polypeptidesformulated in 1% CHAPS compared with the commercially available protein from R&D systems (the commercial protein contains a carrier protein that results in a second, larger peak). **Figure 6c** shows the effective removal of CHAPS from the formulation of the Wnt polypeptides using dialysis over 4 and 20 hours as indicated.

Figure 7 shows a myoblast hypertrophy assay displaying the activity of Wnt7a variants formulated in the presence or absence of detergent. Wnt proteins were constructed with the IgG Kappa secretion signal peptide. Proteins were produced in HEK293 mammalian culture systems and affinity purified. The proteins were formulated in PBS with 1% Chaps detergent. Aliquots of Each protein variant were reformulated by detergent removal using dialysis. Proteins were seen to have equal molar concentration and were applied to the C2C12 Hypertrophy assay.

Figure 8 shows the results of an experiment to determine the average fiber diameter of mouse tibialis anterior (TA) muscles electroporated with expression plasmids encoding modified human Wnt7a polypeptides as discussed elsewhere herein, wild type human Wnt7a, or a LacZ control.

Figure 9 shows the results of an experiment to determine the weight of mouse tibialis anterior (TA) muscles electroporated with expression plasmids encoding modified human Wnt7a polypeptides as discussed elsewhere herein, or a saline control.

Figure 10 shows the results of an experiment to determine the number of Pax7+ satellite stem cells in mouse tibialis anterior (TA) muscles electroporated with

expression plasmids encoding modified human Wnt7a polypeptides as discussed elsewhere herein, wild type human Wnt7a, or a LacZ control.

Figure 11 immunoglobulin Fc fusion proteins. **Figure 11a** shows a western blot of a Wnt7a protein with its native secretion signal peptide replaced with the signal peptide from immunoglobulin Kappa and constructed as a immunoglobulin Fc domain fusion protein. The secretion from mammalian culture systems is shown in figure 11a in comparison to Fc domain-alone control. **Figure 11b** shows the relative molecular weight differences between Wnt7a and Wnt7a-Fc fusion protein by SDS-PAGE western blot using an anti-Wnt7a detection antibody.

BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFIERS

SEQ ID NO: 1 sets forth a cDNA sequence of human Wnt7a.

SEQ ID NO: 2 sets forth the amino acid sequence of the human Wnt7a polypeptide encoded by SEQ ID NO: 1.

SEQ ID NO: 3 sets forth the amino acid sequence of the human Wnt7a polypeptide of SEQ ID NO: 2, having an alanine mutation at amino acid position 73.

SEQ ID NO: 4 sets forth the amino acid sequence of the human Wnt7a polypeptide of SEQ ID NO: 2, having an alanine mutation at amino acid position 206.

SEQ ID NO: 5 sets forth the amino acid sequence of the human Wnt7a polypeptide of SEQ ID NO: 2, having an alanine mutation at amino acid position 73 and at position 206.

SEQ ID NO: 6 sets forth the amino acid sequence of a mouse Wnt7a polypeptide.

SEQ ID NO: 7 sets forth the amino acid sequence of a rat Wnt7a polypeptide.

SEQ ID NO: 8 sets forth the amino acid sequence of a chicken Wnt7a polypeptide.

SEQ ID NO: 9 sets forth the amino acid sequence of a zebrafish Wnt7a polypeptide.

SEQ ID NO: 10 sets forth the amino acid sequence of a porcine Wnt7a polypeptide.

SEQ ID NO: 11 sets forth the amino acid sequence of a bovine Wnt7a polypeptide.

SEQ ID NO: 12 sets forth the amino acid sequence of a human Wnt7a polypeptide with the native secretion signal peptide replaced with the signal peptide of Human Immunoglobulin Kappa Chain.

SEQ ID NO: 13 sets forth the amino acid sequence of a human Wnt7a polypeptide having an alanine mutation at amino acid position 73 and at position 206,with the native secretion signal peptide replaced with the signal peptide of Human Immunoglobulin Kappa Chain.

SEQ ID NO: 14 sets forth a cDNA sequence of human Wnt5a.

SEQ ID NO: 15 sets forth the amino acid sequence of the human Wnt5a polypeptide encoded by SEQ ID NO: 14.

SEQ ID NO: 16 sets forth the amino acid sequence of the human Wnt5a polypeptide of SEQ ID NO: 15, having an alanine mutation at amino acid position 104.

SEQ ID NO: 17 sets forth the amino acid sequence of the human Wnt5a polypeptide of SEQ ID NO: 15, having an alanine mutation at amino acid position 244.

SEQ ID NO: 18 sets forth the amino acid sequence of the human Wnt5a polypeptide of SEQ ID NO: 15, having an alanine mutation at amino acid position 104 and at position 244.

SEQ ID NO: 19 sets forth the amino acid sequence of a mouse Wnt5a polypeptide.

SEQ ID NO: 20 sets forth the amino acid sequence of a rat Wnt5a polypeptide.

SEQ ID NO: 21 sets forth the amino acid sequence of a chicken Wnt5a polypeptide.

SEQ ID NO: 22 sets forth the amino acid sequence of a zebrafish Wnt5a polypeptide.

SEQ ID NO: 23 sets forth the amino acid sequence of a bovine Wnt5a polypeptide.

SEQ ID NO: 24 sets forth the amino acid sequence of a human Wnt1 polypeptide.

SEQ ID NO: 25 sets forth the amino acid sequence of a human Wnt2 polypeptide.

SEQ ID NO: 26 sets forth the amino acid sequence of a human Wnt2b polypeptide.

SEQ ID NO: 27 sets forth the amino acid sequence of a human Wnt3 polypeptide.

SEQ ID NO: 28 sets forth the amino acid sequence of a human Wnt3a polypeptide.

SEQ ID NO: 29 sets forth the amino acid sequence of a human Wnt4 polypeptide.

SEQ ID NO: 30 sets forth the amino acid sequence of a human Wnt5b polypeptide.

SEQ ID NO: 31 sets forth the amino acid sequence of a human Wnt6 polypeptide.

SEQ ID NO: 32 forth the amino acid sequence of a human Wnt7b polypeptide.

SEQ ID NO: 33 sets forth the amino acid sequence of a human Wnt8a polypeptide.

SEQ ID NO: 34 sets forth the amino acid sequence of a human Wnt8b polypeptide.

SEQ ID NO: 35 sets forth the amino acid sequence of a human Wnt9a polypeptide.

SEQ ID NO: 36 sets forth the amino acid sequence of a human Wnt9b polypeptide.

SEQ ID NO: 37 sets forth the amino acid sequence of a human Wnt10a polypeptide.

SEQ ID NO: 38 sets forth the amino acid sequence of a human Wnt10b polypeptide.

SEQ ID NO: 39 sets forth the amino acid sequence of a human Wnt11 polypeptide.

SEQ ID NO: 40 sets forth the amino acid sequence of a human Wnt16 polypeptide.

SEQ ID NOs: 41-46 set forth oligonucleotide sequences.

DETAILED DESCRIPTION

A. Overview

While post-translational lipidation of Wnts is believed to be required for biological activity and protein secretion, the invention provides novel Wnt polypeptides having the amino acid sites of lipidation altered so that no post-translational lipidation occurs. The proteins of the invention retain Wnt biological activity, and the invention thus provides modified Wnt compositions having improved biologic drug-like properties such as enhanced solubility, production, and formulation, and therapeutic uses for such Wnt compositions. The invention provides a novel solution to the problem posed by the insolubility of Wnt polypeptides and further, provides inventive Wnt polypeptides, including fusion polypeptides, that are suitable for clinical scale production and therapeutic use. Therapeutic uses for the Wnt compositions of the invention include, for example, promoting tissue formation, regeneration, repair or maintenance.

The practice of the invention will employ, unless indicated specifically to the contrary, conventional methods of chemistry, biochemistry, organic chemistry, molecular biology, microbiology, recombinant DNA techniques, genetics, immunology, and cell biology that are within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual (3rd Edition, 2001); Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (1982); Ausubel *et al.*, Current Protocols in Molecular Biology (John Wiley and Sons, updated July 2008); Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-interscience; Glover, DNA Cloning: A Practical Approach, vol. I & II (IRL Press, Oxford, 1985);

Anand, Techniques for the Analysis of Complex Genomes, (Academic Press, New York, 1992); Transcription and Translation (B. Hames & S. Higgins, Eds., 1984); Perbal, A Practical Guide to Molecular Cloning (1984); and Harlow and Lane, Antibodies, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1998).

All publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety.

B. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred embodiments of methods and materials are described herein. For the purposes of the present invention, the following terms are defined below.

The articles "a," "an," and "the" are used herein to refer to one or to more than one (*i.e.,* to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

As used herein, the term "about" or "approximately" refers to a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 30, 25, 20, 25, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 % to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In particular embodiments, the terms "about" or "approximately" when preceding a numerical value indicates the value plus or minus a range of 15%, 10%, 5%, or 1%.

As used herein, the term "substantially" refers to a quantity, level, concentration, value, number, frequency, percentage, dimension, size, amount, weight or length that is 95%, 96%, 97%, 98%, 99% or 100% of a reference value. For example, a composition that is substantially free of a substance, *e.g.,* a detergent, is 95%, 96%, 97%, 98%, 99% or 100% free of the specified substance, or the substance is undetectable as measured by conventional means. Similar meaning can be applied to

the term "absence of," where referring to the absence of a particular substance or component of a composition.

As used herein, the term "stem cell" refers to a cell which is an undifferentiated cell capable of (1) long term self-renewal, or the ability to generate at least one identical copy of the original cell, (2) differentiation at the single cell level into multiple, and in some instance only one, specialized cell type and (3) of *in vivo* functional regeneration of tissues. Stem cells are subclassified according to their developmental potential as totipotent, pluripotent, multipotent and oligo/unipotent.

As used herein, the term "adult stem cell" refers to a stem cell found in a developed organism. Adult stem cells include, but are not limited to, ectodermal stem cells, endodermal stem cells, mesodermal stem cells, neural stem cells, hematopoietic stem cells, muscle stem cells, and the like. A muscle stem cell is an example of stem cell that is traditionally thought to be unipotent, giving rise to muscle cells only.

As used herein, the term "satellite stem cell" refers to a type of adult stem cell that gives rise to cells of the myogenic lineage, *e.g.,* myoblasts and myocytes.

As used herein, the term "progenitor cell" refers to a cell that has the capacity to self-renew and to differentiate into more mature cells, but is committed to a lineage (*e.g.*, hematopoietic progenitors are committed to the blood lineage), whereas stem cells are not necessarily so limited. A myoblast is an example of a progenitor cell, which is capable of differentiation to only one type of cell, but is itself not fully mature or fully differentiated. A myoblast may differentiate into a myocyte.

As used herein, the term "myocyte" or "myofiber" refers to a differentiated type of cell found in muscles. Each myocyte contains myofibrils, which are long chains of sarcomeres, the contractile units of the muscle cell. There are various specialized forms of myocytes: cardiac, skeletal, and smooth muscle cells, with various properties known in the art.

As used herein, the term "self-renewal" refers to a cell with a unique capacity to produce unaltered daughter cells and to generate specialized cell types (potency). Self-renewal can be achieved in two ways. Asymmetric cell division produces one daughter cell that is identical to the parental cell and one daughter cell that is different from the parental cell and is a progenitor or differentiated cell.

Asymmetric cell division thus does not increase the number of daughter cells identical to the parental cell, but maintains the number of cells of the parental cell type. Symmetric cell division, in contrast, produces two daughter cells that are each identical to the parental cell. Symmetric cell division thus increases the number of cells identical to the parental cell, expanding the population of parental cells. In particular embodiments, symmetric cell division is used interchangeably with "cell expansion."

As used herein, the term "differentiation" refers to a developmental process whereby cells become specialized for a particular function, for example, where cells acquire one or more morphological characteristics and/or functions different from that of the initial cell type. The term "differentiation" includes both lineage commitment and terminal differentiation processes. States of undifferentiation or differentiation may be assessed, for example, by assessing or monitoring the presence or absence of biomarkers using immunohistochemistry or other procedures known to a person skilled in the art.

As used herein, the term "lineage commitment" refers to the process by which a stem cell becomes committed to forming a particular limited range of differentiated cell types. Lineage commitment arises, for example, when a stem cell gives rise to a progenitor cell during asymmetric cell division. Committed progenitor cells are often capable of self-renewal or cell division.

As used herein, the term "terminal differentiation" refers to the final differentiation of a cell into a mature, fully differentiated cell. Usually, terminal differentiation is associated with withdrawal from the cell cycle and cessation of proliferation.

As used herein, the term "muscle hypertrophy" refers to an increase in muscle size, and may include an increase in individual fiber volume and/or an increase in the cross-sectional area of myofibers, and may also include an increase in the number of nuclei per muscle fiber. Muscle hypertrophy may also include an increase in the volume and mass of whole muscles; however, muscle hypertrophy can be differentiated from muscle hyperplasia, which is an increased number of muscle fibers. In one embodiment, muscular hypertrophy refers to an increase in the number of actin and myosin contractile proteins.

As used herein, the terms "promoting," "enhancing," "stimulating," or "increasing" generally refer to the ability of a Wnt composition of the invention to produce or cause a greater physiological response (*i.e.,* measurable downstream effect), as compared to the response caused by either vehicle or a control molecule/composition. One such measurable physiological response includes, without limitation, an increase in symmetrical stem cell division compared to asymmetrical cell division, *e.g.,* increase in satellite stem cells, and/or an increase muscle hypertrophy compared to normal, untreated, or control-treated muscle cells. For example, the physiological response may be increased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, or greater. In another non-limiting example, muscle hypertrophy in response to administration of a Wnt composition of the invention may be increased by at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 125%, 150%, 175%, 200%, or greater, compared to normal, untreated, or control-treated muscle. An "increased" or "enhanced" response is typically a "statistically significant" response, and may include an increase that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.,* 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.,* 1.5, 1.6, 1.7. 1.8, etc.) the response produced by vehicle (the absence of an agent) or a control composition.

As used herein, the terms "retaining" or "maintaining," or "retain" or "maintain", generally refer to the ability of a Wnt composition of the invention (*i.e.*, a composition of a modified Wnt) to produce or cause a physiological response (*i.e.,* measurable downstream effect) that is of a similar nature to the response caused by a Wnt composition of the naturally occurring Wnt amino acid or nucleic acid sequence. For example, the Wnt compositions of the invention exhibit Wnt biological activity, and thus retain Wnt activity. The compositions of the invention also produce a physiological response, such as muscle hypertrophy, that is of a similar nature to the response caused by a naturally occurring Wnt polypeptide. A Wnt composition of the invention that elicits a similar physiological response may elicit a physiological response that is at least 5%, at least 10% , at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least

80%, at least 85%, at least 90%, at least 95% or about 100% of the level of physiological response elicited by a composition comprising a naturally occurring Wnt amino acid or nucleic acid sequence.

A modified or engineered Wnt7a polypeptide of the invention that retains the "naturally occurring Wnt7a activity" refers to a modified Wnt7a polypeptide having one or more amino acid mutations, additions, deletions, and/or substitutions that reduce lipidation of the protein, wherein the polypeptide generates a physiological response that is at least 100%, at least 90%,at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the physiological response generated by the corresponding naturally occurring Wnt7a polypeptide.

A modified or engineered Wnt5a polypeptide of the invention that retains the "naturally occurring Wnt5a activity" refers to a modified Wnt5a polypeptide having one or more amino acid mutations, additions, deletions, and/or substitutions that reduce lipidation of the protein, wherein the polypeptide generates a physiological response that is at least 100%, at least 90%,at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the physiological response generated by the corresponding naturally occurring Wnt5a polypeptide.

As used herein, the terms "decrease" or "lower," or "lessen," or "reduce," or "abate" refers generally to the ability of a Wnt composition of the invention to produce or cause a lesser physiological response (*i.e.,* downstream effects), as compared to the response caused by either vehicle or a control molecule/composition, *e.g.,* decreased apoptosis. In one embodiment, the decrease can be a decrease in gene expression or a decrease in cell signaling that normally is associated with a reduction of cell viability. A "decrease" or "reduced" response is typically a "statistically significant" response, and may include an decrease that is 1.1, 1.2, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30 or more times (*e.g.,* 500, 1000 times) (including all integers and decimal points in between and above 1, *e.g.,* 1.5, 1.6, 1.7. 1.8, etc.) the response produced by vehicle (the absence of an agent) or a control composition.

C. Wnt Signaling Pathways

The Wnt signaling pathway is an ancient and evolutionarily conserved pathway that regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during development and throughout adult life. Wnt signaling pathways downstream of the Fz receptor have been identified, including canonical or Wnt/β-catenin dependent pathways and non-canonical or β-catenin-independent pathways, which can be further divided into Planar Cell Polarity, Wnt/Ca^{2+} pathways, and others.

Wnt proteins bind to the N-terminal extra-cellular cysteine-rich domain of the Frizzled (Fz) receptor family of which there is ten Fz in humans. The Fz protein is a seven-transmembrane-span protein with topological homology to G-protein coupled receptors. In addition, to the interaction between Wnt and Fz, co-receptors are also required for mediating Wnt signaling. For example the low-density-lipoprotein-related protein5/6 (LRP5/6) is required to mediate the canonical Wnt signal whereas receptor tyrosine kinase RYK may be required for non-canonical functions. Another level of regulation of Wnt signaling occurs in the extra-cellular milieu with the presence of a diverse number of secreted Wnt antagonists. After Wnt binds to a receptor complex, the signal is transduced to cytoplasmic phosphoprotein Dishevelled (Dsh/Dvl). Dsh can directly interact with Fz. At the level of Dsh, the Wnt signal branches into at least three major cascades, canonical (β-catenin), Planar Cell Polarity and Wnt/Ca^{2+}. Further, G protein coupled receptor signaling may also stimulate growth and survival pathways such as PI3K.

1. The Canonical Wnt Signaling Pathway

The canonical Wnt signaling pathway was first identified and delineated from genetic screens in *Drosophila* and intensive studies in the fly, worm, frog, fish and mouse have led to the identification of a basic molecular signaling framework. The hallmark of the canonical Wnt pathway is the accumulation and translocation of the adherens junction associated-protein β-catenin into the nucleus. In the absence of Wnt signaling, cytoplasmic β-catenin is degraded by a β-catenin destruction complex, which includes Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A),

glycogen synthase kinase 3 β (GSK3β) and casein kinase 1α (CK1α). Phosphorylation of β-catenin within this complex by CK1α and GSK3β targets it for ubiquitination and subsequent proteolytic destruction by the proteosomal machinery. Binding of Wnt to its receptor complex composed of the Fz and the LRP5/6 induces the dual phosphorylation of LRP6 by CK1 and GSK3-β and this allows for the translocation of a protein complex containing Axin from the cytosol to the plasma membrane. Dsh is also recruited to the membrane and binds to Fz and Axin binds to phosphorylated LRP5/6. This complex formed at the membrane at Fz/LRP5/6 induces the stabilization of β-cat via either sequestration and/or degradation of Axin. B-catenin translocates into the nucleus where it complexes with Lef/Tcf family members to mediate transcriptional induction of target genes.

Canonical Wnt signaling affects formation of anterior head structure and neuroectodermal pattering, posterior patterning and tail formation, as well as for formation of various organ systems including the heart, lungs, kidney, skin and bone.

Wnts that can signal through the canonical Wnt signaling pathway include, but are not limited to, Wnt1, Wnt2, Wnt2b/13, Wnt3, Wnt3a, Wnt8, Wnt8a, Wnt8b, Wnt10a, Wnt10b, and Wnt16.

2. *The Non-Canonical Wnt Signaling Pathway*

The non-canonical pathway is often referred to as the β-catenin-independent pathway. This pathway can be further divided into at least two distinct branches, the Planar Cell Polarity pathway (or PCP pathway) and the Wnt/Ca2+ pathway, of which only the PCP is discussed in further detail herein. The PCP pathway emerged from genetic studies in *Drosophila* in which mutations in Wnt signaling components including Frizzled and Dishevelled were found to randomize the orientation of epithelial structures including cuticle hairs and sensory bristles. Cells in the epithelia are known to possess a defined apical-basolateral polarity but, in addition, they are also polarized along the plane of the epithelial layer. This rigid organization governs the orientation of structures including orientation of hair follicles, sensory bristles and hexagonal array of the ommatidia in the eye. In vertebrates, this organization has been shown to underlie the organization and orientation of muscle

cells, stereo-cilia in the sensory epithelium of the inner ear, the organization of hair follicles, and the morphology and migratory behavior of dorsal mesodermal cells undergoing gastrulation.

Wnt signaling is transduced through Fz independent of LRP5/6 leading to the activation of Dsh. Dsh through Daam1 mediates activation of Rho which in turn activates Rho kinase (ROCK). Daam1 also mediates actin polymerization through the actin binding protein Profilin. Dsh also mediates activation of Rac, which in turn activates JNK. The signaling from Rock, JNK and Profilin are integrated for cytoskeletal changes for cell polarization and motility during gastrulation.

Wnts that can signal through the non-canonical Wnt signaling pathway include, but are not limited to, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11.

3. Wnt Signaling in Muscle Cell Development

Satellite stem cells are adult stem cells that give rise to muscle cells. Satellite cells in adult skeletal muscle are located in small depressions between the sarcolemma of their host myofibers and the basal lamina. Upon damage, such as physical trauma, repeated exercise, or in disease, satellite cells become activated, proliferate and give rise to a population of myogenic precursor cells (myoblasts) expressing the myogenic regulatory factors (MRF) MyoD and Myf5. In the course of the regeneration process, myoblasts undergo multiple rounds of division before committing to terminal differentiation, fusing with the host fibers or generating new myofibers to reconstruct damaged tissue (Charge and Rudnicki, 2004). During skeletal muscle regeneration, the satellite cell population is maintained by a stem cell subpopulation, thus allowing tissue homeostasis and multiple rounds of regeneration during the lifespan of an individual (Kuang *et al.*, 2008). Satellite stem cells (Pax7+/Myf5-) represent about 10% of the adult satellite cell pool, and give rise to daughter satellite myogenic cells (Pax7+/Myf5+) through asymmetric apical-basal cell divisions.

Wnt signaling plays a key role in regulating developmental programs through embryonic development, and in regulating stem cell function in adult tissues (Clevers, 2006). Wnts are necessary for embryonic myogenic induction in the paraxial

mesoderm (Borello *et al.*, 2006; Chen *et al.*, 2005; Tajbakhsh *et al.*, 1998), as well in the control of differentiation during muscle fiber development (Anakwe *et al.*, 2003). Recently, the Wnt planar cell polarity (PCP) pathway has been implicated in regulating the orientation of myocyte growth in the developing myotome (Gros *et al.*, 2009). In the adult, Wnt signaling is thought to be necessary for the myogenic commitment of adult stem cells in muscle tissue following acute damage (Polesskaya *et al.*, 2003; Torrente *et al.*, 2004). Other studies suggest that Wnt/β-catenin signaling regulates myogenic differentiation through activation and recruitment of reserve myoblasts (Rochat *et al.*, 2004). In addition, the Wnt/β-catenin signaling in satellite cells within adult muscle appears to control myogenic lineage progression by limiting Notch signaling and thus promoting differentiation (Brack *et al.*, 2008).

Recently, it was determined that the Wnt receptor Fzd7 was markedly upregulated in quiescent satellite stem cells. In addition, further studies revealed that Wnt7a is expressed during muscle regeneration and acts through its receptor Fzd7 and Vangl2, a component of the planar cell polarity (PCP) pathway, to induce symmetric satellite stem cell expansion and dramatically enhance muscle regeneration.

Inhibition of receptor or effector molecules in the PCP pathway, *e.g.*, Fzd7 or Vangl2, is believed to abrogate the effects of Wnt7a on satellite stem cells (Le Grand *et al.*, 2009). It has further been demonstrated that administration of lipidated Wnt7a polypeptide, or a polynucleotide encoding a Wnt7a polypeptide that is subsequently post-translationally modified by lipidation, significantly increased satellite stem cell numbers *in vitro* and *in vivo*, and promoted tissue formation *in vivo*, leading to enhanced repair and regeneration in injured and diseased muscle tissue (Le Grand *et al.*, 2009).

Without wishing to be bound to any particular theory, it is contemplated that the mechanism of action of Wnt7a that leads to enhanced repair and regeneration in injured and diseased muscle tissue has two paths: Wnt7a may stimulate the symmetrical expansion of muscle satellite (stem) cells through a PCP pathway, resulting in a larger pool of cells that can subsequently differentiate into myoblasts; and secondly, Wnt7a signaling via the G protein coupled receptor (Frizzled) may stimulate phosphatidylinositol 3-kinase/Akt (protein kinase B)/mammalian target of rapamycin

(PI3K/Akt/mTOR) pathway signaling in myoblasts and myofibers, which has been shown to stimulate hypertrophy (Bodine *et al.*, Nature Cell Biology. 2001; vol. 3; pp. 1014-1017; Glass *et al.*, Nature Cell Biology. 2003; vol. 5; pp. 87-90; Ciciliot and Schiaffino, Current Pharmaceutical Design. 2010; 16(8); pp. 906-914). Wnt7a can signal via the G-protein coupled receptor Frizzled 7 and this Wnt/Frz interaction may contribute to both biological effects.

In various embodiments, the invention contemplates, in part, using Wnt compositions comprising one or more modified Wnts that signal through the non-canonical Wnt signaling pathway to repair and regenerate injured muscle tissue. In particular embodiments, the inventive compositions comprise a modified non-canonical Wnt selected from the group consisting of: Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11. In preferred embodiments, the inventive compositions comprise a modified Wnt5a or Wnt7a polypeptide. In another preferred embodiment, the inventive compositions comprise a modified Wnt5a or Wnt7a polypeptide lacking one or more lipidation sites.

In certain embodiments, the invention compositions comprise a fusion polypeptide comprising a native, heterologous, or hybrid signal peptide, and a non-canonical Wnt polypeptide, optionally lacking one or more lipidation sites.

Although the importance of the PI3K/Akt/mTOR pathway for muscle cell hypertrophy has been described, the therapeutic challenge to specifically stimulate this pathway in muscle cells poses significant obstacles to enhancing repair and regeneration in injured and diseased muscle tissue. Early studies with potent PI3-kinase activators such as IGF-1 produced hypertrophy *in vitro* but the possibility exists for "off-target" metabolic effects (*i.e.*, IGF-1 and PI3K are key regulators of housekeeping metabolic, survival and metabolic processes). Thus, the potential for a muscle-specific stimulation of a non-canonical Wnt pathway, *e.g.,* Wnt7a-Fzd7 stimulation of PI3K/Akt/mTOR pathway, would represent an important and unique therapeutic breakthrough.

As described in further detail below, the present invention contemplates, in part, inventive Wnt compositions that provide an unexpected solution to this

technological hurdle as well as other obstacles to the therapeutic use of Wnt compositions to enhance repair and regeneration in injured and diseased muscle tissue.

D. Polypeptides

Wnt signaling pathways are key components of cell signaling networks. The human Wnt gene family consists of 19 members, encoding evolutionarily conserved glycoproteins with 22 or 24 Cys residues and several conserved Asn and Ser residues. Exemplary human Wnt proteins include Wnt1, Wnt2, Wnt2b/13, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, and Wnt16.

The Wnts are secreted glycoproteins that are heavily modified prior to transport and release into the extra-cellular milieu. After signal sequence cleavage and translocation into the endoplasmic reticulum (ER), Wnts are transported through the endomembrane system to the cell surface and undergo several modifications. Wnts undergo N-linked glycosylation (Burrus and McMahon 1995; Kadowaki *et al.*, 1996; Komekado *et al.*, 2007; Kurayoshi *et al.*, 2007; Mason *et al.*, 1992; Smolich *et al.*, 1993; Tanaka *et al.* 2002). Many Wnts also are palmitoylated at the first conserved cysteine, *e.g.*, C93 in Wnt1, C77 in Wnt3a, and C104 in Wnt5a (Galli *et al.*, 2007; Kadowaki *et al.*, 1996; Komekado *et al.*, 2007; Willert *et al.* 2003). In addition, Wnt3a is modified with palmitoleic acid at a conserved serine, S209, which is also conserved in Wnt1 (S224) Wnt5a (Takada *et al.*, 2006). Furthermore, these conserved cysteine and serine residues are present in many Wnts, *e.g.,* Wnt1, Wnt3a, Wnt4, Wnt5a, Wnt6, Wnt7a, Wnt9a, wnt10a, and Wnt 11, among others (Takada *et al.*, 2006; see also Figure 1).

Wnt acylation is widely accepted to cause the notoriously hydrophobic nature of secreted Wnts (Willert *et al.*, 2003). In addition, post-translational lipidation of mammalian Wnts is believed to be important for function. Mutating a conserved N-terminal cysteine of Wnt1, Wnt3a, or Wnt5a prevented palmitoylation in cell culture. These mutant Wnts were secreted but were shown to have little or no signaling activity (Galli *et al.*, 2007; Komekado *et al.*, 2007; Kurayoshi *et al.*, 2007; Willert *et al.*, 2003), and unpalmitoylated Wnts are believed to be unable to bind Fz receptors (Komekado *et*

al., 2007; Kurayoshi *et al.* 2007). Mutating the conserved serine in the central portion of Wnt3a prevented palmitoleic acid addition and blocked secretion and thus, activity (Takada *et al.*, 2006). Research on Drosophila Wg confirmed the importance of acylation (Franch-Marro *et al.*, 2008a; Nusse 2003; van den Heuvel *et al.*, 1993).

Further, these data are supported by the porcupine (porc) phenotype in *Drosophila*, which shows a strong loss of Wg signaling (van den Heuvel *et al.*, 1993). Porc is an ER-localized integral membrane O-acyl transferase (Kadowaki *et al.*, 1996) required for Wg palmitoylation (Zhai *et al.*, 2004), and for Wg ER exit (Tanaka *et al.*, 2002). Vertebrate Porc also promotes Wnt lipidation and is required for Wnt signaling and Wnt biological activity (Galli *et al.*, 2007).

These studies establish a model in which palmitoleic acid-modification is required for secretion, and palmitate for Fz binding. Thus, Wnt polypeptides lacking either or both of these lipid modifications would be expected to lack biological activity.

In various embodiments, the invention contemplates, in part, Wnt polypeptides that have been modified or engineered to decrease or remove canonical lipidation sites, but that unexpectedly retain Wnt biological activity. In particular embodiments, the inventive Wnt polypeptides promote cell expansion and muscle hypertrophy, and promote tissue formation, regeneration, maintenance and repair. As used herein, the term "canonical" when used in reference to an amino acid sequence, refers to an amino acid or group of amino acids present in the naturally occurring polypeptide. In some contexts, "canonical" is used interchangeably with "native" when referring to amino acids present in the naturally occurring polypeptide.

In certain embodiments, a Wnt polypeptide has been modified or engineered to lack one or more of the native amino acids for lipidation of the Wnt polypeptide. In certain particular embodiments, a Wnt polypeptide has been modified or engineered to lack all of the native amino acids for lipidation of the Wnt polypeptide. In some embodiments, the Wnt polypeptide is a non-canonical Wnt polypeptide, a Wnt polypeptide that signals through a non-canonical Wnt signaling pathway. In particular embodiments, the non-canonical Wnt is selected from the group consisting of: Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11. In preferred embodiments, the Wnt polypeptide is a Wnt5a or Wnt7a polypeptide that is modified or engineered as

discussed herein to lack canonical or native lipidation sites, but that retains or has increased canonical and/or non-canonical Wnt signaling activity.

As noted above, the invention, in embodiments, provides compositions comprising engineered Wnt polypeptides or polynucleotides encoding such engineered Wnt polypeptides, using techniques known and available in the art. In particular embodiments, the Wnt polypeptides are engineered to remove one or more, or all, lipidation sites.

As used herein, the terms "polypeptide," "peptide," and "protein" are used interchangeably, unless specified to the contrary, and according to conventional meaning, *i.e.*, as a sequence of amino acids linked by peptide bonds or modified peptide bonds. In particular embodiments, the term "polypeptide" includes fusion polypeptides. Polypeptides are not limited to a specific length, *e.g.*, they may comprise a full length protein sequence or a fragment of a full length protein, and may include post-translational modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. Polypeptides of the invention may be prepared using any of a variety of well known recombinant and/or synthetic techniques, illustrative examples of which are further discussed below. However, in particular embodiments, Wnt polypeptides of the invention have been engineered such that they have one or more amino acid substitutions, deletions, insertions, or mutations that remove or eliminate one, two, or more or all lipidation sites on the Wnt polypeptide. In certain embodiments, the Wnt polypeptide is a non-canonical Wnt polypeptide, *i.e.,* a Wnt polypeptide that signals through a non-canonical Wnt signaling pathway.

In various embodiments, the Wnt polypeptide is selected from the group consisting of: Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11, wherein the Wnt polypeptide lacks, *e.g.*, by amino acid substitution, deletion, or mutation, one or more or all lipidation sites. In preferred embodiments, the Wnt polypeptide is a Wnt5a or Wnt7a polypeptide that lacks, *e.g.*, by amino acid substitution, deletion, or mutation, one or more or all lipidation sites.

As used herein, the term "non-canonical Wnt polypeptide," refers to a Wnt polypeptide that generally or predominantly signals through non-canonical Wnt

signaling pathways. Exemplary non-canonical Wnt polypeptides include, but are not limited to Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11. In some embodiments, the term "non-canonical Wnt polypeptide," refers to a modified or engineered non-canonical Wnt polypeptide having a sequence that is at least about 70%, more preferably about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or about 100%, identical to a naturally occurring non-canonical Wnt polypeptide sequence. Identity may be assessed over at least about 10, 25, 50, 100, 200, 300, or more contiguous amino acids, or may be assessed over the full length of the sequence. Methods for determining % identity or % homology are known in the art and any suitable method may be employed for this purpose. Illustrative examples of non-canonical Wnt polypeptides are set forth in SEQ ID Nos: 2-13 and 15-23, 29-32, and 39.

As used herein, the term "Wnt7a polypeptide," refers to a Wnt7a protein having a polypeptide sequence corresponding to a wild type Wnt7a sequence. In some embodiments, the term "Wnt7a polypeptide," refers to a modified or engineered Wnt7a polypeptide having a sequence that is at least about 70%, more preferably about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or about 100%, identical to a naturally occurring Wnt7a sequence. Identity may be assessed over at least about 10, 25, 50, 100, 200, 300, or more contiguous amino acids, or may be assessed over the full length of the sequence. Illustrative examples of Wnt7a polypeptides are set forth in SEQ ID Nos: 2-13.

As used herein, the term "Wnt5a polypeptide," refers to a Wnt5a protein having a polypeptide sequence corresponding to a wild type Wnt5a sequence. In some embodiments, the term "Wnt5a polypeptide," refers to a modified or engineered Wnt5a polypeptide having a sequence that is at least about 70%, more preferably about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or about 100%, identical to a naturally occurring Wnt5a sequence. Identity may be assessed over at least about 10, 25, 50, 100, 200, 300, or more contiguous amino acids, or may be assessed over the full length of the sequence. Illustrative examples of Wnt5a polypeptides are set forth in SEQ ID Nos: 15-23.

As used herein, the terms "modified Wnt polypeptide," "modified or engineered Wnt polypeptide," and "engineered Wnt polypeptide," are used interchangeably and refer to a Wnt polypeptide, biologically active fragments or variants thereof, or homolog, paralog, or ortholog thereof that comprises one or more amino acid mutations, additions, deletions, or substitutions. In particular embodiments of the invention, modified Wnt polypeptides comprise one or more amino acid mutations, additions, deletions, and/or substitutions of conserved lipidation sites in order to prevent lipidation of the Wnt polypeptide but that also result in a Wnt polypeptide that retains Wnt biological activity. In particular embodiments, the modified Wnt polypeptide lacks one or more or all lipidation sites but retains Wnt activity. Preferably, modified Wnt polypeptides of the invention retain at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the naturally occurring Wnt activity.

As used herein, the terms "modified non-canonical Wnt polypeptide," "modified or engineered non-canonical Wnt polypeptide," and "engineered non-canonical Wnt polypeptide," are used interchangeably and refer to a non-canonical Wnt polypeptide, biologically active fragments or variants thereof, or homolog, paralog, or ortholog thereof that comprises one or more amino acid mutations, additions, deletions, or substitutions. In particular embodiments of the invention, modified non-canonical Wnt polypeptides comprise one or more amino acid mutations, additions, deletions, and/or substitutions of conserved lipidation sites in order to prevent lipidation of the non-canonical Wnt polypeptide but that also result in a non-canonical Wnt polypeptide that retains non-canonical Wnt biological activity, *e.g.,* signaling through the non-canonical Wnt pathway. In particular embodiments, the modified non-canonical Wnt polypeptide lacks one or more or all lipidation sites but retains non-canonical Wnt activity. Preferably, modified non-canonical Wnt polypeptides of the invention retain at least 100%, at least 90%, at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the naturally occurring non-canonical Wnt activity.

As used herein, the terms "modified Wnt7a polypeptide," "modified or engineered Wnt7a polypeptide," and "engineered Wnt7a polypeptide," are used

interchangeably and refer to a Wnt7a polypeptide, biologically active fragments or variants thereof, or homolog, paralog, or ortholog thereof that comprises one or more amino acid mutations, additions, deletions, or substitutions. In particular embodiments, modified Wnt7a polypeptides of the invention comprise one or more amino acid mutations, additions, deletions, and/or substitutions of conserved lipidation sites in order to prevent lipidation of the Wnt7a polypeptide but that also result in a Wnt7a polypeptide that retains or has increased Wnt7a biological activity. In particular embodiments, the modified Wnt7a polypeptide lacks one or more or all lipidation sites but retains Wnt biological activity. Preferably, Wnt7A polypeptide variants of the invention retain at least 100%, at least 90%,at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the naturally occurring Wnt7a activity. Illustrative examples of modified Wnt7a polypeptides are set forth in SEQ ID Nos: 3-5 and 12-13.

As used herein, the terms "modified Wnt5a polypeptide," "modified or engineered Wnt5a polypeptide," and "engineered Wnt5a polypeptide," are used interchangeably and refer to a Wnt5a polypeptide, biologically active fragments or variants thereof, or homolog, paralog, or ortholog thereof that comprises one or more amino acid mutations, additions, deletions, or substitutions. In particular embodiments, modified Wnt5a polypeptides of the invention comprise one or more amino acid mutations, additions, deletions, and/or substitutions of conserved lipidation sites in order to prevent lipidation of the Wnt5a polypeptide but that also result in a Wnt5a polypeptide that retains or has increased Wnt5a biological activity. In particular embodiments, the modified Wnt5a polypeptide lacks one or more or all lipidation sites but retains Wnt biological activity. Preferably, Wnt5A polypeptide variants of the invention retain at least 100%, at least 90%,at least 80%, at least 70%, at least 60%, at least 50%, at least 40%, at least 30%, at least 20%, at least 10%, or at least 5% of the naturally occurring Wnt5a activity. Illustrative examples of modified Wnt5a polypeptides are set forth in SEQ ID Nos: 16-18.

In particular embodiments, the modified Wnt polypeptides of the invention comprise amino acid mutations, additions, deletions, and/or substitutions that decrease or prevent lipidation of the polypeptide, yet such polypeptides have Wnt

biological activity. In particular embodiments, the Wnt polypeptide is a canonical Wnt polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at one or more of the amino acid positions identified in Table 1, wherein the amino acid mutation, addition, deletion, and/or substitution prevents lipidation at at the identified position, and wherein the canonical Wnt polypeptide retains or has increased levels of canonical Wnt biological activity.

Table 1

Wnt	AA positions	Ref.SEQ ID
Wnt1	93; 224	24
Wnt2	76; 212	25
Wnt2b	88; 224	26
Wnt3	80; 212	27
Wnt3a	77; 209	28
Wnt8a	54; 186	33
Wnt8b	54; 186	34
Wnt9a	93; 221	35
Wnt9b	89; 216	36
Wnt10a	96; 268	37
Wnt10b	83; 253	38
Wnt16	81; 227	40

In particular embodiments, the Wnt polypeptide is a non-canonical Wnt polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at one or more of the amino acid positions identified in Table 2, wherein the amino acid mutation, addition, deletion, and/or substitution prevents lipidation at at the identified position, and wherein the non-canonical Wnt polypeptide retains or has increased levels of non-canonical Wnt biological activity.

Table 2

Wnt	AA positions	Ref.SEQ ID
Wnt4	78; 212	29
Wnt5a	104; 244	15
Wnt5b	83; 223	30
Wnt6	76; 228	31
Wnt7a	73; 206	2
Wnt7b	73; 206	32
Wnt11	80; 215	39

In particular embodiments, the Wnt polypeptide is a Wnt7a polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at amino acid 73 and/or 206 that prevents lipidation at such position(s), wherein the Wnt7a

polypeptide retains or has increased levels of Wnt7a biological activity. In one embodiment, the polypeptide is a Wnt7a polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at amino acid position 73 that prevents lipidation at this position, wherein the Wnt7a polypeptide retains or has increased levels of Wnt7a biological activity. In some embodiments, the Wnt polypeptide of the invention is a Wnt7a polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at amino acid position 206 that prevents lipidation of Wnt7a at this position, wherein the Wnt7a polypeptide retains or has increased levels of Wnt7a biological activity. In some embodiments, the polypeptide is a Wnt7a polypeptide comprising amino acid mutations, additions, deletions, and/or substitutions at amino acid positions 73 and 206, wherein the Wnt7a polypeptide lacks post-translational lipidation and has Wnt biological activity.

In certain embodiments, the C73 and/or S206 of a Wnt7a polypeptide are substituted with Ala or another amino acid that prevents lipidation of these residues. In other embodiments, C73 and/or S206 are mutated or deleted to prevent lipidation of these residues, *e.g.*, SEQ ID Nos: 3 – 5. In some embodiments, C73 and S206 are substituted with Ala, and the Wnt7a polypeptide of the invention lacks lipidation sites and retains some level of Wnt biological activity *e.g.*, SEQ ID NO: 5.

In particular embodiments, the Wnt polypeptide is a Wnt5a polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at amino acid 104 and/or 244 that prevents lipidation at such position(s) , wherein the Wnt5a polypeptide retains or has or increased levels of Wnt5a biological activity. In one embodiment, the polypeptide is a Wnt5a polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at amino acid position 104 that prevents lipidation at this position, wherein the Wnt5a polypeptide retains or has increased levels of Wnt5a biological activity. In some embodiments, the Wnt polypeptide of the invention is a Wnt5a polypeptide comprising an amino acid mutation, addition, deletion, and/or substitution at amino acid position 244 that prevents lipidation of Wnt5a at this position, wherein the Wnt5a polypeptide retains or has increased levels of Wnt5a biological activity. In some embodiments, the polypeptide is a Wnt5a polypeptide comprising amino acid mutations, additions, deletions, and/or substitutions

at amino acid positions 104 and 244, wherein the Wnt5a polypeptide lacks post-translational lipidation and has Wnt biological activity.

In certain embodiments, the C104 and/or S244 of a Wnt5a polypeptide are substituted with Ala or another amino acid that prevents lipidation of these residues. In other embodiments, C104 and/or S244 are mutated or deleted to prevent lipidation of these residues, *e.g.*, SEQ ID Nos: 16-18. In some embodiments, C104 and S244 are substituted with Ala, and the Wnt5a polypeptide of the invention lacks lipidation sites and retains some level of Wnt biological activity *e.g.*, SEQ ID NO: 18.

As used herein, the term "naturally occurring", refers to a polypeptide or polynucleotide sequence that can be found in nature. For example, a naturally occurring polypeptide or polynucleotide sequence would be one that is present in an organism, and can be isolated from the organism, and which has not been intentionally modified by man in the laboratory. The term "wild-type" is often used interchangeably with the term "naturally occurring."

In the context of the invention, a polypeptide, a biologically active fragment or variant thereof, or homolog, paralog, or ortholog thereof, is considered to have at least substantially the same activity as the wild-type protein when it exhibits about 10%, 20%, 30%, 40% or 50% of the activity of the wild-type protein, preferably at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, or at least 80% of the activity of the wild type protein. In particular embodiments, the polypeptide, a biologically active fragment or variant thereof, or homolog, paralog, or ortholog thereof, exhibits at least 70%, at least 80%, at least 90%, at least 95% or about 100% of the activity of the wild-type protein. In certain embodiments, an activity greater than wild type activity may be achieved. Activity of a non-canonical Wnt polypeptide, *e.g.,* a Wnt 5a or Wnt7a polypeptide, a biologically active fragment or variant thereof, or homolog, paralog, or ortholog thereof, for example, can be determined by measuring its ability to mimic wild-type Wnt biological activity by, for example, stimulating the Wnt signaling pathway, such as by promoting symmetrical stem cell expansion or cell growth, and comparing the ability to the activity of a wild type protein. Methods of measuring and characterizing stem cell division, *e.g.,* satellite stem cell division, and cell growth, *e.g.,* muscle hypertrophy are known in the art.

As used herein, the term "biologically active fragment," as applied to fragments of a reference polynucleotide or polypeptide sequence, refers to a fragment of a modified Wnt polypeptide that has at least about 5, 10, 15, 20, 25, 30, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, 100, 110, 120, 150, 200, 300, 400, 500, 600, 700, 800, 900, or 1000% or more of the biological activity of a Wnt reference sequence, such as its biological activity to stimulate the Wnt signaling pathway. Certain embodiments of the present invention contemplate, in part, biologically active fragments of a modified Wnt polypeptide of at least about 20, 50, 100, 150, 200, 250, or 300 contiguous amino acid residues in length or polynucleotide sequences encoding the same, including all integers in between, which comprise or encode a polypeptide having the biological activity of a reference Wnt polypeptide, *e.g.,* a naturally occurring Wnt polypeptide.

Modified polypeptides include polypeptide variants. The term "variant" as used herein, refers to polypeptides that are distinguished from a reference polypeptide by the modification, addition, deletion, or substitution of at least one amino acid residue, as discussed elsewhere herein and as understood in the art. In certain embodiments, a polypeptide variant is distinguished from a reference polypeptide by one or more amino acid substitutions (*e.g.*, 1, 2, 3, 4, 5 or more substitutions), which may be conservative or non-conservative. For example, in various embodiments, one or more conservative or non-conservative substitutions can be made in any amino acid residue that is targeted for lipidation in the naturally occurring Wnt polypeptide.

In other particular embodiments, Wnt polypeptide variants comprise one or more amino acid additions, deletions, or substitutions in order to prevent lipidation, to increase Wnt pathway signaling activity, and/or to increase stability of the modified Wnt polypeptide compared to the naturally occurring Wnt polypeptide.

In other particular embodiments, non-canonical Wnt polypeptide variants comprise one or more amino acid additions, deletions, or substitutions in order to prevent lipidation, to increase Wnt pathway signaling activity, and/or to increase stability of the modified Wnt polypeptide compared to the naturally occurring non-canonical polypeptide.

In other particular embodiments, Wnt7a polypeptide variants comprise one or more amino acid additions, deletions, or substitutions in order to prevent lipidation, to increase Wnt pathway signaling activity, and/or to increase stability of the modified Wnt polypeptide compared to the naturally occurring Wnt7a polypeptide.

In other particular embodiments, Wnt5a polypeptide variants comprise one or more amino acid additions, deletions, or substitutions in order to prevent lipidation, to increase Wnt pathway signaling activity, and/or to increase stability of the modified Wnt polypeptide compared to the naturally occurring Wnt5a polypeptide.

To generate such variants, one skilled in the art, for example, can change one or more of the codons of the encoding DNA sequence, *e.g.*, according to Table 3.

TABLE 3 - Amino Acid Codons

Amino Acids	**Codons**					
Alanine	GCA	GCC	GCG	GCU		
Cysteine	UGC	UGU				
Aspartic acid	GAC	GAU				
Glutamic acid	GAA	GAG				
Phenylalanine	UUC	UUU				
Glycine	GGA	GGC	GGG	GGU		
Histidine	CAC	CAU				
Isoleucine	AUA	AUC	AUU			
Lysine	AAA	AAG				
Leucine	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	AUG					
Asparagine	AAC	AAU				
Proline	CCA	CCC	CCG	CCU		
Glutamine	CAA	CAG				
Arginine	AGA	AGG	CGA	CGC	CGG	CGU
Serine	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	ACA	ACC	ACG	ACU		
Valine	GUA	GUC	GUG	GUU		
Tryptophan	UGG					

Amino Acids	Codons	
Tyrosine	UAC	UAU

Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological or immunological activity can be found using computer programs well known in the art, such as DNASTAR™ software. If desired, amino acid substitutions can be made to change and/or remove functional groups from a polypeptide. Alternatively, amino acid changes in the protein variants disclosed herein can be conservative amino acid changes, *i.e.,* substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and uncharged polar (glycine, asparagine, glutamine, cystine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. See TABLE 4.

TABLE 4 — Conservative Amino Acid Substitutions

Original residue	**Conservative substitution**
Ala (A)	Gly; Ser
Arg I	Lys
Asn (N)	Gln; His
CI(C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr

Original residue	Conservative substitution
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions also are permissible and can be determined empirically or in accord with other known conservative (or non-conservative) substitutions.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.,* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity.

Variants of the polypeptides of the invention include glycosylated forms, aggregative conjugates with other molecules, and covalent conjugates with unrelated chemical moieties (*e.g.*, pegylated molecules). Covalent variants can be prepared by linking functionalities to groups which are found in the amino acid chain or at the N- or C-terminal residue, as is known in the art. Variants also include allelic variants, species variants, and muteins. Truncations or deletions of regions which do not affect functional activity of the proteins are also variants.

Amino acids in polypeptides of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* *244*:1081-1085, 1989). Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or

photoaffinity labeling (Smith *et al.*, *J. Mol. Biol. 224*:899-904, 1992 and de Vos *et al. Science 255*:306-312, 1992).

Certain changes do not significantly affect the folding or activity of the protein. The number of amino acid substitutions a skilled artisan would make depends on many factors, including those described above. Generally speaking, the number of substitutions for any given polypeptide will not be more than 50, 40, 30, 25, 20, 15, 10, 5 or 3.

In addition, pegylation of polypeptides and/or muteins is expected to provide improved properties, such as increased half-life, solubility, and protease resistance. Pegylation is well known in the art.

E. Fusion Polypeptides

In various embodiments, the present invention contemplates, in part, fusion polypeptides, and polynucleotides encoding fusion polypeptides. In one embodiment, the fusion polypeptide comprises a modified Wnt polypeptide, a biologically active Wnt polypeptide fragment, and/or such peptides further comprising one or more amino acid mutations, substitutions, and/or additions, as described elsewhere herein. In a particular embodiment, the fusion polypeptide comprises a non-canonical Wnt polypeptide selected from the group consisting of: Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11. In preferred embodiments, the Wnt polypeptide is a Wnt5a or Wnt7a polypeptide that is modified or engineered as discussed herein to lack canonical or native lipidation sites, but that retains or has increased Wnt signaling activity.

Fusion polypeptides may comprise a signal peptide at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the Wnt polypeptides. Fusion polypeptides may also comprise linkers or spacers, one or more protease cleavage sites, one or more epitope tags or other sequence for ease of synthesis, purification or production of the polypeptide.

Fusion polypeptide and fusion proteins refer to a polypeptide of the invention that has been covalently linked, either directly or via an amino acid linker, to one or more heterologous polypeptide sequences (fusion partners). The polypeptides

forming the fusion protein are typically linked C-terminus to N-terminus, although they can also be linked C-terminus to C-terminus, N-terminus to N-terminus, or N-terminus to C-terminus. The polypeptides of the fusion protein can be in any order.

The fusion partner may be designed and included for essentially any desired purpose provided they do not adversely affect the desired activity of the polypeptide. For example, in one embodiment, fusion partners may be selected so as to increase the solubility or stability of the protein, to facilitate production and/or purification of a Wnt polypeptide, and/or to facilitate systemic delivery and/or tissue uptake of Wnts. Fusion polypeptides may be produced by chemical synthetic methods or by chemical linkage between the two moieties or may generally be prepared using other standard techniques. In one embodiment, a Wnt fusion polypeptide comprises one or more of, or all of: a signal peptide, a Wnt polypeptide, *e.g.,* a non-canonical Wnt such as Wnt5a or Wnt7a, or a biologically active fragment thereof, a protease cleavage site, and an epitope tag.

As used herein, the term "signal peptide" refers to a leader sequence ensuring entry into the secretory pathway. For industrial production of a secreted protein, the protein to be produced needs to be secreted efficiently from the host cell or the host organism. The signal peptide may be, *e.g.*, the native signal peptide of the protein to be produced, a heterologous signal peptide, or a hybrid of native and heterologous signal peptide. Numerous signal peptides are used for production of secreted proteins.

Thus, in various embodiment, the present invention contemplates a method of improving the production and secretion of Wnt polypeptides, including non-canonical Wnt polypeptides such asWnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11, comprising expressing in cells, *e.g.,* mammalian, insect, or bacterial, a fusion polypeptide having a signal peptide and a non-canonical Wnt polypeptide that has been modified or engineered as discussed herein to lack canonical or native lipidation sites, wherein the polypeptide retains or has increased canonical and/or non-canonical Wnt signaling activity. In preferred embodiments, a method of improving the production and secretion of Wnt5a or Wnt7a comprises expressing in cells a fusion polypeptide having a signal peptide and a Wnt5a or Wnt7a polypeptide that has been modified or

engineered as discussed herein to lack canonical or native lipidation sites, but that retains or has increased canonical and/or non-canonical Wnt signaling activity.

Illustrative examples of signal peptides for use in fusion polypeptides of the invention include, but are not limited to: a CD33 signal peptide; an immunoglobulin signal peptide, *e.g.,* an IgGκ signal peptide or an IgGμ signal peptide; a growth hormone signal peptide; an erythropoietin signal peptide; an albumin signal peptide; a secreted alkaline phosphatase signal peptide, and a viral signal peptide, *e.g.,* rotovirus VP7 glycoprotein signal peptide.

In particular embodiments, the inventive fusion polypeptides comprise protease cleavage sites and epitope tags to facilitate purification and production of non-canonical Wnt polypeptides, *e.g.,* Wnt5a and Wnt7a. The position of the protease cleavage site is typically between the C-terminus of the Wnt polypeptide and the epitope tag to facilitate removal of heterologous sequences prior to delivery of the Wnt to a cell or tissue.

Illustrative examples of heterologous protease cleavage sites that can be used in fusion proteins of the invention include, but are not limited to: a tobacco etch virus (TEV) protease cleavage site, a heparin cleavage site, a thrombin cleavage site, an enterokinase cleavage site and a Factor Xa cleavage site.

Illustrative examples of epitope tags that can be used in fusion proteins of the invention include, but are not limited to: a HIS6 epitope, a MYC epitope, a FLAG epitope, a V5 epitope, a VSV-G epitope, and an HA epitope.

A peptide linker sequence may also be employed to separate the fusion polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures, if desired. Such a peptide linker sequence is incorporated into the fusion protein using standard techniques well known in the art. Certain peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be

used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea *et al.*, *Gene 40*:39 46 (1985); Murphy *et al.*, *Proc. Natl. Acad. Sci. USA 83*:8258 8262 (1986); U.S. Pat. No. 4,935,233 and U.S. Pat. No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference. The two coding sequences can be fused directly without any linker or by using a flexible polylinker composed of the pentamer Gly-Gly-Gly-Gly-Ser repeated 1 to 3 times. Such linker has been used in constructing single chain antibodies (scFv) by being inserted between VH and VL (Bird *et al.*, 1988, Science 242:423-426; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. U.S.A. 85:5979-5883). The linker is designed to enable the correct interaction between two beta-sheets forming the variable region of the single chain antibody. Other linkers which may be used include Glu-Gly-Lys-Ser-Ser-Gly-Ser-Gly-Ser-Glu-Ser-Lys-Val-Asp (Chaudhary *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:1066-1070) and Lys-Glu-Ser-Gly-Ser-Val-Ser-Ser-Glu-Gln-Leu-Ala-Gln-Phe-Arg-Ser-Leu-Asp (Bird *et al.*, 1988, Science 242:423-426).

In one embodiment, fusion polypeptides of the invention comprise a portion of an antibody, such as an immunoglobulin "Fc region", and a modified Wnt polpeptide, such as a Wnt5a or Wnt7a polypeptide, that has been modified or engineered as discussed herein to lack canonical or native lipidation sites, but that retains or has increased canonical and/or non-canonical Wnt signaling activity. The Fc region of the antibody is composed of two heavy chains that contribute two or three constant domains depending on the class of the antibody. The Fc region can be obtained from any of the classes of immunoglobulin, IgG, IgA, IgM, IgD and IgE. In some embodiments, the Fc region is a wild-type Fc region. In some embodiments, the Fc region is a mutated Fc region. In some embodiments, the Fc region is truncated at the N-terminal end by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids, (e.g., in the hinge domain). Wnt fusion polypeptides of the invention comprising an Fc region may have improved production and/or purification efficiencies.

In one embodiment, the Wnt fusion polypeptide of the invention comprises a Wnt7a polypeptide modified to lack native lipidation sites, but that retains non-canonical Wnt signaling activity, and a human IgG Fc region. In a specific embodiment, the Wnt7a polypeptide comprises an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 73 or 206 of SEQ ID NO: 2, and a human IgG Fc region. In a specific embodiment, the Wnt7a polypeptide comprises amino acid deletions, insertions, or substitutions at the amino acid positions corresponding to positions 73 and 206 of SEQ ID NO: 2, and a human IgG Fc region. In a specific embodiment, the Wnt7a polypeptide comprises alanine at the amino acid position corresponding to position 73 or 206 of SEQ ID NO: 2, and a human IgG Fc region. In one embodiment, the Wnt7a polypeptide comprises alanine at the amino acid positions corresponding to positions 73 and 206 of SEQ ID NO: 2, and a human IgG Fc region.

Fusion polypeptides comprising an Fc region and a modified non-canonical Wnt polypeptide, *e.g.,* Wnt5a or Wnt7a, may further comprise one or more of, or all of a native or heterologous signal peptide, protease cleavage sites and epitope tags.

In preferred embodiments, a method of improving the half-life, pharmacokinetic properties, solubility, and production efficiency of a modified Wnt5a or Wnt7a polypeptide comprises expressing in cells a fusion polypeptide having a an Fc region and/or signal peptide and a Wnt5a or Wnt7a polypeptide that has been modified or engineered as discussed herein to lack canonical or native lipidation sites, but that retains or has increased canonical and/or non-canonical Wnt signaling activity.

For example, a modified Wnt5a or Wnt7a polypeptide fused to an immunoglobulin Fc region has increased systemic half-life, improved pharmacokinetic properties, solubility and production efficiency. In one embodiment, fusing a Wnt polypeptide to an Fc portion of an antibody optimizes the pharmacokinetic and pharmacodynamic properties of the fusion polypeptide. For example, the Fc portion of the polypeptide may protect the polypeptide from degradation, keeping the polypepitde in circulation longer. In general, polypeptides, fusion polypeptides (as well as their encoding polynucleotides), and cells are isolated. An "isolated" polypeptide or

polynucleotide is one that is removed from its original environment. For example, an "isolated peptide" or an "isolated polypeptide" and the like, as used herein, refer to in vitro isolation and/or purification of a peptide or polypeptide molecule from a cellular environment, and from association with other components of the cell, *i.e.*, it is not significantly associated with in vivo substances. Similarly, an "isolated polynucleotide," as used herein, refers to a polynucleotide that has been purified from the sequences which flank it in a naturally-occurring state, *e.g.*, a DNA fragment that has been removed from the sequences that are normally adjacent to the fragment. A polynucleotide is considered to be isolated if, for example, it is cloned into a vector that is not a part of the natural environment. An "isolated cell" refers to a cell that has been obtained from an *in vivo* tissue or organ and is substantially free of extracellular matrix. Preferably, a polypeptide, polynucleotide, or cell is isolated if it is at least about 60% pure, at least about 70% pure, at least about 80% pure, at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure.

As used herein, the term "obtained from" means that a sample such as, for example, a polynucleotide or polypeptide is isolated from, or derived from, a particular source, such as a recombinant host cell. In another embodiment, the term "obtained from" refers to a cell isolated from or derived from a source such as an in vivo tissue or organ.

F. Polynucleotides

The present invention also provides isolated polynucleotides that encode Wnt polypeptides of the invention. In various embodiments, the present invention contemplates, in part, Wnt polynucleotides that encode polypeptides that lack canonical lipidation sites, but that retain Wnt biological activity, and in some embodiments have increased Wnt signaling activity. In particular embodiments, the inventive Wnt polynucleotides encode Wnt polypeptides that promote stem cell expansion and promote tissue formation, regeneration, maintenance and repair.

The inventive Wnt polynucleotides are suitable for clinical scale production of Wnt polypeptides and for use in methods of enhancing repair and regeneration in injured and diseased muscle tissue in humans. In certain embodiments,

a Wnt polynucleotide encodes a Wnt polypeptide that lacks one or more of the native amino acids for lipidation of the Wnt polypeptide. In certain particular embodiments, a Wnt polynucleotide encodes a Wnt polypeptide that lacks all of the native amino acids for lipidation of the Wnt polypeptide. In preferred embodiments, the Wnt polynucleotide encodes a non-canonical Wnt polypeptide that lacks canonical lipidation sites, but retains or has increased Wnt biological activity. In other preferred embodiments, the Wnt polynucleotide encodes a Wnt5a or Wnt7a polypeptide that lacks canonical lipidation sites, but retains or has increased Wnt biological activity, such as non-canonical Wnt signaling activity.

Nucleic acids can be synthesized using protocols known in the art as described in Caruthers *et al.*, 1992, Methods in Enzymology 211, 3-19; Thompson *et al.*, International PCT Publication No. WO 99/54459; Wincott *et al.*, 1995, Nucleic Acids Res. 23, 2677-2684; Wincott *et al.*, 1997, Methods Mol. Bio., 74, 59-68; Brennan *et al.*, 1998, Biotechnol Bioeng., 61, 33-45; and Brennan, U.S. Pat. No. 6,001,311).

By "nucleotide" is meant a heterocyclic nitrogenous base in N-glycosidic linkage with a phosphorylated sugar. Nucleotides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other (see for example, Usman and McSwiggen, supra; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, (1994, Nucleic Acids Res. 22, 2183-2196).

As used herein, the terms "DNA" and "polynucleotide" and "nucleic acid" refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding a polypeptide refers to a DNA segment that contains one or more coding sequences yet is substantially isolated away from, or purified free from, total genomic DNA of the species from which the DNA

segment is obtained. Included within the terms "DNA segment" and "polynucleotide" are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phagemids, phage, viruses, and the like.

As will be understood by those skilled in the art, the polynucleotide sequences of this invention can include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides, and the like. Such segments may be naturally isolated, recombinant, or modified synthetically by the hand of man.

As will be recognized by the skilled artisan, polynucleotides may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Polynucleotides may comprise a native sequence (*i.e.*, an endogenous sequence that encodes a polypeptide of the invention or a portion thereof) or may comprise a variant, or a biological functional equivalent of such a sequence. Polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions, as described elsewhere herein, preferably such that the variant encodes a polypeptide that lacks canonical lipidation sites, but retains, and in some embodiments, has increased biological activity, such as pathway signaling activity.

Also included are polynucleotides that hybridize to polynucleotides that encode a polypeptide of the invention. To hybridize under "stringent conditions" describes hybridization protocols in which nucleotide sequences at least 60% identical to each other remain hybridized. High stringency hybridization conditions are conditions that enable a probe, primer or oligonucleotide to hybridize only to its target sequence. Stringent conditions are sequence-dependent and will differ. Moderately stringent conditions are conditions that use washing solutions and hybridization conditions that are less stringent (Sambrook, 1989) than those for high stringency, such that a polynucleotide will hybridize to the entire, fragments, derivatives or analogs of

nucleic acids of the present invention. Moderate stringency conditions are described in (Ausubel *et al.*, 1987; Kriegler, 1990). Low stringent conditions are conditions that use washing solutions and hybridization conditions that are less stringent than those for moderate stringency (Sambrook, 1989), such that a polynucleotide will hybridize to the entire, fragments, derivatives or analogs of nucleic acids of the present invention. Conditions of low stringency, such as those for cross-species hybridizations are described in (Ausubel *et al.*, 1987; Kriegler, 1990; Shilo and Weinberg, 1981).

In additional embodiments, the invention provides isolated polynucleotides comprising various lengths of contiguous stretches of sequence identical to or complementary to a polynucleotide encoding a polypeptide as described herein. For example, polynucleotides provided by this invention encode at least about 50, 100, 150, 200, 250, 300, or about 350 or more contiguous amino acid residues of a polypeptide of the invention, as well as all intermediate lengths. It will be readily understood that "intermediate lengths", in this context, means any length between the quoted values, such as 56, 57, 58, 59, etc., 101, 102, 103, etc.; 151, 152, 153, etc.; 201, 202, 203, etc.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein, including polynucleotides that are optimized for human and/or primate codon selection. Further, alleles of the genes comprising the polynucleotide sequences provided herein may also be used.

Polynucleotides compositions of the present invention may be identified, prepared and/or manipulated using any of a variety of well established techniques (see generally, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989, and other like references).

A variety of expression vector/host systems are known and may be utilized to contain and express polynucleotide sequences. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (*e.g.,* baculovirus); plant cell systems transformed with virus expression vectors (*e.g.,*

cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (*e.g.,* Ti or pBR322 plasmids); or animal cell systems.

As used herein, the terms "control elements" or "regulatory sequences" refer to those sequences present in an expression vector that are non-translated regions of the vector, e.g., enhancers, promoters, 5' and 3' untranslated regions, and interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the PBLUESCRIPT phagemid (Stratagene, La Jolla, Calif.) or PSPORT1 plasmid (Gibco BRL, Gaithersburg, Md.), pET plasmid (Novagen) and the like may be used. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter that is recognized by the host organism, and a transcription termination sequence. Specific initiation signals may also be used to achieve more efficient translation of sequences encoding a polypeptide of interest.

In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. Also included are *Pichia pandoris* expression systems (*see, e.g.,* Li *et al., Nature Biotechnology.* 24, 210 – 215, 2006; and Hamilton *et al.*, *Science*, 301:1244, 2003).

In cases where plant expression vectors are used, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, *EMBO J. 6*:307-311 (1987)). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection.

An insect system may also be used to express a polypeptide of interest. Exemplary baculovirus expression systems, include, but are not limited to those that utilize SF9, SF21, and Tni cells (*see, e.g.,* Murphy and Piwnica-Worms, *Curr Protoc Protein Sci*. Chapter 5:Unit5.4, 2001).

In mammalian host cells, a number of viral-based expression systems are generally available. In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells. Examples of useful mammalian host cell lines include COS-7 cells, 293 or 293T cells, BHK cells, VERO-76 cells, HELA cells, and CHO cells, including DHFR-CHO cells. Mammalian expression systems can utilize attached cell lines, for example, in T-flasks, roller bottles, or cell factories, or suspension cultures, for example, in 1L and 5L spinners, 5L, 14L, 40L, 100L and 200L stir tank bioreactors, or 20/50L and 100/200L WAVE bioreactors, among others known in the art.

Also included is cell-free expression of proteins. These and related embodiments typically utilize purified RNA polymerase, ribosomes, tRNA and ribonucleotides; these reagents may be produced by extraction from cells or from a cell-based expression system.

In particular embodiments, polypeptides of the invention are expressed and purified from bacteria. Exemplary bacterial expression vectors include, BLUESCRIPT (Stratagene); pIN vectors (Van Heeke & Schuster, *J. Biol. Chem. 264*:5503 5509 (1989)); and pGEX Vectors (Promega, Madison, Wis.) which may be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). Certain embodiments may employ *E. coli*-based expression systems.

In specific embodiments, protein expression may be controlled by a T7 RNA polymerase (*e.g.,* pET vector series). These and related embodiments may utilize the expression host strain BL21(DE3), a λDE3 lysogen of BL21 that supports T7-mediated expression and is deficient in lon and ompT proteases for improved target protein stability. Also included are expression host strains carrying plasmids encoding tRNAs rarely used in *E. coli*, such as Rosetta™ (DE3) and Rosetta 2 (DE3) strains. Cell lysis and sample handling may also be improved using reagents such as Benzonase® nuclease and BugBuster® Protein Extraction Reagent. For cell culture, auto-inducing media can improve the efficiency of many expression system, including high-throughput expression systems. Media of this type (*e.g.,* Overnight Express™ Autoinduction System) gradually elicit protein expression through metabolic shift without the addition of artificial inducing agents such as IPTG. Certain embodiments

may employ a cold-shock induced *E. coli* high-yield production system, because over-expression of proteins in *Escherichia coli* at low temperature improves their solubility and stability (*see, e.g.,* Qing *et al.*, *Nature Biotechnology*. 22:877-882, 2004).

The protein produced by a recombinant cell can be purified and characterized according to a variety of techniques. Exemplary systems for performing protein purification and analyzing protein purity include fast protein liquid chromatography (FPLC) (*e.g.,* AKTA and Bio-Rad FPLC systems), high-pressure liquid chromatography (HPLC) (*e.g.,* Beckman and Waters HPLC). Exemplary chemistries for purification include ion exchange chromatography (*e.g.,* Q, S), size exclusion chromatography, salt gradients, affinity purification (*e.g.,* Ni, Co, FLAG, maltose, glutathione, protein A/G), gel filtration, reverse-phase, ceramic HyperD® ion exchange chromatography, and hydrophobic interaction columns (HIC), among others known in the art. Also included are analytical methods such as SDS-PAGE (*e.g.,* coomassie, silver stain), immunoblot, Bradford, and ELISA, which may be utilized during any step of the production or purification process, typically to measure the purity of the protein composition.

In certain embodiments, clinical grade proteins can be isolated from *E. coli* inclusion bodies. In particular embodiments, the present invention contemplates methods for producing a recombinant Wnt polypeptide that is suitable for therapeutic uses, as described elsewhere herein.

In one embodiment, a method for producing a recombinant Wnt polypeptide includes one or more of the following steps: i) expression of a Wnt polynucleotide in a host; ii) culturing the host cell to express the Wnt polypeptide as inclusion bodies; iii) one or more steps of washing the inclusion bodies; iv) solubliizing the polypeptide; v) refolding the polypeptide; vi) purifying the polypeptide; and vii) dializing the polypeptide in a desired buffer.

In certain embodiments, Wnt polynucleotide sequences are codon optimized for expression in a bacterial host.

In addition to recombinant production methods, polypeptides of the invention, and fragments thereof, may be produced by direct peptide synthesis using solid-phase techniques (Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963)). Protein

synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Alternatively, various fragments may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

G. Compositions

In various embodiments, the invention contemplates, in part, novel compositions of Wnt polypeptides and polynucleotides encoding the same. As discussed elsewhere herein, one of the major limitations or obstacles to the therapeutic use of Wnts is their low solubility, which makes them impracticable to generate on a clinical scale. The inventors have engineered novel Wnt polypeptides that have increased solubility, stability, and that retain or have increased Wnt biological activity compared to naturally occurring Wnts. In particular embodiments, the invention provides aqueous formulations of soluble Wnt polypeptides to promote stem cell expansion and muscle hypertrophy, and promote tissue formation, regeneration, maintenance and repair. In certain embodiments, the invention provides aqueous formulations of soluble Wnt polypeptides to promote stem cell expansion and muscle hypertrophy, and promote tissue formation, regeneration, maintenance and repair, wherein detergents are substantially absent from the formulations.

The compositions of the invention may comprise one or more polypeptides, polynucleotides, vectors comprising same, etc., as described herein, and one or more pharmaceutically-acceptable salts or carriers and/or physiologically-acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. It will also be understood that, if desired, the compositions of the invention may be administered in combination with other agents as well, such as, *e.g.,* other proteins, polypeptides, small molecules or various pharmaceutically-active agents. There is virtually no limit to other components that may also be included in the compositions, provided that the additional agents do not adversely affect the therapeutic potential of the Wnt composition, such as the ability

of the composition to promote muscle hypertrophy and promote tissue formation, regeneration, maintenance and repair.

Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and those formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

In certain circumstances it will be desirable to deliver the compositions disclosed herein parenterally, intravascularly, *e.g.*, intravenously or intraarterially, intramuscularly, or even intraperitoneally as described, for example, in U.S. Pat. No. 5,543,158; U.S. Pat. No. 5,641,515 and U.S. Pat. No. 5,399,363 (each specifically incorporated herein by reference in its entirety).

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human. The preparation of an aqueous composition that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared.

In certain embodiments, the compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering genes, polynucleotides, and peptide compositions directly to the lungs via nasal aerosol

sprays has been described *e.g.,* in U.S. Pat. No. 5,756,353 and U.S. Pat. No. 5,804,212 (each specifically incorporated herein by reference in its entirety). Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, 1998) and lysophosphatidyl-glycerol compounds (U.S. Pat. No. 5,725,871, specifically incorporated herein by reference in its entirety) are also well-known in the pharmaceutical arts. Likewise, transmucosal drug delivery in the form of a polytetrafluoroetheylene support matrix is described in U.S. Pat. No. 5,780,045 (specifically incorporated herein by reference in its entirety).Particular embodiments of the invention may comprise other formulations, such as those that are well known in the pharmaceutical art, and are described, for example, in Remington: The Science and Practice of Pharmacy, 20th Edition. Baltimore, MD: Lippincott Williams & Wilkins, 2000.

H. Methods of Delivery

In one embodiment, cells, *e.g.*, stem cells such as satellite stem cells, are contacted with a composition comprising one or more inventive Wnt polypeptides and/or polynucleotides. It is contemplated that the cells of the invention may be contacted *in vitro*, *ex vivo*, or *in vivo*. In other embodiments, the Wnt compositions of the invention are administered to a subject.

The compositions of the invention can be administered (as proteins/polypeptides, or in the context of expression vectors for gene therapy) directly to the subject or delivered *ex vivo*, to cells derived from the subject (*e.g.,* as in *ex vivo* gene therapy). Direct *in vivo* delivery of the compositions will generally be accomplished by parenteral injection, *e.g.,* subcutaneously, intraperitoneally, intravenously myocardial, intratumoral, peritumoral, or to the interstitial space of a tissue. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal applications, needles, and gene guns or hyposprays.

The compositions of the invention may also be administered by direct injection into a tissue, such as a muscle. In some embodiments of the invention, a composition of the invention is administered by directly injecting the composition into muscle tissue to prevent a loss of muscle in the injected muscle or to promote

regeneration or repair of the injected muscle, for example by promoting expansion of the muscle cells or hypertrophy of the injected muscle.

Generally, delivery of nucleic acids for both *ex vivo* and *in vitro* applications can be accomplished by, for example, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, direct microinjection of the DNA into nuclei, and viral-mediated, such as adenovirus (and adeno-associated virus) or alphavirus, all well known in the art.

In certain embodiments, it will be preferred to deliver one or more modified Wnts using a viral vector or other *in vivo* polynucleotide delivery technique. In a preferred embodiment, the viral vector is a non-integrating vector or a transposon-based vector. This may be achieved using any of a variety of well-known approaches, such as vectors including adenovirus, retrovirus, lentivirus, adeno-associated virus vectors (AAV), or the use of other viral vectors as expression constructs (including without limitation vaccinia virus, polioviruses and herpes viruses).

Non-viral methods may also be employed for administering the polynucleotides of the invention. In one embodiment, a polynucleotide may be administered directly to a cell via microinjection or a tissue via injection, such as by using techniques described in Dubensky *et al.*, (1984) or Benvenisty & Reshef (1986). It is envisioned that DNA encoding a gene of interest may also be transferred in a similar manner *in vivo* and express the gene product.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA-coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). In another embodiment, polynucleotides are administered to cells via electroporation.

I. Methods of Treatment

The modified Wnt polypeptides and compositions of the invention are useful for various therapeutic applications. For example, the compositions and methods

described herein are useful for promoting tissue formation, regeneration, repair or maintenance in a subject in need thereof.

Some relevant therapeutic applications for the Wnt compositions of the invention include situations where there is a need to prevent muscle loss or regenerate lost or damaged muscle tissue by increasing muscle size, volume or strength. Such situations may include, for example, after chemotherapy or radiation therapy, after muscle injury, or in the treatment or management of diseases and conditions affecting muscle. In certain embodiments, the disease or condition affecting muscle may include a wasting disease (*e.g.,* cachexia, which may be associated with an illness such as cancer or AIDS), muscular attenuation or atrophy, or a muscle degenerative disease. Muscular attenuation and atrophy may be associated with, for example, sarcopenia (including age-related sarcopenia), ICU-induced weakness, disuse of muscle (for example disuse of muscle due to coma paralysis, injury, or immobilization), surgery-induced weakness (*e.g.,* following hip or knee replacement), or a muscle degenerative disease (*e.g.,* muscular dystrophies). This list is not exhaustive.

In certain embodiments, the polypeptides and compositions of the invention may be used to stimulate symmetrical expansion of muscle satellite cells, thereby increasing the proportion of resident satellite cells, or committed precursor cells, in a muscle tissue. The polypeptides and compositions may also be used to promote muscle hypertrophy, such as by increasing the size of individual muscle fibers. The polypeptides and compositions of the invention may thus increase both the number of muscle cells and the size of muscle cells, and as a result may be useful for example, to replace damaged or defective tissue, or to prevent muscle atrophy or loss of muscle mass, in particular, in relation to diseases and disorders affecting muscle, such as muscular dystrophy, neuromuscular and neurodegenerative diseases, muscle wasting diseases and conditions, atrophy, cardiovascular disease, stroke, heart failure, myocardial infarction, cancer, HIV infection, AIDS, and the like.

In additional embodiments, the compositions and methods are useful for repairing or regenerating dysfunctional skeletal muscle, for instance, in subjects having muscle degenerative diseases. The subject can be suspected of having, or be at risk of at having skeletal muscle damage, degeneration or atrophy. The skeletal muscle

damage may be disease related or non-disease related. The human subject may have or be at risk of having muscle degeneration or muscle wasting. The muscle degeneration or muscle wasting may be caused in whole or in part by a disease, for example aids, cancer, a muscular degenerative disease, or a combination thereof.

Illustrative examples of muscular dystrophies include, but are not limited to Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), myotonic dystrophy (also known as Steinert's disease), limb-girdle muscular dystrophies, facioscapulohumeral muscular dystrophy (FSH), congenital muscular dystrophies, oculopharyngeal muscular dystrophy (OPMD), distal muscular dystrophies and Emery-Dreifuss muscular dystrophy. See, *e.g.*, Hoffman *et al.*, *N. Engl. J. Med.*, 318.1363-1368 (1988); Bonnemann, C. G. *et al.*, *Curr. Opin. Ped.*, 8: 569-582 (1996); Worton, R., *Science*, 270: 755-756 (1995); Funakoshi, M. *et al.*, *Neuromuscul. Discord.*, 9 (2): 108-114 (1999); Lim, L. E. and Campbell, K. P., *Cure. Opin. Neurol.*, 11 (5): 443-452 (1998); Voit, T., *Brain Dev.*, 20 (2): 65-74 (1998); Brown, R. H., *Annu. Rev. Med.*, 48: 457-466 (1997); Fisher, J. and Upadhyaya, M., *Neuromuscul. Disord.*, 7 (1): 55-62 (1997).

In certain embodiments, a use of a composition as described herein for the manufacture of a medicament for promoting muscle formation, maintenance, repair, or regeneration of muscle in a subject in need thereof is provided. In particular embodiments, a composition as described herein is provided for use in the manufacture of a medicament for promoting muscle formation, maintenance, repair, or regeneration of muscle in a subject in need thereof is provided. The Wnt polypeptides may be used for preventing or treating muscle atrophy, such as by increasing the size or number of myofibers.

The composition may be administered in an effective amount, such as a therapeutically effective amount. For *in vivo* treatment of human and non-human subjects, the subject is usually administered a composition comprising an effective amount of one or more modified Wnt polypeptides of the present invention. An "effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

A "therapeutically effective amount" of a Wnt polypeptide of the invention, or a composition comprising the same, may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of a Wnt polypeptide to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of a Wnt polypeptide are outweighed by the therapeutically beneficial effects. The term "therapeutically effective amount" refers to an amount of a Wnt polypeptide or composition comprising the same that is effective to "treat" a disease or disorder in a mammal (*e.g.*, a patient).

A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically but not necessarily, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount is less than the therapeutically effective amount.

In various embodiments, the invention provides for methods of increasing the division symmetry of adult stem cells, such as satellite stem cells compared to untreated stem cell populations. The methods disclosed herein are further capable of promoting symmetrical stem cell division without altering the rate of stem cell division and can promote the survival of a population of stem cells. The methods may be performed *in vitro, ex vivo,* or *in vivo.*

In particular embodiments, compositions comprising one or more modified Wnt polypeptides and/or polynucleotides are administered *in vivo* to a subject in need thereof. As used herein, the term "subject" includes, but is not limited to, a mammal, including, *e.g.*, a human, non-human primate (*e.g.*, baboon, orangutan, monkey), mouse, pig, cow, goat, dog, cat, rabbit, rat, guinea pig, hamster, horse, monkey, sheep, or other non-human mammal; a non-mammal, including, *e.g.*, a non-mammalian vertebrate, such as a bird (*e.g.*, a chicken or duck) or a fish, and a non-mammalian invertebrate. In preferred embodiments, the subject is human. Subjects in need of treatment for a disease or condition include subjects exhibiting symptoms of such disease or condition, such as those having a disease or condition, as well as those at risk of having a disease or condition.

In particular embodiments, a method for expanding a population of satellite stem cells *in vivo, ex vivo,* or *in vitro* comprising contacting the stem cells with an effective amount of a composition comprising a modified non-canonical Wnt polypeptide or a polynucleotide encoding such a modified non-canonical Wnt polypeptide. In particular embodiments, the non-canonical Wnt is selected from the group consisting of: Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11. In preferred embodiments, the Wnt polypeptide is a Wnt5a or Wnt7a polypeptide or an active fragment or variant thereof, or ortholog, paralog, or homolog thereof, that binds to and activates a Wnt receptor.

Without being bound to any particular theory, it is believed that increasing the number of satellite cells in a tissue, provides enhanced regeneration potential of the tissue.

In particular embodiments, stem cells are isolated or maintained, and expanded *ex vivo* or *in vitro* and subsequently administered to a subject in need thereof. For example, stem cells can be cultured and expanded *ex vivo* or *in vitro* and contacted with an effective amount of a Wnt composition of the invention and then administered to a patient as a therapeutic stem cell composition according to methods known to skilled persons. In certain embodiments, the expanded stem cell population is administered to the patient in combination with a therapeutic Wnt composition.

The methods of promoting stem cell expansion can be used to stimulate the *ex vivo* or *in vitro* expansion of stem cells and thereby provide a population of cells suitable for transplantation or administration to a subject in need thereof.

In some forms of urinary continence, the dysfunctional muscle can be treated with a composition or method of the invention, for example, by direct protein injection into the muscle. Thus, in one embodiment, the method is useful for treating urinary incontinence.

In further embodiments, damaged or dysfunctional muscle tissue may be cardiac muscle. For instance, the damaged muscle tissue may be cardiac muscle damaged by a cardiovascular event such as myocardial infarct, or heart failure, where the target stem cell would be a cardiac stem cell. In accordance with another aspect of the present invention, there is provided a method of promoting cardiac stem cell

expansion or cardiac muscle hypertrophy in a mammal comprising administering to the mammal an effective amount of a composition as described herein.

Further, in addition to using the stem cells in transplants, stem cells, or compositions comprising stem cells may be used as a research tool and/or as part of a diagnostic assay or kit. Without wishing to be limiting a kit may comprise muscle stem cells, one or more modified Wnt polypeptides, cell culture or growth medium, cell cryopreservation medium, one or more pharmaceutically acceptable delivery media, one or more modified Wnt polynucleotide sequences or genetic constructs, one or more devices for implantation or delivery of cells to a subject in need thereof, instructions for using, delivering, implanting, culturing, cryopreserving or any combination thereof the cells as described herein.

Indicators of cell expansion and/or muscle hypertrophy may be monitored qualitatively or quantitatively and include, for example, changes in gross morphology, total cell number, histology, histochemistry or immunohistochemistry, or the presence, absence or relative levels of specific cellular markers. The presence, absence or relative levels of cellular markers can be analyzed by, for example, histochemical techniques, immunological techniques, electrophoresis, Western blot analysis, FACS analysis, flow cytometry and the like. Alternatively the presence of mRNA expressed from the gene encoding the cellular marker protein can be detected, for example, using PCR techniques, Northern blot analysis, the use of suitable oligonucleotide probes and the like.

All publications, patent applications, and issued patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or issued patent were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the

art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

EXAMPLES

EXAMPLE 1

WNT POLYPEPTIDES HAVE CONSERVED SITES FOR POST-TRANSLATIONAL MODIFICATION

Wnt proteins are secreted signaling proteins involved in cell survival, proliferation, division and migration. Wnts are required for effective tissue patterning during embryogenesis and tissue regeneration in the adult. Certain Wnt proteins drive skeletal muscle regeneration through stimulation of satellite stem cell symmetric expansion and muscle fiber hypertrophy.

19 human Wnts have been identified and grouped based of discrete regions of homology. The Wnt proteins have complex post-translational modifications including both glycosylation and lipidation. Protein glycosylation is required for effective protein folding and secretion. Figure 1 is an alignment of all 19 human Wnt polypeptides. The amino acid residues modified by either glycosylation or lipidation are well conserved (see shaded residues). Further, these same residues are conserved across species as can be seen from the alignment of Wnt7a polypeptides in Figures 2 and 11. Lipidation has historically been thought to be required for effective activity by fixing the mature secreted protein to the plasma membrane; effectively localizing the Wnt to its frizzled receptors. For this reason, Wnts are thought to be autocrine or local paracrine signaling molecules rather than fully systemic growth factor/cytokines.

As described elsewhere herein, protein lipidation is not a requirement for the activity of all Wnt polypeptides. The selective mutation of lipidated cysteine or serine residues in a wild type Wnt (wtWnt) sequence, *e.g.,* Wnt7a, SEQ ID NO: 2, were replaced with non-lipidated alanine residues. In the specific example of Wnt7a, the cysteine residue at position 73 and/or the serine residue at position 206 were mutated to alanine residues. This resulted in proteins comprising the sequences listed in SEQ ID Nos: 3-5, which lacked post translational lipidation at the mutated residues.

EXAMPLE 2

NON-CANONICAL WNTS INDUCE MYOBLAST HYPERTROPHY

Wnt polypeptides signal via frizzled receptors and co-receptors to stimulate several intracellular pathways. Wnts are generally categorized as either "canonical" or "non-canonical" signaling molecules where canonical signaling results in the nuclear localization of the protein β-Catenin and subsequent expression of target genes. Non-canonical signaling generally includes cellular functions of Wnts that do not directly involve the nuclear localization of β-Catenin, such as the activation of the planar cell polarity (PCP) or Calcium/PLC/PKC pathways. Receptors and coreceptors for canonical and non-canonical pathway activation are different; with the canonical signaling pathway showing dependence for the co-receptor LRP. Wnt7a is a non-canonical signaling molecule and has been shown to drive symmetrical expansion of muscle satellite stem cells via the activation of the PCP pathway (Le Grand *et al., Wnt7a activates the planar cell polarity pathway to drive the symmetric expansion of satellite stem cells. Cell Stem Cell* ***4****, 535–547, 2009*). More recently Wnt7a was shown to drive the hypertrophy of myoblasts in culture, potentially via a G-protein-depended activation of the PI3-Kinase/mTOR pathway (Julia von Maltzahn, C. Florian Bentzinger and Michael A. Rudnicki, *Nature Cell Biology, Dec. 11, 2011; epub*).

The ability of several Wnt polypeptides to induce hypertrophy of myoblast cells was tested. The Wnt polypeptides tested initially were obtained from R&D systems and represented canonical (Wnt3a) and non-canonical (Wnt5a and Wnt7a) signaling polypeptides. As shown in Figure 3, while buffer control or canonical Wnt polypeptide Wnt3a had no myoblast hypertrophy effect, both non-canonical Wnt polypeptides (Wnt7a and Wnt5a) produced significant myoblast hypertrophy effect *in vitro*.

Methods

C2C12 mouse myoblasts were obtained from ATCC (#CRL-1772) and grown on gelatin-coated tissue culture plates in DMEM (MediaTech #10-017-CV) medium supplemented with 10% 50 FBS. The cells remained less than 20% confluent

throughout the experiment. 96 well tissue culture plates were coated with 0.1% gelatin for at least 15 minutes at room temperature (RT) and 2,000 cells (in 0.2 mL of growth medium) were plated in each well of the 96-well plate. The plates were then incubated for 24 hours at 37°C. The following day, the media was aspirated and replaced with 0.2 mL of a differentiation media having DMEM (MediaTech #10-017-CV) supplemented with 2% horse serum (Fisher, Hyclone SH30074). After 3 days of differentiation, Wnt polypeptides (rhWnt7a #3008-WN/CF, rhWnt3a #5036-WN/CF or rhWnt5a #645-WN/CF (from R&D systems) were added to the cell culture and incubated for an additional 2 days.

The cells were fixed, washed, permeablized, and stained with myosin slow and fast myosin antibodies (Sigma # M4276-.2ML, Sigma # M8421-.2ML). Cells were visualized; myofiber diameter was calculated for 100 fibers per experiment; and the data from 3 independent biological replicates was collated for a total of 300 data points per treatment group. The median fiber diameter for each biological replicate group is shown in Figure 3. The mean of the median across the three biological replicates for each group was 17.5 μm for medium alone, 18.8 μm (Wnt3a), 27 μm (Wnt7a), 24.6 μm (Wnt5a), and 25.8 μm (insulin growth factor (IGF)). The increase in hypertrophy for cells treated with Wnt7a, Wnt5a and IGF was statistically significant compared with either media control or Wnt3a treatment.

EXAMPLE 3

CONSTRUCTION AND EXPRESSION OF MODIFIED WNT7A POLYPEPTIDES

Non-canonical Wnts induce muscle satellite stem cell expansion and muscle hypertrophy. Induction of both processes would be of great benefit therapeutically: for the treatment of cachexia, muscle atrophy, and muscular dystrophy. The use of Wnt as a therapeutic requires effective scaled production, and purification and formulation applicable for therapeutic use while retaining the specific Wnt activity and receptor specificity. The post-translational lipidation of Wnt polypeptides represents a potential complication to these requirements of manufacture. Wnts were generally thought to require lipid for effective activity, lipidated proteins are

challenging to purify at high concentrations and require the use of detergent formulation for solubility and stability.

To address these challenges, several variants of Wnt7a were constructed. Specifically, the amino acid residues targeted for post-translational lipidation (Cys 73 and Ser206 in Wnt7a) were mutated to Alanine residues using the following molecular biology techniques. The wild type human Wnt7a was PCR amplified using forward primer 5'-GCATGGATCCACCATGAACCGGAAAGCGCGG-3' (SEQ ID NO: 41) and reverse primer 5'-GCATGCGGCCGCTCACTTGCACGTGTACATCTCC-3' (SEQ ID NO: 42). The PCR product was inserted into pcDNA3.1(+) vector between the BamHI and Not I sites. The modified Wnt7a constructs were prepared using the QuikChange® site-directed mutagenesis method. The human Wnt7a C73A construct (cysteine at amino acid 73 substituted with alanine) was made using the human wild type Wnt7a as a template with forward primer 5'-ATGGGCCTGGACGAGGCCCAGTTTCAGTTCCGC-3' (SEQ ID NO: 43) and reverse primer 5'-GCGGAACTGAAACTGGGCCTCGTCCAGGCCCAT-3' (SEQ ID NO: 44). The human Wnt7a S206A construct (serine at amino acid 206 substituted with alanine) was made using the human wild type Wnt7a as a template using forward primer 5'-GTGCCACGGCGTGGCAGGCTCGTGCACC-3' (SEQ ID NO: 45) and reverse primer 5'-GGTGCACGAGCCTGCCACGCCGTGGCAC-3' (SEQ ID NO: 46). The human Wnt7a C73A/S206A constructs were made using the reagents for the individual C73A and S206A constructs. Final vector DNA was prepared using Qiagen Endo-free purification kits. The Wnt cDNAs in the pcDNA3 vector were expressed in HEK293 cells for 48-72 hrs. Wnt polypeptides were subsequently purified from the HEK293 culture media by affinity chromatography using an antibody specific for all variants of Wnt7a produced (Antibody: Santa Cruz K15 #26361). Activity of the purified modified Wnt polypeptides was tested using *in vitro* hypertrophy assay as seen in subsequent examples. A schematic of all Wnt7a constructs built is shown in Figure 4 (see also SEQ ID NOs: 1, 2, 3, 4, 5, 12 and 13).

EXAMPLE 4

HETEROLOGOUS SIGNAL PEPTIDES IMPROVE WNT SECRETION AND PRODUCTION

To improve production, secretion, and solubility of Wnt proteins showing poor secretion from the mammalian culture in HEK293 cells-- with the majority of expressed protein remaining within the cell -- Wnt fusion poylpeptides were constructed in which the endogenous Wnt secretion signal peptide was replaced by the signal peptide of human immunoglobulin G Kappa chain (IgGK) or that of human protein CD33. A schematic of Wnt7a fusion polypeptides comprising heterologous signal peptides is shown in Figure 4 (see also SEQ ID NOs: 12 and 13).

As shown in Figure 5, the Wnt fusion polypeptides having heterologous signal peptides performed significantly better than Wnt polypeptides comprising a native signal peptide, when compared for expression and secretion in HEK293 culture.

EXAMPLE 5

MODIFIED WNT POLYPEPTIDES CAN BE FORMULATED IN THE ABSENCE OF DETERGENT AND RETAIN STABILITY AND ACTIVITY

Wnt protein production and formulation has traditionally relied on formulation in detergent to retain solubility of these lipidated proteins. The effective therapeutic delivery of a Wnt polypeptide requires formulation in the absence of detergent. Wnt polypeptides without lipidation sites were constructed as described in Example 3, expressed in mammalian culture systems, purified from the culture media, and formulated in 1% CHAPS detergent. A HPLC-based assay was configured to allow the effective measurement of CHAPS detergent in the Wnt polypeptide formulations.

As shown in Figure 6a, a titration of CHAPS detergent in solution allowed for effective calibration of the assay. Various preparations of pure Wnt polypeptide were tested and final formulation was shown to be ~1% CHAPS solution in PBS (Figure 6b). Subsequent dialysis of the Wnt polypeptide solution against PBS alone effectively removed the detergent below the level of detection (Figure 6c). The dialyzed polypeptides were then tested for both stability and activity either in the presence or absence of the CHAPS detergent.

Incubation of the protein formulations at either 4°C or 37°C over a 7 day period showed Wnt with native lipidation sites was relatively stable when formulated in detergent but unstable when formulated in the absence of detergent. Conversely,

modified Wnts with lipidation sites removed and replaced with Alanine (C73A, S206A) were seen to have improved stability in the absence of detergent when compared to native, lipidated protein.

Wnt variants formulated with or without detergent were then tested for activity in the C2C12 hypertrophy assay as described in Example 2. Wnt polypeptides were produced in HEK293 mammalian culture systems and affinity purified. The Wnt polypeptides were formulated in PBS with 1% CHAPS detergent. Aliquots of each Wnt polypeptides variant were reformulated by detergent removal using dialysis. Wnt proteins had equal molar concentration and were applied to the C2C12 hypertrophy assay.

Wnt polypeptides produced in the HEK293 culture system with the use of heterologous signal peptides retained their activity when compared to a positive control, native Wnt sequence (Figure 7). Further, Wnt7a C73A and S206A mutants retained specific hypertrophic activity (Figure 7). All modified Wnt retained activity when formulated in detergent.

When Wnts were reformulated in the absence of detergent, only the modified Wnts comprising Alanine substituted lipidation sites retained activity, whereas native Wnts lost myoblast hypertrophic activity (Figure 7). Thus, Wnts specifically altered at conserved lipidation sites, retained biological activity. The modified Wnts also retained activity when formulated in the absence of detergent. Accordingly, the modified Wnt polypeptides of the invention represent useful therapeutic versions of the native protein.

EXAMPLE 6

MODIFIED WNT7A INCREASES MUSCLE HYPERTROPHY AND SATELLITE STEM CELL EXPANSION

To demonstrate the ability of delipidated Wnts, *e.g.*, Wnts that have one or more lipidation sites removed, to stimulate muscle regeneration *in vivo*, the modified Wnt7a were over-expressed by electroporation of CMV-Wnt7a expression plasmids into TA muscles of 3-month old mice.

1. In vivo Electroporation

Plasmids constructs encoding a LacZ, wild type Wnt7a, Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A were electroporated *in vivo* into mice. 40 μg of each plasmid DNA in 0.9 % NaCl or 0.9 % NaCl (saline) was injected directly into a left TA muscle that had been exposed by an incision through the skin of an anesthetized mouse. Immediately after injection, electric stimulation was applied directly to the TA by a pulse generator (ECM 830, BTX) of 100-150 volts for 6 pulses, with a fixed duration of 20 ms and an interval of 200 ms using 5 mm needle electrodes (BTX). Experimental and contralateral TA muscles were isolated and embedded in OCT-15% Sucrose (Tissue-Tek) and frozen with isopentane cooled by cold nitrogen.

2. Histology and Quantification

Transverse sections (8 μm) of experimental and contralateral muscles were cut with a cryostat (Leica CM1850). The entire TA muscles were sectioned, in order to compare experimental and contralateral muscles at the same level on serial sections (around 400 sections were obtained from each TA muscle). For LacZ reaction, cryosections were fixed with 0.1% gluteraldehyde and exposed to X-gal solution. For H&E and immunostaining, sections were fixed with 4% paraformaldehyde. For enumeration of fibers, pictures of laminin-stained cryosections were assembled and counted on Adobe Photoshop CS2. Quantification of myofibers caliber was performed with ImageJ. The satellite cell enumeration was performed on Photoshop, on pictures of Pax7 and Laminin co-immunostained cryosections taken in regenerated areas where all the fibers had centrally located nuclei. "Percent Pax-7+ Cells" represents the number of sub-laminar Pax7+ve satellite cells normalized per fiber number, and to the contralateral leg.

3. Statistical Analysis

A minimum of 2 and up to 5 replicates was done for experiments presented. Data are presented as standard error of the mean. Results were

assessed for statistical significance using Student's T Test (Microsoft Excel) and differences were considered statistically significant at the $p<0.05$ level.

4. Results

Electroporation of WT Wnt7a, Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A constructs produced a statistically significant increase in the average fiber diameter of mouse TA muscles compared to a LacZ control plasmid. Moreover, the Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A constructs retained Wnt biological activity of the wild type Wnt construct, as the increased the average fiber diameter of the TA muscles produced by the Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A constructs was comparable to that produced by the wild type Wnt construct. These results are shown in Figure 1.

Notably, Figure 2 shows that TA muscles electroporated with Wnt7a S206A and Wnt7a C73A/S206A constructs also exhibited a comparable increase in TA muscle mass to TA muscles electroporated with the wild type Wnt construct.

To assess whether Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A similarly stimulated the expansion of satellite stem cells *in vivo*, the numbers of satellite cells and satellite stem cells in regenerated muscle were assessed following electroporation of the modified Wnt7a expression plasmids. Over-expression of Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A resulted in statistically significant increases in the number of Pax7+ satellite cells per myofiber on sections at 3 weeks after electroporation (Wnt7a C73A, $p=0.001$, $n=4$; Wnt7a S206A, $p=0.01$, $n=2$; Wnt7a C73A/S206A, $p=0.05$, $n=2$). The increase in the number of Pax7 satellite cells induced by over-expression of Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A was comparable to the increase induced by wild type Wnt7a. These results are shown in Figure 3.

Taken together, these results shown in Figures 1-3 indicate that over-expression of Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A markedly enhances muscle regeneration, as evidenced by the presence of

increased numbers of larger fibers and the increased mass of muscle and further, increases the numbers of satellite stem cells *in vivo*. In addition, these results show that the effect produced by Wnt7a C73A, Wnt7a S206A, and Wnt7a C73A/S206A was comparable to the effect produced by wild type Wnt7a.

In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

EXAMPLE 7

WNT PROTEINS CAN BE EXPRESSED AS IMMUNOGLOBULIN FC FUSIONS

Immunoglobulin fusion proteins and/or peptibodies have been used to improve the pharmaceutical properties of the Wnt polypeptides, such as their circulating half life *in vivo*. Wnt proteins of the present invention were constructed in mammalian expression vectors (pcDNA3+) with either amino-terminal or carboxyterminal Fc-fusion domains as schematically represented in Figure 4. Amino acid residues 31-349 of native human Wnt7a or the same with C73A and/or S206A mutations were subcloned in frame with the IgG Kappa secretion signal peptide and Human IgG1e3-Fc1 domain as either a N- or C-terminal fusion. This Fc domain comprised amino acid changes that are different from native IgG1 sequence (E233P / L234V / L235A / deltaG236 + A327G / A330S / P331S) to reduce antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) effects. A 17 amino acid linker (GT(GGGGS)3) was added between the Wnt protein sequence and the Fc-fusion sequence to reduce steric hindrance and prevent reduction of Wnt specific activity. These vectors were transfected into HEK293 cells and protein expression continued for 48 hours. Protein expression and secretion was monitored by western blot and can be seen in Figures 11a and 11b. Intact fusion proteins of the expected molecular weight were seen when immune-detected with either anti-Wnt7a antibodies

or anti-Fc detection. Effective secretion was observed for the fusion proteins. Secreted proteins were subsequently purified by Protein A or Protein G affinity chromatography.

CLAIMS

1. A modified Wnt polypeptide comprising one or more amino acids that reduce lipidation of the Wnt polypeptide.

2. A Wnt polypeptide comprising one or more amino acid deletions, insertions, or substitutions that reduce lipidation of the Wnt polypeptide.

3. The Wnt polypeptide of claim 1 or claim 2, wherein the Wnt polypeptide activates a non-canonical Wnt signaling pathway.

4. The Wnt polypeptide of claim 3, wherein the Wnt polypeptide is selected from the group consisting of: Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, and Wnt11.

5. The Wnt polypeptide of claim 3, wherein the Wnt polypeptide is a Wnt7a or Wnt5a polypeptide.

6. A modified Wnt7a polypeptide having decreased lipidation relative to the lipidation of the Wnt7a polypeptide corresponding to any one of SEQ ID NOs: 2 and 6-11.

7. The modified Wnt7a polypeptide of claim 6, wherein the polypeptide comprises an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 73 of any one of SEQ ID NOs: 2 and 6-11.

8. The modified Wnt7a polypeptide of claim 6, wherein the polypeptide comprises an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 206 of any one of SEQ ID NOs: 2 and 6-11.

9. The modified Wnt7a polypeptide of claim 6, wherein the polypeptide comprises amino acid deletions, insertions, or substitutions at the amino acid positions corresponding to positions 73 and 206 of any one of SEQ ID NOs: 2 and 6-11.

10. The modified Wnt7a polypeptide of claim 6, wherein the polypeptide comprises an Alanine at the amino acid position corresponding to position 73 or 206 of any one of SEQ ID NOs: 2 and 6 - 11.

11. The modified Wnt7a polypeptide of claim 6, wherein the polypeptide comprises Alanine at the amino acid positions corresponding to positions 73 and 206 of any of SEQ ID NOs: 2 and 6-11.

12. A modified Wnt5a polypeptide having decreased lipidation relative to the lipidation of the Wnt7a polypeptide corresponding to any one of SEQ ID NOs: 15 and 19-23.

13. The modified Wnt5a polypeptide of claim 12, wherein the polypeptide comprises an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 104 of any one of SEQ ID NOs: 15 and 19-23.

14. The modified Wnt5a polypeptide of claim 12, wherein the polypeptide comprises an amino acid deletion, insertion, or substitution at the amino acid position corresponding to position 244 of any one of SEQ ID NOs: 15 and 19-23.

15. The modified Wnt5a polypeptide of claim 12, wherein the polypeptide comprises amino acid deletions, insertions, or substitutions at the amino acid positions corresponding to positions 104 and 244 of any one of SEQ ID NOs: 15 and 19-23.

16. The modified Wnt5a polypeptide of claim 12, wherein the polypeptide comprises an Alanine at the amino acid position corresponding to position 104 or 244 of any one of SEQ ID NOs: 15 and 19-23.

17. The modified Wnt5a polypeptide of claim 12, wherein the polypeptide comprises Alanine at the amino acid positions corresponding to positions 104 and 244 of any of SEQ ID NOs: 15 and 19-23.

18. A Wnt polypeptide comprising an amino acid sequence as set forth in any one of SEQ ID NOs: 3-5, 12-13, and 16-18.

19. A fusion polypeptide comprising a Wnt polypeptide according to claim 18.

20. The fusion polypeptide of claim 19, comprising a native signal peptide, a heterologous signal peptide, or a hybrid of a native and a heterologous signal peptide.

21. The fusion polypeptide of claim 20, wherein the heterologous signal peptide is selected from the group consisting of: a CD33 signal peptide, an immunoglobulin signal peptide, a growth hormone signal peptide, an erythropoietin signal peptide, an albumin signal peptide, a secreted alkaline phosphatase signal peptide, and a viral signal peptide.

22. The fusion polypeptide of claim 20, wherein the heterologous signal peptide is a CD33 signal peptide, an IgGκ signal peptide, or an IgGμ signal peptide.

23. The fusion polypeptide of any one of claims 19-22, comprising a heterologous protease cleavage site.

24. The fusion polypeptide of claim 23, wherein the heterologous protease cleavage site is selected from the group consisting of: a tobacco etch virus (TEV) protease cleavage site, a heparin cleavage site, a thrombin cleavage site, an enterokinase cleavage site and a Factor Xa cleavage site.

25. The fusion polypeptide of any one of claims 19-24, comprising an epitope tag selected from the group consisting of: a HIS6 epitope, a MYC epitope, a FLAG epitope, a V5 epitope, a VSV-G epitope, and an HA epitope.

26. The fusion polypeptide of any one of claims 19-24, wherein the fusion polypeptide comprising an amino acid sequence as set forth in any one of SEQ ID NOs: 3-5 and 12-13, has increased production, secretion, or solubility compared to a corresponding native Wnt polypeptide as set forth in SEQ ID NOs: 2 and 6-11.

27. The fusion polypeptide of any one of claims 19-24, wherein the fusion polypeptide comprising an amino acid sequence as set forth in any one of SEQ ID NOs: 16-18, has increased production, secretion, or solubility compared to a corresponding native Wnt polypeptide as set forth in SEQ ID NOs: 15 and 19-23.

28. A polynucleotide encoding the polypeptides of any one of claims 1-27.

29. A vector comprising the polynucleotide of claim 28.

30. A host cell comprising the vector of claim 29.

31. A polypeptide produced by the host cell of claim 30.

32. A composition comprising the polypeptide of any one of claims 1-27; a polynucleotide encoding the polypeptide of any one of claims 1-27; or a vector comprising a polynucleotide encoding the polypeptide of any one of claims 1-27.

33. The composition of claim 32, comprising a pharmaceutically-acceptable salt, carrier, or excipient.

34. The composition of claim 32, wherein the composition is soluble in an aqueous solution.

35. The composition of claim 32, wherein the composition is formulated for injection.

36. The composition of claim 32, wherein the composition is formulated without detergent.

37. The composition of claim 32, wherein the composition is formulated for one or more of intravenous injection, intracardiac injection, subcutaneous injection, intraperitoneal injection, or direct injection into a muscle.

38. The composition of claim 32, wherein the composition promotes tissue formation, regeneration, maintenance or repair.

39. The composition of claim 38, wherein the tissue is muscle.

40. The composition of claim 38, wherein the muscle is skeletal, cardiac, or smooth muscle.

41. The composition of claim 32, wherein the composition promotes stem cell expansion.

42. The composition of claim 41, wherein the stem cell is an adult stem cell.

43. The composition of claim 42, wherein the adult stem cell is a satellite stem cell.

44. The composition of claim 32, wherein the composition promotes muscle hypertrophy or prevents atrophy.

45. A method for treating or preventing muscle loss comprising administering to a subject a composition according to claim 32.

46. The method of claim 45, wherein the composition comprises a pharmaceutically-acceptable salt, carrier, or excipient.

47. The method of claim 45, wherein the composition is soluble in an aqueous solution.

48. The method of claim 45, wherein the composition is not formulated in a detergent.

49. The method of claim 45, wherein the composition is formulated for injection.

50. The method of claim 45, wherein the composition is formulated for one or more of intravenous injection, intracardiac injection, subcutaneous injection, intraperitoneal injection, or direct injection into muscle.

51. The method of claim 45, wherein the subject has or is at risk of having a disease or condition affecting muscle.

52. The method of claim 51, wherein the disease is a degenerative disease.

53. The method of claim 52, wherein the degenerative disease is muscular dystrophy.

54. The method of claim 53, wherein the muscular dystrophy is selected from Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Emery-Dreifuss muscular dystrophy, Landouzy-Dejerine muscular dystrophy, facioscapulohumeral muscular dystrophy (FSH), Limb-Girdle muscular dystrophies, von Graefe-Fuchs muscular dystrophy, oculopharyngeal muscular dystrophy (OPMD), Myotonic dystrophy (Steinert's disease) and congenital muscular dystrophies.

55. The method of claim 51, wherein the disease or condition affecting muscle is a wasting disease, muscular attenuation, muscle atrophy, ICU-induced weakness, prolonged disuse, surgery-induced weakness, or a muscle degenerative disease.

56. The method of claim 55, wherein the condition is muscle atrophy associated with muscle disuse, immobilization, surgery-induced weakness, or injury.

57. The method of claim 45, wherein administering the composition promotes muscle hypertrophy.

58. The method of claim 57, wherein the muscle is skeletal muscle or cardiac muscle.

59. The method of claim 45, wherein administering the composition promotes satellite cell expansion.