EUROPEAN PATENT APPLICATION

FUSION PROTEINS FOR TREATING METABOLIC DISORDERS

The invention relates to the identification of fusion proteins comprising polypeptide and protein variants of fibroblast growth factor 21 (FGF21) with improved pharmaceutical properties. Also disclosed are methods for treating FGF21-associated disorders, including metabolic conditions.
Description

FIELD OF THE INVENTION

[0001] The present invention relates to new fusion proteins comprising fibroblast growth factor 21 (FGF21) known to improve metabolic profiles in subjects to whom they are administered.

BACKGROUND OF THE INVENTION

[0002] The fibroblast growth factor (FGF) family is characterized by 22 genetically distinct, homologous ligands, which are grouped into seven subfamilies. FGF-21 is most closely related to, and forms a subfamily with, FGF-19 and FGF-23. This FGF subfamily regulates diverse physiological processes uncommon to classical FGFs, namely energy and bile acid homeostasis, glucose and lipid metabolism, and phosphate as well as vitamin D homeostasis. Moreover, unlike other FGFs, this subfamily acts in an endocrine fashion. (Moore, D.D. (2007) Science 316, 1436-8)(Beenken et al. (2009) Nature Reviews Drug Discovery 8, 235).

[0003] FGF21 is a 209 amino acid polypeptide containing a 28 amino acid leader sequence (SEQ ID NO:5). Human FGF21 has about 79% amino acid identity to mouse FGF21 and about 80% amino acid identity to rat FGF21. Fibroblast growth factor 21 (FGF21) has been described as a treatment for ischemic vascular disease, wound healing, and diseases associated with loss of pulmonary, bronchia or alveolar cell function. (Nishimura et al. (2000) Biochimica et Biophysica Acta, 1492:203-206; patent publication WO01/36640; and patent publication WO01/18172) Although FGF-21 activates FGF receptors and downstream signaling molecules, including FRS2a and ERK, direct interaction of FGFRs and FGF-21 has not been detected. Studies have identified β-klotho, which is highly expressed in liver, adipocytes and pancreas, as a determinant of the cellular response to FGF-21 and a cofactor which mediates FGF-21 signaling through FGFRs (Kurosu, H. et al. (2007) J Biol Chem 282, 26687-95). FGF21 is a potent agonist of the FGFR1(IIIc), FGFR2(IIIc) and FGFR3(IIIc) β-klotho signaling complexes.

[0004] FGF-21 has been shown to induce insulin-independent glucose uptake. FGF-21 has also been shown to ameliorate hyperglycemia in a range of diabetic rodent models. In addition, transgenic mice over-expressing FGF-21 were found to be resistant to diet-induced metabolic abnormalities, and demonstrated decreased body weight and fat mass, and enhancements in insulin sensitivity (Badman, M.K. et al. (2007) Cell Metab 5, 426-37). Administration of FGF-21 to diabetic non-human primates caused a decline in fasting plasma glucose, triglycerides, insulin and glucagon levels, and enhancements in insulin sensitivity (Badman, M.K. et al. (2007) Cell Metab 5, 426-37). Administration of FGF-21 to diabetic non-human primates caused a decline in fasting plasma glucose, triglycerides, insulin and glucagon levels, and enhancements in insulin sensitivity (Badman, M.K. et al. (2007) Cell Metab 5, 426-37). Administration of FGF-21 to diabetic non-human primates caused a decline in fasting plasma glucose, triglycerides, insulin and glucagon levels, and enhancements in insulin sensitivity (Badman, M.K. et al. (2007) Cell Metab 5, 426-37).

[0005] FGF21 regulates adipocyte homeostasis through activation of an AMPK/SIRT1/PGC1α pathway to inhibit PPARγ expression and increase mitochondrial function. (Chau et al. (2010) PNAS 107, 12553) FGF21 also increases glucose uptake by skeletal muscle as measured in cultured human myotubes and isolated mouse tissue. FGF21 treatment of rodent islet cells leads to improved function and survival through activation of ERK1/2 and Akt pathways. (Wente et al. (2006) Diabetes 55, 2470) FGF21 treatment also results in altered gene expression for lipogenesis and fatty acid oxidation enzymes in rodent livers, likely through HNF4α and Foxa2 signaling.

[0006] A difficulty associated with using FGF-21 directly as a biotherapeutic is that its half-life is very short. (Kharitonenkov, A. et al. (2005) Journal of Clinical Investigation 115:1627-1635) In mice, the half-life of human FGF21 is 0.5 to 1 hours, and in cynomolgus monkeys, the half-life is 2 to 3 hours. FGF21 may be utilized as a multi-use, sterile pharmaceutical formulation. However, it has been determined that preservatives, i.e., m-cresol, have an adverse affect on its stability under these conditions.

[0007] In developing an FGF21 protein for use as a therapeutic in the treatment of type 1 and type 2 diabetes mellitus and other metabolic conditions, an increase in half-life and stability would be desirable. FGF21 proteins having enhanced half-life and stability would allow for less frequent dosing of patients being administered the protein. Clearly, there is a need to develop a stable aqueous protein formulation for the therapeutic protein FGF21.

[0008] Furthermore, significant challenge in the development of FGF21 as a protein pharmaceuticals, is to cope with its physical and chemical instabilities. The compositional variety and characteristics of proteins define specific behaviors such as folding, conformational stability, and unfolding/denaturation. Such characteristics should be addressed when aiming to stabilize proteins in the course of developing pharmaceutical formulation conditions utilizing aqueous protein solutions (Wang, W., Int. J. of Pharmaceutics, 18, (1999)). A desired effect of stabilizing therapeutic proteins of interest, e.g., the proteins of the present invention, is increasing resistance to proteolysis and enzymatic degradation, thereby...
improving protein stability and reducing protein aggregation.

SUMMARY OF THE INVENTION

[0009] The invention relates to the identification of new fusion proteins which comprise fibroblast growth factor 21 (FGF21) and which have improved pharmaceutical properties over the wild-type FGF21 and variants thereof under pharmaceutical formulation conditions, e.g., are more stable, possess the ability to improve metabolic parameters for subjects to whom they are administered, are less susceptible to proteolysis and enzymatic degradation, and are less likely to aggregate and form complexes. The fusion proteins of the invention comprise truncations, mutations, and variants of FGF21.

[0010] Also disclosed are methods for treating FGF21-associated disorders, as well as other metabolic, endocrine, and cardiovascular disorders, such as obesity, type 1 and type 2 diabetes mellitus, pancreatitis, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, metabolic syndrome, acute myocardial infarction, hypertension, cardiovascular disease, atherosclerosis, peripheral arterial disease, stroke, heart failure, coronary heart disease, kidney disease, diabetic complications, neuropathy, gastroparesis, disorders associated with severe inactivating mutations in the insulin receptor, and other metabolic disorders, and in reducing the mortality and morbidity of critically ill patients.

[0011] The fusion proteins of the present invention may be used as a once weekly injectable either alone or in combination with oral anti-diabetic agents which will improve the glycemic control, body weight and lipid profile of type 1 and type 2 diabetes mellitus patients. The proteins may also be used for the treatment of obesity or other FGF21-associated conditions.

[0012] The fusion proteins of the invention overcome the significant hurdles of physical instabilities associated with protein therapeutics, including, for instance, with the administration of the wild-type FGF21, by presenting proteins which are more stable, less susceptible to proteolysis and enzymatic degradation, and less likely to aggregate and form complexes, than wild-type FGF21 under pharmaceutical formulation conditions.

[0013] In a first aspect, the invention provides Fibroblast Growth Factor 21 (FGF21) fusion proteins comprising one or more of the sequences listed in Table 1, and further described herein. The FGF21 sequences listed in Table 1 may be variants of the wild-type FGF21 sequence, e.g., the wild-type FGF21 sequence with NCBI reference number NP_061986.1, and found in such issued patents as, e.g., US 6,716,626 B1, assigned to Chiron Corporation.

[0014] Said fusions may be, for example, between the variant FGF21 sequences, e.g., the sequences of Table 1, and other molecules (a non-FGF21 portion), e.g., an IgG constant domain or fragment thereof (e.g., the Fc region), Human Serum Albumin (HSA), or albumin-binding polypeptides. In a preferred embodiment, the non-FGF21 portion of the molecule is an Fc region.

[0015] Other embodiments are drawn to polynucleotides encoding the fusion proteins of the invention, a vector containing said polynucleotides and a host cell carrying said vector.

[0016] Provided herein are methods used to generate the fusion proteins of the invention, wherein such methods involve modification of the wild-type FGF21 protein, via e.g., the site-specific incorporation of amino acids at positions of interest within the wild-type FGF21 protein, as well as the fusion between the FGF21 portion of the molecule to other molecules, e.g., an IgG constant domain or fragment thereof (e.g., the Fc region), Human Serum Albumin (HSA), or albumin-binding polypeptides. Said modifications and fusions enhance the biological properties of the fusion proteins of the invention relative to the wild-type versions of the proteins as well as, in some cases, serving as points of attachment for, e.g., labels and protein half-life extension agents, and for purposes of affixing said variants to the surface of a solid support. Related embodiments of the invention are methods to produce cells capable of producing said proteins of the invention, and of producing vectors containing DNA encoding said variants and fusions.

[0017] In various embodiments, the fusion proteins of the invention disclosed herein can comprise one or more fragments of the FGF21 wild-type sequences, including fragments as small as 8-12 amino acid residues in length, and wherein the polypeptide is capable of lowering blood glucose in a mammal. In various embodiments, the fusion proteins of the invention disclosed herein can comprise one or more variant of the FGF21 wild-type sequences, e.g., with one or more amino acid deletion, insertion, addition, or substitution relative to the wild-type sequences thereof.

[0018] In some embodiments, the fusion proteins of the invention disclosed herein can be covalently linked to one or more polymers, such as polyethylene glycol (PEG) or polysialic acid, whether at the position of site-specific amino acid modifications made relative to the wild-type FGF21, or at the position of amino acids commonly shared with the wild-type versions of those proteins. The PEG group is attached in such a way so as enhance, and/or not to interfere with, the biological function of the constituent portions of the fusion proteins of the invention, e.g., the FGF21 protein variants.

In other embodiments, the polypeptides of the invention can be fused to a heterologous amino acid sequence, optionally via a linker, such as GS, GGGSSGGSSGGGSS (SEQ ID NO:6). The heterologous amino acid sequence can be an IgG constant domain or fragment thereof (e.g., the Fc region), Human Serum Albumin (HSA), or albumin-binding polypeptides. Such fusion proteins disclosed herein can also form multimers.
In some embodiments, a heterologous amino acid sequence (e.g., HSA, Fc, etc.) is fused to the amino-terminal of the fusion proteins of the invention. In other embodiments, the fusion heterologous amino acid sequence (e.g., HSA, Fc, etc.) is fused to the carboxyl-terminal of the fusion proteins of the invention.

Yet another embodiment is drawn to methods of treating a patient exhibiting one or more FGF21-associated disorders, such as obesity, type 2 diabetes mellitus, type 1 diabetes mellitus, pancreatitis, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, metabolic syndrome, acute myocardial infarction, hypertension, cardiovascular disease, atherosclerosis, peripheral arterial disease, stroke, heart failure, coronary heart disease, kidney disease, diabetic complications, neuropathy, gastroparesis, disorders associated with inactivating mutations in the insulin receptor, and other metabolic disorders, comprising administering to said patient in need of such treatment a therapeutically effective amount of one or more proteins of the invention or a pharmaceutical composition thereof.

The invention also provides pharmaceutical compositions comprising the fusion proteins of the invention disclosed herein and a pharmaceutically acceptable formulation agent. Such pharmaceutical compositions can be used in a method for treating a metabolic disorder, and the method comprises administering to a human patient in need thereof a pharmaceutical composition of the invention. Non-limiting examples of metabolic disorders that can be treated include type 1 and type 2 diabetes mellitus and obesity.

These and other aspects of the invention will be elucidated in the following detailed description of the invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Figures 1A-1D show V188 has improved efficacy in the ob/ob diabetic mouse model over V76. V188 shows superior results when administered at 1 milligram per kilogram (mpk), compared to the 5 milligram per kilogram at which V76 was administered. Figure 1A shows fed plasma glucose as a readout (circles represent vehicle (PBS - phosphate buffered saline), squares represent V76 at 5 mpk, and triangles represent V188 at 1 mpk). Figure 1B shows plasma insulin as a readout (from left to right: vehicle, V76 at 5 mpk, and V188 at 1 mpk). Figure 1C shows body weight as a readout (from left to right: vehicle, V76 at 5 mpk, and V188 at 1 mpk). Figure 1D shows liver lipid content as a readout (from left to right: vehicle, V76 at 5 mpk, and V188 at 1 mpk).

Figures 2A-2D show V101 has improved efficacy in the ob/ob diabetic mouse model over V76. V101 shows superior results when administered at 1 milligram per kilogram (mpk), compared to the 5 milligram per kilogram at which V76 was administered. Figure 2A shows fed plasma glucose as a readout (circles represent vehicle (PBS - phosphate buffered saline), squares represent V76 at 5 mpk, and triangles represent V101 at 1 mpk). Figure 2B shows fed plasma insulin as a readout (from left to right: vehicle, V76 at 5 mpk, and V101 at 1 mpk). Figure 2C shows body weight as a readout (from left to right: vehicle, V76 at 5 mpk, and V101 at 1 mpk). Figure 2D shows liver lipid content as a readout (from left to right: vehicle, V76 at 5 mpk, and V101 at 1 mpk).

Figures 3A-3D show V103 has improved efficacy in the ob/ob diabetic mouse model over V76. V103 shows superior results when administered at 1 milligram per kilogram (mpk), compared to the 5 milligram per kilogram at which V76 was administered. Figure 3A shows fed plasma glucose as a readout (circles represent vehicle (PBS - phosphate buffered saline), squares represent V76 at 5 mpk, and triangles represent V103 at 1 mpk). Figure 3B shows fed plasma insulin as a readout (from left to right: vehicle, V76 at 5 mpk, and V103 at 1 mpk). Figure 3C shows body weight as a readout (from left to right: vehicle, V76 at 5 mpk, and V103 at 1 mpk). Figure 3D shows liver lipid content as a readout (from left to right: vehicle, V76 at 5 mpk, and V103 at 1 mpk).

Figures 4A-4D demonstrate the superior pharmacokinetic and thermocynamic properties possessed by the fusion proteins of the invention over FGF21 fusion proteins in the art. Figure 4A shows the plasma concentrations of fusion proteins of the invention in PCT Publication WO10/129600 described as Fc-L(15)-FGF21 (L98R, P171G) and Fc-L(15)-FGF21 (L98R, P171G, A180E), following the IV injection of said fusion in mice. Figure 4B shows pharmacokinetic properties of the fusion proteins of the invention (V101, V103 & V188) after a single IV dose in the mouse as assayed by anti-Fc-ELISA compared with pharmacokinetic data generated in the mouse for V76 in a previous study using an anti-FGF21 antibody ELISA. Figure 4C shows a spot check of the fusion proteins of the invention in an anti-FGF21 Western blot, consistent with anti-Fc-ELISA data at 120 hours and 15 days. The samples in the blot are as follows: A represents V101, B represents V103, and C represents V188. Control is V101 and serum. Figure 4D demonstrates the significantly increased thermodynamic stability of the fusion proteins of the invention compared to V76. From top to bottom, the figure represents V101, V103, and V188, all of which have improved melting temperatures (Tm) compared to V76 (Tm < 50 °C (not shown)) and wild-type FGF21 (Tm = 46.5°C±0.3 (not shown)).

**DETAILED DESCRIPTION OF THE INVENTION**

The fusion proteins of the present invention represent modified versions of the full length, wild-type FGF21 polypeptide, as known in the art. FGF21 wild-type sequence will serve as a reference sequence (SEQ ID NO:1), for
instance, when comparisons between the FGF21 wild-type sequence and the protein variants are necessary. The FGF21 wild-type sequence has NCBI reference sequence number NP_061986.1, and can be found in such issued patents as, e.g., US 6,716,626B1, assigned to Chiron Corporation (SEQ ID NO:1).

```plaintext
Met Asp Ser Asp Glu Thr Gly Phe Glu His Ser Gly Leu Trp Val Ser
1 5 10 15
Val Leu Ala Gly Leu Leu Leu Gly Ala Cys Glu Ala His Pro Ile Pro
20 25 30
Asp Ser Ser Pro Leu Leu Gln Phe Gly Gly Gln Val Arg Gln Arg Tyr
35 40 45
Leu Tyr Thr Asp Ala Gln Thr Glu Ala His Leu Glu Ile Arg
50 55 60
Glu Asp Gly Thr Val Gly Ala Ala Asp Gln Ser Pro Glu Ser Leu
65 70 75 80
Leu Gln Leu Lys Ala Leu Lys Pro Gly Val Ile Glu Ile Leu Gly Val
85 90 95
Lys Thr Ser Arg Phe Leu Cys Gln Arg Pro Asp Gly Ala Leu Tyr Gly
100 105 110
Ser Leu His Phe Asp Pro Glu Ala Cys Ser Phe Arg Glu Leu Leu
115 120 125
Glu Asp Gly Tyr Asn Val Tyr Gln Ser Glu Ala His Gly Leu Pro Leu
130 135 140
His Leu Pro Gly Asn Lys Ser Pro His Arg Asp Pro Ala Pro Arg Gly
145 150 155 160
Pro Ala Arg Phe Leu Pro Leu Pro Gly Leu Pro Pro Ala Leu Pro Glu
165 170 175
Pro Pro Gly Ile Leu Ala Pro Gln Pro Pro Asp Val Gly Ser Ser Asp
180 185 190
Pro Leu Ser Met Val Gly Pro Ser Gln Gly Arg Ser Pro Ser Tyr Ala
195 200 205
Ser
209
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[0028] The corresponding mRNA sequence coding for the full-length FGF21 polypeptide (NCBI reference sequence number NM_019113.2) is shown below (SEQ ID NO:2)

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1 tctgtcagctg aggatccagc cgaagagagag aacaacaccct tctgctactc
61 acctggcaca cttgaacctg gacatcacttc taacacccact cgtttctccgcc gactctcaccac
121 ccggacatca cctagagccac cagacaccattc atgagactgg agaaggcggc acagccagcgac
181 tcagagctct gcattttctgt cattcagtctg ctgcgactgca ggcacacccc
241 atccctgact ccaagtctcct cctgcacacttc gcggccaccagg cttccagctg cttcctcactac
301 acagatgatg ccggacagac acgaagaccac ccagagatgct cagcagcttga aacccttggag gcggggagttt
361 gcggcctgtct ggaggagcct ctcgactcattc tgggagctca aacccttggag gcggggagttt
421 attcctagact ttcctatagc gacatcaccag ggtctgtgacct gcggccagct gcggccacag
481 tatgtagagc tccatctgct cccgagcttc ttctcgctgc ggcgctgccct gcggccagct gcggccacag
541 ggatcataag tttcactagt ccagacagac gcgcctctccgcc gcccgcagct gcggccagct gcggccacag
601 tccccagacct gcggccagct gccgcgcttc tctcgtgctc aacgagcctg
661 ccccgcgacc cttcgcctttc gccgcttgcgc gcccgcagct gcggccagct gcggccacag
721 tcggcccttt cgggacccct ggggaccccc ggccccagct gcggccagct gcggccacag
781 agccagagcc cttcctatagc gacatcacttc tttcttatttt tagtttat gacatcacttc tttctttatttt
tttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt

901                        
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The mature FGF21 sequence lacks a leader sequence and may also include other modifications of a polypeptide such as proteolytic processing of the amino terminus (with or without a leader sequence) and/or the carboxyl terminus, cleavage of a smaller polypeptide from a larger precursor, N-linked and/or O-linked glycosylation, and other post-translational modifications understood by those with skill in the art. A representative example of a mature FGF21 sequence has the following sequence (SEQ ID NO:3, which represents amino acid positions 29-209 of full length FGF21 protein sequence (NCBI reference sequence number NP_061986.1)):

His Pro Ile Pro Asp Ser Ser Pro Leu Leu Gln Phe Gly Gly Gln Val
Arg Gln Tyr Leu Tyr Thr Asp Asp Ala Gln Gln Thr Glu Ala His
Leu Glu Ile Arg Glu Asp Gly Thr Val Gly Gly Ala Ala Asp Gln Ser
Pro Glu Ser Leu Leu Gln Leu Lys Ala Leu Lys Pro Gly Val Ile Gln
Ile Leu Gly Val Lys Thr Ser Arg Phe Leu Cys Glu Arg Pro Asp Gly
Ala Leu Tyr Gly Ser Leu His Phe Asp Pro Glu Ala Cys Ser Phe Arg
Glu Leu Leu Glu Gly Tyr Asn Val Tyr Gln Ser Glu Ala His
Gly Leu Pro Leu His Leu Pro Gly Asn Lys Ser Pro His Arg Asp Pro
Ala Pro Arg Glu Pro Ala Arg Phe Leu Pro Leu Pro Gly Leu Pro
Ala Leu Pro Glu Pro Pro Gly Ile Leu Ala Pro Pro Asp Val
Gly Ser Ser Asp Pro Leu Ser Met Val Gly Pro Ser Glu Gly Arg Ser

The corresponding cDNA sequence coding for the mature FGF21 polypeptide (SEQ ID NO:3) is shown below (SEQ ID NO:4):

1 caccccatcc tctgactccg tgtctctcctg caatttgggg gccaagtcgg gcacgggtac
61 ctctacacag atgatcgcaca gcagcagcaat gccacacttg agatcggaga ggtgagggac
121 gtggggggcg ctgctgacca gacgccggaa agtctgctgc agtggaaagc cttgaagccg
181 gcagggcttc aactttgggg tcacaggccc tggcagtcgg ccaccggagtgg
240 gcctcgtatg gatgctgccc accttgagctg gctgtcggga gctgtcgtctt
301 gagaggcttc acaatgttta caagtcgcaga gcccacgggc ttccgtctga cctgcaaggg
360 aacaagttcc cacacccggga cccgtgaccc cgagagccag cttgctctctt gccactacca
421 gcgctgcccc ccgcatcccc gcggaccccc ggaatctctgg ccccccaggc ccccggtggtg
481 gcgtctctcg acctctctgg acatggtggc cctttcaggga gcggagagcc cagctagcgt
541 tcctgga

The fusion proteins of the invention may comprise protein variants or mutants of the wild-type proteins listed herein, e.g., FGF21 variants. As used herein, the terms "protein variant," "human variant," "polypeptide or protein variant," "variant," "mutant," as well as any like terms or specific versions thereof (e.g., "FGF21 protein variant," "variant," "FGF21 mutant," etc.) define protein or polypeptide sequences that comprise modifications, truncations, other variants of naturally occurring (i.e., wild-type) protein or polypeptide counterparts or corresponding native sequences. "Variant FGF21" or "FGF21 mutant," for instance, is described relative to the wild-type (i.e., naturally occurring) FGF21 protein as described
Representative fusion protein sequences of the invention are listed in Table 1. The descriptions of said fusions include the FGF21 variant and, where applicable, a linker. The changes or substitutions employed by the FGF21 variant are numbered and described relative to wild-type FGF21. By way of example, "Variant 101 (V101)" (SEQ ID NO:10) is Fc-FGF21 fusion with a two amino acid linker and the following substitutions made relative to wild type FGF21: Q55C, A109T, G148C, K150R, P158S, P174L, S195A, P199G, G202A.

<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Sequence</th>
<th>Name*</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>DKTHTCPFCP APEAGGGPSV FLPPPKPD T</td>
<td>Full Length N-term Fc-Fusion with 2 AA Linker (GS) and WT FGF21</td>
</tr>
<tr>
<td>8</td>
<td>DKTHTCPFCP APEAGGGPSV FLPPPKPD T</td>
<td>Full Length N-term Fc-Fusion with 15 AA Linker (GGGGS x 3) between Fc and WT FGF21</td>
</tr>
<tr>
<td>9</td>
<td>DSSPLLGFF GVRQRYLTD DAEQTEAHLE</td>
<td>Variant #76 = Protein with 9 total mutations relative to wild-type FGF21 (as in WO01/018172)</td>
</tr>
<tr>
<td>SEQ ID NO:</td>
<td>Sequence</td>
<td>Name*</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
<td>-------</td>
</tr>
</tbody>
</table>
| 10        | DKTHTCPPCP APEAAGGPSV FLFPFPKPD T
LMISRTP EVT CVVVDVSHED PEVKPNYV YD
GVEVHNA KTK PREEQYNSTY RVVSVLTVLH
QDWLNG K REY CKVSNKALPA PIEKTIS KAK
GQPQE POQ VYT LPPSREEMTK NQVSLTCLV K
GYFPSDIA VE WESNGQEFNN YKTTPVFLDS
DGSSFLYSK L TVDKSRWRQG NVFSQSVHNE
ALHNHYTQ KS LSLSPGKGS D SSSLQQFGQ G
VQRQNYLTD A CQTEAHLEI REDGTVEGAA
DQSPESLQL Q KALKPGVQI Q LGVYTSRFLEC
QRPQDGALYGS LHFDEPEAC SF RELLLEDGYN
VYQSEAHGLP LHLPCNRS PH RDPA RGFAR
FLPLGLPLPA LPEPPGILAP QPPDVGSDDP
| 11        | DKTHTCPPCP APEAAGGPSV FLFPFPKPD T
LMISRTP EVT CVVVDVSHED PEVKPNYV YD
GVEVHNA KTK PREEQYNSTY RVVSVLTVLH
QDWLNG K REY CKVSNKALPA PIEKTIS KAK
GQPQE POQ VYT LPPSREEMTK NQVSLTCLV K
GYFPSDIA VE WESNGQEFNN YKTTPVFLDS
DGSSFLYSK L TVDKSRWRQG NVFSQSVHNE
ALHNHYTQ KS LSLSPGKGS D SSSLQQFGQ G
VQRQNYLTD A CQTEAHLEI REDGTVEGAA
DQSPESLQL Q KALKPGVQI Q LGVYTSRFLEC
QRPQDGALYGS LHFDEPEAC SF RELLLEDGYN
VYQSEAHGLP LHLPCNRS PH RDPA RGFAR
FLPLGLPLPA LPEPPGILAP QPPDVGSDDP
| 12        | DKTHTCPPCP APEAAGGPSV FLFPFPKPD T
LMISRTP EVT CVVVDVSHED PEVKPNYV YD
GVEVHNA KTK PREEQYNSTY RVVSVLTVLH
QDWLNG K REY CKVSNKALPA PIEKTIS KAK
GQPQE POQ VYT LPPSREEMTK NQVSLTCLV K
GYFPSDIA VE WESNGQEFNN YKTTPVFLDS
DGSSFLYSK L TVDKSRWRQG NVFSQSVHNE
ALHNHYTQ KS LSLSPGKGS D SSSLQQFGQ G
VQRQNYLTD A CQTEAHLEI REDGTVEGAA
DQSPESLQL Q KALKPGVQI Q LGVYTSRFLEC
QRPQDGALYGS LHFDEPEAC SF RELLLEDGYN
VYQSEAHGLP LHLPCNRS PH RDPA RGFAR
FLPLGLPLPA LPEPPGILAP QPPDVGSDDP
The variants or mutants used in the fusion proteins of the invention, e.g., variants of wild-type FGF21, feature at least one substituted, added, and/or removed amino acid relative to the wild-type protein. Additionally, the variants may include N- and/or C-terminal truncations relative to the wild-type proteins. Generally speaking, a variant possesses some modified property, structural or functional, of the wild-type protein. For example, the variant may have enhanced or improved physical stability in concentrated solutions (e.g., less hydrophobic mediated aggregation), enhanced or improved plasma stability when incubated with blood plasma or enhanced or improved bioactivity while maintaining a favorable bioactivity profile.

Acceptable amino acid substitutions and modifications which constitute differences between the portions of the fusion proteins of the invention and their wild-type comparator proteins include, but are not limited to, one or more amino acid substitutions, including substitutions with non-naturally occurring amino acid analogs, and truncations. Thus, the fusion proteins of the invention (e.g., the fusion proteins of the invention) include, but are not limited to, site-directed mutants, truncated polypeptides, proteolysis-resistant mutants, aggregation-reducing mutants, combination mutants, and fusion proteins, as described herein.

One skilled in the art of expression of proteins will recognize that methionine or methionine-arginine sequence can be introduced at the N-terminus of any of the fusion proteins of the invention, for expression in E. coli, and are contemplated within the context of this invention.

The fusion proteins of the invention may possess increased compatibility with pharmaceutical preservatives (e.g., m-cresol, phenol, benzyl alcohol), thus enabling the preparation of a preserved pharmaceutical formulation that maintains the physiochemical properties and biological activity of the protein during storage. Accordingly, variants with enhanced pharmaceutical stability relative to wild-type, have improved physical stability in concentrated solutions under both physiological and preserved pharmaceutical formulation conditions, while maintaining biological potency. By way of non-limiting example, the fusion proteins of the invention may be more resistant to proteolysis and enzymatic degradation; may have improved stability; and may be less likely to aggregate, than their wild-type counterparts or corresponding native sequence. As used herein, these terms are not mutually exclusive or limiting, it being entirely possible that a given variant has one or more modified properties of the wild-type protein.

The invention also encompasses nucleic acid molecules encoding the fusion proteins of the invention, comprising, for example, an FGF21 amino acid sequence that is at least about 95% identical to the amino acid sequence of SEQ ID NO:1 (SEQ ID NO:1) unless otherwise specified. All mutations in the FGF21 moiety and corresponding amino acid numbering of said mutations refers back to (SEQ ID NO:1) not to the full-length sequences in this table which may also include Fc and linker regions.

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<table>
<thead>
<tr>
<th>SEQ ID NO:</th>
<th>Sequence</th>
<th>Name*</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>LMSPRTEFY WESNGQPENNY YTTPPVLDS</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>GQPREPQVYT LPPSREMTK NQVSLTCLV</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>SQHDPFRASLG PARFLPLPLPG PPALEPPGI</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>LAPPQPDVGS SDPIANVGS QARSPYSAS</td>
<td></td>
</tr>
</tbody>
</table>

* Note that the FGF21 wild-type sequence in this table refers to NCBI reference sequence number NP_061986.1 (SEQ ID NO:1) unless otherwise specified. All mutations in the FGF21 moiety and corresponding amino acid numbering of said mutations refers back to (SEQ ID NO:1) not to the full-length sequences in this table which may also include Fc and linker regions.
The invention also encompasses a nucleic acid molecule comprising a nucleotide sequence that is at least about 85%, identical, and more preferably, at least about 90 to 95% identical to the nucleotide sequence of SEQ ID NO:2 or SEQ ID NO:4, but wherein the nucleotides encoding amino acid residues conferring the encoded protein’s proteolyis-resistance, aggregation-reducing or other properties have not been further modified. In other words, with the exception of nucleotides that encode residues in the FGF21 mutant sequences that have been modified in order to confer proteolyis-resistance, aggregation-reducing, or other properties, about 15%, and more preferably about 10 to 5% of all other nucleotides in the mutant sequence can be modified. Such nucleic acid molecules encode proteins possessing at least one activity of their wild-type counterparts.

Provided herein are methods used to generate the fusion proteins of the invention, wherein such methods involve site-specific modification and non-site-specific modification of the wild-type versions of the proteins (e.g., the FGF21 wild-type protein as described herein), e.g., truncations of the wild-type proteins, and the site-specific incorporation of amino acids at positions of interest within the wild-type proteins. Said modifications enhance the biological properties of the fusion proteins of the invention relative to the wild-type proteins, as well as, in some cases, serving as points of attachment for, e.g., labels and protein half-life extension agents, and for purposes of affixing said variants to the surface of a solid support. Related embodiments of the invention are methods of producing cells capable of producing said Fusion Proteins of the invention, and of producing vectors containing DNA encoding said variants.

In certain embodiments, such modifications, e.g., site-specific modifications, are used to attach conjugates, e.g., PEG groups to proteins, polypeptides, and/or peptides of the invention, for purposes of, e.g., extending half-life or otherwise improving the biological properties of said proteins, polypeptides, and/or peptides. Said techniques are described further herein.

In other embodiments, such modifications, e.g., site-specific modifications, are used to attach other polymers, small molecules and recombinant protein sequences that extend half-life of the protein of the invention. One such embodiment includes the attachment of fatty acids or specific albumin binding compounds to proteins, polypeptides, and/or peptides. In other embodiments, the modifications are made at a particular amino acid type and may be attached at one or more sites on the protein.

In other embodiments, such modifications, e.g., site-specific modifications, are used as means of attachment for the production of wild-type and/or variant multimers, e.g., dimers (homodimers or heterodimers) or trimers or tetramers. These multimeric protein molecules may additionally have groups such as PEG, sugars, and/or PEG-cholesterol conjugates attached or be fused either amino-terminally or carboxy-terminally to other proteins such as Fc, Human Serum Albumin (HSA), etc.

In other embodiments, such site-specific modifications are used to produce proteins, polypeptides and/or peptides wherein the position of the site-specifically incorporated pyrrolysine or pyrrolysine analogue or non-naturally occurring amino acids (para-acetyl-Phe, para-azido-Phe) allows for controlled orientation and attachment of such proteins, polypeptides and/or peptides onto a surface of a solid support or to have groups such as PEG, sugars and/or PEG-cholesterol conjugates attached.

In other embodiments, such site-specific modifications are used to site-specifically cross-link proteins, polypeptides and/or peptides thereby forming hetero-oligomers including, but not limited to, heterodimers and heterotrimers. In other embodiments, such site-specific modifications are used to site-specifically cross-link proteins, polypeptides and/or peptides thereby forming protein-protein conjugates, protein-polypeptide conjugates, protein-peptide conjugates, polypeptide-polypeptide conjugates, polypeptide-peptide conjugates or peptide-peptide conjugates. In other embodiments, a site specific modification may include a branching point to allow more than one type of molecule to be attached at a single site of a protein, polypeptide or peptide.

In other embodiments, the modifications listed herein can be done in a non-site-specific manner and result in protein-protein conjugates, protein-polypeptide conjugates, protein-peptide conjugates, polypeptide-polypeptide conjugates, polypeptide-peptide conjugates or peptide-peptide conjugates of the invention.

Definitions

Various definitions are used throughout this document. Most words have the meaning that would be attributed to those words by one skilled in the art. Words specifically defined either below or elsewhere in this document have the meaning provided in the context of the present invention as a whole and as are typically understood by those skilled in the art.

As used herein, the term “FGF21” refers to a member of the fibroblast growth factor (FGF) protein family. An amino acid sequence of FGF21 (GenBank Accession No. NP_061986.1) is set forth as SEQ ID NO:1, the corresponding polynucleotide sequence of which is set forth as SEQ ID NO:2 (NCBI reference sequence number NM_019113.2).

“FGF21 variant,” “FGF21 mutant,” and similar terms describe modified version of the FGF21 protein, e.g., with constituent amino acid residues deleted, added, modified, or substituted.

As used herein, the term “FGF21 receptor” refers to a receptor for FGF21 (Kharitonenko,A, et al. (2008)

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carboxy-lysine, or Pcl. "Pcl," as used herein, refers to either Pcl-A or Pcl-B. Pyrrolysine (Pyl) is an amino acid naturally found within methylamine methyltransferases of methanogenic archaea of the family Methanosarcina. The pyrroline-carboxy-lysine (Pcl) in E. coli resulted in the formation of a "demethylated pyrrolysine," referred to herein as pyrroline-carboxy-lysine, or Pcl. "Pcl," as used herein, refers to either Pcl-A or Pcl-B.
The terms "non-natural amino acid" and "unnatural amino acid," as used herein, are interchangeably intended to represent amino acid structures that cannot be generated biosynthetically in any organism using unmodified or modified genes from any organism, whether the same or different. The terms refer to an amino acid residue that is not present in the naturally occurring (wild-type) FGF21 protein sequence or the sequences of the present invention. These include, but are not limited to, modified amino acids and/or amino acid analogues that are not one of the 20 naturally occurring amino acids, selenocysteine, pyrrolysine (Pyl), or pyrroline-carboxy-lysine (Pcl, e.g., as described in PCT patent publication WO2010/48582). Such non-natural amino acid residues can be introduced by substitution of naturally occurring amino acids, and/or by insertion of non-natural amino acids into the naturally occurring (wild-type) FGF21 protein sequence or the sequences of the invention. The non-natural amino acid residue also can be incorporated such that a desired functionality is imparted to the FGF21 molecule, for example, the ability to link a functional moiety (e.g., PEG). When used in connection with amino acids, the symbol "U" shall mean "non-natural amino acid" and "unnatural amino acid," as used herein.

In addition, it is understood that such "unnatural amino acids" require a modified tRNA and a modified tRNA synthetase (RS) for incorporation into a protein. These "selected" orthogonal tRNA/RS pairs are generated by a selection process as developed by Schultz et al. or by random or targeted mutation. As way of example, pyrroline-carboxy-lysine is a "natural amino acid" as it is generated biosynthetically by genes transferred from one organism into the host cells and as it is incorporated into proteins by using natural tRNA and tRNA synthetase genes, while p-aminophenylalanine (See, Generation of a bacterium with a 21 amino acid genetic code, Mehli RA, Anderson JC, Santoro SW, Wang L, Martin AB, King DS, Horn DM, Schultz PG. J Am Chem Soc. 2003 Jan 29;125(4):935-9) is an "unnatural amino acid" because, although generated biosynthetically, it is incorporated into proteins by a "selected" orthogonal tRNA/tRNA synthetase pair.

Modified encoded amino acids include, but are not limited to, hydroxyproline, \(\gamma\)-carboxyglutamate, O-phosphoserine, azetidine carboxylic acid, 2-aminoacipic acid, 3-aminoacipic acid, beta-alanine, aminopropionic acid, 2-aminothyric acid, 4-aminobutyric acid, 6-aminocaproic acid, 2-aminoheptanoic acid, 2-aminoisobutyric acid, 3-aminoisobutyric acid, 2-aminoimipelic acid, tertiary-butyglycine, 2,4-diaminoisobutyric acid, desmosine, 2,2'-diaminopropionic acid, 2,3-diaminopropionic acid, N-ethylglycine, N-methylglycine, N-ethylasparagine, homoproline, hydroxyllysine, allo-hydroxylsine, 3-hydroxyproline, 4-hydroxyproline, isodesmosine, allo-isoleucine, N-methylalanine, N-methylglycine, N-methylisoleucine, N-methylpentylglycine, N-methylvaline, naphthalanine, norvaline, norleucine, ornithine, pentyglycine, piperocic acid and thiprolpine. The term "amino acid" also includes naturally occurring amino acids that are metabolites in certain organisms but are not encoded by the genetic code for incorporation into proteins. Such amino acids include, but are not limited to, ornithine, D-ornithine, and D-arginine.

The term "amino acid analogue," as used herein, refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, by way of example only, an \(\alpha\)-carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group. Amino acid analogues include the natural and unnatural amino acids which are chemically blocked, reversibly or irreversibly, or their C-terminal carboxy group, their N-terminal amino group and/or their side-chain functional groups are chemically modified. Such analogues include, but are not limited to, methionine sulfone, methionine sulfone, (S-carboxymethyl)-cysteine, (S-carboxymethyl)-cysteine sulfone, (S-carboxymethyl)-cysteine sulfone, aspartic acid-(beta-methyl ester), N-ethylglycine, alanine carboxamide, homoserine, norleucine, and methionine methyl sulfoxide.

The term "amino acid mimetics," as used herein, refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but functions in a manner similar to a naturally occurring amino acid.

The term "biologically active variant" refers to any polypeptide variant used in the fusion proteins of the invention, e.g., as a constituent protein of the fusions, that possesses an activity of its wild-type (e.g., naturally-occurring) protein or polypeptide counterpart, such as the ability to modulate blood glucose, HbA1c, insulin, triglyceride, or cholesterol levels; increase pancreatic function; reduce lipid levels in liver; reduce body weight; and to improve glucose tolerance, energy expenditure, or insulin sensitivity, regardless of the type or number of modifications that have been introduced into the polypeptide variant. Polypeptide variants possessing a somewhat decreased level of activity relative to their wild-type versions can nonetheless be considered to be biologically active polypeptide variants. A non-limiting representative example of a biologically active polypeptide variant of the invention is an FGF21 variant, which is modified after, and possesses similar or enhanced biological properties relative to, wild-type FGF21.

The terms "effective amount" and "therapeutically effective amount" each refer to the amount of a fusion protein of the invention used to support an observable level of one or more biological activities of the wild-type polypeptide or protein counterparts, such as the ability to lower blood glucose, insulin, triglyceride or cholesterol levels; reduce liver triglyceride or lipid levels; reduce body weight; or improve glucose tolerance, energy expenditure, or insulin sensitivity. For example, a "therapeutically-effective amount" administered to a patient exhibiting, suffering, or prone to suffer from FGF21-associated disorders (such as type 1 or type 2 diabetes mellitus, obesity, or metabolic syndrome), is such an amount which induces, ameliorates or otherwise causes an improvement in the pathological symptoms, disease pro-
The term "FGF21-associated disorders," and terms similarly used herein, includes obesity, type 1 and type 2 diabetes mellitus, pancreatitis, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, metabolic syndrome, acute myocardial infarction, dyslipidemia, and possibly related disorders.

The term "pharmacologically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of a fusion protein of the invention.

The term "antigen" refers to a molecule or a portion of a molecule that is capable of being bound by an antibody, and additionally that is capable of being used in an animal to produce antibodies that are capable of binding to an epitope of that antigen. An antigen may have one or more epitopes.

The term "native Fc" refers to a molecule or sequence comprising the sequence of a non-antigen-binding fragment resulting from digestion of whole antibody or produced by other means, whether in monomeric or multimeric form, and can contain the hinge region. The original immunoglobulin source of the native Fc is preferably of human origin and can be any of the immunoglobulins, although IgG1 and IgG2 are preferred. Native Fc molecules are made up of monomeric polypeptides that can be linked into dimeric or multimeric forms by covalent (i.e., disulfide bonds) and noncovalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, and IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, and IgG2A). One example of a native Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG (see Ellison et al., 1982, Nucleic Acids Res. 10: 4071-9). The term "native Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

The term "Fc variant" refers to a molecule or sequence that is modified from a native Fc but still comprises a binding site for the salvage receptor, FcRn (neonatal Fc receptor). International Publication Nos. WO 97/34631 and WO 96/32478 describe exemplary Fc variants, as well as interaction with the salvage receptor, and are hereby incorporated by reference. Thus, the term "Fc variant" can comprise a molecule or sequence that is humanized from a non-human native Fc. Furthermore, a native Fc comprises regions that can be removed because they provide structural features or biological activity that are not required for the fusion molecules of the fusion proteins of the invention. Thus, the term "Fc variant" comprises a molecule or sequence that lacks one or more native Fc sites or residues, or in which one or more Fc sites or residues has been modified, that affect or are involved in: (1) disulfide bond formation, (2) incompatibility with a selected host cell, (3) N-terminal heterogeneity upon expression in a selected host cell, (4) glycosylation, (5) interaction with complement, (6) binding to an Fc receptor other than a salvage receptor, or (7) antibody-dependent cellular cytotoxicity (ADCC). Fc variants are described in further detail hereinafter.

The term "Fc domain" encompasses native Fc and Fc variants and sequences as defined above. As with Fc variants and native Fc molecules, the term "Fc domain" includes molecules in monomeric or multimeric form, whether digested from whole antibody or produced by other means. In some embodiments of the present invention, an Fc domain can be fused to FGF21 or a FGF21 mutant (including a truncated form of FGF21 or a FGF21 mutant) via, for example, a covalent bond between the Fc domain and the FGF21 sequence. Such fusion proteins can form multimers via the association of the Fc domains and both these fusion proteins and their multimers are an aspect of the present invention.

The term "modified Fc fragment", as used herein, shall mean an Fc fragment of an antibody comprising a modified sequence. The Fc fragment is a portion of an antibody comprising the CH2, CH3 and part of the hinge region. The modified Fc fragment can be derived from, for example, IgG1, IgG2, IgG3, or IgG4. FcLALA is a modified Fc fragment with a LALA mutation (L234A, L235A), which triggers ADCC with lowered efficiency, and binds and activates human CD16 (an Fc receptor). Additional modifications to the Fc fragment are described in, for example, U.S. Patent No. 7,217,798.

The term "heterologous" means that these domains are not naturally found associated with constant regions of an antibody. In particular, such heterologous binding domains do not have the typical structure of an antibody variable domain consisting of 4 framework regions, FR1, FR2, FR3 and FR4 and the 3 complementarity determining regions (CDRs) in-between. Each arm of the fusobody therefore comprises a first single chain polypeptide comprising a first binding domain covalently linked at the N-terminal part of a constant Cγ1 heavy chain region of an antibody, and a second single chain polypeptide comprising a second binding domain covalently linked at the N-terminal part of a constant Cγ1 light chain of an antibody. The covalent linkage may be direct, for example via peptidic bond or indirect, via a linker, for example a peptidic linker. The two heterodimers of the fusobody are covalently linked, for example, by at least one disulfide bridge at their hinge region, like an antibody structure. Examples of molecules with a fusobody structure have been described in the art, in particular, fusobodies comprising ligand binding region of heterodimeric receptor (see for example international patent publications WO01/46261 and WO11/076781).

The term "polyethylene glycol" or "PEG" refers to a polyalkylene glycol compound or a derivative thereof, with or without coupling agents or derivatization with coupling or activating moieties.

The term "FGF21-associated disorders," and terms similarly used herein, includes obesity, type 1 and type 2 diabetes mellitus, pancreatitis, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, metabolic syndrome, acute myocardial infarction, dyslipidemia, and possibly related disorders.
dial infarction, hypertension, cardiovascular disease, atherosclerosis, peripheral arterial disease, stroke, heart failure, coronary heart disease, kidney disease, diabetic complications, neuropathy, gastroparesis, disorders associated with severe inactivating mutations in the insulin receptor, and other metabolic disorders.

The term "disorders associated with severe inactivating mutations in the insulin receptor," and terms similarly used herein, describe conditions in subjects afflicted with mutations in the insulin receptor (or possible proteins directly downstream from it) which cause severe insulin resistance but are often (though not always) seen without the obesity common in Type 2 diabetes mellitus. In many ways, subjects afflicted with these conditions manifest hybrid symptoms of Type 1 diabetes mellitus and Type 2 diabetes mellitus. Subjects thereby afflicted fall into several categories of roughly increasing severity, including: Type A Insulin Resistance, Type C Insulin Resistance (AKA HAIR-AN Syndrome), Rabson-Mendenhall Syndrome and finally Donohue's Syndrome or Leprechaunism. These disorders are associated with very high endogenous insulin levels, and very often, hyperglycemia. Subjects thereby afflicted also present with various clinical features associated with "insulin toxicity," including hyperandrogenism, polycystic ovarian syndrome (PCOS), hirsutism, and acanthosis nigricans (excessive growth and pigmentation) in the folds of the skin.

"Type 1 diabetes mellitus" is a condition characterized by total lack of insulin. This occurs when the body's immune system attacks the insulin-producing beta cells in the pancreas and destroys them. The pancreas then produces little or no insulin.

"Glucose intolerance" or Impaired Glucose Tolerance (IGT) is a pre-diabetic state of dysglycemia that is associated with increased risk of cardiovascular pathology. The pre-diabetic condition prevents a subject from moving glucose into cells efficiently and utilizing it as an efficient fuel source, leading to elevated glucose levels in blood and some degree of insulin resistance.

"Hyperglycemia" is defined as an excess of sugar (glucose) in the blood.

"Hypoglycemia", also called low blood sugar, occurs when your blood glucose level drops too low to provide enough energy for your body's activities.

"Insulin resistance" is defined as a state in which a normal amount of insulin produces a subnormal biologic response.

"Obesity," in terms of the human subject, can be defined as that body weight over 20 percent above the ideal body weight for a given population (R. H. Williams, Textbook of Endocrinology, 1974, p. 904-916).

"Diabetic complications" are problems, caused by high blood glucose levels, with other body functions such as kidneys, nerves (neuropathies), feet (foot ulcers and poor circulation) and eyes (e.g. retinopathies). Diabetes also increases the risk for heart disease and bone and joint disorders. Other long-term complications of diabetes include skin problems, digestive problems, sexual dysfunction and problems with teeth and gums.

"Metabolic syndrome" can be defined as a cluster of at least three of the following signs: abdominal fat--in most men, a 40-inch waist or greater; high blood sugar--at least 110 milligrams per deciliter (mg/dl) after fasting; high triglycerides--at least 150 mg/dL in the bloodstream; low HDL--less than 40 mg/dl; and, blood pressure of 130/85 mmHg or higher.

"Pancreatitis" is inflammation of the pancreas.

"Dyslipidemia" is a disorder of lipoprotein metabolism, including lipoprotein overproduction or deficiency. Dyslipidemias may be manifested by elevation of the total cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride concentrations, and a decrease in high-density lipoprotein (HDL) cholesterol concentration in the blood.

"Nonalcoholic fatty liver disease (NAFLD)" is a liver disease, not associated with alcohol consumption, characterized by fatty change of hepatocytes.

"Nonalcoholic steatohepatitis (NASH)" is a liver disease, not associated with alcohol consumption, characterized by fatty change of hepatocytes, accompanied by intralobular inflammation and fibrosis.

"Hypertension" or high blood pressure that is a transitory or sustained elevation of systemic arterial blood pressure to a level likely to induce cardiovascular damage or other adverse consequences. Hypertension has been arbitrarily defined as a systolic blood pressure above 140 mmHg or a diastolic blood pressure above 90 mmHg.

"Cardiovascular diseases" are diseases related to the heart or blood vessels.

"Atherosclerosis" is a vascular disease characterized by irregularly distributed lipid deposits in the intima of large and medium-sized arteries, causing narrowing of arterial lumens and proceeding eventually to fibrosis and calci-
Stroke is any acute clinical event, related to impairment of cerebral circulation, that lasts longer than 24 hours. A stroke involves irreversible brain damage, the type and severity of symptoms depending on the location and extent of brain tissue whose circulation has been compromised.

"Heart failure", also called congestive heart failure, is a condition in which the heart can no longer pump enough blood to the rest of the body.

"Coronary heart disease", also called coronary artery disease, is a narrowing of the small blood vessels that supply blood and oxygen to the heart.

"Kidney disease" or nephropathy is any disease of the kidney. Diabetic nephropathy is a major cause of morbidity and mortality in people with type 1 or type 2 diabetes mellitus.

"Neuroapathies" are any diseases involving the cranial nerves or the peripheral or autonomic nervous system.

"Gastroparesis" is weakness of gastric peristalsis, which results in delayed emptying of the bowels.

"Kidney disease", nephropathy, and "Neuroapathies" are part of the systemic inflammatory response syndrome (SIRS), a condition in which the body's response to inflammation becomes overwhelming and injurious.

Heart failure, a condition in which the heart can no longer pump enough blood to the rest of the body, is a major cause of morbidity and mortality in people with type 1 or type 2 diabetes mellitus. Diabetic nephropathy, a condition in which the kidneys are damaged by diabetes, is a major cause of morbidity and mortality in people with type 1 or type 2 diabetes mellitus.

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chemically, or within a cell to which the gene is native. In some embodiments, if the gene product is proteinaceous, it exhibits a biological activity. In some embodiments, if the gene product is a nucleic acid, it can be translated into a proteinaceous gene product that exhibits a biological activity.

"Modulation of FGF21 activity," as used herein, refers to an increase or decrease in FGF21 activity that can be a result of, for example, interaction of an agent with an FGF21 polynucleotide or polypeptide, inhibition of FGF21 transcription and/or translation (e.g., through antisense or siRNA interaction with the FGF21 gene or FGF21 transcript, through modulation of transcription factors that facilitate FGF21 expression), and the like. For example, modulation of a biological activity refers to an increase or a decrease in a biological activity. FGF21 activity can be assessed by means including, without limitation, assaying blood glucose, insulin, triglyceride, or cholesterol levels in a subject, assessing FGF21 polypeptide levels, or by assessing FGF21 transcription levels. Comparisons of FGF21 activity can also be accomplished by, e.g., measuring levels of an FGF21 downstream biomarker, and measuring increases in FGF21 signaling. FGF21 activity can also be assessed by measuring: cell signaling; kinase activity; glucose uptake into adipocytes; blood insulin, triglyceride, or cholesterol level fluctuations; liver lipid or liver triglyceride level changes; interactions between FGF21 and an FGF21 receptor; or phosphorylation of an FGF21 receptor. In some embodiments phosphorylation of an FGF21 receptor can be tyrosine phosphorylation. In some embodiments modulation of FGF21 activity can cause modulation of an FGF21-related phenotype.

Comparisons of FGF21 activity can also be accomplished by, e.g., measuring levels of an FGF21 downstream biomarker, and measuring increases in FGF21 signaling. FGF21 activity can also be assessed by measuring: cell signaling; kinase activity; glucose uptake into adipocytes; blood insulin, triglyceride, or cholesterol level fluctuations; liver lipid or liver triglyceride level changes; interactions between FGF21 and a receptor (FGFR-1c, FGFR-2c, or FGFR-3c); or phosphorylation of an FGF21 receptor. In some embodiments phosphorylation of an FGF21 receptor can be tyrosine phosphorylation. In some embodiments modulation of FGF21 activity can cause modulation of an FGF21-related phenotype.

A "FGF21 downstream biomarker," as used herein, is a gene or gene product, or measurable indicia of a gene or gene product. In some embodiments, a gene or activity that is a downstream marker of FGF21 exhibits an altered level of expression, or in a vascular tissue. In some embodiments, an activity of the downstream marker is altered in the presence of an FGF21 modulator. In some embodiments, the downstream markers exhibit altered levels of expression when FGF21 is perturbed with an FGF21 modulator of the present invention. FGF21 downstream markers include, without limitation, glucose or 2-deoxy-glucose uptake, pERK and other phosphorylated or acetylated proteins or NAD levels.

As used herein, the term "up-regulates" refers to an increase, activation or stimulation of an activity or quantity. For example, in the context of the present invention, FGF21 modulators may increase the activity of an FGF21 receptor. In one embodiment, one or more FGFR-1c, FGFR-2c, or FGFR-3c may be upregulated in response to an FGF21 modulator. Upregulation can also refer to an FGF21-related activity, such as e.g., the ability to lower blood glucose, insulin, triglyceride, or cholesterol levels; to reduce liver lipid or triglyceride levels; to reduce body weight; to improve glucose tolerance, energy expenditure, or insulin sensitivity; or to cause phosphorylation of an FGF21 receptor; or to increase an FGF21 downstream marker. The FGFR21 receptor can be one or more of FGFR-1c, FGFR-2c, or FGFR-3c. Upregulation may be at least 25%, at least 50%, at least 75%, at least 100%, at least 150%, at least 200%, at least 250%, at least 400%, or at least 500% as compared to a control.

As used herein, the term "N-terminus" refers to at least the first 20 amino acids of a protein.

As used herein, the terms "N-terminal domain" and "N-terminal region" are used interchangeably and refer to a fragment of a protein that begins at the first amino acid of the protein and ends at any amino acid in the N-terminal half of the protein. For example, the N-terminal domain of FGF21 is from amino acid 1 of SEQ ID NO:1 to any amino acid between about amino acids 10 and 105 of SEQ ID NO:1.

As used herein, the term "C-terminus" refers to at least the last 20 amino acids of a protein.

As used herein, the terms "C-terminal domain" and "C-terminal region" are used interchangeably and refer to a fragment of a protein that begins at any amino acid in the C-terminal half of the protein and ends at the last amino acid of the protein. For example, the C-terminal domain of FGF21 begins at any amino acid from amino acid 105 to about amino acid 200 of SEQ ID NO:1 and ends at amino acid 209 of SEQ ID NO:1.

The term "domain" as used herein refers to a structural part of a biomolecule that contributes to a known or suspected function of the biomolecule. Domains may be co-extensive with regions or portions thereof and may also incorporate a portion of a biomolecule that is distinct from a particular region, in addition to all or part of that region.

As used herein, the term "signal domain" (also called "signal sequence" or "signal peptide") refers to a peptide domain that resides in a continuous stretch of amino acid sequence at the N-terminal region of a precursor protein (often a membrane-bound or secreted protein) and is involved in post-translational protein transport. In many cases the signal domain is removed from the full-length protein by specialized signal peptidases after the sorting process has been completed. Each signal domain specifies a particular destination in the cell for the precursor protein. The signal domain of FGF21 is amino acids 1-28 of SEQ ID NO:1.
As used herein, the term "receptor binding domain" refers to any portion or region of a protein that contacts a
membrane-bound receptor protein, resulting in a cellular response, such as a signaling event.

As used herein, the term "ligand binding domain" refers to any portion or region of a fusion protein of the
invention retaining at least one qualitative binding activity of a corresponding native sequence.

The term "region" refers to a physically contiguous portion of the primary structure of a biomolecule. In the case
of proteins, a region is defined by a contiguous portion of the amino acid sequence of that protein. In some embodiments
a "region" is associated with a function of the biomolecule.

The term "fragment" as used herein refers to a physically contiguous portion of the primary structure of a
biomolecule. In the case of proteins, a portion is defined by a contiguous portion of the amino acid sequence of that
protein and refers to at least 3-5 amino acids, at least 8-10 amino acids, at least 11-15 amino acids, at least 17-24 amino
acids, at least 25-30 amino acids, and at least 30-45 amino acids. In the case of oligonucleotides, a portion is defined
by a contiguous portion of the nucleic acid sequence of that oligonucleotide and refers to at least 9-15 nucleotides, at
least 18-30 nucleotides, at least 33-45 nucleotides, at least 48-72 nucleotides, at least 75-90 nucleotides, and at least
90-130 nucleotides. In some embodiments, portions of biomolecules have a biological activity. In the context of the
present invention, FGF21 polypeptide fragments do not comprise the entire FGF21 polypeptide sequence set forth in
SEQ ID NO:1.

A "native sequence" polypeptide is one that has the same amino acid sequence as a polypeptide derived from
nature. Such native sequence polypeptides can be isolated from nature or can be produced by recombinant or synthetic
means. Thus, a native sequence polypeptide can have the amino acid sequence of naturally occurring human polypeptide,
murine polypeptide, or polypeptide from any other mammalian species.

As used herein, the phrase "homologous nucleotide sequence," or "homologous amino acid sequence," or
variations thereof, refers to sequences characterized by a homology, at the nucleotide level or amino acid level, of at
least a specified percentage and is used interchangeably with "sequence identity." Homologous nucleotide sequences
include those sequences coding for isoforms of proteins. Such isoforms can be expressed in different tissues of the
same organism as a result of, for example, alternative splicing of RNA. Alternatively, isoforms can be encoded by different
genes. Homologous nucleotide sequences include nucleotide sequences encoding for a protein of a species other than
humans, including, but not limited to, mammals. Homologous nucleotide sequences also include, but are not limited to,
naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. Homologous amino acid
sequences include those amino acid sequences which contain conservative amino acid substitutions and which polypep-
tides have the same binding and/or activity. In some embodiments, a nucleotide or amino acid sequence is homologous
if it has at least 60% or greater, up to 99%, identity with a comparator sequence. In some embodiments, a nucleotide
or amino acid sequence is homologous if it shares one or more, up to 60, nucleotide/amino acid substitutions, additions,
or deletions with a comparator sequence. In some embodiments, the homologous amino acid sequences have no more
than 5 or no more than 3 conservative amino acid substitutions.

Percent homology or identity can be determined by, for example, the Gap program (Wisconsin Sequence
Analysis Package, Version 8 for UNIX, Genetics Computer Group, University Research Park, Madison WI), using default
settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489). In some embodiments,
homology between the probe and target is between about 75% to about 85%. In some embodiments, nucleic acids have
nucleotides that are at least about 95%, about 97%, about 98%, about 99% and about 100% homologous to SEQ ID
NO:2, or a portion thereof.

Homology may also be at the polypeptide level. In some embodiments, constituent polypeptides of the fusion
proteins of the invention may be at least 95% homologous to their full length wild-type counterparts or corresponding
native sequences, or to portions thereof. The degree or percentage identity of Fusion Proteins of the invention, or portions
thereof, and different amino acid sequences is calculated as the number of exact matches in an alignment of the two
sequences divided by the length of the "invention sequence" or the "foreign sequence", whichever is shortest. The result
is expressed as percent identity.

As used herein, the term "mixing" refers to the process of combining one or more compounds, cells, molecules,
and the like together in the same area. This may be performed, for example, in a test tube, petri dish, or any container
that allows the one or more compounds, cells, or molecules, to be mixed.

As used herein, the term "substantially purified" refers to a compound (e.g., either a polynucleotide or a polypep-
tide or an antibody) that is removed from its natural environment and is at least 60% free, at least 75% free, and at least
90% free from other components with which it is naturally associated.

The term "pharmacologically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such
as antibodies or a polypeptide, genes, and other therapeutic agents. The term refers to any pharmaceutical carrier that
does not itself induce the production of antibodies harmful to the individual receiving the composition, and which can be
administered without undue toxicity. Suitable carriers can be large, slowly metabolized macromolecules such as proteins,
polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates
and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmacologically acceptable
Naturally occurring disulfide bonds, as provided by cysteine residues, generally increase thermodynamic stability of proteins. Successful examples of increased thermodynamic stability, as measured in increase of the melting temperature, are multiple disulfide-bonded mutants of the enzymes T4 lysozyme (Matsumura et al., PNAS 86:6562-6566 (1989)) and barnase (Johnson et al., J. Mol. Biol. 268:198-208 (1997)). An aspect of the present invention is an enhancement of the physical stability of FGF21 in the presence of a preservative, achieved by the presence of disulfide bonds within the variants, which constrain the flexibility of wild-type FGF21 and thereby limit access of the preservative to the hydrophobic core of the protein.

The second aspect of the present invention therefore provides variants of human FGF21, or a biologically active peptide thereof, with enhanced pharmaceutical stability engendered by the incorporation of additional disulfide bonds, e.g., via incorporating or substituting cysteine residues into the wild-type FGF21 protein or the polypeptide and protein variants of the invention. One skilled in the art will recognize that the native cysteines, cysteine 103 and cysteine 121, could be utilized as loci to introduce a novel disulfide bond that may impart improved properties, in addition to the suggested embodiments described herein.

These include fusion proteins which incorporate wild-type FGF21 with the substitution of a cysteine for two or more of the following: glutamine 46, arginine 47, tyrosine 48, leucine 49, tyrosine 50, threonine 51, aspartate 52, aspartate 53, alanine 54, glutamine 55, glutamine 56, threonine 57, glutamate 58, alanine 59, histidine 60, leucine 61, glutamate 62, isoleucine 63, valine 69, glycine 70, glycine 71, alanine 72, alanine 73, leucine 144, histidine 145, leucine 146, proline 147, glycine 148, asparagine 149, lysine 150, serine 151, proline 152, histidine 153, arginine 154, aspartate 155, proline 156, alanine 157, proline 158, arginine 159, glycine 160, proline 161, alanine 162, arginine 163, phenylalanine 164, wherein the numbering of the amino acids is based on the full length 209 amino acid hFGF21 sequence SEQ ID NO:1.

Furthermore, fusion proteins of the invention may incorporate variants of wild-type human FGF21, or a biologically active peptide thereof, which are enhanced with engineered disulfide bonds, in addition to the naturally occurring one at Cys103-Cys121, as are follows: Gin46Cys-Ala59Cys, Gin46Cys-His60Cys, Gin46Cys-Glu62Cys, Gin46Cys-Ile63Cys, Arg47Cys-Ala59Cys, Arg47Cys-His60Cys, Arg47Cys-Glu62Cys, Arg47Cys-Ile63Cys, Tyr48Cys-Ala59Cys, Tyr48Cys-His60Cys, Tyr48Cys-Glu62Cys, Tyr48Cys-Ile63Cys, Pro147Cys-Ala59Cys, Pro147Cys-His60Cys, Pro147Cys-Glu62Cys, Pro147Cys-Ile63Cys, Pro152Cys, Ala54Cys-Pro152Cys, Gln55Cys-Pro152Cys, Gln56Cys-Pro152Cys, Thr57Cys-Pro152Cys, Glu58Cys-Pro152Cys, Leu49Cys-Pro152Cys, Tyr50Cys-Pro152Cys, Arg47Cys-Ala59Cys-Ala73Cys, His60Cys-Ala73Cys, Ala59Cys-Val69Cys, Ala59Cys-Gly70Cys, Ala59Cys-Gly71Cys, Ala59Cys-Ala72Cys, Ala59Cys-Ala73Cys, His60Cys-Val69Cys, His60Cys-Gly70Cys, His60Cys-Gly71Cys, His60Cys-Ala72Cys, His60Cys-Ala73Cys, Leu61Cys-Val69Cys, Leu61Cys-Gly70Cys, Leu61Cys-Gly71Cys, Leu61Cys-Ala72Cys, Leu61Cys-Ala73Cys, Arg47Cys-Ser151Cys, Tyr48Cys-Ser151Cys, Tyr48Cys-Ser151Cys, Leu49Cys-Ser151Cys, Pro147Cys-Ser151Cys, Ser151Cys-Asn149Cys, Ser151Cys-Lys150Cys, Ser151Cys-Arg154Cys, Ser151Cys-Pro152Cys, Asp52Cys-Ser151Cys, Ala54Cys-Ser151Cys, Gln55Cys-Ser151Cys, Gln56Cys-Ser151Cys, Thr57Cys-Ser151Cys, Tyr50Cys-Ser151Cys, Arg47Cys-Ser151Cys, Tyr48Cys-Ser151Cys, Leu49Cys-Ser151Cys, Pro147Cys-Ser151Cys, Ser151Cys-Glu62Cys, Ser151Cys-Gly148Cys, Ser151Cys-Gly148Cys, Ser151Cys-Glu62Cys, Ser151Cys-Gly148Cys, Ser151Cys-Pro152Cys, Ser151Cys-Pro152Cys.
Arg154Cys, Gln55Cys-Arg154Cys, Gin56Cys-Arg154Cys, Thr57Cys-Arg154Cys, Glu58Cys-Arg154Cys, Arg47Cys-
Asp155Cys, Tyr48Cys-Asp155Cys, Leu49Cys-Asp155Cys, Tyr50Cys-Asp155Cys, Thr51Cys-Asp155Cys, Asp52Cys-
Asp155Cys, Gin55Cys-Asp155Cys, Ala54Cys-Asp155Cys, Gin55Cys-Asp155Cys, Gin56Cys-Asp155Cys, Thr57Cys-
Asp155Cys, Glu58Cys-Asp155Cys, Arg47Cys-Pro156Cys, Tyr48Cys-Pro156Cys, Leu49Cys-Pro156Cys, Thr50Cys-
Pro156Cys, Thr51Cys-Pro156Cys, Asp52Cys-Pro156Cys, Asp53Cys-Pro156Cys, Ala54Cys-Pro156Cys, Gin55Cys-
Pro156Cys, Gin56Cys-Pro156Cys, Thr57Cys-Pro156Cys, Glu58Cys-Pro156Cys, Arg47Cys-Ala157Cys, Tyr48Cys-
Ala157Cys, Leu49Cys-Ala157Cys, Thr50Cys-Ala157Cys, Thr51Cys-Ala157Cys, Asp52Cys-Ala157Cys, Asp53Cys-
Ala157Cys, Ala54Cys-Ala157Cys, Gin55Cys-Ala157Cys, Gin56Cys-Ala157Cys, Thr57Cys-Ala157Cys, Glu58Cys-
Ala157Cys, Arg47Cys-Pro158Cys, Tyr48Cys-Pro158Cys, Leu49Cys-Pro158Cys, Thr50Cys-Pro158Cys, Thr51Cys-
Pro158Cys, Arg52Cys-Pro158Cys, Asp53Cys-Pro158Cys, Ala54Cys-Pro158Cys, Gin55Cys-Pro158Cys, Gin56Cys-
Pro158Cys, Thr57Cys-Pro158Cys, Glu58Cys-Pro158Cys, Arg47Cys-Arg159Cys, Tyr48Cys-Arg159Cys, Leu49Cys-
Arg159Cys, Thr50Cys-Arg159Cys, Thr51Cys-Arg159Cys, Asp52Cys-Arg159Cys, Asp53Cys-Arg159Cys, Ala54Cys-
Arg159Cys, Gin55Cys-Arg159Cys, Gin56Cys-Arg159Cys, Thr57Cys-Arg159Cys, Glu58Cys-Arg159Cys, Arg47Cys-
G160Cys, Tyr48Cys-G160Cys, Leu49Cys-G160Cys, Thr50Cys-Gly160Cys, Thr51Cys-Gly160Cys, Asp52Cys-Gly-
160Cys, Arg53Cys-Gly160Cys, Ala54Cys-Gly160Cys, Gin55Cys-Gly160Cys, Gin56Cys-Gly160Cys, Thr57Cys-
Gly160Cys, Glu58Cys-Gly160Cys, Arg47Cys-Pro161 Cys, Tyr48Cys-Pro161 Cys, Leu49Cys-Pro161 Cys, Thr50Cys-
Pro161 Cys, Thr51Cys-Pro161 Cys, Asp52Cys-Pro161 Cys, Asp53Cys-Pro161 Cys, Ala54Cys-Pro161 Cys, Gin55Cys-
Pro161 Cys, Gin56Cys-Pro161 Cys, Thr57Cys-Pro161 Cys, Glu58Cys-Pro161 Cys, Arg47Cys-Ala162Cys, Tyr48Cys-
Ala162Cys, Leu49Cys-Ala162Cys, Thr50Cys-Ala162Cys, Thr51Cys-Ala162Cys, Asp52Cys-Ala162Cys, Asp53Cys-
Ala162Cys, Ala54Cys-Ala162Cys, Gin55Cys-Ala162Cys, Gin56Cys-Ala162Cys, Thr57Cys-Ala162Cys, Glu58Cys-
Ala162Cys, Arg47Cys-Ala163Cys, Tyr48Cys-Ala163Cys, Leu49Cys-Ala163Cys, Thr50Cys-Ala163Cys, Thr51Cys-
Arg163Cys, Asp52Cys-Arg163Cys, Asp53Cys-Arg163Cys, Ala54Cys-Arg163Cys, Gin55Cys-Arg163Cys, Gin56Cys-
Arg163Cys, Thr57Cys-Arg163Cys, Glu58Cys-Arg163Cys

[0139] Another aspect of the present invention provides fusion proteins comprising variants of wild-type human FGF21, or a biologically active peptide thereof, comprising a substitution of any charged and/or polar but uncharged amino acid at any of the amino acid positions indicated in the first embodiment of the present invention combined with the substitution of a cysteine at two or more amino acid positions indicated in the second embodiment of the invention.

Improvements of the fusion proteins of the Invention Over Wild Type Protein Comparators and Variants Thereof

[0140] It is well known in the art that a significant challenge in the development of protein pharmaceuticals is to deal with the physical and chemical instabilities of proteins. This is even more apparent when a protein pharmaceutical formulation is intended to be a multiple use, injectable formulation requiring a stable, concentrated and preserved solution, while maintaining a favorable bioactivity profile. Biophysical characterization of wild-type FGF21 in the literature established that a concentrated protein solution (>5 mg/ml), when exposed to stress conditions, such as high temperature or low pH, lead to accelerated association and aggregation (i.e., poor physical stability and biopharmaceutical properties). Exposure of a concentrated protein solution of FGF21 to pharmaceutical preservatives (e.g., m-cresol) also had a negative impact on physical stability. The fusion proteins of the invention were developed as a way to achieve the desirable effects of FGF21 treatment at a higher potency and in a half-life-extended formulation.

[0141] Therefore, an embodiment of the present invention is to enhance physical stability of concentrated solutions, while maintaining chemical stability and biological potency, under both physiological and preserved formulation conditions. It is thought that association and aggregation may result from hydrophobic interactions, since, at a given protein concentration, temperature, and ionic strength have considerable impact on physical stability. For the most part, non-conserved, presumed surface exposed amino acid residues were targeted. The local environment of these residues was analyzed and, those that were not deemed structurally important were selected for mutagenesis. One method to initiate specific changes is to further decrease the pI of the protein by introducing glutamic acid residues ("glutamic acid scan"). It is hypothesized that the introduction of charged substitutes would inhibit hydrophobic-mediated aggregation via charge-charge repulsion and potentially improve preservative compatibility. In addition, one skilled in the art would also recognize that with sufficient degree of mutagenesis the pi could be shifted into a basic pH range by the introduction of positive charge with or without concomitant decrease in negative charge, thus allowing for charge-charge repulsion.

[0142] An additional difficulty associated with therapeutic applications of wild-type FGF21 as a biotherapeutic, for instance, is that its half-life is very short in vivo (on the order of 0.5 and 2 h, respectively, in mouse and primate). There is hence a need to develop follow-up compounds that are more efficacious either through higher potency or longer half-life. The fusion proteins of the invention were developed as a way to achieve the desirable effects of FGF21 treatment at a higher potency and in a half-life-extended formulation.

[0143] As described further herein, the fusion proteins of the invention have half-lives of greater than two weeks in the mouse, compared to the much shorter half-life of wild-type FGF21 and the 17 hour half-life of fusion protein Fc-L(15)-FGF21 (L98R, P171G, A180E) in PCT Publication WO10/129600. The fusion proteins of the invention also demonstrate improved half-life and pharmacokinetic properties compared to PEGylated V76, as described herein and in US
Further, the Fc-FGF21 fusion proteins of the invention at 1 mpk are more efficacious than V76 at 5 mpk on reducing glucose, insulin, body weight and liver lipid. In a 12-day treatment study in ob/ob mice, the fusion proteins show the following % changes from vehicle (all of the fusions are administered at 1.0 mg/kg, and V76 is administered at 5.0 mg/kg):

- Total glucose (AUC) % change from vehicle: V76 is -42%; V101 is -53%, V103 is -46%, and V188 is -42%;
- Total plasma insulin % change from vehicle: V76 is -46%; V101 is -82%, V103 is -69%, and V188 is -59%;
- Total body weight % change from vehicle: V76 is -7%; V101 is -12%, V103 is -12%, and V188 is -11%; and
- Total liver lipid % change from vehicle: V76 is -30%; V101 is -44%, V103 is -50%, and V188 is -51%.

Similarly, in vitro assays reveal the same 5-fold or greater potency of the fusion proteins of the invention over V76:

- In the pERK in human adipocytes assay (mean EC50 ± SEM), V76 is 21 ± 2 nM (n=3); V101 is 1.0 ± 0.1 nM (n=3); V103 is 1.3 ± 0.2 nM (n=3), and V188 is 1.4 ± 0.4 nM (n=3);
- In the pERK in HEK293 with human klotho assay (mean EC50 ± SEM), V76 is 13 ± 4 nM (n=5), V101 is 0.60 ± 0.06 nM (n=5), V103 is 0.9 ± 0.3 nM (n=5), and V188 is 0.4 ± 0.1 nM (n=3); and
- In the glucose uptake in mouse adipocytes assay (mean EC50 ± SEM), V76 is 5 ± 1 nM (n=3), V101 is 0.60 ± 0.06 nM (n=3), V103 is 0.60 ± 0.07 nM (n=3), and V188 is 0.48 ± 0.14 nM (n=3).

Although the embodiments of the present invention concern the physical and chemical stability under both physiological and preserved pharmaceutical formulation conditions, maintaining the biological potency of the fusion proteins of the invention as compared to, e.g., wild-type FGF21 is an important factor of consideration as well. Therefore, the biological potency of the proteins of the present invention is defined by the ability of the proteins to affect glucose uptake and/or the lowering of plasma glucose levels, as shown herein in the examples.

The proteins, polypeptides, and/or peptides of the invention administered according to this invention may be generated and/or isolated by any means known in the art. The most preferred method for producing the variant is through recombinant DNA methodologies and is well known to those skilled in the art. Such methods are described in Current Protocols in Molecular Biology (John Wiley & Sons, Inc.), which is incorporated herein by reference.

Additionally, the preferred embodiments include a biologically active peptide derived from the variant described herein. Such a peptide will contain at least one of the substitutions described and the variant will possess biological activity. The peptide may be produced by any and all means known to those skilled in the art, examples of which included but are not limited to enzymatic digestion, chemical synthesis or recombinant DNA methodologies.

It is established in the art that fragments of peptides of certain fibroblast growth factors are biologically active. See for example, Baird et al., Proc. Natl. Acad. Sci (USA) 85:2324-2328 (1988), and J. Cell. Phys. Suppl. 5:101-106 (1987). Therefore, the selection of fragments or peptides of the variant is based on criteria known in the art. For example, it is known that dipeptidyl peptidase IV (DPP-IV, or DPP-4) is a serine type protease involved in inactivation of neuropeptides, endocrine peptides, and cytokines (Damme et al. Chem. Immunol. 72: 42-56, (1999)). The N-terminus of FGF21 (HisProllePro) contains two dipeptides that could potentially be substrates to DPP-IV, resulting in a fragment of FGF21 that shares the same 5-fold or greater potency of the fusion proteins of the invention over V76.
insertion variants as long as at least one of the indicated amino acid substitutions of the first or second embodiments is present.

The polynucleotides of the invention will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline, neomycin, and dihydrofolate reductase, to permit detection of those cells transformed with the desired DNA sequences. The FGF21 variant can be expressed in mammalian cells, insect, yeast, bacterial or other cells under the control of appropriate promoters. Cell free translation systems can also be employed to produce such proteins using RNAs derived from DNA constructs of the present invention.

E. coli is a prokaryotic host useful particularly for cloning the polynucleotides of the present invention. Other microbial hosts suitable for use include Bacillus subtilis, Salmonella typhimurium, and various species of Serratia, Pseudomonas, Streptococcus, and Staphylococcus, although others may also be employed as a matter of choice. In some prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any of a number of well-known promoters may be present, such as the lactose promoter system, a tryptophan (Trp) promoter system, a beta-lactamase promoter system, or a promoter system from phages lambda or T7. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

One skilled in the art of expression of proteins will recognize that methionine or methionine-arginine sequence can be introduced at the N-terminus of the mature sequence (SEQ ID NO: 3) for expression in E. coli and are contemplated within the context of this invention. Thus, unless otherwise noted, proteins of the present invention expressed in E. coli have a methionine introduced at the N-terminus.

Other microbes, such as yeast or fungi, may also be used for expression. Pichia pastoris, Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Pichia angusta are examples of preferred yeast hosts, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired. Aspergillus niger, Trichoderma reesei; and Schizophyllum commune, are examples of fungi hosts, although others may also be employed as a matter of choice.

Mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention. Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact variants have been developed in the art, and include the CHO cell lines, various COS cell lines, NSO cells, Syrian Hamster Ovary cell lines, HeLa cells, or human embryonic kidney cell lines (i.e. HEK293, HEK293EBNA).

Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from SV40, adenovirus, bovine papilloma virus, cytomegalovirus, Raus sarcoma virus, and the like. Preferred polyadenylation sites include sequences derived from SV40 and bovine growth hormone.

The vectors containing the polynucleotide sequences of interest (e.g., the fusion proteins of the invention and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts.

Various methods of protein purification may be employed and such methods are known in the art and described, for example, in Deutscher, Methods in Enzymology 182: 83-9 (1990) and Scopes, Protein Purification: Principles and Practice, Springer-Verlag, NY (1982). The purification step(s) selected will depend, for example, on the nature of the production process used for the fusion proteins of the invention.

The proteins, polypeptides, and/or peptides of the invention, e.g., the dual activity fusion proteins of the invention, should be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the patient, the site of delivery of the protein compositions, the method of administration, the scheduling of administration, and other factors known to practitioners. The “therapeutically effective amount” of the fusion proteins of the invention for purposes herein is thus determined by such considerations.

The pharmaceutical compositions of the proteins of the present invention may be administered by any means that achieve the generally intended purpose: to treat type 1 and type 2 diabetes mellitus, obesity, metabolic syndrome, or critically ill patients. Non-limiting permissible means of administration include, for example, by inhalation or suppository or to mucosal tissue such as by lavage to vaginal, rectal, urethral, buccal and sublingual tissue, orally, nasally, topically, intranasally, intraperitoneally, parenterally, intravenously, intramuscularly, intraternally, by intraarticular injection, intra-lymphatically, intestinally, intra-arterially, subcutaneously, intrasynovial, transepithelial, and transdermally. In some embodiments, the pharmaceutical compositions are administered by lavage, orally or inter-arterially. Other suitable methods of introduction can also include rechargeable or biodegradable devices and slow or sustained release polymeric
The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent
treatment, if any, frequency of treatment, and the nature of the effect desired. Compositions within the scope of the
invention include all compositions wherein an FGF21 variant is present in an amount that is effective to achieve the
desired medical effect for treatment type 1 or type 2 diabetes mellitus, obesity, or metabolic syndrome. While individual
needs may vary from one patient to another, the determination of the optimal ranges of effective amounts of all of the
components is within the ability of the clinician of ordinary skill.

The proteins of the present invention can be formulated according to known methods to prepare pharmaceutically
useful compositions. A desired formulation would be one that is a stable lyophilized product that is reconstituted
with an appropriate diluent or an aqueous solution of high purity with optional pharmaceutically acceptable carriers,
preservatives, excipients or stabilizers [Remington’s Pharmaceutical Sciences 16th edition (1980)]. The proteins of the
present invention may be combined with a pharmaceutically acceptable buffer, and the pH adjusted to provide acceptable
stability, and a pH acceptable for administration.

For parenteral administration, in one embodiment, the fusion proteins of the invention are formulated generally
by mixing one or more of them at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or
emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concen-
ctrations employed and is compatible with other ingredients of the formulation. Preferably, one or more pharmaceuti-
cally acceptable anti-microbial agents may be added. Phenol, m-cresol, and benzyl alcohol are preferred pharmaceuti-
cally acceptable anti-microbial agents.

Optionally, one or more pharmaceutically acceptable salts may be added to adjust the ionic strength or toxicity.
One or more excipients may be added to further adjust the isotonicity of the formulation. Glycerin, sodium chloride, and
mannitol are examples of an isotonicity adjusting excipient.

Those skilled in the art can readily optimize pharmaceutically effective dosages and administration regimens
for therapeutic compositions comprising Proteins of the invention, as determined by good medical practice and the
clinical condition of the individual patient. A typical dose range for the proteins of the present invention will range from
about 0.01 mg per day to about 1000 mg per day (or about 0.05 mg per week to about 5000 mg per week administered
once per week) for an adult. Preferably, the dosage ranges from about 0.1 mg per day to about 100 mg per day (or
about 0.5 mg per week to about 500 mg per week administered once per week), more preferably from about 1.0 mg/day
to about 10 mg/day (or about 5 mg per week to about 50 mg per week administered once per week). Most preferably,
the dosage is about 1-5 mg/day (or about 5 mg per week to about 25 mg per week administered once per week). The
appropriate dose of an FGF21 variant administered will result in lowering blood glucose levels and increasing energy
expenditure by faster and more efficient glucose utilization, and thus is useful for treating type 1 and type 2 diabetes
mellitus, obesity and metabolic syndrome.

In addition, because hyperglycemia and insulin resistance are common in critically ill patients given nutritional
support, some ICUs administer insulin to treat excessive hyperglycemia in fed critically ill patients. In fact, recent studies
document the use of exogenous insulin to maintain blood glucose at a level no higher than 110 mg per deciliter reduced
morbidity and mortality among critically ill patients in the surgical intensive care unit, regardless of whether they had a
are uniquely suited to help restore metabolic stability in metabolically unstable critically ill patients. Proteins of the
invention such as those containing variants of FGF21 are unique in that they stimulate glucose uptake and enhances
insulin sensitivity but do not induce hypoglycemia.

In another aspect of the present invention, proteins of the invention for use as a medicament for the treatment
of obesity, type 1 and type 2 diabetes mellitus, pancreatitis, dyslipidemia, nonalcoholic fatty liver disease (NAFLD),
nonalcoholic steatohepatitis (NASH), insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, meta-
abolic syndrome, acute myocardial infarction, conditions associated with severe inactivating mutations in the insulin
receptor, and other metabolic disorders is contemplated.

Site-Specific FGF21 Mutants

In some embodiments, the fusion proteins of the invention include additional FGF21 mutants or FGF21 ana-
logues with unnatural amino acids.

In some embodiments, the fusion proteins of the invention comprise FGF21 agonists with one or more of the
following additional modifications of wild-type FGF21:

(i) additional disulfides, unnatural amino acids, or modifications to promote dimerization such as formation of a
disulfide at R154C or introduction of a cysteine at another site, or dimerization through a fused Fc domain, or dimer
formation through a cross-linker such as a bifunctional PEG;
In some embodiments, the fusion proteins of the invention comprise one or more of the following linkers: a simple amide bond, short peptides (particularly Ser/Gly repeats), additional residues from the FGF21 translated sequence, or a larger linker up to an entire protein (such as an Fc domain, an HSA-binding helix bundle, HSA, etc.). The two moieties can also be linked by other chemical means, such as through unnatural amino acids or standard chemical linkers (maleimide-Cys, NHS-Lys, click, etc.)

Other embodiments of the invention include but are not limited to the following attachments, for half-life extension:

- HSA-binding lipid or small molecule or micelle to either the monomeric or a dimeric version of the fusion.

In certain embodiments of the invention, other attachments may be made to proteins, polypeptides, and/or peptides of the invention, to achieve half-life extension and other improved biological properties. They can include attaching PEG-cholesterol conjugates (including micelles and liposomes) to the proteins, polypeptides, and/or peptides of the invention, and/or attaching sugars (glycosylate) to the proteins, polypeptides, and/or peptides of the invention. In still other embodiments, similar techniques are employed to add conjugates of, e.g., polysialic acid (PSA), hydroxyethyl starch (HES), albumin-binding ligands, or carbohydrate shields to proteins, polypeptides, and/or peptides.

The HESylation technique, for example, couples branched hydroxyethylstarch (HES) chains (60 kDa or 100 kDa, highly branched amylopectin fragments from corn starch) to a protein, polypeptides, and/or peptides via reductive alkylation. Polysialation conjugates proteins, polypeptides, and/or peptides of interest with polysialic acid (PSA) polymers in a manner similar to PEGylation. PSA polymers are negatively charged, non-immunogenic polymers that occur naturally in the body and are available in molecular weights of 10-50kDa.

In still other embodiments of the invention, other attachments or modifications may be made to proteins, polypeptides, and/or peptides of the invention, to achieve half-life extension and other improved biological properties. These include the creation of recombinant PEG (rPEG) groups, and their attachment to the proteins, polypeptides, and/or peptides of the invention. As developed by the company Amunix, Inc. the rPEG technology is based on protein sequences with PEG-like properties that are genetically fused to biopharmaceuticals, avoiding the extra chemical conjugation step. rPEGs are extended half-life exenatide constructs that contain a long unstructured tail of hydrophilic amino acids, and which are capable of both increasing a protein or peptide’s serum half-life and slowing its rate of absorption, thus reducing the peak-trough ratio significantly. rPEGs have an increased hydrodynamic radius and show an apparent molecular weight that is about 15-fold their actual molecular weight, mimicking the way PEGylation achieves a long serum half-life.

Truncated FGF21 Polypeptides

One embodiment of the present invention is directed to truncated forms of the mature FGF21 polypeptide (SEQ ID NO:3). This embodiment of the present invention arose from an effort to identify truncated FGF21 polypeptides that are capable of providing an activity that is similar, and in some instances superior, to untruncated forms of the mature FGF21 polypeptide.

As used herein, the term "truncated FGF21 polypeptide" refers to an FGF21 polypeptide in which amino acid residues have been removed from the amino-terminal (or N-terminal) end of the FGF21 polypeptide, amino acid residues have been removed from the carboxyl-terminal (or C-terminal) end of the FGF21 polypeptide, or amino acid residues have been removed from both the amino-terminal and carboxyl-terminal ends of the FGF21 polypeptide. The various truncations disclosed herein were prepared as described herein.

The activity of N-terminally truncated FGF21 polypeptides and C-terminally truncated FGF21 polypeptides can be assayed using an in vitro phospho-ERK assay. Specific details of the in vitro assays that can be used to examine the activity of truncated FGF21 polypeptides can be found in the examples.

The activity of the truncated FGF21 polypeptides of the present invention can also be assessed in an in vivo assay, such as ob/ob mice. Generally, to assess the in vivo activity of a truncated FGF21 polypeptide, the truncated FGF21 polypeptide can be administered to a test animal intraperitoneally. After a desired incubation period (e.g., one hour or more), a blood sample can be drawn, and blood glucose levels can be measured.

a. N-Terminal Truncations

In some embodiments of the present invention, N-terminal truncations comprise 1, 2, 3, 4, 5, 6, 7, or 8 amino acid residues from the N-terminal end of the mature FGF21 polypeptide. Truncated FGF21 polypeptides having N-terminal truncations of fewer than 9 amino acid residues retain the ability of the mature FGF21 polypeptide to lower blood glucose in an individual. Accordingly, in particular embodiments, the present invention encompasses truncated forms of the mature FGF21 polypeptide or FGF21 protein variants having N-terminal truncations of 1, 2, 3, 4, 5, 6, 7, or...
In some embodiments of the present invention, C-terminal truncations comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acid residues from the C-terminal end of the mature FGF21 polypeptide. Truncated FGF21 polypeptides having C-terminal truncations of fewer than 13 amino acid residues exhibited an efficacy of at least 50% of the efficacy of wild-type FGF21 in an in vitro ELK-luciferase assay (Yie J. et al. FEBS Letts 583:19-24 (2009)), indicating that these FGF21 mutants retain the ability of the mature FGF21 polypeptide to lower blood glucose in an individual. Accordingly, in particular embodiments, the present invention encompasses truncated forms of the mature FGF21 polypeptide or FGF21 protein variants having C-terminal truncations of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acid residues.

b. C-Terminal Truncations

In some embodiments of the present invention, C-terminal truncations comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acid residues from the C-terminal end of the mature FGF21 polypeptide. Truncated FGF21 polypeptides having C-terminal truncations of fewer than 13 amino acid residues exhibited an efficacy of at least 50% of the efficacy of wild-type FGF21 in an in vitro ELK-luciferase assay (Yie J. et al. FEBS Letts 583:19-24 (2009)), indicating that these FGF21 mutants retain the ability of the mature FGF21 polypeptide to lower blood glucose in an individual. Accordingly, in particular embodiments, the present invention encompasses truncated forms of the mature FGF21 polypeptide or FGF21 protein variants having C-terminal truncations of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 amino acid residues.

c. N-Terminal and C-Terminal Truncations

As with all FGF21 variants of the present invention, truncated FGF21 polypeptides can optionally comprise an amino-terminal methionine residue, which can be introduced by directed mutation or as a result of a bacterial expression process.

The truncated FGF21 polypeptides of the present invention can be prepared as described in the examples described herein. Those of ordinary skill in the art, familiar with standard molecular biology techniques, can employ that knowledge, coupled with the instant disclosure, to make and use the truncated FGF21 polypeptides of the present invention. Standard techniques can be used for recombinant DNA, oligonucleotide synthesis, tissue culture, and transformation (e.g., electroporation, lipofection). See, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, supra, which is incorporated herein by reference for any purpose. Enzymatic reactions and purification techniques can be performed according to manufacturer’s specifications, as commonly accomplished in the art, or as described herein. Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques can be used for chemical syntheses; chemical analyses; pharmaceutical preparation, formulation, and delivery; and treatment of patients.

The truncated FGF21 polypeptides of the present invention can also be fused to another entity, which can impart additional properties to the truncated FGF21 polypeptide. In one embodiment of the present invention, a truncated FGF21 polypeptide can be fused to an IgG constant domain or fragment thereof (e.g., the Fc region), Human Serum Albumin (HSA), or albumin-binding polypeptides. Such fusion can be accomplished using known molecular biological methods and/or the guidance provided herein. The benefits of such fusion polypeptides, as well as methods for making such fusion polypeptides, are discussed in more detail herein.

FGF21 Fusion Proteins

As used herein, the term “FGF21 fusion polypeptide” or “FGF21 fusion protein” refers to a fusion of one or more amino acid residues (such as a heterologous protein or peptide) at the N-terminus or C-terminus of any FGF21 protein variant described herein.

FGF21 fusion proteins can be made by fusing heterologous sequences at either the N-terminus or at the C-terminus of, for example, an FGF21 protein variant, as defined herein. As described herein, a heterologous sequence can be an amino acid sequence or a non-amino acid-containing polymer. Heterologous sequences can be fused either directly to the FGF21 protein variant or via a linker or adapter molecule. A linker or adapter molecule can be one or more amino acid residues (or -mers), e.g., 1, 2, 3, 4, 5, 6, 7, 8, or 9 residues (or -mers), preferably from 10 to 50 amino acid residues (or -mers), e.g., 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 residues (or -mers), and more preferably from 15 to 35 amino acid residues (or -mers). A linker or adapter molecule can also be designed with a cleavage site for a DNA restriction endonuclease or for a protease to allow for the separation of the fused moieties.
Heterologous peptides and polypeptides include, but are not limited to, an epitope to allow for the detection and/or isolation of an FGF21 protein variant; a transmembrane receptor protein or a portion thereof, such as an extracellular domain or a transmembrane and intracellular domain; a ligand or a portion thereof which binds to a transmembrane receptor protein; an enzyme or portion thereof which is catalytically active; a polypeptide or peptide which promotes oligomerization, such as a leucine zipper domain; a polypeptide or peptide which increases stability, such as an immunoglobulin constant region; a functional or non-functional antibody, or a heavy or light chain thereof; and a polypeptide which has an activity, such as a therapeutic activity, different from the FGF21 protein variants of the present invention. Also encompassed by the present invention are FGF21 mutants fused to human serum albumin (HSA).

a. Fc Fusions

In one embodiment of the present invention, an FGF21 protein variant is fused to one or more domains of an Fc region of human IgG. Antibodies comprise two functionally independent parts, a variable domain known as “Fab,” that binds an antigen, and a constant domain known as “Fc,” that is involved in effector functions such as complement activation and attack by phagocytic cells. An Fc has a long serum half-life, whereas a Fab is short-lived (Capon et al., 1989, Nature 337: 525-31). When joined together with a therapeutic protein, an Fc domain can provide longer half-life or incorporate such functions as Fc receptor binding, protein A binding, complement fixation, and perhaps even placental transfer (Capon et al., 1989).

Throughout the disclosure, Fc-FGF21 refers to a fusion protein in which the Fc sequence is fused to the N-terminus of FGF21. Similarly, throughout the disclosure, FGF21-Fc refers to a fusion protein in which the Fc sequence is fused to the C-terminus of FGF21.

Preferred embodiments of the invention are Fc-FGF21 fusion proteins comprising FGF21 variants as defined herein. Particularly preferred embodiments are Fc-FGF21 fusion proteins comprising a modified Fc fragment (e.g., an Fc LALA) and FGF21 variants as defined herein.

Fusion protein can be purified, for example, by the use of a Protein A affinity column. Peptides and proteins fused to an Fc region have been found to exhibit a substantially greater half-life in vivo than the unfused counterpart. Also, a fusion to an Fc region allows for dimerization/multimerization of the fusion polypeptide. The Fc region can be a naturally occurring Fc region, or can be altered to improve certain qualities, such as therapeutic qualities, circulation time, or reduced aggregation.

Useful modifications of protein therapeutic agents by fusion with the “Fc” domain of an antibody are discussed in detail in PCT Publication No. WO 00/024782. This document discusses linkage to a “vehicle” such as polyethylene glycol (PEG), dextran, or an Fc region.

b. Fusion Protein Linkers

When forming the fusion proteins of the present invention, a linker can, but need not, be employed. When present, the linker’s chemical structure may not critical, since it serves primarily as a spacer. The linker can be made up of amino acids linked together by peptide bonds. In some embodiments of the present invention, the linker is made up of from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids. In various embodiments, the 1 to 20 amino acids are selected from the amino acids glycine, serine, alanine, proline, asparagine, glutamine, and lysine. In some embodiments, a linker is made up of a majority of amino acids that are sterically unhindered, such as glycine and alanine. In some embodiments, linkers are polyglycines, polyalanines, combinations of glycine and alanine (such as poly(Gly-Ala)), or combinations of glycine and serine (such as poly(Gly-Ser)). While a linker of 15 amino acid residues has been found to work particularly well for FGF21 fusion proteins, the present invention contemplates linkers of any length or composition.

The linkers described herein are exemplary, and linkers that are much longer and which include other residues are contemplated by the present invention. Non-peptide linkers are also contemplated by the present invention. For example, alkyl linkers such as can be used. These alkyl linkers can further be substituted by any non-sterically hindering group, including, but not limited to, a lower alkyl (e.g., C1-C6), lower acyl, halogen (e.g., Cl, Br), CN, NH2, or phenyl. An exemplary non-peptide linker is a polyethylene glycol linker, wherein the linker has a molecular weight of 100 to 5000 kD, for example, 100 to 500 kD.

Chemically-Modified Fusion Proteins

Chemically modified forms of the fusion proteins described herein, including, e.g., truncated and variant forms of the FGF21 fusions described herein, can be prepared by one skilled in the art, given the disclosures described herein. Such chemically modified Fusion Proteins are altered such that the chemically modified mutant is different from the unmodified mutant, either in the type or location of the molecules naturally attached to the mutant. Chemically modified
mutants can include molecules formed by the deletion of one or more naturally-attached chemical groups.

In one embodiment, proteins of the present invention can be modified by the covalent attachment of one or more polymers. For example, the polymer selected is typically water-soluble so that the protein to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Included within the scope of suitable polymers is a mixture of polymers. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. Non-water soluble polymers conjugated to proteins of the present invention also form an aspect of the invention.

Exemplary polymers each can be of any molecular weight and can be branched or unbranched. The polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa (the term "about" indicating that in preparations of a water-soluble polymer, some molecules will weigh more and some less than the stated molecular weight). The average molecular weight of each polymer is preferably between about 5 kDa and about 50 kDa, more preferably between about 12 kDa and about 40 kDa, and most preferably between about 20 kDa and about 35 kDa.

Suitable water-soluble polymers or mixtures thereof include, but are not limited to, N-linked or O-linked carbohydrates, sugars, phosphates, polyethylene glycol (PEG) (including the forms of PEG that have been used to derivatize proteins, including mono-(C1-C10), alkoxy-, or aryloxy-polyethylene glycol), monomethoxy-polyethylene glycol, dextran (such as low molecular weight dextran or, for example, about 6 kDa), cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polysols (e.g., sucrose), and polyvinyl alcohol. Also encompassed by the present invention are bifunctional crosslinking molecules that can be used to prepare covalently attached FGF21 protein variant multimers. Also encompassed by the present invention are FGF21 mutants covalently attached to polysialic acid.

Polysaccharide polymers are another type of water-soluble polymer that can be used for protein modification. Therefore, the fusion proteins of the invention fused to a polysaccharide polymer form embodiments of the present invention. Dextrans are polysaccharide polymers comprised of individual subunits of glucose predominantly linked by alpha 1-6 linkages. The dextran itself is available in many molecular weight ranges, and is readily available in molecular weights from about 1 kDa to about 70 kDa. Dextran is a suitable water-soluble polymer for use as a vehicle by itself or in combination with another vehicle (e.g., Fc). See, e.g., International Publication No. WO 96/11953. The use of dextran conjugated to therapeutic or diagnostic immunoglobulins has been reported. See, e.g., European Patent Publication No. 0 315 456, which is hereby incorporated by reference. The present invention also encompasses the use of dextran of about 1 kDa to about 20 kDa.

In general, chemical modification can be performed under any suitable condition used to react a protein with an activated polymer molecule. Methods for preparing chemically modified polypeptides will generally comprise the steps of: (a) reacting the polypeptide with the activated polymer molecule (such as a reactive ester or aldehyde derivative of the polymer molecule) under conditions whereby a FGF21 protein variant becomes attached to one or more polymer molecules, and (b) obtaining the reaction products. The optimal reaction conditions will be determined based on known parameters and the desired result. For example, the larger the ratio of polymer molecules to protein, the greater the percentage of attached polymer molecule. In one embodiment of the present invention, chemically modified FGF21 mutants can have a single polymer molecule moiety at the amino-terminus (see, e.g., U.S. Pat. No. 5,234,784)

In another embodiment of the present invention, Proteins of the invention can be chemically coupled to biotin. The biotin/Proteins of the invention are then allowed to bind to avidin, resulting in tetravalent avidin/biotin/Proteins of the invention. Proteins of the invention can also be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugates precipitated with anti-DNP or anti-TNP-IgM to form decameric conjugates with a valency of 10.

Generally, conditions that can be alleviated or modulated by the administration of the present chemically modified FGF21 mutants include those described herein for Proteins of the invention. However, the chemically modified FGF21 mutants disclosed herein can have additional activities, enhanced or reduced biological activity, or other characteristics, such as increased or decreased half-life, as compared to unmodified FGF21 mutants.

Therapeutic Compositions of Fusion Proteins and Administration Thereof

The present invention also provides therapeutic compositions comprising one or more of the fusion proteins of the invention described herein and in admixture with a pharmaceutically or physiologically acceptable formulation agent or pharmaceutically acceptable carrier selected for suitability with the mode of administration. The compositions are specifically contemplated in light of, e.g., the identification of fusions proteins exhibiting enhanced properties.

In some embodiments the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier. Pharmaceutically acceptable salts can also be present in the pharmaceutical composition, e.g., mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates,
acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations em-
employed. Acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed.

The pharmaceutical composition can contain formulation materials for modifying, maintaining, or preserving, for example, the pH, osmolality, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption, or penetration of the composition. Suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine, or lysine), antimicrobials, antioxidants (such as ascorbic acid, sodium sulfite, or sodium hydrosulfite), buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates, or other organic acids), bulking agents (such as mannitol or glycine), chelating agents (such as ethylenediamine tetraacetic acid (EDTA)), complexing agents (such as caffeine, polyvinylpyrrolidone, beta-cyclodextrin, or hydroxypropyl-beta-cyclodextrin), fillers, monosaccharides, disaccharides, and other carbohydrates (such as glucose, mannose, or dextrins), proteins (such as serum albumin, gelatin, or immunoglobulins), coloring, flavoring and diluting agents, emulsifying agents, hydrophilic polymers (such as polyvinylpyrrolidone), low molecular weight polypeptides, salt-forming counterions (such as sodium), preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid, or hydrogen peroxide), solvents (such as glycerin, propylene glycol, or poly-
ethylene glycol), sugar alcohols (such as mannitol or sorbitol), suspending agents, surfactants or wetting agents (such as pluronics; PEG; sorbitan esters; polysorbates such as polysorbate 20 or polysorbate 80; triton; tromethamine; lecithin; cholesterol or tyloxapal), stability enhancing agents (such as sucrose or sorbitol), toxicity enhancing agents (such as alka-
lic metal halides; preferably sodium or potassium chloride; or mannitol sorbitol), delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants (see, e.g., Remington’s Pharmaceutical Sciences (18th Ed., A. R. Gennaro, ed., Mack Publishing Company 1990), and subsequent editions of the same, incorporated herein by reference for any purpose).

The optimal pharmaceutical composition will be determined by a skilled artisan depending upon, for example, the intended route of administration, delivery format, and desired dosage (see, e.g., Remington’s Pharmaceutical Sciences (18th Ed., A. R. Gennaro, ed., Mack Publishing Company 1990), and subsequent editions of the same, incorporated herein by reference for any purpose).

The pharmaceutical compositions containing the fusion proteins of the invention can be selected for parenteral delivery. Alternatively, the compositions can be selected for inhalation or for delivery through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art.

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

When parenteral administration is contemplated, the therapeutic compositions for use in this invention can be in the form of a pyrogen-free, parenterally acceptable, aqueous solution comprising the desired dual function protein in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which a dual function protein is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polyactic acid or polyglycolic acid), beads, or liposomes, that provides for the controlled or sustained release of the product which can then be delivered via a depot injection. Hyaluronic acid can also be used, and this can have the effect of promoting sustained duration in the circulation. Other suitable means for the introduction of the desired molecule include implantable drug delivery devices.

In one embodiment, a pharmaceutical composition can be formulated for inhalation. For example, a dual function protein of the invention can be formulated as a dry powder for inhalation. Dual function protein inhalation solutions can also be formulated with a propellant for aerosol delivery. In yet another embodiment, solutions can be nebulized. Pulmonary administration is further described in International Publication No. WO 94/20069, which describes the pulmonary delivery of chemically modified proteins.
capsule can be designed to release the active portion of the formulation at the point in the gastrointestinal tract where 
bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate 
asorption of the fusion proteins of the invention. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, 
suspending agents, tablet disintegrating agents, and binders can also be employed.

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

Additional pharmaceutical compositions comprising Fusion Proteins of the invention will be evident to those 
skilled in the art, including formulations involving Fusion Proteins of the invention in sustained- or controlled-delivery 
formulations. Techniques for formulating a variety of other sustained- or controlled-delivery means, such as liposome 
carriers, bio-erodible microparticles or porous beads and depot injections, are also known to those skilled in the art (see, 
e.g., International Publication No. WO 93/15722, which describes the controlled release of polymeric microparticles 
for the delivery of pharmaceutical compositions, and Wischke & Schwendeman, 2008, Int. J Pharm. 364: 298-327, 

Additional examples of sustained-release preparations include semipermeable polymer matrices in the form of 
shaped articles, e.g., films, or microcapsules. Sustained release matrices can include polyesters, hydrogels, polyacrylates 
(U.S. Pat. No. 3,773,919 and European Patent No. 0 058 481), copolymers of L-glutamic acid and gamma ethyl-L-
leucine (Sidman et al., 1983, Biopolymers 22: 547-56), poly(2-hydroxyethyl-methacrylate) (Langer et al., 1981, J. 
supra) or poly-D-3-hydroxybutyric acid (European Patent No. 0 133 988). Sustained-release compositions can also 
include liposomes, which can be prepared by any of several methods known in the art. See, e.g., Epstein et al., 1985, 

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

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

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

Dosages of Fusion Proteins and Administration Thereof

The effective amount of an pharmaceutical composition of the invention to be employed therapeutically will 
depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate 
dosage levels for treatment will thus vary depending, in part, upon the molecule delivered, the indication for which the 
fusion protein variant is being used, the route of administration, and the size (body weight, body surface, or organ size) 
and condition (the age and general health) of the patient. Accordingly, the clinician can titler the dosage and modify the 
route of administration to obtain the optimal therapeutic effect. A typical dosage can range from about 0.1 µg/kg up to 
to about 100 mg/kg or more, depending on the factors mentioned above. In other embodiments, the dosage can range 
from 0.1 µg/kg up to about 100 mg/kg; or 1 µg/kg up to about 100 mg/kg.

The frequency of dosing will depend upon the pharmacokinetic parameters of the dual function protein in the 
formulation being used. Typically, a clinician will administer the composition until a dosage is reached that achieves the 
desired effect. The composition can therefore be administered as a single dose, as two or more doses (which may or 
may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation 
device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art 
and is within the ambit of tasks routinely performed by them. Appropriate dosages can be ascertained through use of 
appropriate dose-response data.

The route of administration of the pharmaceutical composition is in accord with known methods, e.g., orally; 
through injection by intravenous, intraperitoneal, intracerebral (intraparenchymal), intracerebroventricular, intramuscular, 
intrarterial, intraportal, or intrallesional routes; by sustained release systems (which may also be injected); or by im-
Alternatively or additionally, the composition can be administered locally via implantation of a membrane, sponge, or other appropriate material onto which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device can be implanted into any suitable tissue or organ, and delivery of the desired molecule can be via diffusion, timed-release bolus, or continuous administration.

**Therapeutic Uses of Fusion Proteins**

Proteins of the invention can be used to treat, diagnose, ameliorate, or prevent a number of diseases, disorders, or conditions, including, but not limited to metabolic disorders. In one embodiment, the metabolic disorder to be treated is diabetes, e.g., type 2 diabetes mellitus. In another embodiment, the metabolic disorder is obesity. Other embodiments include metabolic conditions or disorders such as type 1 diabetes mellitus, pancreatitis, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), insulin resistance, hyperinsulinemia, glucose intolerance, hyperglycemia, metabolic syndrome, cardiovascular disease, acute myocardial infarction, atherosclerosis, peripheral arterial disease, stroke, heart failure, coronary heart disease, kidney disease, diabetic complications, neuropathy, disorders associated with severe inactivating mutations in the insulin receptor, gastroparesis and other metabolic disorders.

In application, a disorder or condition such as type 1 or type 2 diabetes mellitus or obesity can be treated by administering an FGF21 protein variant as described herein to a patient in need thereof in the amount of a therapeutically effective dose. The administration can be performed as described herein, such as by IV injection, intraperitoneal injection, intramuscular injection, or orally in the form of a tablet or liquid formation. In most situations, a desired dosage can be determined by a clinician, as described herein, and can represent a therapeutically effective dose of the FGF21 mutant polypeptide. It will be apparent to those of skill in the art that a therapeutically effective dose of FGF21 mutant polypeptide will depend, inter alia, upon the administration schedule, the unit dose of antigen administered, whether the nucleic acid molecule or polypeptide is administered in combination with other therapeutic agents, the immune status and the health of the recipient. The term "therapeutically effective dose," as used herein, means that amount of FGF21 mutant polypeptide that elicits the biological or medicinal response in a tissue system, animal, or human being sought by a researcher, medical doctor, or other clinician, which includes alleviation of the symptoms of the disease or disorder being treated.

Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

**EXAMPLES**

**Example 1: Preparation of FGF21 Variant Proteins**

**Expression construct for FGF21 V76:** The FGF21 variants were cloned into the modified E.coli expression vector pET30a, described by Achmuller et al. (2007) (Nature Methods 4:1037-1043), to generate in-frame fusions to a hexa-histidine tag followed by the Npro-EDDIE tag at the N-terminus of FGF21 (aa 33-209).

**Expression and purification of FGF21 V76:** The pET30a-His-Npro-EDDIE-FGF21 expression plasmid was transformed into E. coli BL21 Star (DE3) competent cells (Invitrogen). Overnight growth from a single colony of freshly transformed cells was carried out in 50 mL of Terrific Broth (TB) containing 50 μg/mL of kanamycin at 37°C. The pre-culture was transferred into 1 L of TB medium with kanamycin and cultured in baffled flasks at 37°C with shaking at 250 rpm. After 6 hour of culture, expression of FGF21 was induced by the addition of IPTG at a final concentration of 1 mM, and the cultures were grown overnight at 37°C. The cells were then harvested and resuspended into 50 mL of ice-cold lysis buffer; 50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, followed by lysis using a microfluidizer™.
pH and adding dithiothreitol (DTT) at a concentration of 20 mM. The conditioned eluate was slowly diluted into 1 L of refolding buffer; 50 mM Tris-HCl, pH 8, 0.5 M arginine, 20 mM DTT, followed by incubation for 2 days at 4°C. The diluted sample was concentrated and buffer-exchanged into 20 mM Tris-HCl, pH 9 using an ultrafiltration method. The concentrated sample was loaded onto a 10 mL column of Q sepharose fast flow resin (GE Healthcare) equilibrated with 20 mM Tris-HCl (pH9).

[0233] After washing the resin with the equilibration buffer, proteins bound to the resin were eluted with 20 mM Tris-HCl, pH 9, 500 mM NaCl. To remove the cleaved off His-N\(^\text{pro}\) fusion fragment and any uncleaved fusion protein from the refolded FGF21 protein, the eluate was loaded onto a 5 mL column of Ni-NTA high performance resin equilibrated with 20 mM Tris, pH 8.0, 50 mM imidazole, and the flow-through fraction containing FGF21 was collected. To reduce endotoxin levels, the FGF21 fraction was treated with an EndoTrap HD resin (Hyglos) equilibrated with 10 mM Tris, pH 8, 50 mM imidazole, 500 mM NaCl, 1 mM CaCl\(_2\). The low-endotoxin sample was dialyzed against PBS and then sterilized with a 0.22 \(\mu\)m filter. The purified FGF21 protein was snap-frozen in liquid nitrogen and stored at -80°C. Protein concentration was determined by absorbance at 280 nm using 9362 M-1 cm-1 as the molar extinction coefficient for FGF21. Protein purity and integrity were determined by HPLC, SDS-PAGE and liquid chromatography-mass spectrometry.

[0234] Cysteine PEGylation of FGF21 variants: FGF21 Variant V76 (R154C) variant has the tendency to dimerize via the engineered cysteine; therefore, prior to PEGylation the protein solution (typically 5 mg/mL in Tris buffer) was mildly reduced with 5 mM mercaptoethanolamine for 30 minutes on ice and immediately desalted in 20 mM Tris, pH 7. The freshly reduced protein (typically 3 mg/mL) was then immediately PEGylated with 1.5 equivalent of 40 kDa branched maleimido-PEG reagent (NOF, Cat. # GL2-400MA from the Sunbright series) for 3 hours on ice. The PEGylated protein was finally purified by anion exchange chromatography (MonoQ) with overall yields of about 25%.

[0235] Expression constructs for Fc-FGF21 fusion variants: The cDNAs for human FGF21 variants encoding amino acids 33-209 were cloned into a mammalian expression vector downstream of the cytomegalovirus (CMV) promoter in-frame with N-terminal sequences including a leader peptide (immunoglobulin kappa-chain) to direct secretion of the proteins, followed by an Fc domain and a short linker.

[0236] Expression and purification of Fc-FGF21 variants: The Fc-FGF21 variant proteins were expressed into HEK293T cells (American Type Culture Collection). Cells were grown in suspension culture at 37°C, 8% CO\(_2\), in Freestyle 293 Expression Medium (Invitrogen, Cat. #12338-018) until day of transfection. Cells were centrifuged at 1000xg for 7 min in a swinging bucket rotor and counted using an automated cell counter. Cells were diluted in 900 mL of Freestyle 293 media to a final concentration of 1.4x10\(^6\) cells/mL and placed into a 3 L non-baffled flask (Corning, Cat. #431252). Cells were transfected using a mixture of polyethyleneimine (PEI) and plasmid as follows. Three mL of a sterile 1 mg/mL stock of linear, M.W. 25,000, PEI (Alfa Aesar, Cat.#43896) was added to 50 mL of Freestyle 293 media to a final concentration of 1.4x10\(^6\) cells/mL and placed into a 3 L flask containing 293 media and sterile filtered using a 0.22 \(\mu\)M filter (Pall Corporation, Cat. #4628). On day 6 post-transfection, the cells were centrifuged at 2000xg for 10 minutes and the supernatant was harvested. The supernatant was further clarified by filtration through a 0.8/0.2 \(\mu\)M filter (Pall Corporation, Cat. #4628).

[0238] Batch purification of the FGF21 protein was done by adding 1 mL of recombinant Protein A Sepharose Fast Flow (GE, Cat. #17-5138-03), per 20 mg of expected protein to be purified, directly to the clarified supernatant and incubating for 1 hour at 4°C with gentle rotation. The supernatant mixture was then poured over a disposable Poly-Prep Chromatography Column (Bio-Rad, Cat. #731-1550) and the flow through was discarded. The retained beads were washed with 5 column volumes of DPBS, pH7.4 (Invitrogen, Cat. #14190-144). Elution of the protein from the Protein A beads was done by adding 20 column volumes of 50 mM Sodium Citrate buffer, pH 3.0. The elution buffer was neutralized by the addition of 20 mM Tris-HCl buffer, pH 9.0. Size exclusion chromatography was performed as a secondary polishing step by running the Protein A batch purified material over a High Load 26/600 Superdex 200pg column (GE, Cat.#28-9893-36). The purified protein yield was quantified by A280. SDS-PAGE was run to verify purity and molecular weight. Endotoxin level was quantified by using the Endosafe PTS system (Charles River Labs).

Example 2: Measuring FGF21 Dependent 2-Deoxyglucose (2-DOG) Uptake

[0239] FGF21 has been shown to stimulate glucose-uptake in mouse 3T3-L1 adipocytes in the presence and absence of insulin, and to decrease fed and fasting blood glucose, triglycerides, and glucagon levels in ob/ob and db/db mice and 8 week old ZDF rats in a dose-dependent manner, thus, providing the basis for the use of FGF21 as a therapy for treating diabetes and obesity (see, e.g., patent publication WO03/011213, and Kharitonenkov et al., (2005) Jour. of Clinical Invest. 115:1627-1635). Also, FGF21 was observed to stimulate tyrosine phosphorylation of FGFR-1 and FGFR-2 in 3T3-L1 adipocytes.
Bovine Serum and 1% penicillin-streptomycin for an additional 4 days. Cells were then differentiated in the above media supplemented with 4 μg/mL insulin (Sigma, Cat. # I-5500), 115 μg/mL IBMX (Sigma, Cat. # I5879) and 0.0975 μg/mL dexamethasone (Sigma, Cat. #D1756) for 3 days after which the differentiation media was replaced with complete DMEM. One plate of differentiated 3T3-L1 adipocytes were seeded into four 96-well plates the day after medium replacement.

[0241] The adipocytes were then treated with FGF21-WT and FGF21 variant protein (see Table 2 for list of variants; 30 pM to 100 nM is the typical concentration range used) overnight in complete medium. The adipocytes treated with FGF21 samples were serum starved in 50 μL per well KRH buffer (0.75% NaCl; 0.038% KCl; 0.0196% CaCl2; 0.032% MgSO4; 0.025M HEPES, pH 7.5; 0.5% BSA; 2 mM sodium pyruvate) for 2 hours. The wells for blank were added with 1 μL (final concentration 5 μg/ml) cytochalasin B for 15 min. [3H]-2-DOG (20.6 mCi/mmol, 1 mCi/mL) was diluted 1:20 in 5.1 mM cold 2-DOG and 1 μL diluted 2-DOG was added per well and the cells were incubated for 5 min. The cells were washed with 100 μL/well KRH buffer three times. 40 μL/well 1% SDS was added to cells and the cells were shaken for at least 10 minutes. 200 μL/well scintillation fluid was added and the plates were shaken overnight and read in beta-microplate reader. The values obtained from an entire column/row, which were treated with cytochalasin B, was averaged and subtracted from all other values. The data were analyzed by GraphPad Prism software, the results of which are summarized in Table 2. Fc-FGF21 Fusion Variants V101, V103 and V188 are superior to PEGylated FGF21 Variant V76 in for induction of 2-deoxyglucose uptake by mouse 3T3L1 adipocytes.

Example 3: pERK In Cell Western (ICW) Assay

[0242] HEK293 cells stably transfected with human β-klotho were cultured in DMEM high glucose, 10% FBS, 1% PS and 600 ng/mL G418 are seeded in poly-D-lysine coated 96-well plates (BD bioscience, Cat. #356640) at 30,000 cells per well overnight. The cells were serum starved in DMEM high glucose, 0.5% BSA and 10 mM HEPES for 4 hours. WT FGF21 and the FGF21 variants (see Table 3 for list of variants) were diluted to various concentrations (100 pM to 300 nM is the typical concentration range used) in starvation medium. The cells were stimulated with FGF21 for 10 minutes. Following FGF21 or FGF21 Variant protein stimulation, the media was aspirated from the wells and the cells were washed once with 100 μL cold PBS and then fixed with 100 μL of 4% formaldehyde for 15 minutes at room temperature and followed by an additional 10 minute incubation with 100 μL ice-cold methanol.

[0243] After fixation, the cells were washed with 0.3% Triton X-100 in PBS four times, 5 minutes each. 150 μL Odyssey Blocking Buffer was added to the permeabilized cells at room temperature for 1.5 hours. Phospho-ERK (pERK) antibody was diluted to a concentration of 0.17 μg/mL (1:200 dilution, or the dilutions indicated), and total-ERK (tERK) antibody was diluted to a concentration of 2.2 μg/mL (1:200 dilution, or the dilutions indicated) in Odyssey Blocking Buffer. 50 μL was added to every well, omitting one column which was only treated with secondary antibody to normalize for background. The plate was covered with the wet paper tower and lid to prevent evaporation and then incubated at 4°C overnight.

[0244] Afterwards, the primary antibody was aspirated and the cells were washed four times with 0.3% Tween 20 in PBS for 5 minutes each. During the washing, the secondary antibody reaction mixture was prepared in Odyssey Blocking Buffer containing 1:1000-diluted (or the dilutions indicated) goat anti-mouse Alexa 680 and 1:1000-diluted (or the dilutions indicated) IRDye800 goat anti-rabbit antibody. Once the washing was completed, 40 μL of the reaction mixture was added to each well. Plates were covered with black lid to protect the secondary antibody from light, and plates were incubated at room temperature for 1 hour on a shaker. Finally, the cells were washed again four times with 0.3% Tween 20 in PBS for 5 minutes each and then scanned on the LI-COR Bioscience Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) in the 700 nm (red) and 800 nm (green) channels. Alexa 680 stained the tERK with far-red background. The plate was covered with the wet paper tower and lid to prevent evaporation and then incubated at 4°C overnight.

Table 2: Summary of ERK in cell Western and Mouse 3T3L1 Adipocyte Glucose Uptake Assay Results

<table>
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<tr>
<th>FGF21 Variant ID</th>
<th>pERK (HEK293/human β-klotho) EC50 ± SEM</th>
<th>Glucose Uptake (Mouse 3T3L1 adipocytes) EC50 ± SEM</th>
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<td>V76</td>
<td>13 ± 4 nM (n=5)</td>
<td>5 ± 1 nM (n=3)</td>
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<td>V101</td>
<td>0.60 ± 0.06 nM (n=5)</td>
<td>0.60 ± 0.06 nM (n=3)</td>
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Example 4: In vivo Tests of FGF21 Variants

[0245] The ob/ob mouse is a mouse model for type 2 diabetes. The mice lack functional leptin and are characterized by hyperglycemia, insulin resistance, hyperphagia, hepatic steatosis and obesity. Male ob/ob mice (10-13 weeks old) were used to measure the effect on blood glucose of the following PEGylated FGF21 variant V76 and Fc-FGF21 fusion variants V101, V103 and V188.

[0246] FGF21 variants or PBS vehicle were administered s.c. at 1 mg/kg (V101, V103 and V188) or s.c at 5 mg/kg V76 twice per week 12 days (4 doses total). On the first day of the study, tail blood glucose and body weight were measured and mice were allocated into different groups (n=8 per group) with mean glucose and body weight matched among the groups. Blood glucose was measured using a glucometer (OneTouch). Plasma insulin was measured on day 1 before dosing and on day 12, 24 hours post the last dose. The results of these studies are summarized in Table 5.

[0247] The results of these studies are summarized in Table 3 and Figures 1-3. Fc-FGF21 Fusion Variants V101, V103 and V188 are superior to PEGylated FGF21 Variant V76 on every endpoint measured in these studies and at a five-fold lower dose.

Table 3. % changes versus vehicle in plasma glucose, insulin, body weight (BW) gain, liver TG/lipid by FGF21 variants during 12-day studies in ob/ob mice.

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<th>FGF21 Variant ID</th>
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Example 5: Pharmacokinetics of FGF21 Fusion Variants in Mice

[0248] To determine the pharmacokinetic profile of Fc-FGF21 Fusion Variants V101, V103 and V188, C57BL/6J mice were injected IV with 1 mg/kg test article and bled at various time points out to 16 days (384 hours). Blood samples were collected into EDTA-coated microtainer tubes from either the submandibular or retro-orbital plexus. Approximately 50 μL of blood was collected at each time point, yielding -25 μL of plasma.

[0249] To measure plasma concentrations of test articles by ELISA, 384-well plates were coated overnight at room temperature (RT) with 2 μg/mL of anti-Human Fc-gamma goat polyclonal antibody (30 μL/well) and then blocked with a casein-based diluent for 2 hour at RT (100 μL/well). Diluted samples, standards, and controls were added to the plate (30 μL/well) and incubated for 2 hour at RT. After the samples were removed, the wells were washed 3 times with a phosphate-based wash solution (100 μL/well). The detection antibody, an HRP-labeled version of the capture antibody, was added to the plate and incubated for 1 hour at RT (30 μL/well). After the plate was again washed 3 times with a phosphate-based wash solution (100 μL/well), a chemiluminescent substrate was added (30 μL/well) and the plate luminescence was read within 5 minutes using an appropriate plate reader. As shown in Figures 4A and 4B the Fc-FGF21 fusion variants had a greatly extended plasma half-life relative to known Fc-FGF21 fusions in the art (Figure 4A) and relative to PEGylated FGF21 variant V76 (Figure 4B).

[0250] Serum levels of Fc-FGF21 test articles were validated by Western blot for comparison to levels measured by ELISA to ensure that full length Fc-FGF21 variant and not Fc alone was being detected in the ELISA. Two μL of mouse serum was combined with 2.5 μL of 4X loading buffer, 1 μL of 10X denaturant and 4 μL of dH2O, heated to 95°C for 5 minutes and loaded onto a 4-12% gradient polyacrylamide gel and electrophoresed for 1 hour at 100 Volts (constant voltage). Samples were transferred to nitrocellulose filter paper by Western blot using the iblot system (Invitrogen, Cat.
The nitrocellulose filters were blocked with 30 mL of Rockland blocking solution (Cat. #MB-070), probed following the snap iblot system protocol with a goat anti-FGF21 primary antibody at a 1:2000 dilution (R&D systems, Cat. # BAF2539) and fluorescently labeled streptavidin as a secondary at a 1:10000 dilution (Licor, Cat. # 926-68031). Protein levels were imaged on the Licor Odyssey system at 700 nm and compared with 2 nM control V101 run on the same gel. As shown in Figure 4C full-length Fc-FGF21 variants V101, V103 and V188 are detectible using on a Western Blot using anti-FGF21 antibody out to 15 days from mouse serum from the pharmacokinetic study.

**Example 6: Fc-FGF21 Fusion Variants V101, V103 and V188 are extremely thermodynamically stable**

Proteins can be unfolded at specific temperature range. The temperature of protein unfolding is an intrinsic parameter to describe thermal stability of proteins. Differential Scanning Calorimetry (DSC) is used to detect the unfolding temperature of protein. This characteristic temperature is described as melting temperature (Tm), which is the peak temperature during protein unfolding.

Original protein samples are diluted in PBS to a concentration of ~1mg/ml (0.5mg/ml to 1.2 mg/ml) for a total volume of 0.5ml. An aliquot of 0.4ml per well diluted protein sample, standard, PBS, and DI water are added to DSC 96-well plate. The plate is then covered by a seal. Samples were analyzed in a 96 well Differential Scanning Calorimeter from MicroCal. The temperature was scanned from 10 - 110 degrees C at a rate of 1 degree per minute.

As shown in Figure 4D the melting temperatures of FGF21 variants V101, V103 and V188 are extremely high. This is in contrast to the lower melting temperatures of FGF21 variant V76 and wild-type FGF21 (not shown). We attribute the improved stability of V101, V103 and V188 to the specific addition of a second disulfide bond from the novel Q55C and G148C mutations. This type of thermodynamic stability is known to protect proteins from proteolysis and can in addition translate into significantly prolonged stability in vivo and the improved pharmacokinetic profiles exemplified by the data in Figures 4B and 4C.
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EP 3 321 276 A2

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41
Claims

1. A fusion protein comprising an FGF21 variant and an Fc region.

2. The fusion protein of claim 1, wherein the sequence of said protein is selected from a sequence listed in Table 1.
**Fed plasma glucose**

![Graph of fed plasma glucose levels showing a trend over time with significant differences indicated by asterisks.](image)

* p<0.05 vs. Vehicle, two-way ANOVA

**FIG 1A**

**Fed plasma insulin**

![Bar graph of fed plasma insulin levels showing a significant difference between groups.](image)

* p<0.05 vs. Vehicle, one-way ANOVA

**FIG 1B**
Body weight

* p<0.05 vs. Vehicle, one-way ANOVA

FIG 1C

Liver lipid content

* p<0.05 vs. Vehicle, one-way ANOVA
# p<0.05 vs. LJN285, one-way ANOVA

FIG 1D
Body weight

* p<0.05 vs. Vehicle, one-way ANOVA
# p<0.05 vs. LiN295, one-way ANOVA

FIG 2C

Liver lipid content

FIG 2D
Fed plasma glucose

* p<0.05 vs Vehicle, two-way ANOVA

FIG 3A

Fed plasma insulin

* p<0.05 vs Vehicle, one-way ANOVA

FIG 3B
Body weight

* p<0.05 vs. Vehicle, one-way ANOVA

FIG 3C

Liver lipid content

* p<0.05 vs. Vehicle, one-way ANOVA
# p<0.05 vs. LJN285, one-way ANOVA

FIG 3D
FIG 4A
Single dose mouse PK IV 1 mg/kg

FIG 4B
REFERENCES CITED IN THE DESCRIPTION

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