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(54) **Title:** SECRETED SPLICING VARIANT OF KLOTHO FOR TREATING MUSCLE DISORDERS

(57) **Abstract:** The present invention provides a polypeptide consisting of sequence SEQ ID NO: 1, or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, for use in the prevention and/or treatment of a muscle disease or disorder. The invention also provides nucleic acid sequence that encodes the polypeptide, a gene construct comprising the nucleic acid sequence, or an expression vector comprising the gene construct, for said use. The polypeptide, nucleic acid sequence, gene construct, or expression vector of the invention may be administered in the form of a pharmaceutical composition together with at least one pharmaceutically acceptable excipient, diluent or carrier. The invention also provides a non-therapeutic method for improving muscle function and/or increasing muscle mass of a subject.

Secreted splicing variant of Klotho for treating muscle disorders

This application claims the benefit of European Patent Application EP22383173.6 filed on December 2nd, 2022.

Technical Field

The present invention relates to the field of medicine, in particular, it relates to medical approaches for preventing and/or treating muscle disorders. The compounds of the invention are particularly useful for the treatment of age-related muscle disorders.

Background Art

Senescence represents a stage in life associated with elevated incidence of morbidity and increased risk of mortality due to the accumulation of molecular alterations and tissue dysfunction, promoting a decrease in the organism's protective systems.

Muscular tissue is one of the systems highly affected during aging, characterized by phenotypes like reduced muscular strength and resistance, sarcopenia, muscular fibrosis, and reduction of the muscular regenerative capacity. Skeletal muscle state is an important indicator of the health status of an animal and can directly affect survival by increasing the chances of falls and corporal damage in aged animals.

Tissue-level features of sarcopenia have been described extensively, and include a decreased myofiber size, increased intramuscular fat accumulation, a preferential atrophy of type II (fast-twitch) muscle fibers, and compromised function. However, the understanding of the cellular mechanisms underlying sarcopenia is still lacking, a shortcoming that has hindered the development of targeted and specific interventions.

Currently, approaches to the treatment and prevention of sarcopenia largely focus on the prescription of physical activity and dietary modifications, strategies that have shown moderate success. Furthermore, there are no pharmacological interventions for sarcopenia currently on the market yet.

Previous reports have studied a possible relation between full-length Klotho and muscle function. However, very little is known about the secreted isoform of Klotho, s-KL, and its possible role in muscle function and muscle disease.

Thus, in spite of the efforts made so far, there is still a need for efficient and safe treatments for muscle disorders, in particular age-related muscle disorders.

Summary of Invention

The present inventors have developed a novel therapy for the prevention and/or treatment of muscle disorders based on the administration of secreted splicing isoform of Klotho, s-KL.

The Klotho gene presents two main transcripts —full-length Klotho mRNA and alternatively spliced Klotho mRNA. Full-length Klotho mRNA transcribes a single-pass transmembrane protein called m-KL, of 135KDa. The extracellular domains of m-KL can be released from the membrane by protease-mediated shedding, generating soluble, circulating processed Klotho (p-KL, of 130KDa) which is sometimes simply called soluble Klotho, and has two active domains. The alternatively spliced Klotho mRNA shows a premature stop codon and generates a secreted protein, s-KL (70KDa), containing just one of the active domains and an extra 15 amino acids at its C-terminus. Although a similar abbreviation (s-KL) is sometimes used in the prior art to name soluble Klotho —which is the processed version of transmembrane full-length Klotho— and secreted Klotho —which is the splicing isoform—, these two isoforms present a completely different structure, size, and biological activities. In fact, while full-length Klotho has been described to be involved in FGF23 receptor binding, PTH synthesis, regulation of parathyroid gland growth, and alterations in Vitamin D metabolism and Calcium ion blood levels, the secreted splicing isoform of Klotho (s-KL) has not.

The present invention is exclusively based on the use of the splicing isoform, secreted Klotho, whose biological functions remain largely unknown, and the abbreviation s-KL is used herein to refer to secreted Klotho only.

As shown in the Examples below, the present inventor surprisingly found that the administration of s-KL improved not only the physical performance of aged animals (Fig. 2a-d), but also the state of the aged muscular tissue (Fig. 3a-b). Particularly, s-KL administration increased the number of muscular fibers and reduced the percentage of fibrotic tissue in the muscle.

Furthermore, the present inventors found that the muscle of animals treated with s-KL presented higher number of muscular stem cells and with increased proliferative capacities, especially when the treatment was performed in the adult developmental stage, which provided the muscle a higher regenerative capacity (Fig. 4a-h).

All these effects of s-KL were highly unexpected in light of the prior art as it was previously shown that the intracerebroventricular administration of s-KL in mice had no effect in their locomotor functions (Massó A. et al., "Secreted αKlotho isoform protects against age-dependent memory deficits" Mol Psychiatry, 2018, vol. 23(9), pp. 1937-1947).

The extraordinary combination of effects that s-KL exerts in the muscle tissue found by the present inventors clearly positions s-KL as a useful therapeutic agent to improve muscle function and to treat muscle disorders, particularly age-associated muscle degeneration.

Thus, in a first aspect, the present invention provides a polypeptide consisting of sequence SEQ ID NO: 1, or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, for use in the prevention and/or treatment of a muscle disease or disorder, particularly by improving muscle function.

In a second aspect, the present invention provides a nucleic acid sequence that encodes the polypeptide or the variant thereof as defined in the first aspect, which is for use in the prevention and/or treatment of a muscle disease or disorder, particularly by improving muscle function.

In a third aspect, the present invention provides a gene construct comprising a nucleic acid sequence as defined in the second aspect, operatively linked to an expression promoter, which is for use in the prevention and/or treatment of a muscle disease or disorder, particularly by improving muscle function.

In a fourth aspect, the present invention provides and expression vector comprising the gene construct as defined in the third aspect, which is for use in the prevention and/or treatment of a muscle disease or disorder, particularly by improving muscle function.

In a fifth aspect, the invention provides a host cell which is transformed or transfected with the nucleic acid sequence as defined in the second aspect, the gene construct as defined in the third aspect, or the expression vector as defined in the fourth aspect, which is for use in the prevention and/or treatment of a muscle disease or disorder, particularly by improving muscle function.

In a sixth aspect, the invention provides a polypeptide consisting of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, or a nucleic acid sequence that encodes the polypeptide or the variant thereof, for use in

reducing muscle fibrosis.

In a seventh aspect, the invention provides a non-therapeutic method for improving muscle function and/or increasing muscle mass in a subject, the method comprising administering to the subject a polypeptide consisting of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, or a nucleic acid sequence that encodes the polypeptide or the variant thereof..

In an eighth aspect, the invention provides a non-therapeutic method for increasing the muscle regenerative capacity of a subject, the method comprising administering to the subject a polypeptide consisting of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, or a nucleic acid sequence that encodes the polypeptide or the variant thereof.

In a nineth aspect, the invention provides a non-therapeutic method for improving the physical state or performance of a subject, the method comprising administering to the subject a polypeptide consisting of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, or a nucleic acid sequence that encodes the polypeptide or the variant thereof.

Brief Description of Drawings

Fig. 1. s-KL treatment efficiently increased s-KL protein concentration. a) Schematic representation of the experimental design. b) s-KL gene expression analysis in liver of males (left panel) and females (right panel). c) Quantification of total s-KL protein concentration in serum of males (left panel) and females (right panel). Analysis done with samples of a subset of animals euthanized at the age of 24 months old. Data represented in (b) as fold change expression respect Null treated animals. Mean ± Standard error of the mean (SEM), n=4; *p<0.05; **p<0.01; ***p<0.001; ****p<0.001.

Fig. 2. s-KL treatment improved physical performance of aged animals in physical tests. a) Results of acceleration Rotarod test, showed as maximum speed that animals were able to run. b-c) Results of horizontal bar test, represented as time animals took to fall from the bar in males (b) and females (c). d) Results of grip strength test. Results represent average force animals were able to do in each of the three trials done. Data represented as mean ± Standard error of the mean (SEM), n=8-11; *p<0.05; **p<0.01; ***p<0.001.

Fig. 3. s-KL treatment reduced age-associated muscular fibrosis in mice. a) Quantification of fibre size of soleus muscle. b) Quantification of percentage of fibrotic area in soleus

muscle. Data represented as mean ± Standard error of the mean (SEM), n=4; *p<0.05; **p<0.01.

Fig. 4. Aged muscle of treated animals presented higher regenerative capacity. a-b) Quantification of graft muscular fibers as average fiber size (a) and frequency of the different fiber size (b). c-e) Quantification of the Pax7, Ki67 and double Pax7 and Ki67 positive cells found in the grafts. F-h) Quantification of the MyoD, Ki67 and double MyoD and Ki67 positive cells found in the grafts. Data represented as mean ± Standard error of the mean (SEM), n=8-11; *p<0.05; **p<0.01; ***p<0.001.

Detailed description of the invention

All terms as used herein in this application, unless otherwise stated, shall be understood in their ordinary meaning as known in the art. Other more specific definitions for certain terms as used in the present application are as set forth below and are intended to apply uniformly through-out the specification and claims unless an otherwise expressly set out definition provides a broader definition.

As used herein, the indefinite articles “a” and “an” are synonymous with “at least one” or “one or more.” Unless indicated otherwise, definite articles used herein, such as “the” also include the plural of the noun.

As above discussed, in a first aspect, the invention provides a polypeptide consisting of sequence SEQ ID NO: 1, or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, for use in the prevention and/or treatment of a muscle disease or disorder, particularly by improving muscle function.

This aspect can also be formulated as the use of a polypeptide as defined above for the manufacture of a medicament for the prevention and/or treatment of a muscle disease or disorder. The present invention also relates to a method for the treatment and/or prevention of a muscle disease or disorder, comprising administering a therapeutically effective amount of a polypeptide as defined above, together with pharmaceutically acceptable excipients or carriers, in a subject in need thereof, including a human.

In a more particular embodiment of the first aspect of the invention, the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85%, 86%, 87%, 88%, 88.5%, 89%, 89.5%, 90%, 90.5%, 91%, 91.5%, 92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99%, or 99.5% identical to SEQ ID NO: 1. In an even more particular embodiment, the polypeptide

consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 88% or 98% identical to SEQ ID NO:1.

In a more particular embodiment of the first aspect of the invention, the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85%, 86%, 87%, 88%, 88.5%, 89%, 89.5%, 90%, 90.5%, 91%, 91.5%, 92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99%, or 99.5% identical to SEQ ID NO: 1, wherein the variant thereof substantially maintains or improves the muscle therapeutic effect of SEQ ID NO: 1.

In another embodiment of the first aspect of the invention, the polypeptide consists of sequence SEQ ID NO: 1 or SEQ ID NO: 2.

Protein and polypeptide variants are well understood to those of skill in the art and can involve amino acid sequence modifications. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional, or deletional variants.

In the present invention the term "identity" refers to the percentage of residues that are identical in the two sequences when the sequences are optimally aligned. If, in the optimal alignment, a position in a first sequence is occupied by the same amino acid residue as the corresponding position in the second sequence, the sequences exhibit identity with respect to that position. The percentage of identity determines the number of identical residues over a defined length in a given alignment. Thus, the level of identity between two sequences or ("percent sequence identity") is measured as a ratio of the number of identical positions shared by the sequences with respect to the number of positions compared (i.e., percent sequence identity = (number of identical positions/total number of positions compared) x 100). A gap, i.e., a position in an alignment where a residue is present in one sequence but not in the other, is regarded as a position with non-identical residues and is counted as a compared position.

As an illustration, by a polypeptide having an amino acid sequence having at least, for example, 95% identity to a reference amino acid sequence of SEQ ID NO:1 is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of SEQ ID NO: 1. In other words, to obtain a polypeptide having an amino acid sequence of at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino

acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

A number of mathematical algorithms for rapidly obtaining the optimal alignment and calculating identity between two or more sequences are known and incorporated into a number of available software programs. For purposes of the present invention, the sequence identity between two amino acid sequences is preferably determined using algorithms based on global alignment, such as the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453), preferably implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277); or the BLAST Global Alignment tool (Altschul et al., "Basic local alignment search tool", 1990, J. Mol. Biol, v. 215, pages 403-410), using default settings. Local alignment also can be used when the sequences being compared are substantially of the same length.

The polypeptides with a percentage of identity of at least 88 % with any of SEQ ID NO: 1 or SEQ ID NO: 2 encompass s-KL of mammals other than mice and human.

SEQ ID NO: 1 is the amino acid sequence of the transcript from alternative splicing of α-klotho human gene, comprising the KL1 domain sequence, with an approximate weight of 70 kDa and a specific secretion signal consisting of 15 amino acid tail that is not found in the m-KL transcript. α-klotho human gene is the one located in Chromosome 13 NC_000013.11 (33016063..33066145) of the assembly GRCh38 (24.12.2013) for the human genome maintained by the Genome Reference Consortium. SEQ ID NO: 1 derives from the corresponding cDNA of SEQ ID NO: 3, deriving from the alternative splicing transcript of the mRNA sequence with the GenBank database accession number NM_004795 of 5012 base pairs, version 3 of 03.May.2014.

SEQ ID NO: 2 is the amino acid sequence of the transcript from alternative splicing of α-klotho mouse gene, comprising the KL1 domain sequence, with an approximate weight of 70 kDa with a specific secretion signal consisting of 15 amino acid tail that is not found in the m-KL transcript. α-klotho mouse gene is the one located in Chromosome 5 (150,952,607-150,993,809) of UCSC Genome Browser on Mouse July 2007 (NCBI37/mm9) Assembly for the mouse genome. SEQ ID NO: 2 derived from the corresponding cDNA of SEQ ID NO: 4, deriving in turn from the alternative splicing

transcript of the mRNA sequence with the GenBank database accession number NM_013823 of 5124 base pairs, version 2 of 15.February.2015.

In another embodiment of the first aspect of the invention, the polypeptide variant consists of sequence SEQ ID NO: 5 or SED ID NO: 6.

In another embodiment of the first aspect of the invention, the polypeptide has a length equal to or lower than 645 amino acids, 600 amino acids, or 550 amino acids. In an even more particular embodiment, the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1 and has a length equal to or lower than 645 amino acids, 600 amino acids, or 550 amino acids. In a particular embodiment, the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, and said variant has a length selected from the group consisting of 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 584, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, and 600 amino acids, or a length from 545 to 600 amino acids.

In one embodiment of the first aspect of the invention, the polypeptide is the secreted splicing isoform of mammalian klotho protein (s-KL). In an even more particular embodiment, the polypeptide is the human s-KL. The secreted splicing isoform of mammalian Klotho protein (s-KL) has been disclosed in the prior art (see, for example, WO2017085317A1). Thus, the invention can be in particular formulated as secreted splicing isoform of mammalian Klotho protein (s-KL), in particular human s-KL, or nucleic acid sequence coding therefor, for use in the prevention and/or treatment of a muscle disease or disorder.

The term "secreted splicing isoform of mammalian Klotho" abbreviated as "s-KL", refers to the protein resulting from the transcript from alternative splicing, which generates a truncated form of the protein (s-KL) that is formed by the KL1 domain, with an approximate weight of 70 kDa, together with a specific secretion signal consisting of 15 amino acid tail that is not found in the m-KL transcript, and for this reason is also called the secreted isoform of klotho, s-KL, or the secreted splicing isoform of klotho protein. s-KL is different from other forms of soluble klotho, namely p-KL, p-KL1 and p-KL2. In this description, m-KL stands for the full-length transmembrane form; p-KL stands for the soluble proteolyzed klotho, which is generated by cleavage of the m-KL; and p-KL1 and p-KL2 stand for the soluble klotho forms consisting on the KL1 domain and the KL2 domain of p-KL, respectively. m-KL comes from the full-length transcript encoding a single pass

transmembrane protein with a molecular weight of approximately 130 kDa (m-KL). The protein contains three domains: a short transmembrane domain at the C-terminal, an extracellular domain composed of two internal repeated sequences of about 550 amino acids called KL1 and KL2 respectively, and a very short intracellular domain of 10 amino acids. The extracellular domain of the transmembrane form can be cleaved by metalloproteinases ADAM10 and ADAM17 resulting in another form of soluble Klotho of about 130 kDa (abbreviated p-KL for proteolyzed membrane isoform. Moreover, there is a second recognition site for the proteases ADAM10 and 17 located between the KL1 and KL2 domains, which generates two new 70 kDa isoforms, one contained the KL1 domain only (like the one generated from alternative splicing but without the specific amino acid tail), and the other one contained the KL2 domain. However, it has not been demonstrated *in vivo* that p-KL is proteolyzed into p-KL1 and p-KL2.

In another embodiment of the invention, the polypeptide is for use in the prevention and/or treatment of a muscle disease or disorder by improving muscle function; by increasing muscle mass; by increasing the number of muscle fibers; by decreasing muscle fibrosis; by increasing muscle regenerative capacity; and/or by improving physical state or performance.

In another embodiment of the invention, the polypeptide is for use in the prevention and/or treatment of a muscle disease or disorder by directly improving muscle function.

In another embodiment of the invention, the prevention and/or treatment of a muscle disease or disorder comprises improving muscle function; increasing muscle mass; increasing the number of muscle fibers; decreasing muscle fibrosis; increasing muscle regenerative capacity; and/or improving physical state or performance. In a more particular embodiment, the prevention and/or treatment of a muscle disease or disorder comprises improving muscle function; increasing muscle mass; increasing the number of muscle fibers; decreasing muscle fibrosis; increasing muscle regenerative capacity; and/or improving physical state or performance, by a direct effect on the muscle (i.e., muscle cells).

As used herein, "muscle disease or disorder" refers to a disease, disorder, or condition in muscle-containing animals characterized by the deterioration or weakening or reduction of skeletal and/or smooth muscle such that normal muscular function is reduced. Typically, this leads to a worsened physical state or performance of the animal. Muscular function may be measured as described in the examples below, for example, by rotarod, horizontal or grip strength tests.

In one embodiment of the first aspect of the invention, the muscle disease or disorder is a skeletal muscle disease or disorder.

In one embodiment of the invention, the muscle disease or disorder is muscle degeneration and/or muscle loss. In a more particular embodiment, wherein the muscle disease or disorder is not associated with a cognitive and/or behaviour impairment, and/or with neurodegenerative and/or neuropathological diseases. In an even more particular embodiment, the muscle degeneration and/or muscle loss is not associated with a cognitive and/or behaviour impairment, and/or with neurodegenerative and/or neuropathological diseases.
As used herein, "muscle degeneration" refers to any condition where the structural integrity of the muscle, particularly skeletal muscle, is altered. As used herein, the term "muscle loss" refers to any condition where the muscle mass, particularly the skeletal muscle mass, is reduced.

In one embodiment of the first aspect of the invention, the muscle disease or disorder is age-related muscle degeneration and/or muscle loss.

In one embodiment of the first aspect of the invention, the muscle disease or disorder is selected from the group consisting of sarcopenia, muscle dystrophy, muscle atrophy, muscle wasting syndrome, cachexia, and combinations thereof. The term "sarcopenia" refers to an age-related loss of skeletal muscle mass and function.

In another embodiment of the first aspect of the invention, optionally in combination with any one of the embodiments provided below, the muscle disease or disorder is caused by achondroplasia, cleidocranial dysostosis, enchondromatosis, fibrous dysplasia, Gaucher's Disease, hypophosphatemic rickets, Marfan's syndrome, multiple hereditary exotoses, neurofibromatosis, osteogenesis imperfecta, osteopoikilosis, sclerotic lesions, pseudoarthrosis, pyogenic osteomyelitis, periodontal disease, anti-epileptic drug induced muscle loss, primary and secondary hyperparathyroidism, familial hyperparathyroidism syndromes, weightlessness induced muscle loss, osteoporosis in men, postmenopausal muscle loss, osteoarthritis, renal osteodystrophy, infiltrative disorders of muscle, oral muscle loss, osteonecrosis of the jaw, juvenile Paget's disease, melorheostosis, metabolic muscle diseases, mastocytosis, sickle cell anemia/disease, organ transplant related muscle loss, kidney transplant related muscle loss, systemic lupus erythematosus, ankylosing spondylitis, epilepsy, juvenile arthritides, thalassemia, mucopolysaccharidoses, fabry disease, turner syndrome, Down Syndrome, Klinefelter Syndrome, leprosy, Perthes' Disease, adolescent idiopathic scoliosis, infantile onset multi-system inflammatory disease, Winchester Syndrome, Menkes Disease, Wilson's Disease, ischemic muscle

disease (such as Legg-Calve-Perthes disease, regional migratory osteoporosis), anemic states, conditions caused by steroids, glucocorticoid-induced muscle loss, heparin-induced muscle loss, muscle marrow disorders, scurvy, malnutrition, calcium deficiency, idiopathic osteopenia or osteoporosis, congenital osteopenia or osteoporosis, alcoholism, chronic liver disease, postmenopausal state, chronic inflammatory conditions, rheumatoid arthritis, inflammatory bowel disease, ulcerative colitis, inflammatory colitis, Crohn's disease, oligomenorrhea, amenorrhea, pregnancy, diabetes mellitus, hyperthyroidism, thyroid disorders, parathyroid disorders, Cushing's disease, acromegaly, hypogonadism, immobilization or disuse, reflex sympathetic dystrophy syndrome, regional osteoporosis, osteomalacia, muscle loss associated with joint replacement, HIV-associated muscle loss, muscle loss associated with loss of growth hormone, muscle loss associated with cystic fibrosis, fibrous dysplasia, chemotherapy-associated muscle loss, tumor induced muscle loss, cancer-related muscle loss, hormone-ablative muscle loss, multiple myeloma, drug-induced muscle loss, anorexia nervosa, disease associated facial muscle loss, disease associated cranial muscle loss, disease associated muscle loss of the jaw, disease associated muscle loss of the skull, or muscle loss associated with space travel.

In a particular embodiment of the first aspect, optionally in combination with any of the embodiments provided above and below, the polypeptide is linked to a heterologous moiety.

As used herein, “heterologous moiety” refers to any molecule coupled to the polypeptide via either a covalent or non-covalent bond. In a particular embodiment, the heterologous moiety is located in either the N-terminal or the C-terminal end of the polypeptide. In a particular embodiment, the heterologous moiety is located in both the N-terminal and the C-terminal ends of the polypeptide.

The heterologous moiety can be, for example, a molecule that facilitates the purification of the polypeptide. In a particular embodiment, the heterologous moiety is a peptide. In an even more particular embodiment, the heterologous moiety is a poly histidine track. As the skill in the art would understand, small peptides that assist in the purification of the protein can be maintained in the final compound without affecting its functionality.

The heterologous moiety can also be any vehiculization agent to facilitate the absorption, transport and delivery of the polypeptide.

These polypeptides resulting from KL protein, such as in particular s-KL, may be used directly in the form of the protein, or they can be expressed inside target cells of the tissue of interest by means of gene therapy. To this aim the invention also provides, in a second

aspect, a nucleic acid sequence that encodes the polypeptide or the variant thereof as defined in the first aspect, which is for use in the prevention and/or treatment of a muscle disease or disorder.

The term "a nucleic acid sequence that encodes the polypeptide" is to be understood, in particular, as the mRNA coding for said polypeptide or the cDNA sequence resulting from the reverse transcription (RT-PCR) of the mRNA coding for said polypeptide.

This aspect can also be formulated as the use of the nucleic acid sequence as defined above for the manufacture of a medicament for the prevention and/or treatment of a muscle disease or disorder. The present invention also relates to a method for the treatment and/or prevention of a muscle disease or disorder, comprising administering a therapeutically effective amount of the nucleic acid sequence as defined above, together with pharmaceutically acceptable excipients or carriers, in a subject in need thereof, including a human.

In a particular embodiment of the second aspect, the nucleic acid sequence comprises SEQ ID NO: 3 or SEQ ID NO: 4. In an even more particular embodiment, the nucleic acid sequence consists of SEQ ID NO: 3 or SEQ ID NO: 4.

In a more particular embodiment of the second aspect of the invention, the nucleic acid sequence consists of sequence SEQ ID NO: 3 or SEQ ID NO: 4 or a variant thereof consisting of a sequence at least 85%, 86%, 87%, 88%, 88.5%, 89%, 89.5%, 90%, 90.5%, 91%, 91.5%, 92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99%, or 99.5% identical to SEQ ID NO: 3 or SEQ ID NO: 4.

In a more particular embodiment of the second aspect of the invention, the nucleic acid sequence consists of sequence SEQ ID NO: 3 or SEQ ID NO: 4 or a variant thereof consisting of a sequence at least 85%, 86%, 87%, 88%, 88.5%, 89%, 89.5%, 90%, 90.5%, 91%, 91.5%, 92%, 92.5%, 93%, 93.5%, 94%, 94.5%, 95%, 95.5%, 96%, 96.5%, 97%, 97.5%, 98%, 98.5%, 99%, or 99.5% identical to SEQ ID NO: 3 or SEQ ID NO: 4, wherein the variant thereof substantially maintains or improves the muscle therapeutic effect of SEQ ID NO: 3 or SEQ ID NO: 4.

In a third aspect, the invention provides a gene construct comprising a nucleic acid sequence as defined in the second aspect operatively linked to an expression promoter, which is for use in the prevention and/or treatment of a muscle disease or disorder.

This aspect can also be formulated as the use of a gene construct as defined above for

the manufacture of a medicament for the prevention and/or treatment of a muscle disease or disorder. The present invention also relates to a method for the treatment and/or prevention of a muscle disease or disorder, comprising administering a therapeutically effective amount of a gene construct as defined above, together with pharmaceutically acceptable excipients or carriers, in a subject in need thereof, including a human.

In a particular embodiment of the third aspect, the expression promoter operatively linked is selected from the group consisting of a constitutive expression promoter, an inducible promoter, a muscle-specific expression promoter, and a neuron-specific expression promoter. In a more particular embodiment, the gene construct according to the invention comprises the cytomegalovirus intermediate-early (CMV IE) promoter, the sequence coding for s-KL (cDNA of mouse or human s-KL) and a polyadenylation chain (poly A). In another particular embodiment, the gene construct according to the invention comprises the CAG promoter, the sequence coding for s-KL (cDNA of mouse or human s-KL) and a polyadenylation chain (poly A).

In a particular embodiment of the third aspect, optionally in combination with any of the embodiments provided above and below, the gene construct comprises or consists of SEQ ID NO: 7 or SEQ ID NO: 8.

All these gene constructs are able to express the protein of interest once in the cell.
In order to facilitate administration of the constructs, the invention also provides, in a fourth aspect, an expression vector comprising the gene construct as defined in the third aspect and thus comprising the nucleic acid sequence of the second aspect coding for the polypeptide of the first aspect operatively linked to an expression promoter, and particularly to a constitutive expression promoter, for use in the prevention and/or treatment of a muscle disease or disorder.

This aspect can also be formulated as the use of the expression vector as defined above for the manufacture of a medicament for the prevention and/or treatment of a muscle disease or disorder. The present invention also relates to a method for the treatment and/or prevention of a muscle disease or disorder, comprising administering a therapeutically effective amount of the expression vector as defined above, together with pharmaceutically acceptable excipients or carriers, in a subject in need thereof, including a human.

In a particular embodiment of the fourth aspect, optionally in combination with any of the embodiments provided above and below, the expression vector is a viral vector.

In a particular embodiment of the third aspect, optionally in combination with any of the embodiments provided above and below, the expression vector consists of sequence SEQ ID NO: 9.

In a particular embodiment of the fourth aspect, optionally in combination with any of the embodiments provided above and below, the viral vector is an adeno-associated virus. In a particular embodiment, it an adeno-associated virus of serotype selected from the group consisting of AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh10, PHPeB, and 9P31. In a more particular embodiment, it an adeno-associated virus of serotype AAV9.

In another embodiment of the first, second, third and fourth aspects, optionally in combination with any of the embodiments provided above and below, the polypeptide for use according to the first aspect, the nucleic acid sequence for use according to the second aspect, the gene construct for use according to the third aspect, or the expression vector for use according to the fourth aspect, is administered in the form of a pharmaceutical composition together with at least one pharmaceutically acceptable excipient, diluent or carrier.

The expression “pharmaceutical composition” encompasses both compositions intended for human as well as for non-human animals. The skilled in the art understands that a pharmaceutical composition must comprise a therapeutically effective amount of the compound. The expression "therapeutically effective amount" as used herein, refers to the amount of polypeptide, nucleic acid sequence, gene construct, or expression vector that, when administered, is sufficient to prevent development of, or alleviate to some extent, one or more of the symptoms of the disease which is addressed. The particular dose of compound administered according to this invention will of course be determined by the particular circumstances surrounding the case, including the compound administered, the route of administration, the particular condition being treated, and the similar considerations.

The expression "pharmaceutically acceptable excipient, diluent or carrier" refers to pharmaceutically acceptable materials, compositions or vehicles. Each component must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the pharmaceutical composition. It must also be suitable for use in contact with the tissue or organ of humans and non-human animals without excessive toxicity, irritation, allergic response, immunogenicity or other problems or complications commensurate with a reasonable benefit/risk ratio.

Examples of suitable pharmaceutically acceptable excipients are solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

The relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition of the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered.

Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Excipients such as coloring agents, coating agents, sweetening, and flavoring agents can be present in the composition, according to the judgment of the formulator.

The pharmaceutical compositions containing the protein or nucleic acid of the invention can be presented in any dosage form, for example, solid or liquid, and can be administered by any suitable route, for example, oral, parenteral, rectal, topical, intranasal, intraocular, intraperitoneal or sublingual route, for which they will include the pharmaceutically acceptable excipients necessary for the formulation of the desired dosage form, for example, topical formulations (ointment, creams, lipogel, hydrogel, etc.), eye drops, aerosol sprays, injectable hydrogels, injectable solutions, osmotic pumps, etc.

Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, corn-starch, powdered sugar, and combinations thereof.

Exemplary granulating and/or dispersing agents include, but are not limited to, potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked

polyvinylpyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (Veegum), sodium lauryl sulfate, quaternary ammonium compounds, and combinations thereof.

Exemplary binding agents include, but are not limited to, starch (e.g., corn-starch and starch paste); gelatin; sugars (e.g., sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol); natural and synthetic gums (e.g., acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, polyvinylpyrrolidone), magnesium aluminium silicate (Veegum), and larch arabogalactan); alginates; polyethylene oxide; polyethylene glycol; inorganic calcium salts; silicic acid; polymethacrylates; waxes; water; alcohol; and combinations thereof.

Exemplary preservatives may include antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and other preservatives. Exemplary antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, ascorbyl palmitate, ascorbyl stearate, ascorbyl oleate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and sodium sulfite. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and trisodium edetate.

Exemplary buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's

solution, ethyl alcohol, and combinations thereof.

Exemplary lubricating agents include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, and combinations thereof.

In another embodiment of the first, second, third and fourth aspects, optionally in combination with any of the embodiments provided above and below, the pharmaceutical composition is for being administered to the patient via mucosa (e.g., nasal, sublingual, vaginal, buccal, or rectal), parenterally (e.g., subcutaneous, intravenous, intramuscular, or intraarterial injection, either bolus or infusion), orally, transdermally or via inhalation by means e.g. of an aerosol. In a particular embodiment, the pharmaceutical composition is for being administered systemically. Formulations suitable for parenteral administration, such as, for example, by intraarticular, intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Injection solutions and suspensions can also be prepared from sterile powders, granules, and tablets. In some embodiments, the composition is administered by injection e.g subcutaneous, intraperitoneal, intravesically, intravenous, intracerebroventricular, by infusion, e.g., using a reservoir or osmotic minipump or intramuscular. The formulation can be provided in unit-dose or multi-dose sealed containers, such as ampoules and vials. In an even more particular embodiment, the pharmaceutical composition is for intraventricular administration or for intravenous administration; even more particularly systemic intravenous administration.

In another embodiment of the first, second, third and fourth aspects, optionally in combination with any of the embodiments provided above and below, the polypeptide for use according to the first aspect, the nucleic acid sequence for use according to the second aspect, the gene construct for use according to the third aspect, or the expression vector for use according to the fourth aspect, is administered in combination with another active agent. Suitable active agents to be administered in combination with a compound of the invention are, without limitation, vitamin D, ACE inhibitors, myostatin inhibitors, growth hormone, testosterone, metformin, or creatin.

All embodiments of the first aspect are also meant to apply to the second to sixth aspects of the invention.

The positive effects that s-KL exerts on muscle can also be applied to healthy subjects in order to improve muscle function or general physical state, for example to athletes.

Thus, as above indicated, the invention provides in a seventh, eighth and nineth aspects non-therapeutic methods based in s-KL administration for improving muscle function and/or increasing muscle mass, for increasing the muscle regenerative capacity, and method for improving the physical state or performance of a subject. The embodiments of the first and second aspects, particularly those related to the sequence of the polypeptide or variant thereof or the nucleic acid sequence, are also meant to apply to the seventh, eighth, and nineth aspects of the invention.

As used herein, the term "subject" is meant to encompass human and non-human animals.

In a particular embodiment of the seventh aspect of the invention, the improving muscle function and/or increasing muscle mass comprises increasing the number and/or size of muscular fibers.

In an embodiment of the seventh, eighth, and nineth aspects of the invention, the subject is a healthy subject.

In another embodiment of the seventh, eighth, and nineth aspects of the invention, the nucleic acid sequence is comprised in a gene construct operatively linked to an expression promoter. In a more particular embodiment, the gene construct is comprised in an expression vector. The embodiments of the second, third, and fourth aspects related to the nucleic acid sequence, expression construct, and vector, are also meant to apply to the seventh, eighth, and nineth aspects of the invention.

In another embodiment of the aspects above, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered for a particular time period or for a chronic treatment period, which is, for an extended period of time, including throughout the duration of the subject's life. Within the treatment period, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered on a particular time schedule. In further embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered one, two, three, or four times daily. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or

expression vector is administered once daily. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered twice daily. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered in the morning and evening. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered one, two, three, or four times weekly. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered once a week. In some embodiments the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered one, two, three, or four times monthly. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered once a month. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered for at least three months of every one year. In some embodiments, the polypeptide, nucleic acid sequence, gene construct, or expression vector is administered one month of every six months.

Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Furthermore, the word “comprise” encompasses the case of “consisting of”. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration, and they are not intended to be limiting of the present invention. Reference signs related to drawings and placed in parentheses in a claim, are solely for attempting to increase the intelligibility of the claim, and shall not be construed as limiting the scope of the claim. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein.

For reasons of completeness, various aspects of the invention are set out in the following numbered clauses:

1. Polypeptide consisting of sequence SEQ ID NO: 1, or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, for use in the prevention and/or treatment of a muscle disease or disorder.

2. The polypeptide for use according to clause 1, wherein muscle disease or disorder is muscle degeneration and/or muscle loss.

3. The polypeptide for use according to any of clauses 1-2, wherein the muscle disease or disorder is age-related muscle degeneration and/or muscle loss.

4. The polypeptide for use according to any of clauses 1-3, wherein the muscle disease or disorder is selected from the group consisting of sarcopenia, muscle dystrophy, muscle atrophy, muscle wasting syndrome, cachexia, and combinations thereof.

5. The polypeptide for use according to any of clauses 1-4, wherein the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 88 % identical to SEQ ID NO: 1.

6. The polypeptide for use according to any of clauses 1-5, wherein the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 98 % identical to SEQ ID NO: 1.

7. The polypeptide for use according to any of clauses 1-5, wherein the polypeptide consists of SEQ ID NO: 1 or SEQ ID NO: 2.

8. Nucleic acid sequence that encodes the polypeptide or the variant thereof as defined in any of clauses 1-7, which is for use in the prevention and/or treatment of a muscle disease or disorder.

9. Gene construct comprising a nucleic acid sequence as defined in clause 8, operatively linked to an expression promoter, which is for use in the prevention and/or treatment of a muscle disease or disorder.

10. Expression vector comprising the gene construct as defined in clause 9, which is for use in the prevention and/or treatment of a muscle disease or disorder.

11. The expression vector for use according to clause 10, which is a viral vector.

12. The expression vector for use according to clause 11, which is an adeno-associated virus of serotype selected from the group consisting of AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh10, PHPeB, and 9P31.

13. The polypeptide for use according to any of clauses 1-7, the nucleic acid sequence for use according to clause 8, the gene construct for use according to clause 9, or the expression vector for use according to any of clauses 10-12, which is administered in the form of a pharmaceutical composition together with at least one pharmaceutically acceptable excipient, diluent or carrier.

14. The polypeptide for use according to any of clauses 1-7 and 13, the nucleic acid sequence for use according to clause 8 and 13, the gene construct for use according to clause 9 and 13, or the expression vector for use according to any of clauses 10-13, which is administered in combination with another active agent.

15. A non-therapeutic method for improving muscle function and/or increasing muscle mass of a subject, the method comprising administering to a subject a polypeptide consisting of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, or a nucleic acid sequence that encodes the polypeptide or the variant thereof.

Examples

Materials and methods

Animal housing

C57BL/6J male (n=48) and female mice (n=48) were purchased from Charles River. These animals were randomly divided into 3 groups per sex. Two of those were treated when they were 7 months old with a Null control vector (SEQ ID NO: 10) (n=16) or a s-KL expressing vector (SEQ ID NO: 9) (n=16). The third group was treated when animals were 12 months old with the s-KL expressing vector (SEQ ID NO: 9) (n=16). The main part of these animals was followed during the life span to study health and viability and a second subset of 4 animals per group were randomly selected and euthanatized when they were 24 months old, to study viral vector function.

Mice had free access to food and water and were kept under standard temperature conditions (22±2°C) and a 12-h light/dark cycle (300 lux/0 lux). Mice were periodically checked to address general health status until natural death or euthanasia. Euthanasia protocol was cervical dislocation, applied when a blinded veterinary at the animal house facility considered an animal had reached the end point criteria.

Treatment generation and administration

Gene therapy treatment consisted in expression cassettes under the control of the CAG promoter containing a control-null sequence or the secreted isoform of mouse α-KL gene (SEQ ID NO: 2).

Two adeno-associated viral vectors serotype 9 (AAV9), containing independently those constructs, were generated following the triple transfection method as disclosed in Piedra J. X et al., 2015. Animals were administered simultaneous by intracerebroventricular and intravenous injection, to transduce most mice tissues as possible. Intracerebroventricular stereotaxic injections of AAV vectors were performed as previously described (Massó A. et al., *supra)*. Briefly, the treatment was administered into the right hemisphere at coordinates, −0.2 mm antero-posterior, −2 mm dorso-ventral, and +1 mm medio-lateral from bregma. The vector dose was $1x10^{11}$ viral genomes per animal in 6 µl, administered at a 0.5 µl/min speed using an ultramicropump (WorldPrecision Instruments). The intravenous injection consisted in a dose of $4x10^{11}$ viral genomes per animal diluted with NaCl 0.9% to a final volume of 200 µL and injected manually with a syringe into the lateral tail vein of the mice.

Serum biochemical analysis

Blood samples were obtained by decapitation of deeply anesthetized animals, with a SST serum collection tube (BD microtainer). Blood was left at room temperature for 5 minutes and then placed on ice. Blood serum was isolated by 15 minutes tube centrifugation at 3000 rpm for 10 minutes, and finally aliquoted and kept frozen at -80 °C. KL serum levels were measured using an ELISA kit specific for mouse KL (IBL) following manufacturers indications.

RotaRod

Previous to the Rotarod experiment, three consecutive days of training was done to the animals. Training consisted in two trials of the accelerated Rotarod test to each animal to reduce stress and improve coordination for the new task, and to be able to see differences due to physical state. The day of the test animals were carefully placed in the previously cleaned Rotarod apparatus and the time to fall was quantified. The test was done twice to each animal and the represented results are the averages of both trials per animal.

Horizontal bar

A circular wooden bar with 1 cm of diameter was horizontally placed 40 cm above a soft floor made of expanded polystyrene. Mice were carefully suspended on the bar by their upper limbs, a maximum test length of 40 s. Time spent on the bar (resistance) and distance walked along the bar (coordination) were recorded. Two trials per animal were done, and the best performance was selected.

Grip strength

Animals were let to grip from the front legs to the grip strength platform and were pulled backwards from the tail slowly so the animal could present resistance, until reaching the end of the platform. This procedure was repeated three times and data is represented as each group's average strength for each trial in order to see the exhaustion effect in the animals.

Muscle histology and immunohistochemistry

Mice were euthanized and the indicated muscles were dissected, embedded in OCT solution (TissueTek), frozen in isopentane cooled with liquid nitrogen and stored at −80 °C until analysis or fixed in a 2% PFA solution in PBS. 10 µm muscle cryosections were collected and stained for hematoxylin/eosin (H/E) or Sirius red (Sigma-Aldrich). For immunohistochemistry, the following primary antibodies were used: anti-MyoD (Dako; M3512), anti-Pax7 (DSHB), anti-Ki67 (Abcam; ab15580). Digital images were acquired using the Leica DMR600B microscope equipped with a DFC300FX camera. Fiber type distribution, CSA, and percentage of muscle area positive for Sirius red staining were quantified using Image J software following manufacturer's instructions.

Extensor digitorum longus (EDL) muscle transplantation

Heterografting experiments were performed by removing the extensor digitorum longus muscle from its anatomical bed and transplanted it onto the surface of the tibialis anterior muscle of WT recipient mouse. Muscle grafts were collected for analysis on day 7 after transplantation.

Gene expression

Total RNA isolation was carried out using TRIsure™ reagent following the manufacturer's instructions (Bioline Reagent). The tissue used for the RNA extraction was liver. Samples were homogenized using TissueLyser LT sample disruption apparatus (QIAGEN). RNA quantity and purity were measured with NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). RNA retrotranscription was done using iScript™ Advanced cDNA Synthesis Kit (Bio-rad). Gene expression was analyzed by Real-Time quantitative PCR (RT-qPCR) on a Bio-Rad CFX-384 PCR machine at the Analysis and Photodocumentation Service of the Universitat Autonòma de Barcelona following manufacturer's instructions. Each reaction contained 25 ng of cDNA, 7.5 µL of iTaqTM Universal SYBR Green Supermix (Bio-Rad)

and a primer concentration of 0.2 nM, with a final reaction volume of 15 μL. Primers used are listed in Table 1.

Table 1

Target	Product size (bp)	Forward primer (5'-3')	Reverse primer (5'-3')
s-KL	315	TCATAATGGAAACCTTAAAAGCAA (SEQ ID NO: 11)	CACTGGGTTTTGTCAAAGGA (SEQ ID NO: 12)
β-actin	190	CAACGAGCGGTTCCGAT (SEQ ID NO: 13)	GCCACAGGTTCCATACCCA (SEQ ID NO: 14)

Statistical analysis

Statistical analysis and graphic representation were done with GraphPad Prism ver.8 (GraphPad Software). Statistical differences between groups were analyzed with a two-tailed unpaired Student's t-test when comparing two groups, one-way analysis of variance (ANOVA), followed by Tukey as a post-hoc analysis for comparing all treatment groups. Data were expressed as mean ± standard error of the mean (SEM). Statistical difference was accepted when p values were ≤0.05.

Results

s-KL treatment efficiently increased s-KL protein concentration

Viral vector administration was done by consecutive intravenous and intra cerebroventricular injection (fig. 1a). Out of the 96 animals treated, one died just after the intervention. At 24 months of age, a randomly selected subset of 4 animals of each group were euthanatized in order to assess viral vector function. Gene expression of s-KL was studied in liver because this organ is transfected after AAV9 serotype injection and is the main secreting organ in adult animals (fig. 1b). Expression of the s-KL cDNA was significantly increased in all KL-treated groups, being higher in males than in females, and in the 12MO (12 months old) group compared to the 6MO (6 months old) treated animals. Additionally, efficient protein production and secretion to bloodstream was confirmed by ELISA (fig. 1c). Significant higher KL protein levels in serum were detected in mice administered both at 6 and 12 months of age, compared to animals treated with the AAV9 containing the Null. Again, the concentration was much higher in males than females. In the case of the males, it was also significantly increased in 12MO males compared to the 6MO group, presenting more than double of the s-KL concentration.

s-KL treatment improved performance of aged animals in physical tests

Different behavioral tests were done to assess the physical state of the aged animals. Accelerating rotarod test gives information of the coordination and resistance state of the animals. As can be seen in fig. 2a, s-KL treated females improved performance in this test in both administration points. Moreover, both males and females improved horizontal bar performance, reaching statistical significance for the s-KL treated animals at 12 months of age (fig. 2b, c). This parameter also indicates increased resistance compared to Null-treated animals. Finally, grip strength was measured and an improvement in this parameter was observed for s-KL treated males (fig. 2d).

s-KL treatment reduced age-associated muscular fibrosis in male mice

Two muscles of the animals were analyzed to check the state of the aged muscular tissue. The parameters analyzed were the number of muscular fibers, measured with the hematoxylin and eosin staining, and the muscular fibrosis levels, quantifying the percentage of connective tissue in a Sirius red staining. As can be seen in fig. 3a, there was a tendency to increase the number of fibers in soleus in aged male mice treated with s-KL 6MO. Importantly, there was a significant and consistent reduction in the percentage of fibrotic tissue in muscles of male mice treated with s-KL at the age of 12MO (fig 3b).

Aged muscle of treated animals present higher regenerative capacity

The regenerative capacity of the aged muscles was assessed by extensor digitorum lengus (EDL) muscle transplantation into young mice receivers as explained above. Fibers of the muscular graft were quantified and an increase in the amount and size of these was observed. Animals treated with s-KL at 6MO highly increased the number of fibers and animals treated with s-KL at 12MO increased both amount and percentage of thicker fibers (fig. 4a-b). In order to assess the regenerative capacity, different cellular proliferation markers were analyzed. Paired Box 7 (Pax7) marker was used to quantify the amount of satellite cells. As can be seen in fig. 4c-e, the amount of these cells increased in grafts from treated animals. These satellite cells presented increased proliferative capacity as the number of cells also expressing the cellular proliferation marker Ki67. Myoblast determination protein 1, marker of myogenic commitment of muscular satellite cells, increased in the grafts of s-KL treated animals, reaching statistical differences when colocalization with stem cells markers (fig. 4f-h).

All these results suggest that s-KL administration may provide a useful therapeutic approach for the prevention and/or treatment of muscle disorders, in particular age-related

muscle disorders.

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Claims

1. Polypeptide consisting of sequence SEQ ID NO: 1, or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, for use in the prevention and/or treatment of a muscle disease or disorder by improving muscle function.

2. The polypeptide for use according to claim 1, wherein muscle disease or disorder is muscle degeneration and/or muscle loss.

3. The polypeptide for use according to any of claims 1-2, wherein the muscle disease or disorder is age-related muscle degeneration and/or muscle loss.

4. The polypeptide for use according to any of claims 1-3, wherein the muscle disease or disorder is selected from the group consisting of sarcopenia, muscle dystrophy, muscle atrophy, muscle wasting syndrome, cachexia, and combinations thereof.

5. The polypeptide for use according to any one of claims 1-4, wherein the muscle disease or disorder is not associated with a cognitive and/or behaviour impairment, and/or with neurodegenerative and/or neuropathological diseases.

6. The polypeptide for use according to any of claims 1-5, wherein the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 88 % identical to SEQ ID NO: 1.

7. The polypeptide for use according to any of claims 1-6, wherein the polypeptide consists of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 98 % identical to SEQ ID NO: 1.

8. The polypeptide for use according to any of claims 1-7, wherein the polypeptide consists of SEQ ID NO: 1 or SEQ ID NO: 2.

9. Nucleic acid sequence that encodes the polypeptide or the variant thereof as defined in any of claims 1-8, wherein the nucleic acid is for use in the prevention and/or treatment of a muscle disease or disorder by improving muscle function.

10. Gene construct comprising a nucleic acid sequence as defined in claim 9, operatively linked to an expression promoter, wherein the gene construct is for use in the prevention and/or treatment of a muscle disease or disorder by improving muscle function.

11. Expression vector comprising the gene construct as defined in claim 10, wherein the expression vector is for use in the prevention and/or treatment of a muscle disease or disorder by improving muscle function.

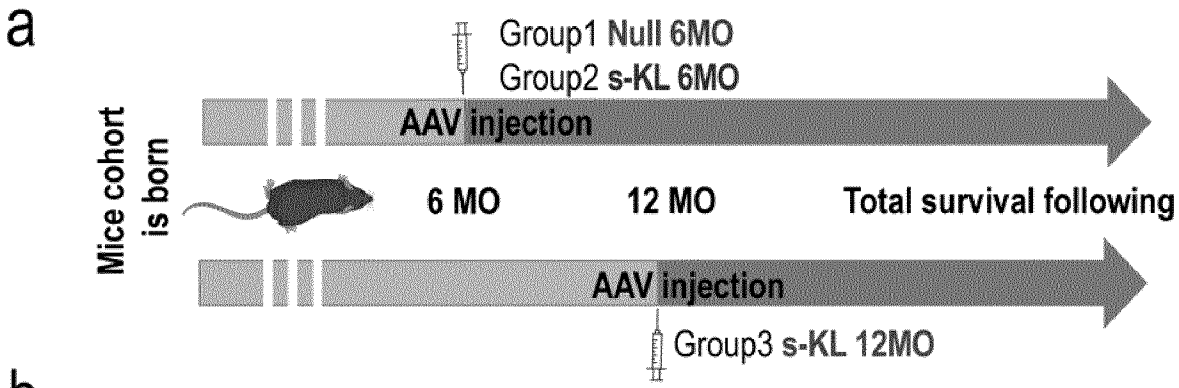
12. The expression vector for use according to claim 11, which is a viral vector.

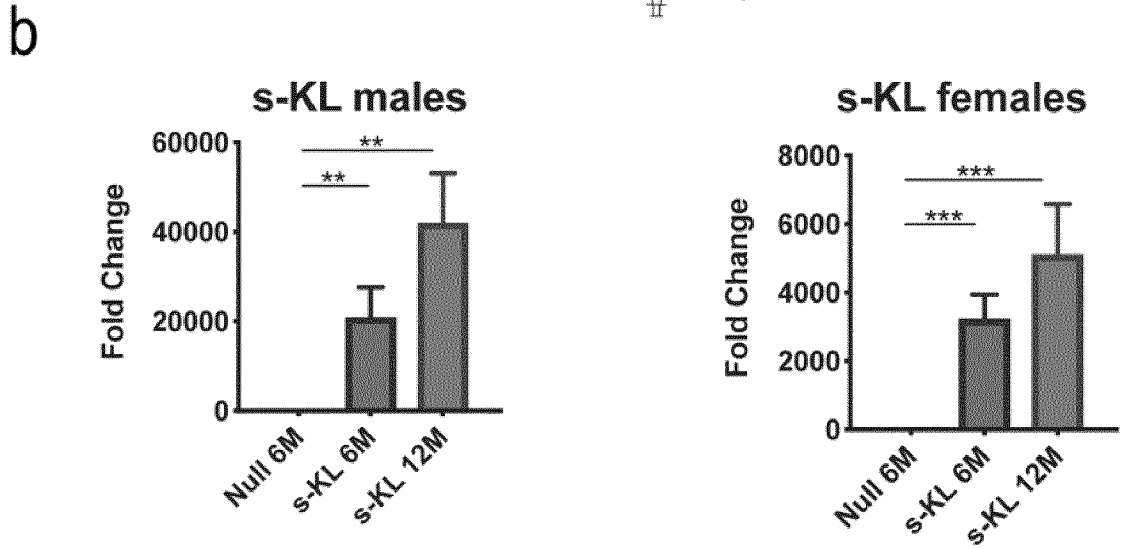
13. The expression vector for use according to claim 12, which is an adeno-associated virus of serotype selected from the group consisting of AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAVrh10, PHPeB, and 9P31.

14. The polypeptide for use according to any of claims 1-8, the nucleic acid sequence for use according to claim 9, the gene construct for use according to claim 10, or the expression vector for use according to any of claims 11-13, which is administered in the form of a pharmaceutical composition together with at least one pharmaceutically acceptable excipient, diluent or carrier.

15. The polypeptide for use according to any of claims 1-8 and 14, the nucleic acid sequence for use according to claim 9 and 14, the gene construct for use according to claim 10 and 14, or the expression vector for use according to any of claims 11-14, which is administered in combination with another active agent.

16. A non-therapeutic method for improving muscle function and/or increasing muscle mass of a subject, the method comprising administering to a subject a polypeptide consisting of sequence SEQ ID NO: 1 or a variant thereof consisting of a sequence at least 85% identical to SEQ ID NO: 1, or a nucleic acid sequence that encodes the polypeptide or the variant thereof.


a
Group1 Null 6MO
Group2 s-KL 6MO
AAV injection
Mice cohort is born
6 MO
12 MO
Total survival following
AAV injection
Group3 s-KL 12MO


b
s-KL males
Fold Change
60000
40000
20000
0
**
**
Null 6M
s-KL 6M
s-KL 12M
s-KL females
Fold Change
8000
6000
4000
2000
0
***
***
Null 6M
s-KL 6M
s-KL 12M

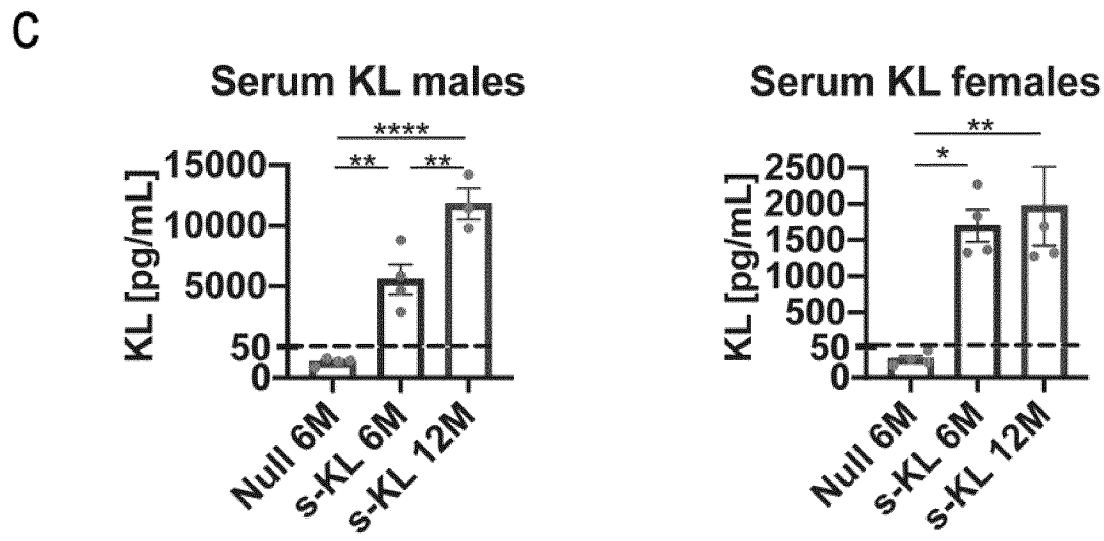

c
Serum KL males
KL [pg/mL]
15000
10000
5000
50
0
****
**
**
Null 6M
s-KL 6M
s-KL 12M
Serum KL females
KL [pg/mL]
2500
2000
1500
1000
500
50
0
**
*
Null 6M
s-KL 6M
s-KL 12M

Fig. 1

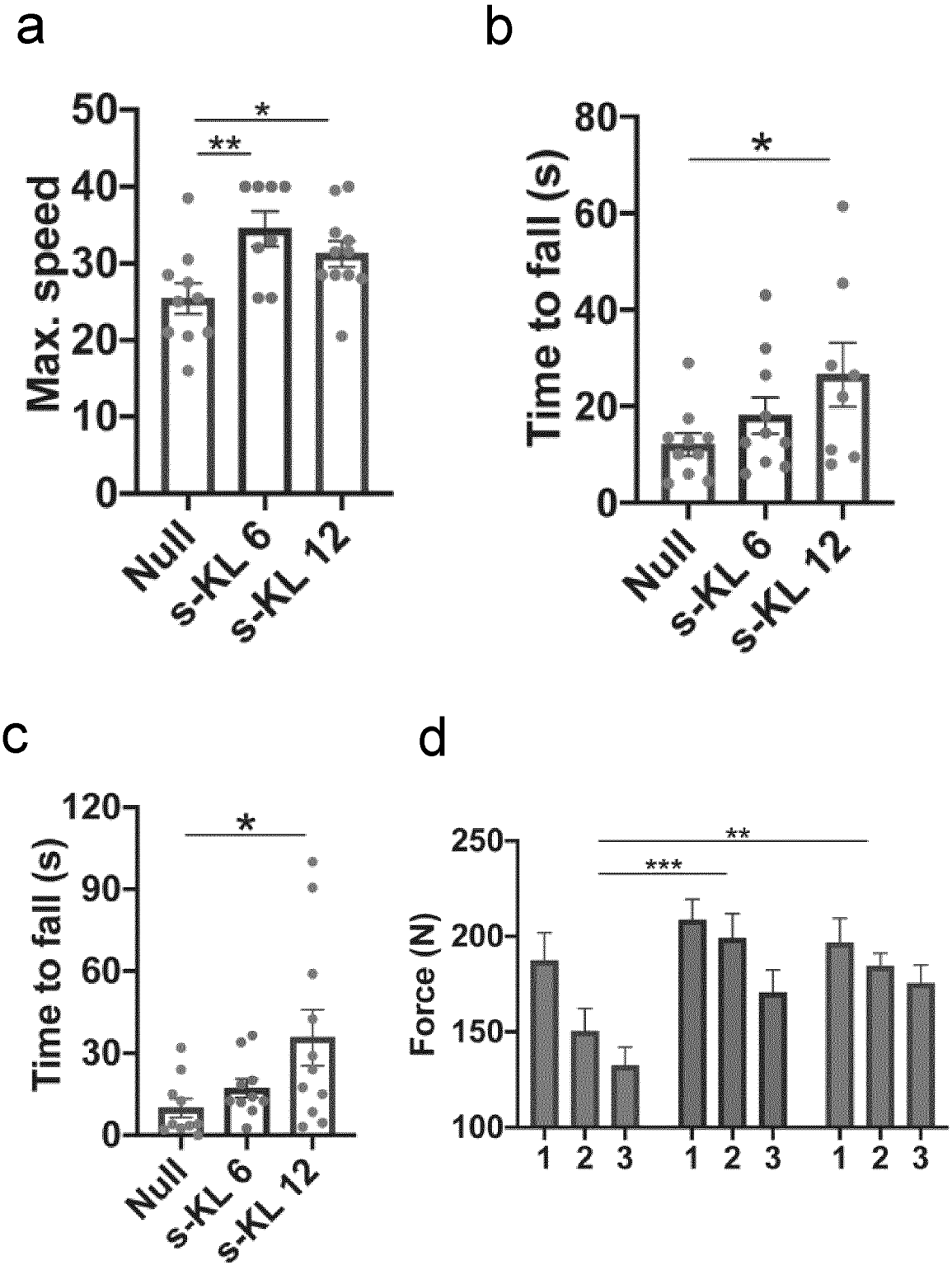
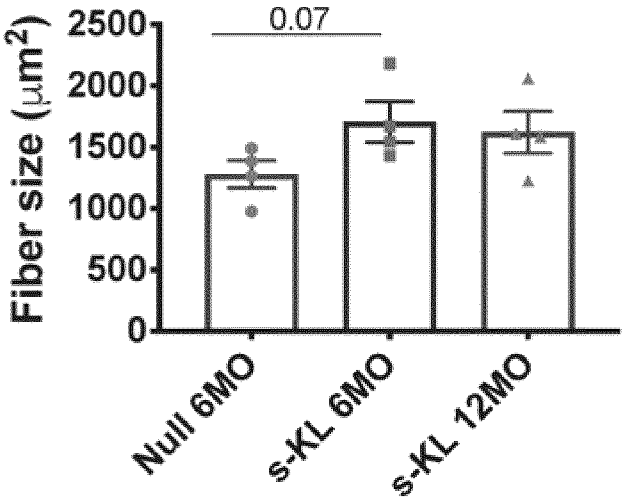

a
Max. speed
50
40
30
20
10
0
*
**
Null
s-KL 6
s-KL 12
b
Time to fall (s)
80
60
40
20
0
*
Null
s-KL 6
s-KL 12
c
Time to fall (s)
120
90
60
30
0
*
Null
s-KL 6
s-KL 12
d
Force (N)
250
200
150
100
**
***
1 2 3 1 2 3 1 2 3

Fig. 2

a


Fiber size (μm²)
2500
2000
1500
1000
500
0
0.07
Null 6MO
s-KL 6MO
s-KL 12MO

b

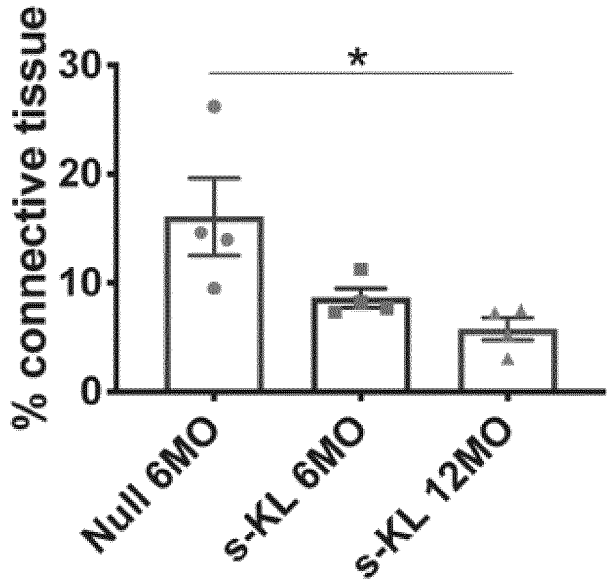

% connective tissue
30
20
10
0
*
Null 6MO
s-KL 6MO
s-KL 12MO

Fig. 3

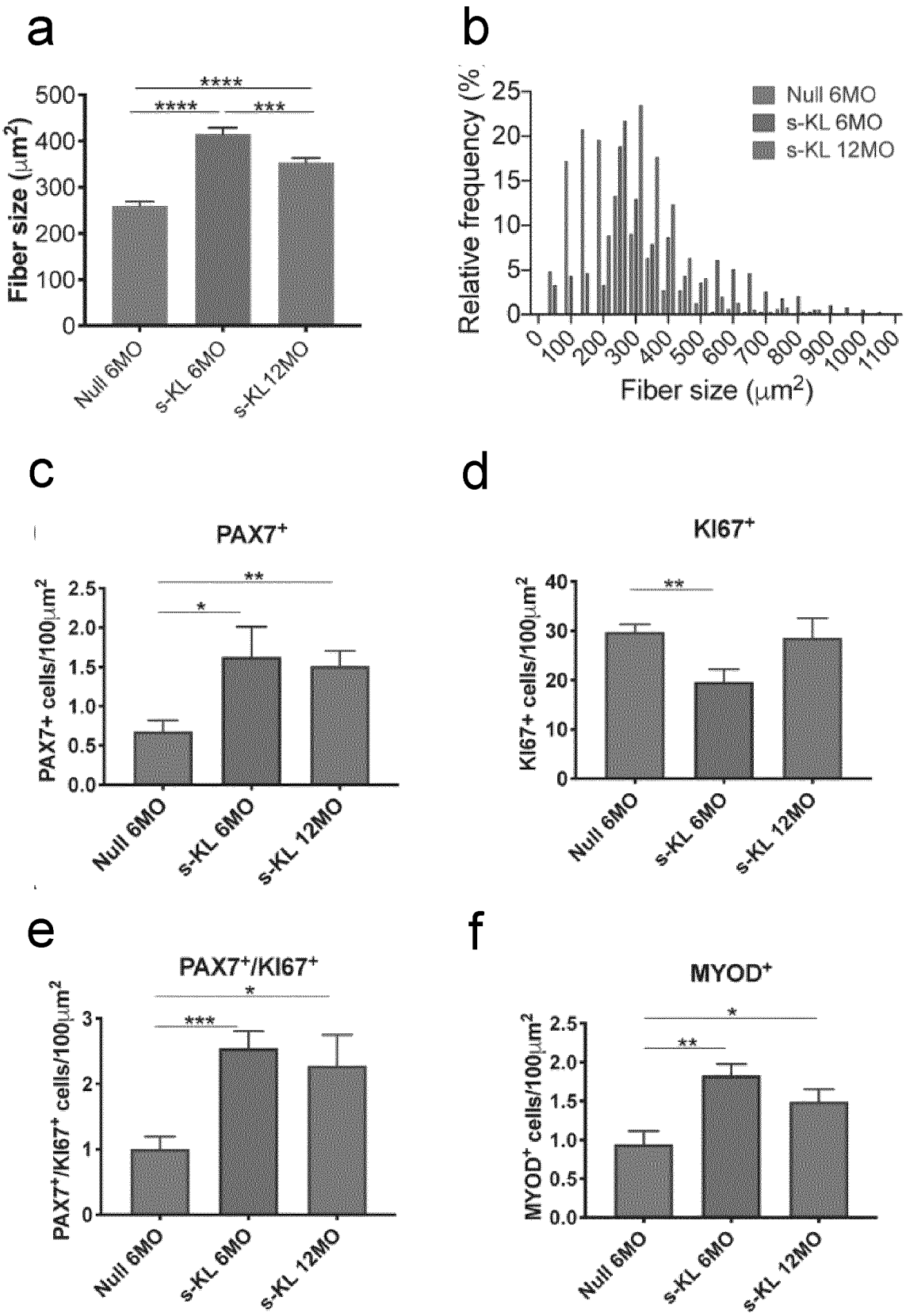

a
Fiber size (μm²)
Null 6MO
s-KL 6MO
s-KL12MO
b
Relative frequency (%)
Null 6MO
s-KL 6MO
s-KL 12MO
Fiber size (μm²)
c
PAX7⁺
PAX7+ cells/100μm²
d
KI67⁺
KI67+ cells/100μm²
e
PAX7⁺/KI67⁺
PAX7⁺/KI67⁺ cells/100μm²
f
MYOD⁺
MYOD⁺ cells/100μm²

Fig. 4

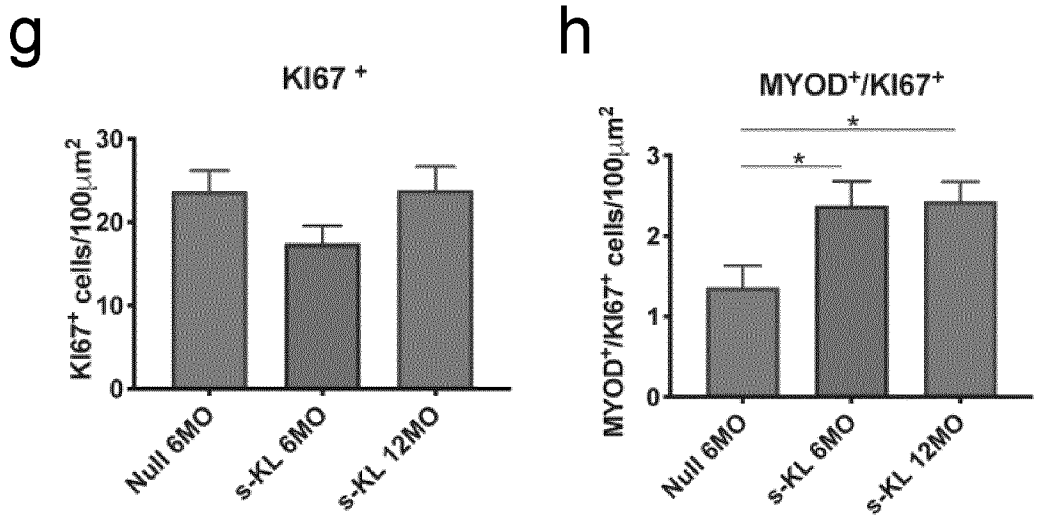

g
KI67 +
KI67+ cells/100μm2
30
20
10
0
Null 6MO
s-KL 6MO
s-KL 12MO
h
MYOD+/KI67+
MYOD+/KI67+ cells/100μm2
3
2
1
0
*
*
Null 6MO
s-KL 6MO
s-KL 12MO

Fig.4 (cont.)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/083910

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/47 C12N9/24 A61P21/00 A61P21/06 C07K14/71
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C12N A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2017/085317 A1 (UNIV BARCELONA AUTONOMA [ES] ET AL.) 26 May 2017 (2017-05-26) claims 1, 8-9, 12, 14 claims 4-5 claims 15-16 page 25, paragraph 6 - page 26, paragraph 1; example 2 page 9, paragraph 6 - page 10, paragraph 2 page 27, line 3, paragraph 1 - line 5 page 28, line 8, paragraph 1 - line 12 page 30, line 26, paragraph 3 - line 28 -----	1-16
A	WO 2022/243519 A1 (UNIV AUTÒNOMA DE BARCELONA [ES]; UNIV BARCELONA [ES] ET AL.) 24 November 2022 (2022-11-24) claims 1, 7 page 17, line 12, paragraph 3 - line 16 claims 8-13, 15 -----	1-16

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 January 2024	12/02/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Böhmerova, Eva

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2018/098375 A1 (KLOTHO THERAPEUTICS INC [US]) 31 May 2018 (2018-05-31) paragraph [0014]; claim 17 paragraph [0033]; claim 18 paragraph [0010]; claims 1, 27 claim 19	1-16
A	ZIELONKA DANIEL ET AL: "Skeletal muscle pathology in Huntington's disease", FRONTIERS IN PHYSIOLOGY, vol. 5, 6 October 2014 (2014-10-06), XP093027071, CH ISSN: 1664-042X, DOI: 10.3389/fphys.2014.00380 abstract	1-16
A	Brotman RG, Moreno-Escobar MC, Joseph J, et al: "Amyotrophic Lateral Sclerosis - StatPearls - NCBI Bookshelf", , 22 August 2022 (2022-08-22), pages 1-9, XP093027073, Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/books/NBK556151/ [retrieved on 2023-02-26] page 1, paragraph 1 - page 2, paragraph 1	1-16
A	WO 2017/210607 A1 (KLOTHO THERAPEUTICS INC [US]) 7 December 2017 (2017-12-07) paragraphs [0050], [0218], [0219], [0226]; claims 42,50	1-16

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/083910

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/083910

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017085317 A1	26-05-2017	AU 2016354988 A1	07-06-2018
		CA 3005398 A1	26-05-2017
		CN 108289933 A	17-07-2018
		CN 117126829 A	28-11-2023
		DK 3377091 T3	02-01-2024
		EP 3377091 A1	26-09-2018
		HK 1259628 A1	06-12-2019
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		US 2019030138 A1	31-01-2019
		WO 2017085317 A1	26-05-2017
WO 2022243519 A1	24-11-2022	CA 3218655 A1	24-11-2022
		WO 2022243519 A1	24-11-2022
WO 2018098375 A1	31-05-2018	AU 2017363321 A1	16-05-2019
		BR 112019010250 A2	17-09-2019
		CA 3041910 A1	31-05-2018
		CN 109996555 A	09-07-2019
		EP 3551212 A1	16-10-2019
		JP 7244923 B2	23-03-2023
		JP 2019535740 A	12-12-2019
		KR 20190088988 A	29-07-2019
		KR 20230093531 A	27-06-2023
		US 2020181224 A1	11-06-2020
		WO 2018098375 A1	31-05-2018
WO 2017210607 A1	07-12-2017	AU 2017272349 A1	22-11-2018
		BR 112018073909 A2	26-02-2019
		CA 3025461 A1	07-12-2017
		CN 109219663 A	15-01-2019
		CN 116478907 A	25-07-2023
		EP 3464608 A1	10-04-2019
		JP 2019526272 A	19-09-2019
		JP 2023123565 A	05-09-2023
		KR 20190015711 A	14-02-2019
		KR 20230125857 A	29-08-2023
		WO 2017210607 A1	07-12-2017