VECTORS AND SEQUENCES FOR THE TREATMENT OF DISEASES

FIELD OF THE INVENTION

The present invention relates to vectors useful for the expression of proteins of interest and their utilization in gene therapy. The present invention also relates to vectors and nucleic acid sequences helpful for the treatment of mucopolysaccharidoses (MPS), and in particular, for the treatment of mucopolysaccharidoses type III or Sanfilippo syndrome.

BACKGROUND OF THE INVENTION

The lysosome is an organelle founded in the cytoplasm of eukaryotic cells, which serves as storage for many hydrolytic enzymes and as a center for degrading and recycling cellular components. This organelle contains several types of hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases and sulfatases. All enzymes are acid hydrolases.

Lysosomal storage diseases (LSDs) are caused by genetic defects that affect one or more lysosomal enzymes. These genetic diseases result generally from a deficiency in a particular enzyme activity present in the lysosome. To a lesser extent, these diseases may be due to deficiencies in proteins involved in lysosomal biogenesis.

LSDs are individually rare, although as a group these disorders are relatively common in the general population. The combined prevalence of LSDs is approximately 1 per 5,000 live births. *See* Meikle P, *et al.*, JAMA 1999; 281:249-254. However, some groups within the general population are particularly afflicted by a high occurrence of LSDs. For instance, the prevalence rates of the Gaucher and Tay-Sachs diseases in descendants from Jewish Central and Eastern European (Ashkenazi) individuals is 1 per 600 and 1 per 3,900 births, respectively. The Finnish population is also afflicted by an uncommonly high LSDs prevalence rate.

Type III mucopolysaccharidoses (MPSIII), known collectively as Sanfilippo syndrome, are LSDs caused by a deficiency in one of the enzymes involved in the degradation of heparan sulfate, leading to its pathological accumulation. MPSIII is classified into four subtypes depending on the enzyme deficiency. Loss of sulfamidase activity causes subtype IIIA and has been reported to be the most severe, with the earliest disease onset and shortest survival. Symptoms of MPSIIIA occur in the first years of life, and are characterized by severe neurodegeneration that leads to deep mental retardation, aggressiveness, hyperactivity, and sleep alterations. Patients progressively lose the capacity of speech, swallow, and basic motor coordination. In addition to the neurological symptoms, MPSIIIA patients suffer non-neurological alterations, including hepato- and splenomegaly, skeletal and joint malformations, as well as frequent diarrhoea and respiratory tract infections. The progressive worsening of symptoms results in the death of the patient during adolescence. *See* Neufeld E, Muenzer J, "The mucopolysaccharidoses" in Scriver C, *et al.*, Eds., "The metabolic and molecular basis of inherited disease" (McGraw-Hill Publishing Co., New York, NY, US, 2001, pp. 3421-3452).

There is no cure for MPSIIIA currently and, therefore, existing treatments are aimed at controlling symptoms of the disease in order to improve the poor quality of life of the patients. MPS disorders can be treated by bone marrow transplantation or enzyme replacement therapy (ERT). Both approaches rely in the endocytosis of lysosomal enzymes from extracellular medium and their targeting to lysosomes via the mannose-6-phosphate receptor (M6PR) present at the cell membrane. Nevertheless, bone marrow transplantation has demonstrated to be inefficient in the treatment of MPSIII patients. *See* Sivakamur P, Wraith J, J. Inherit. Metab. Dis. 1999; 22:849-850. ERT has been extensively proven to be effective in counteracting the non-neurological accumulation in other lysosomal storage diseases, including MPSI, II and VI. *See* Harmatz P, *et al.*, J. Mol. Genet. Metab. 2008; 94:469-475; Muenzer J, *et al.*, Genet. Med. 2006; 8:465-473 and Wraith J, *et al.*, J. Pediatr. 2004; 144:581-588. In addition to the high cost of these treatments, it has been shown that

ERT does not result in correction or preservation of neuronal function due to the insufficient delivery of the exogenously provided enzyme through the blood-brain barrier (BBB). *See* Enns G, Huhn S, Neurosurg. Focus 2008; 24:E12. More recently, it has been demonstrated that high-dose ERT is partially successful in clearing CNS storage in MPS VII, possibly due to the saturation of M6PR and mannose receptors that lead to a longer half-life of the protein in circulation. *See* Vogler C, *et al.*, Proc. Natl. Acad. Sci. USA 2005; 102:14777-14782. This study demonstrates that high levels of the enzyme in circulation during long periods of time correlate with a better correction of the pathology. Intracerebral and intra-CSF delivery of the enzyme have also been proved to be efficient in reducing CNS pathology in MPS IIIA mice. *See* Hemsley K, *et al.*, Genes Brain Behav. 2008; 53(2):161-8 and Savas P, *et al.*, Mol. Genet. Metab. 2004; 82:273-285. However, this approach is highly invasive due to the need for multiple repeated injections and could increase the risk of damage and/or infections in the brain.

Given the limitations of current therapeutic options for MPSIII, alternative approaches are needed. Gene transfer could provide the means to achieve a permanent production of the missing enzyme from a single intervention. Adeno-associated vectors (AAV) are rapidly emerging as the vector of choice for many gene therapy applications, due to their high transduction efficiency and their lack of pathogenicity. AAV vectors efficiently transduce post-mitotic cells and several pre-clinical and clinical studies demonstrated the potential of AAV vector-mediated gene transfer to efficiently drive sustained expression of therapeutic transgenes for a variety of diseases. *See* Daya S, Berns K, Clin. Microbiol. Rev. 2008; 21:583-593.

It has been shown that the administration of an AAV5 vector co-expressing sulfamidase and the sulfatase activator SUMF1 in lateral ventricles of newborn MPSIIIA mice is able to correct many neurological and behavioral alterations. *See* Fraldi A, *et al.*, Hum. Mol. Genet. 2007; 16:2693-2702. However, this proposed course of action has several shortcomings. First, the CMV promoter utilized has been reported to silence. Second, the long term effects of the co-expression of sulfamidase and SUMF1 have not been

assessed yet. It is not clear if the co-expression of SUMF is even necessary and provides any additional permanent benefits in comparison to the treatment with sulfamidase only. Third, AAV5 vectors have a low distribution within the parenchyma, and more importantly, the delivery of sulfamidase into the brain by using these vectors does not result in any transduction of the cerebral tissue, thus, no correction of somatic phenotype is achieved by following this approach. Finally, Fraldi, 2007, *supra* demonstrated the efficacy of gene transfer in only newborn MPSIIIA mice. No experiments were reported in older mice. Since MPSIIIA is usually diagnosed after 3-4 years of age, the newborn animal model is not adequate for predicting the effects of this treatment in human beings.

In view of the difficulties for diagnosing MPSIIIA at birth, the development of therapeutic interventions starting in early adulthood has been proposed. It has been reported that the intravenous delivery of a lentiviral vector expressing sulfamidase in adult mice resulted in little amelioration of the CNS phenotype, likely due to the relatively poor transduction performance of these vectors *in vivo*. *See* McIntyre C, *et al.*, Mol. Genet. Metab. 2008; 93:411-418. Thus, the use of viral vectors with higher transduction efficacy *in vivo*, such as AAV vectors, may provide higher circulating levels of sulfamidase, which could potentially ameliorate or correct the neurological pathology.

The treatment of MPSIIIA via gene therapy requires more efficient vectors and sulfamidase coding sequences. Therefore, there is a long-felt need for an effective treatment of MPSIIIA. There is also the need for novel approaches to the treatment of this disease that would have enhanced security features. MPSIIIA is a rare disease and is therefore an orphan disease. The pharmaceutical agents developed specifically to treat this rare medical condition will be orphan drugs.

SUMMARY OF THE INVENTION

The present invention provides a new nucleotide sequence for the treatment of diseases, preferably for the treatment of mucopolysaccharidoses (MPS). Therefore, the first aspect of the invention refers to a nucleotide

sequence that is a codon optimized sequence of human sulfamidase that allows the transcription of a more stable mRNA. This sequence is transcribed at higher rates, and therefore, produces higher yields of the sulfamidase enzyme. The sequence has a better expression profile and is more effective therapeutically than other attempts to codon optimize the sulfamidase nucleotide sequence. These increased levels in the enzyme expression are followed by an increase in the serum sulfamidase activity, allowing the reduction of the glyocosaminoglycan (GAG) accumulation that causes the disease. Said sequence is SEQ ID NO: 1 or a sequence having at least 85% sequence identity to SEQ ID NO: 1 that codifies for the protein SEQ ID NO: 2.

In a second aspect, the invention relates to a gene construction comprising the nucleotide sequence of the first aspect of the invention.

The present invention also provides new AAV vectors with serotype 9 that are capable of passing across the blood-brain barrier (BBB) and show more tropism for different brain structures. This allows the sulfamidase activity to be increased specifically in the brain, reducing the GAG accumulation and therefore improving the neurological symptoms of MPS. The AAV serotype 9 shows also an unexpectedly high tropism for heart, pancreas and muscle tissue, thus potentiating the overall therapeutic benefits of the invention.

For example, after administrating AAV serotypes 8 and 9 (AAV8 and AAV9) vectors to adult MPSIIIA mice by intravenous (iv) injection, to target the liver, or by intramuscular (im) injection, to target the skeletal muscle, or intracisteranally (ic), to target the central nervous system, the levels of sulfamidase expression achieved with im- vector delivery were not therapeutic. The intracisternal administration was able, not only to increase the level of circulating sulfamidase, but also, to revert the somatic phenotype of MPSIIIA in several types of tissue, including brain tissue. The liver-directed approach was also able to produce high levels of circulating sulfamidase activity, which surprisingly corrected the somatic storage phenotype of MPSIIIA in full and significantly the neuropathology associated with the disease. These results provide evidence of the efficacy of AAV-mediated gene transfer of sulfamidase in adult MPSIIIA mice, a disease model closely resembling a human clinical

setting. The inventors were able to completely correct both somatic and neurological alterations of MPSIIIA.

The gene constructions of the present invention may further comprise adequate promoters, such the CAG or hAAT promoters, to control and potentiate the expression of sulfamidase. For instance, the CAG promoter is more stable than the CMV promoter, and is thus more amenable to induce the expression of sulfamidase for longer periods of time. On the other hand, the safety and efficacy of the hAAT promoter make it an ideal vehicle for delivering follow-up or maintenance doses of sulfamidase. The control of the expression of SEQ ID NO: 1 by the CAG or hAAT promoters has potentiated significantly its therapeutic effects.

Also, the AAV vectors of the present invention increase the sulfamidase activity, which reduces the GAG accumulation and improves the clinical outcome of individuals suffering from MPS. Only one administration may be sufficient because the promoter and the nucleotide sequence of the sulfamidase, located between the inverted terminal repeats (ITR) are incorporated in the genome of the cells of the individual. Therefore, a single parenteral administration is enough to get a long-term effect.

In a third aspect, the invention relates to a pharmaceutical composition comprising the nucleotide sequence of the first aspect of the invention, the gene construction or the expression vector of the invention.

In a fourth aspect, the invention relates to the nucleotide sequence, the gene construction, the expression vector or the pharmaceutical composition of the invention for use as a medicament. The medicament may be used for increasing the sulfamidase activity in the body, for enzyme replacement therapy, for gene therapy or for the treatment of MPS.

In a fifth aspect, the invention relates to a method for the production of the expression vectors of the first and second aspect of invention.

In a sixth aspect, the invention relates to a method for manufacturing the pharmaceutical compositions of the third aspect of the invention.

In a seventh aspect, the invention relates to a method for treating a subject having mucopolysaccharidosis type IIIA with the first, second and third aspects of the invention.

The present invention also relates to the use of a nucleotide sequence, gene construction, expression vector or pharmaceutical compositions of the invention in the manufacture of a medicament for increasing the sulfamidase activity in the body, for enzyme replacement therapy, for gene therapy or for the treatment of mucopolysaccharidoses or mucopolysaccharidosis type IIIA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Intramuscular delivery of AAV1-CAG-mu-SFMD-WPRE. (A) Sulfamidase activity in skeletal muscle of control, MPS and treated mice. (B) Sulfamidase activity in serum of control, MPS and treated mice. (C) Glycosaminoglycan (GAG) quantification in liver of control, MPS and treated mice. Values are means ± SEM of 4 to 8 mice per group. ¥ $P<0.05$ vs. control, ♯$P<0.05$ vs. males, * $P<0.05$ vs. untreated MPS. ND: not detected.

Figure 2. Intramuscular delivery of AAV8-CAG-mu-SFMD-WPRE. (A) Sulfamidase activity in skeletal muscle of control, MPS and treated mice. (B) Sulfamidase activity in serum of control, MPS and treated mice. (C) Glycosaminoglycan (GAG) quantification in liver of control, MPS and treated mice. Values are means ± SEM of 4 to 8 mice per group. ¥ $P<0.05$ vs. control, ♯$P<0.05$ vs. males, * $P<0.05$ vs. untreated MPS. ND: not detected.

Figure 3. Intravenous delivery of AAV8-CAG-mu-SFMD-WPRE. (A) Sulfamidase activity in liver of control, MPS and treated mice. (B) Sulfamidase activity in serum of control, MPS and treated mice. (C) Glycosaminoglycan (GAG) quantification in liver of control, MPS and treated mice. Values are means ± SEM of 4 to 8 mice per group. ¥ $P<0.05$ vs. control, ♯$P<0.05$ vs. males, * $P<0.05$ vs. untreated MPS. ND: not detected

Figure 4. Intravenous delivery of AAV8-hAAT-mu-SFMD. (A) Sulfamidase activity in liver of control, MPS and treated mice. (B) Sulfamidase activity in serum of control, MPS and treated mice. (C) Glycosaminoglycan (GAG)

quantification in liver of control, MPS and treated mice. Values are means ± SEM of 4 to 8 mice per group. ¥ P<0.05 vs. control, ♯P<0.05 vs. males, * P<0.05 vs. untreated MPS. ND: not detected.

Figure 5. Amelioration of the neurological pathology of MPSIIIA mice after intravenous delivery of AAV8-hAAT-mu-SFMD. (A) Sulfamidase activity in different parts of the brain (depicted in the diagram) of control, MPS and treated males. (B) Glycosaminoglycan (GAG) quantification in the same parts of the brain. Values are means ± SEM of 4 to 8 mice per group. ¥ P<0.05 vs. control, * P<0.05 vs. untreated MPS. ND: not detected. (C) Transmission electron microscopy of Purkinje cells in the cerebellum. Somas of Purkinje neurons of non-treated MPSIIIA mice were filled with many large electron-dense inclusions (white arrows), whereas in iv-AAV8-hAAT treated males less and smaller inclusions were found (black arrows).

Figure 6. Intravenous AAV9-CAG-mu-SFMD. (A) Sulfamidase activity in different parts of the brain (depicted in the diagram) of control, MPS and treated mice. (B) Glycosaminoglycan (GAG) quantification in the same parts of the brain. (C) Assessment of motor function by accelerating Rotarod test. Values are means ± SEM of 4 to 8 mice per group. ¥ P<0.05 vs. control, * P<0.05 vs. untreated MPS.

Figure 7. Brain transduction after intracisternal delivery of adeno-associated virus serotypes 1, 2, 5, 7, 8 and 9 GFP vectors. A dose of 5 x 10^{10} vector genomes of the appropriate vector was administered intracisternally to 2 month old animals, which were sacrificed and analyzed 2 weeks later. Adeno-associated virus serotype 9 demonstrated the highest efficiency of transduction in all analyzed areas. Syringe indicates the route of vector delivery, the cisterna magna. P: Pons, Cb: cerebellum, OB: olfactory bulb, Ht: hypothalamus, Cx: cerebral cortex.

Figure 8. Intracisternal delivery of AAV9-CAG-mu-SFMD vectors. Glycosaminoglycan (GAG) quantification in different parts of the brain (depicted in the diagram) of control, MPS and treated mice. Values are means ± SEM of 3 mice per group. ¥ P<0.05 vs. control, * P<0.05 vs. untreated MPS.

Figure 9. Intravenous delivery of AAV9-CAG-hu-co-SFMD. Sulfamidase activity in the liver of control mice, MPS mice and mice treated either with AAV9-CAG-mu-SFMD (non-optimized gene) or AAV9-CAG-hu-co-SFMD (optimized gene).

Figure 10. Reduction of lysosomal pathology in perineuronal glial cells of the occipital cortex. Transmission electron microscopy depicting cortical neurons of the occipital cortex and their associated glial cells. MPSIIIA lysosomal pathology was much more evident in perineuronal glial cells than neurons. The presence of large electro-lucent vacuoles in the glial cells from MPSIIIA untreated male samples (white arrows, upper right panel) and not in WT samples (upper left panel) is shown. This enlargement of the lysosomal compartment was greatly reduced in iv-AAV8hAAT-treated mice, and most of the perineuronal glial cells in these samples presented an aspect similar to that of WTs (bottom panels). (1) neuron, (2) perineuronal glial cell.

Figure 11. Survival in intravenous AAV8-hAAT-SFMD treated males and females. (A) Kaplan-Meier survival analysis in WT, MPSIIIA and intravenous AAV8-hAAT-SFMD treated males. Treatment with AAV-mediated liver-directed gene therapy considerably extended the lifespan of MPSIIIA animals ($p<0.001$). (B) Kaplan-Meier survival analysis in WT, MPSIIIA and iv-AAV8-hAAT-SFMD treated females. Treatment with AAV-mediated liver-directed gene therapy did not extend the lifespan of MPSIIIA females ($p= 0.467$).

Figure 12. Survival in intracisternal and intravenous AAV9-CAG-mu-SFMD treated males and females. Kaplan-Meier survival analysis in WT, MPSIIIA and AAV9-treated males (A) and females (B). Both intracisternal and intravenous treatment with AAV-mediated gene therapy extended the lifespan of MPSIIIA animals.

Figure 13. Intrascisternal administration of AAV9 vectors to dogs leads to transduction of widespread CNS areas and liver. Immunohistochemichal detection of GFP in CNS and liver sections of a dog injected with AAV9-GFP through the cisterna magna. The images correspond to: (a) spinal cord, (b) olivary body of the medulla oblongata, (c) raphe nuclei of the pons, (d) hypothalamic nuclei (e) rhinencephalon (f) occipital cortex, (h) frontal cortex, (i)

cerebellum, (j) dentate gyrus of the hippocampus. Scale bar: 1mm for (a), 500 µm for (b)-(h), 100 µm for (i)-(j).

Figure 14. Liver transduction after intracisternal delivery of AAV9-GFP vector in healthy Beagle dogs. Immunohistochemic detection of GFP in liver sections counterstained with hematoxilin. Representative images of Dog 1 (A) and Dog 2 (B) are shown. Original magnification 200X.

Figure 15. Serum sulfamidase activity and liver GAG content in intravenous AAV9-co-hu-SFMD injected animals. (A) Sulfamidase activity in the serum measured with a fluorogenic substrate. (B) GAG storage in the liver 2 months after vector administration.

DEPOSIT OF MICROORGANISMS

The plasmid pAAV-CAG-co-hu-SFMD was deposited on May 16th, 2011, under access number DSM 24817 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

The plasmid pAAV-CAG-mu-SFMD was deposited on May 16th, 2011, under access number DSM 24818 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

The plasmid pGG2-hAAT-mu-SFMD was deposited on May 16th, 2011, under access number DSM 24819 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

DEFINITIONS

The term “nucleotide sequence” refers to a nucleic acid molecule, either DNA or RNA, containing deoxyribonucleotides or ribonucleotides. The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence.

The term "% sequence identity" refers to the percentage of nucleotides of a candidate sequence that are identical to the nucleotides in SEQ ID NO: 1, after aligning the sequences to achieve the maximum % sequence identity. The % sequence identity can be determined by any methods or algorithms established in the art, such as the ALIGN, BLAST and BLAST 2.0 algorithms. *See* Altschul S, *et al.*, Nuc. Acids Res. 1977; 25:3389-3402 and Altschul S, *et al.*, J. Mol. Biol. 1990; 215:403-410.

Herein, the % sequence identity is calculated dividing the number of nucleotides that are identical after aligning SEQ ID NO: 1 and the candidate sequence, by the total number of nucleotides in SEQ ID NO: 1 and multiplying the result by 100.

The term "codify" refers to the genetic code that determines how a nucleotide sequence is translated into a polypeptide or a protein. The order of the nucleotides in a sequence determines the order of amino acids along a polypeptide or a protein.

The term "protein" refers to a linear chain of amino acids or a polypeptide that is folded into a globular form. Proteins can suffer post-translational modifications, like the conversion of a cysteine residue to 3-oxoalanine, glycosylation or metal binding. Glycosilation of a protein is the addition of different carbohydrates that are linked covalently to the amino acid chain.

The term "effective amount" refers to an amount of a substance sufficient to achieve the intended purpose. For example, an effective amount of an expression vector to increase sulfamidase activity is an amount sufficient to reduce glycosaminoglycan accumulation. A "therapeutically effective amount" of an expression vector to treat a disease or disorder is an amount of the expression vector sufficient to reduce or remove the symptoms of the disease or disorder. The effective amount of a given substance will vary with factors such as the nature of the substance, the route of administration, the size and species of the animal to receive the substance and the purpose of giving the substance. The effective amount in each individual case may be determined empirically by a skilled artisan according to established methods in the art.

The term “individual” refers to an arbitrary animal, preferably human or non-human mammal, more preferably mouse, rat, other rodents, rabbit, dog, cat, pig, cow, horse or primate, further more preferably human.

The term “operably linked” refers to the functional relation and the location of the promoter sequence with respect to the gene of interest (e.g. a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence). Generally, a promoter operably linked is contiguous to the sequence of interest. However, an enhancer does not have to be contiguous to the sequence of interest to control its expression.

The term “tropism” refers to the way in which different viruses have evolved to preferentially target specific host species, or specific cell types within those species.

The term “gene therapy” refers to the transfer of genetic material (e.g. DNA or RNA) of interest into a host to treat or prevent a genetic or acquired disease or condition. The genetic material of interest encodes a product (e.g. a protein polypeptide, peptide or functional RNA) whose production *in vivo* is desired. For example, the genetic material of interest can encode an enzyme, hormone, receptor, or polypeptide of therapeutic value.

The term “CAG promoter” refers to the combination formed by the cytomegalovirus early enhancer element and chicken β-actin promoter (i.e. SEQ ID NO: 3). *See* Alexopoulou A, *et al.*, BMC Cell Biology 2008; 9(2): 1-11.

The term “hATT promoter” refers to the human alpha1-antitrypsin promoter (i.e. SEQ ID NO: 4). *See* Hafenrichter H, *et al.*, Blood 1994; 84: 3394-3404.

The term “viral vector particle” refers to the genetically modified virus used for the delivery of genes into an organism. The viral vector particles carry the viral genome. The viral genome comprises the nucleotide sequence that is located between the ITRs in the expression vector used for the production of the viral vector particles. The adeno-associated viral vector particles are called AAV. The term “AAV vector” refers to the adeno-associated viral vector particles.

DETAILED DESCRIPTION OF THE INVENTION

In a preferred embodiment, the sequence of the first aspect of the invention has at least 85%, sequence identity to SEQ ID NO: 1 that codifies for the protein SEQ ID NO: 2. Preferably, the sequence identity is at least 87%. More preferably, the sequence identity is at least 90%. Much more preferably, the sequence identity is at least 95%. Even more preferably, the sequence identity is at least 98%. Most preferably, the sequence identity is at least 99%. In a more preferred embodiment, the sequence of the first aspect of the invention is nucleotide sequence SEQ ID NO: 1. In another embodiment, the invention relates to a nucleotide sequence SEQ ID NO: 1 or a biologically active variant of this sequence. A biologically active variant includes a molecule having the same biological activity as SEQ ID NO: 1 and at least 85% sequence identity. Biological activity refers to the fact that the nucleotide sequence SEQ ID NO: 1 can be transcribed into a messenger RNA that has increased stability and therefore presents high translation rates, therefore allowing the expression of high levels of active human sulfamidase.

In a preferred embodiment of the second aspect, the gene construction comprises a nucleotide sequence having at least 85%, preferably 87%, 90%, 95%, 98%, 99% sequence identity to SEQ ID NO: 1. In a more preferred embodiment, the gene construction comprises nucleotide sequence SEQ ID NO: 1. A gene construction is nucleic acid molecule where different elements have been engineered in a specific and desired manner. These elements may be, among others, replicating sequences, control sequences, codifying sequences, multicloning sequences or recombination sequences. In a preferred embodiment, the gene construction is a vector. A vector is a nucleic acid molecule used to transfer genetic material into a cell. Apart form the said genetic material, a vector may also contain different functional elements that include control elements for transcription, like promoters or operators, transcription factor binding regions or enhancers, and control elements for the initiation or termination of translation. Vectors include, but are not limited to: plasmids, cosmids, viruses, phages, recombinant expression cassettes and

transposons. The adeno-associated vectors (AAV) are the viral vector particles, therefore they are not a nucleic acid molecule but a genetically modified virus used for the delivery of genes into an organism.

In a preferred embodiment of the second aspect of the invention, the gene construction is a vector that is used for the translation and transcription of a gene of interest, usually controlled by a promoter. A promoter is a nucleotide sequence that controls the translation of the gene of interest. The promoter is operably linked to the gene of interest.

Another preferred vector is an adeno-associated vector. In a preferred embodiment, the adeno-associated vector is used to produce adeno-associated particles where the serotype is 1, 2, 5, 7, 8 or 9. In a more preferred embodiment, the serotype is 9. An adeno-associated vector is a vector derived from the genome of an adeno-associated virus (AAV) of the family *Parvoviridae*. The AAV genome is built of single-stranded deoxyribonucleic acid (ssDNA). AAV infect humans but are non-pathogenic (i.e. do not cause a disease). They can infect dividing and non-dividing cells, and their tropism changes depending on the serotype. The serotype is the classification of the viruses in groups, depending on their capsid antigens. The serotype of the AAV, determined by its capsid proteins, defines the virus tropism and allows its entry into a specific cell type. The production of the adeno-associated vector particles is described below.

In a first preferred embodiment of the second aspect, the expression vector comprises the CAG promoter operably linked to SEQ ID NO: 1.

A preferred vector is an expression vector comprising the CAG promoter, the promoter sequence being SEQ ID NO: 3. Therefore, one embodiment of the second aspect of the invention is an expression vector comprising CAG promoter, the promoter sequence being SEQ ID NO: 3 suitable for treating MPS.

In a second preferred embodiment of the second aspect, the expression vector comprises the liver-specific hAAT promoter operable linked to SEQ ID NO: 1.

A preferred vector is an expression vector comprising the liver-specific hAAT promoter, the promoter sequence being SEQ ID NO: 4. Therefore, one embodiment of the second aspect of the invention is an expression vector comprising the liver-specific hAAT promoter, the promoter sequence being SEQ ID NO: 4 suitable for treating MPS.

Another aspect of the present invention refers to a viral vector particle, also called expression vector, which carries the nucleotide sequences of the first aspect of the invention, or the gene construction or expression vector of the second aspect of the invention.

A preferred expression vector has serotype 1, 2, 5, 7, 8 or 9. A more preferred viral vector particle has serotype 9.

A preferred expression vector has serotype 9 and comprises a viral genome comprising a CAG promoter operably linked to SEQ ID NO: 1.

A preferred expression vector has serotype 8 or 9 and comprises a viral genome comprising a hAAT promoter operably linked to SEQ ID NO: 1.

A preferred expression vector has serotype 9 and comprises a viral genome comprising a hAAT promoter operably linked to SEQ ID NO: 1.

In a preferred embodiment, the expression vector is AAV-CAG-co-hu-SFMD and more preferably, AAV9-CAG-co-hu-SFMD.

In yet another preferred embodiment, the expression vector is AAV-hAAT-co-hu-SFMD, and more preferably, AAV8-hAAT-co-hu-SFMD or pAAV9-hAAT-co-hu-SFMD. The most preferred vector utilized when the hAAT promoter is used is AAV9-hAAT-co-hu-SFMD.

In a preferred embodiment of the third aspect, the pharmaceutical composition is administered by parenteral administration. Parenteral administration refers to the route of administration of a pharmaceutical composition as an injection or infusion. Examples of parenteral administration are intravenous, subcutaneous, intracisternal and intramuscular injections. Preferably, the pharmaceutical composition is administered by intravenous or intracisternal administration

In another preferred embodiment, the pharmaceutical composition comprises a therapeutically effective amount of the nucleotide sequence, the

gene construction, the viral vector particle or the expression vector of the invention.

In a preferred embodiment of the fourth aspect, the nucleotide sequence, the gene construction, the expression vector, the viral vector particle or the pharmaceutical composition of the invention are used as a medicament. In a preferred embodiment, they are used for increasing the sulfamidase activity in the body.

In another preferred embodiment, the nucleotide sequence, the gene construction, the expression vector, the viral vector particle or the pharmaceutical composition of the invention are used as a medicament for enzyme replacement or gene therapy, preferably gene therapy. The inventors propose a new gene therapy approach for the treatment of MPSIIIA therapeutic that is more efficacious than other known in the art. This approach is based on AAV vectors expressing sulfamidase. The enzyme replacement therapy (ERT) is a medical treatment that consists in replacing an enzyme in patients where a particular enzyme is deficient or absent. The enzyme is usually produced as a recombinant protein and administrated to the patient.

In a further embodiment, the nucleotide sequence, the gene construction, the expression vector, the viral vector particles or the pharmaceutical composition of the invention are preferably used for the treatment of mucopolysaccharidoses, more preferably of mucopolysaccharidosis type III or Sanfilippo syndrome, preferably through gene therapy. Within the mucopolysaccharidosis type III syndrome the subtype A is especially amenable to respond to treatment with the present invention.

In a preferred embodiment of the fifth aspect, a method for the production of the expression vectors of the invention is claimed. The process comprises the steps of:

i) providing a first vector comprising the SEQ ID NO: 1 interposed between a first AAV terminal repeat and a second AAV terminal repeat, a CAG or hAAT promoter operably linked to the SEQ ID NO: 1; a second vector comprising an AAV *rep* gene and a AAV *cap* gene; and a third vector comprising the adenovirus helper function gene;

ii) co-transfecting competent cells with the vectors of step i);

iii) culturing the transfected cells of step ii); and

iv) purifying the expression vectors from the culture of step iii).

In a preferred embodiment, the AAV first and second terminal repeats of the first vector are ITRs from the AAV serotype 2. In another preferred embodiment, the AAV ***rep*** genes of the second vector are from the AAV serotype 2. In yet another preferred embodiment, the AAV ***cap*** genes of the second vector are from the AAV serotypes 1, 2, 5, 7, 8 or 9. More preferably, the AAV ***cap*** genes of the second vector are from the AAV serotype 9. In another preferred embodiment, the competent cells are HEK293 cells.

The viral vectors are administered in sufficient amounts to transfect the cells and to provide sufficient levels of gene transfer and expression to provide a therapeutic benefit without undue adverse effects, or with medically acceptable physiological effects, which can be determined by those skilled in the medical arts.

In a preferred embodiment of the sixth aspect, a method for manufacturing the pharmaceutical compositions of the invention is claimed. This method comprises combining any of the nucleotide sequences, gene constructions, viral vector particles or expression vectors of the invention and a pharmaceutically acceptable vehicle or carrier to facilitate administration to yield the pharmaceutical compositions of the invention. The carrier is, for instance, water, or a buffered saline solution, with or without a preservative. The pharmaceutical compositions may be lyophilized for re-suspension at the time of administration or in solution.

In a preferred embodiment of the seventh aspect, a method for treating a subject having mucopolysaccharidosis type IIIA with the nucleotide sequences, gene constructions, viral vector particles, expression vectors or pharmaceutical compositions of the invention is claimed. Schedules and dosages for the administration of the nucleotide sequences, gene constructions, vectors, expression vectors or pharmaceutical compositions according to the present invention can be determined in accordance to dosage protocols known in the art. In a preferred embodiment, the nucleotide sequences, gene constructions,

viral vector particles, expression vectors or pharmaceutical compositions according to the present invention are administered once.

In one additional embodiment, a pharmaceutical composition for gene therapy treatment of MPS consists in the parenteral administration of an expression vector comprising a nucleotide sequence having 90% sequence identity to SEQ ID NO: 1.

In another additional embodiment, a viral vector comprising the CAG promoter and a nucleotide sequence having 95% sequence identity to SEQ ID NO: 1 is used for gene therapy for the treatment of a lysosomal storage disease (LSD) by an intramuscular injection.

In another additional embodiment, an AAV vector with serotype 1 comprising the CAG promoter and a nucleotide sequence having 87% sequence identity to SEQ ID NO: 1 is used as a medicament to treat MPS and is administered intravenously.

In another additional embodiment, a pharmaceutical composition comprising a nucleotide sequence having 98% sequence identity to SEQ ID NO: 1 and an ubiquitous promoter is administered parenterally to treat a disease.

Having described the invention in general terms, it will be more easily understood by reference to the following examples which are presented as an illustration and are not intended to limit the present invention.

GENERAL PROCEDURES

1. Recombinant AAV Vectors

The AAV vectors described herein were constructed by triple transfection. The materials required for making the vectors were: HEK293 cells (expressing E1 genes), helper plasmid providing adenovirus function, helper plasmid providing AAV ***rep*** genes from serotype 2 and ***cap*** genes from the desired serotype (i.e. AAV1, AAV2, AAV5, AAV7, AAV8, AAV9) and, finally, the backbone plasmid with ITRs and the construct of interest.

To generate sulfamidase-expressing AAV vectors, the cDNA of murine sulfamidase was cloned into an AAV backbone plasmid under the control of the ubiquitous hybrid CAG promoter or the liver-specific hAAT promoter.

Vectors (viral vector particles) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. *See* Matsushita T, *et al.*, Gene Ther. 1998; 5:938-945 and Wright J, *et al.*, Mol. Ther. 2005; 12:171-178. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM (Dulbeccos's Modified Eagle Medium) supplemented with 10% FBS (Foetal Bovine Serum) and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by the viral ITRs (described above); 2) a helper plasmid carrying the AAV rep2 and the correspondent cap (cap1 and cap9 genes; and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutives cesium chloride gradients using either a standard protocol or an optimized protocol as previously described. *See* Ayuso E, *et al.*, Gene Ther. 2010; 17:503-510. Vectors were dialyzed against PBS, filtered, titred by qPCR (quantitative Polymerase Chain Reaction) and stored at -80ºC until use.

a. Construction of pAAV-CAG-mu-SFMD-WPRE

Murine sulfamidase cDNA was utilized as starting material (Clone ID: D330015N16; Riken, Saitama, JP). The cDNA was received inside the plasmid pFLCI-Sgsh. High-fidelity PCR was performed to amplify the sulfamidase codifying region with primers that included MluI restriction sites at both ends. The sequences of the respective sense and antisense primers were: SEQ ID NO: 5 (Fw) CTTACTTATGACGCGTATGCACTGCCCGGGACTG and SEQ ID NO: 6 (Rv) TATCCTATCGACGCGTTCAGAGTTCATTGTGAAGCGGTC.

The backbone plasmid pAAV-CAG-WPRE was generated previously and contained both ITRs from the AAV2 genome, the CAG promoter, the WPRE element and the polyA signal of rabbit β-globin. The CAG promoter is a hybrid promoter composed of the CMV early/intermediate enhancer and the chicken β-actin promoter. This promoter is able to drive a potent expression ubiquitously.

The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is a hepadnavirus sequence that is widely used as a *cis*-acting regulatory module in various types of plasmid or viral gene vectors. When placed in the 3' untranslated region of gene transfer cassettes, the WPRE enhances the production of the transgene by increasing both nuclear and cytoplasmic mRNA levels. *See* Zanta-Boussif M, *et al.*, Gene Ther. 2009; 16:605-619.

The PCR-amplified sulfamidase coding region was cloned into the MluI restriction site of the AAV backbone plasmid pAAV-CAG-WPRE, and the resulting plasmid was named pAAV-CAG-mu-SFMD-WPRE. *See* SEQ ID NO: 7.

b. Construction of pAAV-CAG-mu-SFMD

The WPRE element in the pAAV-CAG-mu-SFMD-WPRE plasmid is flanked by two EcoRI restriction sites. To generate the pAAV-CAG-mu-SFMD plasmid (accession number DSM 24818), the pAAV-CAG-mu-SFMD-WPRE plasmid was digested with EcoRI, to eliminate the WPRE sequence, and was then subsequently religated. *See* SEQ ID NO: 8.

c. Construction of pAAV-CAG-co-hu-SFMD

The pAAV-CAG-mu-SFMD plasmid was digested with MluI and EcoRI to remove the murine sulfamidase codifying region. Subsequently, the cDNA of the codon optimized human sulfamidase (co-hu-SFMD) was digested and cloned at the same restriction sites to generate the pAAV-CAG-co-hu-SFMD plasmid (accession number DSM 24817). *See* SEQ ID NO: 9.

d. Construction of pAAV9-CAG-hu-SFMD

The pAAV9-CAG-hu-SFMD plasmid was obtained by co-transfecting 293HEK cells with the pAAV-CAG-co-hu-SFMD plasmid, a plasmid encoding

the adenovirus helper function and a plasmid encoding the AAV2 ***rep*** and AAV9 ***cap*** genes.

e. Construction of pGG2-hAAT-mu-SFMD

The murine sulfamidase codifying region was excised from the pAAV-CAG-mu-SFMD-WPRE plasmid by digestion with MluI. This region was then cloned into the MluI site in the AAV backbone plasmid pGG2-hAAT to yield the pGG2-hAAT-mu-SFMD plasmid (accession number DSM 24819). *See* SEQ ID NO: 10.

f. Construction of pGG2-hAAT-co-hu-SFMD

The codon optimized human sulfamidase codifying region was excised from the pAAV-CAG-co-hu-SFMD plasmid (accession number DSM 24817) by digestion with MluI-EcoRI. The pGG2-hAAT-mu-SFMD plasmid (accession number DSM 24819) was digested with MluI to remove mu-SFMD gene and, afterwards, the codon optimized human sulfamidase codifying region was cloned in this site by blunt-end ligation. The resulting plasmid was named as pGG2-hAAT-co-hu-SFMD. *See* SEQ ID NO: 11.
The pGG2-hAAT-co-hu-SFMD plasmid contained both AAV2-ITRs, the hAAT promoter and the SV40-derived poly-adenylation signal.

g. Construction of pAAV9-hAAT-co-hu-SFMD and pAAV8-hAAT-co-hu-SFMD.

Vectors were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. *See* Matsushita, 1998, *supra* and Wright, 2005, *supra*. Cells were cultured to 70% confluence in roller bottles (RB) (Corning, Corning, NY, US) in DMEM supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by the viral ITRs (pGG2-hAAT-co-hu-SFMD); 2) a helper plasmid carrying the AAV

rep2 and the correspondent cap genes (cap8 or 9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutives cesium chloride gradients using either a standard protocol or an optimized protocol as previously described. *See* Ayuso, 2010, *supra*. Vectors were dialyzed against PBS, filtered, titred by qPCR and stored at -80ºC until use.

The pGG2-hAAT-mu-SFMD plasmid contained bothAAV2-ITRs, the hAAT promoter and the SV40-derived poly-adenylation signal. The hAAT promoter is a hybrid promoter composed of the 4 tandem repeats of the hepatocyte control region (HCR) enhancer from the apolipoprotein E and the human α-anti-trypsin promoter. Its expression is restricted to hepatocytes. *See* Mingozzi F, *et al.*, J. Clin. Invest. 2003; 111:1347-1356.

The vectors of the present invention were constructed according to molecular biology techniques well known in the art. *See* Brown T, "Gene Cloning" (Chapman & Hall, London, GB, 1995); Watson R, *et al.*, "Recombinant DNA", 2nd Ed. (Scientific American Books, New York, NY, US, 1992); Alberts B, *et al.*, "Molecular Biology of the Cell" (Garland Publishing Inc., New York, NY, US, 2008); Innis M, *et al.*, Eds., "PCR Protocols. A Guide to Methods and Applications" (Academic Press Inc., San Diego, CA, US, 1990); Erlich H, Ed., "PCR Technology. Principles and Applications for DNA Amplification" (Stockton Press, New York, NY, US, 1989); Sambrook J, *et al.*, "Molecular Cloning. A Laboratory Manual" (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, US, 1989); Bishop T, *et al.*, "Nucleic Acid and Protein Sequence. A Practical Approach" (IRL Press, Oxford, GB, 1987); Reznikoff W, Ed., "Maximizing Gene Expression" (Butterworths Publishers, Stoneham, MA, US, 1987); Davis L, *et al.*, "Basic Methods in Molecular Biology" (Elsevier Science Publishing Co., New York, NY, US, 1986), Schleef M, Ed., "Plasmid for Therapy and Vaccination" (Wiley-VCH Verlag GmbH, Weinheim, DE, 2001).

2. Animals

A congenic C57Bl/6 sulfamidase-deficient mouse colony (MPSIIIA) was used. *See* Crawley A, *et al.*, Brain Res. 2006; 1104:1-17. Affected MPSIIIA and

healthy control mice were inbred from heterozygous founders. Genotype was determined by PCR analysis on genomic DNA from tail-clipped samples, which amplifies a sequence encompassing the mutation, and its subsequent digestion with MspA1I restriction enzyme, as previously described. *See* Bhattacharyya R, *et al.*, Glycobiology 2001; 11:99-103. Mice were fed *ad libitum* with a standard diet (Panlab, Barcelona, ES) and maintained under a light-dark cycle of 12 h (lights on at 9:00 A.M.).

3. Vector administration and sample collection

For intravenous delivery of AAV vectors, a total dose of 10^{12} vector genomes of the appropriate AAV vector were injected into 2-month-old MPSIIIA animals via tail vein. For intramuscular injection, 2-month-old MPSIIIA animals were anesthetized with a mixture of ketamine (100 mg/kg) and xylacine (10 mg/kg), and a total dose of 10^{12} vector genomes of the appropriate AAV vector were injected into 6 muscles of the hind limbs (quadriceps, gastrocnemius and tibialis anterior from both legs). At 10 months of age, mice were anesthetized and then transcardially perfused with 10 ml of PBS to completely clear blood from tissues. The entire brain and multiple somatic tissues (including liver, spleen, pancreas, kidney, lung, heart, skeletal muscle and testicles) were collected and either frozen in liquid nitrogen and stored at -80ºC or immersed in formalin for subsequent histological analyses.

4. RNA analysis

Total RNA was obtained from skeletal muscle and liver samples by using TriPure Isolation Reagent (Roche Diagnostics, Barcelona, ES) and analyzed by Northern blot. Blots were hybridized with a murine sulfamidase probe, labeled with ^{32}P-dCTP by random priming with Ready-to-Go DNA Labelling Beads (Amersham Biosciences, Piscataway, NJ, US).

5. Sulfamidase activity and glycosaminoglycan quantification

Liver, skeletal muscle and brain samples were sonicated in water and sulfamidase activity was assayed in supernatants with a 4-methylumbelliferone-derived fluorogenic substrate (Moscerdam Substrates, Oegstgeest, NL) as described previously. *See* Karpova E, *et al.*, J. Inherit. Metab. Dis. 1996; 19:278-285. Sulfamidase activity levels were normalized against the total amount of protein, quantified using the Bradford protein assay (Bio-Rad, Hercules, CA, US).

For glycosaminoglycan (GAG) quantification, tissue samples were weighted and then digested with proteinase K and extracts were clarified by centrifugation and filtration. GAG levels in tissue extracts and urine were determined using Blyscan sulfated glycosaminoglycan kit (Biocolor, Carrickfergus, County Antrim, GB) with chondroitin 4-sulfate as the standard. GAG levels in tissues were normalized to wet tissue weight and in urine to creatinine concentration, measured with a specific kit (Horiba ABX, Irvine, CA, US).

6. Histological analyses

Tissues were fixed for 12-24 h in formalin, embedded in paraffin and sectioned followed by heat-induced epitope retrieval (citrate buffer, pH 6). For immunohistochemical detection of LAMP1, paraffin sections were incubated overnight at 4ºC with rat anti-LAMP1 antibody (1D4B; Santa Cruz Biotechnology, Santa Cruz, CA, US) diluted at 1:100 and subsequently incubated with biotinylated rabbit anti-rat antibody (Dako, Glostrup, DK) at 1:300. LAMP1 signal was amplified by incubating sections with ABC-Peroxidase staining kit (Thermo Scientific, Waltham, MA, US) at 1:100 and visualized by using 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, US) as a chromogen. Brightfield images were obtained with an optical microscope (Eclipse E800; Nikon, Tokyo, JP). For parvalbumin and calbindin immunostaining, paraffin sections were incubated overnight at 4ºC with rabbit anti-calbindin D28k (Swant, Marly, CH) diluted at 1:2000 or with rabbit anti-

parvalbumin (Swant, Marly, CH) diluted at 1:100. Afterwards, samples were incubated with the biotinylated goat anti-rabbit IgG (Vector Labs., Burlingame, CA, US), and then with streptavidin-Alexa 488 (1:100, Molecular Probes, Invitrogen, Carlsbad, CA, US), and nuclei were stained with TOPRO-3. Images were obtained with a confocal microscope (Leica Microsystems, Heidelberg, DE).

For double immunostaining LAMP1 and Mac2, sections were first incubated overnight at 4°C with rat anti-LAMP1 antibody at 1:100, then with biotinylated rabbit anti-rat antibody at 1:300 followed by an incubation with streptavidin-Alexa 488 (1:300). Afterwards, sections were incubated with rabbit anti-Mac2 at 1:50, then with biotinylated goat anti-rabbit at 1:300 followed by incubation with streptavidin-Alexa 568 (1:300; Molecular Probes, Invitrogen, Carlsbad, CA, US). Finally, nuclei were stained with Hoechst (1:100; Sigma-Aldrich, St. Louis, MO, US).

7. Western blot analysis

Halves of cerebellum were homogenized in protein lysis buffer. Ten micrograms of protein were run on a 10% (wt/vol) SDS-PAGE, transferred to polyvinylidene difluoride membranes and probed overnight at 4°C with primary antibodies against calbindin (Swant, Marly, CH) and α-tubulin (Abcam, Cambridge, MA, US). Detection was performed using horseradish peroxidase-labelled swine anti-rabbit antibody (Dako, Glostrup, DK) and ECL Plus Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ, US).

8. Transmission electron microscopic analysis

Mice were sacrificed by an overdose of isofluorane (Isofluo, Labs. Esteve, Barcelona, ES) and perfused via inferior vena cava with 1 ml of 2.5% glutaraldehyde and 2% paraformaldehyde. A small portion (approximately $1mm^3$) of the lateral lobe of the liver and of the culmen of the cerebellum were sectioned and incubated for 2 hours at 4°C in the same fixative. After washing

in cold cacodylate buffer, the specimens were postfixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, and then dehydrated through a graded ethanol series and embedded in epoxy resin. Ultrathin sections (600–800 Å) from the resin blocks were stained using lead citrate and examined in a transmission electron microscope (H-7000; Hitachi, Tokyo, JP).

9. Statistical analysis

All results are expressed as mean ± SEM. Statistical comparisons were made using either t-test or one-way ANOVA. Statistical significance was considered if $P < 0.05$.

EXAMPLES

Example 1:
Intramuscular delivery of AAV1-CAG-mu-SFMD-WPRE

A total dose of 10^{12} vector genomes of the AAV1-CAG-mu-SFMD-WPRE vector was injected into 6 muscles of the hind limbs (quadriceps, gastrocnemius and tibialis anterior from both legs) of 2-month-old male and female MPSIIIA mice.

Eight months after the administration, the injected muscles displayed high levels of vector-derived sulfamidase expression and activity, but very low levels of sulfamidase activity were observed in serum (6-7% of control mice), suggesting low secretion efficiency from the skeletal muscle. *See* Figures 1A and 1B. In addition, very low but significant vector-derived sulfamidase expression was observed in the liver of these mice, indicating that, at the moment of injection, the vector leaked from the skeletal muscle into the circulation and transduced the liver. Even with the low levels of circulating sulfamidase activity achieved, correction of GAG accumulation was seen in liver, and a significant reduction in some other somatic tissues (spleen, heart,

pancreas), but not in others (kidney, lung). *See* Figure 1C. No reduction of the GAG storage was achieved in the brain.

Example 2:

Intramuscular delivery of AAV8-CAG-mu-SFMD-WPRE

A total dose of 10^{12} vector genomes of the AAV8-CAG-mu-SFMD-WPRE vector was injected into 6 muscles of the hind limbs (quadriceps, gastrocnemius and tibialis anterior from both legs) of 2-month-old male and female MPSIIIA mice.

Eight months after the administration, the injected muscles displayed sulfamidase activity levels similar to those of a healthy control animal. *See* Figure 2A. Low levels of sulfamidase activity were observed in serum (10-15% of control mice). *See* Figure 2B. Vector leakage to the liver was also observed, since vector-derived sulfamidase expression and activity was seen in the liver, even at higher levels than in mice treated with intramuscular AAV1. *See* Example 1. Correction of GAG accumulation was seen in liver and spleen, and a greater reduction was observed in other somatic tissues (heart, pancreas, urinary bladder), but kidney and lungs remained largely uncorrected. *See* Figure 2C. No reduction of the GAG storage was achieved in the brain.

Example 3:

Intravenous delivery of AAV8-CAG-mu-SFMD-WPRE

A total dose of 10^{12} vector genomes of the AAV8-CAG-mu-SFMD-WPRE vector was injected into 2-month-old MPSIIIA mice via tail vein.

Eight months after administration, the treated males displayed sulfamidase activity in the liver at levels similar to those of control mice, but 4-fold lower in females. *See* Figure 3A. Consistently, circulating sulfamidase activity was high in males (similar levels to those of control mice), and lower in females (25% of control mice). *See* Figure 3B. Those high levels of circulating sulfamidase were able to correct GAG accumulation in liver, heart, spleen,

pancreas and urinary bladder, and significantly reduce it in lungs, but not in kidney. *See* Figure 3C for liver GAG quantification. No reduction of GAG storage was observed in the brain.

Example 4:

Intravenous delivery of AAV8-hAAT-mu-SFMD

A total dose of 10^{12} vector genomes of the AAV8-hAAT-mu-SFMD vector was injected into 2-month-old MPSIIIA mice via tail vein.

Eight months after administration, the treated males displayed a sulfamidase activity level in the liver 500% higher than in control animals. In the female subjects, the liver sulfamidase level reached the same level of the control subjects. *See* Figure 4A. Circulating sulfamidase activity was consistently higher in males than in females (500% in males vs. 160% in females). *See* Figure 4B. These supraphysiological levels of circulating sulfamidase were able to correct GAG accumulation in all somatic organs, including the kidney. *See* Figure 4C for liver GAG quantification.

Treated males showed low levels of sulfamidase activity, and reduced GAG accumulation in brain. *See* Figures 5A and B. Purkinje cells of the cerebellum of treated males displayed less electron-dense inclusions when examined by electron microscopy. *See* Figure 5C. The intravenous treatment with AAV8-hAAT-mu-SFMD vector ("iv-AAV8-hAAT-mu-SFMD") achieved the correction of the somatic pathology but only ameliorated the neurodegeneration characteristic of MPSIIIA mice.

The cortex ultrastructure was analyzed by transmission electron microscopy. No distinguishable differences were noted in the ultrastructure of occipital cortical neurons of MPSIIIA treated and untreated subjects. A clear enlargement of the lysosomal compartment was observed in the perineuronal glial cells of the MPSIIIA untreated mice which was practically absent in treated animals. *See* Figure 10. These results suggest that sustained high circulating sulfamidase activity prevents neuronal degeneration in MPSIIIA subjects.

By 17 months of age, all untreated MPSIIIA males had died while 100% of iv-AAV8-hAAT-mu-SFMD treated males were still alive (Median survival = 14.2 ± 0.5 vs 18.8 ± 0.9 months for MPSIIIA untreated and treated males respectively, p= 0.001) This improvement was not evident in the female group, where both treated and untreated subjects showed similar survival rates (Median survival = 13.1 ± 0.5 *vs.* 13.9 ± 1.2 months for MPSIIIA untreated and treated females, respectively, p= 0.467). This result is consistent with the lower levels of sulfamidase activity measured in serum and brain and the lower degree of GAG reduction observed in female animals. *See* Figure 11.

The greater survival of iv-AAV8-hAAT-mu-SFMD treated MPSIIIA males further demonstrated the therapeutic potential of sustained supraphysiological levels of circulating sulfamidase obtained through liver-directed gene transfer. Treatment with iv-AAV8-hAAT-mu-SFMD extended the lifespan of MPSIIIA male subjects. *See* Figure 11.

Example 5:

Intravenous delivery of AAV9-CAG-mu-SFMD

A total dose of 10^{12} vector genomes of the AAV9-CAG-mu-SFMD vector was injected into 2-month-old MPSIIIA mice via tail vein.

Both treated males and females showed high levels of circulating sulfamidase (500% of control levels in males and 150% in females), which efficiently corrected all somatic tissues in both genders. In addition, given the high efficiency of brain transduction of the AAV serotype 9, significant sulfamidase activity was observed in the brains of both genders, which efficiently corrected GAG storage in all areas of the brain. *See* Figures 6A and 6B. Neuroinflammation (astrogliosis and microgliosis), characteristic of MPSIIIA, was completely normalized in AAV9-treated mice. In addition, AAV9-treated mice performed better in the Rotarod test than untreated animals. *See* Figure 6C.

The intravenous treatment with the AAV9-CAG-mu-SFMD vector ("iv-AAV9-CAG-mu-SFMD") extended the lifespan of MPSIIIA animals. *See* Figure

12. By 17 months of age, all untreated MPSIIIA males had died while 100% of iv-AAV9-CAG-mu-SFMD treated males were still alive at 20 months of age ($p<$ 0.001 and p= 0.037 for MPSIIIA treated versus untreated males, respectively). *See* Figure 12. The female group showed a similar but less impressive improvement (p= 0.063 and p= 0.057 for MPSIIIA treated versus untreated females, respectively). This result is consistent with the lower levels of sulfamidase activity measured in serum of female animals after iv-AAV9-CAG-mu-SFMD treatment.

Example 6:

Intracisternal delivery of AAV9-CAG-mu-SFMD

A total dose of $5x10^{10}$ vector genomes of the AAV9-CAG-mu-SFMD vector was injected into the cisterna magna of 2-month-old anaesthetized MPSIIIA animals in a total volume of 5 µl.

Three months after the administration, a complete correction of the GAG accumulation was achieved throughout the entire brain of treated animals. *See* Figure 8. Vector-derived sulfamidase expression was also found in the liver of treated animal, suggesting that, after an intracisternal delivery, some vectors reach circulation and are taken up by the liver. In agreement with this result, GAG accumulation was also normalized in the liver.

The intracisternal delivery of the AAV9-CAG-mu-SFMD vector ("ic-AAV9-CAG-mu-SFMD") extended the lifespan of MPSIIIA animals. *See* Figure 12. By 17 months of age, all untreated MPSIIIA males had died while 100% of ic-AAV9-CAG-mu-SFMD treated males were still alive at 20 months of age ($p<$ 0.001 and p= 0.037 for MPSIIIA treated versus untreated males, respectively). *See* Figure 12. The female group showed a similar but less impressive improvement (p= 0.063 and p= 0.057 for MPSIIIA treated versus untreated females, respectively). This result is consistent with the lower levels of sulfamidase activity measured in serum of female animals after ic-AAV9-CAG-mu-SFMD treatment.

Example 7:

Intravenous delivery of AAV9-CAG-co-hu-SFMD (codon optimized human sulfamidase)

The codon usage of the human sulfamidase was optimized in order to reduce the vector administered dose. The object of this approach was to stabilize the sulfamidase mRNA and increase its translation, thus favoring a higher production of sulfamidase from the same vector dose.

10^{12} viral genomes (vg) of the AAV9-CAG-hu-co-SFMD vector were administered intravenously to 2-month-old MPSIIIA mice via tail vein. At least a 3-fold increase in the sufamidase level in the liver was obtained in comparison with the non-optimized gene. *See* Figure 9.

Example 8:

Intravenous delivery of AAV9-hAAT-co-hu-SFMD

Following the same procedure than the one described in example 7, two month-old MPSIIIA mice are treated with 10^{12} vg of AAV9-hAAT-co-hu-SFMD vector by intravenous administration into the tail vein. The sulfamidase level is measured in the same way than for example 7. The results show a substantial increase with respect to the non optimized gene.

Example 9:

Intracisternal delivery of different serotypes of AAV-CAG-GFP-WPRE

To evaluate the tropism in the brain of the different AAV serotypes when administered into the cerebrospinal fluid, $5x10^{10}$ vector genomes of AAV1, AAV2, AAV5, AAV7, AAV8 and AAV9 vectors carrying the GFP reporter gene (CAG-GFP-WPRE construct) were administered to MPSIIIA mice intracisternally.

Significant transduction of cells was achieved in the pons by all serotypes, with the highest and lowest efficacies achieved by AAV9 and AAV1,

respectively. In the cerebellum, the reporter signal was located mostly in axons identified morphologically as mossy fibers, and especially with AAV1 and AAV9, but also with the other serotypes, except for AAV8, in Purkinje neurons. Greater differences in gene transfer efficiency were observed amongst serotypes in distant brain regions. Many cells were transduced in the cerebral cortex, olfactory bulb and hippocampus in the AAV9-injected group, and to a lesser extent in the AAV7 group, whereas no GFP-positive cell bodies were observed with AAV1, AAV2, AAV5 or AAV8 serotypes in these areas. In the hypothalamus, the AAV9 serotype transduced efficiently neurons, and the AAV1 serotype led to a few scattered GFP+ cells. Occasional GFP-positive axons could be observed throughout the whole brain in all groups, which possibly projected from neurons infected near the cisterna magna. AAV9 vectors showed the highest transduction efficiency among the different serotypes. *See* Figure 7.

Example 10:

Scalability of the intracisternal delivery of AAV9-CAG-co-hu-SFMD for clinical use

As a first step toward the potential clinical application of AAV9 intracisternal delivery, whether the pattern of transduction observed in mice was maintained in an animal with a more relevant brain size was evaluated. To this end, $1.5x10^{12}$ vg/kg of AAV9-CAG-GFP-WPRE were delivered to the cisterna magna of healthy Beagle dogs. A total of 4 dogs were injected; in two animals (dogs nos. 1 and 4) a pump was used to infuse the viral vector solution at a flux similar to the rate of CFS formation (1 ml/10 minutes), in the other two (dogs nos. 2 and 3) the vector was infused in a few seconds. Figure 13 shows the immunological detection of GFP in samples from dog 1. A strong labelling was observed in regions close to the cisterna magna, such as the medulla oblongata, the pons and the hypothalamus. *See* Figure 13b, c and d. In the cerebellum, despite being close to the point of injection, only few isolated Purkinje cells were transduced, whereas in the hippocampus, a region far from

the cisterna, efficient transduction of the dentate gyrus occurred. *See* Figure 13 i and j. The distribution of the virus through the CSF allowed the transduction of areas distant to the point of injection, such as the rhinencephalon and the frontal, parietal and occipital cortex, where more superficial areas showed greater transduction. *See* Figure 13e, h and f. Finally, the vector reached also the spinal cord and GFP signal was detected in ventral motoneurons and astrocytes from the nearby ganglia. *See* Figure 13a. The semiquantitative comparison of GFP localization in all four dogs suggested that the rate of infusion of the viral solution did not influence significantly the efficacy or the distribution of the AAV9 vector. *See* Table 1.

Finally, similar to the observations made in mice after intracisternal delivery of AAV9, GFP was also detected in the liver of Beagle dogs, where an average of 3.7% of hepatocytes was transduced. *See* Figure 14. These results suggested systemic distribution of the AAV9 vector after intracisternal delivery.

Brain Area	Dog 1	Dog 2	Dog 3	Dog 4
Frontal cortex	+++	+++	++	+++
Parietal cortex	++++	+++	+++	+++
Occipital cortex	++++	++++	++++	++
Hippocampus	++++	++++	+++	+++
Hypothalamus	++++	N.D.	++++	+
Cerebelum	+	++	++	N.D.
Brainstem	+++	+++	+++	++
Medula oblongata	+++	++++	+++	+++
Spinal cord	+++	++	++	N.D.

Table 1. Semi-quantitative analysis of brain transduction after ic delivery of AAV9-GFP vectors in healthy beagle dogs. Several pictures of each brain area were counted by three independent observers and the average is represented. The semi-quantitative criteria was the following: (+) less than 10 GFP-positive cells/10X microscopic field; (++) 10-30 GFP-positive cells/10X microscopic field;

(+++) 30-60 GFP-positive cells/10X microscopic field and (++++) more than 60 GFP-positive cells/10Xmicroscopic field. N.D. not determined.

Example 11:

Functional efficacy of codon-optimized human sulfamidase (co-hu-SFMD)

Expression cassettes including a codon optimized version of the human sulfamidase cDNA sequence (co-hu-SFMD) were designed and obtained. Codon optimization was performed to increase the efficiency of SFMD protein production in human beings by utilizing the most abundant tRNAs to the species and by also taking into account its particular translation profile. Mice were utilized for experimental purposes due to their similarity to human beings and the predictive capacity of the mouse animal model.

To ensure that this sequence led to the production of active sulfamidase, male MPSIIIA mice were intravenously injected with $1x10^{12}$ vg of an AAV9 vector in which co-hu-SFMD was expressed under the control of the ubiquitous CAG promoter. The activity of sulfamidase in the serum of these mice reached levels similar to that of healthy wild-type animals and was maintained for the duration of the study (2 months). *See* Figure 15a. This sustained sulfamidase activity led to the normalization of the GAG content in the livers of these animals, similar to what had been observed with the AAV9-delivered murine transgene. *See* Figure 15b.

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

sequence list PCT2212.2_ST25

SEQUENCE LISTING

<110> Laboratorios del Dr. Esteve, S.A.
Universitat Autònoma de Barcelona

<120> Vectors and sequences for the treatment of diseases

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<150> EP10382169
<151> 2010-06-10

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CLAIMS

1. An isolated nucleotide sequence having at least 85% sequence identity to SEQ ID NO: 1 that codifies for the protein SEQ ID NO: 2.

2. The isolated nucleotide sequence according to claim 1, wherein said sequence is SEQ ID NO: 1.

3. A gene construction comprising the nucleotide sequence according to any of claims 1 or 2.

4. The gene construction according to claim 3, wherein said gene construction is a vector.

5. The gene construction according to claim 4, wherein said vector is an expression vector.

6. The expression vector according to claim 5, wherein said vector is an adeno-associated vector.

7. The expression vector according to claim 6, wherein the serotype is 1, 2, 5, 7, 8 or 9.

8. The expression vector according to claim 7, wherein the serotype is 9.

9. An expression vector according to claim 6 comprising a CAG promoter operably linked to SEQ ID NO: 1.

10. The expression vector AAV9-CAG-co-hu-SFMD according to claim 9 wherein the adeno-associated vector is serotype 9.

11. The plasmid vector pAAV-CAG-co-hu-SFMD according to claim 10 with accession number DSM 24817.

12. An expression vector according to claim 5 comprising a hAAT promoter operably linked to SEQ ID NO: 1.

13. The plasmid vector pAAV-hAAT-co-hu-SFMD according to claim 12 wherein the adeno-associated vector is serotype 8 or 9.

14. The plasmid vector pAAV9-hAAT-co-hu-SFMD according to claim 13 wherein the adeno-associated vector is serotype 9.

15. A pharmaceutical composition comprising: the nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14.

16. The pharmaceutical composition according to claim 15 for parenteral administration, preferably for intravenous or intracisternal administration.

17. The pharmaceutical composition according to any of claims 15 or 16 comprising a therapeutically effective amount of: the nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14.

18. The pharmaceutical composition according to claim 15 comprising a therapeutically effective amount of the adeno-associated vector of serotype 9 according to claim 9 for intracisternal administration.

19. The nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector

according to any of claims 6 to 14; or the pharmaceutical composition according to any of claims 15 to 18 for use as a medicament.

20. The nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14; or the pharmaceutical composition according to any of claims 15 to 18 for increasing the sulfamidase activity in the body.

21. The nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14; or the pharmaceutical composition according to any of claims 15 to 18 for use as medicament for enzyme replacement or gene therapy, preferably for gene therapy.

22. The nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14; or the pharmaceutical composition according to any of claims 15 to 18 for the treatment of mucopolysaccharidoses, preferably of mucopolysaccharidosis type IIIA or Sanfilippo syndrome.

23. A method of producing the expression vectors according to claim 6 comprising the steps of:

i) providing a first vector comprising the SEQ ID NO: 1 interposed between a first AAV terminal repeat and a second AAV terminal repeat, a CAG or hAAT promoter operably linked to the SEQ ID NO: 1; a second vector comprising an AAV rep gene and a AAV cap gene; and a third vector comprising the adenovirus helper function;

ii) co-transfecting competent cells with the vectors of step i);

iii) culturing the transfected cells of step ii); and

iv) purifying the expression vectors from the culture of step iii).

24. An isolated cell transfected with: the nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14.

25. A method of manufacturing the pharmaceutical compositions of any of claims 15 to 18 which comprises combining the nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14; and a pharmaceutically acceptable vehicle or carrier.

26. A method for treating mucopolysaccharidosis type IIIA comprising administering to a subject having mucopolysaccharidosis type IIIA the nucleotide sequence according to any of claims 1 or 2; the gene construction according to any of claims 3 to 5; or the expression vector according to any of claims 6 to 14; or the pharmaceutical composition according to any of claims 15 to 18.