ADENOASSOCIATED VIRUS VECTORS FOR THE TREATMENT OF MUCOPOLYSACCHARIDOSES.

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 II or Hunter syndrome.

BACKGROUND OF THE INVENTION

The lysosome is an organelle found in the cytoplasm of animal cells that contains more than 50 hydrolases that break down biomolecules during the recycling of worn-out cellular components or after the engulfment of viruses and bacteria. 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 of 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.

Type II mucopolysaccharidoses (MPSII), known also as Hunter syndrome and first descrived by Dr. Charles Hunter, is a chronic, progressive and multisystemic LSDs caused by deficiency or absence of activity of the iduronate-2-sulfatase (IDS) enzyme, encoded by the *IDS* gene and involved in the lysosomal stepwise degradation of the glycosaminoglycans (GAG) heparan sulfate (HS) and dermatan sulfate (DS), leading to their pathological accumulation. See Hunter, Proc R Soc Med. 1917;10(Sect Study Dis Child):104-16. Due to the X-linked recessive inheritance, almost all Hunter patients are males, although some women with Hunter syndrome have been reported in the literature. *See* Mossman *et al.*, Arch Dis Child. 1983;58:911-915, Gullén-Navarro *et al.*, Orphanet J Rare Dis. 2013;25(8):92, Valstar *et al.*, J. Inherit. Metab. Dis. 2008;31(2):240-52.

MPSII is characterized clinically as a childhood-onset, progressive neuropathy of the Central Nervous System (CNS). Hunter children are usually normal at birth and develop symptoms before the age of 2 years. *See* Schwartz *et al.*, Acta Paediatr Suppl. 2007;96:63-70. The clinical course generally begins with slow-progressive cognitive impairment followed by behavioural problems and progressive intellectual decline. Loss of locomotion occurs later. In addition to the neurological symptoms, MPSII patients suffer from non-neurological alterations, including recurrent ear, nose, throat and chest infections, frequent diarrhoea and constipation, cardiac failure, coarse facial features, short stature, progressive joint stiffness and degeneration, skeletal abnormalities which affect mobility, as well as hepato and splenomegaly. *See* Neufeld and Muenzer, "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. The spectrum of clinical manifestations of the disease varies considerably depending on the residual levels of IDS activity that the patient has, which in turn is determined by the underlying mutation of the *IDS* gene, with >300 mutations of the *IDS* gene described to date (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=IDS). In general, two clinical forms of MPSII have been described. The most severe form, with an onset between 18 months and 4 years of age, is three times more common than the mild form, and, is characterized by coarse facial features, skeletal deformities, hepatosplenomegaly and neurological involvement which leads to mental retardation. *See* Wraith *et al.*, Eur J Pediatr. 2008;167(3):267-277. Patients usually die during the second decade of life due to obstructive airway disease and cardiac failure. *See* Wraith *et al.*, Eur J Pediatr. 2008;167(3):267-277, Neufeld and Muenzer, *supra*. A more slowly progressive form of the disease, with later onset, longer survival and minimal

neurological dysfunction, known as the attenuated phenotype, has also been reported in a subset of MPSII patients. *See* Wraith *et al.*, Eur J Pediatr. 2008;167(3):267-277, Neufeld and Muenzer, *supra*.

Until recently there were no specific approved therapies for MPSII syndrome and the only treatment available was symptomatic using a wide range of unspecific drugs for the prevention and management of disease complications. In the last few years, two main therapeutic options have become available: Enzyme Replacement Therapy (ERT) and hematopoietic stem cell transplantation (HSCT). The design of both therapeutic strategies relies on the possibility of cross-correction, based on the fact that normal cells secrete significant amounts of mannose-6-phosphate (M6P)-tagged soluble lysosomal enzymes, such as IDS, which can be subsequently taken up from the extracellular compartment by other cells via M6P receptors on the plasma membrane and targeted to the lysosomes. *See* Enns *et al.*, Neurosurg Focus. 2008;24(3-4):E12. In addition, there is a threshold of residual enzymatic activity, generally very low, above which the cell is capable of coping with substrate influx and subjects are not affected by the disease, suggesting that restoration of normal activity is not a requisite to modify the clinical course. *See* Neufeld, Annu Rev Biochem. 1991;60:257-80.

Since its approval by the Food and Drug Administration (FDA) in 2006 and by the European Medicines Agency (EMA) in 2007, recombinant human iduronate-2-sulfatasa (Idursulfase, ELAPRASE®, Shire Pharmaceuticals) has been indicated for the treatment of patients with MPSII. The treatment is administered weekly at a dose of 0.5 mg/kg by intravenous infusion, with an average infusion time of 1-3 hours. *See* Giugliani *et al.*, Genet Mol Biol. 2010;33(4):589-604. ELAPRASE® was approved after a randomized, double-blind, placebo-controlled study of 96 Hunter patients with no cognitive decline at baseline and with moderately advanced disease. See Muenzer et al., Genet Med. 2006;8(8):465-73, Muenzer et al., Genet Med. 2011;13(2):95-101. After one year of treatment, ELAPRASE®-treated patients showed an increase in the distance walked in six minutes (six-minute walk test) compared to patients on placebo. *See* Muenzer *et al.,* Genet Med. 2011;13(2):95-101. ERT with ELAPRASE® has also been shown to improve joint range of motion (ROM) and to reduce liver and spleen volumes. *See* Muenzer *et al.,* Genet Med. 2011;13(2):95-101. Furthermore, there is evidence of improved pulmonary function when neutralizing antibodies against Idursulfase are not present; development of anti-IDS antibodies was reported in 50% of

the long-term treated patients. *See* Muenzer *et al.,* Genet Med. 2011;13(2):95-101.

A phase I/II study in 31 MPSII patients compared the efficacy of ELAPRASE® with that of a second product based on the beta isoform of Idursulfase with a proposed commercial name of Hunterase® (NCT01301898, http://clinicaltrials.gov). Both proteins were administered intravenously at a dose of 0.5 mg/kg/week for ELAPRASE® and 0.5 and 1.0 mg/kg/week for Hunterase® during 24 weeks. The results from Hunterase® treatment showed reduced urine GAG excretion and improved performance in the 6-minute walking test, but none of the doses was able to mediate therapeutic efficacy in pulmonary function, cardiac function or joint mobility. *See* Sohn *et al.*, Orphanet J Rare Dis. 2013;8:42 . Hunterase® infusions were generally safe and well-tolerated, although a few adverse events, such as urticaria and skin rash, were reported. *See* Sohn *et al.*, Orphanet J Rare Dis. 2013;8:42. A pivotal PIII study has recently been completed (http://clinicaltrials.gov, NCT01645189), but results are not yet available.

Due to hypersensitivity to ELAPRASE®, medical support has to be available during product administration. During the trial, the most severe adverse events described were anaphylactic reactions that could appear anytime during ELAPRASE® infusion or up to 24 hours after product administration. *See* Muenzer *et al.*, Genet Med. 2006;8(8):465-73, Muenzer *et al.,* Genet Med. 2011;13(2):95-101. These anaphylactic reactions, that can compromise the patient's life, include respiratory distress, hypoxia, hypotension, urticaria and/or angioedema of throat or tongue (http://elaprase.com/) and may require interventions such as resuscitation or emergency tracheotomy, and treatment with inhaled beta-adrenergic agonists, epinephrine or intravenous corticosteroids. *See* Burton *et al.,* Mol Genet Metab. 2011;103(2):113-20. Other disadvantages of ERT include: 1) the difficulty of performing 1-3 hour-long intravenous infusions in paediatric patients, many of whom suffer from mental illness (http://elaprase.com/), 2) the fact that 50% of patients treated with ELAPRASE® in clinical studies became positive for antibodies to Idursulfase of yet unknown clinical significance, but which might limit product efficacy in the long-term, as suggested by tests of pulmonary function. *See* Muenzer *et al.*, Mol Genet Metab. 2007;90(3):329-37, Muenzer *et al.*, Genet Med. 2006;8(8):465-73, Muenzer *et al.,* Genet Med. 2011;13(2):95-101, and 3) the high cost of the therapy, which includes also the costs of home-care. *See* Wyatt *et al.*, Health Technol Asses. 2012;16(39):1-543.

Regardless of the safety concerns or the cost of ELAPRASE® administration,

the inability of intravenously administered recombinant IDS to reach the CNS, at least at the currently recommended dose of 0.5 mg/kg per week, likely limits the potential applicability of ERT to the treatment of the severe neurodegeneration observed in Hunter patients. Only a partial rescue of IDS brain activity was achieved by weekly intravenous administration of 1.2 or 10 mg ELAPRASE®/kg to 2 or 7 month-old MPSII mice, respectively. *See* Polito *et al.*, Hum Mol Genet. 2010;19(24):4871-85. Furthermore, even at these high doses, IDS activity in circulation returned to pre-treatment levels 72 hours post-administration of the protein. *See* Polito *et al.*, Hum Mol Genet. 2010;19(24):4871-85. Indeed, intravenous ERT failed to correct GAG accumulation in the brains of a murine model of MPSII. *See* Garcia *et al.*, Mol Genet Metab. 2007;91(2):183-90. Therefore, the indication of ELAPRASE® is limited to the treatment of non-neurological symptoms of the disease.

An alternative to the intravenous delivery of ERT is the provision of the exogenous enzyme directly to the CNS. The administration of 20 µg of recombinant human IDS to the lateral ventricle of 5-month-old MPSII mice every 3 weeks increased IDS activity in cerebrum, cerebellum and somatic organs, such as liver, heart, kidney and testis. *See* Higuchi *et al.,* Mol Genet Metab. 2012;107(1-2):122-8. The restoration of IDS activity led to the recovery of short-term memory and locomotor activity and to a reduction in cellular vacuolation and lysosomal distension in cerebellum, liver and testis. However, therapeutic efficacy was partial, GAG content was not completely normalized and some behavioural alterations remained refractory to the treatment. *See* Higuchi *et al.,* Mol Genet Metab. 2012;107(1-2):122-8. A recent safety and dose ranging study of administration of Idursulfase to the cerebrospinal fluid (CSF) via an intrathecal drug delivery device to directly treat CNS pathology in Hunter patients has demonstrated reductions of approximately 80-90% in CSF GAG levels after 6 months of treatment. *See* Muenzer *et al.*, Genet. Med. 2015; doi:10.1038/gim.2015.36 and www.clincialtrials.gov (NCT00920647). However, the implantation of the permanent intrathecal delivery device that the therapy requires is associated with substantial risks and shortcomings and the therapy itself has a very high economic cost per patient/year.

Another way to reach the CNS by systemic administration is using a molecular Trojan horse. An example of that approach it the insulin Receptor Antibody-Iduronate 2-Sulfatase fusion protein (HIRMAb-IDS), which can cross the blood-brain barrier (BBB) via receptor-mediated transport. Intravenous administration of 3, 10 and 30

mg/kg of HIRMAb-IDS to male juvenile Rhesus monkeys weekly, for 26 weeks, resulted in a HIRMAb-IDS brain uptake of 1% of the total injected dose. *See* Boado *et al.,* Biotechnol Bioeng. 2014;111(11):2317-25. The study also demonstrated safety of the fusion protein, as no infusion-related reaction or immune response was observed.

Hematopoietic stem cell transplantation (HSCT) using bone marrow-derived stem cells (Bone marrow transplantation, BMT) has proven efficient in the treatment of both somatic and neurological pathology in patients with other MPSs. *See* Peters *et al.*, Blood. 1996;87(11):4894-902, Peters and Steward, Bone Marrow Transplant. 2003;31(4):229-39 and Yamada *et al.*, Bone Marrow Transplant. 1998;21(6):629-34. The principle underlying the correction by HSCT is that donor monocytes are able to cross the capillary wall, even at the blood-brain barrier, after which they differentiate into tissue macrophages, microglia in the case of the CNS, and secrete the deficient enzyme for delivery to the various cells. *See* Krivit *et al.*, Cell Transplant. 1995;4(4):385-92. BMT performed in MPSII mice reduced GAG accumulation in a variety of somatic tissues, including liver, spleen and lung, but not in the CNS. *See* Akiyama *et al.*, Mol Genet Metab. 2014;111(2):139-46. When BMT is combined with ERT (0.5 mg Idursulfase/kg/weekly), an additive effect on GAG levels in heart, kidney and lung was observed 7 months after treatment of MPSII mice, but accumulation of GAGs in the CNS remained at pathological levels. *See* Akiyama *et al.*, Mol Genet Metab. 2014;111(2):139-46. However, the evidence for clinical efficacy is not very strong in MPSII patients. The follow-up of 10 Hunter patients who received BMT between 1982 and 1991 showed highly varying degrees of success. *See* Vellodi *et al.*, J Inherit Metab Dis. 1999;22(5):638-48. Four of those patients died before 100 days post-BMT, and 3 more before 7 years after the procedure. Of the 3 patients that survived for more than 7 years after BMT, one of them reported no clinical benefit; a second showed a minimal increase of IDS activity in plasma and the third failed to normalize GAG content despite having a slight increase in IDS activity in plasma. *See* Vellodi *et al.*, J Inherit Metab Dis. 1999;22(5):638-48. The Magnetic Resonance Imaging (MRI) of the brain showed a slight decrease in the number of cystic lesions 2.5 years after BMT in a patient with mild MPSII phenotype. *See* Seto *et al.*, Ann Neurol. 2001;50(1):79-92. However, the same study provided data on another patient with mild phenotype that did not show any improvement under MRI. *See* Seto *et al.*, Ann Neurol. 2001;50(1):79-92. Clinical outcomes appears to be highly variable among Hunter patients, presumably due to various factors; genotype, age at HSCT, patient's clinical status at HSCT, such as degree of neurological impairment, donor status, donor

chimerism, stem cell source, and enzyme activity have all been suggested to influence the long-term outcome. *See* Giugliani *et al.*, Genet Mol Biol. 2010;33(4):589-604, Valayannopoulos *et al.*, Rheumatology. 2011;5:v49-59.

When successful, HSCT can contribute to some degree of clinical benefit at somatic level, decrease behavioural problems and better sleeping patterns, but whether the treatment can mediate any significant improvement of cognitive impairment remains unclear. *See* Giugliani *et al.*, Genet Mol Biol. 2010;33(4):589-604, Valayannopoulos *et al.*, Rheumatology. 2011;5:v49-59. In general, this approach is not recommended for Hunter patients, due to the high rate of morbidity and mortality and the variable neurocognitive benefits. *See* Giugliani *et al.*, Genet Mol Biol. 2010;33(4):589-604.

A plausible explanation to the failure of HSCT is the limited IDS expression in engrafted cells, leading to an insufficient IDS cross-correction in the CNS. Lentiviral vectors encoding for the human IDS gene were used to transduce bone marrow cells prior to their transplantation into MPSII mice. Treated MPSII mice showed improved performance in the T-maze memory test 14 weeks post-transplant. *See* Podetz-Pedersen et al., Mol Ther. 2013;21:s1-s285.

Given the limitations of current therapeutic options for MPSII, alternative approaches are needed. In vivo gene therapy offers the possibility of a one-time treatment for MPSII and other inherited diseases, with the prospect of lifelong beneficial effects. Several gene therapy approaches based on the use of different viral vectors combined with different routes of administration have been tested in animal models of MPSII disease.

Adenoassociated virus (AAV) vector–mediated gene transfer, in particular, is rapidly emerging as the approach of choice for many *in vivo* gene therapy applications, due to the high transduction efficiency and the lack of pathogenicity of these vectors. AAV vectors can transduce post-mitotic cells and several pre-clinical and clinical studies have demonstrated the potential of AAV vector-mediated gene transfer to efficiently drive sustained expression of therapeutic transgenes for a variety of diseases. *See* Bainbridge *et al.*, N Engl J Med. 2008;358(21):2231-9, Hauswirth *et al.*, Hum Gene Ther. 2008;19(10):979-90, Maguire *et al.*, N Engl J Med. 2008;358(21):2240-8, Niemeyer *et al.*, Blood 2009;113(4):797-806, Rivera *et al.*, Blood

2005;105(4):1424-30, Nathawani *et al.*, N Engl J Med. 2011;365(25):2357-65 and Buchlis *et al.*, Blood 2012;119(13):3038-41.

Systemic administration of AAV5-CMV-human IDS vectors to the temporal vein of MPSII mouse pups (p2) resulted in an increase in IDS activity in heart, kidney, liver, lung, muscle and spleen, and a moderated increase in IDS activity in the brain, which led to a reduction in somatic tissue and urinary GAG content up to 18 months post a single vector administration. *See* Polito *et al.*, Am J Hum Genet. 2009;85(2):296-301. Also, this treatment prevented the development of CNS pathology by preventing neurodegeneration, and correcting astrogliosis and inflammation. The evaluation of mice in the Open Field Test 18 moths post AAV injection demonstrated the improvement with treatment in the gross motor phenotype of MPSII mice. *See* Polito *et al.*, Am J Hum Genet. 2009;85(2):296-301.

AAVs of serotype 8 encoding for the human IDS gene under the control of the liver-specific TBG promoter have also been used to treat MPSII. Up to 7 months following the intravenous administration of vectors to 2 month-old MPSII mice, an increase in serum, liver, spleen, lung, heart, kidney and muscle IDS activity was observed, resulting in complete correction of GAG storage in these somatic tissues. *See* Cardone *et al.*, Hum Mol Genet. 2006;15(7):1225-36. However, very high doses (4 x 10^{12} viral genomes/mouse) were required to achieve a slight increase in IDS activity and partial clearance of GAG accumulation in the brain when the vectors were administered intravenously. *See* Cardone *et al.*, Hum Mol Genet. 2006;15(7):1225-36. Similarly, the intravenous administration of AAV8 vectors in which the human IDS gene is under the control of the ubiquitous elongation factor 1-a (EF) promoter to adult MPSII mice demonstrated an increase in IDS activity in liver, heart, spleen and kidney up to 24 weeks after administration, with full correction of GAG accumulation in those organs. *See* Jung *et al.*, Mol Cells. 2010;30(1):13-8. IDS activity in the brain was only increased in the group of animals sacrificed at short-term (6 weeks post-injection); however, this was not sufficient to normalize GAG content in the CNS. *See* Jung *et al.*, Mol Cells. 2010;30(1):13-8.

None of aforementioned approaches has fully restored Iduronate-2-sulfatase activity, achieved full eradication of intracytoplasmic inclusions in the CNS and somatic tissues, or corrected all clinical signs of MPSII. Thus, there is a need for novel approaches to the treatment of MPSII that have better efficacy and safety profiles.

SUMMARY OF THE INVENTION

The present invention provides new nucleotide sequences for the treatment of mucopolysaccharidoses, in particular mucopolysaccharidoses type II (MPSII), or Hunter syndrome.

In a first aspect, the present invention relates to an isolated nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1 and having between 75 % and 90 % identity with SEQ ID NO:2. In particular, the isolated nucleotide sequences according to this first aspect of the invention are selected from SEQ ID NO:5 and SEQ ID NO:8.

In a second aspect, the present invention relates to a plasmid containing a nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1, and in particular a nucleotide sequence having at least 75 % identity with SEQ ID NO:2, preferably having between 75 % and 90 % identity with SEQ ID NO:2.

In a third aspect, the invention provides new recombinant vectors for the treatment of mucopolysaccharidoses type II. Said recombinant vectors are in particular Adeno-associated Virus Vectors (AAV) containing a nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1, and in particular a nucleotide sequence having at least 75 % identity with SEQ ID NO: 2, preferably having between 75 % and 90 % identity with SEQ ID NO:2. In a preferred embodiment, the Adenoassociated Virus Vectors are of serotype 9 (AAV9). The AAV9 vectors of the present invention may further contain a promoter linked to the coding nucleotide sequence in order to control the expression of IDS. A suitable promoter is the CAG promoter, SEQ ID NO: 14.

A further aspect of the present invention relates to a pharmaceutical composition comprising a therapeutically effective amount of the nucleotide sequence or the plasmid or the recombinant vector described herein.

Still, a further aspect of the invention relates to the nucleotide sequences of the invention or a plasmid described herein, or a recombinant vector described herein for use as a medicament, in particular for the treatment of mucopolysaccharidoses type II.

The present invention also provides a method for the production of the plasmids according to the invention, as well as a method for the production of the recombinant vectors according to the invention.

In a further aspect, the invention relates to isolated cells comprising the nucleotide sequence coding for IDS, and in particular a nucleotide sequence having at least 75 % identity with SEQ ID NO: 2, preferably having between 75 % and 90 % identity with SEQ ID NO:2.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Generation of pAAV-CAG-hIDS and AAV-CAG-hIDS. (A) Schematic representation of the plasmid pAAV-CAG-hIDS and its components. (B) Schematic representation of the genome of an Adeno-associated vector containing the hIDS coding sequence.

Figure 2. Generation of pAAV-CAG-ohIDS-version1 and AAV-CAG-ohIDS-version1. (A) Schematic representation of the plasmid pAAV-CAG-ohIDS-version1 and its components. (B) Schematic representation of the genome of an Adeno-associated vector containing the ohIDS-version1 coding sequence.

Figure 3. Generation of pAAV-CAG-ohIDS-version2 and AAV-CAG-ohIDS-version2. (A) Schematic representation of the plasmid pAAV-CAG-ohIDS-version2 and its components. (B) Schematic representation of the genome of an Adeno-associated vector containing the ohIDS-version2 coding sequence.

Figure 4. Generation of pAAV-CAG-omIDS and AAV-CAG-omIDS. (A) Schematic representation of the plasmid pAAV-CAG-omIDS and its components. (B) Schematic representation of the genome of an Adeno-associated vector containing the omIDS coding sequence.

Figure 5. Hydrodynamic delivery of pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2 to healthy mice. Histograms depict iduronate-2-sulfatase (IDS) activity in liver (A) and serum (B) measured 48 hours post administration of 30 µg of the human IDS-encoding plasmids pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2. IDS activity of saline-injected WT mice was set to 100%. Values are means ± SEM of 5 mice per group. * $P<0.05$.

Figure 6. Hydrodynamic delivery of pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2 to MPSII mice. (A, B) Histograms depict iduronate-2-sulfatase (IDS) activity in liver (A) and serum (B) measured 1 week after administration of the human IDS-encoding plasmids pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2. Wild-type and saline-injected MPSII mice were used as controls. IDS activity of WT mice was set to 100%. (C) Quantification of glycosaminoglycan (GAG) content in liver, spleen, heart, urinary bladder and testis 1 week after the administration of the different human IDS-coding plasmids. Values are means ± SEM of 5 mice per group. * $P<0.05$, *** $P<0.001$, **** $P<0.0001$.

Figure 7. Intravascular delivery of AAV9-CAG-hIDS, AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2 to MPSII mice. MPSII mice were injected intravenously with $1x10^{10}$ vg of AAV9 vectors encoding wild-type human IDS (AAV9-CAG-hIDS) or two different versions of optimized human IDS (AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2). Wild-type and untreated MPSII mice were used as controls. (A, B) Histograms depict iduronate-2-sulfatase (IDS) activity in liver (A) and serum (B). IDS activity of WT mice was set to 100%. (C) Quantification of glycosaminoglycan (GAG) content in liver in animals administered with the different human IDS-coding vectors. Values are means ± SEM of 5 mice per group. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

Figure 8. Intra-CSF delivery of AAV9-CAG-hIDS, AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2 to MPSII mice. MPSII mice aged 2 months were injected in the cisterna magna with $5x10^{10}$ vg of AAV9 vectors encoding wild-type human IDS (AAV9-CAG-hIDS) or two different versions of optimized human IDS (AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2). Wild-type (WT), untreated MPSII (MPSII) and MPSII mice administered with non-coding (Null) AAV9 vectors (MPSII-

Null) were used as controls. (A) Iduronate-2-sulfatase activity analysed 1.5 months after vector delivery in different parts of the brain (Sections I-V, in which Section I represents the most rostral part of the brain and Section V represents the most caudal portion). IDS activity of WT mice was set to 100%. (B) Quantification of glycosaminoglycan (GAG) content in the same regions of the brain as in (A). Results are shown as mean ± SEM of 5 mice per group. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Figure 9. Intra-CSF delivery of AAV9-CAG-hIDS, AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2 to MPSII mice. MPSII mice aged 2 months were injected in the cisterna magna with $5x10^{10}$ vg of AAV9 vectors encoding wild-type human IDS (AAV-CAG-hIDS) or two different versions of optimized human IDS (AAV-CAG-ohIDS-version1 and AAV-CAG-ohIDS-version2). Wild-type (WT), untreated MPSII (MPSII) and MPSII mice receiving non-coding (Null) AAV9 vectors (MPSII-Null) were used as controls. (A, B) Iduronate-2-sulfatase activity, expressed as % of WT activity, in liver (A) and serum (B), measured 1.5 months after treatment. (C) Quantification of glycosaminoglycan (GAG) content in somatic organs. Results are shown as mean ± SEM of 5 mice per group. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Figure 10. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). (A) Iduronate-2-sulfatase activity in different parts of the brain (sections I-V) of wild-type (healthy) mice, untreated MPSII mice and MPSII mice administered in the CSF, via intracisternal (IC) injection, with $5x10^{10}$ vg of control non-coding vector (AAV9-Null) or AAV9-CAG-omIDS. WT IDS activity was set to 100%. Analysis was performed 4 months after vector delivery. (B) Quantification of glycosaminoglycans (GAGs) in the same parts of the brain as in (A). Results are shown as means ± SEM of 4-5 mice per group. *** P<0.001, **** P<0.0001 vs. MPSII-Null.

Figure 11. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). (A) Quantification of the signal intensity obtained in different areas of the brain following staining for the lysosomal marker LAMP-2, in wild-type (healthy) mice and MPSII mice administered in the cisterna magna with either

$5x10^{10}$ vg of control non-coding vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS. Analysis was performed 4 months after vector delivery. (B) Activity of other lysosomal enzymes in brain extracts. IDUA, *iduronidase, alpha-L-*, SGSH, *N-sulfoglucosamine sulfohydrolase*, NAGLU, *N-acetylglucosaminidase, alpha*, HGSNAT, *heparan-alpha-glucosaminide N-acetyltransferase*, GALNS *galactosamine (N-acetyl)-6-sulfatase*, GUSB, *glucuronidase, beta*, HEXB, *hexosaminidase B*. Values are means ± SEM of 4-5 mice per group. ** P<0.01, *** P<0.001, **** P<0.0001 vs. MPSII-Null.

Figure 12. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). Quantification of the signal intensity obtained in different areas following staining of brain sections for the lysosomal marker LAMP2. Analysis was performed in wild-type (WT) mice and MPSII littermates 8 months after the latter received an intra-CSF administration of either $5x10^{10}$ vg of control non-coding vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS. Results are shown as mean ± SEM of 2-6 mice per group. *P<0.05 and **P<0.01 vs. MPSII-Null.

Figure 13. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). (A, B) Histograms represent the signal intensity measured following immunostaining for the astrocyte marker GFAP (A) and for the microglial marker BSI-B4 (B) in sections of frontal, parietal, and occipital cortex, superior colliculus, and thalamus from wild-type (healthy) mice, and MPSII mice administered 4 months before in the cisterna magna with either $5x10^{10}$ vg of control non-coding vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS. Results are shown as means ± SEM of 5 mice per group. ** P<0.01, *** P<0.001, **** P<0.0001 vs. MPSII-Null.

Figure 14. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). (A, B) Histograms represent the signal intensity measured following immunostaining for the astrocyte marker GFAP (A) and for the microglial marker BSI-B4 (B) in sections of the encephalon from healthy wild-type (WT) mice and MPSII littermates that received intra-CSF administration of either $5x10^{10}$ vg of control non-coding vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS. Analysis was

performed 8 months after vector delivery. Results are shown as mean ± SEM of 2-6 mice per group. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. MPSII-Null

Figure 15. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). (A,B) Iduronate-2-sulfatase activity, expressed as % of WT activity, in liver (A) and serum (B) of wild-type (healthy) mice, untreated MPSII mice and MPSII mice administered in the CSF with $5x10^{10}$ vg of control non-coding vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS vector at 2 months of age and analysed 4 months later. WT IDS activity was set to 100%. (C) Quantification of glycosaminoglycans (GAGs) in somatic organs. Results are shown as means ± SEM of 4-5 mice per group. ** P<0.01, *** P<0.001, **** P<0.0001 vs. MPSII-Null.

Figure 16. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). (A, B) Iduronate-2-sulfatase activity, expressed as % of WT activity, in lung (A) and heart (B) of healthy wild-type (WT) mice, untreated MPSII (MPSII) mice and MPSII mice administered in the CSF with $5x10^{10}$ vg of control non-coding vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS vector at 2 months of age and analysed 4 months later. (C) Quantification of vector genome copy number/diploid genome (vg/dg) in liver, lung and heart in MPSII mice administered intra-CSF with AAV9-CAG-omIDS. Tissues obtained from an untreated MPSII mouse were used as controls. Results are shown as mean ± SEM of 4-5 mice per group in (A and B). *P<0.05, **P<0.01 and ***P<0.001 MPSII-Null.

Figure 17. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). (A) Wet weight of the liver relative to whole body weight of wild-type (healthy) mice, untreated MPSII mice and MPSII mice administered in the CSF with $5x10^{10}$ vg of control vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS vector at two months of age and analysed 4 months later. (B) Activity of other lysosomal enzymes in liver extracts obtained from the same cohorts of animals as in (A). (C) β-hexosaminidase (β-HEXO) activity, expressed as % of WT activity, in serum of the same animal cohorts as in (A). IDUA, *iduronidase, alpha-L-*, SGSH, *N-sulfoglucosamine sulfohydrolase*, NAGLU, *N-acetylglucosaminidase, alpha*, HGSNAT,

heparan-alpha-glucosaminide N-acetyltransferase, GALNS *galactosamine (N-acetyl)-6-sulfatase*, GUSB, *glucuronidase, beta*, HEXB, *hexosaminidase B*. WT enzyme activities were set to 100%. Values are means ± SEM of 4-5 mice per group. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 vs. MPSII-Null.

Figure 18. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). Locomotor and exploratory activity evaluation thorough the Open Field test in naïve wild-type (healthy) mice, untreated MPSII mice and MPSII mice administered in the CSF with $5x10^{10}$ vg of control vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS vector at two months of age and analysed 4 months later. (A) Time in center, (B) Time in periphery, (C) Entries in center, (D) Latency to center, (E) Total movements, (F) Lines crossed. Values are means ± SEM of 17-22 mice per group. * P<0.05, ** P<0.01 vs. MPSII-Null.

Figure 19. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS). Kaplan-Meier analysis of survival in healthy wild-type (WT) mice, untreated MPSII (MPSII) mice and MPSII mice administered in the CSF with $5x10^{10}$ vg of control non-coding vector (AAV9-Null) or $5x10^{10}$ vg of AAV9-CAG-omIDS vector. N=24 for WT, N=22 for untreated MPSII, N=27 for Null-injected MPSII and N=91 for mice receiving therapeutic vector.

Figure 20. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS) at different doses ($1.58x10^9$, $5x10^9$, $1.58x10^{10}$ and $5x10^{10}$ vg/mouse). (A) Iduronat-2-sulfatase activity in different parts of the brain (sections I-V) of wild-type (healthy) mice, untreated MPSII mice and MPSII mice administered in the CSF via intracisternal (IC) injection with different doses of AAV9-CAG-omIDS at 2 months of age and analysed 1.5 months later. WT IDS activity was set to 100%. (B) Quantification of glycosaminoglycans (GAGs) in the same parts of the brain as in (A). Results are shown as means ± SEM of 5 mice per group. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 vs. untreated MPSII.

Figure 21. Intra-CSF delivery of AAV9 vectors coding for optimized murine Iduronate-2-sulfatase (AAV9-CAG-omIDS) at different doses ($1.58x10^9$, $5x10^9$, $1.58x10^{10}$ and $5x10^{10}$ vg/mouse). (A, B) Iduronate-2-sulfatase activity, expressed as % of WT activity, in liver (A) and serum (B) of wild-type (healthy) mice, untreated MPSII mice and MPSII mice administered in the CSF with different doses of AAV9-CAG-omIDS vector at 2 months of age and analysed 1.5 months later. WT IDS activity was set to 100%. (C) Quantification of glycosaminoglycans (GAGs) in somatic organs. Results are shown as means ± SEM of 5 mice per group. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 vs. untreated MPSII.

DEPOSIT OF MICROORGANISMS

The plasmids pAAV-CAG-hIDS (SEQ ID NO: 3), pAAV-CAG-ohIDS-version1 (SEQ ID NO: 6) and pAAV-CAG-ohIDS-version2 (SEQ ID NO: 9) were deposited on December 18th, 2014, under access number DSM 29866, DSM 29867 and DSM 29868 at the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstraße 7 B, D-38124 Braunschweig, Federal Republic of Germany.

DEFINITIONS

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

The term “% sequence identity” or “% identity” refer to the percentage of nucleotides of a candidate sequence that are identical to the nucleotides in the sequence of reference, 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 or "% identity" is calculated dividing the number of nucleotides that are identical after aligning the sequence of reference and the candidate sequence, by the total number of nucleotides in the sequence of reference and multiplying the result by 100.

The terms "codify" or "coding" refer 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 macromolecule composed of one or more linear chains of amino acids or polypeptides. 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 AAV9 vector to increase iduronate-2-sulfatase (IDS) 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 eradicate the signs and 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 a mammal, 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 cell 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 “recombinant viral vector” or “viral vector” refers to an agent obtained from a naturally-occurring virus through genetic engineering techniques capable of transferring genetic material (e.g. DNA or RNA) of interest to a cell, which results in production of the product encoded by that said genetic material (e.g. a protein polypeptide, peptide or functional RNA) in the target cell.

The term “recombinant plasmid” or “plasmid” refers to a small, circular, double-stranded, self-replicating DNA molecule obtained through genetic engineering techniques capable of transferring genetic material of interest to a cell, which results in production of the product encoded by that said genetic material (e.g. a protein polypeptide, peptide or functional RNA) in the target cell. Furthermore, the term “recombinant plasmid” or “plasmid” also refers to a small, circular, double-stranded, self-replicating DNA molecule obtained through genetic engineering techniques used during the manufacturing of viral vectors as carriers of the recombinant vector genome.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides new nucleotide sequences for the treatment of mucopolysaccharidoses, in particular mucopolysaccharidoses type II (MPSII), or Hunter syndrome.

The nucleotide sequences according to the present invention codify for the protein Iduronate-2-sulfatase (referred to as IDS) as set forth in SEQ ID NO:1, enzyme

involved in the stepwise degradation of the glycosaminoglycans heparan sulphate and dermatan sulphate. The “natural IDS” or “wild type IDS” terms refer in the context of the invention to a nucleotide sequence obtained or produced from host cells using methods known to those skilled in the art, or alternatively chemically synthetized using as starting material the coding sequence (CDS) for IDS of each species. Preferably, “natural IDS” or “wild type IDS” are chemically synthetized from the murine CDS (referred to as mIDS) or from the humans CDS (referred to as hIDS). More preferably the nucleotide sequence hIDS is chemically synthetized from human CDS and corresponds to SEQ ID NO: 2.

According to the present invention, evidence establishes that modified nucleotide sequences, also referred to as optimized nucleotide sequences, mediate the production of greater amounts of protein of interest, likely due to efficient transcription of mRNA or the transcription of a more stable mRNA, or the more efficient usage of codons. These sequences are referred herein also as “optimized sequences”; omIDS and ohIDS, when obtained from murine and human CDS, respectively. Advantageously, optimized sequences according to the present invention are codon optimized sequences.

Thus a first aspect of the invention relates to an isolated nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1 and having between 75 % and 90 % identity with SEQ ID NO:2. In particular, the isolated nucleotide sequence according to the invention has 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 82 %, 85 %, 87 % or 90 % identity with SEQ ID NO:2. In a preferred embodiment, the isolated nucleotide sequences of the present invention are selected from ohIDS-version1 as set forth in SEQ ID NO:5 and ohIDS-version2 as set forth in SEQ ID NO:8.

In accordance with the present invention, the isolated sequences described hereinbefore may be inserted into a multiple cloning site (MCS) of a backbone plasmid. In particular the backbone plasmid is a plasmid that contains the ITRs of the Adeno-associated Virus (AAV), referred to as pAAV herein.

A second aspect of the invention provides a plasmid containing a nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1 and having at least 75 % identity with SEQ ID NO:2. Advantageously, the plasmid according to the present invention has between 75 % and 90 % identity with SEQ ID NO:2. In particular, the plasmid according to the invention contains an isolated

nucleotide sequence having 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 82 %, 85 %, 87 % or 90 % identity with SEQ ID NO:2. In a preferred embodiment, the plasmid of the present invention contains a nucleotide sequence selected from hIDS as set forth in SEQ ID NO:2, ohIDS-version1 as set forth in SEQ ID NO:5 and ohIDS-version2 as set forth in SEQ ID NO:8.

In addition to the isolated sequences mentioned above, the plasmids according to the present invention also include conventional control elements which may be operably linked to the nucleotide sequence in a way that allows its transcription, translation and/or expression in a cell transfected with the plasmids. In particular, the plasmids according to the present invention contain a promoter as control element operably linked to the nucleotide sequence of interest. A great number of promoters, which are native or chimeric, constitutive or inducible, ubiquitous and/or tissue- specific are known in the art. Advantageously, the promoter used in the context of the present invention is the CAG promoter which refers to the combination comprising the cytomegalovirus early enhancer element and the chicken □-actin promoter. It further includes portions of the chicken B-actin and rabbit □-globin introns that confer stability to the mRNA derived from the nucleotide sequence of interest, *See* Alexopoulou A, *et al.*, BMC Cell Biology 2008; 9(2): 1-11. The CAG promoter included in the pAAV plasmids of the present invention has a sequence SEQ ID NO:14. This specific CAG promoter allows a long-lasting expression of the missing enzyme in all areas of the brain and in the liver. As a consequence the lysosomal accumulation of glycosaminoglycan (GAG) is corrected, preventing in this way the neurological and somatic alterations characteristic of MPSII.

In a particularly advantageous embodiment, the plasmid according to the invention is the plasmid pAAV-CAG-hIDS, as set forth in SEQ ID NO:3 with access number DSM 29866.

In another particularly advantageous embodiment, the plasmid according to the invention is the plasmid pAAV-CAG-ohIDS-version1, as set forth in SEQ ID NO:6 with access number DSM 29867.

In another particularly advantageous embodiment, the plasmid according to the invention is the plasmid pAAV-CAG-ohIDS-version2, as set forth in SEQ ID NO:9 with access number DSM 29868.

A third aspect of the invention relates to new recombinant vectors for the treatment of mucopolysaccharidoses type II. It has to be understood that a vector of the present invention is a capsid protein as well as a vector genome contained within, used to transfer a genetic material of interest into a cell. Apart from said genetic material of interest, the genome of the vector may also contain different functional elements that include control elements for transcription such as promoters or operators, transcription factors binding regions or enhancers and control elements for the initiation or termination of translation.

The vectors according to the invention are derived from Adeno-associated viruses (AAV) and are used to transfer the nucleotide sequence of interest into a target cell. They have proved to have a high efficiency in transducing post-mitotic cells in a wide range of tissues. In the context of the present invention, the vectors are used to deliver the human Iduronate-2-sulfatase coding sequence (hIDS of SEQ ID NO: 2) or an optimized version of the human Iduronate-2-sulfatase coding sequence, (ohIDS-version1 of SEQ ID NO: 5 or ohIDS-version2 of SEQ ID NO: 8). An adeno-associated vector is a vector derived from an adeno-associated virus of the family of *parvoviridae*. The adenoassociated virus genome is built of single-stranded deoxyribonucleic acid (ssDNA). These viruses infect mammals but are non-pathogenic (i.e. do not cause disease). They can infect dividing or non-dividing cells, and their tropism changes depending on the serotype. The serotype is the classification of the viruses groups, depending on their capsid antigens. The serotype of adeno-associated virus, determined by its capsid protein, defines the virus tropism and allows its entry into a specific cell type. In the context of the present invention, the AAV has a serotype 1, 2, 5, 7, 8, 9 or 10. Preferably, the AAV is of serotype 9 (AAV9), since it shows the best ability to deliver the genetic material to the brain as well as to peripheral organs upon a single administration to the CSF. The AAV9 vectors of the present invention are composed of the viral capsid of the serotype 9 of human adenoassociated virus and a modified genome, containing the Inverted Terminal Repeats (ITRs) of human adenoassociated virus serotype 2, the CAG promoter, the coding sequence of the human Iduronate-2-sulfatase (hIDS) gene or an optimized version of it (also referred to as nucleotide sequence according to the present invention), and the polyA from the rabbit beta-globin gene.

Thus in this aspect the invention relates to a recombinant AAV containing a nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in

SEQ ID NO:1 and having at least 75 % identity with SEQ ID NO:2. Advantageously, the recombinant AAV according to the invention contains between 75 % and 90 % identity with SEQ ID NO:2. In particular the recombinant AAV according to the invention contains a nucleotide sequence according to the invention having 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 82 %, 85 %, 87 % or 90 % identity with SEQ ID NO:2. In a preferred embodiment, the isolated nucleotide sequences contained in the recombinant AAV of the present invention are selected from hIDS as set forth in SEQ ID NO:2, ohIDS-version1 as set forth in SEQ ID NO:5 and ohIDS-version2 as set forth in SEQ ID NO:8.

In an advantageous embodiment of this aspect, the present invention relates to a recombinant AAV9 containing a nucleotide sequence coding for the protein Iduronate-2-sulfatase IDS as set forth in SEQ ID NO:1 and having at least 75 % identity with SEQ ID NO:2. Preferably, the recombinant AAV9 of the invention contains a nucleotide sequence having between 75 % and 90 % identity with SEQ ID NO:2. In particular the recombinant AAV9 of the invention contain a nucleotide sequence according to the invention having 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 82 %, 85 %, 87 % or 90 % identity with SEQ ID NO:2. In a preferred embodiment, the recombinant AAV9 of the invention contains the nucleotide sequences selected from hIDS as set forth in SEQ ID NO:2, ohIDS-version1 as set forth in SEQ ID NO:5 and ohIDS-version2 as set forth in SEQ ID NO:8.

It has further been surprisingly found that the association, in the same entity, of the AAV9 capsid with a nucleotide sequence coding for the Iduronate-2-sulfatase (IDS), together with a chosen promoter, especially the CAG promoter, allows a long-lasting expression of the missing enzyme in all areas of the brain, in particular when the entity is delivered to the cerebrospinal fluid (CSF) through intracisternal injection. As a consequence the lysosomal accumulation of glycosaminoglycan (GAG) is corrected, preventing by that way the neurological alterations characteristic of the MSPII disease. This effect has been observed even in the olfactory bulb, which is distant from the point of administration of the vectors (cisterna magna). Further the AAV9 vectors according to the invention delivered into the CSF were able to reach the systemic circulation to transduce the liver. The production and secretion of the enzyme by liver cells resulted in an increase of Iduronate-2-sulfatase (IDS) activity in serum, ultimately leading to the reduction of lysosomal pathology in many somatic tissues. This represents a clear advantage of the vectors according to the invention over the

existing approaches that only partially corrected the clinical signs of the disease and usually exert their effect either in the brain or in the systemic circulation, but not in both.

Accordingly the present invention relates to AAV9 vectors containing a CAG promoter linked to a nucleotide sequence coding for protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1.

In particular the AAV9 vectors of the present invention contain a CAG promoter linked to a nucleotide sequence coding for the protein iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1 and having at least 75 % identity with SEQ ID NO:2. Advantageously, the AAV9 vectors of the invention contain a CAG promoter linked to a nucleotide sequence coding for the protein iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1 and having between 75 % and 90 % identity with SEQ ID NO:2. In particular the nucleotide sequence contained in the AAV9 vector according to the invention has 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 82 %, 85 %, 87 % or 90 % identity with SEQ ID NO:2.

In a preferred embodiment, the recombinant vector of the present invention is the AAV9-CAG-hIDS (SEQ ID NO: 4) containing the nucleotide sequence SEQ ID NO:2 operably linked to the CAG promoter of SEQ ID NO:14.

In another preferred embodiment, the recombinant vector of the present invention is the AAV9-CAG-ohIDS-version1 (SEQ ID NO: 7) containing the nucleotide sequence SEQ ID NO:5 operably linked to the CAG promoter of SEQ ID NO:14.

In another preferred embodiment, the recombinant vector of the present invention is the AAV9-CAG-ohIDS-version2 (SEQ ID NO: 10) containing the nucleotide sequence SEQ ID NO:8 operably linked to the CAG promoter of SEQ ID NO:14.

The recombinant vectors of the invention as defined hereinbefore may be obtained from the corresponding plasmids also described hereinbefore by transfection of HEK293 cells using methods known in the state of the art.

Thus the present invention further provides a method for the production of the adenoassociated viral vectors AAV according to the invention, and especially a AAV9. The process comprises the steps of:

i) providing a first plasmid comprising the sequence coding for the protein of interest interposed between a first AAV terminal repeat and a second AAV

terminal repeat, a CAG promoter operably linked to the sequence coding for the protein of interest; a second vector comprising an AAV *rep* gene and a AAV *cap* gene, and a third vector comprising the adenovirus helper function genes;

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

iii) culture of the transfected cells of step ii) for a period of time sufficient to produce viral particles; and

iv) purification of the 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 another preferred embodiment, the competent cells are HEK293 cells. In another preferred embodiment, the AAV ***cap*** genes of the second vector are from the AAV serotype 9.

The invention also provides a method for the preparation of the plasmid according to the invention, comprising the steps of:

i) excising the sequence coding for the protein of interest from the starting plasmid, by digestion, in particular using MluI/EcoRI,

ii) cloning the sequence coding for the protein of interest between two restriction sites of the AAV backbone plasmid pAAV-CAG, hereby obtaining the corresponding plasmid including the sequence coding for the protein of interest.

The present invention contemplates, in an additional aspect, pharmaceutical compositions containing a therapeutically effective amount of the isolated nucleotide sequences described herein, the plasmids as described herein, or the AAV vectors, especially AAV9 vectors, described herein.

Pharmaceutical compositions of the invention comprise the isolated nucleotide sequences described herein, the plasmids as described herein, or the AAV vectors described herein in a pharmaceutically acceptable carrier. The composition may also comprise at least one auxiliary substance. The auxiliary substances can be selected among carriers, excipients, solvents, diluents, or adjuvants. Acceptable carriers, diluent or adjuvants are non-toxic and are preferably inert at the dosage and concentrations employed and include buffers such as phosphate, citrate or other organic acids; antioxidants; low molecular weight polypeptides, proteins such as serum albumin, gelatin or immunoglobulins; hydriophilic polymers; aminoacids; monosaccharides,

disaccharides and other carbohydrates including glucose, mannose or dextrins; chelating agents; sugar alcohols such as mannitol or sorbitol, salt forming couterions such as sodium; and/or non-ionic surfactants such as polyethylene-polyoxypropylene block copolymer (Pluronic F68®) and polyethylene glycol (PEG).

In a preferred embodiment, the pharmaceutical compositions according to the invention are suitable for parenteral administration. Examples of parenteral administration are intravenous, subcutaneous, intracisternal and intramuscular injections. Preferably, the pharmaceutical composition according to the invention is suitable for intravenous or intracisternal administration. Compositions suitable for such parenteral administration include sterile aqueous solutions or dispersions, sterile powders for extemporaneous preparation of sterile solutions or dispersions. Advantageously the pharmaceutical compositions according to the invention are preserved from contaminating action of bacteria and fungi.

The dosage for humans and animals may vary depending on factors that have their basis in the respective species or other factors, such as age, sex, weight or degree of illness and so forth.

Still a further aspect of the present invention relates to the therapeutical use of the isolated nucleotide sequences described herein, the plasmids as described herein, or the AAV vectors, especially AAV9 vectors, described herein. As mentioned above, the isolated nucleotide sequences, the plasmids as described herein, or the AAV vectors, especially AAV9 vectors according to the invention mediate expression of the missing IDS enzyme, thus correcting the lysosomal accumulation of GAGs. This allows correcting all clinical signs of mucopolysaccharidoses type II (MPSII). In this respect, the present invention also concerns the isolated nucleotide sequences described herein, the plasmids as described herein, or the AAV vectors, especially AAV9 vectors, described herein for use as a medicament.

In particular, the invention relates to the isolated nucleotide sequences described herein, the plasmids as described herein, or the AAV vectors, especially AAV9 vectors, described herein for increasing iduronate-2-sulfatase (IDS) activity in the body.

In a further preferred aspect, the present invention relates to the isolated nucleotide sequences described herein, the plasmids as described herein, or the AAV

vectors, especially AAV9 vectors, described herein for treatment of mucopolysaccharidoses type II (MPSII).

In a still further embodiment, the present invention relates to the use of the isolated nucleotide sequences described herein, the plasmids as described herein, or the AAV vectors, especially AAV9 vectors, described herein for the manufacture of a medicament useful for the treatment of mucopolysaccharidoses type II (MPSII).

Another embodiment of the present invention is directed to the method of treatment of mucopolysaccharidoses type II (MPSII), comprising the step of administering at least an isolated nucleotide sequences described herein, a plasmid as described herein, or an AAV vectors, especially AAV9 vectors, described herein to a subject in need thereof.

The present invention further provides an isolated cell comprising the nucleotide sequence coding for Iduronate-2-sulfatase SEQ ID NO: 1. In particular the cell according to the invention comprises a nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO:1 and having at least 75 % identity with SEQ ID NO:2., and advantageously between 75 % and 90 % identity with SEQ ID NO:2. In particular the nucleotide sequence contained in the isolated cell according to the invention has 75 %, 76 %, 77 %, 78 %, 79 %, 80 %, 82 %, 85 %, 87 % or 90 % identity with SEQ ID NO:2.

In a preferred embodiment the cells of the invention comprise the nucleotide sequence SEQ ID NO: 2 coding for Iduronate-2-sulfatase (IDS) SEQ ID NO: 1.

In another preferred embodiment the cells of the invention comprise the nucleotide sequence SEQ ID NO: 5 coding for Iduronate-2-sulfatase (IDS) SEQ ID NO: 1.

In another preferred embodiment the cells of the invention comprise the nucleotide sequence SEQ ID NO: 8 coding for Iduronate-2-sulfatase (IDS) SEQ ID NO: 1.

The following examples are merely illustrative of certain embodiments of the invention and cannot be considered as restricting in any way.

GENERAL PROCEDURES

1. Recombinant AAV Vectors

The AAV vectors described herein were obtained by triple transfection. The materials required for making the vectors were: HEK293 cells (expressing adenoviral E1 genes), helper plasmid providing adenovirus function, plasmid providing AAV ***rep*** genes from serotype 2 and ***cap*** genes from serotype 9 (AAV9) and, finally, the backbone plasmid with AAV2 ITRs and the construct of interest.

To generate Iduronate-2-sulfatase-expressing AAV vectors, the optimized or non-optimized coding sequences of human or murine Iduronate-2-sulfatase were cloned into an AAV backbone plasmid under the control of the ubiquitous hybrid CAG promoter. Large-scale production of plasmids was done using an EndoFree Plasmid Megaprep Kit (Qiagen).

Vectors 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 supplemented with 10% FBS and then co-transfected with: 1) a plasmid carrying the expression cassette flanked by the viral ITRs of serotype 2 AAV (described above); 2) a plasmid carrying the AAV rep2 and the cap9 genes; and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutives cesium chloride gradients using an optimized protocol as previously described. *See* Ayuso E, *et al.*, Gene Ther. 2010;17:503-510. Vectors were dialyzed against PBS + 0.001% Pluronic® F68, filtered, titred by qPCR and stored at -80°C until use.

The vectors of the present invention were constructed according to molecular biology techniques well known in the art.

2. Animals

An Iduronate-2-sulfatase-deficient mouse (MPSII) model was purchased from Taconic (Germantown, NY 12526 USA, Stock TF1838). Affected MPSII and healthy

control mice were inbred from hemizygous males and heterozygous female founders. Genotype was determined on genomic DNA from tail-clipped samples with a PCR analysis that amplifies a sequence encompassing the targeted mutation. The sequences of the respective sense and antisense primers were: Forward Primer: 5'-TTT TGT GTA CTC CAA CCC CG -3' (SEQ ID NO:15), Reverse Primer: 5'- TGT CTC CAT AAC AGC CCA GG -3' (SEQ ID NO:16), Reverse Primer Mutation: 5'- GCC CTC ACA TTG CCA AAG GA -3' (SEQ ID NO:17). Mice were fed ad libitum with a standard diet (Harlan, Tekland) and maintained under a light-dark cycle of 12 h (lights on at 9:00 A.M.).

3. Hydrodynamic delivery of IDS-encoding plasmids to mice

For hydrodynamic delivery of pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2 plasmids, 3-month-old MPSII and wild-type animals received through tail vein injection in <5 seconds a total dose of 30 µg of plasmid in a volume equal to 10% of the body weight of the animal. This technique results in expression of plasmid-encoded transgenes mainly in the liver. *See* Liu *et al.,* Gene Ther. 1990;6(7):1258-66. As control, a cohort of mice received and equal volume of saline solution. Mice were divided into two cohorts, and sacrificed either at 48 hours or 1 week after hydrodynamic injection of the plasmids. Organs were harvested as described in the following section.

4. Vector administration to mice

For intra-CSF delivery of AAV9-CAG-omIDS vectors to mice, a total dose of $5x10^{10}$ vg were injected to the cisterna magna of 2-month-old MPSII animals. A similar cohort of animals was injected with $5x10^{10}$ vg control non-coding (AAV9-Null) vector. At 6 and 10 months of age, i.e. 4 and 8 months post vector administration, mice were anesthetized and tissues were harvested.

For the intravenous delivery of AAV9 vectors containing the wild-type hIDS or either of the optimized versions of the IDS coding sequence to mice, a total dose of

$1x10^{10}$ vg of vector were injected in the tail vein of 3.5-month-old MPSII animals. WT and untreated MPSII animals served as controls. Three weeks after vector administration, mice were anesthetized and tissues were harvested.

5. Sample collection

At sacrifice, animals were deeply anesthetized and then transcardially perfused with 12 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, testicles, urinary bladder, intestine and adipose tissue) were collected and either frozen in liquid nitrogen and stored at -80°C or immersed in formalin for subsequent histological analyses.

6. Iduronate-2-sulfatase activity and glycosaminoglycan quantification

Brain, liver, lung and heart samples were sonicated in Mili-Q water. Serum was analysed unprocessed. Iduronate-2-sulfatase activity was determined with a 4-methylumbelliferone-derived fluorogenic substrate (Moscerdam Substrates, Oegstgeest, NL), as described previously. *See* Voznyi *et* al., J Inher Metab Diss 2001;24:675-680. Brain, liver, lung and heart activity levels were normalized against the total amount of protein, quantified using Bradford protein assay (Bio-Rad, Hercules, CA, US). Serum activity was normalized against volume.

For glycosaminoglycan (GAG) quantification, tissue samples were weighted and then digested with proteinase K and extracts were clarified by centrifugation and filtration. GAG levels were determined in tissue extracts with the Blyscan sulfated glycosaminoglycan kit (Biocolor, Carrickfergus, County Antrim, GB), using chondroitin 4-sulfate as standard. The levels of GAG were normalized to wet tissue weight.

7. Activity of other lysosomal enzymes

Brain and liver samples were sonicated in 500 µl of Mili-Q water and enzyme activities were determined in supernatants using 4-methylumbelliferone-derived fluorogenic substrates. Serum was analysed unprocessed. IDUA activity was assayed in 15 µg of protein incubated for 1 h at 37 °C with 4-methylumbelliferyl α-L-iduronide (Glycosynth). *See* Bacter *et al.,* Blood 2002;99(5)1857-9. SGSH activity was measured as previously described. *See* Karpova *et al.*, J Inherit Metab Dis. 1996;19(3):278–285, Haurigot *et al.*, *supra*. Briefly, 30 µg of protein were first incubated with 4-MU-αGlcNS for 17 hours at 47°C. The second incubation was carried out in the presence of 10 U/ml of α-glucosidase (Sigma-Aldrich) in 0.2% BSA for 24 hours at 37°C. For NAGLU activity, 30 µg of tissue protein extract were incubated with 4-methylumbelliferyl-α-N-acetyl-D-glucosaminide (Moscerdam Substrates) for 3 h at 37°C, as previously described. *See* Marsh *et al.*, Clin Genet. 1985;27(3):258-62, Ribera *et al.*, *supra*. HGSNAT activity was determined from 30 µg of protein extract incubated with Acetylcoenzyme A and 4-methylumbelliferyl-β-D-glucosamine (MU-βGlcNH$_2$, Moscerdam Substrates) for 17 h at 37 °C. *See* Voznyi *et al.*, J Inh Metab Dis 1993;16:465-72. GALNS activity was assayed by a 2-step protocol using 10 µg of protein extract and *4-Methylumbelliferyl β-D-Galactopyranoside-6-sulfate Sodium Sal* (MU-βGal-6S) during the first incubation for 17 h at 37 °C. The second step was carried out adding *P_i-buffer* (0.9M Na_2HPO_4/0.9M NaH_2PO_4 buffer, pH4.3 + 0.02% (w/v) Na-azide) and β-Galactosidase (β-Gal-Ao, Sigma) and incubating the mix for 2 h at 37 °C. *See* van Diggelen *et al.,* Clin Chim Acta 1990;187:131-40. The activity of GUSB enzyme was determined from 10 µg of protein extract incubated with 4-methylumbelliferyl-β-D-glucuronide (Sigma) at 37°C for 1 h. HEXB activity was assayed by incubation of 0.1 µg of protein extract with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma) for 1 h at 37°C. After stopping reactions by increasing the pH, released fluorescence was measured with FLx800 fluorimeter (BioTek Instruments). All brain and liver activities levels were normalized against the total amount of protein, quantified using Bradford protein assay (Bio-Rad, Hercules, CA, US).

8. Histological analysis

Tissues were fixed for 12-24 h in formalin, embedded in paraffin and sectioned. For immunohistochemical detection of LAMP2 in brain, paraffin sections were

subjected to heat-induced epitope retrieval in citrate buffer, pH 6, and then incubated overnight at 4°C with rat anti-LAMP2 antibody (Ab13524; Abcam, Cambridge, UK) diluted at 1:500 and subsequently incubated with biotinylated rabbit anti-rat antibody (Dako, Glostrup, DK) at 1:300. For GFAP immunostaining in brain samples, paraffin sections were incubated overnight at 4°C with rabbit anti-GFAP antibody (Ab6673; Abcam, Cambridge, UK) diluted at 1:1000 and subsequently incubated with biotinylated goat anti-rabbit antibody (31820; Vector Laboratories, Burlingame, CA, USA) at 1:300. LAMP2, and GFAP signals were amplified by incubating sections with ABC-Peroxidase staining kit (Thermo Scientific, Waltham, MA, US) at 1:100 dilution and visualized using 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, US) as a chromogen.

To stain microglial cells in brain samples, paraffin sections were incubated overnight at 4°C with BSI-B4 lectin (L5391; Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:100. BSI-B4 signal was visualized using 3,3-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, US) as a chromogen. Brightfield images were obtained with an optical microscope (Eclipse 90i; Nikon, Tokyo, JP).

The NIS Elements Advanced Research 2.20 software was used to quantify LAMP2, GFAP, and BSI-B4 signals in 3-4 images of each brain region (original magnification, ×20) per animal, using the same signal threshold settings for all animals. Then, the percentage of positive area was calculated, i.e., the area, in pixels, with a positive signal over the total tissue area in the image.

9. Quantification of vector genome copy number in tissues

After an overnight tissue digestion in Proteinase K (0.2 mg/ml), total DNA was isolated with MasterPureDNA Purification Kit (Epicenter). Quantitative PCR with primers and probe specific for the HBB2 sequence (contained in the polyA segment) were used to quantify the vector genome copy numbers in 20 ng of total DNA. Forward primer: 5'-CTT GAG CAT CTG ACT TCT GGC TAA T-3'; reverse primer: 5'-GAT TTG CCC TCC CAT ATG TCC-3'; probe: 5'-CCG AGT GAG AGA CAC AAA AAA TTC CAA CAC-3'. Reference standard curve built by serial dilutions of the linearized plasmid containing polyA sequence was used to interpolate the final values of vg/sample.

10. Open field test

The behavior of 6-month-old mice was analyzed by the Open Field test performed between 9:00 am and 1:00 pm. Animals were placed in the lower left corner of a brightly lit chamber (41 x 41 x 30 cm) crossed by 2 bundles of photobeams (SedaCom32; Panlab) that detected horizontal and vertical movements of the mice. The area surface was divided into three squared concentric regions: center (14 x 14 cm), periphery (27 x 27 cm) and border (41 x 41 cm). Exploratory and motor activities were recorded during the first 3 minutes of the test using a video-tracking system (SmartJunior, Panlab).

11. Statistical analysis

All results are expressed as mean ± SEM. Statistical comparisons were made using one-way ANOVA. Multiple comparisons between control and treatment groups will be made using Dunnett's post test, and between all groups using Tukey's post test. Statistical significance was considered if $P < 0.05$. Kaplan-Meier curves were used to estimate survival and the long-rank test was used for comparisons.

EXAMPLES

Example 1: Construction of pAAV-CAG-hIDS

The CDS for human Iduronate-2-sulfatase was utilized as starting material (NCBI Reference Sequence: NM_000202.6) and chemically synthetized for this purpose (GenScript Inc). The CDS was received cloned inside the plasmid pUC57 (AmpR) flanked by SwaI restriction sites.

The SwaI-SwaI human Iduronate-2-sulfatase CDS fragment was excised from the pUC57 plasmid and subsequently cloned between the MluI and EcoRI restrictions sites of the AAV backbone plasmid pAAV-CAG after rendering the 5' and 3' overhangs

blunt with Klenow fragment (Fermentas). The resulting plasmid was named pAAV-CAG-hIDS (accession number DSM 29866). *See* Figure 1A and SEQ ID NO:3.

The pAAV-CAG plasmid had been previously generated and contained the ITRs from the AAV2 genome, the CAG promoter, and the polyA signal from rabbit β-globin, as well as a multicloning site for cloning of CDSs of interest. 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. *See* Sawicki J *et al.*, Exper Cell Res. 1998;244:367–369, Huang J *et al.*, J Gene Med. 2003;5:900–908, Liu Y *et al.*, Exp Mol Med. 2007; 39(2):170-175.

Example 2: Construction of pAAV-CAG-ohIDS-version1

Expression cassettes including an optimized version of human Iduronate-2-sulfatase cDNA sequence (ohIDS) were designed and obtained. The sequence optimization was performed to maximize the efficiency of Iduronate-2-sulfatase protein production in human beings through elimination of cryptic splice sites and RNA destabilizing sequence elements for increased RNA stability, addition of RNA stabilizing sequence elements, codon optimization and G/C content adaptation, avoidance of stable RNA secondary structures amongst others changes. The CDS for human Iduronate-2-sulfatase (NCBI Reference Sequence: NM_000202.6) was used as starting point for sequence optimization (DNA 2.0 Inc). The optimized CDS was received cloned inside the plasmid pJ204:191476 (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

The MluI/EcoRI optimized human Iduronate-2-sulfatase CDS fragment was excised from the pJ204:191476 plasmid and subsequently cloned between the MluI and EcoRI restrictions sites of the AAV backbone plasmid pAAV-CAG. The resulting plasmid was named pAAV-CAG-ohIDS-version1 (accession number DSM 29867). *See* Figure 2A and SEQ ID NO: 6.

Example 3: Construction of pAAV-CAG-ohIDS-version2

The CDS for human Iduronate-2-sulfatase (NCBI Reference Sequence: NM_000202.6) was subjected to sequence optimization (GeneScript Inc). The optimized CDS was received cloned inside the plasmid pUC57 (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

The pUC57-ohIDS plasmid was digested with MluI and EcoRI to excise the optimized Iduronate-2-sulfatase CDS. Subsequently, this fragment was cloned between the same restriction sites of the pAAV-CAG backbone plasmid to generate the pAAV-CAG-ohIDS-version2 plasmid (accession number DSM 29868). *See* Figure 3A and SEQ ID NO:9.

Example 4: Construction of pAAV-CAG-omIDS

The CDS for murine Iduronate-2-sulfatase (NCBI Reference Sequence: NM_010498.3) was subjected to sequence optimization (GeneArt; Life Technologies). The optimized CDS was received cloned inside the plasmid pMA-RQ (AmpR) flanked by MluI and EcoRI restriction sites at 5' and 3', respectively.

The MluI/EcoRI optimized murine Iduronate-2-sulfatase CDS fragment (SEQ ID NO:11) was excised from the pMA-RQ plasmid and subsequently cloned between the MluI and EcoRI restrictions sites of the AAV backbone plasmid pAAV-CAG. The resulting plasmid was named pAAV-CAG-omIDS. *See* Figure 4A and SEQ ID NO:12.

Example 5: Production of AAV9-CAG-hIDS

Vectors AAV9-CAG-hIDS (SEQ ID NO:4) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. *See* Matsushita *et al.*, Gene Ther. 1998;5(7):938-45, Wright *et al.,* Mol Ther. 2005;12(1)171-8. 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 AAV2 ITRs (pAAV-CAG-hIDS); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a

plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutives cesium chloride gradients using an optimized protocol as previously described. *See* Ayuso *et al.*, Gene Ther. 2010;17(4):503-10. Vectors were dialyzed against PBS + 0.001% Pluronic® F68, filtered, titred by qPCR and stored at -80°C until use. *See* Figure 1B.

Example 6: Production of AAV9-CAG-ohIDS-version1

Vectors AAV9-CAG-ohIDS-version1 (SEQ ID NO:7) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. *See* Matsushita *et al.*, and Wright *et al., 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 AAV2 ITRs (pAAV-CAG-ohIDS-version1); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutives cesium chloride gradients using an optimized protocol as previously described. *See* Ayuso *et al.*, *supra*. Vectors were dialyzed against PBS + 0.001% Pluronic® F68, filtered, titred by qPCR and stored at -80°C until use. *See* Figure 2B.

Example 7: Production of AAV9-CAG-ohIDS-version2

Vectors AAV9-CAG-ohIDS-version2 (SEQ ID NO:10) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. *See* Matsushita *et al.*, and Wright *et al., 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 AAV2 ITRs (pAAV-CAG-ohIDS-version2); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutives cesium chloride gradients using an optimized protocol as previously described. *See* Ayuso *et al.*, *supra*. Vectors were

dialyzed against PBS + 0.001% Pluronic® F68, filtered, titred by qPCR and stored at -80°C until use. *See* Figure 3B.

Example 8: Production of AAV9-CAG-omIDS

Vectors AAV9-CAG-omIDS (SEQ ID NO:13) were generated by helper virus-free transfection of HEK293 cells using three plasmids with modifications. *See* Matsushita *et al.*, and Wright *et al., 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 AAV2 ITRs (pAAV-CAG-omIDS); 2) a plasmid carrying the AAV2 *rep* and the AAV9 *cap* genes (pREP2CAP9); and 3) a plasmid carrying the adenovirus helper functions. Vectors were purified by two consecutives cesium chloride gradients using an optimized protocol as previously described. *See* Ayuso *et al.*, *supra*. Vectors were dialyzed against PBS + 0.001% Pluronic® F68, filtered, titred by qPCR and stored at -80°C until use. *See* Figure 4B.

Example 9: Hydrodynamic injection of pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2 to healthy mice

A total dose of 30 μg of the plasmids pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2 containing different versions of the Iduronate-2-sulfatase expressing cassette were administered to 2-month-old WT mice via tail hydrodynamic tail vein injection. This technique targets expression of the delivered plasmid to the liver. *See* Liu *et al.,* Gene Ther. 1990;6(7):1258-66.

Forty-eight hours post plasmid delivery, a considerable increase over basal levels in Iduronate-2-sulfatase activity was documented in the livers and serums of all the animals administered with Iduronate-2-sulfatase-coding plasmids. In both liver and serum, the levels of activity reached with the expression cassettes containing optimized versions of the Iduronate-2-sulfatase gene were higher than those obtained with the

wild-type gene. Furthermore, in serum, the animals that received the pAAV-CAG-ohIDS-version2 plasmid showed levels of Iduronate-2-sulfatase activity that were statistically higher than those documented with the other 2 plasmids. *See* Figures 5A and 5B.

Example 10: Hydrodynamic injection of pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2 to MPSII mice

A total dose of 30 μg of the plasmids pAAV-CAG-hIDS, pAAV-CAG-ohIDS-version1 and pAAV-CAG-ohIDS-version2 containing different versions of the Iduronate-2-sulfatase expressing cassette were administered to 3-month-old MPSII-affected mice via tail hydrodynamic tail vein injection.

Tissues were harvested 1 week after plasmid delivery. All three Iduronate-2-sulfatase-containing plasmids mediated a substantial increase in Iduronate-2-sulfatase activity with respect to MPSII animals that received saline injection; activities ranged from 1200% to 2200% of WT levels in liver and 2000% to 5700% of WT in serum. The levels of activity reached with the expression cassettes containing codon-optimized versions of the Iduronate-2-sulfatase gene were statistically higher than those mediated by the plasmid containing the wild-type gene. *See* Figures 6A and 6B.

Consistent with the high levels of Iduronate-2-sulfatase activity documented in liver and serum, GAG content was completely normalized in all tissues analysed with all plasmid constructs. *See* Figures 6C.

Example 11: Intravenous delivery of AAV9-CAG-hIDS, AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2 to MPSII mice

MPSII mice (3.5-month-old) received an intravenous injection through the tail vein of $1x10^{10}$ vg of AAV9 vectors containing either the wild-type or the optimized human Iduronate-2-sulfatase sequences. Separate cohorts of age-matched WT and

untreated MPSII mice served as controls. Three weeks after the treatment, animals were sacrificed and blood and liver samples were collected and analysed.

The intravenous administration of AAV9 vectors at a dose of $1x10^{10}$ vg/mouse targets transgene expression mainly to the liver. *See* Wu *et al.*, Mol. Ther. 2006; 14(3):316-27, Inagaki *et al.*, Mol. Ther. 2006; 14(1):45-33. When the activity of Iduronate-2-sulfatase was measured in liver extracts 3 weeks after vector delivery, a clear increase in enzymatic activity was documented in MPSII males that received any of the human IDS-coding vectors. *See* Figure 7A. The increase was, however, significantly higher in the case of the MPSII animals that received the AAV9 vectors containing optimized human Iduronate-2-sulfatase sequences (AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2). The levels of Iduronate-2-sulfatase activity reached in the animals that received vectors with the wild-type sequence were approximately the double of those observed in healthy WT animals. *See* Figure 7A. With the vectors that contain the optimized human IDS sequences the levels of iduronate-2-sulfatase activity were several fold higher than those observed in healthy animals or in MPSII animals treated with vectors containing the wild-type IDS sequence. *See* Figure 7A.

Iduronate-2-sulfatase is a secretable lysosomal enzyme, and as such its expression in the liver is a source of circulating enzyme. *See* Haurigot *et al*, *supra*. Similar to the observations made in liver extracts, the levels of Iduronate-2-sulfatase activity achieved in circulation of MPSII animals treated with the AAV9 vectors containing the optimized human IDS sequences were several fold higher than those observed in healthy animals or in MPSII animals treated with vectors containing the wild-type human IDS sequence. *See* Figure 7B.

Consistent with the high levels of Iduronate-2-sulfatase activity documented in liver and serum, GAG content was completely normalized in the liver of the MPSII mice treated with AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2. GAG levels were, however, only partially reduced in the liver of animals treated with an equal dose of AAV9 vectors carrying the wild-type IDS coding sequence. *See* Figure 7C.

Example 12: Intracisternal delivery of AAV9-CAG-hIDS, AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version 2 to MPSII mice

Two-month-old MPSII mice received an intracisternal injection of $5x10^{10}$ vg of AAV9 vectors containing either the wild-type or the optimized human Iduronate-2-sulfatase sequence in a total volume of 5 µl. Separate cohorts of age-matched WT, untreated MPSII mice and MPSII mice receiving $5x10^{10}$ vg of a non-coding vector (AAV9-CAG-Null) served as controls. At 3.5 months of age, i.e. 1.5 months after treatment, animals were sacrificed and samples were collected and analysed.

When the activity of Iduronate-2-sulfatase was measured in brain extracts, a clear increase in enzymatic activity was documented in MPSII males that received any of the human IDS-coding vectors. *See* Figure 8A. The increase was, however, several folds higher in the case of the MPSII animals that received the AAV9 vectors containing optimized human Iduronate-2-sulfatase sequences (AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2) than in those MPSII animals treated with vectors containing the wild-type IDS sequence. *See* Figure 8A. The levels of Iduronate-2-sulfatase activity reached in the animals that received vectors with optimized human Iduronate-2-sulfatase sequences were almost as high as those of WT, or even higher; in the most rostral part of the brain (Section I) the IDS activity derived from vectors containing optimized human Iduronate-2-sulfatase sequences nearly doubled the levels of WT. *See* Figure 8A.

In agreement with the increase in IDS activity throughout the brain, the accumulation of substrate that characterizes the disease was corrected in the brains of treated MPSII mice, as indicated by the significant reduction in the GAG content. *See* Figure 8B. A full normalization of GAG levels was observed with all constructs in all regions analysed, except for Section V in which the effect of the expression of IDS derived from vectors containing the wild-type IDS sequence was not apparent. *See* Figure 8B.

AAV9 vectors administered to the CSF leak to the periphery and transduce the liver. *See* Haurigot et al., Clin Invest. 2013;123(8):3254-3271, Ribera et al., Hum Mol Genet. 2014;24(7):2078-2095. Accordingly, an increase in Iduronate-2-sulfatase activity was documented in the liver and serum of MPSII mice treated with any of the human IDS-coding vectors. *See* Figures 9A and 9B. The increase was, again, significantly higher in the case of the MPSII animals that received the AAV9 vectors containing optimized human Iduronate-2-sulfatase sequences (AAV9-CAG-ohIDS-version1 and AAV9-CAG-ohIDS-version2) than in those MPSII animals treated with vectors containing the wild-type IDS sequence. *See* Figures 9A and 9B. When the

efficacy of the therapy was evaluated through quantification of the GAG content in different somatic organs, a full normalization of the GAG levels was observed in all tissues of MPSII mice treated with any of the optimized human IDS-coding vectors. *See* Figure 9C. The MPSII mice that received the wild-type human IDS sequence showed, however, a full normalization of GAG content in liver, heart, lung and spleen but only a partial correction in testis, kidney, urinary bladder and adipose tissue. *See* Figure 9C.

Example 13: Intracisternal delivery of AAV9-CAG-omIDS

A total dose of $5x10^{10}$ vector genomes of AAV9-CAG-omIDS vector was injected into the cisterna magna of 2-month-old MPSII animals in a total volume of 5 µl. First, mice were analysed at 6 months of age, i.e. 4 months after vector administration. The intra-CSF delivery of AAV9-CAG-omIDS vectors led to restoration of Iduronate-2-sulfatase activity in all brain areas analysed, reaching levels that averaged 40% of those observed in healthy animals in the different regions. *See* Figure 10A. Four months post vector delivery the substrate accumulation characteristic of the disease was completely reverted in the brains of treated MPSII mice, as indicated by the normalization of GAG content in all brain areas analysed. *See* Figure 10B. Likewise, the size of the lysosomal compartment was completely normalized, as indicated by the quantification of the signal intensity for the immune detection of the lysosomal marker LAMP2. LAMP2 signal is proportional to the size of the lysosomal compartment, which in turn, depends on the amount of accumulated undegraded heparan and dermatan sulphate. *See* Figure 11A. Moreover, the effect of the treatment on lysosomal distension remained stable 8 months after AAV9-CAG-omIDS delivery, indicating long-term efficacy of the therapy. *See* Figure 12.

The disruption of normal lysosomal homeostasis due to the accumulation of undegraded substrate can alter the activity of other lysosomal enzymes different from the one directly affected by the mutation. *See* Ribera *et al.,* Hum Mol Genet. 2014;*doi: 10.1093/hmg/ddu727*. In the brains of untreated MPSII mice or MPSII mice treated with control "Null" vector, the activities of IDUA (iduronidase, alpha-L-), SGSH (N-sulfoglucosamine sulfohydrolase), NAGLU (N-acetylglucosaminidase, alpha), HGSNAT

(heparan-alpha-glucosaminide N-acetyltransferase), GALNS (galactosamine (N-acetyl)-6-sulfatase), GUSB (glucuronidase, beta), HEXB (hexosaminidase B) were altered, but treatment with AAV9-CAG-omIDS returned those activities to the levels observed in healthy WT animals indicating that the vector was capable of restoring lysosomal homeostasis. *See* Figure 11B.

In agreement with the correction of the lysosomal pathology, all signs of inflammation disappeared from the brains of treated MPSII mice. Four months post treatment, the signal intensities for the stainings used to detect astrocytosis (GFAP) and microgliosis (BSI-B4) were similar in treated MPSII mice and in healthy animals in different brain regions, as opposed to the signal documented in MPSII mice administered with the control "Null" AAV9 vector that showed a clear upregulation of these markers of neuroinflammation. *See* Figures 13A and 13B. Furthermore, at 10 months of age, i.e. 8 months after gene transfer, the beneficial impact of AAV9-CAG-omIDS treatment on neuroinflammation -evaluated through staining for both GFAP and BSI-B4- persisted, indicating long-term eradication of neuroinflammation. *See* Figures 14A and 14B.

AAV9 vectors administered to the CSF leak to the periphery and transduce the liver. *See* Haurigot *et al.*, Clin Invest. 2013;123(8):3254-3271, Ribera *et al.,* Hum Mol Genet. 2014;*doi: 10.1093/hmg/ddu727*. Accordingly, an increase in Iduronate-2-sulfatase activity was documented 4 months after gene transfer in the liver and serum of MPSII mice treated with AAV9-CAG-omIDS, reaching levels of approximately 1700% and 700% of the levels observed in healthy animals, respectively. *See* Figures 15A and 15B. When the somatic efficacy of the therapy was evaluated through quantification of the GAG content in different organs, a full normalization was observed in most tissues, including liver, heart, lung, testis, spleen and, adipose tissue, with the exception of kidney and urinary bladder, in which a >50% reduction of GAGs was observed. *See* Figure 15C.

Four months after treatment IDS activity was also increased in lung and was particularly high in heart. *See* Figure 16A and 16B. These two organs, lung and heart, presented very low values of vector genome copy number/diploid genome, indicating lack of efficient transduction of these organs following intra-CSF AAV9-CAG-omIDS delivery at the dose of $5X10^{10}$ vg. *See* Figure 16C. This finding suggested cross-correction of IDS deficiency by uptake of IDS from the circulation.

In agreement with the GAG content data, the weight of the liver was normalized in 6-month-old MPSII mice treated with AAV9-CAG-omIDS at the age of 2 months. *See* Figure 17A. Further demonstration of the potential of intra-CSF AAV9-CAG-omIDS treatment to counteract lysosomal pathology in MPSII mice was provided by the measurement of activity of other lysosomal enzymes in liver extracts. IDUA, SGSH, NAGLU, HGSNAT, GALNS, GUSB, HEXB were considerably altered with respect to WT levels in untreated MPSII mice or in MPSII mice treated with control "Null" vector. Treatment with AAV9-CAG-omIDS completely normalized the activities of all these enzymes. *See* Figure 17B. Furthermore, serum HEXB activity also increases as a consequence of lysosomal pathology, and it was completely normalized following AAV9-CAG-omIDS treatment, *See* Figure 17C, providing evidence of whole-body correction of lysosomal functionality.

The impact of the intra-CSF administration of AAV9-CAG-omIDS on behaviour was assessed at 6 months of age with the Open Field test, which evaluates the general locomotor and exploratory activity of mice in unknown surroundings. Untreated and AAV9-null–treated MPSII mice displayed reduced exploratory activity compared with healthy mice in terms of the time spent in the centre and in the periphery, the number of entries in the centre and in the periphery and the total number of fast movements. Intracisternal administration of AAV9-CAG-omIDS completely corrected behavioural deficits in MPSII mice. *See* Figures 18A-F.

Finally, the therapeutic efficacy of intra-CSF AAV9-CAG-omIDS treatment was evaluated by comparing the survival of untreated and treated MPSII mice. AAV9-CAG-omIDS gene therapy considerably extended the lifespan of MPSII mice. *See* Figure 19. Whilst at 17 months of age all untreated or AAV9-CAG-Null-treated MPSII mice were dead, 76% of MPSII mice receiving AAV9-CAG-omIDS treatment were alive. Moreover, 65% of treated animals were still alive at 22 months of age. The % of wild-type animals alive at this age was 79%. *See* Figure 19.

Example 14: Intracisternal delivery of different doses of AAV9-CAG-omIDS: dose-response study

Four different doses ($1.58x10^9$, $5x10^9$, $1.58x10^{10}$ and $5x10^{10}$ vg/mouse) of AAV9-CAG-omIDS vectors were administered to 2-month-old MPSII animals via intracisternal injection in a total volume of 5 µl.

One and a half months post vector administration, animals were sacrificed and tissues harvested. Iduronat-2-sulfatase activity was measured in different parts of the brain (sections I-V). Activity increased with dose and ranged from 0.8 and 53% of WT activity in the different regions. *See* Figures 20A. A similar dose-response was observed when the effect of the treatment on GAG storage was analysed. However, a complete correction of GAG accumulation was documented only at the two highest doses $1.58x10^{10}$ and $5x10^{10}$ vg/mouse. *See* Figure 20B.

In the liver and serum, activity also increased with dose, ranging from 20% to 4300% in the liver and 0.4% and 1100% in serum. No IDS activity was detectable in serum with the 2 lowest doses ($1.58x10^9$, $5x10^9$ vg/mouse). *See* Figures 21A and 21B. In agreement with the activity data, the measurement of GAG content in peripheral tissues demonstrated a dose-response decrease in GAG content in liver, heart, lung, testis, spleen, kidney, urinary bladder and adipose tissue. Complete or almost complete normalization of GAG levels was achieved in most tissues with the 2 highest doses ($1.58x10^{10}$ and $5x10^{10}$ vg/mouse), with the exceptions of lung and urinary bladder, in which >70% reductions were documented, and kidney, in which an approximately 50% decrease was observed. *See* Figure 21C.

CLAIMS

1. An isolated nucleotide sequence coding for the protein Iduronate-2-sulfatase (IDS) as set forth in SEQ ID NO: 1, wherein said nucleotide sequence is selected from SEQ ID NO: 5 and SEQ ID NO: 8.

2. A plasmid containing a nucleotide sequence according to claim 1.

3. The plasmid according to claim 2, which is pAAV-CAG-ohIDS-version1 with accession number DSM 29867, as set forth in SEQ ID NO: 6.

4. The plasmid according to claim 2, which is pAAV-CAG-ohIDS-version2 with accession number DSM 29868, as set forth in SEQ ID NO: 9.

5. A recombinant adeno-associated Virus Vector of serotype 9, AAV9 vector containing a nucleotide sequence according to claim 1.

6. The recombinant vector according to claims 5, which is the AAV9-CAG-ohIDS-version1 containing the nucleotide sequence SEQ ID NO: 5 linked to the CAG promoter of SEQ ID NO: 14.

7. The recombinant vector according to claim 5, which is the AAV9-CAG-ohIDS-version2 containing the nucleotide sequence SEQ ID NO: 8 linked to the CAG promoter of SEQ ID NO: 14.

8. A pharmaceutical composition comprising a therapeutically effective amount of the nucleotide sequence according to claim 1, the plasmid according to any of claims 2 to 4 or the recombinant vector according to any of claims 5 to 7.

9. The nucleotide sequence according to claim 1, the plasmid according to any of claims 2 to 4 or the recombinant vector according to any of claims 5 to 7 for use in the treatment of mucopolysaccharidosis type II.

10. A method of producing the vectors defined in any of claims 5 to 7 comprising the steps of:
i) providing a first vector comprising the sequence coding for the protein of interest interposed between a first AAV terminal repeat and a second AAV terminal repeat, a CAG promoter operably linked to the sequence coding for the protein of interest; a second vector comprising an AAV rep gene and a AAV cap gene from serotype 9; 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).

11. A method for the preparation of the plasmid according to any of claims 2 to 4, comprising the step of:
i) excising the sequence coding for the protein of interest from the starting plasmid, by digestion, in particular using MluI/EcoRI,
ii) cloning the sequence coding for the protein of interest between two restriction sites of the AAV backbone plasmid pAAV-CAG, hereby obtaining the corresponding plasmid including the sequence coding for the protein of interest.

12. An isolated cell comprising the nucleotide sequence according to claim 1.