NUCLEIC ACID CONSTRUCTS AND GENE THERAPY VECTORS FOR USE IN THE TREATMENT OF WILSON DISEASE

FIELD OF THE INVENTION

The present invention relates to nucleic acid constructs and gene therapy vectors for use in the treatment of Wilson's disease and other conditions.

BACKGROUND ART

The state of the art regarding gene therapy of Wilson's disease was reviewed by Merle et al. (*Current Gene Therapy* 2007; 7: 217-220) and is here summarized and completed with later disclosed references.

Wilson's disease (WD) is an autosomal recessively inherited disorder of copper metabolism with an average prevalence of 1:30,000. WD is caused by mutations of the ATP7B gene coding for a P-type copper transporting ATPase, which is located on chromosome 13. ATP7B is expressed mainly in hepatocytes and functions in the transmembrane transport of copper. Absent or reduced function of ATP7B protein leads to decreased hepatocellular excretion of copper into bile and results in copper accumulation primarily in the liver and subsequently in the neurologic system and other tissues. Failure to incorporate copper into ceruloplasmin is an additional consequence of the loss of functional ATP7B protein.

WD can present clinically as liver disease, as a progressive neurologic disorder, or as psychiatric illness. Patients with hepatic WD usually present in late childhood or adolescence, and exhibit features of acute hepatitis, fulminant hepatic failure, or progressive chronic liver disease. Neurologic manifestations of WD typically present later than the liver disease, most often in the second or third decade and include extrapyramidal, cerebellar and cerebral-related symptoms.

The aim of medical treatment of WD is to remove the toxic deposit of copper from the body and to prevent its reaccumulation. Three anti-copper drugs are currently approved for WD: D-penicillamine, trientine, and zinc salts. Medical therapy is effective in most, but not all patients with WD. Liver transplantation is a therapeutic option in WD patients presenting with fulminant liver failure or progressive liver failure. It has been shown to correct the WD phenotype and provides excellent long-term survival.

However, an interruption of therapy or inadequate treatment can lead to fatalities within few months. Because WD medication has to be taken regularly, adherence to treatment in some patients, especially in adolescent WD patients, is poor.

Under therapy residual neurological symptoms are relatively common and even progressive symptoms can occur. Because current medical treatment options are not in all WD patients effective and adherence to therapy is a problem, a more comprehensive solution could involve gene therapy.

Theoretically, expression of wild type ATP7B in hepatocytes would reverse all disease-related abnormalities and rescue the liver and the neurological symptoms. The ultimate goal of an ideal gene therapy for WD would be to deliver ATP7B, in sufficient quantity, specifically to hepatocytes for a lifelong duration.

All published studies on adenoviral gene transfer for WD have used early-generation adenoviral vectors producing only transient transgene expression. Terada et al. [Terada et al. *J. Biol. Chem.* 1998; 273:1815-1820; Terada et al. *FEBS Lett.* 1999; 448: 53-56] demonstrated successful gene transfer by adenovirus mediated gene delivery in the LEC rat model. Restoration of holoceruloplasmin synthesis, of serum ceruloplasmin oxidase activity, and of copper excretion in bile was shown, indicating a therapeutic effect of the gene transfer. These effects were of a very limited duration, with a maximum level at day three and a decline thereafter. Ha-Hao et al. [*Z. Gastroenterol.* 2002; 40: 209-216] also demonstrated an increased copper content in stool of LEC rats after adenovirus-mediated ATP7B gene transfer, indicating increased copper excretion into the bile. The therapeutic effect was in addition demonstrated by restoration of holoceruloplasmin and of its ferroxidase activity. However, once again the duration of the therapeutic effect in these experiments was only transient with a limited duration of a few days.

Gutless adenoviral vectors have not been tested for this application so far.

Other commonly used non-integrating viral vector system, the adeno-associated virus (AAV), has neither been tested for WD so far, mainly because the ATP7B gene (approximately 4.4 kb large) leaves minimum space for allocating the rest of required sequences (e.g. promoter, poly A signal sequence, etc) within the AAV vector, whose packaging capacity is 4.4-4.7 kb. German patent application DE 100156121A1 (published 2003) proposed a recombinant adeno-associated viral vector for the gene therapy of WD that possesses a shortened metal-sensitive promoter (metallothionein-I promoter) to produce copper or zinc inducible expression of ATP7B transgene. Nevertheless, this document does not provide, nor has been later disclosed, any information regarding the therapeutic efficiency and performance of the vector.

On the other hand, several lentiviral vectors carrying wild type ATP7B have been tested in animal models of WD. Merle et al. [*Scan. J. Gastroenterol.* 2006; 41: 974-982] reported systemic gene therapy in LEC rats with lentiviral vectors expressing ATP7B under the control of a phosphoglycerokinase promoter. Twenty-four weeks after gene transfer liver copper content was lowered significantly and liver histology improved in treated rats compared to untreated controls, but the effect was only partial. Serum ceruloplasmin oxidase activity was increased two weeks after gene transfer when compared to controls, however, it declined to lower levels 24 weeks after treatment. More recently, Roybal et al. [*Gene Therapy* 2012; 19: 1085-1094] have reported early gestational gene transfer in ATP7B$^{-/-}$ mice with a lentivirus carrying human ATP7B under transcriptional control of a liver-specific promoter which contained element of apolipoprotein E and alpha-1 antitrypsin. In utero administration of the vector provided a decrease in liver copper levels, preservation of normal hepatic histology, restoration of copper incorporation into ceruloplasmin and improved cholesterol biosynthesis. However, the efficiency of the treatment was very variable from mice to mice and declined with time and never reached full correction of the different pathologically altered parameters.

SUMMARY OF THE INVENTION

The inventors have engineered and tested several viral vectors carrying transgenes encoding different truncated forms of the enzyme ATP7B: e.g. vector AAV2/8-AAT-ATP7B(d223-366), encoding ATP7B(d223-366) [ATP7B-T1]; and vector AAV2/8-AAT-ATP7B(d57-486), encoding ATP7B(d57-486) [ATP7B-T2]. When administered to ATP7B knockout mice (a recognized animal model of Wilson's disease), the AAV vector carrying ATP7B-T2 corrected main Wilson's disease pathological characteristics for at least 24 weeks after treatment while the AAV vector carrying ATP7B-T1 had only a partial effect. Cu excretion (Cu urine content), and liver Cu content were significantly reduced in Wilson's disease mice treated with the AAV2/8-AAT-ATP7B(d57-486) vector, and ceruloplasmin activity was significantly restored. On the other hand, the administration of the vector resulted in the normalization of serum transaminases levels and of liver histology, together with a marked reduction of the inflammatory infiltrate, biliary duct proliferation and fibrosis.

Furthermore, a dose of 1 x 10^{10} vg / mouse of the AAV2/8-AAT-wtATP7B vector was shown to be a "suboptimal dose" for the wt construct both for the obtaining of a normalization of the serum ceruloplasmin activity and a reduction of Cu accumulation in the liver (Figures 10A and 11A); whereas the vector carrying the truncated form was shown to provide statistically

significant therapeutic effects (vs untreated) at said suboptimal dose (Figure 10B and 11B). Moreover, the observed difference in activity between the full length ATP7B and T2 constructs at a dose of 1×10^{10} vg / mouse was also shown to be statistically significant for these two therapeutic effects (Fig. 12 and Fig.14).

These observations indicated that both a nucleic acid construct encoding the truncated form ATP7B(d57-486) and vectors that carry it, in particular AAV vectors, enable to overcome the most relevant pathological effects of an accumulation of copper linked to a deficiency or dysfunction of ATP7B and thus can be very suitable for gene therapy applied to a condition caused by a deficiency or dysfunction of Copper-transporting ATPase 2, such as Wilson's disease, or a disease and/or condition associated with a decrease of ATP7B-dependent lysosomal exocytosis and copper accumulation. Moreover, unexpectedly the truncated form ATP7B(d57-486) and vectors that carry it, were shown to achieve normalization of some of these pathological manifestations of the disease at dosages where the full length ATP7B protein and vectors encoding the same proved to be less effective.

Therefore, in a first aspect the invention relates to a nucleic acid construct (hereinafter also referred as "nucleic acid construct of the invention"), that comprises: a) a nucleotide sequence of an eukaryotic promoter; b) a nucleotide sequence encoding a truncated Copper-transporting ATPase 2 (ATP7B) in which the N-terminal heavy metal associated sites HMA 1, HMA 2, HMA 3, and HMA 4 are totally deleted and HMA 5 and HMA 6 remain undeleted; and c) a polyadenylation signal sequence.

In another aspect, the invention relates to an expression vector (hereinafter also referred as "expression vector of the invention"), that comprises a nucleic acid construct of the invention.

In another aspect, the invention relates to a host cell comprising a nucleic acid construct or an expression vector of the invention.

In another aspect, the invention relates to a viral particle (hereinafter also referred as "viral particle of the invention"), that comprises a nucleic construct or an expression vector of the invention. Preferably, the nucleic acid construct constitutes the genomic sequence of the viral vector.

In another aspect, the invention relates to a pharmaceutical composition that comprises a product of the invention, i.e. a product that comprises a nucleic acid construct of the invention, and a pharmaceutically acceptable carrier. The term "product of the invention" as used herein refers to and indistinctively covers any of: a) the nucleic acid construct of the invention; b) the

expression vector of the invention, c) the host cell of the invention and d) the viral particle of the invention.

In another aspect, the invention further relates to a kit comprising a nucleic acid construct, vector, host cell, viral particle or pharmaceutical composition of the invention in one or more containers.

In another aspect, the invention relates to a product of the invention for use in medicine (as a medicament or medicinal composition). This use in medicine includes the treatment of a condition caused by a deficiency or dysfunction of Copper-transporting ATPase 2. Said another way, the invention relates to: the use of a product of the invention in the preparation of a medicament for use in the treatment of a condition caused by a deficiency or dysfunction of Copper-transporting ATPase 2; and to a method for the treatment of a condition caused by a deficiency or dysfunction of Copper-transporting ATPase 2 in a subject or a patient, that comprises administering to the subject or patient a therapeutically effective amount of a product of the invention. In a more particular aspect, the product of the invention is used for the treatment of Wilson's disease.

In another aspect, the invention further relates to a pharmaceutical composition comprising a product of the invention as described above, for the proposed uses in medicine and therapeutic methods herein described.

In an even further aspect, the invention relates to a process of producing viral particles of the invention comprising the steps of:

a) culturing a host cell containing a nucleic acid construct or expression vector of the invention in a culture medium; and

b) harvesting the viral particles in the cell culture supernatant and/or inside the cells.

In a related aspect, the present invention relates to the use of the nucleic acid construct of the invention or the expression vector of the invention for the production of viral particles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Schematic representation of the nucleic acid construct of vector AAV2/8-AAT-wtATP7B which carries human ATP7B; vector AAV2/8-AAT-ATP7B(d223-366) which carries truncated form ATP7B(d223-366) [ATP7B-T1]; and vector AAV2/8-AAT-ATP7B(d57-486) which carries truncated ATP7B(d57-486) [ATP7B-T2]. The elements of the constructs are: a) alpha-1-antitrypsin gene promoter (AAT); b) nucleotide sequence encoding respectively human ATP7B, ATP7B-T1, or ATP7B-T2; c) the polyadenylation signal (pA), and

flanking the vector genome d) the inverted terminal repeat sequences of AAV2 (ITRs).

Figure 2: Serum alanine transaminase (ALT) levels in wild type male mice [WT], ATP7B deficient male mice [Wilson's Disease mice, WD], and WD male mice treated with the vectors AAV2/8-AAT-wtATP7B [WD AAV-ATP7B], AAV2/8-AAT-ATP7B(d223-366) [WD AAV-T1]; or AAV2/8-AAT-ATP7B(d57-486) [WD AAV-T2]. A vector dose of 3 $x10^{10}$ vg / mouse was administered when the animals were 6 weeks old. ALT levels were measured 4, 9, 14 and 24 weeks after treatment [Weeks] and is expressed as IU / L (IU: international units). ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 3: Total urine copper content in wild type male mice [WT], Wilson's Disease male mice [WD], and WD male mice treated with the vectors AAV2/8-AAT-wtATP7B [WD AAV-ATP7B], AAV2/8-AAT-ATP7B(d223-366) [WD AAV-T1]; or AAV2/8-AAT-ATP7B(d57-486) [WD ATP7B-T2]. Vector dose: 3 $x10^{10}$ vg / mouse. Copper content was measured 4, 9, 14 and 24 weeks after treatment [Weeks] in 24 hours urine and expressed as nanograms of Cu (ngr / 24h).

Figure 4: Serum ceruloplasmin activity in wild type male mice [WT], Wilson's Disease male mice [WD], and WD male mice treated with the vectors AAV2/8-AAT-wtATP7B [WD AAV-ATP7B], AAV2/8-AAT-ATP7B(d223-366) [WD AAV-T1]; or AAV2/8-AAT-ATP7B(d57-486) [WD ATP7B-T2]. Vector dose: 3 x 10^{10} vg / mouse. Ceruloplasmin activity was measured 4 weeks after treatment and expressed as the absorbance measured at 570 nm of wave-length [Abs (570 nm)]. ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 5. Liver Cu content in wild type male mice [WT], Wilson's Disease male mice [WD], and WD male mice treated with the vectors AAV2/8-AAT-wtATP7B [WD AAV-ATP7B], AAV2/8-AAT-ATP7B(d223-366) [WD AAV-T1]; or AAV2/8-AAT-ATP7B(d57-486) [WD ATP7B-T2]. Vector dose: 3 $x10^{10}$ vg / mouse. Cupper content was determined after sacrificing the animals 24 weeks after treatment by atomic absorption spectroscopy; and expressed as µg / g (Cu µg / g of dry liver tissue). ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 6: Histological images of livers of wild type animals male mice [WT], Wilson's Disease male mice [WD], and WD male mice treated with the vectors AAV2/8-AAT-wtATP7B [WD AAV-ATP7B], AAV2/8-AAT-ATP7B(d223-366) [WD AAV-T1]; or AAV2/8-AAT-ATP7B(d57-486) [WD ATP7B-T2]. Vector dose: 3 $x10^{10}$ vg / mouse. Images were taken after sacrificing the animals (30 weeks of age). **A**: Images of liver sections stained with hematoxylin

and eosin. **B**: Images of histological samples stained by Timm's sulphide silver technique for detection of copper deposits.

Figure 7: Analysis of liver inflammation, Bile duct proliferation and fibrosis. Images of livers of wild type male mice [WT], Wilson's Disease male mice [WD], and WD male mice treated with the vectors AAV2/8-AAT-wtATP7B [WD AAV-ATP7B], AAV2/8-AAT-ATP7B(d223-366) [WD AAV-T1]; or AAV2/8-AAT-ATP7B(d57-486) [WD ATP7B-T2]. Vector dose: 3 x10^{10} vg / mouse. Analysis was performed after sacrificing the animals (30 weeks of age). **CD45**: Images of liver sections immunostained with anti-CD45 for detecting liver inflammatory infiltrates. **PANCK**: Images of liver sections immunostained with anti-PANCK for detecting bile duct proliferation. **SR**: Images of liver sections stained with Sirius red for detecting fibrosis.

Figure 8: Serum alanine transaminase (ALT) levels in wild type female mice [WT], WD female mice [WD], and WD female mice treated with the vector AAV2/8-AAT-ATP7B(d57-486) [WD AAV-T2]. Different groups of 6 weeks old WD female mice were administered different doses of the vectors (respectively 1 x 10^{10}, 3 x 10^{10}, 1 x 10^{11} vg / mouse). ALT levels were measured 4, 9, 14 and 24 weeks after treatment [Weeks] and is expressed as IU / L. ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 9: Urinary Cu Content levels in wild type female mice [WT], WD female mice [WD], and WD female mice treated with the vector AAV2/8-AAT-ATP7B(d57-486) [WD AAV-T2]. Different groups of 6 weeks old WD female mice were administered different doses of the vector (respectively 1 x 10^{10}, 3 x 10^{10}, 1 x 10^{11} vg / mouse). Urinary copper levels were measured 4, 9, 14 and 24 weeks after treatment [Weeks] and is expressed as ngr of Cu in 24 hours urine (ngr / 24h). ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 10: Ceruloplasmin activity in serum was measured in wild type female mice [WT], WD female mice [WD], and WD female mice treated with the vector AAV2/8-AAT-ATP7B(d57-486) [WD+AAV-T2] or the vector AAV2/8-AAT-wtATP7B [WD+AAV-ATP7B]. For each experimental group, different groups of 6 weeks old WD female mice were administered different doses of the vector (respectively 1 x 10^{10}, 3 x 10^{10}, 1 x 10^{11} vg / mouse). Ceruloplasmin activity was measured 4 weeks after treatment and is expressed as the absorbance measured at 570 nm of wave-length [Abs (570 nm)]. ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 11: Liver Cu Content was measured in wild type female mice [WT], WD mice

[WD], and WD female mice treated with the vector AAV2/8-AAT-wtATP7B [WD AAV ATP7B] or the vector AAV2/8-AAT-ATP7B(d57-486) [WD AAV T2]. For each experimental group, different groups of 6 weeks old WD female mice were administered different doses of the vector (respectively 1 x 10^{10}, 3 x 10^{10}, 1 x 10^{11} vg / mouse). Copper concentration was measured 24 weeks after treatment and is expressed as µg / g of dry tissue ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 12: Liver Cu Content in wild type male mice [WT, n=15], WD male mice [WD; n=25], and WD male mice treated with the vector AAV2/8-AAT-wtATP7B [WD AAV ATP7B; n=7] or the vector AAV2/8-AAT-ATP7B(d57-486) [WD AAV T2; n=7]. For each experimental group, WD mice were administered a suboptimal dose of the vector (1 x 10^{10} vg / mouse) when the animals were 6 weeks old. Copper concentration was measured 24 weeks after treatment and is expressed as µg / g of dry tissue ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 13: Liver Cu Content in wild type male mice [WT, n=15], WD male mice [WD; n=25], and WD male mice treated with the vector AAV2/8-AAT-ATP7B(d57-486) [WD AAV T2; n=13] or the vector AAV2/8-AAT-coATP7B(d57-486) [WD AAV coT2; n=4]. For each experimental group, 6 weeks old WD male mice were administered a suboptimal dose of the vector (1 x 10^{10} vg / mouse). Copper concentration was measured 24 weeks after treatment and is expressed as µg / g of dry tissue ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

Figure 14: Ceruloplasmin activity in serum of wild type male mice [WT, n=15], WD male mice [WD; n=25], and WD male mice groups treated with one of the vectors AAV2/8-AAT-wtATP7B [WD AAV ATP7B; n=10], AAV2/8-AAT-coATP7B [WD AAV coATP7B; n=8], AAV2/8-AAT-ATP7B(d57-486) [WD AAV T2; n=13] and AAV2/8-AAT-coATP7B(d57-486) [WD AAV coT2; n=4]. For each experimental group, 6 weeks old WD male mice were administered a suboptimal dose of the vector (1 x 10^{10} vg / mouse). Oxidase activity of ceruloplasmin was measured 4 weeks after treatment and is expressed as the absorbance measured at 570 nm of wave-length [Abs (570 nm)]. ns: no significant; *: $p<0.05$, **: $p<0.01$; ***: $p<0.001$ [Mann-Whitney unpaired test].

DETAILED DESCRIPTION OF THE INVENTION

All terms as used herein in this application, unless otherwise stated, shall be understood in their ordinary meaning as known in the art. Other more specific definitions for certain terms as

used in the present application are as set forth below and are intended to apply uniformly through-out the specification and claims unless an otherwise expressly set out definition provides a broader definition.

The terms "nucleic acid sequence" and "nucleotide sequence" may be used interchangeably to refer to any molecule composed of or comprising monomeric nucleotides. A nucleic acid may be an oligonucleotide or a polynucleotide. A nucleotide sequence may be a DNA or RNA. A nucleotide sequence may be chemically modified or artificial. Nucleotide sequences include peptide nucleic acids (PNA), morpholinos and locked nucleic acids (LNA), as well as glycol nucleic acids (GNA) and threose nucleic acid (TNA). Each of these sequences is distinguished from naturally-occurring DNA or RNA by changes to the backbone of the molecule. Also, phosphorothioate nucleotides may be used. Other deoxynucleotide analogs include methylphosphonates, phosphoramidates, phosphorodithioates, N3'P5'-phosphoramidates and oligoribonucleotide phosphorothioates and their 2'-0-allyl analogs and 2'-0-methylribonucleotide methylphosphonates which may be used in a nucleotide of the invention.

The term "nucleic acid construct" as used herein refers to a man-made nucleic acid molecule resulting from the use of recombinant DNA technology. A nucleic acid construct is a nucleic acid molecule, either single- or double-stranded, which has been modified to contain segments of nucleic acids sequences, which are combined and juxtaposed in a manner, which would not otherwise exist in nature. A nucleic acid construct usually is a "vector", i.e. a nucleic acid molecule which is used to deliver exogenously created DNA into a host cell.

The term "expression vector" or "vector" as used herein refers to a recombinant nucleotide sequence that is capable of effecting expression of a gene (transgene) in host cells or host organisms compatible with such sequences. Together with the transgene, expression vectors typically include at least suitable transcription regulatory sequences and optionally, 3' transcription termination signals. Additional factors necessary or helpful in effecting expression may also be present, such as expression enhancer elements able to respond to a precise inductive signal (endogenous or chimeric transcription factors) or specific for certain cells, organs or tissues.

The term "subject" or "patient" as used herein, refers to mammals. Mammalian species that can benefit from the disclosed methods of treatment include, but are not limited to, humans, non-human primates such as apes; chimpanzees; monkeys, and orangutans, domesticated animals, including dogs and cats, as well as livestock such as horses, cattle, pigs, sheep, and

goats, or other mammalian species including, without limitation, mice, rats, guinea pigs, rabbits, hamsters, and the like.

The term "packaging cells" as used herein, refers to a cell or cell line which may be transfected with a helper vector or virus or a DNA construct, and provides in *trans* all the missing functions which are required for the complete replication and packaging of a viral vector. Typically, the packaging cells express in a constitutive or inducible manner one or more of said missing viral functions.

A NUCLEIC ACID CONSTRUCT OF THE INVENTION

Nucleotide sequence of eukaryotic promoter

As used herein, the term "eukaryotic promoter" refers to a DNA sequence region that initiates transcription of a particular gene, or one or more coding sequences, in eukaryotic cells. A promoter can work in concert with other regulatory regions or elements to direct the level of transcription of the gene or coding sequence/s. These regulatory elements include, without limitation, transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one of skill in the art to act directly or indirectly to regulate the amount of transcription from the promoter, including e.g. attenuators, enhancers, and silencers. The promoter is located near the transcription start site of the gene or coding sequence to which is operably linked, on the same strand and upstream of the DNA sequence (towards the 5' region of the sense strand). A promoter can be about 100–1000 base pairs long. Positions in a promoter are designated relative to the transcriptional start site for a particular gene (i.e., positions upstream are negative numbers counting back from -1, for example -100 is a position 100 base pairs upstream).

The term "core promoter" or "minimal promoter" refers to the minimal portion of a promoter sequence required to properly initiate transcription. It includes the transcription start site (TSS) and elements directly upstream; a binding site for RNA polymerase (RNA polymerase II); and general transcription factors binding sites. Commonly a promoter also comprises a proximal promoter sequence (upstream of the core promoter), that contains other primary regulatory elements (such as enhancers, silencers, boundary elements/insulators); and a distal promoter sequence (downstream of core promoter), that may contain additional regulatory elements, normally with a weaker influence on the level of transcription of the gene.

According to the invention, the eukaryotic promoter sequence is operably linked to the nucleotide sequence encoding the truncated Copper-transporting ATPase 2. As used herein, the

term "operably linked" refers to a linkage of polynucleotide (or polypeptide) elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or transcription regulatory sequence is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous; where it is necessary to join two protein encoding regions, they are contiguous and in reading frame.

According to the invention, the eukaryotic promoter sequence of the nucleic acid construct comprises at least the core promoter and, optionally other regulatory regions or elements of the same gene or of different genes (i.e. hybrid or chimeric promoters).

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the eukaryotic promoter is a constitutive promoter, a tissue specific promoter, or an inducible promoter.

As used herein, a "constitutive" promoter is a promoter that is active in most eukaryotic tissues under most physiological and developmental conditions.

A "tissue specific promoter" is a promoter only active in specific types of tissues or cells. That is to say a tissue specific promoter, in the context of this invention, is one which is more active in one or several particular tissues (for example two, three or four) than in other tissues (i.e. the promoter is capable of driving a higher expression of the coding sequence to which it is operably linked in the tissue(s) for which it is specific than in the others). Typically, the gene down-stream of a "tissue specific" promoter is active to a much higher degree in the tissue(s) for which the promoter is specific than in any other tissue(s). In this case, there may be little or substantially no activity of the promoter in any tissue other than the one(s) for which it is specific.

An "inducible" promoter is a promoter that is physiologically or developmentally regulated, e.g. by the application of a chemical inducer.

Many promoters are known in the art [Sambrook and Russell (Molecular Cloning: a Laboratory Manual; Third Edition; 2001 Cold Spring Harbor Laboratory Press); and Green and Sambrook (Molecular Cloning: a Laboratory Manual, cuarta edición, 2012 Cold Spring Harbor Laboratory Press)].

Suitable tissue specific promoters may be found in the Tissue-Specific Promoter Database, TiProD (Nucleic Acids Research 2006; J4: D104-D107).

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the eukaryotic promoter is a liver specific promoter. In the context of this invention, a "liver specific promoter" is a promoter which is more active in the liver than in any other tissue of the body. Typically, the activity of a liver specific promoter will be considerably greater in the liver than in other tissues. For example, such a promoter may be at least 2, at least 3, at least 4, at least 5 or at least 10 times more active (for example as determined by its ability to drive the expression in a given tissue in comparison to its ability to drive the expression in other cells or tissues). Accordingly, a liver specific promoter allows an active expression in the liver of the gene linked to it and prevents its expression in other cells or tissues.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the eukaryotic promoter is a nucleotide sequence of the α1-antitrypsin gene promoter (AAT), or a chimeric promoter sequence EalbPa1AT that comprises an α1-antitrypsin gene promoter sequence (AAT or Pa1AT) combined with an albumin gene enhancer element (Ealb). Both promoter sequences have properties of liver specific promoters.

In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the eukaryotic promoter sequence is the sequence delimited by bases 156..460 of SEQ.ID.NO.1 (AAT); or SEQ.ID.NO.5 (EalbPa1AT).

Truncated Copper-transporting ATPase 2 (ATP7B)

Copper-transporting ATPase 2 (ATP7B) is a P-type cation transport ATPase that functions exporting copper out of the cells.

The gene that encodes human enzyme is located at chromosome 13 (chromosome location 13q14.3; gene name ATP7B). Information on human ATP7B polypeptide (amino acid sequences, structure, domains and other features) is for example available at Uniprot with Accession number: P35670 (http://www.uniprot.org/uniprot/P35670; Entry version 168 (03 Sep 2014), Sequence version 4 (16 Jun 2009)). Information on the ATP7B gene encoding this enzyme is available at Entrez with accession number Gene ID: 540 (http://www.ncbi.nlm.nih.gov/gene/540; updated on 19-Sep-2014). 4 isoforms produced by alternative splicing have been described for ATP7B; isoform 1 (identifier P35670-1, 1465 amino acids long) is chosen as the canonical sequence.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleotide acid construct of the invention comprises a nucleotide sequence that encodes a truncated form of a human ATP7B, preferably a human ATP7B whose amino acid sequence is the canonical sequence (SEQ.ID.NO.2), herein also referred to as wtATP7B.

Several conserved motifs are present in ATP7B that are characteristic for the P-type ATPase protein family. These motifs are required for ATP catalysis and include the nucleotide binding domain (N-domain), the phosphorylation domain (P-domain) and the actuator domain (A-domain). Highly conserved signature residues are present in these motifs; SEHPL in the N-domain, DKTG in the P-domain, and TGE in the A-domain. The amino terminal tail of human ATP7B contains "six metal binding sites" (MBS), also indistinctively named as "heavy metal associated (HMA)" sites or domains, each containing the core sequence MxCxxC. These HMA bind Cu(I) in a stoichiometry of one atom of Cu(I) per HMA. These amino-terminal HMAs of ATP7B are required for several aspects of its function, including copper translocation, incorporation of copper in cuproenzymes, ATPase activity, localization and trafficking, and protein–protein interactions. The HMA sites are identified starting at the amino end, as domains HMA 1 (amino acids 59 – 125 in the canonical sequence), HMA 2 (amino acids 144 – 210), HMA 3 (258 – 327), HMA 4 (360 – 426), HMA 5 (489 – 555), and HMA 6 (565 – 631).

According to the invention, optionally in combination with one or more features of the various embodiments described above or below, the nucleic acid construct comprises a nucleotide sequence that encodes a truncated ATP7B in which the N-terminal heavy metal associated sites HMA 1, HMA 2, HMA 3, and HMA 4 are totally or partially deleted.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleotide sequence that encodes truncated ATP7B keeps the 56 amino acids of N-terminal signal sequence of ATP7B.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the deletion in truncated ATP7B comprises amino acids 57 to 486 of the canonical sequence.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleotide sequence encodes a truncated ATP7B whose amino acids sequence is SEQ.ID.NO.7.

Because of the codons redundancy, there are numerous nucleotide sequences that can be generated encoding ATP7B polypeptides with same amino acids sequence.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleotide sequence encoding the truncated Copper-transporting ATPase 2 is the coding sequence CDS of SEQ.ID.NO.6, bases 473..3580.

In another embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleotide sequence encoding the truncated Copper-transporting ATPase 2 is SEQ.ID.NO.8, a sequence with an optimized codon usage bias for the human cells.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleotide sequence encoding the truncated Copper-transporting ATPase 2 is a sequence wherein at least 827, at least 879, at least 931, or at least 983 of the codons encoding truncated Copper-transporting ATPase 2 are identical to the codons of coding sequence SEQ.ID.NO.8.

Polyadenylation signal sequence

As used herein, the term "polyadenylation signal" or "poly(A) signal" refers to a specific recognition sequence within 3' untranslated region (3' UTR) of the gene, which is transcribed into precursor mRNA molecule and guides the termination of the gene transcription. Poly(A) signal acts as a signal for the endonucleolytic cleavage of the newly formed precursor mRNA at its 3'-end, and for the addition to this 3'-end of a RNA stretch consisting only of adenine bases (polyadenylation process; poly(A) tail). Poly(A) tail is important for the nuclear export, translation, and stability of mRNA. In the context of the invention, the polyadenylation signal is a recognition sequence that can direct polyadenylation of mammalian genes and/or viral genes, in mammalian cells.

Poly(A) signals typically consist of a) a consensus sequence AAUAAA, which has been shown to be required for both 3′-end cleavage and polyadenylation of premessenger RNA (pre-mRNA) as well as to promote downstream transcriptional termination, and b) additional elements upstream and downstream of AAUAAA that control the efficiency of utilization of AAUAAA as a poly(A) signal. There is considerable variability in these motifs in mammalian genes.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the polyadenylation signal sequence of the nucleic acid construct of the invention is a polyadenylation signal sequence of a mammalian gene or a viral gene. Suitable polyadenylation signals include, among others, a SV40 early polyadenylation

signal, a SV40 late polyadenylation signal, a HSV thymidine kinase polyadenylation signal, a protamine gene polyadenylation signal, an adenovirus 5 EIb polyadenylation signal, a growth hormone polydenylation signal, a PBGD polyadenylation signal, *in silico* designed polyadenylation signal (synthetic) and the like.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the polyadenylation signal sequence of the nucleic acid construct is a synthetic poly(A) signal sequence which is also capable of directing and effecting the endonucleolytic cleavage and polyadenylation of the precursor mRNA resulting from the transcription of nucleotide sequence coding for truncated ATP7B.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the polyadenylation signal sequence of the nucleic acid construct is the synthetic poly(A) signal sequence delimited by bases 4877..4932 of SEQ.ID.NO.1.

Other nucleotide elements

In one embodiment, the nucleic acid construct of the invention constitutes the recombinant genome of an expression vector for gene therapy, the expression vector of the invention; and more particularly of a viral vector for gene therapy.

Thus, in one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleic acid construct of the invention further comprises a 5'ITR and a 3'ITR of a virus.

As used herein the term "inverted terminal repeat (ITR)" refers to a nucleotide sequence located at the 5'-end (5'ITR) and a nucleotide sequence located at the 3'-end (3'ITR) of a virus, that contain palindromic sequences and that can fold over to form T-shaped hairpin structures that function as primers during initiation of DNA replication. They are also needed for viral genome integration into host genome; for the rescue from the host genome; and for the encapsidation of viral nucleic acid into mature virions. The ITRs are required in *cis* for the vector genome replication and its packaging into the viral particles.

In one embodiment, the nucleic acid construct comprises a 5'ITR, a ψ packaging signal, and a 3'ITR of a virus. "ψ packaging signal" is a cis-acting nucleotide sequence of the virus genome, which in some viruses (e.g. adenoviruses, lentiviruses ...) is essential for the process of packaging the virus genome into the viral capsid during replication.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleic acid construct comprises a 5'ITR and a 3'ITR of a virus selected from the group consisting of parvoviruses (in particular adeno-associated viruses), adenoviruses, alphaviruses, retroviruses (in particular gamma retroviruses, and lentiviruses), herpesviruses, and SV40; in a preferred embodiment the virus is an adeno-associated virus (AAV), an adenovirus (Ad), or a lentivirus.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleic acid construct comprises a 5'ITR and a 3'ITR of an AAV.

The AAV genome is composed of a linear, single-stranded DNA molecule which contains 4681 bases (Berns and Bohenzky, (1987) Advances in Virus Research (Academic Press, Inc.) 32:243-307). The genome includes inverted terminal repeats (ITRs) at each end which function in *cis* as origins of DNA replication and as packaging signals for the virus. The ITRs are approximately 145 bp in length. The internal non-repeated portion of the genome includes two large open reading frames, known as the AAV rep and cap genes, respectively. These genes code for the viral proteins involved in replication and packaging of the virion. In particular, at least four viral proteins are synthesized from the AAV rep gene, Rep 78, Rep 68, Rep 52 and Rep 40, named according to their apparent molecular weight. The AAV cap gene encodes at least three proteins, VP1, VP2 and VP3. For a detailed description of the AAV genome, see, e.g., Muzyczka, N. (1992) Current Topics in Microbiol. and Immunol. 158:97-129.

The construction of recombinant AAV virions is generally known in the art and has been described for instance in US 5,173,414 and US5,139,941; WO 92/01070, WO 93/03769, (Lebkowski et al. (1988) Molec. Cell. Biol. 8:3988-3996; Vincent et al. (1990) Vaccines 90 (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) Current Opinion in Biotechnology 3:533-539; Muzyczka, N. (1992) Current Topics in Microbiol. and Immunol. 158:97-129; and Kotin, R. M. (1994) Human Gene Therapy 5:793-801.

The invention may be carried out by using ITRs of any AAV serotype, including AAV1, AAV2, AAV3 (including types 3A and 3B), AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12, avian AAV, bovine AAV, canine AAV, equine AAV, ovine AAV, and any other AAV serotype now known or later discovered.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleic acid construct comprises a 5'ITR and a 3'ITR of an AAV of a serotype selected from the group consisting of an AAV1, an AAV2, and

an AAV4. In a preferred embodiment the nucleic acid construct comprises the ITR sequences delimited by bases 1..141, and bases 4968..5107 of SEQ.ID.NO.1, that are the ITRs sequences of an AAV2.

The ITRs are the only AAV viral elements which are required in *cis* for the AAV genome replication and its packaging into the viral particles.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the nucleic acid construct comprises a 5'ITR, a ψ packaging signal, and a 3'ITR of an adenovirus of any of the serotypes within any of the classification sub-groups (A-F). In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, these 5'ITR, ψ signal, and 3'ITR sequences come from a sub-group C adenovirus, more preferably from an adenovirus of serotype 2 (Ad2) or serotype 5 (Ad5).

On the other hand, in other embodiments the invention can be carried out by using synthetic 5'ITR and/or 3'ITR; and also by using a 5'ITR and a 3'ITR which come from viruses of different serotype.

All other viral genes required for viral vector replication can be provided in *trans* within the virus-producing cells (packaging cells) as described below. Therefore, their inclusion in the nucleic acid construct of a viral vector genome according to the invention is optional.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the expression vector is an AAV vector.

In a particular embodiment, the nucleic acid construct of the invention constitutes an AAV vector selected from the group of combinations consisting of

a) a vector that comprises a 5'ITR and a 3'ITR nucleotide sequences of an AAV2, an AAT promoter sequence, and a nucleotide sequence encoding truncated human ATP7B(d57-486);

b) a vector that comprises a 5'ITR and a 3'ITR nucleotide sequences of an AAV2, an AAT promoter sequence, and the codon optimized nucleotide sequence SEQ.ID.NO.8 encoding truncated human ATP7B(d57-486);

c) a vector that comprises a 5'ITR and a 3'ITR nucleotide sequences of an AAV2, an EalbPa1AT hybrid promoter sequence, and a nucleotide sequence encoding truncated human ATP7B(d57-486); and

d) a vector that comprises a 5'ITR and a 3'ITR nucleotide sequences of an AAV2, an EalbPa1AT hybrid promoter sequence, and a codon optimized nucleotide sequence SEQ.ID.NO.8 encoding truncated human ATP7B(d57-486).

Each of these AAV vector embodiments also includes a polyadenylation signal sequence, such as synthetic poly(A) signal sequence of SEQ.ID.NO.1 or any other suitable poly(A) signal; together or not with other optional nucleotide elements.

In another embodiment, optionally in combination with one or more features of the various embodiments described above or below, the expression vector is an adenoviral vector. This adenoviral vector according to the invention can be, in particular, a first-, second-, or third-generation adenovirus [see Adenovirus. Methods and Protocols. Chillón M. and Bosch A. (Eds); third Edition; 2014 Springer], or any other adenoviral vector system already known or later described.

In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the viral vector of the invention is a "third generation adenovirus", which may also be referred to as "gutless adenovirus", "helper-dependent adenovirus (HD-Ad)", or "high capacity adenovirus (HC-Ad)". A third generation adenovirus has all viral coding regions removed (gutless); it depends on a helper adenovirus to replicate (helper-dependent); and it can carry and deliver into the host cell up to 36 Kbp inserts of foreign genetic material (high-capacity). A gutless adenovirus keeps the inverted terminal repeats ITRs (5' and 3') and the packaging signal (ψ).

The nucleic acid construct and expression vector of the invention herein described can be prepared and obtained by conventional methods known to those skilled in the art: Sambrook and Russell (Molecular Cloning: a Laboratory Manual; Third Edition; 2001 Cold Spring Harbor Laboratory Press); and Green and Sambrook (Molecular Cloning: a Laboratory Manual; Fourth Edition; 2012 Cold Spring Harbor Laboratory Press).

A VIRAL PARTICLE OF THE INVENTION FOR GENE THERAPY

The terms "viral particle", and "virion" are used herein interchangeably and relate to an infectious and typically replication-defective virus particle comprising the viral genome (i.e. the nucleic acid construct of the expression viral vector) packaged within a capsid and, as the case may be, a lipidic envelope surrounding the capsid.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the virion of the invention is a "recombinant AAV

virion" or "rAAV virion" obtained by packaging of a nucleic acid construct of an AAV vector according to the invention in a protein shell.

Proteins of the viral capsid of an adeno-associated virus (capsid proteins VP1, VP2, and VP3) are generated from a single viral gene (cap gene). Differences among the capsid protein sequences of the various AAV serotypes result in the use of different cell surface receptors for cell entry. In combination with alternative intracellular processing pathways, this gives rise to distinct tissue tropisms for each AAV serotype.

In a particular embodiment, a recombinant AAV virion according to the invention may be prepared by encapsidating the nucleic acid construct of an AAV vector/genome derived from a particular AAV serotype on a viral particle formed by natural Cap proteins corresponding to an AAV of the same particular serotype. Nevertheless, several techniques have been developed to modify and improve the structural and functional properties of naturally occurring AAV viral particles (Bünning H et al. *J Gene Med* 2008; 10: 717–733). Thus, in another AAV viral particle according to the invention the nucleotide construct of the viral vector flanked by ITR(s) of a given AAV serotype can be packaged, for example, into: a) a viral particle constituted of capsid proteins derived from the same or different AAV serotype [e.g. AAV2 ITRs and AAV5 capsid proteins; AAV2 ITRs and AAV8 capsid proteins; etc]; b) a mosaic viral particle constituted of a mixture of capsid proteins from different AAV serotypes or mutants [e.g. AAV2 ITRs with AAV1 and AAV5 capsid proteins]; c) a chimeric viral particle constituted of capsid proteins that have been truncated by domain swapping between different AAV serotypes or variants [e.g. AAV2 ITRs with AAV5 capsid proteins with AAV3 domains]; or d) a targeted viral particle engineered to display selective binding domains, enabling stringent interaction with target cell specific receptors [e.g. AAV4 ITRs with AAV2 capsid proteins genetically truncated by insertion of a peptide ligand; or AAV2 capsid proteins non-genetically modified by coupling of a peptide ligand to the capsid surface].

The skilled person will appreciate that the AAV virion according to the invention may comprise capsid proteins from any AAV serotype. In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the viral particle comprises capsid proteins of an AAV. In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the AAV viral particle comprises capsid proteins from a serotype selected from the group consisting of an AAV1, an AAV5, an AAV7, an AAV8, and an AAV9 which are more suitable for delivery to the liver cells (Nathwani et al. *Blood* 2007; 109: 1414–1421; Kitajima et al.

Atherosclerosis 2006; 186:65-73). In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the viral particle comprises a nucleic acid construct of invention wherein the 5'ITR and 3'ITR sequences of the nucleic acid construct are of an AAV2 serotype and the capsid proteins are of an AAV8 serotype.

In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the AAV viral particle comprises capsid proteins from Anc80, a predicted ancestor of viral AAVs serotypes 1, 2, 8, and 9 that behaves as a highly potent gene therapy vector for targeting liver, muscle and retina (Zinn et al. *Cell Reports* 2015; 12:1–13). In a more particular embodiment, the viral particle comprises the Anc80L65 VP3 capsid protein (Genbank accession number: KT235804).

Viral-glycan interactions are critical determinants of host cell invasion. In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the AAV viral particle comprises capsid proteins comprising one or more amino acids substitutions, wherein the substitutions introduce a new glycan binding site into the AAV capsid protein. In a more particular embodiment, the amino acid substitutions are in amino acid 266, amino acids 463-475 and amino acids 499-502 in AAV2 or the corresponding amino acid positions in AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV 8, AAV9, AAV10 or any other AAV serotype, also included Anc80 and Anc80L65.

The introduced new glycan binding site can be a hexose binding site [e.g. a galactose (Gal), a mannose (Man), a glucose (Glu) or a fucose (fuc) binding site]; a sialic acid (Sia) binding site [e.g. a Sia residue such as is N-acetylneuraminic acid (NeuSAc) or N-Glycolylneuraminic acid (NeuSGc)]; or a disaccharide binding site, wherein the disaccharide is a sialic acid linked to galactose, for instance in the form of Sia(alpha2,3)Gal or Sia(alpha2,6)Gal. Detailed guidance to introduce a new binding site from an AAV serotype into a capsid protein of an AAV of another serotype is given on international patent publication WO2014144229 and in Shen et al. (J. Biol. Chem. 2013; 288(40):28814–28823). In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the Gal binding site from AAV9 is introduced into the AAV2 VP3 backbone resulting in a dual glycan-binding AAV strain which is able to use both HS and Gal receptors for cell entry. Preferably, said dual glycan-binding AAV strain is AAV2G9. Shen et al. generated AAV2G9 by substituting amino acid residues directly involved and immediately flanking the Gal recognition site on the AAV9 VP3 capsid protein subunit onto corresponding residues on the AAV2 VP3

subunit coding region (AAV2 VP3 numbering Q464V, A467P, D469N, I470M, R471A, D472V, S474G, Y500F, and S501A).

In another embodiment, optionally in combination with one or more features of the various embodiments described above or below, the virion of the invention is an adenoviral virion, such as an Ad5 virion. As it is the case for AAV virions, capsid proteins of Ad virions can also be engineered to modify their tropism and cellular targeting properties, alternative adenoviral serotypes can also be employed.

Production of viral particles

Production of viral particles carrying the nucleic acid construct of the expression viral vector of the invention can be performed by means of conventional methods and protocols, which are selected having into account the structural features chosen for the actual embodiment of the nucleic acid construct and viral particle of the vector to be produced.

Briefly, viral particles can be produced in a specific virus-producing cell (packaging cell) which is transfected with the nucleic acid construct of the vector to be packaged, in the presence of a helper vector or virus or other DNA construct(s).

Accordingly, in one aspect the invention concerns the use of the nucleic acid construct or expression vector of the invention for the production of viral particles.

In a related aspect, the invention concerns a process of producing viral particles of the invention comprising the steps of:

a) culturing a host cell comprising a nucleic acid construct or expression vector of the invention in a culture medium; and

b) harvesting the viral particles from the cell culture supernatant and/or inside the cells.

Preferably, said host cell is a packaging cell, as described below. Suitable culture media will be known to a person skilled in the art. The ingredients that compose such media may vary depending on the type of cell to be cultured. In addition to nutrient composition, osmolarity and pH are considered important parameters of culture media. The cell growth medium comprises a number of ingredients well known by the person skilled in the art, including amino acids, vitamins, organic and inorganic salts, sources of carbohydrate, lipids, trace elements (CuS04, FeS04, Fe(N03)3, ZnS04...), each ingredient being present in an amount which supports the cultivation of a cell *in vitro* (i.e., survival and growth of cells). Ingredients may also include different auxiliary substances, such as buffer substances (like sodium bicarbonate, Hepes, Tris...), oxidation stabilizers, stabilizers to counteract mechanical stress, protease inhibitors,

animal growth factors, plant hydrolyzates, anti-clumping agents, anti-foaming agents. Characteristics and compositions of the cell growth media vary depending on the particular cellular requirements. Examples of commercially available cell growth media are: MEM (Minimum Essential Medium), BME (Basal Medium Eagle) DMEM (Dulbecco's modified Eagle's Medium), Iscoves DMEM (Iscove's modification of Dulbecco's Medium), GMEM, RPMI 1640, Leibovitz L-15, CHO, McCoy's, Medium 199, HEK293, Ham (Ham's Media) F10 and derivatives, Ham F12, DMEM/F12, etc.

A HOST CELL OF THE INVENTION

In another aspect, the invention relates to a host cell comprising a nucleic acid construct or expression vector of the invention.

The term "host cell" as used herein refers to any cell line that is susceptible to infection by a virus of interest, and amenable to culture *in vitro*.

The host cell of the invention may be used for *ex vivo* gene therapy purposes. In such embodiments, the cells are transfected with the nucleic acid construct or viral vector of the invention and subsequently transplanted to the patient or subject. Transplanted cells can have an autologous, allogenic or heterologous origin. For clinical use, cell isolation will generally be carried out under Good Manufacturing Practices (GMP) conditions. Before transplantation, cell quality and absence of microbial or other contaminants is typically checked and liver preconditioning, such as with radiation and/or an immunosuppressive treatment, may be carried out. Furthermore, the host cells may be transplanted together with growth factors to stimulate cell proliferation and/or differentiation, such as Hepatocyte Growth Factor (HGF).

In a particular embodiment, the host cell is used for ex vivo gene therapy into the liver. Preferably, said cells are eukaryotic cells such as mammalian cells, these include, but are not limited to, humans, non-human primates such as apes; chimpanzees; monkeys, and orangutans, domesticated animals, including dogs and cats, as well as livestock such as horses, cattle, pigs, sheep, and goats, or other mammalian species including, without limitation, mice, rats, guinea pigs, rabbits, hamsters, and the like. A person skilled in the art will choose the more appropriate cells according to the patient or subject to be transplanted.

Said host cell may be a cell with self-renewal and pluripotency properties, such as stem cells or induced pluripotent stem cells. Stem cells are preferably mesenchymal stem cells. Mesenchymal stem cells (MSCs) are capable of differentiating into at least one of an osteoblast, a chondrocyte, an adipocyte, or a myocyte and may be isolated from any type of tissue.

Generally MSCs will be isolated from bone marrow, adipose tissue, umbilical cord, or peripheral blood. Methods for obtaining thereof are well known to a person skilled in the art. Induced pluripotent stem cells (also known as iPS cells or iPSCs) are a type of pluripotent stem cell that can be generated directly from adult cells. Yamanaka et al. induced iPS cells by transferring the Oct3/4, Sox2, Klf4 and c-Myc genes into mouse and human fibroblasts, and forcing the cells to express the genes (WO 2007/069666). Thomson et al. subsequently produced human iPS cells using Nanog and Lin28 in place of Klf4 and c-Myc (WO 2008/118820).

Said host cells may also be hepatocytes. Hepatocyte transplantation procedures, including cell isolation and subsequent transplantation into a human or mice recipient is described for instance in Filippi and Dhawan, Ann NY Acad Sci. 2014, 1315 50-55; Yoshida et al., Gastroenterology 1996, 111: 1654-1660; Irani et al. Molecular Therapy 2001, 3:3, 302-309; and Vogel et al. J Inherit Metab Dis 2014, 37:165-176. A method for ex vivo transduction of a viral vector into hepatocytes is described for instance in Merle et al., Scandinavian Journal of Gastroenterology 2006, 41:8, 974-982.

In another particular embodiment, the host cell is a packaging cell. Said cells can be adherent or suspension cells. The packaging cell, and helper vector or DNA constructs provide together in *trans* all the missing functions which are required for the complete replication and packaging of the viral vector.

Preferably, said packaging cells are eukaryotic cells such as mammalian cells, including simian, human, dog and rodent cells. Examples of human cells are PER.C6 cells (WO01/38362), MRC-5 (ATCC CCL-171), WI-38 (ATCC CCL-75), HEK-293 cells (ATCC CRL-1573), HeLa cells (ATCC CCL2), and fetal rhesus lung cells (ATCC CL- 160). Examples of non-human primate cells are Vero cells (ATCC CCL81), COS-1 cells (ATCC CRL-1650) or COS-7 cells (ATCC CRL-1651). Examples of dog cells are MDCK cells (ATCC CCL-34). Examples of rodent cells are hamster cells, such as BHK21-F, HKCC cells, or CHO cells.

As an alternative to mammalian sources, cell lines for use in the invention may be derived from avian sources such as chicken, duck, goose, quail or pheasant. Examples of avian cell lines include avian embryonic stem cells (WO01/85938 and WO03/076601), immortalized duck retina cells (WO2005/042728), and avian embryonic stem cell derived cells, including chicken cells (WO2006/108846) or duck cells, such as EB66 cell line (WO2008/129058 & WO2008/142124).

In another embodiment, said host cell are insect cells, such as SF9 cells (ATCC CRL-1711), Sf21 cells (IPLB-Sf21), MG1 cells (BTI-TN-MG1) or High Five™ cells (BTI-TN-5B1-4).

Accordingly, in a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the host cell comprises:

a) a nucleic acid construct or expression vector of the invention (i.e., the recombinant AAV genome), generally as a plasmid;

b) a nucleic acid construct, generally a plasmid, encoding AAV rep and/or cap genes which does not carry the ITR sequences; and/or

c) a nucleic acid construct, generally a plasmid or virus, comprising viral helper genes.

Viral genes necessary for AAV replication are referred herein as viral helper genes. Typically, said genes necessary for AAV replication are adenoviral helper genes, such as E1A, E1B, E2a, E4, or VA RNAs. Preferably, the adenoviral helper genes are of the Ad5 or Ad2 serotype.

Conventional methods can be used to produce viral particles of the AAV vector, which consist on transient cell co-transfection with nucleic acid construct (e.g. a plasmid) carrying the recombinant AAV vector/genome of the invention; a nucleic acid construct (e.g., an AAV helper plasmid) that encodes rep and cap genes, but does not carry ITR sequences; and with a third nucleic acid construct (e.g., a plasmid) providing the adenoviral functions necessary for AAV replication.

Thus, in a particular embodiment, optionally in combination with one or more of the features of the various embodiments described above or below, said host cell is characterized by comprising:

i) a nucleic acid construct or an expression vector of the invention (i.e., the recombinant AAV genome);

ii) a nucleic acid construct encoding AAV rep and cap genes which does not carry the ITR sequences; and

iii) a nucleic acid construct comprising adenoviral helper genes.

Alternatively, the rep, cap, and adenoviral helper genes can be combined on a single plasmid (Blouin Vet al. *J Gene Med.* 2004; 6(suppl): S223-S228; Grimm D. et al. *Hum. Gene Ther.* 2003; 7: 839-850). Thus, in another particular embodiment, optionally in combination with

one or more of the features of the various embodiments described above or below, said host cell is characterized by comprising:

i) a nucleic acid construct or an expression vector of the invention (i.e., the recombinant AAV genome); and

ii) a plasmid encoding AAV rep and cap genes which does not carry the ITR sequences and further comprising adenoviral helper genes.

In a further particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the host cell comprises:

a) a nucleic acid construct or an expression vector of the invention (i.e., the recombinant AAV genome);

b) a plasmid encoding AAV rep and cap genes which does not carry the ITR sequences; and

c) a plasmid comprising adenoviral helper genes E2a, E4, and VA RNAs,

wherein co-transfection is performed in cells, preferably mammalian cells, that constitutively express and transcomplement the adenoviral E1 gene, like HEK-293 cells (ATCC CRL-1573).

Large-scale production of AAV vectors according to the invention can also be carried out for example by infection of insect cells with a combination of recombinant baculoviruses (Urabe et al. *Hum. Gene Ther.* 2002; 13: 1935-1943). SF9 cells are co-infected with three baculovirus vectors respectively expressing AAV rep, AAV cap and the AAV vector to be packaged. The recombinant baculovirus vectors will provide the viral helper gene functions required for virus replication and/or packaging.

By using helper plasmids encoding the rep ORF (open reading frame) of an AAV serotype and cap ORF of a different serotype AAV, it is feasible packaging a vector flanked by ITRs of a given AAV serotype into virions assembled from capsid structural proteins of a different serotype. It is also possible by this same procedure packaging mosaic, chimeric or targeted vectors.

On the other hand, the production of HC-Ad vectors according to the invention can be carried out by means of mammalian cells that constitutively express and transcomplement the adenoviral E1 gene, and also Cre recombinase (e.g. 293Cre cells). These cells are transfected with the HC-Ad vector genome and infected with a first-generation adenoviral helper virus (E1-deleted) in which the packaging signal is flanked by loxP sequences. [Parks RJ et al. *Proc. Natl. Acad. Sci. USA* 1996; 13565-13570; for 293Cre cells, see Palmer and Engel. *Mol. Ther.* 2003; 8:846-852]. Several Cre/loxP-based helper virus systems have been described that can be used

for packaging HC-Ad vectors, such as AdAdLC8cluc, or the optimized self-inactivating AdTetCre helper virus (EP2295591; Gonzalez-Aparicio et al. *Gene Therapy* 2011; 18: 1025-1033).

Further guidance for the construction and production of viral vectors for gene therapy according to the invention can be found in:

Viral Vectors for Gene Therapy, Methods and Protocols. Series: Methods in Molecular Biology, Vol. 737. Merten and Al-Rubeai (Eds.); 2011 Humana Press (Springer).

Gene Therapy. M. Giacca. 2010 Springer-Verlag.

Heilbronn R. and Weger S. Viral Vectors for Gene Transfer: Current Status of Gene Therapeutics. In: Drug Delivery, Handbook of Experimental Pharmacology 197; M. Schäfer-Korting (Ed.). 2010 Springer-Verlag; pp. 143-170.

Adeno-Associated Virus: Methods and Protocols. R.O. Snyder and P. Moulllier (Eds). 2011 Humana Press (Springer).

Bünning H. et al. Recent developments in adeno-associated virus technology. J. Gene Med. 2008; 10:717-733.

Adenovirus: Methods and Protocols. M. Chillón and A. Bosch (Eds.); Third Edition. 2014 Humana Press (Springer).

THERAPEUTIC USES

In a further aspect, the invention relates to the product of the invention as defined within the Summary of the invention for use as a medicament.

In an additional aspect, the invention relates to the product of the invention as defined within the Summary of the invention for use in the treatment of a condition caused by a deficiency or dysfunction of Copper-transporting ATPase 2, and of any other conditions and illnesses in which an upregulation of Copper-transporting ATPase 2 expression and activity may produce a therapeutic benefit or improvement, in particular a disease or condition associated with a decrease of ATP7B-dependent lysosomal exocytosis and accumulation of copper in lysosomes, such as choleostatic disorders, Alzheimer disease and/or cancer (Polishchuck et al. Dev Cell. 2014, 29(6), 686-700; Gupta and Lutsenko, Future Med. Chem. 2009, 1, 1125-1142)..

The subject to be treated can be a mammal, and in particular a human patient.

In a particular embodiment, optionally in combination with one or more features of the various embodiments described above or below, the condition caused by a deficiency or dysfunction of Copper-transporting ATPase is Wilson's disease (WD, Online Mendelian

Inheritance in Man catalog accession number OMIN 277900; http://www.omim.org/entry/277900).

In a related aspect, the invention pertains to the use of the product of the invention, as defined within the Summary of the invention, in the preparation of a medicament for use in the treatment of a condition caused by a deficiency or dysfunction of Copper-transporting ATPase 2, and of any other conditions and illnesses in which an upregulation of Copper-transporting ATPase 2 expression and activity may produce a therapeutic benefit or improvement, preferably for use in the treatment of Wilson's disease.

In a further aspect, the invention relates to the treatment of a condition caused by a deficiency or dysfunction of Copper-transporting ATPase 2, and of any other conditions and illnesses in which an upregulation of Copper-transporting ATPase 2 expression and activity may produce a therapeutic benefit or improvement, preferably for use in the treatment of Wilson's disease, in a patient that comprises administering to the patient a therapeutically effective amount of a nucleic acid construct, an expression vector, a host cell, a viral particle or a pharmaceutical composition of the invention.

The treatment with a product of the invention may alleviate, ameliorate, or reduce the severity of one or more symptoms of WD. For example, treatment may increase and/or restore holoceruplasmin synthesis, ceruloplasmin oxidase activity, and /or copper excretion in the bile (thus reducing copper accumulation in serum, liver, brain and urine); and as a consequence may alleviate, ameliorate, or reduce the severity of abdominal pain, fatigue, jaundice, frequency of uncontrolled movements, muscle stiffness, problems with speech, swallowing or physical coordination.

The product of the invention will be typically included in a pharmaceutical composition or medicament, optionally in combination with a pharmaceutical carrier, diluent and/or adjuvant. Such composition or medicinal product comprises the product of the invention in an effective amount, sufficient to provide a desired therapeutic effect, and a pharmaceutically acceptable carrier or excipient.

Accordingly, in a further aspect, the invention relates to a pharmaceutical composition that comprises a nucleic acid construct, an expression vector, a host cell or a viral particle of the invention, and a pharmaceutically acceptable carrier.

Any suitable pharmaceutically acceptable carrier or excipient can be used in the preparation of a pharmaceutical composition according to the invention (See e.g., Remington:

The Science and Practice of Pharmacy, Alfonso R. Gennaro (Editor) Mack Publishing Company, April 1997). Pharmaceutical compositions are typically sterile and stable under the conditions of manufacture and storage. Pharmaceutical compositions may be formulated as solutions (e.g. saline, dextrose solution, or buffered solution, or other pharmaceutically acceptable sterile fluids), microemulsions, liposomes, or other ordered structure suitable to accommodate a high product concentration (e.g. microparticles or nanoparticles). The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. The product of the invention may be administered in a controlled release formulation, for example in a composition which includes a slow release polymer or other carriers that protect the product against rapid release, including implants and microencapsulated delivery systems. Biodegradable and biocompatible polymers may for example be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic / polyglycolic copolymers (PLG). Preferably, said pharmaceutical composition is formulated as a solution, more preferably as an optionally buffered saline solution.

Supplementary active compounds can also be incorporated into the pharmaceutical compositions of the invention. Guidance on co-administration of additional therapeutics can for example be found in the Compendium of Pharmaceutical and Specialties (CPS) of the Canadian Pharmacists Association.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the pharmaceutical composition of the invention is a parenteral pharmaceutical composition, including a composition suitable for intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular administration. These pharmaceutical compositions are exemplary only and do not limit the pharmaceutical compositions suitable for other parenteral and non-parenteral administration routes.

In the context of the invention, an "effective amount" means a therapeutically effective amount.

As used herein a "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary to achieve the desired therapeutic result, such as an elevation of copper translocation activity, thus increasing copper in bile and reducing copper in serum, liver, brain and urine. The therapeutically effective amount of the product of the invention, or pharmaceutical composition that comprises it may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the product or pharmaceutical composition to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also typically one in which any toxic or detrimental effect of the product or pharmaceutical composition is outweighed by the therapeutically beneficial effects.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the pharmaceutical composition carrying the product of the invention is administered to the subject or patient by a parenteral route.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the pharmaceutical composition is administered by intravenous, intraarterial, subcutaneous, intraperitoneal, or intramuscular route.

In one embodiment, optionally in combination with one or more features of the various embodiments described above or below, the pharmaceutical composition comprising a product of the invention is administered by interstitial route, i.e. by injection to or into the interstices of a tissue. The tissue target may be specific, for example the liver tissue, or it may be a combination of several tissues, for example the muscle and liver tissues. Exemplary tissue targets may include liver, skeletal muscle, heart muscle, adipose deposits, kidney, lung, vascular endothelium, epithelial and/or hematopoietic cells. In a preferred embodiment, optionally in combination with one or more features of the various embodiments described above or below, it is administered by intrahepatic injection, i.e. injection into the interstitial space of hepatic tissue.

The amount of product of the invention that is administered to the subject or patient may vary depending on the particular circumstances of the individual subject or patient including, age, sex, and weight of the individual; the nature and stage of the disease, the aggressiveness of the disease; the route of administration; and/or concomitant medication that has been prescribed to the subject or patient. Dosage regimens may be adjusted to provide the optimum therapeutic response.

For any particular subject, specific dosage regimens may be adjusted over time according to the individual needs and the professional judgment of the person administering or supervising

the administration of the compositions. Dosage ranges set forth herein are exemplary only and do not limit the dosage ranges that may be selected by medical practitioners.

In one embodiment, an AAV vector according to the invention can be administered to the subject or patient for the treatment of Wilson's disease in an amount or dose comprised within a range of 5×10^{11} to 1×10^{14} vg / kg (vg: viral genomes; kg: subject's or patient's body weight). In a more particular embodiment, the AAV vector is administered in an amount comprised within a range of 1×10^{12} to 1×10^{13} vg / kg.

In another embodiment, a HC-Ad vector according to the invention can be administered to the subject or patient for the treatment of Wilson's disease in an amount or dose comprised within a range of 1×10^{9} to 1×10^{11} iu / kg (iu: infective units of the vector).

In another aspect, the invention further relates to a kit comprising a nucleic acid construct, vector, host cell, viral particle or pharmaceutical composition of the invention in one or more containers. A kit of the invention may include instructions or packaging materials that describe how to administer a nucleic acid construct, vector, host cell or viral particle of the invention contained within the kit to a patient. Containers of the kit can be of any suitable material, e.g., glass, plastic, metal, etc., and of any suitable size, shape, or configuration. In certain embodiments, the kits may include one or more ampoules or syringes that contain a nucleic acid construct, vector, host cell, viral particle or pharmaceutical composition of the invention in a suitable liquid or solution form.

Throughout the description and claims the word "comprise" and variations of thereof, are not intended to exclude other technical features, additives, components, or steps. Furthermore, the word "comprise" encompasses the case of "consisting of". Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples are provided by way of illustration, and they are not intended to be limiting of the present invention. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein.

EXAMPLES

Example 1. Construction of recombinant expression vectors

Five different AAV vectors that carry and express human ATP7B, or a truncated form of human ATP7B, were designed and produced for conducting gene therapy of Wilson's Disease

(WD): AAV2/8-AAT-wtATP7B, AAV2/8-AAT-coATP7B, AAV2/8-AAT-ATP7B(d223-366), AAV2/8-AAT-ATP7B(d57-486), and AAV2/8-AAT-coATP7B(d57-486).

1.1 Vector AAV2/8-AAT-wtATP7B [herein also named as AAV-wtATP7B]

Genomic sequence of this vector is identified as SEQ.ID.NO.1.

Firstly, the plasmid pUC-ATP7B was assembled at request (GenScript) by cloning nucleic acid construct into a pUC57 plasmid. Nucleic acid construct contained cDNA sequence encoding human ATP7B (transgene) together with a synthetic polyadenylation signal sequence (Levitt N. et al. *Genes & Development* 1989; 3(7):1019-1025) downstream of the transgene.

Next, the minimal promoter of alpha1 anti-trypsin gene (AAT) was introduced into the plasmid pUC-ATP7B, upstream the ATP7B gene. The minimal promoter consists on the sequence from nucleotide -261 to nucleotide +44 relative to cap site of the AAT promoter (Kramer M.G. et al. *Mol. Therapy* 2003; 7(3): 375-385) and contains the tissue-specific element (TSE), required for liver function, and the distal region (DRI) required for whole promoter activity. The AAT promoter was obtained by PCR amplification using as template the pEnhAlbAAT-luciferase plasmid (provided by M.G. Kramer) and the following primers

Primer AAT-Forward

5' CTGGTCTAGAACGCGTCGCCACCCCCTCCACCTTGG 3' (SEQ.ID.NO.10); and

Primer AAT-reverse

5' ATCATGATGCGGCCGCTTCACTGTCCCAGGTCAGTG 3' (SEQ.ID.NO.11).

The AAT-Forward primer has a restriction site for XbaI and MluI and the 3´ AAT-reverse primer has a restriction site for NotI.

Therefore, in order to obtain plasmid pUC-AAT-ATP7B, the plasmid pUC-ATP7B was digested with XbaI and NotI and ligated to AAT promoter previously digested with the same enzymes.

The expression cassette was subsequently subcloned into the AAV transfer plasmid pAAV-MCS (Agilent technologies) by digestion with restriction enzymes PmlI and MluI, thus producing the plasmid pAAV2-AAT-wtATP7B.

Once the plasmid had been constructed, the AAV vector was made by double transfection into 293 cells of the plasmid pAAV2-AAT-ATP7B and of the plasmid pDP8 (obtained from PlasmidFactory, Bielefeld, Germany; plasmid pDP8 expresses AAV8 capsid protein, AAV2 rep protein and the adenoviral molecules required for production and packaging of AAV).

The vector was finally purified by iodixanol gradient and titrated by quantitative PCR.

1.2 Vector AAV2/8-AAT-coATP7B [herein also named as AAV-coATP7B]

Genomic sequence of this vector is identified as SEQ.ID.NO.3.

To obtain the AAV vector expressing a codon optimized version of the ATP7B gene (coATP7B), the plasmid pUC-coATP7B was firstly assembled at request (GenScript) by cloning nucleic acid construct into a pUC57 plasmid. Next the coATP7B was excised from the pUC-coATP7B by digestion with the restriction enzymes NotI and KpnI and subcloned into the pAAV2-AAT-wtATP7B plasmid previously digested with the same enzymes, NotI and KpnI, to obtain the plasmid pAAV2-AAT-coATP7B.

Once the plasmid had been constructed, the production of vector genome and packaging of viral particles was performed as has been described previously for the vector AAV2/8-AAT-wtATP7B: double transfection of previously obtained plasmid pAAV2-AAT-coATP7B with plasmid pDP8, purification (iodixanol gradient) and titration.

1.3 Vector AAV2/8-AAT-ATP7B(d223-366) [herein also named as AAV-T1]

This vector carries as the transgene a nucleic acid sequence (SEQ.ID.NO.12) encoding ATP7B(d223-366), a truncated form of human ATP7B in which amino acids 223 to 366 have been deleted. The deleted sequence includes HMA 3 domain and seven amino acids of the HMA 4 domain.

To obtain the vector, the plasmid pUC57-wtATP7B was digested with the restriction enzymes MfeI and Nae I, to obtain the plasmid pUC57-ATP7B-T1. This way, the size of the codifying region was reduced in 432 nucleotides and the size of the protein in 144 amino acids.

Once the plasmid pUC57-ATP7B-T1 had been constructed, the production of vector genome and packaging of viral particles was performed as described previously for the vector AAV2/8-AAT-wtATP7B: ligation to AAT promoter, subcloning into plasmid pAAV-MCS, double transfection of previously obtained plasmid pAAV2-AAT-T1 with plasmid pDP8, virus purification (iodixanol gradient) and titration.

1.4 Vector AAV2/8-AAT-ATP7B(d57-486) [herein also named as AAV-T2]

Genomic sequence of this vector is identified as SEQ.ID.NO.6.

This vector carries as the transgene a nucleic acid sequence encoding ATP7B(d57-486) [also named as ATP7B-T2], a truncated form of human ATP7B in which amino acids 57 to 486 have been deleted. This way, the first 4 HMA domains have been eliminated while maintaining the signal sequence that comprises the 56 amino acids of the amino terminal region, reducing the size of the codifying region in 1.29 Kb and the protein in 430 amino acids.

The nucleotide sequence of ATP7B(d57-486) was obtained by PCR amplification using the pUC57-wtATP7B as template and two sets of primers;

a first set of primers amplifying the amino terminal sequence:

Primer F1:

5' CTAGATGCGGCCGCCACCATGCCTG 3' (SEQ.ID.NO.14), and

Primer R1:

5' CTGAGAAGAAGGGCCCAGGCC 3' (SEQ.ID.NO.15); and

a second set of primers amplifying the carboxy terminal region:

Primer F2:

5' GGCCCTTCTTCTCAGCCGCAGAAGTGCTTCTTACAG 3' (SEQ.ID.NO.16), and

Primer R2:

5' ACCAAAATCGATAAAACCGATTACAATCC 3' (SEQ.ID.NO.17).

The 5' terminal sequences of primers R1 and F2 are complementary. Using equimolecular amounts of the two PCR purified fragments as template, and primers F1 and R2, PCR was performed to obtain nucleotide sequence encoding ATP7B(d57-486) . The PCR product was then digested with NotI and ClaI and cloned into the pUC57-AAT-wtATP7B plasmid previously digested with both enzymes obtaining the plasmid pUC57-ATP7B-T2.

Once the plasmid pUC57-ATP7B-T2 had been constructed, the production of vector genome and packaging of viral particles was performed as described previously for the vector AAV2/8-AAT-wtATP7B: ligation to AAT promoter, subcloning into plasmid pAAV-MCS, double transfection of previously obtained plasmid pAAV2-AAT-T2 with plasmid pDP8, purification (iodixanol gradient) and titration.

1.5 Vector AAV2/8-AAT-coATP7B(d57-486) [herein also named as AAV-AAT-coT2]

This vector carries as transgene a codon optimized nucleic acid sequence [SEQ.ID.NO.8; coATP7B(d57-486) or coATP7B-T2] that also encodes ATP7B(d57-486).

The nucleotide sequence of coATP7B(d57-486) was obtained by PCR amplification using the pUC57-coATP7B as template and two sets of primers;

a first set of primers amplifying the amino terminal sequence:

Primer F3:

5' ACGCGTGCGGCCGCCACCATGCCAG 3' (SEQ.ID.NO.18), and

Primer R3:

5' CTGGGAGCTAGGTCCCAGTCC 3' (SEQ.ID.NO.19); and

A second set of primers amplifying the carboxy terminal region:

Primer F4:

5' GGACCTAGCTCCCAGCCTCAGAAGTGTTTTCTGCAG 3' (SEQ.ID.NO.20), and

Primer R4:

5' TGTTCCTCGCGAATGATCAGGTTGTCCTC 3' (SEQ.ID.NO.21).

The 5' terminal sequences of primers R3 and F4 are complementary. Using equimolecular amounts of the two PCR purified fragments as template, and primers F3 and R4, PCR was performed to obtain codon optimized nucleotide sequence encoding ATP7B(d57-486). The PCR product was then digested with NotI and NruI and cloned into the pUC57-AAT-wtATP7B plasmid previously digested with both enzymes obtaining the plasmid pUC57-coATP7B-T2.

Once the plasmid pUC57-coATP7B-T2 had been constructed, the production of vector genome and packaging of viral particles was performed as described previously for the vector AAV2/8-AAT-wtATP7B: ligation to AAT promoter, subcloning into plasmid pAAV-MCS, double transfection of previously obtained plasmid pAAV2-AAT-coT2 with plasmid pDP8, virus purification (iodixanol gradient) and titration.

Example 2. Wilson's disease animal model: ATP7B KO

The therapeutic performance of the vectors AAV2/8-AAT-ATP7B-T1 and AAV2/8-AAT-ATP7B-T2 was tested in ATP7B knockout mice (ATP7B KO, $ATP7B^{-/-}$ or WD mice) which are a representative animal model of WD. This animal model was developed by Buiakova et al., by introducing an early termination codon in the mouse ATP7B mRNA by engineering the substitution of a portion of ATP7B exon 2 with a neomycin cassette oriented in the opposite transcriptional frame (Buikova O.I. et al. *Human Molecular Genetics* 1999; 8(9): 1665-1671). ATP7B knockout mice show no ATP7B expression in the liver and high Cu excretion in the urine, low holoceruloplasmin levels in serum, high transaminase levels, high Cu concentration in the liver and a pathologic liver histology. These mice exhibit the typical biochemical characteristics of human Wilson's disease except for the neurological affectation (Lutsenko S. *Biochemical Society Transactions* 2008; 36(Pt 6): 1233-1238).

Example 3. Determination of the therapeutic effect of viral vectors AAV2/8-AAT-ATP7B-T1 and AAV2/8-AAT-ATP7B-T2 in Wilson's disease mice

Six weeks (6w) old male ATP7B-/- mice were divided in 4 groups of 5 mice each: 1 of the groups were treated intravenously with the vector AAV2/8-AAT-wtATP7B at a dose of $3x10^{10}$ vg / mouse (vg: viral genomes); a second group with the same dose of the vector AAV2/8-AAT-ATP7B-T1; a third group with the same dose of the vector AAV2/8-AAT-

ATP7B-T2; and a fourth group was left untreated. An additional group of wild type mice was kept untreated as a control group (control). Animals were sacrificed twenty-four weeks after vector administration (w30).

Four weeks after vector administration and every five weeks after that up to week 30; serum transaminases (ALT) levels and urine Cu content were determined in all the groups. Serum ceruloplasmin activity was measured 4 weeks after treatment.

Serum transaminases (ALT) levels were determined by the DGKC method (Roche Diagnostics, Mannheim, Germany) using a Hitachi 747 Clinical Analyzer (Hitachi, Tokyo, Japan).

Serum ceruloplasmin activity was determined with o-dianisidine dihydrochloride (4, 4'-diamino-3,3'-dimethoxy-biphenyl) as substrate (Sigma-Aldrich, San Louis, MO, United States) as described by Schosinsky and cols. (*Clinical Chemistry* 1974; 20(12): 1556-1563). Absorbance was measured at 540 nm in a spectrophotometer.

Urine copper content was determined by atomic absorption spectroscopy (SIMAA 6000, from Perkin-Elmer GmbH, Bodenseewerk).

After the sacrifice the liver was excised for histological analyses.

Hepatic copper content was determined in dry liver tissue by atomic absorption spectroscopy (SIMAA 6000, from Perkin-Elmer GmbH, Bodenseewerk), and by Timm's sulphide silver staining (Danscher G. and Zimmer J. Histochemistry 1978; 55(1): 27-40).

Liver structure was assessed in sections stained with hematoxylin and eosin.

Immunohistochemistry with anti-mouse CD45 antibody (BioLegend, San Diego, USA; Catalog Number 103102) was performed to detect inflammatory infiltration in the liver.

Immunohistochemistry with anti-mouse PanCk antibody (Invitrogen/Life Technologies, 18-0132, clon AE1/AE3) was also performed to detect biliary cells.

To determine fibrosis we used conventional Sirius Red staining as a method for collagen determination.

As shown in Figure 2, transaminase levels were normalized in the mice receiving AAV2/8-AAT-wtATP7B or AAV2/8-AAT-ATP7B-T2 but no in animals treated with AAV2/8-AAT-ATP7B-T1. Furthermore, the concentration of Cu in urine was significantly lower in the animals that received AAV2/8-AAT-wtATP7B, AAV2/8-AAT-ATP7B-T1, or AAV2/8-AAT-ATP7B-T2; however AAV2/8-AAT-ATP7B-T1 was less efficient in reducing Cu concentration in urine (Figure 3). Ceruloplasmin activity was restored four weeks after treatment in the animals receiving AAV2/8-AAT-wtATP7B or AAV2/8-AAT-ATP7B-T2 but no in animal treated with

AAV2/8-AAT-ATP7B-T1 (Figure 4). This result was corroborated by western blot analysis. Holoceruloplasmin was detected in mice treated with AAV2/8-AAT-wtATP7B or AAV2/8-AAT-ATP7B-T2 but no in animals treated with AAV2/8-AAT-ATP7B-T1 where as in untreated WD mice only the apoceruloplasmin form could be detected.

On the other hand, the administration of the AAV2/8-AAT-wtATP7B, AAV2/8-AAT-ATP7B-T1, or AAV2/8-AAT-ATP7B-T2 significantly reduced Cu content in the liver; however, AAV2/8-AAT-ATP7B-T1 was less efficient in reducing Cu concentration in the liver (Figure 5). The results were confirmed in the image obtained after Timm's staining (Figure 6B). Regarding liver histology, untreated animals showed an abnormal hepatic architecture with huge hepatocytes containing enormous nuclei. The administration of the vectors AAV2/8-AAT-wtATP7B or AAV2/8-AAT-ATP7B-T2 but no AAV2/8-AAT-ATP7B-T1 resulted in the normalization of liver histology (Figure 6A). Furthermore, WD animals presented a strong liver infiltrate mainly composed by CD45 positive cells; infiltration disappeared after treatment with the recombinant viral vectors (Figure 7). Thus, the administration of AAV vector resulted in a marked reduction of the inflammatory infiltrate. Furthermore, biliary duct proliferation and liver fibrosis were also significantly reduced in AAV2/8-AAT-wtATP7B, AAV2/8-AAT-ATP7B-T2, and AAV2/8-AAT-ATP7B-T1-treated WD mice (Figure 7).

Example 4. Therapeutic effect of viral vector AAV2/8-AAT-ATP7B(d57-486) in Wilson's disease female mice.

Six weeks (6w) old female ATP7B-/- mice were divided in 4 groups of 5 mice each: animals of the groups 1 - 3 were treated intravenously with the viral vector AAV2/8-AAT-ATP7B(d57-486), each group receiving a different dose (respectively 1×10^{10}, 3×10^{10}, and 1×10^{11} vg / mouse); a fourth group were left untreated. An additional group of wild type mice was kept untreated as a control group (WT).

Four weeks after vector administration and every five weeks after that up to 24 weeks after treatment (when the mice were 30 weeks old), serum transaminases (ALT) levels and urine Cu concentration were determined in all the groups, by the same methods as described in Example 3.

As shown in Figure 8, AAV2/8-AAT-ATP7B(d57-486) normalized transaminase levels in WD female mice at the two highest doses (3×10^{10}, and 1×10^{11} vg / mouse); the lowest dose 1×10^{10} vg / mouse significantly reduced transaminase levels but failed to eliminate liver

damage. However, treatment with the three different doses significantly reduced Cu urinary excretion reaching the levels found in WT mice (Figure 9).

Example 5. Comparison of the therapeutic effect of viral vectors AAV2/8-AAT-wtATP7B and AAV2/8-AAT-ATP7B(d57-486) in Wilson's disease female mice.

Two experimental groups were established. For each experimental group, six weeks (6w) old female ATP7B-/- mice were divided in 4 groups of 5 mice each: 3 of the groups were treated intravenously with a viral vector to be tested, each group receiving a different dose (respectively 1×10^{10}, 3×10^{10}, and 1×10^{11} vg / mouse; a fourth group were left untreated. An additional group of wild type mice were kept untreated as a control group (WT).

In first experimental group (experimental group 1), WD mice receiving treatment were administered with the vector AAV2/8-AAT-wtATP7B; in second experimental group (experimental group 2) they were administered with the vector AAV2/8-AAT-ATP7B(d57-486).

Serum ceruloplasmin activity determined 4 weeks after treatment, and hepatic Cu content determined 24 weeks after treatment, were measured by the same methods as described in example 3.

Serum ceruloplasmin activity

Serum ceruloplasmin activity was corrected only by the administration of the highest dose of the AAV2/8-AAT-wtATP7B vector (Figure 10A experimental group 1); no effect being observed after the administration of the two lowest doses.

Conversely, the AAV2/8-AAT-ATP7B(d57-486) vector significantly increased ceruloplasmin levels at the lowest dose of 1×10^{10} vg / mouse; the administration of the medium dose of vector normalized ceruloplasmin levels and the highest dose increased ceruloplasmin activity over the normal levels (Figure 10B experimental group 2).

Cu concentration in the liver

Besides, Cu concentration in the liver was reduced but not normalized by the administration of the two highest doses of AAV2/8-AAT-wtATP7B; and no effect was observed at the lowest dose (Figure 11A experimental group 1). On the contrary, Cu concentration was shown to be reduced after administration of the AAV2/8-AAT-ATP7B(d57-486) vector at all the tested doses, and at the highest dose the levels were close to normal (Figure 11B experimental group 2).

Accordingly, a dose of 1×10^{10} vg / mouse of the AAV2/8-AAT-wtATP7B vector was shown to be a "suboptimal dose" for the wt construct both for the obtaining of a normalization of

the serum ceruloplasmin activity and a reduction of Cu accumulation in the liver; whereas the vector carrying the truncated form unexpectedly provided statistically significant therapeutic effects at said suboptimal dose.

Example 6. Comparison of the therapeutic effect of viral vectors AAV2/8-AAT-wtATP7B and AAV2/8-AAT-ATP7B(d57-486) in WD mice.

Six weeks (6w) old male ATP7B-/- mice were divided in 3 groups of mice: 2 groups of animals were respectively treated with a suboptimal intravenous dose (1×10^{10} vg / mouse) of the vector AAV2/8-AAT-wtATP7B or the vector AAV2/8-AAT-ATP7B(d57-486); a third group were left untreated. An additional group of wild type mice were kept untreated as a control group (WT).

Hepatic Cu content was measured by the same method as described in example 3.

As it is shown in Figure 12, although both AAV2/8-AAT-wtATP7B and AAV2/8-AAT-ATP7B(d57-486) vectors given at a suboptimal dose reduced accumulation of copper in the liver of WD mice, AAV2/8-AAT-ATP7B(d57-486) provided a reduction of hepatic copper content that was significantly greater than the reduction provided by AAV2/8-AAT-wtATP7B.

Example 7. Comparison of the therapeutic effect of viral vectors AAV2/8-AAT-ATP7B(d57-486) and AAV-AAT-coATP7B(d57-486) in WD mice.

Six weeks (6w) old male ATP7B-/- mice were divided in 3 groups of mice: 2 groups of animals were respectively treated with a suboptimal intravenous dose (1×10^{10} vg / mouse) of the vector AAV2/8-AAT-ATP7B(d57-486) and AAV-AAT-coATP7B(d57-486); a third group were left untreated. An additional group of wild type mice were kept untreated as a control group (WT).

Hepatic Cu content was measured by the same method as described in example 3.

As it is shown in Figure 13, although both AAV2/8-AAT-ATP7B(d57-486) and AAV2/8-AAT-coATP7B(d57-486)vectors given at a suboptimal dose reduced accumulation of copper in the liver of WD mice, AAV2/8-AAT-coATP7B(d57-486) provided a reduction of hepatic copper content that was significantly greater than the reduction provided by AAV2/8-AAT-ATP7B(d57-486).

Example 8. Therapeutic effect of codon optimized viral vector AAV2/8-AAT-coATP7B(d57-486) in WD mice.

Six weeks (6w) old male ATP7B-/- mice were divided in 5 groups of mice: 4 groups of animals were respectively treated with a suboptimal intravenous dose (1 x 10^{10} vg / mouse) of the vectors AAV2/8-AAT-wtATP7B, AAV2/8-AAT-coATP7B, AAV2/8-AAT-ATP7B(d57-486) or AAV2/8-AAT-coATP7B(d57-486); a fifth group were left untreated. An additional group of wild type mice were kept untreated as a control group (WT).

Serum ceruloplasmin activity was measured by the same method as described in example 3.

As it is shown in Figure 14, the two vectors carrying nucleotide sequence of truncated ATP7B-T2 restored ceruloplasmin oxidase activity when administered to WD mice at the suboptimal dose, while vectors carrying nucleotide sequences encoding complete human ATP7B did not provide any significant improvement of ceruloplasmin activity when administered at the same treatment conditions.