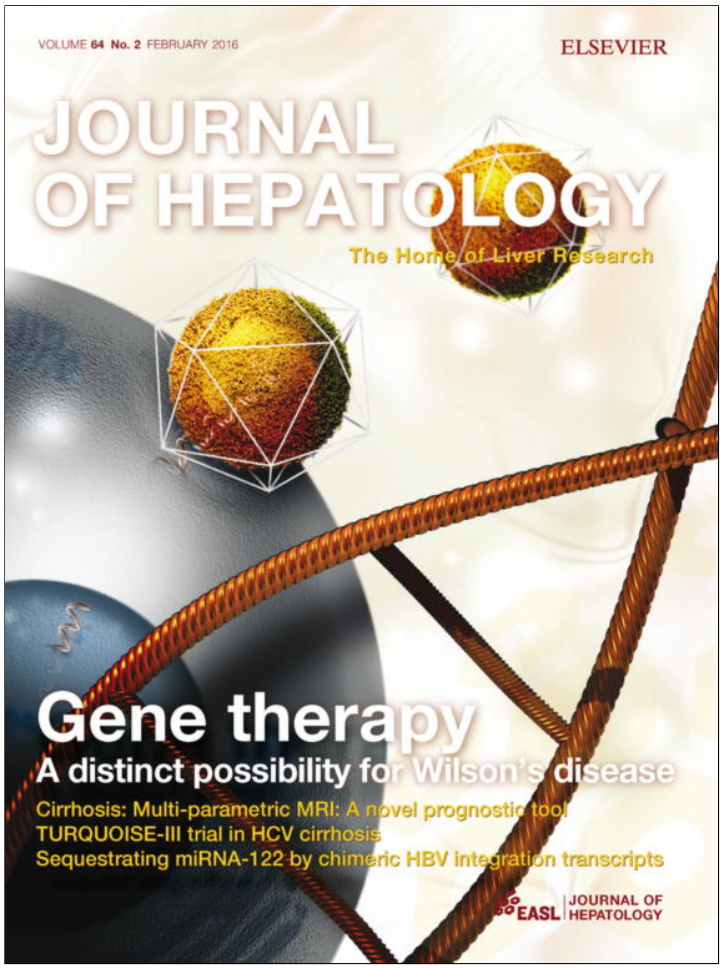

Research Article

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Long-term metabolic correction of Wilson's disease in a murine model by gene therapy

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Background & Aims: Wilson's disease (WD) is an autosomal recessively inherited copper storage disorder due to mutations in the *ATP7B* gene that causes hepatic and neurologic symptoms. Current treatments are based on lifelong copper chelating drugs and zinc salts, which may cause side effects and do not restore normal copper metabolism. In this work we assessed the efficacy of gene therapy to treat this condition.

Methods: We transduced the liver of the *Atp7b*$^{-/-}$ WD mouse model with an adeno-associated vector serotype 8 (AAV8) encoding the human ATP7B cDNA placed under the control of the liver-specific α1-antitrypsin promoter (AAV8-AAT-ATP7B). After vector administration we carried out periodic evaluation of parameters associated with copper metabolism and disease progression. The animals were sacrificed 6 months after treatment to analyze copper storage and hepatic histology.

Results: We observed a dose-dependent therapeutic effect of AAV8-AAT-ATP7B manifested by the reduction of serum transaminases and urinary copper excretion, normalization of serum holoceruloplasmin, and restoration of physiological biliary copper excretion in response to copper overload. The liver of treated animals showed normalization of copper content and absence of histological alterations.

Conclusions: Our data demonstrate that AAV8-AAT-ATP7B-mediated gene therapy provides long-term correction of copper metabolism in a clinically relevant animal model of WD providing support for future translational studies.

Introduction

Wilson's disease (WD) is a rare autosomal recessive inborn error of copper metabolism caused by mutations in the gene that encodes the ATPase copper transporting beta polypeptide, *ATP7B*. Copper is a potentially toxic metal but it is essential for a wide number of physiological functions acting as a co-factor of a variety of enzymes [1]. After its intestinal absorption, copper is transported to hepatocytes where it binds to ATP7B located in the membrane of the trans-Golgi network (TGN). This large transmembrane protein is in charge of transferring the metal to copper-dependent enzymes. Loading of copper into ceruloplasmin is essential for the ferroxidase activity of this enzyme and constitutes an important secretory pathway for the metal, as 95% of copper present in the plasma of healthy individuals is bound to ceruloplasmin [1,2]. In response to increased cytosolic copper concentration, ATP7B translocates from TGN to the apical membrane of hepatocytes to facilitate copper excretion into the bile [3]. WD affects approximately 1 in 30,000 people worldwide. The genetic defect causes progressive copper accumulation in the liver during the first decade of life followed by copper deposition in the brain if the stores continue to grow. The disease is commonly heralded by liver damage which may evolve to neurologic dysfunction if it is left untreated. Current therapy is aimed at reducing copper stores and preventing its re-accumulation. With adequate treatment, the progression of the disease is usually halted and symptoms improve. Drugs approved for WD

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Abbreviations: AAV, Adenoassociated virus; ATP7B, ATPase copper transporting beta polypeptide; TGN, trans-Golgi network.

treatment are zinc salts and copper chelators such as penicillamine and trientine [4,5]. Zinc acts by blocking the absorption of copper in the intestinal tract and by inducing the expression of metallothionein in enterocytes. This cysteine-rich protein is an endogenous chelator of metals that binds copper in the enterocyte and inhibits its entry into the portal circulation. This action both depletes accumulated copper and prevents its reaccumulation. Copper chelators act by ligating copper and promoting its urinary excretion but they do not restore the normal copper metabolism, i.e. they do not increase serum ceruloplasmin nor enhance biliary copper excretion. In fact copper chelators by removing copper from the stores and transferring it to blood before urinary excretion may facilitate deposition of the metal in the brain, resulting in exacerbation of neurological symptoms [6]. Another limitation of current therapies for WD is that they should be given lifelong which implies problems derived from side effects and treatment adherence [5]. Furthermore, a recent study of a large cohort of patients on chelation therapy showed that although half of the patients fully recovered or improved, 15% deteriorated, 8% required liver transplant and 7.4% died [7]. Thus, alternative therapeutic approaches, particularly those capable of offering a permanent correction of the disorder, like gene or cell therapy, are desirable.

Translational research in WD has been aided by the existence of relevant animal models. Long-Evans Cinnamon (LEC) rats harbor a spontaneous deletion in the 3' terminal region of the ATP7B [8]. More recently, *Atp7b* knockout mice were developed [9,10]. These animals show no ATP7B expression in the liver and exhibit the typical biochemical and physiopathological alterations found in human WD, except for neurological manifestations. Abnormalities include high copper excretion in the urine, low holoceruloplasminemia, high serum transaminase levels and increased liver copper concentration with associated hepatocellular damage [9,10].

Proof of concept studies performed in LEC rats showed that introduction of a functional *ATP7B* gene in transgenic rats resulted in hepatic expression of the protein, with restoration of holoceruloplasmin biosynthesis and biliary copper excretion [8]. Early gene therapy approaches using first-generation adenoviral vectors expressing ATP7B to LEC rats achieved therapeutic effects, with restoration of serum active holoceruloplasmin and elevation of copper content in stool [11]. However, improvements were transient due to the short duration of transgene expression achieved by this kind of vectors. So far, *in vivo* use of long-term expression vectors like lentivirus was unable to completely reverse the liver alterations present in adult WD animals, probably due to inefficient hepatic transduction [12]. These evidences suggest that gene therapy could become a curative treatment for WD if sufficient transgene expression of ATP7B can be maintained in the liver for a long period of time. Gene therapy using adeno-associated viral vectors (AAV) is nowadays the most promising therapy for the correction of genetic disorders. Clinical proof of concept has been reported in hemophilia B patients, showing sustained expression of the therapeutic transgene for more than 5 years after a single intravenous administration of an AAV8 vector, with excellent tolerance [13]. In this study we describe the construction and characterization of an AAV8 vector designed for liver-specific expression of ATP7B and its long-lasting therapeutic effects in a relevant mouse model of WD.

Material and methods

Animals and animal manipulation

The generation and characterization of *Atp7b*$^{-/-}$ mice on the hybrid C57BL/6 × 129S6/SvEv background (Jackson Laboratories) was previously described by Dr. Svetlana Lutsenko [9,10]. Mice were bred and maintained under pathogen-free conditions and genotyped at 3 weeks of age according to the original protocol [9]. Treatment with AAV vectors were performed in male mice at 6 weeks of age by intravenous injection. For urine and feces collection, mice were placed for 24 h into metabolic cages (Tecniplast s.p.A.; Buguggiate, VA, Italy) and received food and water ad libitum. Liver samples were collected from euthanized mice for copper determination, histological analysis and for nucleic acid extraction. For the copper overload experiments, mice were fasted overnight and then they received an intraperitoneal injection of 100 μg $CuSO_4$ before starting feces collection. The experimental design was approved by the Ethical Committee for Animal Testing of the University of Navarra.

Construction of AAV vector genomes

The plasmids used in this study were AAV-pA1AT-ATP7B-sPolyA, AAV-pA1AT-dLuciferase-sPolyA and AAV-AlbEnh-pA1AT-EGFP-BGHPolyA. They contain the genome of the AAV vectors AAV8-AAT-ATP7B, AAV8-AAT-dLuc and AAV8-EalbAAT-EGFP, respectively. The expression cassette contained in the AAV8-pA1AT-ATP7B-sPolyA plasmid consisted of: 1) human *ATP7B* coding sequence (CDS) (GenBank accession number U03464); 2) the liver-specific human α1-antitrypsin promoter (pA1AT) [14]; and 3) a synthetic polyadenylation signal (sPolyA) [15]. Gene synthesis of the human *ATP7B* followed by sPolyA was carried out by GenScript (Piscataway, NJ 08854 USA) to obtain the plasmid pUC57-ATP7B-sPolyA. Human pA1AT was isolated from ssAAV8-AlbEnh-pA1AT-EGFP-BGHPolyA plasmid by PCR amplification using the following primers: 1) pA1AT forward primer 5′CTGGTCTAGAACGCGTCGCCACCCCCTCCACCTTGG 3′; 2) pA1AT reverse primer 5′ATCATGATGCGGCCGCTTCACTGTCCCAGGTCAGTG 3′. The PCR product was then digested with XbaI and NotI restriction enzymes and inserted into the same sites of pUC57-ATP7B-sPolyA. Then, the expression cassette containing A1AT-ATP7B-sPolyA was subcloned into the shuttle pAAV-MCS vector using MluI and SmaI sites. The expression cassette contained in the AAV8-pA1AT-dLuciferase-sPolyA plasmid consisted of: 1) the destabilized luciferase gene (the luciferase open reading frame fused to the rapid degradation domain of mouse ornithine decarboxylase); 2) the pA1AT promoter; and 3) the sPolyA. The destabilized luciferase gene (dLuciferase) was released from pdLucFXR plasmid (provided by Dr. Tomas Aragon, CIMA) (GenBank accession number AY603759) using NruI and BamH1. The resulting fragment was ligated in the blunted NotI and BamH1 restriction sites of puc57-pA1AT-sPolyA. Finally, the expression cassette pA1AT-dLuciferase-sPolyA was removed from pUC57 using MluI and SmaI and ligated in the MluI and PmlI restriction sites of the shuttle pAAV-MCS vector. The expression cassette contained in the AAV8-AlbEnh-pA1AT-EGFP-BGHPolyA plasmid consisted of: 1) the enhanced green fluorescent protein gene (EGFP); 2) the mouse albumin enhancer (AlbEnh) linked to the pA1AT promoter [14]; and 3) the bovine growth hormone polyadenylation signal. In all the constructs the expression cassette was flanked by both AAV2 wild-type ITRs.

Serum ceruloplasmin assay

To determine oxidase activity from serum ceruloplasmin, o-dianisidine dihydrochloride was used as substrate. Chloride was removed from the samples prior to analysis to avoid interference with enzymatic activity. This was accomplished by precipitating serum proteins with saturated ammonium sulfate solution. After 10,000 rpm centrifugation for 5 min at room temperature, clear supernatants containing the chloride were removed with a pipette. The pellets were resuspended then in 0.1 M sodium acetate buffer pH5.0 and added to a 96-well plate. Total oxidase activity of the samples was measured by adding 2.5 mg/ml of dimethoxybenzidine dihydrochloride (o-dianisidine; Sigma-Aldrich Co., MO, USA). Then, samples were incubated at 30 °C for 90 min until reaction was stopped by the addition of 9 M sulfuric acid to each well. Finally, absorbance was measured at 540 nm.

Metal-responsive element luciferase reporter assay

A reporter plasmid (pGL3-4MRE-LUC) was used to assess the functional copper export capacity of the ATP7B transgene cloned in our AAV vector. This plasmid

was obtained essentially as described in, [16] and contains 4 metal response elements (MRE) derived from the metallothionein-1 promoter upstream of a minimal E1B promoter. The reporter plasmid responds to bioavailable cytosolic copper inducing luciferase expression. HEK293T cells were co-transfected with pGL3-4MRE-LUC and a plasmid expressing ATP7B under the control of the CMV promoter (pCMV-ATP7B), or the empty pIRES plasmid (Clontech, Mountain View, CA, USA) as a control. All cells were co-transfected with the pR-TK plasmid (Promega, Madison WI, USA) for internal control of transfection efficiency. Cells were incubated with 0 to 150 μM $CuSO_4$ for 24 h. Relative light units were calculated by normalizing Firefly luciferase activities with Renilla luciferase activity.

Copper measurement

A representative solid sample (liver or feces) of each mouse was collected. Special care was taken to minimize the risk of adventitious contamination when handling. Solid samples were weighed (300 and 400 mg for liver and feces, respectively) and later dried in a stove at 70 °C to constant weight. Samples were digested with 10 ml sub-boiling nitric acid (distilled from nitric acid 65% p.a., Merck, Darmstadt, Germany) in an acid-decomposition system. Solutions obtained were then made up to 25 ml with ultrapure deionized water.

Urine samples were collected during 24 h in acid-washed polyethylene containers. Insoluble or suspension particles were removed by centrifugation at 1500 g for 15 min. Appropriate dilution (1:3) with ultrapure water was carried out before analysis. Samples were then assayed for copper content by flame atomic absorption spectrophotometry (Perkin Elmer AAnalyst 800, Norwalk, CT, USA). Measurements were accomplished by direct calibration using working aqueous standards (0–4 mg/L and 0–2 mg/L for solid and urine samples, respectively) made up each day by dilution from stock standard solution (1000 mg/L, Merck) with enough sub-boiling nitric acid to match the acid concentration similar to digested samples. Blank reagents were subjected to similar sample acid-attack procedure. Detection limit (LOD) was calculated according to the criteria established by IUPAC ($X_b \pm 3$ s.d.$_b$) as the average of three times the standard deviation of the reagent blank, setting at 0.002 mg/L equivalent to 5.0 mg/kg and 6 μg/L when expressed in terms of solid and urine samples, respectively. To check the accuracy of analytical procedures, an estimation of copper recovery was performed by spiking on all different assayed samples at different levels of concentration. The percentage recoveries were satisfactory ranging from 96% to 102%. Moreover, throughout the course of the study, both blank reagent and an in-house control were run to satisfy the criteria established in the quality program and to provide on-going quality control information.

For more details and additional methods, see the Supplementary material.

Results

Liver transduction by AAV8 is enhanced in Atp7B$^{-/-}$ mice

We first analyzed whether the presence of liver damage that occurs in WD might affect the intensity or duration of transgene expression following gene therapy with an AAV vector. With this aim we administered AAV8 vectors encoding destabilized luciferase (AAV8-AAT-dLuc) or green fluorescence protein (AAV8-EalbAAT-EGFP) to 6 week old *Atp7B$^{-/-}$* and *Atp7B$^{+/-}$* mice (hereinafter referred to as WD and control mice, respectively). In both cases, transgenes were placed under the control of a liver-specific α1 antitrypsin (AAT) promoter. Luciferase expression was analyzed in animals receiving AAV8-AAT-dLuc by weekly bioluminescence imaging, as shown in Fig. 1A hepatic luciferase expression was significantly higher in WD mice in comparison with their wild-type counterparts (≈10-fold). In both groups, sustained expression was demonstrated for the entire duration of the observation period (100 days). Animals receiving AAV8-EalbAAT-EGFP were sacrificed 4 weeks after vector injection, and GFP expression was analyzed by immunohistochemistry. In concordance with the previous observation, an increase in hepatocyte transduction was observed in WD mice (see representative microphotographs in Fig. 1B). These results indicate that AAV8 vectors are efficient tools for sustained and efficient liver transduction in WD mice.

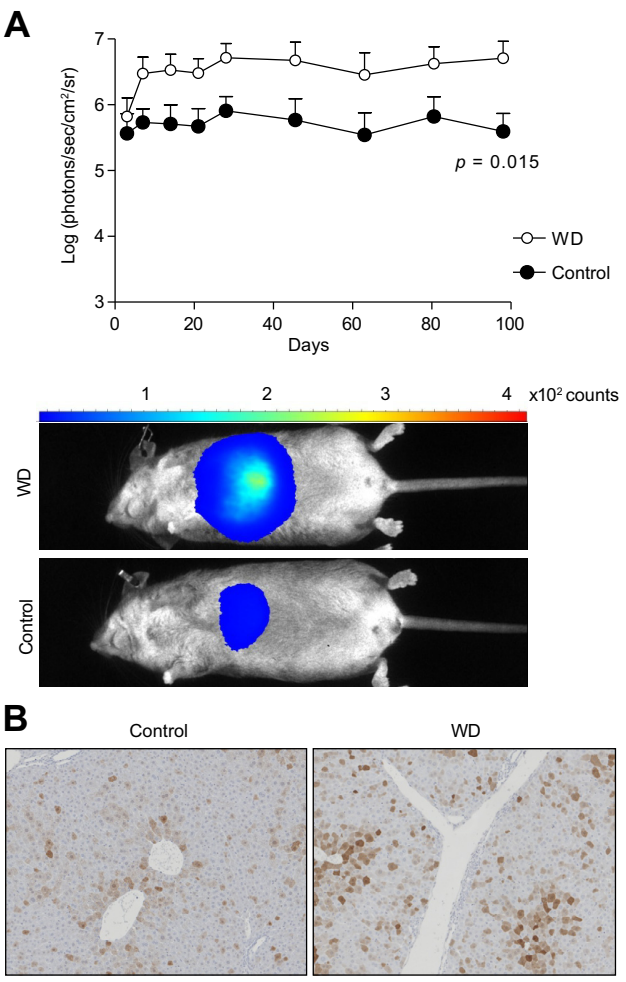

Fig. 1. Liver transduction of *ATP7B*$^{-/-}$ after injection of AAV8 recombinant vectors. (A) Wilson's disease (WD, *ATP7B*$^{-/-}$) and control (heterozygous) mice (n = 5 per group) were intravenously injected with 1×10^{11} vg/mouse AAV8-AAT-dLuc. Bioluminescence imaging was performed 3 days after injection and then weekly for 3 months. Light emission is represented as photons/s/sr/cm^2. A representative picture of control and WD mice 14 weeks after injection is shown in the right panel. (B) Mice (n = 4) were intravenously injected with 3×10^{10} vg/mouse AAV-EalbAAT-EGFP and they were euthanized 4 weeks after virus injection. Immunohistochemistry with anti-GFP antibodies and hematoxylin counterstaining was performed on liver sections (200× microphotographs).

Design of an AAV8 vector for the treatment of WD

The ATP7B transmembrane protein is encoded by a large gene, with 23 exons spanning ~150 kb of genomic DNA, resulting in a 4.4 kb-long cDNA. This sequence was synthetized and introduced in a mammalian expression plasmid containing a ubiquitous CMV promoter (pCMV-ATP7B). HEK293 cells were transfected with this plasmid, and expression of ATP7B was confirmed by Western blot in cell lysates obtained 48 h later. As previously reported [17], the protein was detected as a 165 kDa band coinciding with the expected size (Fig. 2A). To verify the

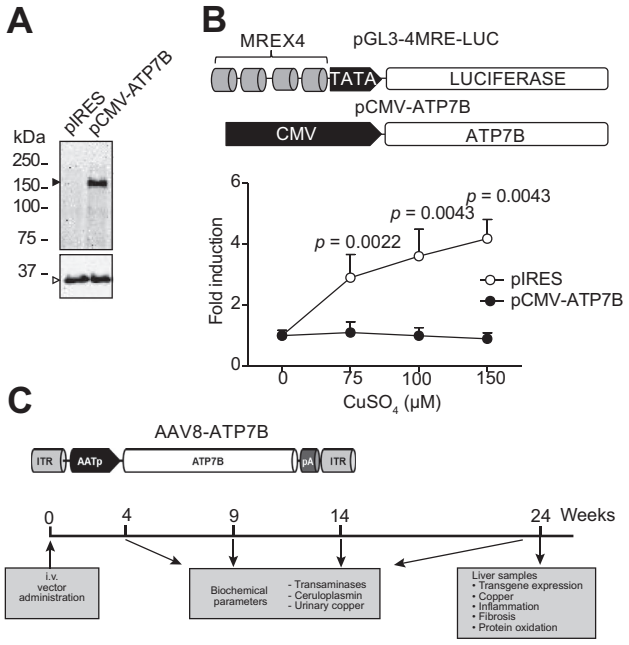

Fig. 2. Biological activity of the transgene, structure of the AAV8-AAV-ATP7B vector and experimental design. (A) HEK293 cells were transfected with the pCMV-ATP7B or empty plasmid (pIRES), and cell extracts were analyzed for ATP7B expression by Western blot 48 h later. GAPDH detection is shown to confirm equivalent sample loading. (B) HEK293 cells were co-transfected with the copper reporter plasmid pGL3-4MRE-Luc and the pCMV-ATP7B or pIRES and then the indicated concentrations of $CuSO_4$ were added to the culture medium. Luciferase expression was determined in cell extracts 24 h later. The graph represents the increase in luciferase activity relative to the basal state in the absence of copper overload. (C) Schematic representation of the vector genome components. i) alpha-1-antitrypsin promoter (AAT); ii) human ATP7B coding sequence; iii) a synthetic polyadenylation signal (sPolyA); iv) the inverted terminal repeat sequences of AAV2 (ITRs) flanking the vector genome. The timeline of *in vivo* experiments is represented in the lower panel. The vector was administered intravenously (i.v.) to 6 week old WD mice. Biochemical parameters were analyzed at the indicated times, and mice were sacrificed 6 months later for evaluation of liver samples.

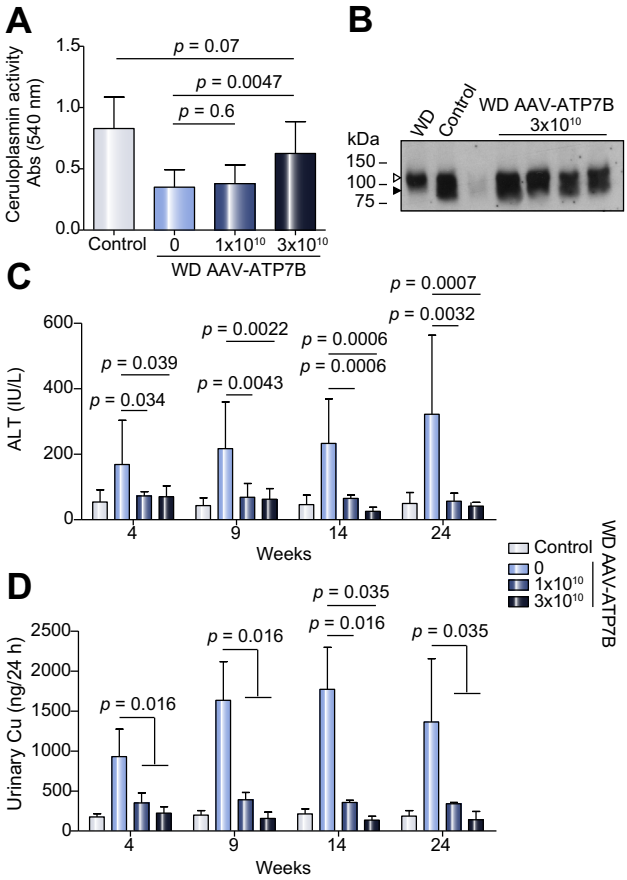

Fig. 3. A single intravenous administration of AAV8-AAT-ATP7B corrects biochemical abnormalities in WD mice. AAV8-AAT-ATP7B (AAV-ATP7B) was administered intravenously to 6 week old WD mice at 1×10^{10} vg/mouse or 3×10^{10} vg/mouse. Other groups include healthy mice (controls) and untreated WD mice. (A) Oxidase activity of ceruloplasmin, expressed as absorbance at 540 nm. (B) Holo- and apo-ceruloplasmin were detected in serum samples obtained 4 weeks after treatment by Western blot under non-reducing, non-denaturing conditions. (C) Serum ALT levels were determined at the indicated times, and expressed as IU/L (IU: international units). (D) Copper content, expressed as ng/24 h, was measured in urine samples at the indicated times by atomic absorption spectrophotometry.

biological activity of ATP7B, pCMV-ATP7B was co-transfected with a reporter plasmid expressing luciferase under the control of a metal-inducible promoter (Fig. 2B). In concordance with the expression of the functional copper transporter, cells transfected with the pCMV-ATP7B plasmid showed no elevation of luciferase signal in response to copper overload, in contrast with cells transfected with an empty plasmid (Fig. 2B). Once expression and activity of the therapeutic gene were confirmed, we designed the therapeutic vector. Given the size constraints imposed by the AAV packaging capacity, a relatively small liver-specific promoter derived from the human α1-antitrypsin promoter [14] and a compact synthetic polyadenylation signal [15] were used to assemble the AAV8-AAT-ATP7B vector, as depicted in Fig. 2C.

AAV8-AAT-ATP7B corrects biochemical alterations of WD mice

Two groups of 6 week old WD mice (n = 5 per group) received AAV8-AAT-ATP7B at a dose of 1×10^{10} or 3×10^{10} vg/mouse and another group of WD mice of the same age was left untreated. Different biochemical parameters including serum transaminases, serum oxidase activity, serum holoceruloplasmin (the copper-loaded serum ceruloplasmin fraction) and urinary copper excretion, were analyzed at different time points post-treatment during 6 months (see experiment timeline in Fig. 2C). In addition we studied sequentially a group of age-matched *Atp7b*$^{+/-}$ littermates taken as a healthy control group since these heterozygous animals showed no alterations of copper metabolism or liver function parameters in comparison to wild-type mice (data not shown). We found that alanine aminotransferase (ALT) was elevated in WD mice at 4 weeks after the initiation of the study (corresponding to 10 weeks of age), and remained elevated during all the observation period (6 months). Treatment with AAV8-AAT-ATP7B achieved complete and sustained normalization of ALT (Fig. 3A) and aspartate aminotransferase levels (not shown), even at the lowest dose tested. Furthermore, complete restoration of serum holoceruloplasmin (Fig. 3B) and of its oxidase activity (Fig. 3C), was only achieved in the high dose group

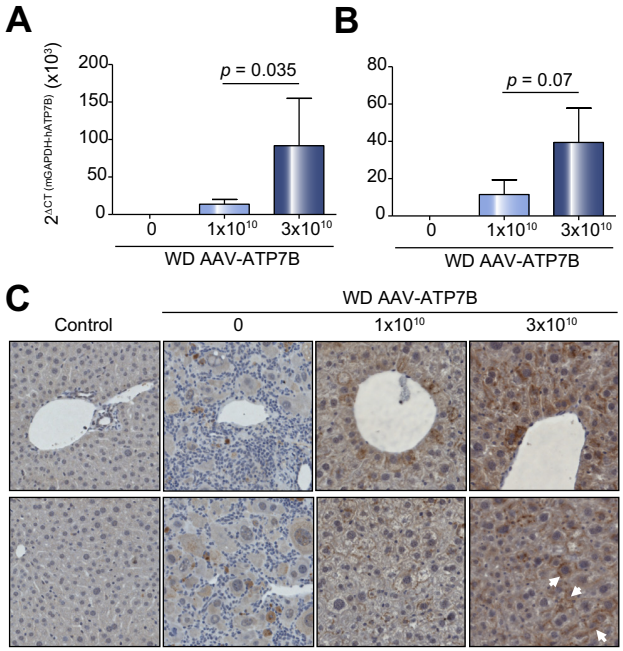

Fig. 4. AAV8-AAT-ATP7B achieves dose-dependent, long-term expression of ATP7B in the liver of WD mice. The vector was administered intravenously to 6 week old control or WD mice at the indicated doses (vg/mouse), and livers were collected 6 month later for analysis of vector genome content by qPCR (A). Hepatic ATP7B expression was analyzed at the mRNA level by qRT-PCR (B), and at the protein level by immunohistochemistry (C). Note the localization of staining in the bile canaliculi (white arrows).

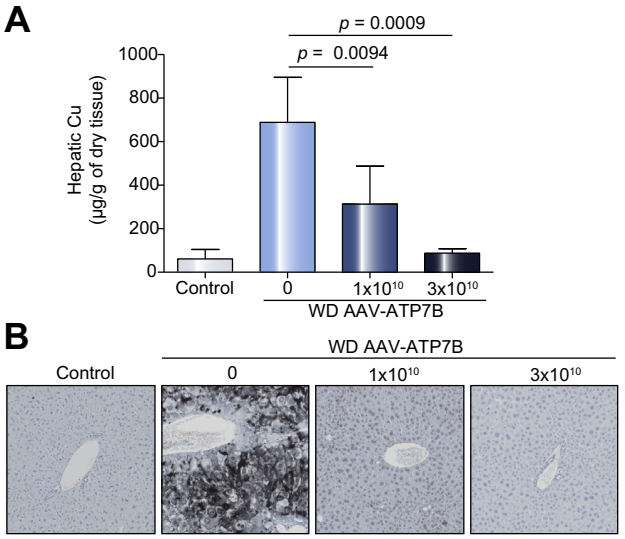

Fig. 5. AAV-AAT-ATP7B prevents the accumulation of copper in the liver of WD mice in a dose-dependent manner. (A) Copper content, expressed as µg/g of dry tissue, was determined by atomic absorption spectrophotometry on livers from WD mice sacrificed 24 weeks after a single injection of AAV-AAT-ATP7B vector at the indicated doses (in vg/mouse). Copper content in healthy mice is included as a control. (B) Timm's sulphide silver staining in liver sections from representative mice in each group (400× microphotographs). (This figure appears in colour on the web.)

of animals but not in the group treated with the lowest dose of vector. The increase in oxidase activity caused by AAV8-AAT-ATP7B treatment was easily evidenced in young WD mice (4 weeks after treatment, 10 weeks of age), before the onset of liver damage. Interestingly, older WD mice showed spontaneous elevation of oxidase activity that coincides with increased transaminase levels (Fig. 3A; Supplementary Fig. 1). We hypothesize that liver damage may activate an ATP7B-independent mechanism for loading copper into ceruloplasmin, although this mechanism does not alleviate hepatic copper overload in WD animals. To prove that liver injury causes an increase in holoceruloplasmin and serum oxidase activity in this animal model, we treated 6 week old WD or control mice with an intravenous dose of a first-generation adenovirus vector encoding GFP in order to cause inflammatory liver damage. Adenovirus administration in wild-type mice induced a rapid elevation of ceruloplasmin oxidase activity, in agreement with its role as an acute phase reactant. This response occurs even at low viral doses, in the absence of ALT elevations (Supplementary Fig. 2). In contrast, WD mice lacked this physiological response. Instead, they experienced a delayed elevation of oxidase activity concomitant with the onset of liver damage (Supplementary Fig. 2). Of note, WD mice were more sensitive than control mice to the hepatotoxic effect of adenovirus. Thus these data support our initial hypothesis, indicating that the unexpected elevation of holoceruloplasmin levels in serum is associated with liver damage in WD mice. Furthermore, the results obtained suggest that in young WD animals treated with AAV8-AAT-ATP7B the normalization of ceruloplasmin oxidase activity in the absence of ALT elevations can be attributed to the therapeutic effect of the vector when used at a dose of 3×10^{10} vg/mouse.

One of the most clinically relevant parameters in WD patients is the elevation of urinary copper excretion. At all times tested, WD mice treated with AAV-AAT-ATP7B showed a significant reduction in urinary copper content, with complete normalization in the high dose group (Fig. 3D).

AAV8-AAT-ATP7B treatment prevents liver copper accumulation and liver injury in WD mice

Six months after the initiation of treatment, all mice were sacrificed and liver samples were collected. The presence of the vector genomes was determined by qPCR (Fig. 4A), and *ATP7B* mRNA content was quantified by qRT-PCR (Fig. 4B). Expression of the therapeutic protein was detected by immunohistochemistry (Fig. 4C). All these determinations indicate a dose-dependent transduction of the liver in WD mice and persistence of functional vector for the entire duration of the experiment. Next, hepatic copper content was measured in liver samples by atomic absorption spectrophotometry. In concordance with the previous observations, a dose-dependent reduction in metal accumulation was detected in treated animals (Fig. 5A). At 1×10^{10} vg/mouse the reduction was partial, but in the 3×10^{10} vg/mouse group copper content was comparable to the levels detected in healthy livers. Reduction of copper deposits was confirmed by Timm's sulphide silver staining in liver sections (Fig. 5B).

One of the most remarkable findings of our study was the complete normalization of liver histology observed in the treated animals. WD mice at 6 months of age showed dramatic

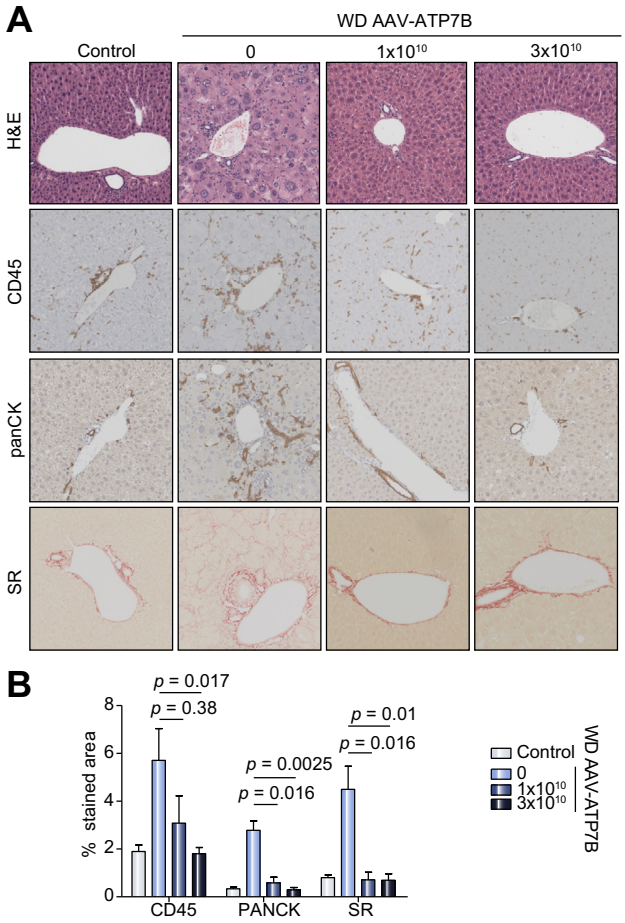

Fig. 6. AAV-AAT-ATP7B prevents liver damage in WD mice. Liver histology in samples from WD mice sacrificed 24 weeks after a single injection of AAV-AAT-ATP7B vector at the indicated doses (in vg/mouse). Healthy mice are included as a control. (A) Representative microphotographs (400×) of Hematoxylin/Eosin staining (H&E), CD45, panCK immunohistochemistry and Sirius Red staining (SR). (B) Automated quantification of the percentage of positive areas for CD45, PanCK and Sirius Red (SR) staining in each group.

alterations in organ architecture. Most hepatocytes were extremely enlarged and presented big nuclei, as revealed by hematoxylin/eosin staining (Fig. 6A). Also, an intense and widespread leukocyte infiltrate was observed, a finding which was confirmed by CD45 immunostaining. In addition we noted an increased number of panCK$^+$ cells, indicating proliferation of small bile ducts. Sirius Red staining revealed increased collagen deposition, compatible with a moderate degree of fibrosis (Fig. 6A, B). All these alterations were absent in WD mice treated with the AAV8-AAT-ATP7B vector at 3×10^{10} vg/mouse (Fig. 6A, B), in concordance with the absence of copper accumulation (Fig. 5). WD mice treated with low vector dose also showed normalization of several histological changes including collagen content and biliary duct proliferation. In addition, the oxidative status of hepatic proteins was measured in liver samples, as an indicator of cell damage caused by free copper excess in hepatocytes. Supplementary Fig. 3A, shows fluorescent staining of oxidized thiol groups in total protein extracts separated by 1 dimension acry-

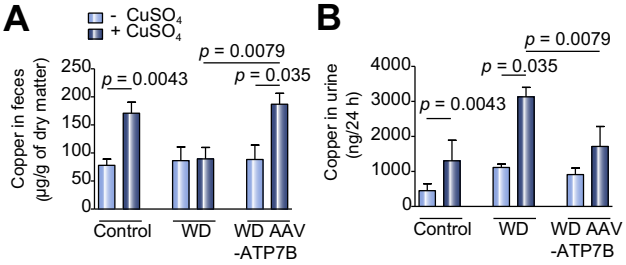

Fig. 7. AAV8-AAT-ATP7B restores physiological excretion of copper following a metal overload. Different groups of 6 week old WD mice (n = 5 per group) were treated or not with AAV8-AAT-ATP7B. Heterozygous littermates were used as control. Two weeks after treatment, some groups received a copper overload by intraperitoneal administration of 100 µg $CuSO_4$. Copper concentration was analyzed in feces (A) and urine (B) collected during the next 24 h.

lamide gel electrophoresis from representative animals in each group. Compared with the oxidation pattern in control mice, WD mice showed differences in proteins of diverse molecular weight, particularly evident in the low molecular weight range. The pattern of protein oxidation was reverted to normal in treated WD mice especially in those given the high vector dose. Densitometric quantification of bands in the low molecular range demonstrated reduced protein oxidation (Supplementary Fig. 3B), in good correlation with the normalization of hepatic copper concentration shown in Fig. 5.

AAV8-AAT-ATP7B treatment restores physiological copper excretion in WD mice

Finally, we investigated whether gene transfer of the *ATP7B* gene to the liver of WD mice was able to restore biliary copper excretion. To this end, 6 week old WD mice (n = 5 per group) were treated with AAV8-AAT-ATP7B or were left untreated, and a group of heterozygous littermates was used as control.

Two weeks after treatment, some animals received a copper overload by intraperitoneal administration of 100 µg $CuSO_4$, or saline as a control and were placed in a metabolic cage for urine and feces collection during 24 h. We found that control animals and AAV8-AAT-ATP7B-treated WD mice were similarly able to excrete the excess of copper in feces (Fig. 7A). In contrast, copper overload did not change fecal copper excretion in untreated WD mice, and as a result urinary copper was significantly higher in this group compared to AAV8-AAT-ATP7B-treated WD mice (Fig. 7B). These data clearly demonstrate that AAV8-AAT-ATP7B restores normal copper metabolism in WD mice.

Discussion

Present treatment of WD is based on lifelong zinc salts and/or copper chelators despite substantial problems associated with their use, including side effects and lack of adherence. An important drawback of these drugs is their inability to promote a physiological elimination of copper through the biliary system. In contrast, on one hand copper chelators promote mobilization of the metal from liver stores into the blood, with subsequent elimination through the urine. This may lead to elevations of circulating copper after the initiation of chelating therapy, with the risk of increasing copper deposition in the brain resulting in

irreversible neurological deterioration [4,5,18]. On the other hand, in children, zinc salts have shown poor efficacy as a first line treatment and a high rate of gastrointestinal side effects (including gastric perforation) making necessary in some cases a switch to copper chelators [19]. Compared to current treatments, gene therapy has two main advantages: first, one single vector dose may have an effect lasting for years; and second, it restores physiological copper homeostasis, facilitating biliary excretion without transit of copper through the blood. Our results support this notion, showing that AAV8-AAT-ATP7B treatment achieves copper depletion from liver, concomitant with reduced urinary excretion and increased biliary elimination into feces.

After decades of intense efforts, the first evidence of clinical benefit of liver-directed gene therapy is coming from hemophilia B patients treated with AAV8 vectors encoding the coagulation factor IX [13]. This study was of considerable interest as it showed that: (i) the human liver is permissive to transduction by AAV8 vectors; and (ii) a disease like hemophilia, which needs only a small increase in the serum concentration of factor IX for correction of the hemostatic defect, can be successfully treated by inducing the liver to secrete this protein to the circulation using a relatively low vector dose. In WD, the essential defect is the altered ATP7B expression in the liver, a notion supported by the fact that all disturbances of copper metabolism are corrected in cirrhotic patients subjected to liver transplantation [20]. Here we have tested whether liver transduction with a safe and efficient long-term expression vector such as AAV8 encoding ATP7B could be an effective treatment for WD. One of the main challenges in WD is the fact that the ATP7B transporter is a large transmembrane protein that should be expressed in the TGN of hepatocytes to be functional. Therefore, no cross-correction of cells is expected, and there is no information about the percentage of transduced hepatocytes required for a clinically relevant effect. In this work we provide preclinical data suggesting that a moderate dose of the AAV8-AAT-ATP7B vector (3×10^{10} vg/mouse, 1.5×10^{12} vg/kg), which is feasible in humans, protects WD mice from copper accumulation in the liver and normalizes all biochemical and histological parameters tested. According to our immunohistochemistry data on livers transduced with AAV8 vectors encoding reporter or therapeutic genes (Figs. 1B and 4C, respectively), protection was obtained with transduction efficiencies below 100%. In principle, this would imply that hepatocytes expressing high levels of ATP7B could work as a sink, excreting copper to the bile and thus preventing copper accumulation in neighboring cells. Previous studies performed in LEC rats support this theory [8,21,22]. When the animals were treated with transplantation of ATP7B-expressing hepatocytes after different liver preconditioning regimes (radiation and ischemia reperfusion [8], two-thirds hepatectomy alone [21] or in combination with retrorsine [22]), prevention of liver damage, copper accumulation and partial restoration of biliary copper excretion was observed with less than 100% hepatocyte repopulation. However, more studies are needed to firmly support this conclusion because we cannot rule out that copper excretion is mediated by low levels of ATP7B expression, undetectable by immunohistochemistry.

Importantly, several lines of evidence demonstrate that the phenotype correction observed in treated animals is not due to spontaneous reversion of the disease in the *Atp7b*$^{-/-}$ model. On one hand, no reversion was observed in any of the untreated mice included in the control groups of our experiments and on the other hand, we have documented a dose-response therapeutic effect in animals treated with the AAV8-AAT-ATP7B vector. Finally, genotyping performed in liver samples from WD animals confirmed that the endogenous *Atp7b* gene remains disrupted (not shown). Interestingly, our data indicate that hepatic transduction with AAV is favored in WD mice compared with control mice (Fig. 1), a phenomenon which was not observed with other vectors such as adenovirus (not shown). This is in line with previous works showing that AAV transduction is enhanced in different situations of cellular stress, including treatment with DNA damaging agents [23], alcohol-induced oxidative stress [24] as well as inflammation and response to misfolded proteins [25]. The mechanism responsible for the enhanced transgene expression in WD mice is currently being investigated.

If these events in mice would also occur in patients with WD, AAV vectors would be privileged tools for the treatment of this condition, although direct extrapolation is not possible because higher heterogeneity in liver damage is expected in humans due to differences in the severity of the disease and their response to standard therapies. Therefore, although gene therapy for WD is more challenging than for other diseases such as hemophilia, we have found that the level of liver transduction required for correction of the disease is compatible with the current state of the technology. So far, we have not detected any side effect associated with the vector at the highest dose tested. Nevertheless, expanded dose escalation and detailed toxicological studies are underway as part of our preclinical evaluation of the treatment. In summary, our data encourage future translational efforts to test AAV-based gene therapy as an option in the management of WD patients.

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Conflict of interest

The authors who have taken part in this study declared that they do not have any conflict of interest with respect to this manuscript.

Authors' contributions

OM, GGA, RH and JP study concept and design; OM, DM and CG acquisition of data; IF, ES, GGA, JP analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; OM, DM, statistical analysis; GGA, JP, obtained funding; AM and FC, proteomic analysis, DME, INB, IM copper determination; RH, JP and GGA study supervision.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.09.014.

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