Human Gene Therapy

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Long-term correction of copper metabolism in WD mice with AAV8 vector delivering truncated ATP7b

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Human Gene Therapy
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Abstract

Wilson's disease is an autosomal recessive disorder of copper metabolism caused by mutations in the ATP7B gene encoding a liver active copper transport enzyme. Gene therapy with adeno-associated virus (AAV) carrying full length ATP7b, which is about 4.4 kb, was shown to rescue copper metabolism disorder in WD mouse model. However, due to its relatively large size, the AAV vector containing full length ATP7b could be oversized for its packaging capacity, which could lead to inefficient packaging. To this purpose, we engineered a truncated ATP7b mutant (tATP7b) that is about 3.3kb in length and used for AAV gene therapy for WD mice. In vitro test showed that the excretion of copper outside the cells could be achieved with tATP7b as efficient as the full-length ATP7b. In vivo delivery of tATP7b to WD mice by AAV8 vectors corrected their copper metabolisms and significantly rescued copper accumulation related syndromes, including reduced urinary copper excretion, increased serum ceruloplasmin and improved liver damages. Thus, our work demonstrated AAV gene therapy based on truncated ATP7b is a promising strategy in the treatment of WD.

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Introduction

Wilson's disease, also named hepatolenticular degeneration, is an autosomal recessive disorder of copper metabolism, resulting in hepatic and neurological degeneration.[1] Gene responsible for WD encodes P-type ATPase B that is mainly expressed in hepatocytes and involved in the excretion of copper outside hepatocytes into the bile. [2-5]Mutations in ATP7B gene lead to excessive accumulation of copper in the liver, brain, and other organs that eventually caused cell malfunction and degeneration. [6, 7]Current therapeutic strategies against WD included limiting dietary intake, inhibiting absorption and promoting urinary excretion for copper.[8-11] However, these strategies did not restore the normal copper metabolism and required lifelong treatment. [12]Besides, promoting urinary excretion by copper chelators frequently led to serious side effects, such as allergic reaction and chronic key damage, making long-term treatment a big challenge.[13, 14]

Recently, gene therapy with full-length wildtype ATP7b gene had been demonstrated to be a promising strategy against WD in animal models. [15]Adenovirus delivered full length ATP7b to LEC rats achieved efficient therapeutic effects, including restoration of serum ceruloplasmin and elevation of feces copper. However, due to short in long-term expression capacity, adenovirus mediated therapy was transient. [16]By contrast, AAV mediated ATP7b transduction to WD mice caused long-term improvement of copper metabolism, including sustained reduction of serum transaminases and urinary copper excretion, normalization of serum ceruloplasmin, and restoration of physiological biliary copper excretion. [15]However, due to its relatively large size, full length ATP7b is difficult to be packaged efficiently because the packaging capacity of AAV vector is less than 5 kb, which is a major hurdle standing in the way of clinical translation. [17]

The aim of this work was to discover a truncated but functional ATP7b protein that is small enough to be easily packaged into AAV vectors. Structurally, full-length mammalian ATP7b contains at least 3 functional domains, the N-terminal metal-binding domains (MBD) containing 6 MBDs responsible for Cu-binding, the transmembrane domain (TMD) encompassing eight transmembrane helices responsible for Cu-coordination, and the C-terminal domain containing two leucine motifs responsible for TGN localization. [18-

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[22]Through protein blast searching, we found several transcriptional or splicing isoforms of ATP7b harboring deletions in MBD or/and transmembrane domains (isob-e), and the shortest isoform is from gorilla, harboring a N-terminal deletion of the first 4 MBDs (isoGx6). In vitro test revealed that among these truncated isoforms, only isoC and isoGx6 retained the ability to excrete copper. Because previous study had demonstrated that the N-terminal peptide was critical for ATP7b to translocate to the apical region, an equivalent structure to bile canalicular, we added this peptide to the shortest isoform to construct a novel isoform, tATP7b.[23] Targeted delivery of tATP7b to the liver of WD mice corrected their copper metabolisms and significantly rescued copper accumulation related syndromes. Just at the time of preparing this manuscript, Aseguinolaza lab in Spain reported similar results using a similar truncated ATP7b to ours. Therefore, together data from Aseguinolaza lab, these results demonstrated the translational potential of truncated functional ATP7b in the treatment of WD.

Materials and methods

Animals and animal manipulation

C3He-ATP7b$^{tx\text{-}j}$/J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were bred and maintained under pathogen-free conditions and genetically identified at 3 weeks of age.[24] Treatment with AAV vectors were performed in male mice at 6-8 weeks of age by intravenous injection via tail vein. Blood was collected from the eyelids as zero point data before injection. For urine 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 assays, histological analysis, and nucleic acid extraction. All animal procedures were performed following the protocol approved by the Institutional Animal Care and Treatment Committee of Sichuan University (Chengdu, China).

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A reporter plasmid (pGL3-7×MRE-LUC) was used to assess the functional copper export capacity of tATP7b transgenes cloned in the AAV vectors. This plasmid contains 7 metal response elements (MRE) derived from the metallothionein-1 promoter upstream of a minimal E1B promoter. The reporter plasmid responds to bioavailable cytosolic copper by activating luciferase expression. HEK293T cells were co-transfected with pGL3-7×MRE-LUC and a plasmid expressing tATP7b under the control of the CMV promoter (pCMV-tATP7b), or the empty pEGFP plasmid as a control. All cells were co-transfected with the pCMV-RL plasmid for internal control of transfection efficiency. Cells were incubated with 75μm CuSO4 for 48 h. Relative light units were calculated by normalizing firefly luciferase activity with Renilla luciferase activity.

Production and purification of rAAV vectors

The plasmid ssAAV8-ApoE/hAAT-tATP7b was constructed for rAAV packaging. hATP7b was expressed under the control of a liver-specific promoter hAAT with an ApoE enhancer (ApoE/hAAT), which has been described previously.[25] All rAAV vectors were produced at Sichuan University using triple plasmid transfection method.[26] Briefly, Adenovirus helper plasmid, Rep/Cap helper plasmid and transgene plasmid were mixed at a ratio of 1:1:1. HEK293 cells were tranfected with the mixture of the three plasmids by calcium phosphate method. The cells and supernatants were harvested at 72 hour post transfection, and viral vectors were purified by double cesium chloride ultracentrifugation followed by dialysis. The vectors were aliquoted and stored at -80°C. The vector titers were determined by qPCR (Transgen Biotech, Beijing, China) using the following primers: hATP7B forward primer 5´CATTCCAGGACTGTCCATTCT3´; hATP7B reverse primer 5´GGCCTGAACGTAGAAGTACCA3´.

Serum ceruloplasmin assay

The mixture of 45μl of 0.1 M acetate buffer solution (pH 6.0) and 15μl of serum was warmed at 37°C for 5 min. After the addition of 60 μl of TMB solution warmed at 37°C, the solution was mixed well and kept at 37°C for 30 min. The reaction was terminated by the addition of 30μl of sodium azide solution. The absorbance of resulting product was measured at 645 nm.[27]

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Quantification of rAAV genome copy in liver cells

To quantify viral genome copies in liver cells, total genome DNA was isolated from the frozen liver tissues using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). Viral genomes were quantified by qPCR using TransStart Tip Green qPCR SuperMix (Transgen Biotech, Beijing, China) using StepOnePlus™ Real-Time PCR System (Applied Biosystems™, Carlsbad, CA). In a 20-ul qPCR system, 100 ng of genome DNA for each sample was used as the template, and the primer pairs were the same as above for hATP7B titration. Data were normalized to mouse Glyceraldehyde-3-phosphate deshidrogenase (mGAPDH) and primer pairs used for mGAPDH were 5´ AACGGATTTGGCCGTATTGG3´(forward primer) and CATTCTCGGCCTTGACTGTG3´(reverse primer).

Quantification of tATP7b transcription

To measure hATP7B gene expression, total RNAs were extracted from the frozen liver tissues using TRIzol® Reagent method (Ambion, Carlsbad, CA), and kept at -80°C. cDNAs were obtained using PrimeScriptTM RT reagent Kit and gRNA Eraser (Takara). Expression of tATP7b were measured by qPCR using TransStart Tip Green qPCR SuperMix, and the primer pairs were the same as above for tATP7b titration. Data were normalized to mGAPDH and primers used were the same as above for the vector genome quantification.

Copper measurement

A representative sample （liver or urine） of each mouse was collected. Special care was taken to minimize the risk of adventitious contamination when handling. Copper measurements were performed in Analytical & Testing Center， Sichuan University, Chengdu, China.

ALT activity measurement

Peripheral blood samples were collected via retro-orbital bleeding at the indicated time points, and the serum was separated from whole blood after centrifugation at 3,000 rpm for 25 minutes at 4 ^{o}C. ALT activity was measured in West China Women's and Children's Hospital, Sichuan University.

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Histological analysis of paraffin embedded section

Livers were harvested at the indicated time points and immediately fixed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde overnight at 4℃ upon sacrifice. The paraffin sections were stained with hematoxylin and eosin (H.E.). Primary antibodies against ATP7b（ab124973， Abcam, human specific）, N Cadherin (ab18203, Abcam) and CD45(ab10558, Abcam) were used to visualization each protein by immuno-histological chemistry (IHC) staining of the tissue sections. For quantification of tATP7b positive cells, IHC stained tissue sections were scanned with a Pannoramic MIDI scanner (3DHISTECH), and the resulting images were analyzed with image-pro plus 6.0 software.

Results

Identification of functional truncated ATP7b proteins

Because the full length of ATP7b is too large for efficient packaging of AAV vectors, we sought to identify functional truncated mutant. We firstly searched naturally occurred transcriptional or splicing ATP7b isoforms, and found several candidates that lacking either MBD or transmembrane domains or both. We selected several representative isoforms, including - isoform b (NCBI Reference Sequence:NM_001005918.2); isoform c (NCBI Reference Sequence: NM_001243182.1); isoform d (NCBI Reference Sequence:NM_001330578.1); isoform e (NCBI Reference Sequence: NM_001330579.1) and gorilla isoform X6 （NCBI Reference Sequence:. XM_019039855.1）. We cloned these hATP7b isoforms and humanized gorilla isoform X6 into mammalian expression vector for in vitro functional analysis.

To test the copper excretion activity of these candidates, we constructed a copper responsive reporter, in which firefly luciferase was driven by a mosaic promoter harboring 7 copies of metal responsive elements (MRE) and an adenovirus derived E1b minimal promoter (Figure 1A). [28, 29] This reporter would be triggered by MRE-binding transcription factor-1 (MTF-1) in response to cellular stress caused by heavy metals, such as copper and zinc. [30] Therefore, upon copper exposure, the expression of firefly luciferase was significantly induced in the cells transfected with this reporter. However, when functional

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ATP7B was introduced to export copper outside the cells, this copper-dependent reporter induction was abrogated. As shown in Fig 1C and 1E, MRE reporter worked very well in determining copper transport activity of the full length ATP7b protein. Among these truncated isoforms, isoC and isoGx6 harboring 4 or 2 MBDs deletion respectively were as active as full-length ATP7b, suggesting that MBDs are functionally redundant in copper transport. This result was in consistent with previous functional analysis of site-directed mutations and deletions in MBDs in ATP7b. The rest isoforms did not show any transport activity, possibly due to they lacked some transmembrane domains, suggesting the integrity of transmembrane region is critical for copper transport.

Previous works have shown that the N-terminal sequence in full length ATP7b protein is critical for its subcellular translocation in response to intracellular copper concentration.[23] In these works, a truncated ATP7b protein that containing N-terminal translocation motif and isoGx6 have been shown to be able to translocate to apical region, which is an equivalent subcellular structure to bile canaliculi formed in cultured polarized hepatic. Therefore, to ensure Cu-dependent trafficking, the N-terminal signal was added to the humanized isoGx6 ATP7b (tATP7b). Compared to full length human ATP7b, tATP7b has a deletion of 425 amino acids (Δ57-481). As shown in Figure 1E, tATP7b had similar copper excretion activity to full length and humanized isoGx6 ATP7b proteins.

tATP7b delivered by AAV8 vector corrects copper metabolism in WD mice

Recombinant AAV vectors pseudotyped with AAV serotype 8 capsid have been demonstrated to improve liver transduction in comparison to other AAV serotypes.[31] Therefore, we constructed an AAV vector containing the tATP7b cDNA under the control of a liver-specific mosaic promoter containing human ApoE enhancer and AAT promoter (Figure 2A). Prior to AAV packaging and production, the transgene expression of recombinant AAV plasmid were tested in vivo by hydrodynamic tail-vein (HTV) injection. As shown in Fig 2B, injection of AAV-tATP7b plasmid indeed expressed tATP7b proteins, suggesting that the tATP7b expression cassette was functional. Then the tATP7b transgene was packaged into AAV8 vector which was named AAV8-tATP7b.

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WD mice at 6-8 weeks old were randomly allocated into four groups and injected with various doses of AAV8-tATP7b as indicated in Table.1（materials and methods）(5×10^{11}, 2.5×10^{12}, and 2×10^{13} GC/kg). A group of age-matched Atp7b+/- littermates were taken as healthy controls and WD mice injected with PBS served as negative control. Two key WD related biochemical parameters, serum ceruloplasmin and urinary copper excretion, were analyzed for evaluation of therapeutic effects at different time points post treatment as scheduled in Fig 3A. As shown in Fig 3B, urinary copper excretion was obviously reduced in WD mice after AAV8-tATP7b transduction. The reduction of urinary excretion was correlated with AAV dosage, indicating a dose-dependent therapeutic effect. Another biochemical parameter, serum ceruloplasmin, was rescued by AAV8-tATP7b transduction. Compared to PBS treatment, AAV8-tATP7b transduction promoted ceruloplasmin activity in WD mice to the level similar to that of wildtype ones. Importantly, these restoration of WD related biochemical parameters were maintained at least 6 months. Together, these results suggested AAV8-tATP7b significantly improved copper metabolism in WD mice.

Effects of AAV8-tATP7B gene therapy on copper accumulation and liver injury in WD mice

In WD patients, copper accumulation in liver resulted in hepatocyte degeneration and cell death. To evaluate whether improved copper metabolism by AAV8-tATP7b gene therapy was accompanied with inhibition of liver degeneration, we tested serum ALT activity, which is a sensitive parameter for hepatocyte damage and frequently elevated in WD patients. As shown in Fig 4A, compared to wild type mice, PBS treated WD mice had significantly higher serum ALT activity. In WD mice injected with AAV8-tATP7b, only those received 2×10^{13} GC/kg viral vectors showed normalized ALT activity. And the ones received 2.5×10^{12} and 5×10^{11} GC/kg viral vectors showed detectable but only mild decrease in ALT activity, which were significantly higher than ATP7b+/- control, indicating that those two dosages failed to completely prevent copper accumulation and relieve liver damage.

To demonstrate this notion and further evaluate the therapeutic effects of AAV8-tATP7b, we sacrificed all the mice at six months post initial treatment and analyzed a panel of pathological features of livers. Tissue section and HE staining analysis revealed that,

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compared to wild type control mice, PBS treated WD mice showed extremely enlarged hepatocytes with abnormally swelling nuclei. Again, only the mice treated with the highest dose of vectors had widespread normal hepatocytes, while mice treated with other dosages had very small portion of normal hepatocytes. In consistent with this, expression of tATP7b were only detected in very restricted regions in the livers of mice treated with 5×10^{11} and 2.5×10^{12} GC/kg vectors as determined by IHC staining (Fig. 4D). In 5×10^{11} GC/kg vectors group, about 3.35% hepatic cells were detected to be transgene positive and in 2.5×10^{12} GC/kg vectors group 7.61% hepatic cells were transgene positive. In the livers of mice treated with 2×10^{13} GC/kg vectors, widespread tATP7b expression was detected (71.8%). The expression of tATP7b was further confirmed by qPCR analysis of the vector genomes and tATP7b mRNAs (Fig. 4B and 4C). Interestingly, we noticed that only tATP7b positive hepatocytes looked morphologically normal while those hepatocytes negative for tATP7b, even the ones neighboring to and surrounded by tATP7b positive cells, were obviously enlarged and harboring big nuclei, suggesting that ATP7b functions in a cell-autonomous manner in protecting hepatocytes from copper induced degeneration.

Next, we tested whether copper accumulation in the liver was reduced by AAV8-tATP7b treatment. Liver samples were analyzed with atomic absorption spectrophotometry for copper content. In consistent with observations of liver degeneration, we found only mild reduction in hepatic liver copper content in mice received 5×10^{11} and 2×10^{13} GC/kg. And mice received 2×10^{13} GC/kg had significant reduced copper content, despite level of which was much higher than that of wild type healthy mice. These results were further confirmed by Timm's sulphide silver staining of liver tissue sections, which is a sign of copper accumulation. In the sections, we also found dose dependent reduction in sliver staining. In sections from 5×10^{11} and 2×10^{13} groups, only mildly reduced silver staining was observed. And 2×10^{13} group had very few but obvious and restricted silver staining. Accompanied with the reduction of copper accumulation, a panel of hepatic cirrhosis related pathological features was also relieved in tATP7b treated mice. As shown in Fig 6, dose-dependently reduced the infiltration of leukocytes, collagen deposition and biliary duct proliferation could be observed after tATP7b injection. Taken together, these data

demonstrated that our tATP7b gene therapy prevent copper accumulation and liver injury in WD mice.

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Discussion

WD is among the very rare inherited diseases that are treatable. However, current strategies for treating WD patients involved lifelong low-copper dietary, plus zinc salts and metal chelators that possess more or less inclination to copper, which often led to serious side effects, such as neutropenia, thrombocytopenia and nephrotoxicity.[13, 14] Recently, gene therapies with wild type, full length ATP7b gene had been demonstrated to be promising for the treatment of WD. [15, 16, 32-36]Among the vectors used to deliver ATP7b, AAV vector is the most promising one because it can achieve long-term expression of transgene and therefore stably correct the copper metabolism and inhibit liver degeneration.[37, 38]

However, the open reading frame (ORF) of full length ATP7b is around 4.4 kb, which is a big burden for AAV packaging, inevitably resulting in inefficient packaging and in vivo transduction of AAV vectors.[39] A promising way to resolves this hurdle is searching for shorter ATP7b isoforms or engineering truncated functional variants. ATP7b belongs to a subfamily of heavy metal transporting P-type ATPase, which are distinguished from other P-type ATPase by the presence of N-terminal metal-binding domain.[40] ATP7b from higher vertebrates possesses six copies of MBDs, while its yeast counterpart, Ccc2p, contains only two copies of MBDs, indicating that MBDs in higher vertebrates might be functional redundant.[20] In supporting to this notion, a compensation assay in Ccc2p deficient yeast, using human ATP7b gene harboring directed mutations or deletions of the MBDs, revealed that retaining only the sixth MBD is sufficient for normal metal transport.[41] However, different from that in yeast, copper metabolisms in vertebrates involved directional absorptions and secretions.[21, 42] The main function of vertebrate ATP7b is to secrete hepatic copper into the bile, which requires ATP7b a proper subcellular localization.[21, 42] Previously studies have demonstrated that the N-terminal signal peptide is essential for the subcellular translocation of ATP7b in respond to copper concentration.[23] In consistent with this observation, mutations in the N-terminal region were found in WD patients,

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suggesting that the N-terminal region was indispensable for normal ATP7b function.[43] In searching for naturally occurred transcriptional or splicing ATP7b isoforms, we found several candidates that lacking either MBD or transmembrane domains or both. Among these isoforms, isoform X6 from gorilla that lacks N-terminal 63 amino acids, including copper translocation motif and 1-4 MBDs was found to be able to secrete copper as efficient as the full length ATP7b. This result was consistent with previously observations in yeast assays.[44] To ensure its copper-responsive translocation, we added the N-terminal signal peptide to this isoform to form our tATP7b. As shown above, the engineered tATP7b retained the ability to corrected copper metabolism in WD mice.

In vivo delivery of tATP7b into the liver through AAV vectors efficiently increased serum ceruloplasmin to the level of healthy control mice, which was also accompanied with a significant reduction in urinary copper excretion. The normalization of WD related biochemical parameters were found in all dosages, without any obviously dose-dependence. However, lower dosages failed to prevent hepatic degeneration. WD mice received 5×10^{11} and 2.5×10^{12} GC/kg showed a dose-dependent but only mild decrease in serum ALT activity as compared to PBS treated WD mice, while their levels were significantly higher than that of healthy wildtype mice. In agreement with this observation, widespread degenerations of hepatocytes were found in liver from these two groups. Only tATP7b positive hepatocytes were morphological normal. In mice received 2×10^{13} GC/kg, the serum ALT activity was significantly reduced to almost normal level. In livers from this group, the degenerated hepatocytes were very few but obvious, scattering around tATP7b positive cells. These observations suggested that copper metabolisms, at least some of copper related biochemical parameters, were relatively easier to be corrected than hepatic copper accumulation and the resulting hepatic degeneration. Therefore, copper parameters were not sufficient to predict clinical outcomes of tATP7b gene therapy and liver damage monitoring must be included. The scenario that only tATP7b positive cells were morphologically normal, even in the high dosage group, also indicated that it is the breadth rather than the amount of transgene expression determined therapeutic effects in WD mice.

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Although the main therapeutic effects we observed were generally similar to those described in a recently published paper by Murillo et al, there is one major difference between the two studies. Murillo et al observed that only a relatively small portion of hepatocytes expressing transgene achieved morphological normalization in hepatocytes, even in the ones that did not express transgene. However, as discussed above, in our study, only transgene expressing hepatocytes were morphological normal. One possible explanation to these inconsistent findings might be the intrinsic difference in the genetic background and ATP7b lesion between WD animal models used. Mouse model used by Murillo et al was a knockout model in the hybrid C57BL/6×129S6/SvEv background that contained a neo cassette replacing part of exon 2 of ATP7b gene, which abolishes the expression of ATP7b. Our study used the tx-j mouse model that was generated from C3H/HeJ strain and harbored a single point mutation at position 2135 in exon 8, which leads to a G712D substitution mutation. [45]Although the two animal models shared main common features that was also frequently observed in WD patients, such as disturbance in copper metabolism, hepatic copper accumulation and liver degeneration, they also have several major differences, including variant degrees in liver regeneration and neurologic symptoms.[46] It is therefore reasonable that these intrinsic differences between the two animal models lead to different therapeutic response. In agreement with this explanation, it is common in WD patients that different mutations lead to different pathological phenotypes, such as age of onset, baseline serum ceruloplasmin and urine copper levels.

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Conflict of interest

The authors declared that they do not have any conflict of interest with respect to this manuscript.

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Table 1. Dosage, Ear-tag number, and Age in AAV8-tATP7b treated ATP7b mice

Dosage(GC/kg)	♂	
	No	Age(weeks)
0	H122	7
	H123	7
	D33	8
5×10^{11}	B6	8
	D74	6
	D78	8
2.5×10^{12}	F1	8
	B15	7
	D35	8
2×10^{13}	C91	8
	C92	8
	D21	6

Metal-responsive element luciferase reporter assay

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Figure Legends

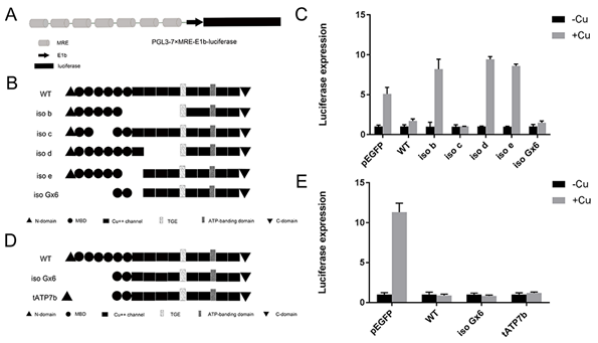

Figure 1. Characterization of truncated ATP7b isoforms

A. A scheme showing copper responsive reporter. B. Structures of natural ATP7b isoforms tested in this study. Important domains or motifs were labelled with symbols as indicated. C. Copper transport activity of various ATP7b isoforms was analyzed with MRE reporter under low or high copper concentrations. Empty pEGFP was used as control plasmid. D. Structures of recombinant truncated ATP7b isoform (tATP7b). E. Copper transport activity of tATP7b was analyzed with MRE reporter.

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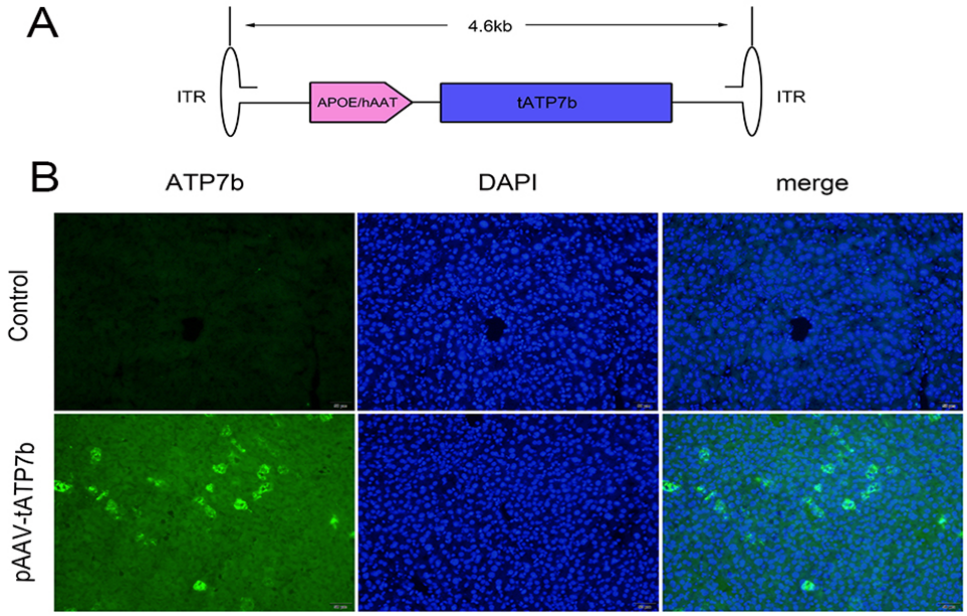

Fig 2. Construction and expression analysis of a liver specific AAV-tATP7b packaging plasmid.

A. Schematic representation of AAV-tATP7b plasmid. In this vector, expression of tATP7b was controlled by a liver specific promoter containing ApoE enhancer and hAAT promoter. B. Expression of HTA injected AAV-tATP7b was analyzed with immunofluorescence with an antibody recognizing C-terminal peptide of ATP7b.

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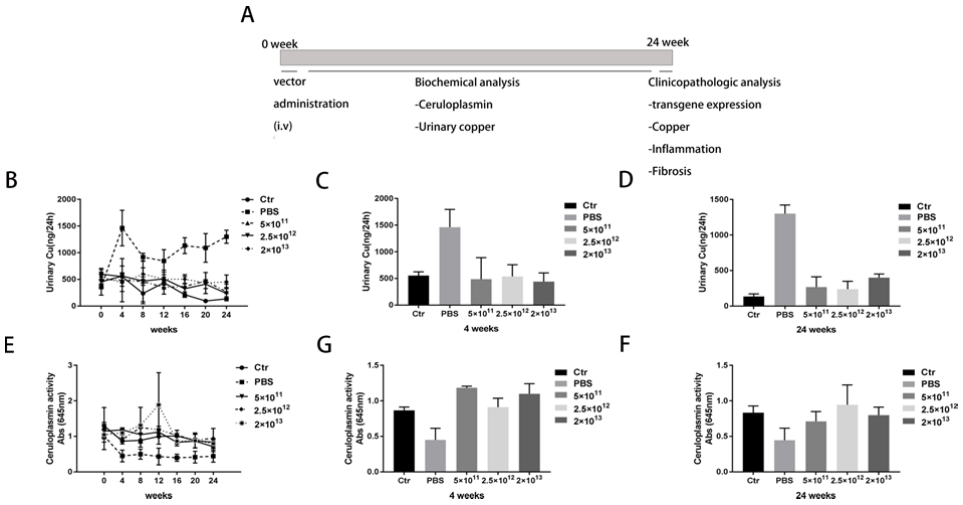

Fig 3. A single dose of AAV8-tATP7b restored serum biochemical parameter in WD mice.

AAV8-tATP7b viruses were injected into 6-8 weeks old WD mice via tail vein. Age matched WD mice received PBS was used as negative control, and heterozygous WD mice was used as healthy control. A. A schedule showing biochemical and histological analysis at different time points in a 24 weeks window. B. Urinary copper excretion was measured by atomic absorption spectrophotometry at indicated time points. C and D. Urinary copper content of mice at 4 and 24 weeks. E. Oxidase activity of serum ceruloplasmin activity was measured at indicated time points. F and G. Oxidase activity of serum ceruloplasmin activity of mice at 4 and 24 weeks.

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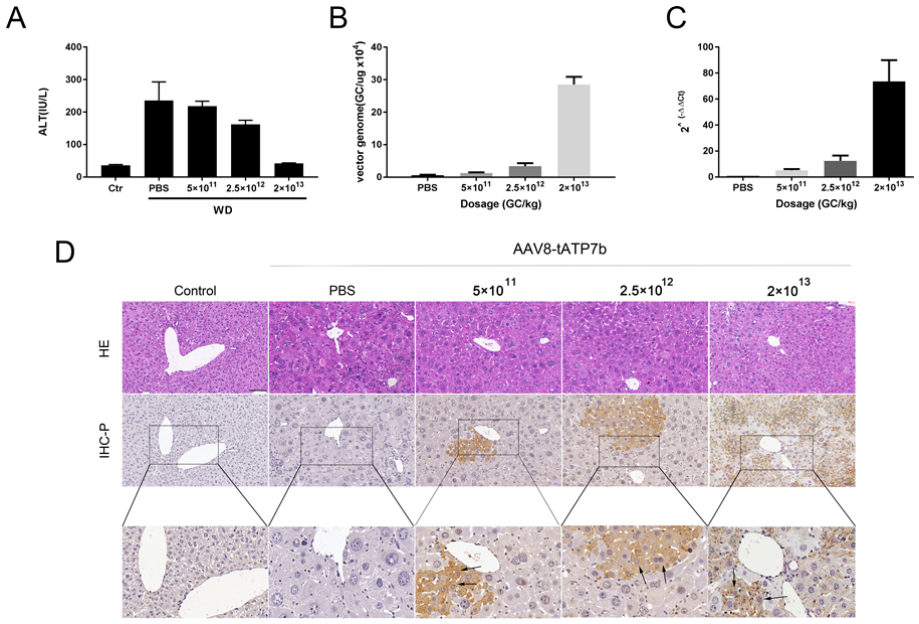

Fig 4. Dose dependent expressions of transgenic tATP7b and their protective effects on liver degeneration.

A. Serum ALT levels were determined at 24 weeks post AAV administration. B. AAV genomes in different samples were quantified by quantitative PCR. C. tATP7b expression was analyzed at the mRNA level by quantitative reverse transcriptional PCR. D. Pathological analysis of liver samples from different groups. Upper panels show HE staining of the tissue sections. Lower panels show IHC staining of the serial sections correspond to each upper panel. Black arrows point to the tATP7b positive cells. Note that only tATP7b positive hepatic cells remain morphologically normal, while tATP7b negative cells were enlarged, harboring swelling nuclei. Scale bar=100μm

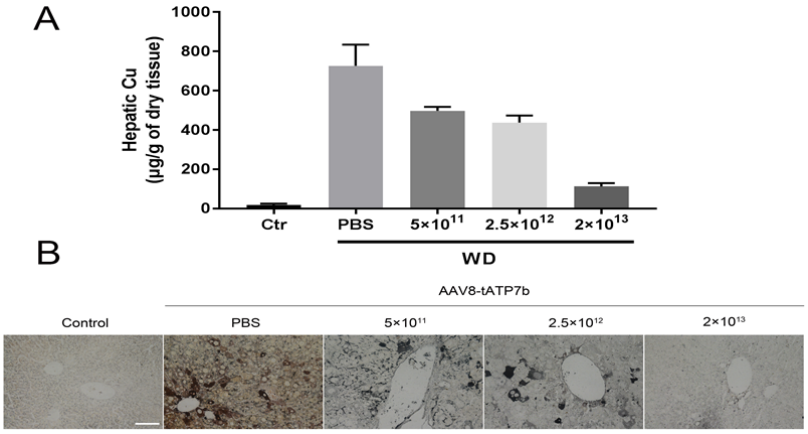

Fig 5. AAV8-tATP7b injection released liver copper accumulation in WD mice.

A. Liver copper content were analyzed with atomic absorption spectrophotometry. Liver samples were collected at 24 weeks post AAV administration. B. In situ copper depositions in liver samples were indicated by Timm's sulphide silver staining. Scale bar=100μm

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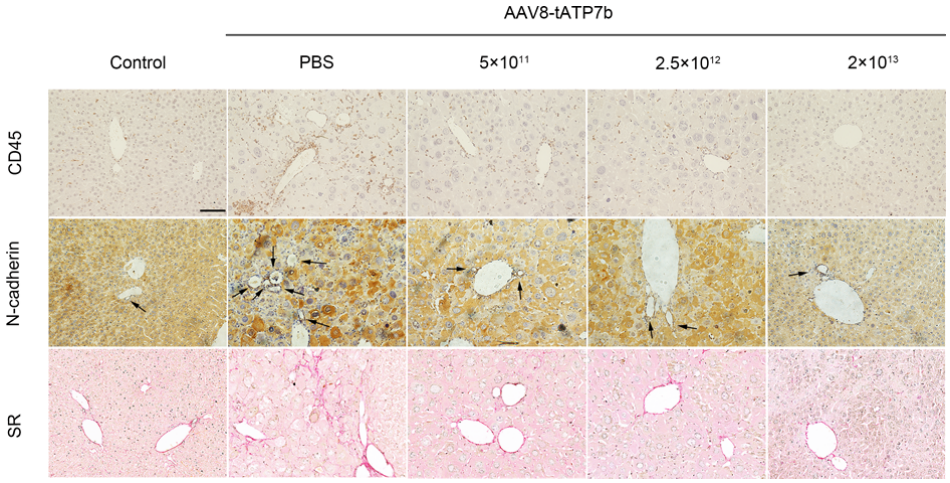

Fig 6. AAV8- tATP7b prevents liver damage in WD mice.

Immunohistochemistry staining of the leukocyte marker, CD45 (upper panels) and bile ducts marker, N Cadherin (middle panels) in liver sections from different groups. Lower panels showed deposited collagen indicated by Sirius red staining (SR). Black arrows point to the bile ducts. Scale bar=100µm