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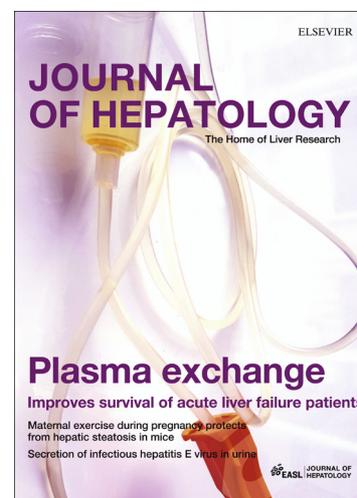
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Gene therapy of Wilson disease: a “golden” opportunity using rAAV on the 50th anniversary of the discovery of the virus

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Wilson disease (WD) was a once progressive and uniformly fatal inherited disorder of copper metabolism. Medical therapy to arrest progression or prevent complications of WD was developed in the 1950's with the introduction of parenterally administered BAL[1], and over the next two decades by oral therapy with d-penicillamine trientine, and zinc [2]. Effective therapy for WD requires life-long administration of daily medication. At least 30-50% of patients on medication for their WD have periods of non-adherence, some suffering liver failure, others developing potentially irreversible neurologic or psychiatric symptoms. Herein lies the justification for developing therapies that would provide correction instead of serial treatment of WD.

Liver transplantation (LT) is curative for WD. Biliary copper excretion is restored, ceruloplasmin and copper levels in the circulation normalize, neurologic and psychiatric disease stabilizes or improves and Kayser Fleischer rings disappear over time. [2] Following LT, patients do not require WD-specific therapy, but rather life-long immunosuppression. Thus LT proved that the defect for WD lay within the liver, consistent with physiologic studies showing reduced biliary copper excretion in WD patients [3]. Therefore targeting the liver to correct the defect in biliary copper excretion would provide a cure for WD.

The WD gene was identified as the trans-membrane copper transporter *ATP7B* in 1993, and studies showed that the *ATP7B* protein was preferentially expressed in hepatocytes [6]. *ATP7B* facilitates copper transport into bile and incorporation of copper into ceruloplasmin. Animal models of WD, the LEC rat and toxic milk mouse, lack *ATP7B* and manifest hepatic copper accumulation due to impaired biliary copper excretion and low circulating ceruloplasmin levels

[4]. *Atp7b* knockout mice develop the same phenotype. Therefore, introduction of ATP7B into hepatocytes should correct the phenotype. Proof of principle for gene therapy came from expression of ATP7B in the liver of LEC rats and murine models of WD using adenoviral and lentiviral vectors, which achieved transient correction of copper excretion and incorporation of copper into ceruloplasmin[5,6]. What was missing was longer term *ATP7B* expression and robust transduction without oncogenesis.

How many hepatocytes must be transduced with *ATP7B* to improve the phenotype? This was answered in part from studies where hepatocyte with normal ATP7B expression were transplanted into congenic LEC rats with hepatic copper accumulation[7]. With ~ 40% hepatocyte repopulation, liver damage from copper accumulation was prevented and circulating ceruloplasmin was increased. This proved that a fraction of hepatocytes with normal copper transport in an affected liver was sufficient to provide a conduit for copper into bile, and suggested that transduction of only a fraction of hepatocytes with *ATP7B* by gene therapy should protect against copper toxicity. In this issue of Journal of Hepatology, gene therapy by systemic administration of a viral vector into a murine model of WD was reported by Murillo et al [8].

Initially, liver directed gene therapy involved ex vivo transduction of hepatocytes isolated from a resected liver segment using recombinant oncoretroviral vectors that integrate permanently in the host genome [9]. Inefficient retroviral transduction of primary hepatocytes and inefficiency of hepatocyte engraftment restricted the success of these efforts. The risk of activation of protooncogenes by randomly integrated transgenes was highlighted by occurrence of leukemia in some children undergoing bone marrow-directed ex vivo gene therapy for severe combined

immunodeficiency. Subsequently, systemic gene therapy utilized episomal recombinant adenovirus that can infect hepatocytes and express the transgene with high efficiency without integrating into the host genome [10]. However, intrinsic host immunity and adaptive immune response shortened the duration of transgene expression and precluded repeated administration of the vector. The death of a recipient of adenoviral gene therapy was a major setback to gene therapy efforts [11]. Although, viral gene-deleted adenoviral vectors permitted prolonged transgene expression, the problem of adaptive immune response remains.

Partly due to setbacks with retroviral and adenoviral vectors, attention was focused on a small (20nm) single stranded non-enveloped parvovirus, termed adeno-associated virus (rAAV), which was employed by Murillo et al [8] for the treatment of the *atp7b*^{-/-} mouse model of WD (WD mice). This year marks the 50th anniversary of the discovery of AAV [12], which has taken the center stage in gene therapy because of many desirable features as a gene delivery vector. Up to 90% of the population has been exposed to wildtype AAV serotype 2 (AAV2), without any deleterious effect, indicating safety of the virus. All viral genes can be deleted in rAAV vectors, enhancing safety and reducing immunogenicity. Eleven AAV serotypes have been characterized that can infect both dividing and quiescent cells in liver, brain, retina, heart, muscle, lung, and pancreas, making rAAV a versatile gene transfer platform.

AAV belongs to the genus *Dependoparvovirus* because its replication is dependent on a "helper virus" like adenovirus or herpes simplex virus. Adenoviral gene products providing this help were identified [14], and rAAV is now generated by co-transfection with helper plasmids into the packaging cell. Specific AAV serotypes differ in their cellular tropism [15]. rAAV lacks the *Rep* gene products required for site-specific integration into the human chromosome 19. After the rAAV genome is converted to double-stranded DNA, the inverted terminal repeats (ITR) of

the rAAV genome mediate the formation of circular concatamers that persist episomally in non-dividing cells, and integrate into the host genome very infrequently and in a semi-random manner [16].

The 4.7 kb single-stranded DNA genome of AAV contains two open reading frames (ORF), *rep* and *cap*, flanked on both ends by ITRs required for packaging the genome into the viral capsid [17]. rAAVs are generated by replacing the *rep* and *cap* ORFs with the gene of interest and providing *rep* and *cap* as helper genes in *trans* to package the transgene inside the capsid. The capsid proteins from different serotypes determine their cell specificity and immunogenicity. Taking advantage of this, the well-characterized AAV2 genome is packaged with capsids of different serotypes to generate pseudotyped rAAVs that can efficiently transduce cells of interest in vivo. Murillo et al [8] inserted their genes of interest (ATP7B, eGFP or luciferase) with hepatocyte-specific promoters between the 5' and 3' ITRs of the AAV2. To achieve liver specificity they used the capsid of the AAV8 serotype to package the rAAV vector. Using such pseudotyped vectors has become a standard procedure to achieve cell type-preferred gene delivery [18]. Different approaches to engineering capsids to achieve enhanced cell specificity and reduce or circumvent an immune response have been reviewed recently [19].

In the paper by Murillo et al [8], a single injection of rAAV at doses (viral genomes/kg body weight) similar to those used in patients with hemophilia B (Factor IX deficiency) resulted in normalization of plasma holoceruloplasmin levels, increased biliary copper excretion as evidenced by increased fecal copper, and reduced hepatic and urinary copper in a dose related manner 6 months after the treatment. Many, but not most host hepatocytes stained positive for ATP7B, confirming the predictions derived from hepatocyte transplantation experiments that

copper excretion by a fraction of hepatocytes can deplete the excessive copper in the remaining ATP7B deficient cells (7). In patients with hemophilia B, Nathwani et al. reported only 1-6% of normal plasma factor IX levels for a median of 3.2 years [20]. Although this level was therapeutically significant, similar levels of ATP7B expression in WD mice would be inadequate to achieve normalization of copper stores as observed by Murillo et al [8]. While there may be a species difference, pre-existing antibodies against wildtype AAV or Factor IX, and/or adaptive immune response might have reduced plasma levels of this protein. Intriguingly, rAAV transduced hepatocytes of WD mice more efficiently than hepatocytes of wildtype mice [8], suggesting a higher transduction efficiency of rAAV in livers with genotoxic hepatic injury, which occurs in WD mice due to excess copper, but not in patients with hemophilia B or inherited metabolic liver diseases without liver injury. In the human hemophilia B trial, a high proportion of patients exhibited increased serum alanine aminotransferase (ALT) levels 7-10 weeks after the rAAV injection, which was ameliorated by prednisolone therapy. This indicated the loss of some rAAV-transduced hepatocytes through an adaptive immune response. Such ALT spikes were not reported in treated WD mice [8], but were perhaps masked by their baseline elevated ALT levels.

What is the prospect of rAAV-mediated gene therapy of WD? Because it required up to 5 weeks for transgene expression to reach peak levels after rAAV injection in canine models of hemophilia B [21], this vector is unlikely to be useful in the treatment of acute liver failure due to WD. Prolonged ATP7B expression after a single rAAV injection would be attractive for treating WD patients as an alternative to life-long daily maintenance therapy. Although the hemophilia B clinical trial showed therapeutic levels of Factor IX persisting for a median period of 3.2 years after one rAAV injection, episomal rAAV will likely be lost eventually through cell

division, and re-administration of the vector may be hindered by humoral and cell-mediated immune responses resulting from prior treatment. This may be addressed by packaging the rAAV genome with the capsid from an alternative serotypes, although antibodies to one AAV serotype can sometimes cross-react with a different capsid of another serotype. However, extensive research on directed evolution of AAV capsids by multiple laboratories is likely to yield rAAVs with capsids engineered to evade preexisting immunity against the originally used rAAV capsid [13]. Thus, on the “golden” anniversary year of the discovery of AAV [12], there is reason for optimism about the application of rAAV vectors in liver-directed gene therapy for WD and other monogenic liver-based disorders.

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Table 1. Desirable features of recombinant adeno-associated virus for gene therapy and goals for genetic correction of Wilson disease

Desirable features of rAAV for gene therapy or Wilson's disease	Goals for gene therapy of Wilson disease with ATP7b
<ul style="list-style-type: none"> • Transductional specificity: targeting tissue of choice using appropriate capsid for pseudotyping 	<ul style="list-style-type: none"> • Increase biliary copper excretion
<ul style="list-style-type: none"> • Transcriptional specificity: using tissue specific promoters 	<ul style="list-style-type: none"> • Normalize hepatic copper
<ul style="list-style-type: none"> • Can be administered parenterally in humans despite low levels of preexisting antibodies against AAV 	<ul style="list-style-type: none"> • Prevent injury of liver cells
<ul style="list-style-type: none"> • High transduction efficiency of both quiescent and dividing cells 	<ul style="list-style-type: none"> • Remove excess circulating copper that causes extrahepatic injury
<ul style="list-style-type: none"> • Low immunogenicity 	<ul style="list-style-type: none"> • Normalization of circulating ceruloplasmin level
<ul style="list-style-type: none"> • Episomal vector, not associated with positional effect or risk of oncogenicity by activation of oncogenes. 	<ul style="list-style-type: none"> • Long-term correction with possibility of re-treatment