Atp7b\(^{-/-}\) mice as a model for studies of Wilson’s disease

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Abstract
Wilson’s disease is a severe human disorder of copper homoeostasis. The disease is associated with various mutations in the \(ATP7B\) gene that encodes a copper-transporting ATPase, and a massive accumulation of copper in the liver and several other tissues. The most frequent disease manifestations include a wide spectrum of liver pathologies as well as neurological and psychiatric abnormalities. A combination of copper chelators and zinc therapy has been used to prevent disease progression; however, accurate and timely diagnosis of the disease remains challenging. Similarly, side effects of treatments are common. To understand better the biochemical and cellular basis of Wilson’s disease, several animal models have been developed. This review focuses on genetically engineered \(Atp7b^{-/-}\) mice and describes the properties of these knockout animals, insights into the disease progression generated using \(Atp7b^{-/-}\) mice, as well as advantages and limitations of \(Atp7b^{-/-}\) mice as an experimental model for Wilson’s disease.

The role of ATP7B in mammalian physiology

The copper-transporting ATPase ATP7B is an ATP-driven copper transporter that was discovered as a protein associated with a WD (Wilson’s disease) phenotype in humans \([1,2]\). WD is caused by various mutations in the gene \(ATP7B\) located on chromosome 13. To date, more than 380 mutations have been reported in patients with WD \([3]\); the up-to-date information on the type of mutations and their location within protein/cDNA sequences can be found at http://www.medicalgenetics.med.ualberta.ca/wilson/index.php. The vast majority of the WD-causing mutations are single amino acid substitutions, frameshift mutations and mutations introducing premature stop codons, all distributed along the entire coding region of \(ATP7B\). The biochemical and cell-biological characterization of some of these mutations revealed their effects on protein synthesis and stability, changes in functional properties of ATP7B and/or mutation-induced alterations in the localization within the cell \([4–9]\). Gain-of-function mutations have not been reported.

In human tissues, ATP7B plays a dual role. The most important function of ATP7B is in the liver, the main organ for homoestastic regulation of copper. Hepatic ATP7B facilitates the delivery of copper to the copper-dependent ferroxidase ceruloplasmin and mediates the export of excess copper into the bile, for eventual removal with faeces \([10]\). ATP7B performs these different functions in distinct intracellular locations. When present in the late compartment of the Golgi \([TGN (\text{trans}-\text{Golgi network})]\), ATP7B transfers copper from the cytosol into the lumen of the TGN for biosynthetic utilization by apo-ceruloplasmin. When intracellular concentration of hepatic copper exceeds a certain threshold, ATP7B trafficks towards the apical membrane \([11–13]\) and sequesters copper into the sub-apical vesicles. It is thought that subsequent fusion of these vesicles with the apical

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Key words: ATP7A, ATP7B, copper, liver, mouse model, Wilson’s disease.
Abbreviations used: ABC, ATP-binding cassette; CNS, central nervous system; DBH, dopamine \(\beta\)-hydroxylase; TGN, trans-Golgi network; WD, Wilson’s disease.
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membranes results in copper excretion. A decrease in copper concentration is accompanied by the return of ATP7B from the vesicles to the TGN.

The level of ATP7B expression is highest in the liver; however, ATP7B is also present in the CNS (central nervous system), placenta, mammary gland, kidney, ovary, lung, heart and other tissues. Recent studies suggest that, in placenta, ATP7B exports excess copper via the apical membrane to the maternal circulation and hence contributes to the maintenance of placental copper homeostasis [14]. In human breast, ATP7B was shown to be present in luminal epithelial cells, consistent with its role in copper excretion [15]. It is interesting that the intracellular localization of ATP7B in mammary cells differs in the resting and lactating tissue, showing the TGN-like and vesicular localization respectively [15]. These results suggest that the primary function of ATP7B in human breast may be tightly controlled by the metabolic needs of the organism and be ‘switched’ from the biosynthetic copper delivery to the secretory pathway (in the resting state) to copper export into milk upon lactation. Studies using polarized PMC42-LA cells showed that recombinant mouse ATP7B expressed in these cells facilitates copper efflux from the apical surface [15], supporting further the role of ATP7B in copper delivery to milk.

The role of ATP7B in other organs is much less clear. Frequent neurological manifestations in WD patients indicate that ATP7B is important for normal CNS function, yet little is known about the specific role of ATP7B in any region of the brain, although basal ganglia seems to be most affected by ATP7B inactivation. Ceruloplasmin, the protein that receives copper from ATP7B in the liver (see above), is also expressed in the brain [16–18]. In mice, both ATP7B and ceruloplasmin are detected in Purkinje neurons [16–19], thus it seems likely that one of the functions of ATP7B is to supply copper to ceruloplasmin. Additional roles may include a fine-tuning of intracellular copper concentrations in co-operation with another copper-transporting ATPase, ATP7A.

ATP7B inactivation alters renal and cardiac function [20–22]. Whether this effect is due to systemic dysregulation of copper metabolism or due to specific disruption of ATP7B activity in respective tissues remains to be determined. As described below, the studies using various animal models including the Atp7b<sup>−/−</sup> mice are providing important insights into the role of ATP7B in copper physiology in normal and diseased tissues. Owing to space limitations, in the present review, only the Atp7b<sup>−/−</sup> animals will be described in detail. For a description of other murine models of WD, see [21–25].

**WD: major manifestations and challenges**

WD is a severe autosomal recessive disorder. The first symptoms of the disease usually appear in young patients (6–20 years old), but disease onset has been also reported in much older patients [26]. The oft-cited prevalence of the disease is 1 per 7000 births, but modern sensitive diagnostic tools may correct this value (recent estimates in the Caucasian population gave a range of 1:18000 to 1:70000 [27]). In some populations, much higher disease incidence has been reported (for example, 1 per 7000 births in the Sardinian population [6]). Intensive genetic studies have not, so far, revealed strong genotype–phenotype correlations, although the hepatic onset of the disease manifests, on average, earlier than neurological symptoms.

There is a significant difference in the frequency of certain WD-causing mutations in world populations. The R778L mutation is frequently observed in Chinese, Japanese and Korean patients (up to 55% frequency has been reported [28]), whereas the H1069Q substitution is the most common mutation in Caucasian patients with WD (38%) [2]. Independently of the type of mutation, the major biochemical symptoms are largely the same. Inactivation of copper-transport activity of ATP7B is associated with the disrupted delivery of copper into the secretory pathway. The lack of copper supply results in low levels of the copper-containing ferroxidase ceruloplasmin in serum (although, in approx. 5% cases of WD, ceruloplasmin levels are normal). In addition, copper efflux into the bile is impaired. Consequently, copper accumulates to very high levels in the liver (more than 250 μg/g of dry weight), leading to a wide spectrum of liver pathologies that range from cirrhosis, chronic hepatitis and steatosis to fulminant liver failure (which is more frequently observed in female patients). In severe cases, the WD patients require liver transplantation. When disease manifestation is less acute, treatment with copper chelators and/or zinc can prevent progressive deterioration and eventual death that would occur without treatment [29].

The hepatic course of the disease presents many challenges. The disease symptoms are not very specific. It remains unclear why the severity and onset of WD differs so drastically. It is also not understood why WD patients with the same mutation sometimes have a very different course of the disease (even siblings with different manifestations of WD have been described). The disease manifestations do not correlate with the amount of copper in the liver (which is nevertheless always elevated), suggesting that the time of exposure to copper, the intracellular copper distribution, the form in which copper is present in the liver and the involvement of additional protein regulators may contribute significantly to WD pathology. It is commonly cited that excess copper triggers liver pathology by stimulating a Fenton-like reaction and inducing oxidation of lipids and proteins, and also causing DNA damage. Numerous studies have yielded evidence for the increased liver peroxidation and DNA changes; however, it remains unknown whether these events are causative or whether they appear when the disease is in its advanced stage, and whether the oxidation targets are specific or random.

The aetiology of neurological symptoms in WD is also not very clear. The neurological symptoms include movement disorders, such as tremor, lack of co-ordination and dystonia. Diagnosis of neurological WD is greatly assisted by commonly present Kayser–Fleischer rings (greenish-gold or -brown colouring of the cornea, which apparently represent copper/sulfur deposits) [30,31]. It is thought that copper
accumulates in the brain after the liver becomes saturated with copper. However, the expression of ATP7B in the CNS suggests that the CNS abnormalities may develop in parallel to liver impairment and that the delay in the appearance of neurological symptoms could be due to partial compensation of ATP7B malfunction by another copper-transporting ATPase, ATP7A, which is expressed in the brain, but not in hepatocytes. Treatment of patients with neurological symptoms using chelators, particularly penicillamine, may have no effect or even worsen the pathology [23], even though such treatment commonly alleviates hepatic symptoms. A better understanding of the mechanism behind WD is clearly needed. Animals with genetic defects in ATP7B have been a remarkably useful tool in dissecting the disease pathology, despite some interspecies differences, the most notable of which is the lack of strong neurological phenotype in all animal models of WD studied to date.

**Generation and major characteristics of \textit{Atp7b}^{−/−} mice**

The \textit{Atp7b}^{−/−} mouse is the only genetically engineered rodent strain to serve as an animal model of WD. Other available experimental models such as LEC (Long–Evans Cinnamon) rat, toxic milk mice and txJ mice are products of spontaneous mutations in the murine \textit{Atp7b} gene that occurred during breeding [23–25]. The \textit{Atp7b}^{−/−} mice were generated by Buiakova et al. [32] on the hybrid C57BL×129S6/SvEv background by inserting the early termination codons in the mouse \textit{Atp7b} mRNA. This was accomplished by replacing a portion of the \textit{Atp7b} exon 2 (the largest out of 21 exons) with a PGK-neo selection cassette encoding multiple stop codons. The procedure preserved the exon/intron boundaries, and, in tissues, the alternative splicing event removes exon 2 with the neomycin cassette, producing a truncated mRNA with a frameshift mutation. As a result, the alternatively spliced mRNA (lacking 1270 bp) is present in the \textit{Atp7b}^{−/−} tissues, but the ATP7B protein is not produced [32].

It should be noted that, in rats, pineal and retinal cells express a short variant of ATP7B (PINA) produced from the alternative promoter downstream of the exon 8 [33]. This form, if present in mice, would not be affected by manipulations within exon 2. Therefore, if PINA or other alternatively-spliced/truncated ATP7B variants are expressed in other CNS regions (either normally or in response to inactivation of the full-length ATP7B), their presence may compensate for the loss of the full-length ATP7B and explain the lack of notable neurological symptoms in adult \textit{Atp7b}^{−/−} mice.

\textit{Atp7b}^{−/−} mice are born copper-deficient [32] and, when nursed by homozygous \textit{Atp7b}^{−/−} mothers, may display neurological symptoms (tremor, ataxia abnormal locomotor behaviour). When neurological symptoms are apparent, such pups, as a rule, do not survive beyond 2 weeks of age. Heterozygous breeding/nursing prevents the copper-deficiency phenotype, which is likely to be due to low copper content in the milk of homozygous \textit{Atp7b}^{−/−} dams [32]. Despite very low initial copper concentration in the liver, copper rapidly accumulates in the tissue and, by 6 weeks, hepatic copper reaches its highest levels [34]. Other organs (brain, placenta, eye and kidney) also accumulate copper, but at a much lower rate and to a significantly lower degree [32]. This difference between liver and other organs is most likely to be due to the presence in non-hepatic tissues of additional copper-transporting ATPase ATP7A (for a comparison of function and regulation of two Cu-ATPases, see [10]).

ATP7A exports copper across the basolateral membrane and thus maintains intracellular copper balance in many tissues [14,35–38]. Studies of \textit{Atp7b}^{−/−} mice has produced supportive evidence for the compensatory role of ATP7A in the case of ATP7B inactivation. Comparison of the intracellular localization of ATP7A in control and \textit{Atp7b}^{−/−} kidneys of adult mice revealed a significant change in the ATP7A localization in response to ATP7B inactivation [39]. In proximal tubules of control kidneys, ATP7A has a mostly intracellular vesicular pattern [39]. In contrast, in mice lacking ATP7B, ATP7A is located in close proximity to the basolateral membrane, a change consistent with the enhanced copper-export function [39]. Presumably, this is necessary to compensate for the lack of transport activity of ATP7B and normalize renal copper levels. This speculation is supported by measurements of copper concentration and phenotypic characterization of kidneys, which indicate that the copper concentration in the 6–8-week-old \textit{Atp7b}^{−/−} kidneys is similar to that of controls. Also, there are no significant morphological changes in the kidney. Nevertheless, renal compensation is not complete: metabolic changes are evident from the appearance of fluorescent deposits in a very distinct region of \textit{Atp7b}^{−/−} kidney, where ATP7A expression is, presumably, low or its function is insufficient [39]. The compensatory function of ATP7A was also suggested by studies in the brain of \textit{Atp7b}^{−/−} mice. In the cerebellum, Purkinje neurons express ATP7A, ATP7B and ceruloplasmin [17,40]. Estimates of the relative abundance of ATP7A in Purkinje neurons at different stages of development vary [17,40,41], while the amount of ATP7B in control mice appears constant. In the \textit{Atp7b}^{−/−} liver, the loss of ATP7B activity is associated with the disrupted delivery of copper to ceruloplasmin and production of inactive apo-protein [34]. In contrast, ceruloplasmin in the cerebellum appears to contain copper [17], presumably due to copper transport into the TGN by ATP7A. Fixing conditions in immunohistochemistry procedures may artificially increase staining intensity in one type of cell over the other [41]. This technical difficulty generates uncertainty as to whether compensatory copper delivery to ceruloplasmin occurs in glial cells (in which both ATP7A and ceruloplasmin were observed in \textit{Atp7b}^{−/−} cerebellum [17]) or in Purkinje neurons (which are morphologically altered and are not efficiently stained in \textit{Atp7b}^{−/−} animals).

**Liver disease in \textit{Atp7b}^{−/−} mice**

The highest level of copper accumulation and the most striking phenotype are observed in the liver of \textit{Atp7b}^{−/−} mice. Depending on whether pups are nursed by a heterozygous or homozygous mother, the onset of symptoms may differ by
several weeks, but the course of the pathology development is invariably the same. At early stages of copper accumulation (up to 6–8 weeks after birth), the morphological changes in the liver are minor, and, in some animals, liver histology appears normal. The only apparent change is the increase in nuclear size that coincides with a significant copper accumulation in this cell compartment [34]. At 12–15 weeks, the disease is always present and easy to detect. Tissue histology shows swollen hepatocytes with huge nuclei, inflammation, necrosis and proliferation of bile ducts [34]. At 20 weeks, fibrotic tissue and nodules of regenerating hepatocytes become apparent. In mice older than 9 months, a large portion of liver regenerates, while extensive atypical proliferation of bile ducts continues. In these proliferating regions, multiple layers of cells with a high nuclear/cytoplasmic ratio, hyperchromasia and lack of cell polarity are indicative of displasia and suggest possible carcinomatous transformation [34].

How does copper induce all these marked transformations? Biochemical studies have provided some interesting new insights. Analysis of subcellular copper distribution has revealed that copper accumulates in high concentrations in cytosol and, unexpectedly, in cell nuclei. In the cytosol, copper is likely to be bound to metallothionein, which is highly up-regulated in the Atp7b−/− mice [42]. It appears that sequestration by metallothionein renders copper inactive, as very little change in protein composition (abundance and modification) is detected in the cytosol of knockout animals [42]. In contrast, both nuclear morphology and nuclear function are significantly affected. Analysis of changes in hepatic mRNAs at the early stages of copper accumulation (at 6 weeks after birth and before noticeable pathology development in the liver) revealed very specific effects in the liver transcriptome.

The observed changes of liver mRNA provided little evidence for the oxidative stress response in Atp7b−/− liver despite a 20–40-fold increase in its copper content. This somewhat unexpected observation suggests that the initial effects of copper on liver function are more selective than was previously thought and that oxidative damage and the subsequent response are more characteristic of the later stage of the disease. This conclusion is supported by a very significant increase in the activity of serum alanine aminotransferase, which is an indicator of liver injury or biliary duct problems [34]. Indeed, the volume of bile in Atp7b−/− mice is usually low.

**Copper in the serum and urine of Atp7b−/− mice**

One of the most characteristic features of WD, both in human and rodents, is the loss of ferroxidase activity of ceruloplasmin in serum (and often the loss of protein altogether owing to instability and degradation). Ceruloplasmin is an abundant serum cuproprotein, which contains approx. 60–70% of total serum copper [43]. Serum ceruloplasmin is produced in the liver and is normally secreted from the liver in a copper-bound form; ferroxidase activity of this protein requires bound copper. Inactivation of ATP7B precludes the biosynthetic incorporation of copper into ceruloplasmin and inactivates the enzyme. The loss of holo-ceruloplasmin decreases the total serum copper content; however, the decrease is less significant than one would expect (10–15% compared with an anticipated 60–70%). In fact, in Atp7b−/− mice, the decrease in serum is not readily detectable [34,39]. Therefore there must be other copper carrier(s) that compensate for the loss of ceruloplasmin. The identity of such carrier(s) is currently unknown; however, markedly increased levels of copper in the urine (in either the WD patients or in Atp7b−/− mice) suggest that such molecules are small and are likely to be filtered into the urine. The origin of the copper excess in urine is also not entirely clear. Recent studies from our group suggest that the hepatocytes of Atp7b−/− do not accumulate copper beyond a certain level (M. Ralle, S. Vogt, J.L. Burkhead, T.R. Capps, R. Linz, C.L. Corless, T. Fukai and S. Lutsenko, unpublished work). Therefore the excess copper in the urine could be either due to absorbed dietary copper that does not enter hepatocytes or to the copper exported from the hepatocytes by currently unknown mechanisms. Identification of the molecular mechanisms that prevent copper accumulation in the liver may have significant implications for the understanding and treatment of WD.

The consequences of ceruloplasmin inactivation on WD progression, if any, are not understood. It is interesting that, in Atp7b−/− animals, the consequences of ATP7B inactivation were also detected in tissues in which ATP7B is not expressed [44]. For example, in the adrenal gland, the activity of DBH (dopamine β-hydroxylase) is decreased even though ATP7B is not normally expressed in this tissue, and the delivery of copper to cuproenzymes such as DBH is mediated by ATP7A [44]. The mRNA levels of adrenal DBH are not affected, suggesting that the diminished DBH activity is most likely to be due to a less efficient copper supply to the tissue. This result illustrates an important point that so far has not been discussed in the WD literature. Specifically, changes in function of some cells or organs in WD could be due to improper supply of copper (as apparently happens in the adrenal gland) or activation of the ATP7A-mediated copper efflux (as observed in kidney). Both events would
lead to temporary or persistent copper deficiency in such tissues, even though there is a great excess of copper in the liver. Perhaps toxic effects of penicillamine in the CNS could be, at least in part, due to exacerbated copper deficiency in certain cell populations. The detailed temporary and spatial analysis of copper distribution, the characterization of tissue transcriptions and the analysis of function of various organs in Atp7b−/− mice at different stages of the disease are necessary to generate a complete picture of WD pathology.

Conclusions
In summary, Atp7b−/− mice represent a valuable model to study hepatic WD. Similarly to human patients, these mice accumulate copper in the liver owing to disrupted export of copper into the bile. Copper transport to the secretory pathway is also impaired, resulting in the generation of inactive ceruloplasmin. As in human patients, in Atp7b−/− mice, copper is elevated in the urine. Liver pathology involves hepatitis, inflammation, fibrosis and the appearance of regenerating nodules. The initial studies of the liver transcriptome suggest that similar molecular pathways are affected in mice and humans [42] and involve changes in lipid homeostasis and cell-cycle regulation. At the same time, neurological symptoms are not generally observed in Atp7b−/− mice and acute liver failure has not been detected. Furthermore, Atp7b−/− mice survive and live without treatment, whereas human patients are much more vulnerable. Dissecting the common effects of copper on liver morphology and function and identifying the molecular basis of interspecies differences is likely to yield new insights into the WD mechanism and facilitate the development of improved treatment protocols.

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References


