Relevance of animal models for understanding mammalian copper homeostasis

Willianne IM Vonk, Cisca Wijmenga, and Bart van de Sluis

ABSTRACT
As a trace element, copper has a crucial role in mammalian metabolism, but it can be toxic in excess. The importance of a balanced copper homeostasis is illustrated by several copper-associated disorders in man, such as Menkes and Wilson disease, and in a wide variety of animal models (eg, mice, dogs, and sheep). Proteins involved in controlling copper metabolism have been well studied in yeast and in vitro. Recently, naturally occurring mutants and transgenic mouse models have been used to study the physiologic role of copper transporters in copper homeostasis. We discuss the most common mammalian animal models used to study copper-related diseases, evaluate what these model systems have recently shown about copper metabolism, and discuss the importance of these models for identifying specific and sensitive biomarkers associated with copper status in the near future. Am J Clin Nutr 2008;88(suppl):840S–5S.

INTRODUCTION
Copper is an essential trace element required for activating multiple cuproenzymes, such as Cu/Zn superoxide dismutase (SOD1), lysyl oxidase, and ceruloplasmin (Table 1). However, in excess, copper can be toxic because of its ability to generate reactive hydroxyl radicals. It is therefore important that copper uptake, distribution, and excretion are properly controlled to maintain copper homeostasis. Yeast (Saccharomyces cerevisiae) has been a powerful eukaryotic model for mammalian copper metabolism because this process is well conserved between different organisms. Currently, a variety of animal models, such as mice, dogs, sheep, and zebrafish, are being used to study the pathophysiology of copper metabolism. This work may identify specific and sensitive molecular biomarkers associated with copper status or new therapies for copper-related disorders such as Menkes disease (OMIM 277900), in which patients suffer from copper deficiency, and Wilson disease (OMIM 309400), in which patients suffer from copper deficiency and copper toxicity, respectively. In this review, we discuss recent achievements in animal studies involving copper homeostasis and the value of these animal models for future disease-associated research.

COPPER HOMEOSTASIS
Daily dietary copper intake is \( \approx 2.5 \) mg in humans, of which 25% is directly lost in the feces. The remaining 75% is taken up in the proximal part of the small intestine, mediated by the copper transporter 1 protein, CTR1, and the P-type ATPase protein ATP7A. ATP7A is involved in copper transport across the basolateral membrane of the enterocyte into the portal circulation after binding to carrier proteins, such as albumin and histidine. Most of the absorbed copper is distributed to the liver, which is the central organ for copper homeostasis. After copper uptake by the hepatocyte, the transport and incorporation of the metal (and thereby activation of different cuproenzymes) is accompanied by a variety of copper transporters and chaperones (Figure 1). When copper is in excess, its excretion into the bile canalculus is promoted by translocation of ATP7B, a highly homologous protein to ATP7A, toward the canalicular membrane (1, 2). Free intracellular copper is sequestered by metallothioneins, and different studies suggest its presence in vesicular copper pools. Recently, a possible role for CTR2 was indicated in the transport of these vesicular copper pools from the endosomal system into the cytosol (3). These data show that copper homeostasis is tightly controlled by a variety of copper transporters and chaperones. The necessity of the homeostatic control of intracellular copper is further emphasized by copper-related disorders such as Menkes disease and Wilson disease.

COPPER DEFICIENCY DISORDER: MENKES DISEASE
Menkes disease is an X-linked disorder characterized by a malabsorption of copper in the gut mucosa, which leads to copper deficiency in the liver and most extrahepatic tissues (2, 4, 5). As a consequence, cuproenzymatic activities are reduced, resulting in hypopigmentation of hair and skin pallor, hypothermia, connective tissues defects, growth retardation, and neurologic abnormalities (Table 1). The phenotypic severity of Menkes disease is associated with an impairment of ATP7A-dependent copper transport and can be divided into 3 distinct forms: classic Menkes disease, mild Menkes disease, and occipital horn syndrome (OMIM 304150). In contrast with patients with classic Menkes disease, mild Menkes disease, and occipital horn syndrome, patients with Menkes disease have a more severe phenotype, characterized by early death before 1 year of age.

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and mild Menkes disease, those with occipital horn syndrome, who predominantly display connective tissue abnormalities, do not show signs of severe neurologic defects, although sometimes mild mental retardation is observed. Treatment of these patients with intramuscular or intravenous copper injections appears to be effective when given very early in life, preferable prenatally, to avoid neurologic damage and connective tissue defects. However, although patients with the milder forms of the disease respond well to treatment, severely affected patients with classic Menkes disease do not benefit much. Therefore, this form of therapy, as well as others (eg, copper replacement therapy), is still being investigated (6).

Mottled mice have been shown to be an excellent animal model for studying Menkes disease. Different mottled mice phenotypes, resulting from various mutations in Atp7a, can be linked to the 3 forms of the human diseases (4, 7, 8). Similar to Menkes disease, connective tissue abnormalities and hypopigmentation are seen in these mice. However, several mottled mutants are embryonic lethal (Table 2). Different studies have identified an association between the severity of the mottled phenotypes and residual activity of Atp7a, thereby providing better insight into the pathogenesis of Menkes disease and into the molecular function of ATP7A (30). Furthermore, these animal models for Menkes disease are essential for developing potential therapeutic strategies (eg, gene therapy).

### TABLE 1
Cuproenzymes: their functions and their consequences

<table>
<thead>
<tr>
<th>Cuproenzyme</th>
<th>Function</th>
<th>Deficiency results in</th>
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<tbody>
<tr>
<td>Ceruloplasmin</td>
<td>Iron transport</td>
<td>Anemia</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>Cellular energy production</td>
<td>Hypothermia</td>
</tr>
<tr>
<td>Hephaestin</td>
<td>Iron transport</td>
<td>X-linked Anemia</td>
</tr>
<tr>
<td>Dopamine β-hydroxylase</td>
<td>Catecholamine production</td>
<td>Neurological defects, hypothermia, hypotension</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td>Cross-linking collagen and elastin</td>
<td>Connective tissue defects, abnormalities in bone formation</td>
</tr>
<tr>
<td>Peptidylglycine α-amidating mono-oxynase</td>
<td>Neuropeptide and peptide hormone processing</td>
<td>Reduced bioactivity of melanocyte-stimulating hormone, widespread physiologic effects</td>
</tr>
<tr>
<td>Cu, Zn superoxide dismutase (SOD1)</td>
<td>Free radical detoxification</td>
<td>Oxidative damage</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Melanin production</td>
<td>Hypopigmentation</td>
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![FIGURE 1. Intracellular copper pathways in hepatocytes.](image-url) After uptake, mediated by high-affinity copper transporter 1 protein (CTR1), copper binds to several cytosolic copper chaperones, such as CCS and COX17, which deliver copper to Cu/Zn superoxide dismutase (SOD1) and the mitochondrial cytochrome (cyt) c oxidase, respectively. However, the role of COX17 in cytosolic copper transport has recently been questioned. The copper chaperone ATOX1 traffics copper to the trans-Golgi network, where ATP7B is localized to incorporate copper into ceruloplasmin. Free intracellular copper is bound to metallothioneins or is stored in vesicular copper pools. Recent data suggest a possible role for the low-affinity copper transporter CTR2 in releasing the copper from these pools. If there is an excess of copper, ATP7B translocates toward the canalicular membrane, thereby promoting copper excretion into the bile canaliculus and eventually into the feces. The interaction of COMMD1 with ATP7B suggests that COMMD1 cooperates with the function of ATP7B. It has been implied that COMMD1 is involved in regulating the protein stability of ATP7B.
In addition to the mottled mice, copper deficiency has also been observed in the Ctr1/H11002/H11002 and Atox1/H11002/H11002 mouse strains. As a consequence of decreased copper availability and thereby diminished cuproenzymatic activities, loss of Ctr1 or Atox1 results in embryonic and perinatal lethal phenotypes, respectively (Table 2; 14–16). Intestine-specific Ctr1 knockout mice also showed copper deficiency in other tissues, but intestinal copper concentrations were highly increased, indicating that copper uptake was not affected in this mouse strain. Remarkably, the bioavailability of these accumulated copper pools was diminished. These findings suggest that Ctr1 is not important for the apical copper uptake but has another, as yet unknown, function in intestinal copper absorption in mammals (17).

COPPER TOXICITY DISORDERS

Wilson disease

In contrast with Menkes disease, the autosomal recessive disorder of Wilson disease is characterized by copper accumulation in the liver, brain, and cornea. Patients with Wilson disease carry small mutations or deletions in the ATP7B gene that result in impaired intracellular copper transport. This is accompanied by decreased biliary copper excretion and leads to liver cirrhosis, pigmental corneal rings (Kayser-Fleischer rings), and in some cases low serum ceruloplasmin concentrations (4, 5). Only one-half of patients manifest progressive neurologic defects like depression, schizophrenia, anxiety, and aggression. Copper chelating agents, such as D-penicillamine or zinc supplementation, have been shown to be successful for treatment, but the use of several agents has been questioned because of side effects (31).

Several animal models have been recognized as outstanding models for Wilson disease, such as naturally occurring mutants like toxic milk mice and Long-Evans Cinnamon (LEC) rats (18, 20, 21). Similar to Wilson disease patients, both rodent mutants are characterized by hepatic copper accumulation and hepatocellular damage as the result of mutations in Atp7b (Table 2). In contrast with human Wilson disease, no signs of neurologic or physiologic defects have been observed in these animals. Interestingly, LEC rats display liver injuries due to copper toxicity similar to those seen in toxic milk mice, but they also go on to

<table>
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<th>Animal</th>
<th>Affected gene</th>
<th>Phenotype</th>
<th>Reference</th>
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<tr>
<td>Copper deficiency</td>
<td>Atp7a</td>
<td>Models for classic MD, mild MD, or OHS</td>
<td>(7–13)</td>
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<tr>
<td>Mottled Mice</td>
<td>Pewter (Mo&lt;sup&gt;wa&lt;/sup&gt;)</td>
<td>Variations in pathology</td>
<td></td>
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<tr>
<td></td>
<td>Blotchy (Mo&lt;sup&gt;bs&lt;/sup&gt;)</td>
<td>Onset of lethality</td>
<td></td>
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<tr>
<td></td>
<td>Viable Brindled (Mo&lt;sup&gt;br&lt;/sup&gt;)</td>
<td></td>
<td></td>
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<td></td>
<td>Brindled (Mo&lt;sup&gt;br&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td>Macular (Mo&lt;sup&gt;ma&lt;/sup&gt;)</td>
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<td>Dappled (Mo&lt;sup&gt;dp&lt;/sup&gt;)</td>
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<td></td>
<td>Tortoiseshell (Mo&lt;sup&gt;t&lt;/sup&gt;)</td>
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<tr>
<td>Atox1 knockout mouse</td>
<td>Atox1</td>
<td>Perinatal lethality</td>
<td>(14)</td>
</tr>
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<td>Ctr1 knockout mouse</td>
<td>Ctr1</td>
<td>Embryonically lethal (E8.5 – 9.5)</td>
<td>(15, 16)</td>
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<td>Conditional Ctr1 knockout mouse</td>
<td>Intestinal Ctr1</td>
<td>Neonatal lethality (at ≈10 d after birth)</td>
<td>(17)</td>
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<tr>
<td>Copper toxicity</td>
<td>Atp7b</td>
<td>Model for WD</td>
<td>(18)</td>
</tr>
<tr>
<td>Toxic milk mouse</td>
<td>Atp7b</td>
<td>Neonatal lethality (at 10 – 14 d after birth)</td>
<td></td>
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<tr>
<td>Atp7b knockout mouse</td>
<td>Atp7b</td>
<td>Model for WD</td>
<td>(19)</td>
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<td>Neurologic problems</td>
<td></td>
</tr>
<tr>
<td>Long-Evans Cinnamon (LEC) rat</td>
<td>Atp7b</td>
<td>Model for WD</td>
<td>(20, 21)</td>
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<td>Rarely neurologic problems</td>
<td></td>
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<tr>
<td>Bedlington terrier</td>
<td>Commd1</td>
<td>Age of onset: 2–6 y</td>
<td>(22)</td>
</tr>
<tr>
<td>Commd1 knockout mouse</td>
<td>Commd1</td>
<td>Embryonically lethal (E9.5 – 10.5)</td>
<td>(23)</td>
</tr>
<tr>
<td>North Ronaldsay sheep</td>
<td>Unknown</td>
<td>Absence of placental vascularization</td>
<td></td>
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<tr>
<td>Other transgenic mice models</td>
<td>Cox17</td>
<td>Embryonically lethal (E8.5 – E10)</td>
<td>(25)</td>
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<tr>
<td>Cox17 knockout mouse</td>
<td>Cox17</td>
<td>Severe reduction in CCO activity (starting at E6.5)</td>
<td></td>
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<tr>
<td>Ccs knockout mouse</td>
<td>Ccs</td>
<td>Decreased SOD1 activity in liver, kidney and brain</td>
<td>(26, 27)</td>
</tr>
<tr>
<td>MT-I and MT-II double knockout mouse</td>
<td>MT-I and MT-II</td>
<td>No detectable defects</td>
<td>(28, 29)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal reproduction</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> MD, Menkes disease; OHS, occipital horn syndrome; WD, Wilson disease; HIF-1, ; ICT, idiopathic copper toxicosis; CCO, cytochrome c oxidase; SOD1, Cu/Zn superoxide dismutase; HIF-1, hypoxia inducible factor-1.
develop hepatocellular carcinoma (HCC). The prevalence of HCC has also been described in Wilson disease patients, but at a very low frequency. This might be explained by a shortened life expectation of untreated patients, which is probably insufficient for HCC to develop. However, other risk factors such as hepatitis B viral infection are not excluded. Additionally, other, as yet unidentified, genetic defects causing HCC in LEC rats have been questioned. Recently, an Atp7b knockout mouse was generated that displays all the Wilson disease characteristics, including neurologic defects, but at an age of ~20 wk, these mice develop cholangiocarcinomas (19). Thus, Atp7b null mice, together with LEC rats, suggest that prolonged copper accumulation is associated with an increased susceptibility to cancer. The strong phenotypic similarities between these rodents and Wilson disease in humans imply that these animals are excellent mammalian models for studying liver pathophysiology and developing novel therapies in relation to copper overload disorders. In addition, a more recent study performed in Atp7b−/− mice has provided more insight into the pathophysiology of copper-overload disorders. Presymptomatic animals showed defects in lipid metabolism and cell cycle machinery (32). Interestingly, these alterations in cholesterol homeostasis were also seen in Wilson disease patients.

**Idiopathic copper toxicity**

Other hepatic copper overload disorders have been described with Indian childhood cirrhosis (OMIM 215600), endemic Tyrolean infantile cirrhosis (OMIM 215600), and sporadic cases occurring worldwide and collectively referred to as idiopathic copper toxicosis (ICT). As seen in Wilson disease, ICT is characterized by liver fibrosis and eventual cirrhosis resulting from copper accumulation. Different population studies suggest that ICT displays an autosomal inherited pattern (4, 33). In contrast with Wilson disease, besides a genetic predisposition, environmental factors such as high copper exposure seem to be important for the manifestation of ICT. In a variety of patients, several candidate genes, including ATP7B, have been excluded, but the causative genes have not yet been identified. It has been suggested that copper toxicosis in North Ronaldsay sheep is a valuable model for ICT because these sheep are sensitive to environmental copper, as illustrated by hepatocellular damage with fibrosis and cirrhosis (24). Although North Ronaldsay sheep are valuable for genetic studies, the underlying gene for copper toxicity is still unknown. At the moment, little is known about the sheep genome and there is no breeding program, making it almost impossible to map this trait locus by use of a genetic study. However, recent proteomics studies have provided more insight into the pathogenesis of copper toxicosis in North Ronaldsay sheep (34).

**Copper toxicosis in Bedlington terriers**

Copper storage disorders have also been observed in several dog breeds, such as Bedlington terriers, Labrador retrievers, West Highland White terriers, and Doberman Pinschers. However, for some breeds it is still unclear whether the affected hepatic copper metabolism is the primary cause of hepatitis. The autosomal recessive disorder of copper toxicosis in Bedlington terriers is at present the best-characterized copper storage disease. Copper toxicosis is caused by defective biliary copper excretion resulting in hepatic copper accumulation after chronic progressive hepatitis and cirrhosis. The onset is between 2 and 6 y of age and the clinical phenotype resembles Wilson disease, although neurologic defects are absent and affected animals have normal serum ceruloplasmin concentrations. By use of a positional cloning strategy, the mutation in dogs affected with copper toxicosis, a deletion of ~40 kb in the COMMD1 gene, has been identified (22), although some Bedlington terriers with copper toxicosis in the United Kingdom and Australia were been identified without this homozygous deletion. No other COMMD1 mutations have been seen in these dogs, which suggests that other, as yet unknown, genetic defects are involved in copper toxicity in Bedlington terriers without a COMMD1 deletion.

Several studies support the role of COMMD1 in copper homeostasis, because the protein has been identified to bind and partially colocalize with ATP7B (35). Together with increased intracellular copper concentrations in Commd1 knockout cells, these data suggest that COMMD1 and ATP7B cooperate in the regulation of biliary copper excretion. The exact molecular mechanism is still unknown, but the latest data suggest that COMMD1 mediates the protein stability of ATP7B (35).

It is not yet known whether mutations in COMMD1 are also the cause of copper storage disorders in other breeds. However, mutation analyses in several dog breeds with copper toxicity have excluded COMMD1 and other known copper transporters as the disease-causing gene. In addition, COMMD1 has also been excluded as a candidate for different human copper storage disorders (36–39). These data imply that an as yet unidentified gene or genes play an essential role in copper homeostasis.

**Transgenic mouse models for copper homeostasis**

Recently, transgenic mice have shown to be a pivotal tool for studying the physiologic role of the copper transporters Ctrl1, Atox1, copper chaperone for Cu/Zn superoxide dismutase (Ccs), and Cox17 (Table 2). Deletion of Ccs and Cox17 results mainly in a marked reduction in cuproenzymatic activities of Cu/Zn superoxide dismutase (Sod1) and cytochrome c oxidase (Cco), respectively (25, 26). Both Cox17−/− and Ctrl1−/− mice are embryonically lethal and it has been suggested that Cox17 participates downstream of Ctrl1 during early embryogenesis (25). However, deficits in Cco activity might have a greater effect during embryonic development than reduced activity of other cuproenzymes resulting from the absence of copper transporters, such as Atox1 and Atp7a.

In contrast with affected Bedlington terriers, Commd1-deficient mice are embryonically lethal and show defects in placental vasculization that are apparently caused by aberrant HIF-1 activity (23). Although COMMD1 is associated with copper toxicity, no clear defects in copper homeostasis were observed. These data and other recent studies clearly demonstrate that, besides copper homeostasis, COMMD1 is also involved in several other cellular processes including NF-κB signaling (40). It has been established that COMMD1 is involved in mediating the stability of the proteins in these different pathways, such as ATP7B, HIF-1α, and RelA. The role of COMMD1 in hepatic copper transport, with the use of a liver-specific Commd1 conditional knockout mouse, is currently being investigated in our laboratory.
Biomarkers of Copper Status

As mentioned previously, a disturbed copper balance can result in copper overload or copper deficiency disorders, of which many (such as ICT) have a yet unknown etiology. In addition, alterations in copper levels have been associated with other diseases, including inflammation, cancer, and atherosclerosis. Therefore, the identification of specific and sensitive biomarkers for copper status will be a valuable aim for patient screening in early stages of copper-related diseases and diagnosis to continuing public health. At present, several studies have attempted to identify specific biomarkers for copper status. Studies in different copper-deficient rodent models have recognized CCS as a potential marker for copper deficiency, because an increased expression of this protein has been identified in brain, heart, liver, and erythrocytes of these animals (41). In contrast, expression and activities of the cuproenzymes ceruloplasmin, SOD1, and peptidylglycine α-amidating monoxygenase were reduced in plasma of copper-deficient animals (41, 42). In addition to decreased serum ceruloplasmin and SOD1 activity, low serum copper concentrations have been considered as an early marker for copper deficiency in humans (43). Interestingly, in a human cohort with mild excess copper exposure, reduced mRNA expression of CCS and SOD1 has been observed and postulated as possible biomarkers for moderate copper overload (44). Although these proteins have the potential to be markers to reflect copper status, further investigation is needed to elucidate their sensitivity and specificity under different physiologic and pathologic conditions.

Conclusion

Over the years, animal models have proved to be useful for studying the physiologic role of copper in many different processes, such as neurologic and embryonic development. Together with the identification of the naturally occurring rodent models for Menkes and Wilson disease, new insights into the pathogenic changes related to copper deficiency and overload have been gained. Animal models have also been shown to be extremely valuable for carrying out genetic studies (22). Our increasing knowledge about the genomes of different species will soon make a significant contribution to improving the mapping of specific traits. In addition to mammalian models, the zebrafish has recently been shown to be a valuable model for studying small molecules that affect copper homeostasis and for performing genetic screens to identify copper regulators (45). Technologic advances in generating transgenic mice will aid our understanding of the physiologic role of copper transporters in a tissue-specific manner, as has been clearly shown for Ctr1. Along with our increasing expertise in studying the whole genome on different levels, such as genomics, proteomics, and metabolomics (described elsewhere in this issue), animal models will play a key role in broadening our understanding of copper metabolism in mammals and the role of copper status in various diseases such as inflammation. Additionally, they will help us to identify specific molecular biomarkers related to copper status and to develop new therapies in the treatment of copper-related disorders.

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