Correction of a mouse model of Menkes disease by the human Menkes gene

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Abstract

The brindled mouse is an accurate model of the fatal human X-linked copper deficiency disorder, Menkes disease. Males carrying the mutant allele of the Menkes gene orthologue *Atp7a* die in the second week of life. To determine whether the genetic defect in the brindled mice could be corrected by expression of the human Menkes gene, male transgenic mice expressing ATP7A from the chicken β-actin composite promoter (CAG) were mated with female carriers of the brindled mutation ($Atp7a^{Mo-br}$). Mutant males carrying the transgene survived and were fertile but the copper defect was not completely corrected. Unexpectedly males corrected with one transgenic line (T25#5) were mottled and resembled carrier females, this effect appeared to be caused by mosaic expression of the transgene. In contrast, males corrected with another line (T22#2) had agouti coats. Copper concentrations in tissues of the rescued mutants also resembled those of the heterozygous females, with high levels in kidney (84.6±4.9 μg/g in corrected males vs. 137.0±44.3 μg/g in heterozygotes) and small intestine (15.6±2.5 μg/g in corrected males vs. 15.7±2.8 μg/g in heterozygotes). The results show that the Menkes defect in mice is corrected by the human Menkes gene and that adequate correction is obtained even when the transgene expression does not match that of the endogenous gene.
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1. Introduction

The mottled mice are an allelic series of mouse mutants that are due to mutations in the murine orthologue of the X-linked Menkes genes (*Atp7a*) [1,2]. The mutant males exhibit a wide range of phenotypes ranging from prenatal lethality to mild symptoms such as a grey coat [3]. The carrier females are readily identified because they have a mottled coat due to the random X-inactivation while the unaffected mice have an agouti coat [4]. As with the human disorder Menkes disease, the various defects in the mice are attributable to copper deficiency affecting a range of copper-dependent enzymes [5]. The affected gene encodes a copper transporting ATPase (Atp7a), which supplies copper to secreted cuproenzymes and also effluxes copper from the cell [6]. This copper transporter has an important role in transporting copper across the placenta, and mutant fetuses are severely copper deficient as a result, but the placenta accumulates copper [7]. The closest mottled mutant to Menkes disease is the brindled mouse, mutant males carrying the brindled allele $Atp7a^{Mo-br}$ survive until about 15 days after birth, and have a markedly hypopigmented coat and curly whiskers due to copper deficiency [4]. The mutation in *Atp7a* in the brindled mouse was found to be an in-frame deletion of 6 nucleotides, resulting in the loss of two highly conserved amino acids [8]. The brindled mutant mouse can be rescued by copper treatment provided this occurs before 10 days postnatal [9]. These mice have been used to investigate the treatment of Menkes disease, however, in contrast with the success with the brindled mice, the treatment of human patients with copper salts or even with copper histidine has not proven to be very successful, with only a handful of successfully treated patients being reported [10].

The wide range of defects in the mottled mice suggests that Atp7a is involved in various aspects of copper physiology in addition to placental transport. These include uptake of copper across the small intestine and transport of copper across the blood brain barrier [11]. The fact that copper accumulates in the

kidney of the brindled mouse indicates that Atp7a has a role in resorption of copper from the urine. Copper is absorbed into the proximal tubules, but because of the lack of Atp7a, the copper is trapped in cells and accumulates in the organ [12]. The Menkes protein is not expressed in hepatocytes [13], where the important copper homeostatic mechanism of biliary copper excretion is carried out by the closely related protein, ATP7B which is defective in the copper toxicity condition, Wilson disease [14].

To investigate the possibility of gene correction of Menkes disease we report here the use of transgenic mice that express the human Menkes protein (ATP7A) to demonstrate that the human Menkes gene (*ATP7A*) allows survival of the brindled mutant, the mouse model of Menkes disease. Despite expression patterns that do not entirely match the endogenous gene, the corrected mice are fertile and survive for more than 2 years, but the phenotype of the mutant males corrected with the transgenic line T25#5 resembled that of the heterozygote females.

2. Materials and methods

2.1. Animals

All animal studies were approved by Deakin University Animal Welfare Committee (A1/1999 and A20/A2001). Animals were housed under constant conditions with a 12-h light and 12-h dark cycle, and given deionised water and standard lab chow (Barastoc, Ridley AgriProducts, Australia) ad libitum. The transgenic mice were maintained in the C57BL/6 background, and the brindled allele of the X-linked mottled mutants, $Atp7a^{Mo-br}$ in our laboratory is maintained on a C3H/CBA background.

2.2. Screening of transgenic lines

Mice were genotyped by PCR of tail DNA using the following primer set: sense oligonucleotide Y2H7 (5′-TCTCTCTTCCTTAAACTTTAC) corresponding to the 3′ end sequence of *ATP7A* and the antisense oligonucleotide SP6 (5′-ATTTAGGTGACACTATAG) from the vector. PCR conditions were: 95 °C for 3 min for one cycle, then 30 cycles of: 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min. Animals shown to be transgenic were subsequently mated to wild-type C57BL/6 mice. Transgenic lines were maintained as heterozygotes.

2.3. Rescue of the brindled males

Transgenic male mice were mated with females carrying the $Atp7a^{Mo-br/+}$ X-linked mottled allele. Pups were screened for presence of the transgene by PCR using the primers Y2H7 and SP6 as described previously and the $Atp7a^{Mo-br}$ mutation was detected by PCR [8]. In brief, sense oligonucleotides MMNK22 (5′-GGCAAAACCTCCGAGGCAAAG) specific for the mutant allele or MMNK23 (5′-CAAAACCTCCGAGGCTCTTGC) specific for the wild type allele and antisense oligonucleotide MMNK24L (5′-AGGAGGAGATTTTCA-GAGTTCAG) were used to amplify products of 83 bp and 87 bp, respectively, in separate reaction tubes. PCR conditions were: 96 °C for 4 min, 63 °C for 1 min, 72 °C for 1 min for two cycles followed by 96 °C for 1 min, 63 °C for 1 min, 72 °C for 30 s for 35 cycles. The products were analysed on 4% agarose 3:1™ HRB gels (Amresco, Solon, Ohio, USA) and detected by staining with ethidium bromide.

2.4. Antibodies

Affinity-purified ATP7A antibody (R17-BX) has been described [15]. Polyclonal anti-calnexin antibody (1:500) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. Western blot analysis

Protein extracts were prepared by homogenisation of lung, heart, liver, kidney, small intestine, brain and spleen in 140 mM Tris–HCl (pH 6.8), 6% SDS, 22.4% glycerol and one Mini-EDTA free protease inhibitor tablets (Roche Diagnostics GmbH, Mannheim, Germany) per 10 ml of homogenisation buffer. The protein concentration was determined by BCA method (Pierce, Rockford, IL, USA). Samples containing 40 μg of protein were separated using 7.5% SDS/PAGE, transferred to Hybond-C (Amersham Biosciences, Bucks, UK) and probed with the R17-BX antibody. Protein bands were detected by donkey anti-sheep/goat horseradish peroxidase-coupled secondary antibody (Chemicon, Melbourne, Vic, Australia) using Lumilight Western Blotting Substrate (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

2.6. Immunohistochemical analysis

Brain, kidney, heart and lung were immersion-fixed in 4% PFA in PBS for 5–24 h at 4 °C, embedded in paraffin and sectioned at 4 μm on a Microm HM330 microtome (Walldorf, Germany). Small intestine and liver were embedded in OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) on dry ice and sectioned on a Leica CM1850 cryostat (Leica, Nussloch, Germany) at 4 μm. The sections were air dried for 24 h at room temperature and fixed in 4% PFA in PBS. All tissue sections were treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase and blocked in 1% normal rabbit serum for 1 h at room temperature. The sections were then incubated with R17-BX antibody diluted 1:2000 in PBS (1:1000 on liver sections) for 1 h at room temperature or 24 h at 4 °C. Binding of the primary antibody was visualized with the Vectastain ABC Kit and Nova Red as the chromogen (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with Harris's heamatoxylin (Amber Scientific, Belmont, WA, Australia) and mounted with DXT (Chem-Supply, Gillman, SA, Australia).

2.7. Real time quantitative polymerase chain reaction

Total RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Clifton Hill, Vic, Australia). cDNA was generated from 5 μg RNA using StrataScript® reverse transcriptase (Stratagene, East Kew, Vic, Australia) following the manufacturer's instructions. The specific primers for *ATP7A* were (5′-GCTACCTTGTCAGACAC-GAATGAG, and 5′-TCTTGAACTGGTGTCATCCCTTT) and for *Atp7a* were (5′-AAACCTTGCGAGAAGCAATTG, and 5′-GGGCAAAA-GAGGTGTTTCCA). Real time PCR was performed in triplicate on 20 ng cDNA (RNA content prior to reverse transcription) using the AB 7500 Real Time PCR System (Applied Biosystems, Scoresby, Vic, Australia) coupled with SYBR Green technology (Applied Biosystems, Scoresby, Vic, Australia). The threshold cycle value (Ct), defined as the cycle number when fluorescence level exceeds the threshold value was determined using the AB 7500 software. The fold change relative to *Atp7a* in the corrected mutant male was calculated using the following equation: $2^{\Delta Ct}$ where ΔCt=mean Ct of endogenous *Atp7a* − mean Ct of *ATP7A*. Values represent mean fold change±standard deviation.

2.8. Determination of tissue copper concentration

Tissues were analysed for trace metals by flame atomic absorption spectrometry, using a Varian GTA 100 spectrophotometer (Mulgrave, Vic, Australia).

2.9. Statistical analysis

All values are expressed as means±S.D., unless otherwise indicated. Mann–Whitney tests or Student's *t*-tests were used to assess statistical differences between two given genotypes. A *P* value <0.05 was considered significant.

3. Results

3.1. Correction of the Menkes defect in the mottled mouse mutant

We used two lines of transgenic mice (T25#5 and T22#2) expressing the human Menkes gene to correct the brindled mutants. The generation and characterization of the lines is described elsewhere [15]. In these lines ATP7A is regulated by the chicken β-actin promoter (CAG) and the transgene was found to be widely expressed with some differences in expression pattern between the lines. To introduce the CAG-*ATP7A* transgene into the mutant brindled males, T25#5 or T22#2 transgenic male mice were mated with non-transgenic females heterozygous for the $Atp7a^{Mo-br}$ X-linked mottled allele. Pups were genotyped by PCR of tail DNA for their transgenic status and for the brindled mutation using allele-specific oligonucleotides, as described in Materials and methods. Amplification of the mutant allele generated a 83 bp PCR product compared to a 87 bp fragment from the wild type allele as previously described [8]. Fig. 1A shows the PCR results from the four possible *Atp7a* genotypes generated from such mating (transgene status is not shown). Out of the total number of 148 pups genotyped at d9 postnatal, 19 were found to be uncorrected brindled mutants and 18 were found to be corrected mutants, the predicted number being 18.5. The uncorrected mutant males were hypopigmented (Fig. 2A) and died between 14 and 20 days. The transgenes permitted survival of the mutant but surprisingly the T25#5 corrected mutants (Fig. 2C and D) had a mottled coat similar to the heterozygous females (Fig. 2B). Apart from the mottling of the

coat of the T25#5 corrected mutant males, corrected mutant males appear in good health and have survived for more than 2 years. Their growth rate was similar to the normal male littermates (Fig. 1B) and no significant difference was found between the weight of the corrected mutant males and their normal littermates at d60 postnatal (Student's *t*-test). To establish the fertility of the corrected mutant males and demonstrate transmission of the mutant allele the corrected males were mated with C57Bl/6 females and pups were genotyped as described in Materials and methods. As expected from X-linked inheritance patterns, all female pups were heterozygote for the brindled allele and no brindled males were produced. Out of a total number of 59 progeny, 12 were $Atp7a^{Mo-br/+}$, Tg+; 15 were $Atp7a^{Mo-br/+}$, Tg−; 11 were $Atp7a^{+/y}$, Tg+ and 21 were $Atp7a^{+/y}$, Tg−. Most of the work described in this paper is on the T25#5 corrected males, as this line was characterized first. Subsequently, we investigated the correction by a different transgenic line, T22#2. Interestingly, T22#2 corrected males had an agouti coat colour similar to their wild type littermates (Fig. 2E and F) but otherwise were similar in health, growth rate and longevity compared with the T25#5 corrected males. The corrected mutant males of both lines had straight whiskers (note Fig. 2C–F) contrasting with the curly whiskers of the uncorrected mutant (Fig. 2A), which are caused by copper deficiency.

3.2. Transgene expression in the corrected mutant males

The expression of the ATP7A protein and *ATP7A* mRNA levels in a range of tissues from T25#5 corrected mutant males were determined by Western blot analysis (Fig. 3A) using an affinity purified anti-ATP7A antibody R17-BX [15] and real time PCR (Fig. 3B). The R17-BX antibody could detect the mouse Atp7a as well as the human protein, however, the sensitivity of the antibody was not sufficient to detect the endogenous protein in most tissues of the non-transgenic animals. Bands at a similar size to ATP7A (178 kDa) were seen in samples from the lung, kidney, brain and spleen with highest levels in kidney (Fig. 3A top panel). The human protein, ATP7A was expressed in a wide range of tissues of the corrected mutant male, albeit to varying extents and migrated in a position consistent with the expected size of 178 kDa (Fig. 3A bottom panel) [16]. A strong signal was observed in heart and lower levels of expression were seen in small intestine, brain, lung, spleen and kidney. Expression in the liver was very low, the band present at a lower Mr in Fig. 3A is an artifact due to the presence of a major liver protein at that position in the gel and is present in both the non-transgenic and transgenic livers. Calnexin, the loading control could not be detected in non-transgenic small intestine, most likely due to degradation in this particular sample, since we routinely are able to visualize it. Nevertheless, we have been unable to detect endogenous Atp7a in small intestine when the calnexin band is visible. Since endogenous Atp7a is not detected in all tissues tested we could not determine the expression levels of ATP7A relative to the endogenous protein. To estimate the relative levels of transgene expression to the endogenous *Atp7a* gene expression, mRNA
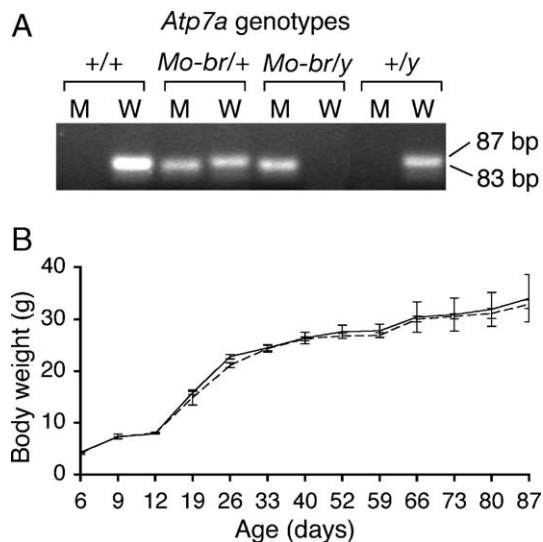

Fig. 1. Characterization of the corrected mutant mice. (A) PCR detection of the brindled mutation in tail DNA from the progeny of transgenic male mice mated with non-transgenic females heterozygous for the $Atp7a^{Mo-br}$ X-linked mottled allele. Primers MMNK22 and MMNK24L detect the mutant allele (M) and primers MMNK23 and MMNK24L detect the wild type allele (W). (B) Growth curves of corrected mutant and normal male mice. Measurements represent the average weight of three corrected mutant mice (dashed line) and two normal male littermates (solid line). Error bars indicate standard deviation.
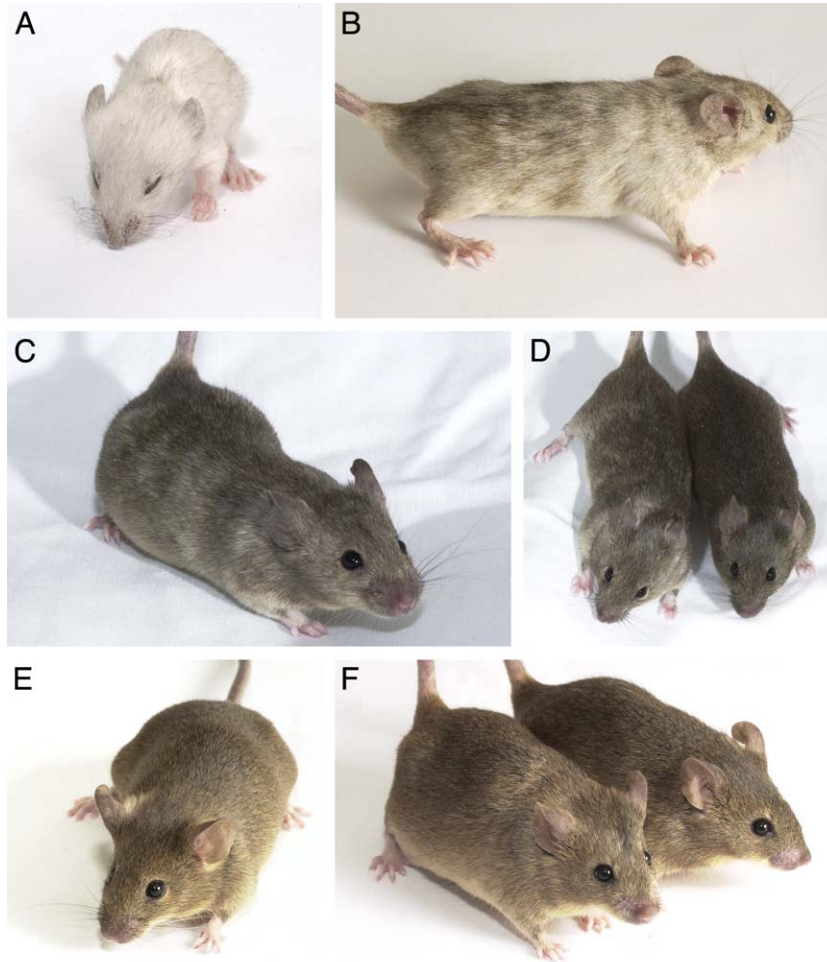
Fig. 2. Appearance of corrected mutant mice. (A) Uncorrected mutant mouse at 12 days of age. (B) Heterozygote female. (C) T25#5 corrected mutant male mouse at 105 days of age. (D) Another T25#5 corrected mutant male mouse at 78 days of age (left) compared to a normal male littermate (right). (E) T22#2 corrected mutant male mouse at 186 days of age. (F) Another T22#2 corrected mutant male mouse at 135 days of age (left) compared to a normal male littermate (right).

levels of *Atp7a* and *ATP7A* were determined by real time quantitative PCR (Fig. 3B) on tissue samples from the same corrected mutant male presented in Fig. 3A. ATP7A mRNA expression levels relative to *Atp7a* levels were markedly increased in heart (244 fold), small intestine (38 fold), brain (11 fold), lung (7 fold), kidney (3 fold) and spleen (2 fold). Lower expression levels were measured in liver (1.7 fold) consistent with the Western blot results.

The cellular distribution of ATP7A in various organs was determined by immunohistochemistry using the R17-BX antibody. Endogenous Atp7a was undetectable in non-transgenic mice under the conditions used (data not shown). The transgene was expressed in a mosaic pattern in most tissues examined (Fig. 4). In the lung ATP7A was detected in smooth muscle of pulmonary vessels, but not in the parenchyma (Fig. 4A). In the heart the transgene was expressed in cardiac muscle (Fig. 4B). In the liver, we found expression of the transgene in clusters of hepatocytes (Fig. 4C), however, the majority of hepatocytes did not appear to express ATP7A. Interestingly the protein appeared to be at the plasma membrane rather than in an intracellular location as was seen in most other cell types. In the small intestine the transgene was expressed in the perinuclear

region on the apical side of the enterocytes (Fig. 4D) consistent with the TGN localization in these cells reported by Monty et al. [17]. In kidney, the transgene was expressed in the macular densa of the distal tubules (Fig. 4E), in tubules in the outer cortex (Fig. 4F), as well as in the thin loops of Henle in the outer medulla (data not shown). In the brain, we found transgene expression in the Purkinje cell layer of the cerebellum (Fig. 4G), in the CA1 and CA2 region of the hippocampus (Fig. 4H), the mitral layer of olfactory bulb and in some instances in the vascular endothelium (data not shown). Some expression was detected in the choroid plexus but we could not detect expression in astrocytes (data not shown).

3.3. Effect of ATP7A expression on Cu concentration in corrected mice

Copper concentrations in the mice were determined at 12 days (Table 1) and 60 days postnatal (Table 2). Copper concentrations in the heart of the uncorrected mutant male at 12 days of age were much lower than normal (8.8 ± 2. μg/g vs. 24.1 ± 9.7 μg/g) but the copper concentrations were restored to normal levels in the heart of the corrected mutants. In the liver,
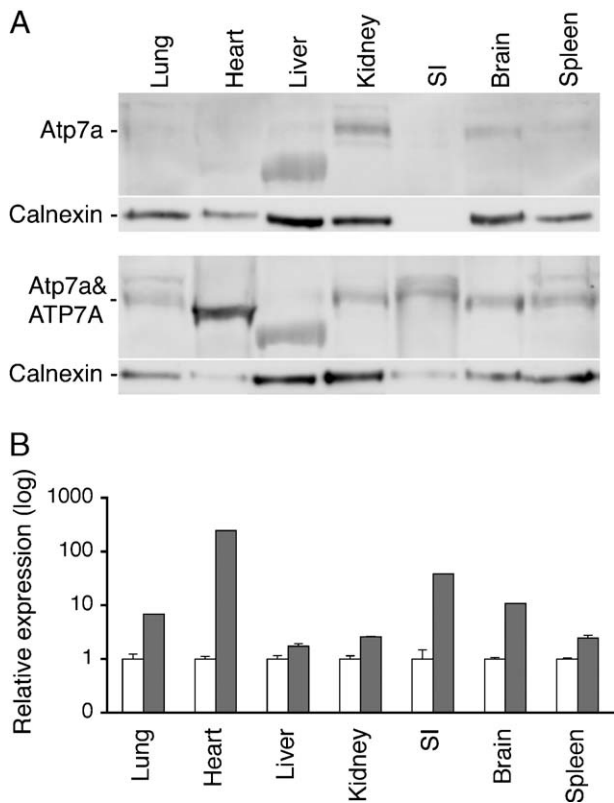
Fig. 3. Transgene expression in mouse tissues. (A) Representative Western blots of 40 μg protein extracts from a normal male, Tg− (top panel) and a corrected mutant male (bottom panel) mouse tissues. The R17-BX antibody detected ATP7A (178 kDa) in lung, heart, spleen, kidney, small intestine (SI), and brain of the corrected mutant male. Endogenous Atp7a was detected in lung, kidney, brain and spleen. Calnexin was used as a loading control and detected using a polyclonal antibody. (B) Gene expression of *ATP7A* in the same corrected mutant male analysed by Western blot. mRNA levels were expressed relative to endogenous *Atp7a*. Real time quantitative PCR data for normal male (open bars) and corrected mutant male (dotted bars) represent mean fold change±standard deviation in the logarithmic scale.

the normal males and female had high copper concentrations typically found in mice prior to weaning [18]. The uncorrected male mutant and the heterozygous females both had very low copper concentrations in their livers. The presence of the transgene in the male mutants increased the hepatic copper concentrations by about 50%, still well below the normal values. Interestingly the expression of ATP7A in the heterozygous females caused a marked increase (250%) in hepatic copper concentrations but not to the levels found in normal animals. Both the heterozygous female and the mutant male had very high copper concentrations in the kidney. The expression of the transgene decreased the kidney copper concentrations in the heterozygous female, but increased the copper in the kidneys of the corrected males. Although ATP7A was well expressed in the small intestine (Fig. 4B), this did not appear to correct the elevation of copper in the small intestine of the mutant male or heterozygous female. The brain of the uncorrected mutant had extremely low copper concentrations (3.3 ± 0.1 μg/g vs. 8.9 ± 1.4 μg/g in normal mice), this severe copper deficiency was completely corrected by the expression of the transgene.

Copper concentrations in the tissues of the 60-day-old animals is shown in Table 2. The uncorrected mutant males had all died by this age. Copper concentrations in the corrected mutants heart and liver were normal. In the kidney of the corrected males, the copper concentrations were almost 6 fold higher than the normal males, and were similar to the values seen in the heterozygous females expressing the transgene; the non-transgenic heterozygous females, however, had renal copper concentrations approximately 9 fold higher than normal females. The small intestine copper levels were slightly elevated in the corrected mutant males, and again resembled the values seen in the heterozygous females. By this age, the brain copper concentrations of all genotypes was similar.

4. Discussion

The expression of the human Menkes protein permitted survival of the brindled mutant males, however, the copper concentrations were not restored to those found in the normal male but were similar to copper concentrations in the heterozygous females. As we found here and previously reported [19,20], the females heterozygous for the *Atp7a*[Mo-br] allele have quite abnormal copper concentrations with pronounced copper accumulation in the small intestine, kidney and low copper concentrations in the liver similar to the values seen in the mutant male, yet the females survive normally. The explanation for the copper accumulation is that, as a result of random X-inactivation, approximately 50% of cells in each tissue are expressing the mutant allele and accumulate copper due to defective efflux. As with cells from patients with Menkes disease, the mutant cells take up copper but cannot efflux it, leading to accumulation [21]. The intestinal enterocytes expressing the normal allele of *Atp7a* transport copper into the body, clearly allowing sufficient copper to reach critical enzymes to permit normal growth and development. The low levels of copper in the liver are an indication that supplies of copper are limited, probably due to a combination of the reduced intestinal transport (only 50% of cells operating transporting copper), and a "copper sink" effect of cells expressing the mutant allele accumulating copper. Thus, the heterozygous female is a genetic mosaic of cells in which copper is normally handled and cells in which copper is accumulating, but is not transported across the *trans*Golgi network to cuproenzymes in the secretory pathway. This mosaicism manifests as a mottled coat; the normally pigmented cells have an active tyrosinase that receives copper from Atp7a, and the hypopigmented patches represent cells containing an inactive tyrosinase due to the mutant Atp7a.

Our data suggests that, like the heterozygous female, the males corrected with the T25#5 transgene are a genetic mosaic of transgene-expressing or non-expressing cells (e.g., Fig. 4). The consequences for coat colour and tissue copper accumulation are similar to that seen in the heterozygous females, however, the exact phenotype will depend on the pattern of transgene inactivation, which is likely to vary between lines of mice and possibly even between individuals of the same line. The observation that the corrected males of the T22#2 line have
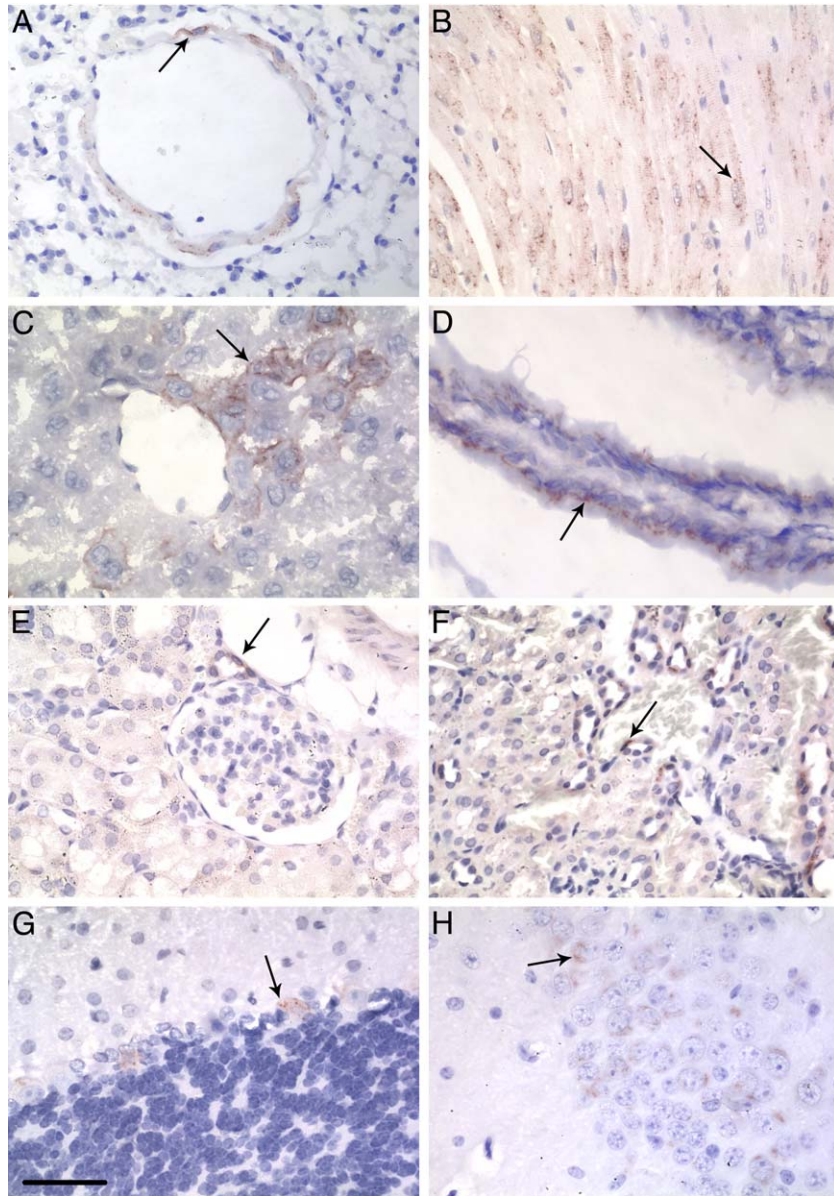
Fig. 4. Immunohistochemical analysis of ATP7A in various tissues of a representative corrected mutant male mouse collected at 60 days postnatal. Tissue sections were prepared as described in Materials and methods, and ATP7A was detected with the R17-BX antibody and immunoperoxidase staining. Endogenous Atp7a was not detected (data not shown). ATP7A expression was detected in lung (A), heart (B), liver (C), SI (small intestine) (D), kidney (E and F), the Purkinje cell layer of the cerebellum (G) and in the CA2 region of the hippocampus (H). Arrows indicate a typical cell expressing ATP7A. In panels B, E and F there is some background staining due to longer development time for those sections. Scale bar=50 μm.

a normal coat suggests that the transgene in this line is uniformly expressed in the skin, but other data has shown mosaic expression in other tissues [15]. Mosaic expression of transgenes is not uncommon and presumably reflects random silencing events as was shown for the keratin 5/*lac*Z constructs which express in a mosaic fashion in the epithelium of transgenic mice [22] and may be a consequence of the chromosomal integration site or transgene copy number [23]. We have not investigated the site of integration of the transgene or the copy number in transgenic lines T25#5 and T22#2, however, PCR screening for presence of the transgene suggests that the copy number of the T22#2 line is higher than that of the T25#5 line (not shown). Variable transgene expression has been

reported for a number of genes regulated by the CAG promoter [24–26]. Although this property could potentially be an issue if one is considering gene correction for Menkes disease, our results demonstrate that even partial correction is sufficient to achieve normal growth and development.

ATP7A expression and *ATP7A* mRNA levels were found to vary widely between the tissues of the corrected mutant mice. Expression was highest in the heart muscle cells where the levels were over two hundred times relative to the endogenous mRNA. In cells where the endogenous protein was detectable, such as the kidney, relative levels were about 3 fold higher than the endogenous gene. The high relative expression in the heart is due to a combination of the high activity of the CAG

Table 1
Tissue copper concentration in organs of 12-day-old progeny generated from the mating of transgenic male and $Atp7a^{Mo-br}$ female mice

Tg and description	Coat colour	Genotype	Cu µg/g dry weight of mice tissue				
			Heart	Liver	Kidney	SI	Brain
Normal female (−)	Ag	Tg− $Atp7a^{+/+}$	17.2±6.6 (6)	222±81.4 (8)	16.9±7.0 (5)	20.8±8.0 (7)	11.1±3.0 (7)
Normal female (+)	Ag	Tg+ $Atp7a^{+/+}$	18.0±4.0 (4)	164.8±92.2 (6)	10.0±1.3 (4)	20.9±6.9 (5)	8.5±1.4 (6)
Heterozygote female (−)	Br	Tg− $Atp7a^{Mo-br/+}$	20.4±9.1 (6)	14.9±3.6 (6)	88.2±7.7 (5)	35.5±3.6 (5)	5.9±1.4 (6)
Heterozygote female (+)	Ag or Br	Tg+ $Atp7a^{Mo-br/+}$	13.4±5.8 (6)	52.8±13.6 (5)[a]	71.2±5.8[a]	48.8±18.6 (5)[a]	7.8±2.4 (5)
Normal male (−)	Ag	Tg− $Atp7a^{+/y}$	24.1±9.7 (5)	164.5±52.4 (6)	18.5±8.1 (5)	24.4±6.9 (6)	8.9±1.4 (5)
Normal male (+)	Ag	Tg+ $Atp7a^{+/y}$	17.1±4.6 (4)	164±70.6 (5)	19.3±5.1 (4)	14.9±2.7 (5)	10.9±2.8 (5)
Uncorrected mutant (−)	Wh	Tg− $Atp7a^{Mo-br/y}$	8.8±2.1 (4)	10.7±2.4 (7)	52.2±10.3 (6)	59.2±43.2 (7)	3.3±0.1 (5)
Corrected mutant (+)	Br	Tg+ $Atp7a^{Mo-br/y}$	21.5±8.2 (3)[b]	15.8±4.5 (4)[c]	66.0±15.6 (4)[c, d]	54.9±27.4 (4)[c]	9.3±3.5 (4)[b]

Results are copper concentration expressed as µg/g dry weight of tissue±standard deviation, number of observations in parentheses. SI, small intestine. Ag=normal agouti coat, Br=mottled brindled coat, Wh=white coat. Tg− and Tg+ refer to non-transgenic and transgenic mice, respectively. $P<0.05$ were found for the following pairs:
[a] Heterozygote female (+) versus normal female (+).
[b] Corrected mutant (+) versus uncorrected mutant (−).
[c] Corrected mutant (+) versus normal male (+).
[d] Corrected mutant (+) versus heterozygote female (−).

promoter in muscle cells [26] and the very low endogenous levels in this tissue.

The over-expression of ATP7A did not result in apparently abnormal intracellular distribution of the protein as it was found in regions consistent with the *trans*Golgi network, the established location of the Menkes protein in a normal copper environment [27]. Elevated levels of ATP7A in cells has shown to cause depletion of copper, due to enhanced efflux of copper [28,29], and this effect is apparent in the trend for reduced copper concentrations in the hearts of the transgenic animals (Table 2) [15]. It has not been established whether this copper depletion has any physiological consequences for the heart.

ATP7A was expressed in patches of hepatocytes in the liver of the corrected mice, and unlike other cell types appeared located on the plasma membrane, rather than in a region consistent with the TGN. It is probably that this region is the basolateral surface of the hepatocyte, as ATP7A has been shown to traffic to the basolateral surface in polarized cells [30] and proteins that are located on the apical surface of hepatocytes give a characteristic discrete circular staining pattern [31,32]. Normally, hepatocytes do not express ATP7A, instead they express the closely related Wilson protein, ATP7B [31]. When the copper levels in the hepatocyte are low, ATP7B is found in the TGN, but the protein traffics to large vesicles in the vicinity of the biliary canalicular membrane when the copper levels are elevated leading to biliary excretion of copper [31]. Our result suggests a physiological reason for the lack of ATP7A expression in the liver in normal animals. If ATP7A were normally expressed in hepatocytes it would presumably pump copper into the sinusoidal space, and hence back into the circulation, rather than delivering excess copper into the bile and act in opposition to the biliary excretion role of ATP7B. It will be of interest to explore further the physiological consequences of this presumed reversal of copper flow in further experiments.

Although the pattern of expression of the transgene in tissues did not precisely match that of the reported expression patterns of the endogenous gene [12], ATP7A was expressed in the tissues critically important for the survival of the mutant mice. For example, ATP7A was well expressed in the intestinal enterocytes where copper accumulates in mutants when the Menkes protein is defective [19], and this expression presumably allows

Table 2
Tissue copper concentration in 60-day-old progeny generated from the mating of transgenic male and $Atp7a^{Mo-br}$ female mice

Tg and description	Coat colour	Genotype	Cu µg/g dry weight of mice tissue				
			Heart	Liver	Kidney	SI	Brain
Normal female (−)	Ag	Tg− $Atp7a^{+/+}$	19.3±2.2 (5)	15.6±2.1 (4)	17.9±1.5 (5)	8.2±2.3 (5)	15.0±1.1 (5)
Normal female (+)	Ag	Tg+ $Atp7a^{+/+}$	17.1±2.6 (4)	13.5±1.5 (4)	15.1±1.4 (4)	9.7±1.5 (4)	14.6±1.4 (4)
Heterozygote female (−)	Br	Tg− $Atp7a^{Mo-br/+}$	22.1±5.0 (5)	14.1±1.8 (5)	137.0±44.3 (4)	15.7±2.8 (5)	11.3±3.2 (5)
Heterozygote female (+)	Ag or Br	Tg+ $Atp7a^{Mo-br/+}$	17.1±3.0 (4)	14.7±3.9 (5)	82.6±14.1 (5)[a]	14.4±1.2 (5)[a]	15.9±1.0 (5)
Normal male (−)	Ag	Tg− $Atp7a^{+/y}$	18.5±2.0 (5)	19.2±2.7 (5)	17.1±2.2 (5)	9.6±1.5 (5)	13.4±1.1 (5)
Normal male (+)	Ag	Tg+ $Atp7a^{+/y}$	13.8±2.3 (7)	18.4±2.5 (6)	14.6±3.1 (7)	9.0±0.9 (7)	15.0±1.4 (7)
Corrected mutant (+)	Br	Tg+ $Atp7a^{Mo-br/y}$	15.5±2.9 (5)[b]	17.7±1.7 (6)[b]	84.6±4.9 (6)[c, b]	15.6±2.5 (6)[c]	15.3±1.9 (6)

Results are copper concentration expressed as µg/g dry weight of tissue±standard deviation, number of observations in parentheses. SI, small intestine. Ag=normal agouti coat, Br=mottled brindled coat. No data are listed for the uncorrected mutants (−) because they all died around 14–20 days of age. $P<0.05$ were found for the following pairs:
[a] Heterozygote female (+) versus normal female (+).
[b] Corrected mutant (+) versus heterozygote female (−).
[c] Corrected mutant (+) versus normal male (+).

the absorption of dietary copper. Recently Monty et al. have shown that ATP7A in the intestinal enterocytes of the ATP7A transgenic mice responded to increasing copper in the lumen of the small intestine by trafficking from a perinuclear region to vesicles in the basolateral region, presumably facilitating copper delivery to the circulation [17]. The fact that the brain copper is restored to normal is of critical importance for the survival of the mice. The Menkes protein is thought to be necessary for the transport of copper across the blood brain barrier, and in mutant mice copper has been found to accumulate in the vascular endothelium and astroglial cells of the mouse brain [33]. Surprisingly, we could not detect consistent expression of the transgene in the vascular endothelial cells or astrocytes, nevertheless we suggest that a low but undetectable amount of ATP7A must have been present, otherwise copper would not have been able to reach the brain. We note that our antibodies are not sensitive enough to detect endogenous levels of Atp7a, so expression of the transgene at levels equivalent to the normal protein may not have been visible in the sections. We detected substantial amounts of ATP7A in the Purkinje cell layer of the cerebellum, in the CA1 and CA2 region of the hippocampus and in the mitral layer of olfactory bulb, which are also reported to express the Atp7a in normal mice [12,34]. This expression of the transgene may have assisted in the restoration of normal brain function, however, the complete role of the Menkes protein and indeed copper in the brain is far from clear.

In Menkes patients copper accumulates in the proximal tubules of the kidney, the place of copper resorption [11]. We found ATP7A expression in the macular densa of the distal tubules, in tubules in the outer cortex, as well as in the thin loops of Henle in the outer medulla, which was sufficient to reduce the copper levels of the corrected mutant males compared to the heterozygote females. Comparison of transgene expression with the endogenous Atp7a is complicated by the differences in published reports of the expression pattern of Atp7a in the kidney. For example, strong endogenous Atp7a expression was found by in situ hybridization studies in the proximal tubules, however low levels of expression were found in the glomeruli [35]. Moore and Cox found strong endogenous expression in the glomeruli using in situ hybridization and slight expression in the interior of various tubules [36]. We have previously detected Atp7a in both the proximal and distal convoluted tubules with little staining in the glomeruli [8].

Normally the livers of fetal mice accumulate copper far above the adult levels, and the high concentrations are maintained throughout the first 10–15 days of life [18]. The uncorrected mutant has an extremely low hepatic copper concentration of 10.7 ± 2.4 μg/g at 12 days compared with the normal male littermate (164.5 ± 70.6 μg/g), consistent with the severe copper deficiency of the mutants. This low liver copper is primarily due to the defective transport of copper across the mutant placenta [7]. The transgene only partially corrects this deficiency, with the hepatic copper being only 15.8 ± 4.5 μg/g. This value suggests that the expression of the transgene must be moderately restricted in the placenta but we have not assessed placental expression. Interestingly the 12-day-old heterozygous females have low hepatic copper as well (14.9 ± 3.6 μg/g) which

may be due to the preferential inactivation of the normal paternal allele in the placenta leaving most of the fetal placenta with the mutant X active [37]. The transgene in the heterozygous females increases hepatic copper concentrations of around 50 μg/g closer to the normal values than the corrected mutant males. It is probable that the expression of the transgene in the placenta supplements the restricted placental copper transport by increasing the number of placental trophoblasts expressing an active Menkes protein. Despite the low hepatic copper concentrations in the corrected mutant males and heterozygous females the pups develop normally.

In conclusion, the rescue of the brindled mutants demonstrates that even partial correction of the Menkes genetic defect in humans would be sufficient for relatively normal development and these mice also will provide further information about the role of the Menkes ATPase in copper physiology.

Acknowledgements

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