Defective localization of the Wilson disease protein (ATP7B) in the mammary gland of the toxic milk mouse and the effects of copper supplementation

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Toxic milk (*tx*) is a copper disorder of mice that causes a hepatic accumulation of copper similar to that seen in patients with Wilson disease. Both disorders are caused by a defect in the *ATP7B* copper-transporting ATPase. A feature of the *tx* phenotype is the production of copper-deficient milk by lactating dams homozygous for the *tx* mutation; the milk is lethal to the pups. It has not been determined whether the production of copper-deficient milk is a direct consequence of impaired expression of ATP7B protein in the mammary gland. With the use of immunohistochemistry, our study demonstrated that the ATP7B protein was mislocalized in the lactating *tx* mouse mammary gland, which would explain the inability of the *tx* mouse to secrete normal amounts of copper in milk. Confocal microscopy analysis showed that, in the lactating *tx* mammary gland, ATP7B was predominantly perinuclear in comparison with the diffuse, cytoplasmic localization of ATP7B in the lactating normal mammary gland. Lactating *tx* mice showed impaired delivery of copper from the mammary gland to the milk and this was not ameliorated by dietary copper supplementation. In contrast, the normal mouse mammary gland responded to increased dietary copper by increasing the amount of copper in milk. A change in the distribution of the ATP7B protein from perinuclear in the non-lactating gland to a diffuse, cytoplasmic localization in the lactating gland of the normal (DL) mouse suggests that the relocalization of APT7B is a physiological process that accompanies lactation. We conclude that the impaired copper transport from the mammary gland into milk in lactating *tx* mice is related to the mislocalization of ATP7B.

Key words: homoeostasis, lactation, subcellular distribution.

INTRODUCTION

Wilson disease is an autosomal recessive defect of copper transport that results in hepatic cirrhosis and neurological disorders. The Wilson gene encodes a transmembrane copper-binding ATPase (ATP7B) that is expressed primarily in the liver and to a smaller extent in the heart, kidney, brain, skeletal muscle and lung [1,2]. Mutations in the *ATP7B* gene lead to impaired biliary excretion of copper from the liver, resulting in excessive accumulation of copper in the liver that can cause liver failure or neurological problems owing to the deposition of excess copper released from the liver [3].

Toxic milk (*tx*) is an autosomal recessive copper disorder of mice that causes the hepatic accumulation of copper similar to that seen in patients with Wilson disease [4]. The *tx* mouse has a single nucleotide change in the *ATP7B* gene from the normal (DL) mouse; this results in the alteration of a highly conserved methionine residue to valine in the eighth transmembrane domain of the predicted protein [5]. In *tx* mice, liver copper levels were 14-fold higher than in DL mice 7 weeks after birth [4] and 55-fold higher in adult *tx* mice compared with adult control mice [6].

An unusual feature of the *tx* disorder is the production of copper-deficient milk by lactating dams homozygous for the *tx* mutation [4]. Pups feeding from mutant dams often die as a result of the decreased copper levels in the milk. It is not known whether the production of copper-deficient milk is a direct consequence of impaired expression of the Wilson disease gene in the mammary gland, which decreases the transfer of copper into milk, or whether it is a secondary consequence of the inappropriate hepatic metabolism of copper. Decreased copper levels in milk from patients with Wilson disease have not been reported.

A significant function for ATP7B protein in the transport of copper into milk in the mammary gland is suggested from studies in *ATP7B*-null mice, which were generated by using homologous recombination to disrupt the normal *ATP7B* gene. The copper concentrations in the mammary gland of lactating *ATP7B*-null mice were twice those in the mammary glands of control mice [7]. This result suggests that the copper uptake pathway in the mammary gland might be functional but that the secretory pathway is impaired.

In *tx* mice, the abnormal accumulation of copper in the liver is not due to decreased levels of expression of *ATP7B* mRNA, because a Northern blot analysis showed no difference between control and *tx* liver mRNA [5]. However, it is likely that the defect in copper transport results from an inactive mutant ATP7B protein. In cultured cells and normal rat hepatocytes, the ATP7B protein is located in the *trans*-Golgi network, but in response to elevated copper concentrations ATP7B relocates to a vesicular compartment within the cell [8,9]. A form of ATP7B with the common disease-causing H1069Q mutation did not relocate in response to copper [10]. A similar effect of disease-causing mutations in blocking copper-induced trafficking has been reported for the closely related Menkes protein, ATP7A [11].

Here we investigate the expression of the Wilson protein in the mammary gland of normal and *tx* mice. We find that the ATP7B protein is expressed at quite high levels in the mammary gland and, interestingly, lactation induces a dispersal similar to that seen in cultured cells exposed to high levels of copper. Signifi-

Abbreviation used: *tx*, toxic milk.

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cantly, the *tx* ATP7B did not show lactation-induced dispersal. Thus our results suggest that ATP7B is important in the delivery of copper into milk, and that trafficking of the protein from the *trans*-Golgi network to a vesicular compartment is part of this process.

MATERIALS AND METHODS

Animals

The *tx* mutation arose in the inbred DL strain and has subsequently been maintained on this background [4]. Toxic-milk pups are routinely fostered to normal mice because they have a very high mortality if allowed to remain suckling with their own mothers for more than 5 days. In the present study, *tx* pups were fostered to normal dams within 3 days. Fostering is considered essential for *tx* pup survival because the paradoxically low copper in *tx* dam milk and a presumed defect in the placental transfer of copper results in a copper-deficient state in the *tx* neonatal period. Normal pups (DL) were fostered within 40 h of birth to either normal or *tx* dams. Females carried and delivered between one and three litters and were culled while still lactating when pups were 5 days old. To investigate the effects of dietary copper supplementation, lactating dams were provided with water containing 0.1 mg/ml copper as copper acetate from the time of mating. The mice tolerated this level of copper with no obvious ill effects over a period of 3–4 months. The transport of copper from the mammary gland of the mother to the pups was estimated by measuring the copper content of the pup stomach. The analysis of stomach contents of pups has previously been used as an indicator of milk copper and zinc concentrations [4,12].

Mouse tissue

Mammary and liver tissue from non-lactating and lactating DL and *tx* mice was excised, frozen immediately and stored at −80 °C until use.

Atomic absorption spectrometry

Tissue was dried at 50 °C for 1 week, weighed and transferred into an acid-washed container to which 0.5 ml of nitric acid had been added. The samples were incubated at room temperature for 1 h, then at 65 °C for 4 h; after this, 2.5 ml of deionized water was added, the samples were centrifuged and the supernatant was analysed with a Varian SpectraAA-880, with direct aspiration.

Preparation of ATP7B antibody

A polyclonal antibody against the mouse ATP7B protein was prepared after the immunization of rabbits with a fusion protein that spanned the copper-binding sites 2, 3 and 4. After precipitation with sodium sulphate, the IgG fraction was affinity-purified by affinity chromatography over the antigen linked to Sepharose 4B (M. Theophilos and J. F. B. Mercer, unpublished work).

Western-blot analysis

Mammary tissue (100 mg) was homogenized in 1.5 ml of 250 mM sucrose/10 mM Hepes (pH 7.5)/2.5 mM EDTA containing protease inhibitors used in accordance with the manufacturer's instructions (Boehringer Mannheim catalogue no. 1836170) and centrifuged for 1 min at 1000 ***g***. The supernatant was centrifuged at 100000 ***g*** for 60 min at 4 °C and the pellet was resuspended in 62.5 mM Tris/HCl (pH 7.5)/2.5 mM EDTA containing protease inhibitors. The extract (60 μg of protein) was fractionated by SDS/PAGE [7.5 % (w/v) polyacrylamide] with a Bio-Rad Mini Protean Gel system. Proteins were transferred to nitrocellulose membranes at 4 mA/cm^2 for 120 min with a Bio-Rad Mini Trans-Blot in 25 mM Tris/HCl (pH 8.3)/192 mM glycine. After being blocked with 1 % (w/v) casein in TBST [0.05 M Tris/HCl/0.15 M NaCl/0.1 % (v/v) Tween 20], the membrane was exposed to the ATP7B primary antibody (dilution 1:500) in blocking buffer for 2 h, then washed in blocking buffer (four times, for 15 min each). ATP7B protein was detected with a 1:1000 dilution of a horseradish-peroxidase-conjugated goat anti-rabbit antibody and a chemiluminescence detection kit (Boehringer catalogue no. 1500798).

Sectioning

Tissue blocks (0.1 cm^3) were immersed in OCT (Tissue Tek) and frozen in liquid nitrogen for 3 min, then sectioned (Leica CM 1800 cryostat) at between −17 °C and −20 °C. Sections 8–10 μm thick were collected on slides coated with gelatin (5 %, w/v).

Staining with haematoxylin and eosin

Tissue sections were air-dried for 20 min, then fixed in 4 % (w/v) paraformaldehyde for 10 min at room temperature [13] and hydrated through an ethanol series [100 %, 70 %, 50 % and 20 % (v/v)] to deionized water, leaving them for 1 min in each solution. Instant haematoxylin stain was applied to the sections for 5 min, followed by a rinse with deionized water. Sections were immersed in Scott's tap water for 20 s, rinsed in deionized water and differentiated in ethanol containing 1 % (v/v) HCl for 2 s. After the sections had been rinsed with deionized water, yellow eosin stain was applied for 2–3 min and washed off in deionized water; the sections were then dehydrated in an ethanol series [20 %, 50 %, 70 % and 100 % (v/v)], leaving them for 1 min in each solution. DPX was added and a coverslip was applied.

Immunohistochemistry

The sections were dried and fixed in paraformaldehyde as described above, rinsed twice for 10 min in PBS, then permeabilized with 5 % (v/v) Triton X-100 in PBS for 5 min and blocked with 3 % (w/v) BSA in PBS for 90 min. ATP7B antibody, diluted 1:250 with 1 % (w/v) BSA in PBS, and pre-immune rabbit antiserum, diluted 1:1000 with 1 % (w/v) BSA in PBS, were applied to tissues for 2 h at room temperature. After three washes with PBS, the second antibody, dichlorotriazinyl amino fluorescein-conjugated AffiniPure Donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories) or FITC-conjugated sheep anti-mouse IgG (Sigma) [dilution 1:200 in 1 % (w/v) BSA in PBS] was applied for 2 h. Sections were washed twice for 10 min in PBS and, where indicated, ethidium bromide (1 μg/ml) was added for 5 min to stain the nuclei lightly. A drop of Fluoroguard (Bio-Rad) was added to the sections and a coverslip was applied. Epifluorescence was viewed with an Olympus BX50 microscope with a PlanApo 60 × magnification, 1.4 numerical aperture oil objective. Confocal images were collected with an Optiscan F900e system in a 512 × 512 pixels format.

RESULTS

Copper concentrations in normal and *tx* mouse stomach contents and livers

The copper concentration of stomach contents of pups was dependent on the genotype of the lactating dam rather than that of

Table 1 Copper concentrations in stomach contents and livers of pups feeding from lactating dams and in the mammary glands of lactating dams

Lactating dams received drinking water with no added copper or with 0.1 mg/ml copper (as copper acetate). Stomach and liver tissues were taken from 5-day-old pups and mammary glands were taken from lactating dams 5 days after the birth of pups. Values shown are means ± S.D.

		Copper concentration (μg/g dry wt)			
		Control (no copper in water)		Copper-loaded water	
Location of copper	Genotype of dam and pup ...	+/+	*tx*/*tx*	+/+	*tx*/*tx*
Pup stomach		6.4 ± 2.0 ($n = 29$)	1.2 ± 0.3 ($n = 20$)	8.0 ± 2.1 ($n = 22$)	0.9 ± 0.2 ($n = 8$)
Pup liver		127 ± 47 ($n = 19$)	6.4 ± 0.7 ($n = 18$)	202 ± 49 ($n = 22$)	6.5 ± 1.6 ($n = 8$)
Dam mammary gland		6.5 ± 0.84 ($n = 6$)	7.8 ± 2.32 ($n = 6$)	8.7 ± 1.53 ($n = 3$)	27 ± 3.51 ($n = 3$)

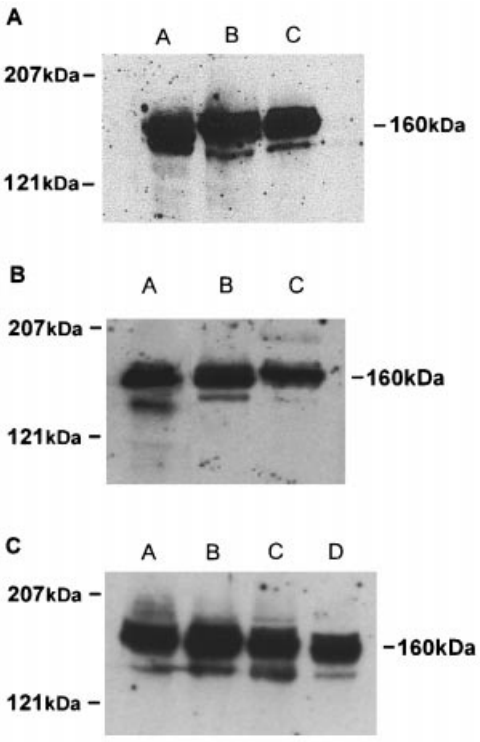

Figure 1 Western-blot analysis of mouse breast tissue and liver extracts

Tissue extracts were prepared as described in the Materials and methods section and analysed by SDS/PAGE [7.5% (w/v) gel]; ATP7B protein was detected with a polyclonal antibody against ATP7B and a chemiluminescence system. (**A**) Lane A, extracts of mammary tissue (60 μg) from non-lactating DL mice; lane B, extracts of mammary tissue (60 μg) from non-lactating *tx* mice; lane C, liver extract (60 μg) from DL mice. (**B**) Lane A, extracts of mammary tissue (60 μg) from lactating DL mice; lane B, extracts of mammary tissue (60 μg) from lactating *tx* mice; lane C, liver extract (60 μg) from DL mice. (**C**) Lane A, extracts of mammary tissue (60 μg) from copper-treated non-lactating DL mice; lane B, extracts of mammary tissue (60 μg) from copper-treated lactating DL mice; lane C, extracts of mammary tissue (60 μg) from copper-treated non-lactating *tx* mice; lane D, extracts of mammary tissue (60 μg) from copper-treated lactating *tx* mice. In all panels the positions of molecular-mass markers are indicated (in kDa) on the left.

the pup and was used as an indicator of the copper content of milk. The copper concentration in the stomach contents of 5-day-old normal (DL) pups fostered on normal mothers was 6.4 ± 2.0 μg of copper/g dry weight but in normal pups on *tx* mothers it was significantly lower (1.2 ± 0.3 μg of copper/g dry weight; *t* test $P = 1.4 \times 10^{12}$) (Table 1). The low copper concentration is consistent with a marked decrease in the delivery of copper to the milk in the *tx* dam, similar to that in a previous study [4]. The low copper concentration in milk led to a marked decrease in the copper concentration in the livers of pups: only 6.4 ± 0.7 μg of copper/g dry weight in normal pups fostered to *tx* dams (*t* test $P = 1.9 \times 10^{-13}$) compared with 127 μg of copper/g dry weight in the livers of the normal pups fostered to normal dams (Table 1).

Copper concentrations in normal and *tx* mouse stomach contents and livers after dietary supplementation with copper

To determine whether dietary supplementation of lactating dams with copper could influence the copper concentration in milk and copper in the livers of pups, lactating normal and *tx* dams were provided with copper-loaded drinking water (0.1 mg/ml copper as copper acetate) from the time of mating.

The copper concentration in the stomach contents of normal pups fostered on copper-treated normal mothers was 8.0 ± 2.0 μg of copper/g dry weight, which was significantly higher (*t* test $P = 1.4 \times 10^{-4}$) than the copper concentration of the stomach contents of pups suckling on dams not supplemented with copper (6.4 ± 2.0 μg of copper/g dry weight) (Table 1). Surprisingly, the copper concentration in the stomachs of pups on *tx* mothers receiving copper-supplemented water was lower (0.9 ± 0.2 μg of copper/g dry weight) than the copper concentration in pups sucking on the dams receiving normal water (1.2 ± 0.3 μg of copper/g dry weight) (*t* test $P = 6 \times 10^{-3}$) (Table 1).

The copper concentration in livers of normal pups fostered on copper-treated normal mothers was 202 ± 49 μg of copper/g dry weight. This result was significantly higher ($P = 5.8 \times 10^{-6}$) than that of normal pups fostered on normal dams not receiving copper-supplemented water (127 ± 47 μg of copper/g dry weight), supporting the observation that the pups were exposed to higher concentrations of copper in milk when the mother was supplemented with copper. In comparison, the copper concentration in livers of DL pups fostered on copper-treated *tx* mothers was 6.5 ± 1.6 μg of copper/g dry weight, which was similar to that of the pups fostered on *tx* mothers not supplemented with copper (6.4 ± 0.7 μg of copper/g dry weight). Thus, even in copper-supplemented *tx* dams, little copper appeared in the milk.

Copper concentrations in the normal and *tx* mammary gland

The copper concentration in the mammary glands of lactating *tx* mice was 7.8 μg/g dry weight, which was not significantly higher than that of normal mice (6.5 μg/g dry weight; Table 1). After dietary copper supplementation, the copper concentration of normal mammary glands did not increase significantly (*t* test $P = 0.03$). In contrast, the mammary copper concentration of the

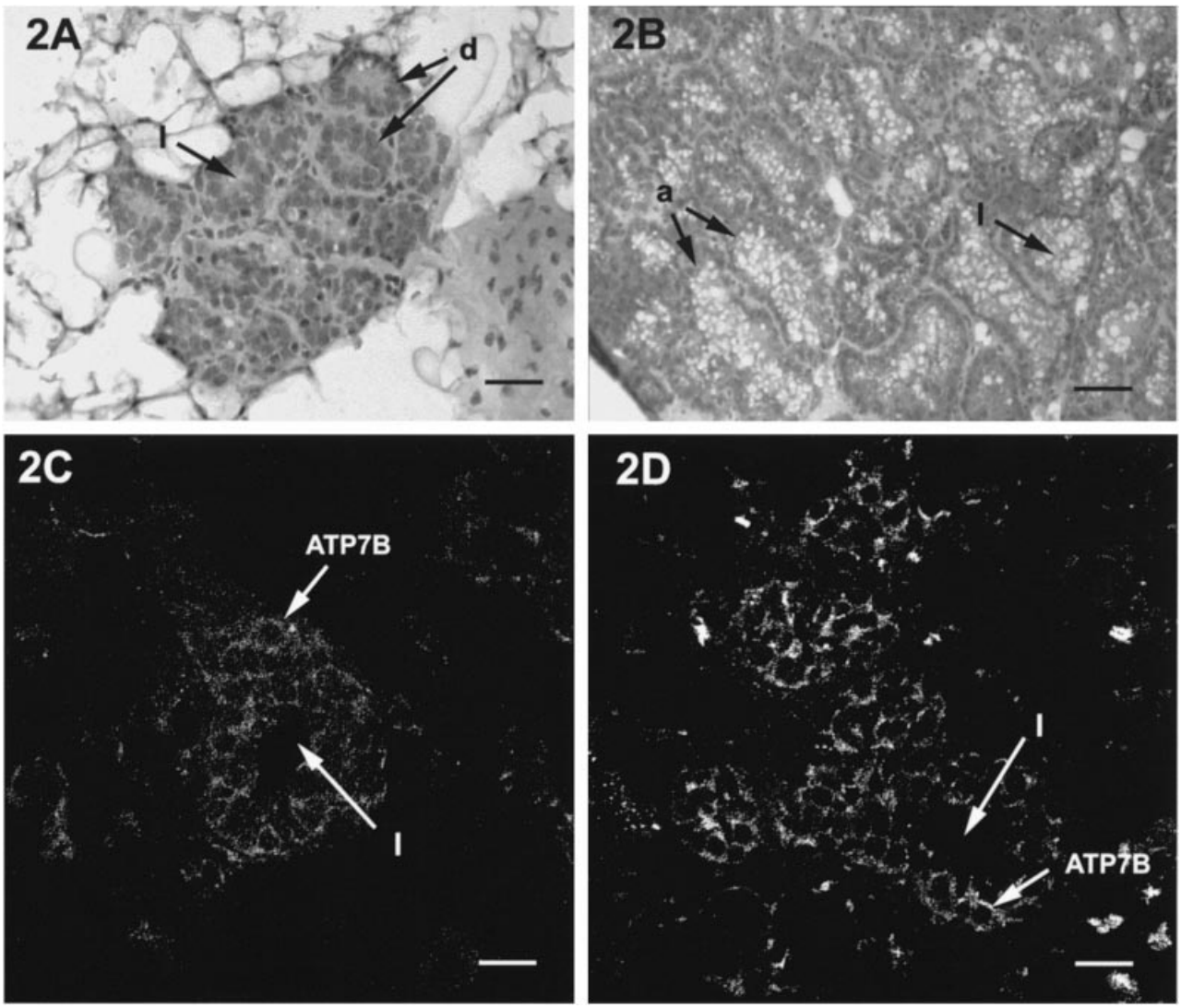

Figure 2 Haematoxalin and eosin staining and immunolocalization of ATP7B in mouse mammary tissue

(**A**) Section, stained with haematoxylin and eosin, from non-lactating DL mouse mammary gland to show ducts (d) and the lumen of a duct (l). Scale bar, 160 μm. (**B**) Section, stained with haematoxylin and eosin, from lactating DL mouse mammary gland to show secretory alveoli (a) and the lumen of an alveolus (l). Scale bar, 160 μm. (**C**) Immunohistochemical localization of ATP7B in a section through non-lactating DL mouse mammary gland showing the lumen of a duct (l) and diffuse ATP7B labelling in the epithelial cells of the ducts. Scale bar, 12 μm. (**D**) Immunohistochemical localization of ATP7B in a section through the non-lactating mammary gland of the *tx* mouse, showing the lumen of an alveolus (l) and ATP7B in the epithelial cells of the alveoli. Scale bar, 12 μm.

tx mice was increased 3-fold to 27 μg/g dry weight after supplementation with copper. This increase, together with the results for copper from stomach contents and from pup livers, suggests that more copper was available to the mammary gland in the copper-supplemented dams and that it was secreted into the milk in normal mice but accumulated in the mammary gland of *tx* dams.

Western-blot analyses of mouse mammary glands

Western blots of non-lactating mammary-gland extracts incubated with the anti-ATP7B antibody detected bands of size 160 kDa in both the control (DL) and the *tx* mouse tissue (Figure 1A). A band of size 160 kDa was also found in a sample of DL liver, which was used as a control. Similar levels of ATP7B protein were found in the control mammary gland, the *tx* mouse mammary gland and the control liver, suggesting that the mammary gland is a major site of expression of ATP7B. The lactating tissue extracts also showed bands of 160 kDa in both the DL mouse and the *tx* mouse (Figure 1B). Comparable levels of ATP7B protein were found in the DL lactating mammary gland and liver and in the *tx* mouse lactating mammary gland. The levels of ATP7B protein were similar in the lactating and the non-lactating gland; supplementation of the lactating dams with copper did not affect the expression of ATP7B in either DL or *tx* mammary glands (Figure 1C).

Immunolocalization of ATP7B in mammary glands

A section through non-lactating *tx* tissue stained with haematoxylin and eosin (Figure 2A) shows a duct, distinguished by its irregular shape and several alveoli, all of which are lined with an

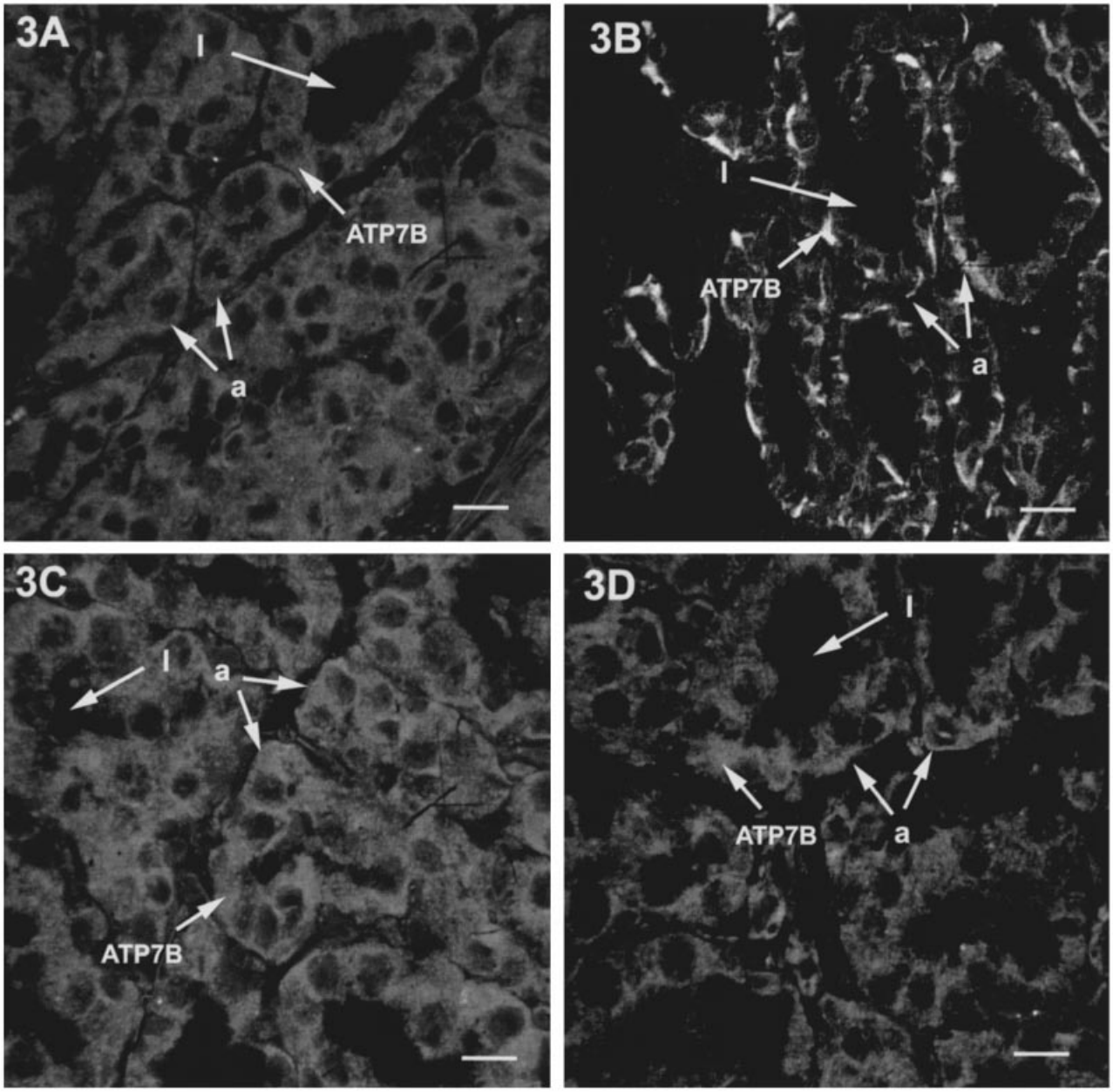

Figure 3 Immunolocalization of ATP7B in the lactating mammary gland of DL and *tx* mice, with or without copper treatment

(**A**) Immunohistochemical localization of ATP7B in a section through lactating DL mouse mammary gland, showing secretory alveoli (a), the lumen of an alveolus (l) and ATP7B in the epithelial cells of the alveoli. Scale bar, 10 μm. (**B**) Immunohistochemical localization of ATP7B in a section through the lactating mammary gland of the *tx* mouse, showing secretory alveoli (a), the lumen of an alveolus (l) and ATP7B in the epithelial cells of the alveoli. Scale bar, 12 μm. (**C**) Immunohistochemical localization of ATP7B in a section from the mammary gland of a lactating DL mouse that had been provided with water containing 0.3 mg/ml copper acetate, showing secretory alveoli (a), the lumen of an alveolus (l) and diffuse labelling of ATP7B in the epithelial cells of the alveoli. Scale bar, 10 μm. (**D**) Immunohistochemical localization of ATP7B in a section through the mammary gland of a lactating *tx* mouse that had been provided with water containing 0.1 mg/ml copper (as copper acetate), showing secretory alveoli (a), the lumen of an alveolus (l) and ATP7B in the epithelial cells of the alveoli. Scale bar, 10 μm.

inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. In lactating *tx* tisssue (Figure 2B), numerous alveoli containing secretory material can be seen. No structural differences between DL and *tx* mammary glands were seen after a microscopic examination of sections of non-lactating or lactating tissue stained with haematoxylin and eosin.

In non-lactating mice, ATP7B was present in the luminal epithelial cells of the mammary gland as a granular perinuclear label; there was no obvious difference in ATP7B distribution between the DL gland (Figure 2C) and the *tx* gland (Figure 2D).

In the lactating DL mouse mammary gland, ATP7B protein was detected as a diffuse, granular, cytoplasmic label (Figure 3A). In contrast, ATP7B protein in the lactating *tx* mouse mammary gland was localized to the perinuclear region; only a minor proportion of the fluorescence was detected in the cytoplasm (Figure 3B).

Effect of dietary copper loading on the subcellular distribution of ATP7B protein in the lactating mammary gland

To determine whether the perinuclear distribution of ATP7B in the mammary epithelial cells of *tx* mice could be influenced by dietary copper treatment, lactating DL and *tx* mice were provided with water containing 0.1 mg/ml copper as copper acetate for up to 3–4 months before the birth of the pups. Treatment of lactating DL mice with copper did not affect the subcellular distribution of ATP7B compared with lactating dams not treated with copper. In the copper-treated lactating DL mice, ATP7B was detected as a granular cytoplasmic label (Figure 3C) and this distribution of ATP7B was similar to that seen in the lactating DL mice not treated with copper (Figure 3A). In contrast, after treatment of the lactating *tx* mouse with copper, ATP7B was detected as a granular label localized to a region of the cytoplasm between the nucleus and the cell membrane (Figure 3D).

DISCUSSION

Our results unequivocally confirm that there is impaired transport of copper into milk in lactating *tx* mice and establish that ATP7B is the major copper transporter in the mammary gland, delivering copper into breast milk. The origin of copper deficiency in *tx* milk is the result of a failure of copper to be secreted into milk rather than of a failure of copper to be delivered to the mammary gland as a secondary consequence of defective ATP7B in the liver. Furthermore, long-term dietary copper supplementation did not overcome the copper deficiency in toxic milk. Our results suggest that the copper transport defect in the *tx* mouse involves defective trafficking of the ATP7B protein in the *tx* mammary gland during lactation.

The localization of ATP7B was found to undergo a marked change in response to lactation in the normal mouse. In the non-lactating gland, the protein was in a perinuclear region consistent with localization to the *trans*-Golgi network, as has been reported for ATP7B in cultured cells [8,10]. However, in the lactating gland the distribution of ATP7B was diffuse and resembled that seen in cells exposed to an elevated concentration of copper [8,9]. This observation shows that the trafficking of ATP7B seen in cultured cells is not simply a non-physiological artefact but is observed under physiological conditions and is presumably important in the secretion of copper into milk.

The most significant finding of our study was that the ATP7B protein in the *tx* mouse mammary gland did not disperse in response to lactation but remained in the perinuclear region of the cell. In other studies, mutant ATP7B molecules show defective trafficking in response to copper; for example, ATP7B with the common disease-causing H1069Q mutation remained in the *trans*-Golgi network even when cells were exposed to an elevated concentration of copper [10]. Results from our laboratory with Chinese hamster ovary cells transiently transfected with the *tx* construct show that copper-induced trafficking of ATP7B protein is abolished (M. Theophilos and J. F. B. Mercer, unpublished work). The link between mutations that prevent copper transport (such as that expected for the *tx* mutation, which is in the eighth transmembrane domain) and blocking trafficking is not clear but it is also found for mutations in the Menkes protein, ATP7A [11]. Whatever the mechanism, the defective trafficking contributes to the failure of the copper efflux mechanism in the gland.

Treatment of the lactating DL mouse with copper did not cause any change in the localization of ATP7B protein compared with lactating mice not treated with copper. It was somewhat unexpected to find that treatment of the lactating *tx* mouse with copper caused a redistribution of the ATP7B protein to a more peripheral region of the cell, between the cytoplasm and the nucleus. However, the distribution was not as extensive as that seen in the normal lactating gland and this result suggests that, in the mammary gland, the mutant ATP7B protein seems to have retained some capacity for trafficking. The difference in trafficking between the systems *in vitro* and *in vivo* might be related to the fact that the more complex system *in vivo* in the mammary gland consists of interacting polarized cells, compared with the non-polarized, transformed lines used in the cell-culture studies. This observation merits further investigation.

It is interesting that the levels of ATP7B protein in the mammary gland were as high as those found in the liver, which suggests that the role of ATP7B in copper efflux is as important in the mammary gland as in the liver, the main organ of whole-body homoeostasis. Our results showed that the expression levels of ATP7B protein were not increased in lactating tissue relative to non-lactating tissue. Thus the relocalization of the ATP7B protein to a peripheral cytoplasmic compartment might be a key factor in providing an increased delivery of copper to the milk during lactation.

Our results showed that the copper concentrations in the *tx* mammary gland were not significantly higher than those of the normal mammary gland. This contrasts with the doubling of mammary copper levels in three lactating *ATP7B* knock-out mice relative to that of three control mice [7]. The difference between the two could be related to the severity of the mutation. We have shown that the *tx* mouse does produce ATP7B protein but, in comparison with the null mutation, the mis-sense mutation in transmembrane 8 of the *tx* mouse might allow some residual copper-transporting activity and maintain lower copper concentrations than the complete knock-out of *ATP7B*.

To determine whether the delivery of copper in the milk of *tx* mice could be restored by dietary copper supplementation, copper-loaded (0.1 mg/ml) water was provided to lactating normal and *tx* mice. This resulted in a 33% increase in the copper concentrations in the stomachs of pups reared by normal mice and a near doubling of the hepatic copper concentration in the livers of the pups, indicating that more copper was secreted into milk and was absorbed by the pups. Thus copper concentrations in milk are increased if the maternal diet contains large amounts of copper; however, the increase is relatively minor compared with the large amounts of copper being consumed by the animals. In contrast, the treatment of *tx* dams with copper did not increase the delivery of copper to milk; neither were the copper concentrations in livers of pups reared by *tx* dams increased. Interestingly, the concentration of copper in the mammary gland of the copper-loaded *tx* mouse was increased approx. 3-fold with copper loading. This result supports our conclusion that the production of copper-deficient milk is not due to a lack of copper reaching the mammary gland but is due to a defect in the capacity of the *tx* mammary gland to transfer copper into milk.

Our results suggest that ATP7B protein has an important function in transporting copper into milk in the mammary gland. However, the observation that even when this protein is defective there is still some copper present in milk (approximately one-sixth the normal level) suggests the presence of another pathway for the secretion of copper. There are several possible additional pathways: one involves the copper protein ceruloplasmin, which is synthesized in the mammary gland and secreted into milk [14] and might account for 25% of the copper in milk [15]. However, this source of copper would also be expected to be decreased in the *tx* mouse, because ATP7B is most probably involved in the delivery of copper to ceruloplasmin in the mammary gland as well as in the liver. The role of ATP7A, the Menkes copper ATPase, in mammary-gland copper homoeostasis is still unclear.

In previous studies we showed that the levels of expression of ATP7A mRNA and ATP7B mRNA in human breast epithelial cells were similar [16] and that there was an increased expression of the Menkes gene in the lactating human mammary gland [17]. It is possible that the residual copper in the *tx* mouse is delivered by means of the Menkes protein. There might be other pathways for the delivery of copper to milk not involving the Cu-ATPases but our results suggest strongly that the primary delivery system is the ATP7B protein.

In conclusion, we have demonstrated that a defective localization of the ATP7B protein is responsible for the low concentration of copper in the milk of the toxic-milk mouse. Our findings suggest that ATP7B is trafficked in the mammary gland in response to physiological stimuli and is directly involved in copper homeostasis in the newborn mouse. Dietary copper supplementation was ineffective in increasing the copper content of milk from the *tx* mouse, implying that normal homoeostatic mechanisms for copper transport in the mammary gland are defective.

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